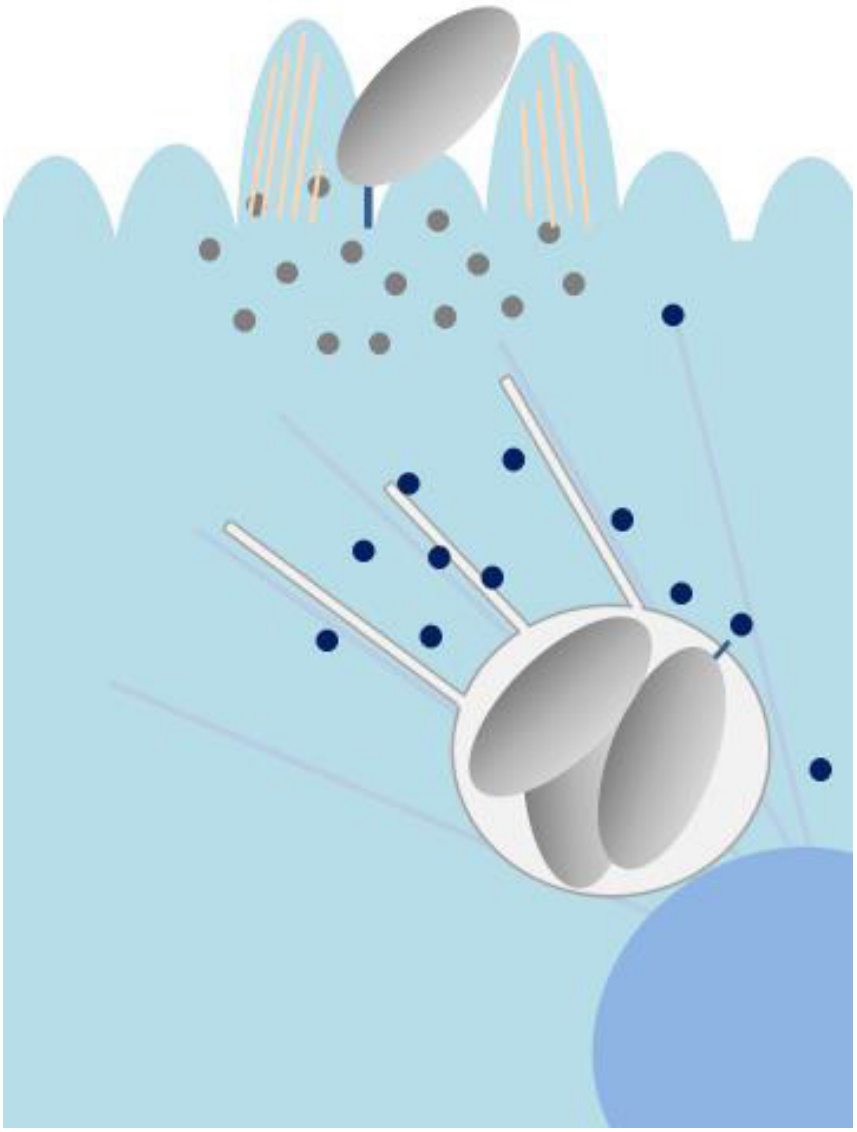
HOST-ADAPTED METABOLISM AND ITS REGULATION IN BACTERIAL PATHOGENS

EDITED BY: Thomas Dandekar and Wolfgang Eisenreich
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



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ISSN 1664-8714
ISBN 978-2-88919-538-1
DOI 10.3389/978-2-88919-538-1

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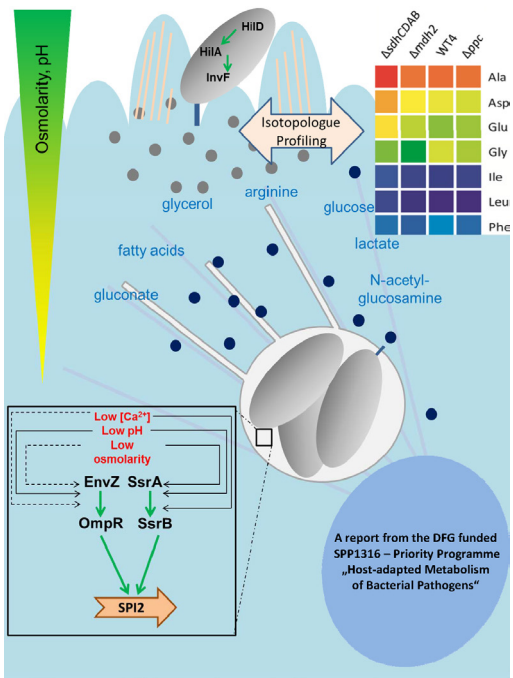
HOST-ADAPTED METABOLISM AND ITS REGULATION IN BACTERIAL PATHOGENS

Topic Editors:
Thomas Dandekar, University of Würzburg, Germany
Wolfgang Eisenreich, Technische Universität München, Germany



Host-adapted metabolism of intracellular *Salmonella typhimurium*

Pathogens adapt their metabolism rapidly to the host. Our topic covers these phenomenon regarding extracellular and intracellular pathogens as well as general methods to elucidate different metabolic adaptation processes - an essential guide for any scientist wanting to keep abreast of recent developments in infection biology.

Citation: Dandekar, T., Eisenreich, W., eds. (2015). Host-Adapted Metabolism and Its Regulation in Bacterial Pathogens. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-538-1

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frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 27 March 2015
doi: 10.3389/fcimb.2015.00028

Host-adapted metabolism and its regulation in bacterial pathogens

Thomas Dandekar[1]* *and Wolfgang Eisenreich*[2]

[1] *Bioinformatics, University of Würzburg, Würzburg, Germany,* [2] *Biochemistry, Technische Universität München, Garching, Germany*

Keywords: bacterial pathogens, enteric pathogens, metabolism, host-pathogen adaption, isotopolog profiling

The mutual interaction between bacterial pathogens and their host organisms is a key feature of virulence. Since bacterial pathogens are highly specific in their infection behavior against different host organisms, cell types and cell compartments, the host-pathogen interplay is crucial in bacterial virulence. There is increasing evidence that basic metabolic pathways and fluxes in pathogens are modulated during the infection process as a response to adapt to the specific and dynamic conditions of the host environments, such as various cell types and compartments. Although a manipulation of these adaptation processes could be a key in the future treatment of bacterial infections, our knowledge about these metabolic features and their controlling factors is still limited.

Hence, the German research council (DFG) funded priority programme SPP1316 (2008–2014) for the investigation of host-adapted metabolism of bacterial pathogens. Within the SPP1316, a number of groups have investigated in considerable detail how bacterial pathogens adapt their metabolism during colonization of their host organisms, how the metabolism of pathogenic bacteria and host organism is interconnected and which mechanisms of control are active.

As a result for these investigations and further studies, various chapters of this "Topic hosted by Frontiers" report on the identification of metabolic pathways that are important for the bacterial pathogen during infection, and on determination of metabolic fluxes. Questions that are addressed by the Topic include: Why are bacteria able to multiply in the body of the host and can cause disease? Which specific toxins and/or invasins are produced? In general, which metabolic adaptations allow bacterial pathogens to colonize their host? Further points that are considered concern the interconnected metabolism of host and pathogen and the regulatory mechanisms that are activated in both pathogen and host. Furthermore, we cover several highly sensitive methods of bioanalytics and high throughput screening approaches developed in recent years. A systematic synopsis of metabolism of bacterial pathogens adapted to host conditions is given here.

Further chapters consider main metabolic pathways and fluxes, metabolic reactions of specific pathogens in their respective host niches as well as genetic and regulatory mechanisms of their metabolic adaptation. As a result, an integrated view on the nature and mechanisms in host-adapted bacterial metabolism is represented by the various articles of this Topic. The data show that host-pathogen metabolic adaptation is highly specific at least for most of the pathogens described in the Topic. Nevertheless, the data also demonstrate that the metabolic networks encountered in host-pathogen systems include essential reactions that could serve as future targets in drug therapy. It can be envisaged that further studies will foster our knowledge about metabolic features in host-adaptation of bacterial pathogens but also vice versa in the metabolic response of host organisms upon bacterial infections.

In summary, the Topic presents an integrated picture of the host- and pathogen adaptation of bacterial metabolism during infection, a bioanalytical characterization of the relevant metabolic pathways as well as insights by functional and analytical investigations, covering both extracellular and intracellular pathogens. We therefore believe that this research Topic

OPEN ACCESS

Edited and reviewed by:
Yousef Abu Kwaik, University of Louisville School of Medicine, USA

***Correspondence:**
Thomas Dandekar, dandekar@biozentrum.uni-wuerzburg.de

Received: *04 March 2015*
Accepted: *09 March 2015*
Published: *27 March 2015*

Citation:
Dandekar T and Eisenreich W (2015) Host-adapted metabolism and its regulation in bacterial pathogens. Front. Cell. Infect. Microbiol. 5:28. doi: 10.3389/fcimb.2015.00028

will contribute in stimulating further studies in the fascinating field of host-pathogen interactions.

We present the following articles (blue font indicates articles of SPP1316 members)

General Aspects of Host-Adapted Metabolism of Bacterial Pathogens

1. Metabolic aspects of bacterial persisters
   Marcel Prax and Ralph Bertram*
2. Interrelationship between type three secretion system and metabolism in pathogenic bacteria
   Gottfried Wilharm* and Christine Heider
3. Small RNA functions in carbon metabolism and virulence of enteric pathogens
   Kai Papenfort* and Jörg Vogel
4. From screen to target: insights and approaches into the development of anti-virulence compounds
   Katherine SH Beckham and Andrew J Roe

Metabolism of Intracellular Bacterial Pathogens

5. Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism
   Stephanie Grubmüller, Kristina Schauer, Werner Göbel, Thilo M Fuchs and Wolfgang Eisenreich
6. Nutrient generation and retrieval from the host cell cytosol by intra-vacuolar *Legionella pneumophila*
   Christopher T. D. Price, Ashley Marie Richards and Yousef Abu Kwaik*
7. *Salmonella*—how a metabolic generalist adopts an intracellular life style during infection
   Thomas Dandekar*, Astrid Fieselmann, Eva Fischer, Jasmin Popp, Michael Hensel and Janina Noster
8. Metabolism of the vacuolar pathogen *Legionella* and implications for virulence
   Christian Manske and Hubert Hilbi
9. The *Mycobacterium avium* ssp. *paratuberculosis* specific *mptD* gene is required for maintaining of the metabolic homeostasis necessary for full virulence in mouse infections Thorsten
   Meißner, Elke Eckelt, Tina Basler, Jochen Meens, Julia Heinzmann, Aabdulhadi Suwandi, Walter Martin Roland Oelemann, Sandra Trenkamp, Otto Holst, Siegfried Weiss, Boyke Bunk, Cathrin Spöer, Gerald-F. Gerlach and Ralph Goethe

Metabolism of Extracellular Bacterial Pathogens

10. Coregulation of host-adapted metabolism and virulence by pathogenic yersiniae
    Ann Kathrin Heroven and Petra Dersch*
11. Defining the metabolic requirements for the growth and colonization capacity of *Campylobacter jejuni*
    Dirk Hofreuter*
12. *Staphylococcus aureus* small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism
    André Kriegeskorte*, Stephanie Grubmüller, Claudia Huber, Barbara C. Kahl, Christof von Eiff, Richard Allan Proctor, Georg Peters, Wolfgang Eisenreich and Karsten Becker*
13. Metabolism and virulence in *Neisseria meningitides*
    Christoph Schoen*, Laura Kischkies, Johannes Elias and Biju Joseph Ampattuu
14. The arginine-ornithine antiporter ArcD contributes to biological fitness of *Streptococcus suis*
    Marcus Fulde, Joerg Willenborg, Claudia Huber, Angela Hitzmann, Daniela Willms, Maren Seitz, Wolfgang Eisenreich, Peter Valentin-Weigand* and Ralph Goethe
15. *Staphylococcus aureus* Small Colony Variants (SCVs): A road map for the metabolic pathways involved in persistent infections
    Richard Allan Proctor*, Andre Kriegeskorte, Barbara Kahl, Karsten Becker, Bettina Löffler and Georg Peters
16. *Yersinia pestis*: mechanisms of entry into and resistance to the host cell
    Yuehua Ke*, Zeliang Chen and Ruifu Yang

Acknowledgments

Funding by DFG, SPP1316—Priority Programme: Host-adapted Metabolism of Bacterial Pathogens is gratefully acknowledged.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

MINI REVIEW ARTICLE
published: 22 October 2014
doi: 10.3389/fcimb.2014.00148

Metabolic aspects of bacterial persisters

Marcel Prax and Ralph Bertram*

Department of Microbial Genetics, Faculty of Science, Interfaculty Institute of Microbiology and Infection Medicine Tübingen (IMIT), University of Tübingen, Tübingen, Germany

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
Alain Charbit, University Paris Descartes, France
Petra Dersch, Helmholtz Center for Infection Research, Germany

****Correspondence:***
Ralph Bertram, Department of Microbial Genetics, Faculty of Science, Interfaculty Institute of Microbiology and Infection Medicine Tübingen (IMIT), University of Tübingen, Waldhäuser Str. 70/8, 72076 Tübingen, Germany
e-mail: ralph.bertram@uni-tuebingen.de

Persister cells form a multi-drug tolerant subpopulation within an isogenic culture of bacteria that are genetically susceptible to antibiotics. Studies with different Gram negative and Gram positive bacteria have identified a large number of genes associated with the persister state. In contrast, the revelation of persister metabolism has only been addressed recently. We here summarize metabolic aspects of persisters, which includes an overview about the bifunctional role of selected carbohydrates as both triggers for the exit from the drug tolerant state and metabolites which persisters feed on. Also alarmones as indicators for starvation have been shown to influence persister levels via different signaling cascades involving the activation of toxin-antitoxin systems and other regulatory factors. Finally, recent data obtained by ^{13}C-isotopolog profiling demonstrated an active amino acid anabolism in *Staphylococcus aureus* cultures challenged with high drug concentrations. Understanding the metabolism of persister cells poses challenges but also paves the way for the development of anti-persister compounds.

Keywords: *Staphylococcus aureus*, persisters, metabolism, toxin-antitoxin system, ppGpp, biofilm

INTRODUCTION

The treatment of recurrent bacterial infections is often a tedious trial due to antibiotic recalcitrance. This is not solely caused by resistance but also implies persister cells which are (multi-) drug-tolerant (Lewis, 2010). Persisters were first described in 1944 when killing of *S. aureus* with penicillin was found to leave a few survivor cells behind (Bigger, 1944). Notably the antibiotic tolerance of persisters is not genetically manifested, as progenies of persisters are as susceptible as the parent strains (Keren et al., 2004a). Consistent with a number of studies, persisters among an isogenic bacterial culture temporarily reside in a slow- or non-growing state and arise both stochastically and in response to environmental cues. For instance, biofilms accommodate a high level of persisters (Lewis, 2005) and their number within a culture depends greatly on the growth stage, with stationary cultures exhibiting much higher persister levels compared to the exponential phase (Keren et al., 2004a; Lechner et al., 2012). This correlation was confirmed by further studies that established a strong influence between inoculum age and persister frequency (Luidalepp et al., 2011). Retarded protein synthesis as well as protein aggregate accumulation were found to affect the persister levels of a culture (Kwan et al., 2013; Leszczynska et al., 2013), as does bacterial compound signaling (Keller and Surette, 2006). Molecules such as indole, 2′ Amino-acetophenone, or CSP pheromone, some of which are quorum sensing (QS) messengers, can induce drug tolerance and the persister state in different bacteria (Leung and Levesque, 2012; Vega et al., 2012, 2013; Que et al., 2013). Akin to QS, some bacteria have been shown to produce so called resuscitation-promoting factors, converting dormant cells back to a more active state. Among several examples apparently based upon similar mechanisms, the addition of spent culture medium to dormant *S. aureus* cells led to accelerated awakening (Mukamolova et al., 1999; Pinto et al., 2013; Pascoe et al., 2014). The retention of a viable state over longer periods of time and particularly the reversion from dormancy to a growing state requires metabolic activity. One major question is how persisters maintain a critical degree of metabolism over extended periods of time without being killed during hostile conditions. Here, we sum up recent findings on the involvement of metabolism in the persister state (**Figure 1**) and illustrate the experimental difficulties and challenges accessing this topic. The reader is also referred to a recent review article by Amato et al. (2014).

GENES LINKING METABOLISM AND THE PERSISTER STATE

The number of identified genes associated with the persister state is steadily increasing and toxin-antitoxin (TA) systems act as key regulators in this regard (Lewis, 2010; Schuster and Bertram, 2013). These systems usually comprise a toxin that blocks or corrupts essential cellular functions and an antitoxin abrogating the toxin's activity. TA systems participate in multiple processes in bacteria, ranging from stress response to regulation of metabolism and survival inside host cells (McKenzie et al., 2012; Helaine et al., 2014). The issue of how TA systems are controlled and how this leads to persister formation has been illuminated in a number of cases. For example, glucose starvation and shortage of amino acids activate RelE-toxin homologs in *E. coli* (Christensen-Dalsgaard et al., 2010). Proteome analysis of starving *Mycobacterium tuberculosis* cells revealed an increased abundance of TA system proteins under nutrient limited conditions (Albrethsen et al., 2013). The alarmone ppGpp is part of the stringent response signaling pathway, which is switched on in response to amino acid depletion. ppGpp abundance and TA system activity appear to be tightly intertwined to control the metabolic state of bacterial cells (Traxler et al., 2008; Bokinsky et al., 2013; Germain et al., 2013; Maisonneuve et al., 2013). For

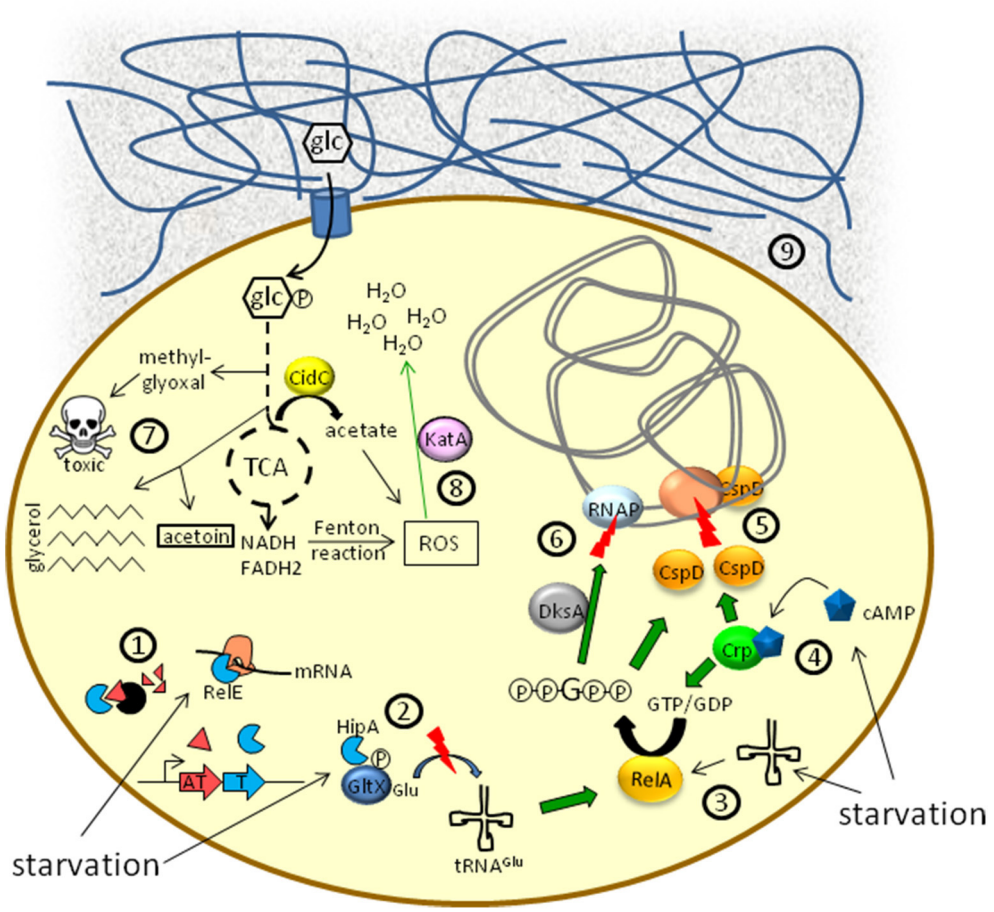

FIGURE 1 | Schematic overview of metabolic aspects associated with the persister state. ①, ② Toxin/antitoxin-systems: In a number of bacteria, Lon or Clp proteases, activated in response to starvation, degrade antitoxin proteins ①. Liberated toxins (e.g., RelE) can cleave mRNA or employ a ppGpp-dependent signal transduction ② to induce growth arrest. ③ Uncharged tRNAs due to amino acid starvation lead to the synthesis of ppGpp via RelA. ④ Nutrient limitation favors the synthesis of the second messenger cAMP by adenylate cyclase. cAMP binds to the cAMP receptor protein (Crp) and the cAMP/Crp complex activates the expression of both *relA* and *cspD*. ⑤ Inhibition of DNA replication by CspD. ⑥ Modulation of RNA polymerase (RNAP) activity by the DksA/ppGpp complex. ⑦ Metabolic flux alterations result in a decreased TCA cycle activity and increased persistence. Synthesis of methylglyoxal leads to growth inhibition. Acetoin and triglyceride synthesis represent alternative pathways for the deprivation of pyruvate and acetyl-CoA from the TCA cycle. ⑧ Different branches of metabolism can produce reactive oxygen species (ROS) as hazardous side products, impairing persister formation. Enzymes counteracting ROS activity (e.g., KatA) are upregulated in persister cells. ⑨ Biofilms containing protein- and/or aminosugar-polymer structures (blue meshwork) may represent environments of low supply of nutrients, such as glucose (glc), which favors persister formation.

example, the TA toxin HipA phosphorylates the glutamyl-tRNA synthetase GltX which inhibits the loading of $tRNA^{Glu}$ and consequently mimics nutrient limitation resulting in ppGpp synthesis. *Pseudomonas aeruginosa* actively responds to nutrient limitation via a ppGpp-dependent mechanism directing cells to a state of increased antibiotic tolerance (Nguyen et al., 2011). Metabolic stress can also lead to a different scenario, in which ppGpp and the cognate hydrolase SpoT influence persister formation (Amato et al., 2013). Low levels of SpoT thus increase ppGpp abundance that is also associated with DNA gyrase inhibition and reduction of RNA polymerase activity. In *S. aureus* a similar role for ppGpp was demonstrated, as its permanent synthesis leads to growth inhibition and impaired virulence, facilitating persistent infections (Gao et al., 2010). Also the *E. coli* cold shock protein CspD that is expressed during stationary phase and is induced by glucose starvation is influenced by ppGpp (Yamanaka and Inouye, 1997). The lack of nutrients leads to a CspD-dependent inhibition of DNA replication, resulting in increased persister formation (Kim and Wood, 2010). Interestingly, also another second messenger, cyclic AMP (cAMP), is part of the regulatory network of CspD. cAMP, whose physiological level is associated with nutrient availability, increases *cspD* transcription in complex with its receptor protein Crp (Uppal et al., 2014). Moreover, the cAMP-Crp complex also activates the expression of *relA*, resulting in a further increase of the intracellular ppGpp level (Nakagawa et al., 2006). This example illustrates how the metabolic state of a cell can be coupled to persister formation via different pathways to achieve a subtle and precise regulation. In the light of these results,

ppGpp seems to be an important mediator between metabolism and persister formation.

Reports about the genetic alterations in the energy metabolism of bacterial cells provide a rather inconsistent picture. *E. coli* mutants lacking *ubiF* or *sucB*, encoding for enzymes involved in ubiquinone biosynthesis, or the TCA cycle, respectively, showed decreased persister levels compared to the wild-type strain (Ma et al., 2010). Both enzymes contribute to the generation of the intracellular ATP pool. However, the inhibition of ATP synthesis by carbonyl cyanide m-chlorophenylhydrazone (CCCP) led to an increased persister formation in another study (Kwan et al., 2013). The same effect was observed for the membrane binding protein TisB of the *tisAB* TA-system. *TisB* expression decreases the proton motive force (PMF) and impedes energy production causing an elevated persister level (Unoson and Wagner, 2008; Dörr et al., 2010).

EXPERIMENTAL APPROACHES AND METABOLIC PECULIARITIES OF PERSISTER CELLS

Major drawbacks in the analysis of persister cells' metabolism are both the natural heterogeneity of the bacterial population and the fact that antibiotics used to isolate persisters destroy their naïve state. Furthermore, the persister state is of temporary nature only and these cells usually merely represent a small subpopulation within a culture. It is therefore of utmost importance to distinguish between results stemming from persister and non-persister cells, which requires efficient means to separate them. Lytic antibiotics or unstable GFP-variants have been used before to address this issue (Keren et al., 2004b; Shah et al., 2006). Obtained transcriptome patterns of suchlike differentiated *M. tuberculosis* or *E. coli* persisters indicated a downregulation of metabolic genes, and therefore a decreased metabolism in persisters (Shah et al., 2006; Keren et al., 2011). Different approaches have been taken to examine the metabolism of persisters more directly. The group of Brynildsen used phenotype microarrays and a fluorescent dye to assay the activity of bacterial reductases as a proxy for metabolic activity (Orman and Brynildsen, 2013a,b). Based on these results, a less active metabolism is apparently not a requirement, but it increases the chance for a cell to enter the persister state. Another powerful technique, termed isotopolog profiling, is based upon feeding of ^{13}C-isotope labeled carbohydrates to cultures and subsequent analysis of labeled intermediates. This allows deducing relative activities of metabolic pathways or even networks in a time-resolved manner by comparing ratios of labeled and unlabeled compounds (Eisenreich et al., 2010). Isotopolog profiling provides information of relative metabolic fluxes but not on the quantities or absolute concentrations of metabolites. Measuring the decrease of energy substrates in the medium over time can be theoretically used to determine the metabolic level. We used isotopolog profiling to investigate, which metabolic pathways are active in stationary growth phase *S. aureus* cells that had been challenged with daptomycin (Lechner et al., 2014). *De novo* biosynthesis of amino acids was observed, and their labeling patterns suggested an active glycolysis, TCA cycle and pentose phosphate pathway. Of note, analysis of ^{13}C-labeling pattern of Asp and Glu indicated an increased activity of the TCA cycle.

Recent studies provided first insights into the metabolic state of persisters associated with biofilms that provide a protective niche for bacteria against antibiotics and other harmful conditions (Mah and O'Toole, 2001; Donlan and Costerton, 2002; Davenport et al., 2014). This is due, in part, to metabolic downshifts in biofilm dwelling cells. Impaired nutrient penetration and consumption by peripheral cells result in decreased nutrient supply in this environment. Genes involved in TCA cycle and energy production were downregulated in tobramycin challenged and biofilm embedded *Burkholderia cenocepacia* persisters (Van Acker et al., 2013). Metabolic activity can lead to H_2O_2 generation by the reduction of molecular oxygen caused by the respiratory chain (Gonzalez-Flecha and Demple, 1995). H_2O_2 can thereby accidentally drive the Fe^{2+}-dependent Fenton reaction leading to the formation of reactive oxygen species (ROS), which attack essential cellular functions (Imlay et al., 1988). Therefore, long-term survival of a bacterial cell could benefit from an impaired metabolism. In addition, a reduced energy level simultaneously prevents the PMF-dependent uptake of the aminoglycoside tobramycin, as detailed below. In *M. tuberculosis*, redirections in the carbon flux were correlated to growth arrest and antibiotic tolerance (Baek et al., 2011). Acyl-CoA is thereby converted to triglycerides, draining the fuel for the TCA cycle. A further example for increased persistence due to the change of metabolic fluxes is the synthesis of methylglyoxal, which impedes growth of *E. coli* (Girgis et al., 2012). Single deletions of the genes encoding the two metabolic enzymes glycerol-3-phosphate dehydrogenase (*glpD*) or transketolase A (*tktA*) lead to accumulation of dihydroxyacetone phosphate (DHAP) which is finally converted to methylglyoxal. Interestingly, the glyoxylate shunt is upregulated in *B. cenocepacia* persister cells, bypassing NADH production and possible ROS formation via the TCA cycle, illustrating an additional protective mechanism. Another link between persister level and ROS formation in biofilm was established in *P. aeruginosa*, where mutants defective in the stringent response were more susceptible toward antibiotic treatment (Nguyen et al., 2011). Starvation apparently leads to increased antioxidant countermeasures by an upregulation of catalase activity and the restriction of the synthesis of pro-oxidant substances. Furthermore, the metabolic regulator catabolite repression control (Crc) protein decreases the metabolic activity of *P. aeruginosa* in biofilms conferring increased tolerance toward ciprofloxacin (Zhang et al., 2012). These results indicate that a metabolic adaptation process especially in regard to the TCA cycle is involved in the maintenance of the persister state. Besides biofilm cells, the importance of a metabolic downshift was also confirmed by long-term survival assays of planktonic *S. aureus* cells, in which mutants lacking the TCA cycle enzymes aconitase or succinate dehydrogenase showed an enhanced stationary-phase survival level (Somerville et al., 2002; Gaupp et al., 2010). Retarded metabolic flux through or disruption of the TCA cycle was found in clinical *S. epidermidis* isolates with enhanced survival during β-lactam treatment (Thomas et al., 2013). Reduced ROS formation was determined as one critical feature in this regard. In line, ROS activity seems to be involved in programmed cell death, as shown in *S. aureus* (Thomas et al., 2014). ROS formation is apparently linked to acetate production which is again tightly

regulated by the two antagonistic factors CidC and AlsSD. CidC is an oxidase converting pyruvate to acetate and is activated by the CidR regulator during the presence of glucose. The CidR regulon also comprises the *alsSD* operon encoding for an α-acetolactate synthase/decarboxylase leading to acetoin synthesis from pyruvate, thereby reducing the amount of acetate by CidC. These data illustrate the strong connection of TCA cycle dependent ROS formation and its negative influence on the long-term survival of cells thereby requiring alternative metabolic pathways to avoid their production.

ANTI-PERSISTER STRATEGIES AND THE INTERPLAY BETWEEN CARBOHYDRATE SUPPLY AND PERSISTER KILLING

The importance of persisters in bacterial infections is more and more corroborated (Fauvart et al., 2011). Recent studies indicate that physiology and metabolism could be an Achilles heel for the development of new anti-persister strategies (Allison et al., 2011). A number of compounds counteract the persister state by targeting indispensable cellular processes or by activating resuscitation. These drugs include the acyldepsipeptide ADEP4 which permanently activates Clp proteases or a biphenyl-derivative termed C10 that reverts cells to an antibiotic susceptible state (Kim et al., 2011; Conlon et al., 2013). Manipulating bacterial signaling via artificial QS inhibitors is another approach (Pan and Ren, 2013) and also ppGpp was identified as a potential anti-persister/-biofilm target. A dodecamer peptide termed 1018 was reported to label the alarmone for degradation, thereby inhibiting formation or dispersal of biofilms as sources for recurrent and persistent infections. This peptide was active against at least seven Gram positive or Gram negative bacterial species (de la Fuente-Nunez et al., 2014). In 2011 the group of James J. Collins described that the addition of selected carbohydrates enhanced the killing of persisters by aminoglycoside antibiotics. They established a relationship between the metabolism of selected sugars, the generation of PMF and the enhanced uptake of the drug (Allison et al., 2011). The rate of increased killing is thereby mainly determined by the rate of substrate utilization. Particularly fructose was an effective compound in combination with gentamicin to eradicate *E. coli* as well as *S. aureus* persisters. Subsequent to this finding on metabolite enabled killing, the non-susceptibility of persisters toward aminoglycosides treatment aided in the identification of the most utilizable substrates for such cells (Orman and Brynildsen, 2013b). Another successful approach of metabolite induced killing was demonstrated by combating *P. aeruginosa* biofilms with a combination of mannitol and tobramycin (Barraud et al., 2013) and also arginine and nitrate were described as useful additives in this regard (Borriello et al., 2006). By contrast, an excess of glucose in *S. epidermidis* led to a higher level of dormant cells in a biofilm, presumably due to the accumulation of acidic degradation products resulting from glucose metabolism (Cerca et al., 2011).

CONCLUSION

Recent studies highlight the importance of investigating the two interconnected fields of persister state and metabolic activity in bacteria in more detail. Adaptation of the metabolism is a key prerequisite for persisters to cope with hostile conditions. In particular, the modulation of TCA cycle activity appears as a hallmark in persister metabolism. This regulation must be precisely controlled to avoid ROS formation with potentially destructive implications for persister cells. Multiple lines of evidence suggest that the metabolism of persisters can be tuned to alter their susceptibility toward antibiotics or to trigger programmed cell-death-like processes (Rice and Bayles, 2003). In a number of cases, this is achieved simply by supplementing selected carbohydrates. Based upon these findings, new effective anti-persister therapies could be developed to reduce the risk of relapsing or chronic infections. This could result in the development of concerted combination therapies, exploiting the natural metabolic activity of persister cells.

ACKNOWLEDGMENTS

We thank Friedrich Götz for support. This work was supported by grant BE4038/2 within the priority programme 1316 "host adapted metabolism of bacterial pathogens" of the Deutsche Forschungsgemeinschaft. Funding for the open access charge was provided by the Open Access Publishing Fund of the University of Tübingen.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 July 2014; paper pending published: 02 September 2014; accepted: 05 October 2014; published online: 22 October 2014.

*Citation: Prax M and Bertram R (2014) Metabolic aspects of bacterial persisters. Front. Cell. Infect. Microbiol. **4**:148. doi: 10.3389/fcimb.2014.00148*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 27 October 2014
doi: 10.3389/fcimb.2014.00150

Interrelationship between type three secretion system and metabolism in pathogenic bacteria

Gottfried Wilharm* and Christine Heider

Project Group P 2, Robert Koch-Institute, Wernigerode, Germany

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
Knut Ohlsen, University of Wuerzburg, Germany
Samuel Wagner, Tübingen University, Germany

****Correspondence:***
Gottfried Wilharm, Project Group P 2, Robert Koch-Institute, Bereich Wernigerode, Burgstr. 37, D-38855 Wernigerode, Germany
e-mail: wilharmg@rki.de

Before the advent of molecular biology methods, studies of pathogens were dominated by analyses of their metabolism. Development of molecular biology techniques then enabled the identification and functional characterisation of the fascinating toolbox of virulence factors. Increasing, genomic and proteomic approaches form the basis for a more systemic view on pathogens' functions in the context of infection. Re-emerging interest in the metabolism of pathogens and hosts further expands our view of infections. There is increasing evidence that virulence functions and metabolism of pathogens are extremely intertwined. Type three secretion systems (T3SSs) are major virulence determinants of many Gram-negative pathogens and it is the objective of this review to illustrate the intertwined relationship between T3SSs and the metabolism of the pathogens deploying them.

Keywords: virulence, metabolism, type three secretion system, T3SS, cross-talk, *Yersinia*, *Salmonella*, *Pseudomonas*

INTRODUCTION

In the introduction to a review on "Metabolism of Microorganisms as related to their pathogenicity" published more than 50 years ago, Panos and Ajl stated, "The problem of how certain aspects of the metabolism of a bacterial cell are related to its pathogenicity is an exceedingly complex and difficult one" (Panos and Ajl, 1963). As will be outlined here, our present view on this subject could hardly be formulated more to the point. Elsewhere in their review, Panos and Ajl state that "This review is not intended to be an exhaustive coverage of the voluminous literature available on this subject," indicating that many forgotten treasures could be raised from that past literature, including important details that newcomers to the field might not know (Panos and Ajl, 1963). Indeed, as will be also illustrated here, many of these early studies can now be linked to more recent ones, mounting increasingly complex regulatory networks in which functions of metabolism and virulence are closely intertwined. Other early studies might serve as stimuli for reinvestigation using state-of-the-art methodology.

It is obvious that a pathogen requires metabolic functions in order to establish an infection and that these metabolic functions adapt to nutritional situation(s) during infection. Consequently, the metabolism of pathogens during infection can differ substantially from that of the *in vitro* situation (Munoz-Elias and McKinney, 2006). It is also known that sensing specific nutrients together with other environmental conditions that are indicative of a host environment stimulates production of virulence factors in many pathogens (Poncet et al., 2009). Further, global regulatory networks such as the stringent response system, which responds to various nutritional and metabolic stresses, or the carbon catabolite repression and carbon storage regulator systems, respectively, play an important role in the control of virulence traits (Romeo, 1998; Gorke and Stulke, 2008; La et al., 2008; Poncet et al., 2009; Dalebroux et al., 2010; Heroven et al., 2012). Though important, these aspects are not the focus of this review, are treated elsewhere and will only be mentioned briefly. The question we address is whether there is anything beyond this global and well-established relationship in the interrelatedness of virulence functions and metabolism in bacterial pathogens.

It will become apparent in this review that the link between virulence and metabolism is so important in many pathogens that we are probably only seeing the tip of the iceberg. Using the example of the type three secretion system (T3SS), a prototypic virulence determinant of many Gram-negatives, we will show in particular that not only metabolism controls and underpins production and functioning of virulence factors but also that, conversely, virulence factors can modulate metabolic functions of pathogens in a coordinate way, as for example, in *Yersinia* (Moncla et al., 1983; Du et al., 2009; Schmid et al., 2009) or pave the way for novel metabolic options as in *Salmonella* (Winter et al., 2010) and *Pseudomonas* (Dacheux et al., 2001b). The following criteria served as indicators to identify similar phenomena in other pathogens: (i) Can it be assumed or is it known that deletion of a T3SS component results in changes of metabolism? (ii) Is there evidence that such metabolic changes are due to a specific interference between T3SS and metabolic functions rather than due to global metabolic effects (e.g., relief from a global metabolic burden due to the loss of a virulence plasmid). (iii) Assuming global effects in general, studies of specific metabolic functions required for full virulence will not been considered unless the metabolic functions can be linked to specific T3SS components (e.g., deletion of a metabolic function specifically interferes with T3SS gene expression). Examples of interrelatedness between T3SS functions and metabolism will be followed by discussion of common principles and their impact on our view of bacterial pathogenesis.

YERSINIA: CROSS-TALK BETWEEN A TYPE THREE SECRETION SYSTEM AND METABOLISM

Studies linking metabolism and virulence in *Yersinia pestis* (originally called *Pasteurella pestis*), the causative agent of plague, go back more than 50 years. After Devignat and Schoetter (1942) reported in 1942 that virulent strains of *Y. pestis* become avirulent upon continued aerobic culturing at 37°C, Fukui et al. (1957) showed that loss of virulence was accompanied by a loss of the so-called V-antigen (Bacon and Burrows, 1956). Only decades later did it become clear that V-antigen (LcrV) was a key component of the plasmid-encoded *Yersinia* type three secretion system (T3SS), a major virulence determinant shared by *Y. pestis* and the enteropathogenic species *Y. pseudotuberculosis* and *Y. enterocolitica*. It was this virulence plasmid, termed pCD in *Y. pestis* and pYV in *Y. pseudotuberculosis* and *Y. enterocolitica*, that was rapidly lost at 37°C *in vitro* (Cornelis et al., 1998). Higuchi and Carlin (Higuchi and Carlin, 1958) found that virulent strains grew faster than avirulent types at 37°C but not at 27°C. A search for media promoting growth of virulent strains of *Y. pestis* revealed a critical role of calcium ions preventing the emergence of avirulent variants (Kupferberg and Higuchi, 1958). Shortly after, Delwiche et al. (1959) noted that bicarbonate, aspartate and glutamate also prevented loss of virulence. How can these early observations be interpreted in the light of the current knowledge and do they point to a specific interrelationship between virulence and metabolism in *Yersinia*? First, production and assembly of the *Yersinia* T3SS machinery occur only at 37°C (Yother et al., 1986), and a low concentration of calcium ions triggers the massive secretion of T3SS substrate proteins called Yops into the culture supernatant (Heesemann et al., 1984). It seemed reasonable to assume that the poor growth of pathogenic *Yersinia* at 37°C under low calcium conditions could be explained by the heavy metabolic burden imposed by the massive production and secretion of Yops (Ramamurthi and Schneewind, 2002). This interpretation was supported by the fact that loss of the virulence plasmid encoding the T3SS relieves from this burden but the effect of bicarbonate, aspartate and glutamate could not be explained.

The fact that bicarbonate prevented the loss of virulence in *Y. pestis* (Delwiche et al., 1959) stimulated Baugh et al. (1964) to compare carbon dioxide fixation reactions in extracts from virulent and avirulent strains. Although Baugh et al. did not observe any significant difference (Baugh et al., 1964), it seems now likely that their hypothesis was correct, since there is a direct linkage between carbon dioxide fixation and T3SS in *Yersinia* (Schmid et al., 2009). Specifically, two regulatory components of the *Y. enterocolitica* T3SS, YscM1 and YscM2, bind directly to the phosphoenolpyruvate carboxylase (PEPC) of *Yersinia*. PEPC catalyses the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate, a replenishing or so-called anaplerotic reaction. The PEPC reaction replenishes the tricarboxylic acid (TCA) cycle that, besides generating reduction equivalents, provides building blocks for amino acid synthesis. More precisely, oxaloacetate is used to form aspartate and other amino acids of the aspartate family, while α-ketoglutarate is used to form glutamate and related amino acids. It is important to remember that aspartate and glutamate both suppress growth restriction in *Y. pestis* (Delwiche et al., 1959), and that activation of the *Yersinia* T3SS requires glutamate, glutamine, aspartate, or asparagine (Lee et al., 2001). Furthermore, alkaline pH, which favors formation of bicarbonate from CO_2, as well as bicarbonate, mitigates growth restriction of pathogenic *Yersinia* (Ogg et al., 1958; Delwiche et al., 1959). So, is the interaction between the T3SS regulators YscM1/YscM2 and the CO_2-fixing PEPC the key to understanding *Yersinia* growth cessation under conditions leading to T3SS activation?

The *Yersinia* virulence plasmid-encoded T3SS comprises about 45 components (Cornelis et al., 1998). The regulatory gene *lcrQ* in *Y. pestis* and *Y. pseudotuberculosis* was presumably duplicated in *Y. enterocolitica* to give *yscM1*, encoding a protein 99% identical to LcrQ, and, *yscM2*, whose product is almost 60% identical to YscM1 and LcrQ. YscM1 and YscM2 are functionally redundant in the control of *yop* gene expression (Stainier et al., 1997). However, while both were shown to interact with PEPC, only YscM1 influenced PEPC activity *in vitro* (Schmid et al., 2009). In fact, YscM1 is a potent inhibitor of PEPC. What is more, YscM1 (LcrQ) and YscM2 are also T3SS secretion substrates and are injected into host cells (Pettersson et al., 1996; Cambronne et al., 2000). Their status as secretion substrates is crucial since induction of type III secretion (accompanied by growth cessation) decreases intracellular YscM/LcrQ levels (Pettersson et al., 1996). In other words, how can inhibition of PEPC activity by YscM1/LcrQ explain growth restriction given that the YscM1/LcrQ level in *Yersinia* is low under these conditions? Perhaps this has to do with the complex protein/protein interaction network in which these regulators are intertwined with at least seven possible interaction partners besides PEPC (Cambronne et al., 2000; Swietnicki et al., 2004; Dittmann et al., 2007; Wilharm et al., 2007; Li et al., 2014a,b). Moreover, there is also evidence that PEPC is not the only junction between the YscM proteins and metabolism in *Yersinia*.

Stable isotope labeling experiments with universally ^{13}C-labeled glucose followed by mass spectrometry analyses of the labeling patterns of amino acids were performed to assess the influence of PEPC, YscM1 and YscM2 on carbon fluxes during Yop secretion (Schmid et al., 2009). The data showed that PEPC replenishes the oxaloacetate pool of the TCA cycle under Yop secretion conditions. Deletion of either *yscM1* or *yscM2* caused slight changes of carbon fluxes in glycolysis and/or the Entner-Doudoroff pathway, the pentose phosphate pathway, the TCA cycle and amino acid biosynthesis. Collectively, these results suggest that not only the PEPC reaction but also one or several other central metabolic reactions are modulated via *yscM1/yscM2*. Strikingly, while purified YscM1 and YscM2 behaved differently with respect to PEPC inhibition *in vitro*, as mentioned above, the *yscM1* and *yscM2* deletion strains were indistinguishable with respect to ^{13}C labeling patterns.

Although it is evident that much has to be done to get a clearer picture of the interrelatedness of virulence and metabolism in *Yersinia*, it is already reasonable to speculate on possible functions of such a cross-talk. *Yersinia* type III secretion is demanding with respect to both synthesis of Yops and provision of energy for their secretion (Wilharm et al., 2007; Schmid et al., 2009). Thus, it seems plausible that functioning of the T3SS under *in vivo* conditions requires the coordinated adaptation

of metabolic pathways. Due to their bifunctionality, YscM/LcrQ proteins are ideally suited to coordinate *yop* gene expression and metabolic requirements. Since YscM/LcrQ interact with multiple T3SS components, and in particular chaperones of the Yop effectors, they might integrate information on the status of the T3SS, e.g., by sensing if the chaperones are charged with Yops, and transduce this information into control of both Yop production and the anaplerotic PEPC reaction, thereby balancing amino acid biosynthesis and energy provision.

A crucial role of the *Yersinia* T3SS is to prevent phagocytosis. The T3SS is therefore fully assembled at 37°C and a presynthesized pool of Yops is "ready-to-go" for microinjection. As the extracellular milieu of the host is rich in calcium, growth restriction will not occur and *Yersinia* is able to replicate. Upon phagocytic attack, a very rapid and concerted reaction is pivotal for *Yersinia*. It seems plausible that maintenance of energy charge for translocation of Yop effectors and replenishment of Yops have to be prioritized over continued replication under these conditions. Sensing an attacking cell might thus trigger a growth cessation programe, accompanied by reprogramming of metabolic pathways in which the PEPC-YscM/LcrQ interaction plays a role.

In support, Meng et al. (2010) demonstrated that phosphoenolpyruvate synthase and glutaminase change phosphorylation/modification status upon induction of the T3SS in *Y. enterocolitica*.

ASPARTASE: A KEY TO UNDERSTANDING *YERSINIA* VIRULENCE

Fully virulent epidemic isolates of *Y. pestis* exhibit a lower aspartase activity than attenuated *Y. pestis* isolates and the enteropathogenic *Y. pseudotuberculosis* and *Y. enterocolitica* (Dreyfus and Brubaker, 1978; Bearden et al., 2009; Bearden and Brubaker, 2010). Aspartase (aspartate ammonia lyase, AspA) catalyses the deamination of aspartate to yield fumarate, an intermediate of the tricarboxylic acid (TCA) cycle. This reaction can thus be regarded as anaplerotic, and its absence from *Y. pestis* results in a net loss of carbon excreted in the form of aspartate (Dreyfus and Brubaker, 1978; Bearden and Brubaker, 2010). Fowler and Brubaker (1994) pointed out a specific role of carbon dioxide fixation reactions in relation to the aspartase deficiency identified in *Y. pestis* (Dreyfus and Brubaker, 1978). Carbon dioxide fixing reactions such as formation of the TCA-cycle intermediate oxaloacetate from phosphoenolpyruvate by the phosphoenolpyruvate carboxylase (PEPC, see previous section) might be especially important to replenish the TCA cycle and to compensate for the aspartase deficiency in *Y. pestis*. The aspartase deficiency might explain why growth restriction associated with low calcium response (LCR) is more prominent in *Y. pestis* compared to the enteropathogenic *Yersinia* (Bearden and Brubaker, 2010). According to Brubaker and colleagues, a key to explaining the exceptional virulence of epidemic *Y. pestis* compared to attenuated enzootic strains and to the enteropathogenic *Yersinia* is to understand how aspartase deficiency increases the virulence of *Y. pestis* (Viola et al., 2008; Bearden et al., 2009; Bearden and Brubaker, 2010). In support of a direct linkage between virulence and aspartase in *Y. pestis*, *aspA* is down-regulated in a mutant strain lacking the T3SS regulator gene *lcrG* (Du et al., 2009).

UPTAKE AND DEGRADATION OF FATTY ACIDS DEPENDS ON THE PRESENCE OF VIRULENCE PLASMIDS IN *Y. PESTIS*

The cross-talk between virulence functions and metabolism in *Y. pestis* is presumably even more complicated. Moncla et al. (1983) demonstrated constitutive uptake and degradation of fatty acids in *Y. pestis* via β–oxidation and the glyoxylate shunt. Intriguingly, fatty acid uptake was found to depend on the T3SS-encoding plasmid pCD1 as well as on the small virulence plasmid encoding the plasminogen activator (Pla) and pesticin (Pst). Unfortunately, these interesting findings were not followed-up. However, transcriptional profiling of wild-type *Y. pestis* and a strongly attenuated mutant lacking *lcrG* indicated up-regulation of *aceA* and *aceB*, encoding the key enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase, in the *lcrG* mutant, which supports the interrelationship between T3SS and fatty acid metabolism (Du et al., 2009). Furthermore, differential expression of *aceA* and *aceB* upon a shift from 26 to 37°C was reported in two studies (Han et al., 2004; Motin et al., 2004), supporting a specific role of fatty acid metabolism during infection.

Another illustration of the complexity of the network is the fact that acetyl-CoA, fatty acids and fatty acyl-CoA are allosteric activators of PEPC, and β–oxidation of fatty acids, which leads to acetyl-CoA, requires oxaloacetate as an acceptor (Morikawa et al., 1980; Sauer and Eikmanns, 2005). Furthermore, L-malate, a product of the glyoxylate shunt reactions, is an allosteric inhibitor of PEPC.

CATABOLITE REPRESSION CONTROLS TYPE III EFFECTOR PRODUCTION IN *YERSINIA*

Zhan et al. (2008, 2009) demonstrated that cyclic AMP receptor protein (CRP) controls expression of the *sycO-ypkA-yopJ* operon in *Y. pestis*. This operon encodes two effectors of the T3SS, YopJ (YopP in *Y. enterocolitica*) and YpkA (YopO in *Y. enterocolitica*), together with SycO, the YpkA chaperone. The cAMP/CRP complex, but not CRP alone, binds to the promoter region of *sycO-ypkA-yopJ*, suggesting that high cAMP levels repress the *sycO-ypkA-yopJ* operon (Zhan et al., 2009). Zhan et al. (2008) defined a minimal CRP regulon by identifying all genes affected by the *crp* deletion. Besides *ypkA* the 37 genes or operons of the minimal CRP regulon include those encoding pesticin (*pst*) and plasminogen activator (*pla*), which are on the small virulence plasmid. Strikingly, expression of aspartase gene *aspA* was also strongly influenced by *crp* (Zhan et al., 2008). While Δ*crp* substantially increased *ypkA* expression, it dramatically lowered expression of *pst*, *pla* and *aspA*, suggesting these inversely regulated factors are required under completely different physiological conditions. These results indicate the effector YpkA (and possibly also YopJ) is only engaged under certain physiological conditions, since other *yop* genes are not part of the CRP regulon (Zhan et al., 2008). It is also worth mentioning that SycO interacts with YscM1 and regulates Yop production (Dittmann et al., 2007). SycO may thus also interfere with the PEPC-YscM1 regulatory network.

Taken together, a densely interwoven regulatory network connects the T3SS along with other virulence factors to the central carbon metabolism in *Yersinia*. The relationships depicted above are graphically summarized in **Figure 1**. It is important to note that very recently a study on the evolution of *Yersinia* identified

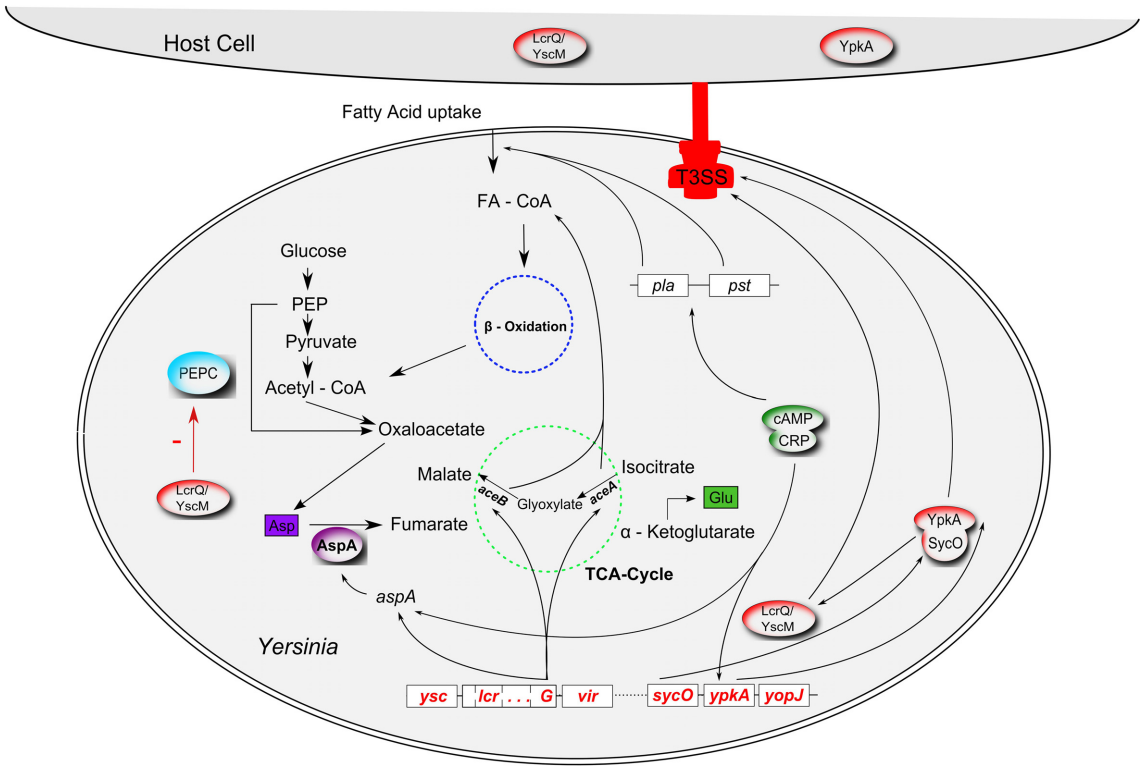

FIGURE 1 | Interrelations between virulence factors and metabolism in *Yersinia*. The type 3 secretion system (T3SS; red), encoded by the pCD virulence plasmid (called pYV in *Y. enterocolitica*) interferes with the anaplerotic enzyme phosphoenolpyruvate carboxylase (PEPC, blue) via the T3SS regulator LcrQ (YscM1 in *Y. enterocolitica*; inhibitor of PEPC); *lcrG*, another component of the low calcium response regulon, controls transcription of *aceAB* and *aspA*. The glyoxylate cycle enzymes isocitrate lyase and malate synthase, encoded by *aceAB*, fulfill an anaplerotic function required for fatty acid degradation (FA, fatty acid; FA-CoA, fatty acyl-CoA). AspA, an aspartase generating fumarate from aspartate can also be regarded as an anaplerotic enzyme. AspA seems to be catalytically inactive in *Y. pestis*. In an unknown way, plasmid pCD seems to be involved in fatty acid uptake together with another virulence plasmid, pPCP. Production of the pPCP-encoded virulence factors Pla (plasminogen activator) and Pst (pesticin) is controlled by cAMP (cyclic adenosine monophosphate) via the cAMP receptor protein CRP ("catabolite repression"). The cAMP/CRP complex represses the T3SS effector gene *ypkA* (*yopO* in *Y. enterocolitica*). SycO, the chaperone of YpkA, directly interacts with LcrQ/YscM1. Cross-talk between fatty acid metabolism, glyoxylate shunt and the PEPC reaction also occurs via allosteric effectors (FA, FA-CoA, malate; interactions not illustrated for reasons of clarity).

that different from the previous view (Carniel, 2002; Wren, 2003) acquisition of the pYV virulence plasmid occurred independently in the *Y. enterocolitica* and *Y. pseudotuberculosis*/*Y. pestis* lineages after their separation (Reuter et al., 2014). Consequently, the regulatory networks in which the T3SSs are embedded can differ for the two lineages as can the specific interrelations between metabolism and the respective T3SS.

PSEUDOMONAS AERUGINOSA T3SS: A HUNTING WEAPON?

The opportunistic pathogen *Pseudomonas aeruginosa* deploys a T3SS with many constituents highly homologous to that of the *Yersinia* T3SS. Their regulatory networks differ considerably, however, because *Pseudomonas* lacks a homolog of the *Yersinia* LcrQ/YscM regulators. Another important difference concerns the use of the respective T3SS. While immune evasion and avoidance of collateral damage seems to be a major issue for *Yersinia*, the situation seems less clear for *P. aeruginosa*. The pore-forming activity of the *P. aeruginosa* T3SS results in macrophage oncosis and release of a chemoattractant from these oncotic cells (Dacheux et al., 2001b). This leads to rapid accumulation of pseudomonads around the cells, a phenomenon called pack swarming. It seems plausible that leakage of the oncotic cells is exploited by the bacteria for their nutrition and, thus, that the *Pseudomonas* T3SS is deployed both for immune evasion and as a weapon to hunt for nutrients.

Several studies have shown that production of the T3SS of *Pseudomonas aeruginosa* is affected by the metabolic state of the cells. The pyruvate dehydrogenase operon *aceAB* (please note the confusing nomenclature, these genes are unrelated to genes with identical names encoding the isocitrate lyase and malate synthase enzymes of the glyoxylate shunt) is required for maximal production of the T3SS (Dacheux et al., 2002). While the growth defect of *aceAB* mutants could be restored by acetate supplementation, the associated defect in T3SS production could be not, suggesting an interaction between *aceAB* gene products and the T3SS signaling cascade, rather than a global metabolic defect restricting expression of the T3SS genes.

Under T3SS-inducing conditions, adenylate cyclases CyaA and CyaB control expression of the T3SS genes via the cAMP binding protein Vfr (Wolfgang et al., 2003; Smith et al., 2004; Rietsch and Mekalanos, 2006), but global catabolite repression control in *Pseudomonas* is not critically dependent on Vfr (Suh et al., 2002),

a close homolog of the cAMP receptor protein (CRP) of *E. coli*. A metabolic signal derived from acetyl-CoA and controlling the *P. aeruginosa* T3SS might also control activity of CyaA and/or CyaB (Rietsch and Mekalanos, 2006).

Also of interest here is the fact that histidine utilization in *P. aeruginosa* interferes with production and translocation of the T3SS cytotoxic effector ExoS (Rietsch et al., 2004), indicated by the observation that a transposon insertion leading to loss of ExoS-dependent cytotoxicity causes over-expression of several histidine utilization (*hut*) genes. Rietsch and colleagues showed that the loss of cytotoxicity could be suppressed by excluding histidine from the medium and by deletion of several *hut* genes. Another suppressing mutation was localized in *cbrA*, encoding the sensor of a two-component system involved in sensing of- and responding to carbon-nitrogen imbalance. Such an imbalance might result from excessive histidine catabolism. Tryptophan catabolism also interferes with type III secretion in *P. aeruginosa* (Shen et al., 2008). This linkage might be explained by the role of tryptophan as a precursor of quorum sensing-like signaling molecules, given that expression of the *P. aeruginosa* T3SS genes is cell density-dependent.

Collectively, several studies suggest that virulence gene expression and the nutritional situation of *P. aeruginosa* are coordinated during infection. It is interesting to note that the broad metabolic adaptation manifested by *P. aeruginosa* associated with long-term colonization of cystic fibrosis (CF) patients is accompanied by a down-regulation of ExoS production (Hogardt et al., 2007; Hoboth et al., 2009). In the light of the interdependence of metabolism and virulence, down-regulation of ExoS in this case might be explained by metabolic adjustments that lead to altered regulation of T3SS gene expression. In line with this explanation, ExoS down-regulation in late CF isolates can be overcome by overproduction of the T3SS transcriptional activator ExsA (Dacheux et al., 2001a; Hoboth et al., 2009). Finally, since *P. aeruginosa* T3SS is apparently designed to provide nutrients to the bacteria, coordination between metabolic states and expression of virulence factor genes is intuitive.

SALMONELLA: T3SS-MEDIATED RECRUITMENT OF NUTRIENTS FOR MY BROTHER

Tetrathionate has long been used as a supplement to media for enrichment of *Salmonella* (Muller, 1923). However, only in recent years has the biological context of tetrathionate usage by *Salmonella* been elucidated in more detail, revealing an intricate relation to T3SS-based virulence. *Salmonella* species causing typhoid fever and gastroenteritis deploy two T3SSs encoded in *Salmonella* pathogenicity islands SPI-1 and SPI-2, here termed T3SS-1 and T3SS-2, respectively. With reference to the link between pathogenicity and metabolism, a sub-population of *Salmonella* Typhimurium seems to use the T3SS-1 altruistically but self-destructive to the nutritional benefit of the surviving subpopulation (Ackermann et al., 2008). During colonization of the mouse gut, only a subpopulation of *S.* Typhimurium expresses the T3SS-1 genes (15% of the salmonellae found in the lumen), which then invades the gut tissue and triggers an inflammatory response. While the invading bacteria seem to be killed, the subpopulation residing in the gut lumen is enabled to compete with the normal gut flora and presumably to acquire the released nutrients. Specifically, Winter et al. (2010) could show that the respiratory burst induced by the T3SS-1 results in formation of the alternative respiratory electron acceptor tetrathionate in the gut. As a consequence, tetrathionate then enables salmonellae to use ethanolamine as a substrate which is abundant and provided by the host yet does not promote growth effectively by fermentation (Thiennimitr et al., 2011) (**Figure 2**). It is interesting to note that the *ttr* genes required for tetrathionate respiration are encoded within SPI-2 in close proximity to T3SS-2 genes (Hensel et al., 1999) although it is hitherto unknown whether there is a linkage between *ttr* genes and T3SS-2 genes beyond that co-localization within SPI-2.

It is worthwhile mentioning here that *Y. enterocolitica* also harbors the tetrathionate pathway suggesting that inflammatory responses are exploited in a similar way (Thomson et al., 2006; Winter et al., 2010).

Another layer of complexity is observable in *Salmonella* Typhimurium strains harboring the SopEΦ prophage. SopE, an effector protein translocated by T3SS-1, can induce increased levels of nitric oxide (NO) within the mouse gut, which as a net result of interaction with reactive oxygen species (ROS) can lead to increased nitrate levels, that is generation of another alternative electron acceptor that is preferred over tetrathionate and promoting growth in the gut lumen (Lopez et al., 2012).

Taken together, the *Salmonella* T3SS-1 is not only deployed to mediate entry into host cells by the manipulation of host cellular signaling but also to unlock novel nutritional resources, suggesting intimate cross-talk between the physiological state and the expression of virulence factor genes. In fact, the central regulator gene of the *Salmonella* T3SS-1, *hilA*, is activated by carbon source deprivation and (p)ppGpp (Pizarro-Cerda and Tedin, 2004; Song et al., 2004). Moreover, *hilA* and many other SPI-1 encoded genes are controlled by metabolic enzyme genes, *adhE*, encoding a bifunctional acetaldehyde-CoA/alcohol dehydrogenase and *pflB*, encoding pyruvate formate lyase I, of the pyruvate metabolism (Abernathy et al., 2013). Furthermore, expression of several virulence factor genes including those of T3SS–1, is controlled by short-chain fatty acids that might serve as nutrients in the intestine (El-Gedaily et al., 1997; Utley et al., 1998; Lawhon et al., 2002). The DNA-binding protein Fis might play a decisive role in coordinating metabolism and virulence in *Salmonella* (Kelly et al., 2004), since it controls expression of genes in several pathogenicity islands on the one hand and of genes encoding metabolic pathway components involved in fatty acid and acetate metabolism including the glyoxylate shunt on the other. The role of the glyoxylate shunt during infection in particular is unclear since it seems to depend on infection stages and differs between *Salmonella* serovars (Utley et al., 1998; Faucher et al., 2006; Tchawa Yimga et al., 2006; Eisenreich et al., 2010; Bowden et al., 2014). In *Salmonella* central metabolic enzymes including key enzymes of glycolysis, gluconeogenesis, TCA cycle and glyoxylate shunt are extensively acetylated to control metabolic fluxes, imposing yet another layer of complexity in understanding regulatory networks (Wang et al., 2010).

Very recently, another striking example of a direct interrelationship between a T3SS and metabolism was discovered in

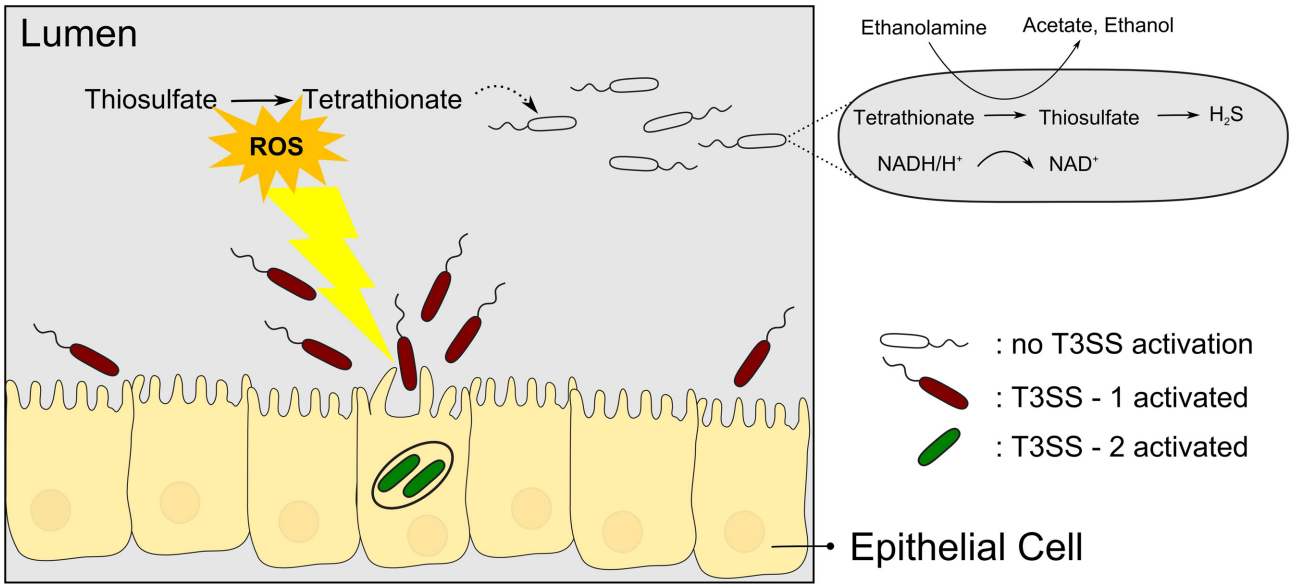

FIGURE 2 | Salmonellae recruit new nutrients for their brothers by means of self-destructive deployment of a T3SS. T3SS-1 mediated invasion of the gut tissue is mostly a kamikaze mission but the inflammatory response induced comprises the release of reactive oxygen species (ROS) which results in the formation of tetrathionate, an alternative electron acceptor. Surviving salmonellae can then use tetrathionate to metabolize ethanolamine to proliferate and outcompete the normal gut flora.

Salmonella (Maze et al., 2014). The phosphotransferase system (PTS) of bacteria catalyses the uptake and concomitant phosphorylation of sugars using phosphoenolpyruvate (PEP) as donor of the phosphoryl group (Deutscher et al., 2006). Now, the PTS component $EIIA^{Glc}$ was shown to associate with the SPI-2 encoded T3SS-2 (Maze et al., 2014). Moreover, effector secretion by the T3SS-2 required $EIIA^{Glc}$ and systemic *Salmonella* virulence was found to critically depend on $EIIA^{Glc}$. Interestingly, however, the phosphorylation-dependent sugar transport function of $EIIA^{Glc}$ and its regulatory impact on adenylate cyclase activity did not account for the phenotype. Rather, a direct interaction between $EIIA^{Glc}$ and T3SS-2 was suggested (Maze et al., 2014). It remains to be determined whether this moonlighting function of $EIIA^{Glc}$ is of purely structural nature or whether it contributes to a coordination of metabolic and virulence functions.

All in all, the literature on *Salmonella* contains numerous arguments of a close interrelation between metabolism and virulence, notably referring to the T3SSs.

FURTHER EXAMPLES

The examples presented and discussed above are systems in which the link between metabolism and T3SS has been most extensively studied. Interested readers will find further examples and hints in studies on the *Aeromonas* T3SS. The regulatory interplay between the pyruvate dehydrogenase complex and T3SS regulator genes *aexT* and *aopN* in *A. hydrophila* reported by Vilches et al. (2009) is strongly reminiscent of the interrelation between *aceAB* and T3SS in *P. aeruginosa* discovered by Dacheux et al. (2002) and discussed above.

In another example, enteropathogenic *E. coli* (EPEC) secrete glyceraldehyde-3-phosphate dehydrogenase (GAPDH) via a T3SS (Kenny and Finlay, 1995). GAPDH might be injected into the host cell cytosol and interfere with host cellular metabolism e.g., by inhibiting host cellular GAPDH complexes through formation of unproductive heterooligomers or by increasing fluxes within the glycolytic pathway. Alternatively, GAPDH has a moonlighting function independent of its enzymatic activity, fulfilling in bacteria and/or the host. For example, GAPDH is also secreted by different *Streptococcus* species, and exhibits ADP-ribosylating activity and immunomodulatory properties (Pancholi and Fischetti, 1992, 1993; Madureira et al., 2007). It is interesting to note in this context, that a T3SS effector, NleB, found in EPEC and EHEC (enterohaemorrhagic *E. coli*), was recently found to act as glycosyltransferase toward host cellular GAPDH to inhibit NF-κB activation, illustrating yet another moonlighting facet of GAPDH (Gao et al., 2013). It remains to be determined whether it is just by chance that EPEC secrete and possibly translocate GAPDH into host cells in which they also target host cellular GAPDH and whether these phenomena are first indications of an interlock of metabolism and T3SS on both the bacterial and the host cellular side.

Finally, AdhE, a bifunctional acetaldehyde-CoA dehydrogenase/alcohol dehydrogenase was shown to control expression of the EHEC T3SS (Beckham et al., 2014), a finding reminiscent of the relation between *adhE* and SPI-1 found in *Salmonella* (Baumler et al., 1994; Abernathy et al., 2013).

CONCLUSIONS AND PERSPECTIVES

A NOVEL INTERRELATIONSHIP BETWEEN VIRULENCE FUNCTIONS AND METABOLISM

In very general terms, the environment within the host determines the physiological state of the pathogen, which in turn

Table 1 | Summary of the metabolic pathways impacted by T3SSs and/or vice versa in the order of appearance in this manuscript.

Species	T3SS component	Gene/Pathway	References
Yersinia	LcrQ/YscM	*ppc*/PEP carboxylase	Schmid et al., 2009
	lcrG	*aspA*/aspartase	Du et al., 2009
	pCD1	β–oxidation, glyoxylate shunt	Moncla et al., 1983
	lcrG	*aceA, aceB*/glyoxylate shunt	Du et al., 2009
	sycO-ypkA-yopJ	cAMP-CRP	Zhan et al., 2009
Pseudomonas	T3SS	*aceAB*/pyruvate dehydrogenase	Dacheux et al., 2002
	T3SS	*cyaA,cyaB*/cAMP-Vfr	Wolfgang et al., 2003
	ExoS	*hut*/histidine utilization	Rietsch et al., 2004
	T3SS	*kynA*/tryptophan metabolism	Shen et al., 2008
Salmonella	T3SS-1 (SPI-1) and SPI-2	*ttr*/tetrathionate respiration	Hensel et al., 1999; Winter et al., 2010
	SopE (T3SS-1)	NO, nitrate respiration	Lopez et al., 2012
	hilA (SPI-1)	(p)ppGpp	Pizarro-Cerda and Tedin, 2004; Song et al., 2004
	T3SS-1 (SPI-1)	*adhE, pflB*/pyruvate metabolism	Abernathy et al., 2013
	T3SS-1 (SPI-1)	Short-chain fatty acids	El-Gedaily et al., 1997
	T3SS-2 (SPI-2)	*crr*/PTS component $EIIA^{Glc}$	Maze et al., 2014
Aeromonas	*aexT, aopN*	*aceA*/pyruvate dehydrogenase	Vilches et al., 2009
E. coli (EPEC)	T3SS	Secretion of glycolytic enzyme GAPDH	Kenny and Finlay, 1995
E. coli (EHEC)	T3SS	*adhE*/pyruvate metabolism	Beckham et al., 2014

governs production and functioning of virulence factors. Our knowledge of the complexity of these regulatory networks is further increasing (as illustrated above; see **Table 1** for summary). Our way of looking at bacterial pathogenesis is still dominated by the question of which virulence factors are produced and how metabolism controls virulence gene expression. However, virulence factors conversely control metabolic functions in a coordinated manner. Furthermore, there is evidence that virulence functions are deployed to manipulate host metabolism, potentially for the benefit of the metabolism in the pathogen. Clearly, we face a novel interrelationship between virulence and metabolic functions. However, do such phenomena reflect the exception or the rule?

The multiple convergent evolution of dense regulatory networks to coordinate virulence and metabolic functions suggests strong selection pressures acting and indicates that such cross-talk could be the rule rather than the exception. From a cybernetic point of view, many regulator circuits seem plausible.

CROSS-TALK VIA ANAPLEROTIC FUNCTIONS: A COMMON THEME

From the point of view of the invader, infection usually means unbalanced diet and shortage. From *in vitro* studies it is known that growing on a limited number of carbon sources requires considerable reprogramming of metabolic fluxes via anaplerotic enzymes. From the above examples, it seems as if such anaplerotic enzymes are preferred nodal points for communication with virulence functions, lending support to the view that "unbalanced diet" is a condition frequently faced by pathogens.

Agrobacterium tumefaciens induces crown gall tumors on its plant hosts by means of a type 4 secretion system (T4SS). The virulence (*vir*) genes are localized on the Ti (tumor-inducing) virulence plasmid and are controlled by the two-component system VirAG. Deletion of *pckA* encoding the anaplerotic enzyme phosphoenolpyruvate carboxykinase (PckA) attenuates virulence through reduced *vir* gene expression (Liu et al., 2005) illustrating that such regulatory circuits are not restricted to pathogens running T3SSs.

This view is further underscored by the fact that anaplerotic reactions play a key role during infection in many pathogens with less well-defined armament. A crucial function of the glyoxylate shunt during infection, for example, has been demonstrated for *Mycobacterium tuberculosis* (McKinney et al., 2000) and for the fungal pathogen *Candida albicans* (Lorenz and Fink, 2001).

METABOLISM OF THE HOST

For reasons of clarity and scope, this review was confined mainly to the central carbon metabolism of bacterial pathogens and its interplay with virulence functions related to T3SSs. Availability of all kind of nutrients (not only of carbon), however, depends on host metabolism, which differs depending on localization within tissues and cells and, moreover, is not static but changes over the course of infection. As an example, sepsis is accompanied by a massive redistribution of carbon and nitrogen fluxes within the infected individual, accompanied by decreased glutamine uptake in the intestine and a switch from glutamine uptake state to release in the kidney. In contrast, proliferating lymphocytes increase glutamine utilization upon sepsis and the liver becomes the major glutamine consumer under these conditions (Karinch et al., 2001). Consequently, many pathogens will have to adapt their metabolism to rearrangements of the host metabolism.

Changes of host metabolism in the context of infection can be viewed as a host response or as directed manipulation of

host metabolism by the pathogen. As discussed above, there are hints that virulence factors are deployed to manipulate host metabolism and to serve the release of nutrients. The function of virulence factors is often discussed in a biased way, neglecting that the primary goal of the pathogen is not to fool the host but to replicate in it. We expect that manipulating host metabolism, on the single host cell level as well as on the tissue, organ and systemic level will turn out to be a major issue of pathogens.

SYSTEMATIC INVESTIGATION OF CROSS-TALK BETWEEN VIRULENCE AND METABOLISM: TOWARD A SYSTEMIC VIEW OF INFECTION

Much of the above discussion is based on incidental observations. So, how should we examine cross-talk between metabolism and virulence functions more systematically? As a first step, literature from the pioneers studying pathogens may still store many valuable hints to follow-up. Further, it is important to screen the transcriptomics, proteomics and metabolomics datasets to identify novel interrelationships. Clearly, a comprehensive view will require other "omics" such as "interactomics" and "fluxosomics," including data on the host. Integration of these data to form a penetrable view of infection will be a major intellectual challenge. Feeding systems biologists with "omics" data is a must to drive modeling toward a systemic view of infection. It is however clear that applicability of modern systems biology approaches is still limited from an economic and data handling perspective but also with regard to sensitivity as well as temporal and spatial resolution. For example, bacterial pathogens practice division of labor as do cells of their hosts so that the different events on which we sum up in most experiments cannot be resolved and the results misguide us. Moreover, type three secretion is not only a very complex but also a highly dynamic process which poses a specific challenge to the temporal resolution of our methodology. Single cell analysis is now rapidly developing on the "omics" level and at least DNA and RNA sequencing will become broadly available (Pan, 2014) to resolve some of the heterogeneity that we are faced with.

In conclusion, cohesive use of physiology and molecular biology is needed to enlighten the complex events of infection: we cannot understand infection without understanding of metabolism of host and the pathogen.

ACKNOWLEDGMENTS

Work in the authors' lab is supported by the DFG priority programme 1316 "Host-adapted metabolism of bacterial pathogens."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 July 2014; paper pending published: 31 August 2014; accepted: 07 October 2014; published online: 27 October 2014.

*Citation: Wilharm G and Heider C (2014) Interrelationship between type three secretion system and metabolism in pathogenic bacteria. Front. Cell. Infect. Microbiol. **4**:150. doi: 10.3389/fcimb.2014.00150*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 15 July 2014
doi: 10.3389/fcimb.2014.00091

Small RNA functions in carbon metabolism and virulence of enteric pathogens

Kai Papenfort*[1] *and* ***Jörg Vogel*[2]**

[1] *Department of Molecular Biology, Princeton University, Princeton, NJ, USA*
[2] *RNA Biology Group, Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Germany*

Edited by:
Thomas Dandekar, University of Würzburg, Germany

Reviewed by:
Michael Shapira, University of California, Berkeley, USA
Petra Dersch, Helmholtz Center for Infection Research, Germany

****Correspondence:***
Kai Papenfort, Department of Molecular Biology, Princeton University, Washington Road, Princeton, NJ 08544, USA
e-mail: kaip@princeton.edu

Enteric pathogens often cycle between virulent and saprophytic lifestyles. To endure these frequent changes in nutrient availability and composition bacteria possess an arsenal of regulatory and metabolic genes allowing rapid adaptation and high flexibility. While numerous proteins have been characterized with regard to metabolic control in pathogenic bacteria, small non-coding RNAs have emerged as additional regulators of metabolism. Recent advances in sequencing technology have vastly increased the number of candidate regulatory RNAs and several of them have been found to act at the interface of bacterial metabolism and virulence factor expression. Importantly, studying these riboregulators has not only provided insight into their metabolic control functions but also revealed new mechanisms of post-transcriptional gene control. This review will focus on the recent advances in this area of host-microbe interaction and discuss how regulatory small RNAs may help coordinate metabolism and virulence of enteric pathogens.

Keywords: sRNA, carbon metabolism, Hfq, CsrA, virulence

INTRODUCTION

Bacteria colonize almost every niche on earth. Accordingly, they have developed complex regulatory systems to respond to their environment. In particular, the right choice of nutrients is crucial to thrive in conditions of stress or competition. Pathogenic bacteria are no different in this respect. At the very heart of most infections, the host presents an exquisite source of nutrients for the pathogen. However, the immune response of the host can create a hostile environment demanding precise coordination of stress-related and metabolic genes.

Transcription factors have long been known to link metabolic pathways and virulence gene expression. The highly conserved cAMP receptor protein (CRP) transcription factor, for example, coordinates the uptake and utilization of alternative carbon sources in a process termed carbon catabolite repression (CCR) (Gorke and Stulke, 2008). Mutations in CCR components often have drastic consequences for virulence gene expression (Poncet et al., 2009) and loss of CRP activity, either by mutation or low intracellular cAMP levels, strongly reduces the virulence of *Salmonella enterica* (Curtiss and Kelly, 1987; Teplitski et al., 2006), *Vibrio cholerae* (Skorupski and Taylor, 1997), and *Yersinia* species (Petersen and Young, 2002; Kim et al., 2007).

Besides protein-dependent transcriptional control, RNA-controlled mechanisms have turned out to play important roles in regulating virulence genes (Papenfort and Vogel, 2010). Regulatory RNAs operate at all layers of gene expression, ranging from transcription initiation to translation control and protein activity (Waters and Storz, 2009). The majority of the regulatory RNAs characterized to date act by base-pairing with target mRNAs and are commonly referred to as small regulatory RNAs (sRNAs). This group can be further divided into sRNAs encoded on the opposite strand of the regulated RNA (*cis*-encoded) and those that are transcribed distantly from their targets (*trans*-encoded). These sRNAs have been documented to regulate numerous important processes in bacterial pathogens including outer membrane homeostasis (Papenfort et al., 2006, 2010; Song et al., 2008; Corcoran et al., 2012; Fröhlich et al., 2012), quorum sensing (Lenz et al., 2004; Shao et al., 2013), iron homeostasis (Murphy and Payne, 2007), biofilm formation (Monteiro et al., 2012; Zhao et al., 2013), host-cell contact (Heroven et al., 2008; Sterzenbach et al., 2013; Gruber and Sperandio, 2014), and amino-acid metabolism (Sharma et al., 2011).

Other classes of riboregulators are riboswitches (Serganov and Nudler, 2013) or RNA thermometers (Kortmann and Narberhaus, 2012). Both describe RNA elements typically found in the 5′ UTR (untranslated region) of mRNAs regulating gene expression via structural rearrangements of the RNA. Whereas riboswitches respond to varying availability of metabolites or metals in the cell, RNA thermometers function by sensing changes in temperature. Riboswitches may also produce small RNAs (Vogel et al., 2003) and act as *trans*-acting regulators on mRNAs (Loh et al., 2009). For many pathogenic bacteria, host body temperature is a central signal activating virulence gene expression. RNA thermometers have been shown to contribute to this regulation in enteric bacteria such as *Yersinia pseudotuberculosis* and *Listeria monocytogenes* (Johansson et al., 2002; Bohme et al., 2012), as well as the non-enteric human pathogen *Neisseria meningitidis* (Loh et al., 2013).

Due to the relatively small size of their genes or simply because of incomplete genome annotations riboregulators were often overlooked in traditional genetic screens for virulence determinants. In addition, the fact that most regulatory RNAs may act to

fine-tune processes and so give milder phenotypes when mutated than regulatory proteins has also disfavored their identification in virulence screens. However, the recent advent of next-generation sequencing (NGS) techniques has begun to remedy some of these limitations: NGS can provide global maps of RNA expression at nucleotide resolution for any bacterial pathogen of interest, and some of the newly identified sRNAs have already been documented to contribute to microbial virulence (Caldelari et al., 2013).

Evidence for regulatory RNAs being important for the control of virulence and metabolism has also come from the loss-of-function phenotypes of two proteins, Hfq (a.k.a. HF-I protein) and CsrA (carbon storage regulator A). The RNA chaperone, Hfq, is required for virulence in diverse bacterial pathogens and *hfq* mutants usually display pleiotropic defects such as reduced growth rates, altered metabolic profiles and changes in virulence gene expression (Chao and Vogel, 2010; Sobrero and Valverde, 2012). At the mechanistic level, Hfq is known to serve as a "molecular matchmaker" by facilitating base-pairing of sRNAs and target mRNAs but it also protect sRNAs from degradation by cellular ribonucleases (Vogel and Luisi, 2011). In the laboratory, Hfq has proven as a useful tool to precipitate bona-fide sRNAs (Chao et al., 2012 and references therein) and therefore frequently served as starting point for the functional characterization of sRNA regulators.

Likewise, the RNA-binding protein CsrA (a.k.a. RsmA in some organisms) is required for virulence of many pathogens (Lucchetti-Miganeh et al., 2008). Originally described as a pleiotropic regulator of glycogen biosynthesis in *Escherichia coli* (Romeo et al., 1993), CsrA homologs have now been annotated in more than 1500 bacterial species (Finn et al., 2014). Binding of CsrA occurs at GGA-rich elements in the mRNA and commonly results in reduced ribosome association and subsequent mRNA decay (Romeo et al., 2013), though CsrA-mediated gene activation has also been reported (Yakhnin et al., 2013). The key regulators of CsrA activity are CsrB-like sRNAs which act as decoys of the protein. These sRNAs, of which many bacteria encode more than one copy, carry multiple high-affinity sites containing the GGA motif and thereby titrate CsrA away from its target mRNAs (Babitzke and Romeo, 2007).

Recent global studies of other gastrointinal pathogens such as *Helicobacter pylori* (Sharma et al., 2010), *Campylobacter jejunii* (Dugar et al., 2013), and *Clostridium difficile* (Soutourina et al., 2013) have suggested a wealth of potential RNA regulators in these organisms, but if and how these are involved in metabolic processes and infection is mostly unclear. Therefore, in this review we concentrated on the functions of established sRNAs in carbon metabolism and virulence of enteric pathogens and, where applicable, outlined the underlying mechanisms of regulation.

GLUCOSE HOMEOSTASIS THROUGH SgrS

The facultative intracellular pathogen *S. enterica* serovar Typhimurium is probably one of the best understood bacteria when it comes to metabolic profiling during infection (Dandekar et al., 2012). Transcriptome analyses of intracellular *Salmonella* suggested a preference for glucose, glucose-6-phosphate (G-6-P), and gluconate as primary carbon sources during infection (Hautefort et al., 2008); the preference for glucose (though not G-6-P) during intracellular growth was also supported by isotopologue profiling experiments (Gotz et al., 2010). In agreement with these observed preferences, glucose and glycolysis are essential for the virulence of *Salmonella* (Bowden et al., 2009).

Glucose uptake and catabolism are strictly controlled, and *Salmonella* shares many of the underlying regulatory mechanisms with its close relative, *E. coli*. The transport of glucose across the bacterial membrane is achieved by so-called phosphotransferase systems (PTS) (Jahreis et al., 2008). Gram-negative model bacteria encode a plethora of PTS with varying substrate specificities (Deutscher et al., 2006). For glucose, the translocation process generates G-6-P (**Figure 1**) which, once in the cytosol, can enter several metabolic pathways including glycolysis or the pentose-phosphate pathway.

Phosphosugars such as G-6-P are a double-edged sword, though. On the one hand, they serve as a primary energy source for generating ATP and NADH via glycolysis. On the other hand, high levels of phosphorylated sugars can impair growth (Irani and Maitra, 1977; Kadner et al., 1992) and may cause DNA damage (Lee and Cerami, 1987). Importantly, many non-metabolizable carbohydrates are invariably imported and phosphorylated by Crr and PtsG, the major proteins for glucose uptake in *E. coli* and *Salmonella*. The accumulation of intracellular G-6-P or other phosphorylated sugars is often referred to as phosphosugar stress and has been observed in many Gram negative bacteria (Bobrovskyy and Vanderpool, 2013). Not surprisingly, intracellular glucose levels are strictly controlled and glucose homeostasis is subject to complex transcriptional and post-transcriptional control. Six transcriptional regulators, including the two alternative sigma-factors σ^S and σ^H, control the *ptsG* gene in *E. coli* (Jahreis et al., 2008). Furthermore, the *ptsG* mRNA is destabilized in response to high intracellular G-6-P levels (Kimata et al., 2001), an effect which could be attributed to the activity of a phosphosugar stress-induced sRNA, SgrS (Vanderpool and Gottesman, 2004). Upon activation by the SgrR transcriptional regulator (Vanderpool and Gottesman, 2004, 2007), SgrS base-pairs with the ribosome binding site (RBS) of the *ptsG* mRNA to inhibit translation initiation. Thereby SgrS reduces *de novo* production of PtsG protein and limits glucose import and intracellular G-6-P levels (Vanderpool and Gottesman, 2004) (**Figures 1**, **2**).

SgrS has many characteristics of an Hfq-dependent sRNA: it co-immunoprecipitates with Hfq (Zhang et al., 2003) and mutation of the *hfq* gene impairs the intracellular stability of SgrS and its ability to repress the *ptsG* mRNA (Kawamoto et al., 2006). Recent work showed that Hfq binds at the Rho-independent transcriptional terminator hairpin at the 3′ end of SgrS (Otaka et al., 2011; Ishikawa et al., 2012). SgrS has also been a model sRNA in establishing general mechanisms of sRNA activity in bacteria. For example, the Aiba group showed that successful repression of *ptsG* by SgrS required a very short seed pairing, involving as few as six essential base-pairs (Kawamoto et al., 2006; Maki et al., 2010); that regulation may occur at the inner membrane (Kawamoto et al., 2005); and crucially involves RNase E (Morita et al., 2005). Interestingly, although SgrS induces *ptsG* mRNA decay (Morita et al., 2005), RNA duplex-formation alone suffices for translational repression (Morita et al., 2006).

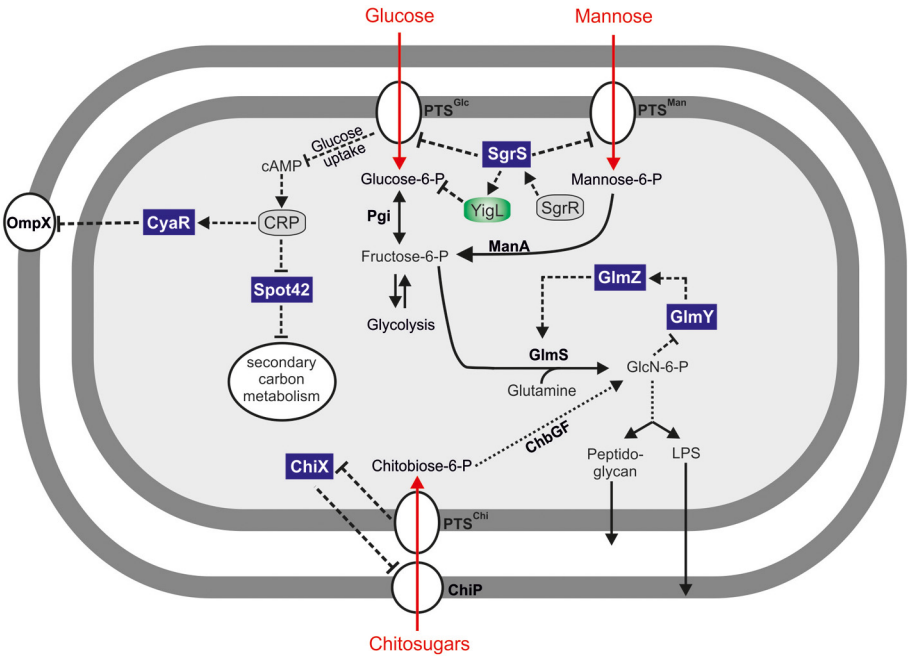

FIGURE 1 | Hfq-dependent sRNAs regulating carbon metabolism. Small RNA controlled carbohydrate metabolic pathways for uptake and metabolism of glucose, mannose, and chitosugars. Spot 42 sRNA acts as a global regulator of secondary carbon metabolism. SgrS sRNA controls uptake and secretion of various carbohydrates. The GlmYZ sRNAs control the expression of glucosamine-6-phosphate synthetase (GlmS) in response to its product, GlcN-6-P. Enzymes and transporters are depicted in bold and the transcriptional regulators SgrR and CRP are shown in gray.

Additional mRNA targets of SgrS have been identified (Papenfort et al., 2012). The *manXYZ* transcript encodes a mannose-specific uptake system and is also repressed by SgrS (**Figures 1**, **2**; Rice and Vanderpool, 2011). However, different from the *ptsG* mRNA, the *manXYZ* transcript contains two functional SgrS binding sites (Rice et al., 2012).

In *Salmonella*, SgrS also represses the *sopD* mRNA (**Figure 2**) (Papenfort et al., 2012) which encodes a *Salmonella*-specific effector protein that is injected into host cells (Brumell et al., 2003). In the host, SopD contributes to phagosome formation (Bakowski et al., 2007) and fluid secretion (Jones et al., 1998). Intriguingly, repression of the *sopD* mRNA requires base-pairing of SgrS to the RBS with the same conserved seed sequence that targets *ptsG* and *manXYZ* (Kawamoto et al., 2006; Papenfort et al., 2012; Rice et al., 2012). Thus, the SgrS seed sequence underlying the regulation of sugar transport mRNAs has been recruited to control the mRNA of a horizontally acquired virulence gene.

The study of this non-sugar stress related *sopD* target has revealed the exceptional fidelity by which SgrS recognizes mRNAs. That is, many *Salmonella* isolates carry a duplication of the *sopD* gene termed *sopD2* (Brumell et al., 2003); the SopD2 protein is also secreted into host cells and is required for virulence in mice (Jiang et al., 2004). However, despite extensive sequence homology between the two virulence factor mRNAs, the *sopD2* mRNA is not regulated by SgrS. Genetic and biochemical analyses of the underlying mechanism revealed that a single-nucleotide variation between the two mRNA sequences is sufficient to prevent SgrS from targeting *sopD2*. This single nucleotide difference renders a stable G-C pair in the productive SgrS-*sopD* interaction into a silent G-U pair which prevents SgrS from regulating *sopD2*. Although the G-U pair is predicted to make only a minor difference in RNA-duplex stability, its crucial location at the proximal end of the RNA seed interaction prevents *sopD2* from becoming an SgrS target (Papenfort et al., 2012). In other words, a single hydrogen bond (G-C vs. G-U pair) determines which of these two virulence factor mRNAs is regulated by SgrS.

The most recent addition to the list of SgrS target genes is the *yigL* mRNA (Papenfort et al., 2013) (**Figure 1**). Different from the hitherto known negative regulations, SgrS activates the synthesis of YigL protein (**Figure 2**). Importantly, the *yigL* gene is expressed as part of a di-cistronic *pldB-yigL* mRNA but the activation by SgrS is restricted to the *yigL* part of the transcript. The underlying activation mechanism involves base-pairing of SgrS to a processed monocistronic *yigL* mRNA species in which SgrS sequesters a RNase E cleavage site. This site-specific inhibition of RNase E-mediated decay increases transcript stability and YigL protein synthesis (Papenfort et al., 2013). This novel mode of post-transcriptional activation complemented previously observed mechanisms of positive regulation (Fröhlich and Vogel, 2009) and was subsequently reencountered in the activation of *cfa* mRNA by RydC sRNA (Fröhlich et al., 2013).

The SgrS-mediated activation of *yigL* plays an important biological role during glucose-phosphate stress. Since it occurs within minutes, it can be considered to be part of an immediate stress response program (Papenfort et al., 2013). The *yigL*

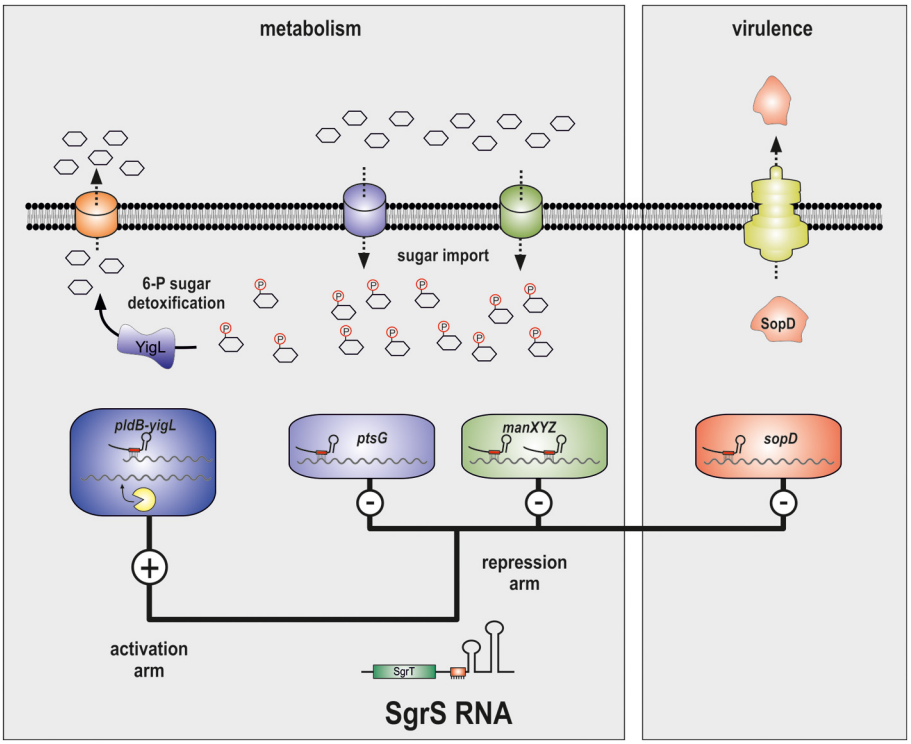

FIGURE 2 | SgrS controls carbon metabolism and virulence factor production. The SgrS sRNA regulates the *ptsG* and *manXYZ, yigL* and *sopD* mRNAs via direct base-pairing with the respective transcripts. Activation of *yigL* requires inhibition of endonucleolytic degradation through sequestration of a RNase E cleavage site. The *ptsG* and *manXYZ* mRNAs encode carbohydrate transporters for glucose and mannose, respectively. The *yigL* gene encodes a potent phosphatase which removes phosphate residues from intracellular carbohydrates which allows export. The *sopD* gene is specific to *Salmonella* and its translation results in a secreted virulence factor that enters the mammalian host cell.

gene encodes a potent phosphatase which catalyzes the removal of phosphate residues from intracellular carbohydrates (Kuznetsova et al., 2006; Papenfort et al., 2013). Since the negative charge of the phosphate normally prevents the toxic carbohydrates from crossing the bacterial membrane, the dephosphorylation by YigL enables efficient export and detoxification (Papenfort et al., 2013; Sun and Vanderpool, 2013). Whether the RNA-based activation of *yigL* is important for *Salmonella* infection remains to be seen. However, we note that the *yigL* gene is required for pathogenicity of the insect pathogen *Xenorhabdus nematophila* (Richards et al., 2009).

Another relevant element for instant stress relief is the SgrT peptide. In contrast to most other Hfq-binding sRNAs, SgrS does not strictly act as a non-coding regulator. The proximal part of the molecule encodes the ~40aa SgrT peptide which can inhibit carbohydrate import, likely by blocking the glucose channel (Wadler and Vanderpool, 2007). SgrT is not required for the regulation of target mRNAs (Balasubramanian and Vanderpool, 2013) and not necessarily conserved in *sgrS* homologs of other species (Horler and Vanderpool, 2009).

In order to fully understand the function of SgrS in metabolism and virulence, it will be important to identify the cause of glucose-phosphate stress and the molecule(s) involved in SgrS induction in pathogenic organisms. Suppressor studies in non-pathogenic *E. coli* have suggested a connection of phosphate metabolism and glucose-phosphate stress (Richards and Vanderpool, 2012) and experiments from Aiba and Vanderpool groups indicated that G-6-P itself is not causing toxicity. Rather, the depletion of glycolytic intermediates induces growth arrest (Morita et al., 2003; Richards et al., 2013) but how this ties in with virulence factor control such as the observed repression of SopD synthesis in *Salmonella* remains to be understood.

Along the same line, robust virulence-related phenotypes of *sgrS* mutants are yet to be identified in *Salmonella* (Santiviago et al., 2009; Papenfort et al., 2012). Given the massive competition for glucose by other microbes in the intestine it is likely that SgrS-mediated gene regulation is most relevant when *Salmonella* has entered the host cell. Here, glucose is plentiful and serves as the primary carbon source for intracellular replication (Dandekar et al., 2012). When *Salmonella* disseminates systemically into the liver and spleen it continues to grow within macrophage where glycolysis and glucose metabolism remain highly relevant (Bowden et al., 2009). Therefore, regulation of glycolytic flux and virulence factor production by SgrS could be important under these conditions. In this context it is interesting to note that in *V. cholerae* a related sRNA, TarA, is required for infant mouse colonization by this pathogen. Similar to SgrS, TarA represses the production of PtsG; different from SgrS, though, the expression

of TarA is directly controlled by a major virulence transcription factor, ToxT (Richard et al., 2010).

CRP-CONTROLLED sRNAs

Spot 42 (encoded by the *spf* gene) was one of the first bacterial riboregulators identified (Ikemura and Dahlberg, 1973) and it is also one of the most conserved sRNAs (Hansen et al., 2012). Transcription of Spot 42 is repressed by cAMP-bound CRP (Polayes et al., 1988) and the over-expression of this sRNA reduces growth on various carbon sources (Rice and Dahlberg, 1982; Beisel and Storz, 2011). Direct targets of Spot 42 remained elusive until in 2002, when the Valentin-Hansen lab discovered that Spot 42 associated with Hfq (Moller et al., 2002a) and regulated galactose metabolism in *E. coli* (Moller et al., 2002b). Specifically, they showed that Spot 42 targets the distal part of the *galETKM* operon mRNA by base-pairing to the RBS of the *galK* cistron, demonstrating for the first time that sRNAs can post-transcriptionally modulate specific genes within multi-cistronic transcripts. Such discoordinate operon expression, resulting in selective repression or activation of internal cistrons, has recently been described for other sRNAs, too (Balasubramanian and Vanderpool, 2013; Papenfort et al., 2013).

Searches for additional Spot 42 target mRNAs have since revealed a more global role for Spot 42 during glucose catabolism (Beisel and Storz, 2011; Beisel et al., 2012). Nineteen more repressed transcripts were discovered, most of which have documented functions in the transport and metabolism of secondary carbon sources. Intriguingly, many of them are regulated by CRP at the transcriptional level, suggesting that CRP and Spot 42 form a complex feed-forward loop which reinforces CCR. Here, Spot 42 directly inhibits the translation of mRNAs involved in the utilization of secondary carbon sources, the same genes which are also regulated by CRP at the transcriptional level (Beisel and Storz, 2011; Papenfort and Vogel, 2011). Since many target interactions of Spot 42 seem conserved in various enteric pathogens (Wright et al., 2013), Spot 42 may be relevant as carbon source composition change rapidly in the course of an infection process.

CyaR is another CRP-controlled sRNA which binds Hfq and is highly conserved among the enterobacteria (Zhang et al., 2003). In contrast to Spot 42, which is repressed by CRP, CyaR is activated by the CRP-cAMP complex. One conserved target of CyaR is the *ompX* mRNA which encodes a major outer membrane protein of *Salmonella* and *E. coli* (Johansen et al., 2008; Papenfort et al., 2008; De Lay and Gottesman, 2009). Additional targets of CyaR include the transcripts of *yqaE*, *nadE*, and *luxS* (De Lay and Gottesman, 2009) as well as *ptsI*, *yobF*, and *sdhA* (Wright et al., 2013), in other words, transcripts of genes that relate directly or indirectly to metabolic functions. For example, the *luxS* gene is required for the production of the common autoinducer AI-2 and repression by CyaR suggests a link between carbon metabolism and population behavior (De Lay and Gottesman, 2009). Recent studies revealed the expression of several CRP-dependent sRNAs (including CyaR) in *Yersinia pestis* -infected lungs, suggesting a potential role for carbon metabolism and sRNAs in pathogenicity (Koo et al., 2011; Yan et al., 2013). Indeed, in *Y. pestis* Crp expression itself depends on the Hfq chaperone which is revelant for the development of pneumonic plague (Lathem et al., 2014).

CHITIN UTILIZATION THROUGH sRNAs

Chitin is a solid polymer made of N-acetylglucosamine (GlcNAc) and one of the most abundant biomaterials on Earth. Thanks to its inert structure chitin requires specialized enzymes, termed chitinases, to be utilized (Bhattacharya et al., 2007). Interaction with chitin can be important during multi-species biofilm formation with fungal partners and may also affect the virulence of individual bacterial pathogens (Brandl et al., 2011 and references therein). Ecologically, chitinases play an important role in the lifestyle of many marine bacteria, e.g. *V. cholerae* (Meibom et al., 2004) where GlcNAc induces the expression of the competence-regulating TfoR sRNA (Yamamoto et al., 2011). Further, chitinases are also encoded by non-marine enteropathogens such as *Salmonella* (McClelland et al., 2001).

In *E. coli* and *Salmonella* chitin utilization is regulated by a complex mechanism involving the sRNA ChiX (a.k.a RybC, MicM, or SroB) and a decoy mRNA transcript (Mandin and Gottesman, 2009). In the absence of chitosugars, ChiX sRNA continuously binds to and represses the *chiP* mRNA which encodes a chitoporin required for the uptake of chitooligosaccharides (Rasmussen et al., 2009) (**Figure 1**). Genetic screens for relief of *chiP* repression by ChiX hinted at another layer of post-transcriptional control (Figueroa-Bossi et al., 2009; Overgaard et al., 2009). Here, expression of the *chb* operon (encoding genes for chitosugar utilization) is induced in the presence of chitobiose via the ChbR transcriptional regulator (Plumbridge and Pellegrini, 2004). Through a base-pairing interaction, the *chb* mRNA titrates the ChiX sRNA, inducing a rapid degradation of this repressor. This decoy function of *chb* indirectly increases the synthesis of the ChiP porin, adjusting its levels to the availability of the enzymes for chitosugar processing (Figueroa-Bossi et al., 2009; Overgaard et al., 2009). In addition, when chitosugar concentrations are low ChiX activity is accompanied by transcriptional repression of the *chiP* and *chb* genes by NagC. However, when chitosugars enter the cell repression by NagC is alleviated and *chb* can act as a decoy for ChiX (Plumbridge et al., 2014).

Chitin utilization is also important in the Gram positive bacterium, *L. monocytogenes*. Recent studies suggested that the chitinolytic activity of this pathogen could have important functions during immune evasion; in addition, mutations in the chitinase-encoding gene *chiA* reduced virulence (Chaudhuri et al., 2013b). Interestingly, expression of the *chiA/B* genes is controlled by the master virulence regulator PrfA (Larsen et al., 2010), but the levels of the *chiA* mRNA are additionally controlled by the Hfq-dependent LhrA sRNA (Nielsen et al., 2011). LhrA represses the translation of at least three genes, i.e., *chiA*, *lmo0302* (hypothetical protein), and *lmo0880* (cell wall associated protein). Expression of LhrA has a negative effect on the chitonolytic activity of *L. monocytogenes*, however, it is not yet clear if this function is also relevant for virulence. Note that LhrA was the first example of a sRNA from a Gram positive bacterium that requires Hfq for target regulation (Nielsen et al., 2010).

THE GlmY/Z sRNAs ACT BY SEQUESTRATION AND BASE-PAIRING

The two homologous sRNAs, GlmY and GlmZ, are highly conserved among the *enterobacteriae*. Both sRNAs activate production of GlmS (**Figure 1**), although only GlmZ directly base-pairs with the *glmS* transcript. The *glmS* mRNA accumulates as the distal part of the *glmUS* dicistronic transcript, and is separated from the *glmU* ORF by an RNase E mediated cleavage event (Kalamorz et al., 2007). Following this processing, the *glmS* mRNA remains translationally inactive because of an intrinsic inhibitory structure within its 5′ UTR. Binding of GlmZ to the *glmS* 5′ UTR resolves this structure, which releases the RBS of this transcripts and increases synthesis of the GlmS protein (Reichenbach et al., 2008; Urban and Vogel, 2008).

The enzymatic product of GlmS is glucosamine-6-phosphate (GlcN6P), a central aminosugar required for cell wall biosynthesis. Low levels of GlcN6P induce the expression of GlmY which indirectly activates GlmS production through GlmZ and the accessory protein, RapZ (a.k.a. YhbJ). Due to its structural similarity with GlmZ, GlmY can function through molecular mimicry to interfere with GlmZ degradation by RNase E and RapZ. The latter protein is a specialized adapter that targets GlmZ for RNase E-mediated decay. Recognition by RapZ is guided by a RNA element shared between GlmZ and GlmY and high levels of GlmY titrate the RapZ protein from GlmZ, thus stabilizing the GlmZ sRNA. GlmY itself does not bind Hfq, suggesting that it acts as a specific decoy for GlmZ rather than regulating mRNAs expression on its own (Gopel et al., 2013). Taken together, these two well-conserved sRNAs act hierarchically in a complex regulatory cascade to adjust the translation of the *glmS* mRNA to physiological needs.

In *Salmonella* and other enterobacteria, transcription of GlmY and GlmZ is regulated by two overlapping promoters controlled by either σ^{70} or σ^{54} although this may vary between species (Urban et al., 2007; Reichenbach et al., 2009; Gopel et al., 2011). GlmY expression also requires binding of the global transcriptional regulator, IHF (Gopel et al., 2011). In addition, the expression of the *glmY/Z* genes by the σ^{54} version of RNA polymerase requires the QseF and QseE proteins (a.k.a. GlrR/GlrK) (Reichenbach et al., 2009; Gopel et al., 2011). Intriguingly, QseF and QseE constitute a two-component system that is important for the virulence of *Y. pseudotuberculosis* (Flamez et al., 2008) and enterohemorrhagic *E. coli* (EHEC) (Reading et al., 2007) indicating that GlmY/Z might have a function in virulence.

Indeed, the Sperandio group recently reported a crucial role of the GlmY/Z sRNAs for the pathogenicity of EHEC, observing that mutations of either *glmY* or *glmZ* increased pedestal formation on host cells by this organisms (Gruber and Sperandio, 2014). Surprisingly, GlmY/Z did not seem to control *glmS* expression in EHEC. Instead, both sRNAs regulated transcripts from the LEE4 and LEE5 pathogenicity islands as well as the mRNA of the secreted effector protein EspFu. This regulation is reminiscent of the above described SgrS-*sopD* example (Papenfort et al., 2012) in that conserved "core" sRNAs are recruited to regulate the mRNAs of horizontally acquired virulence factors through Hfq and base-pairing.

GLOBAL FUNCTIONS FOR THE RNA-BINDING PROTEIN, CsrA

CsrA-like proteins are conserved in most enteric pathogens and deletion of the *csrA* gene often impairs virulence (Lucchetti-Miganeh et al., 2008; Seyll and Van Melderen, 2013). Given the multi-faceted phenotypes of many *csrA* mutant strains, one may argue that reduced pathogenicity primarily resulted from decreased overall fitness rather than the specific virulence functions. Indeed, a *Salmonella csrA* mutant displayed multiple defects in metabolic regulation and virulence factor expression (Altier et al., 2000; Lawhon et al., 2003) and comparable phenotypes were observed in uropathogenic *E. coli* (Mitra et al., 2013). However, CsrA also regulates *Salmonella* pathogenicity more directly. For example, CsrA binds to the 5′ UTR of the mRNA of HilD repressing the synthesis of this master transcriptional regulator of virulence (Martinez et al., 2011). Similarly, CsrA affects biofilm formation through interaction with the mRNA of an phosphodiesterase gene (STM3611) regulating intracellular c-di-GMP levels (Jonas et al., 2010). CsrA was also found to coordinate the expression of two mutually exclusive fimbrial operons in *Salmonella* by a putative novel mechanism of mRNA cross-regulation (Sterzenbach et al., 2013).

The global activity of CsrA in *E. coli* and *Salmonella* is counteracted by the CsrB/C sRNAs whose transcription is under control of the BarA/UvrY TCS (Gudapaty et al., 2001; Suzuki et al., 2002). Transcriptional control of the CsrA antagonists by the BarA/UvrY TCS seems to be a conserved principle in many bacteria (Seyll and Van Melderen, 2013). While some bacteria encode only one CsrB-like RNA, *V. cholerae* species encode three different CsrA antagonists: CsrB, CsrC, and CsrD (Lenz et al., 2005). Here, expression of the Csr-sRNAs affects virulence via regulation of the quorum sensing pathway (Jang et al., 2010). In addition, expression of CsrB-like sRNAs can also be controlled post-transcriptionally. The CsrD RNA-binding protein of *E. coli* (not to be confused with the CsrD sRNA from *V. cholerae*) can bind the CsrB/C sRNAs and target them for degradation by RNase E (Suzuki et al., 2006). CsrA also reduces the expression of CsrD (Jonas et al., 2008) generating a negative feedback loop for robust signaling under conditions of stress (Adamson and Lim, 2013).

The Csr system and its relevance for virulence and metabolism have been studied in greater detail for the human enteropathogen *Y. pseudotuberculosis* where a mutation of the *csrA* gene resulted in complex phenotypic alterations (Heroven et al., 2008). Transcriptomic studies revealed deregulation of ∼500 ORFs in the *csrA* mutants, ∼20% of which are metabolic genes (Heroven et al., 2012a). The *Y. pseudotuberculosis* genome encodes two CsrB-like sRNAs (CsrB and CsrC) and their expression is crucial during the initial phase of infection because sequestration of CsrA is needed to allow the production of the host cell adhesion factor, InvA (Heroven et al., 2008). Induction of InvA involves a complex regulon including the transcriptional factor RovA (Heroven and Dersch, 2006). Regulation via the Csr-system is further controlled via CCR. The CRP protein represses the response regulator UvrY which is required for CsrB activation. A *crp* mutant has increased levels of the CsrB sRNA which promotes CsrC and RovA repression. Not surprisingly, a *Y. pseudotuberculosis* mutant lacking the *crp* gene is strongly impaired in virulence (Heroven et al., 2012b).

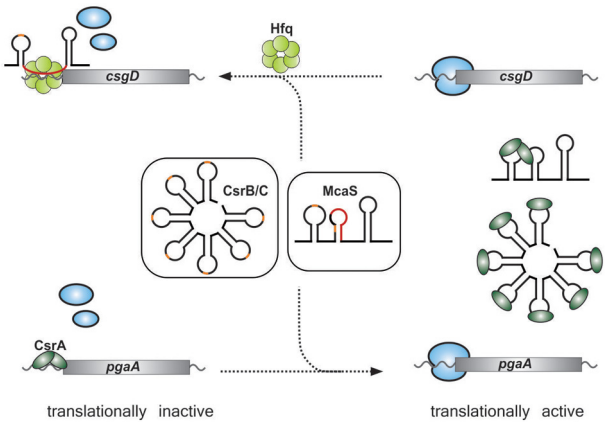

FIGURE 3 | Overlap of the CsrA and Hfq regulons through McaS. Both McaS and the CsrB-like sRNAs bind to the CsrA protein via GGA-rich motifs (orange). Binding of CsrA results in titration of CsrA from its target mRNAs which usually activates their translation (lower panel). In addition, McaS can also directly bind and regulate target mRNAs (e.g. *csgD*) through Hfq-mediated base-pairing (indicated in red).

A new type of CsrA antagonist has recently been reported in *E. coli*. It was observed that the Hfq-binding sRNA, McaS, which regulates the *flhD* and *csgD* mRNAs (encoding regulators of motility and biofilm formation, respectively) by base pairing interactions, impacted expression of the *pgaA* gene by a supposedly indirect mechanism (Jorgensen et al., 2012; Thomason et al., 2012) (**Figure 3**). The PgaA protein is crucial for the production of PGA (poly-β-1,6-N-acetyl-glucosamine), an important factor for biofilm adhesion (Itoh et al., 2008). Expression of *pgaA* had been known to be subject to control by CsrA (Wang et al., 2005), which suggested a link between McaS and CsrA. Indeed, the McaS sRNA was found to bind the CsrA protein via two exposed GGA motifs and thereby indirectly regulate the expression of several CsrA-target genes, including *pgaA* (Jorgensen et al., 2013) (**Figure 3**). In summary, McaS is the first sRNA regulating target gene expression via both Hfq and CsrA. Future studies may reveal additional sRNAs that serve in both of these global post-transcriptional networks.

FUTURE DIRECTIONS

The above examples of sRNA-mediated gene regulation in enteric pathogens serve to illustrate the growing number of potential post-transcriptional links between metabolic and virulence functions in these organisms. To date, many of these links remain inferences from functional studies of sRNA-mRNA interactions, and how these contribute to nutritional adjustment and control of virulence factor expression requires more detailed studies. However, it is important to note that global studies of the RNA targets of Hfq and CsrA, two proteins that each may control up to 20% of all mRNAs in enteric model organisms (Chao and Vogel, 2010; Romeo et al., 2013), revealed a high number of mRNAs from metabolic and virulence pathways, suggesting that many more sRNAs could be involved in these pathways. In addition, the growing depths of NGS will soon allow us to extensively profile bacterial RNA expression in complex tissue and inside host cells, even simultaneously with gene expression of the eukaryotic host to inform details of the pathogen's metabolic environment (Westermann et al., 2012).

There are more potential links between virulence and metabolism in the available sRNA data whose physiological importance needs to be explored. For example, the recent profiling of Hfq-bound *Salmonella* transcripts revealed the DapZ sRNA, which is encoded in the 3′ UTR of the well-conserved metabolic *dapB* gene. In *Salmonella*, the horizontally acquired virulence regulator HilD has been recruited to transcriptionally activate the DapZ sRNA which then acts to repress the synthesis of oligopeptide uptake proteins (Chao et al., 2012). Under regular growth conditions oligopeptide uptake is controlled by the conserved GcvB sRNA (Sharma et al., 2011) and regulation of DapZ by HilD enables the cell to exert a similar function under virulence-related conditions. However, why DapZ is linked to *dapB* and how the metabolic function of the DapB protein, an enzyme that produces the lysine precursor diaminopimelate, may be interwoven with a DapZ-mediated repression of amino acid uptake, is far from obvious. Of note, regulation of oligopeptide uptake through sRNAs has been observed in non-enteric bacteria, too. The RsaE sRNA from *Staphyloccus aureus*, which is also conserved in other Gram positives, directly controls the mRNA encoding the OppB protein (Geissmann et al., 2009) and several other transcripts of metabolic genes (Bohn et al., 2010).

The most recent count for sRNA regulators in *Salmonella* revealed ∼280 sRNAs, many of which are Hfq-dependent and expressed under stress or virulence mimicking conditions (Kroger et al., 2012, 2013). How many of these sRNAs are also relevant for virulence is still an open question but novel approaches such as Tn-Seq (combining transposon mutagenesis and HTPS) could be powerful tools to evaluate the roles of sRNAs during infection (Van Opijnen and Camilli, 2013). The same technology can also be used to identify metabolic genes required for infection. Indeed, two recent studies using Tn-Seq in *Salmonella* or *V. cholerae* identified several genes involved in carbon metabolism to be required for full pathogenicity (Chaudhuri et al., 2013a; Fu et al., 2013).

Probably one of the most exciting areas of host-microbe interaction today is how pathogens deal with the commensal microbiota of the host. It is now understood that the carbohydrate metabolism of the microbiota significantly impacts on the virulence gene expression of enteric pathogens and that carbohydrates can function as signaling molecules in the intestine (Pacheco et al., 2012). In contrast, close to nothing is known about how sRNAs shape the interaction of pathogens with commensals and we are yet to see if such sRNAs would also impact virulence. Again, NGS-based metatranscriptomics of multi-species intestinal communities could provide a valuable starting point to address the relevance of regulatory RNAs and metabolic genes in the context of the host microbiota (Xiong et al., 2012). These new exciting venues at the interface of microbiology and host-microbe interaction might become relevant for the design of alternative anti-microbial compounds which consider both, the pathogen and the host microbiota.

ACKNOWLEDGMENTS

We thank Cari Vanderpool, Chase Beisel, and Kathrin Fröhlich for comments on the manuscript. This work was funded by support from the DFG Priority Program SPP1316 (Vo875/6-1), the Bavarian BioSysNet program, and a BMBF RNASys grant. Kai Papenfort was supported by a postdoctoral fellowship from the Human Frontiers in Science Program (HFSP).

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Conflict of Interest Statement: The Guest Associate Editor Thomas Dandekar declares that, despite being affiliated to the same institution as author Jörg Vogel, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 April 2014; accepted: 19 June 2014; published online: 15 July 2014.
Citation: Papenfort K and Vogel J (2014) Small RNA functions in carbon metabolism and virulence of enteric pathogens. Front. Cell. Infect. Microbiol. ***4****:91. doi: 10.3389/fcimb.2014.00091*
This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 30 September 2014
doi: 10.3389/fcimb.2014.00139

From screen to target: insights and approaches for the development of anti-virulence compounds

Katherine S. H. Beckham [†] ***and Andrew J. Roe*** *[†]

Glasgow Biomedical Research Centre, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
V. K. Viswanathan, University of Arizona, USA
Michael Hensel, Universität Osnabrück, Germany
Gaby Wangorsch, University of Wuerzburg, Germany

***Correspondence:**
Andrew J. Roe, Glasgow Biomedical Research Centre, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, 120 University Place, Glasgow G12 8TA, UK
e-mail: andrew.roe@glasgow.ac.uk

[†] *These authors have contributed equally to this work.*

A detailed understanding of host-pathogen interactions provides exciting opportunities to interfere with the infection process. Anti-virulence compounds aim to modulate or pacify pathogenesis by reducing expression of critical virulence determinants. In particular, prevention of attachment by inhibiting adhesion mechanisms has been the subject of intense research. Whilst it has proven relatively straightforward to develop robust screens for potential anti-virulence compounds, understanding their precise mode of action has proven much more challenging. In this review we illustrate this challenge from our own experiences working with the salicylidene acylhydrazide group of compounds. We aim to provide a useful perspective to guide researchers interested in this field and to avoid some of the obvious pitfalls.

Keywords: infection, anti-virulence, secretion, inhibitor, *Escherichia coli*

INTRODUCTION

The treatment of bacterial infections has become more challenging due to the increased prevalence of antibiotic-resistant strains and a stark reduction in the development of novel anti-bacterials. The current armory of compounds inhibit enzymes that are often essential to the survival of the pathogen, for example β-lactams and aminoglycosides that target bacterial cell wall biosynthesis and translation respectively. As these processes are essential for growth, the selective pressure imposed by antibiotics is strong, and the development of resistance mechanisms high. The identification of novel targets that are not essential for survival *per se* is therefore becoming an active area of research.

The AV approach is one that specifically targets "virulence factors" used by pathogens to facilitate the infection process. The application of AV compounds against factors such as quorum sensing, adhesins, and secretion systems has been tested, however the development of these compounds is still in the early stages. Whether targeting virulence factors will lead to lower selective pressure for the generation of resistance is an interesting question and has been scrutinized recently (Allen et al., 2014). Certainly anti-virulence (AV) approaches can have merit, for example when the use of traditional antibiotics is not appropriate. The clinical symptoms associated with Enterohaemorrhagic *Escherichia coli* (EHEC) infections have been shown to increase in severity following administration of certain antibiotics. This is a result of the release of Shiga-toxin following bacterial lysis (Zhang et al., 2000).

The focus of this review is the development of AV compounds that inhibit the Type Three Secretion System (T3SS), a virulence factor important for the pathogenicity of several Gram-negative pathogens, including *Salmonella* spp., *Yersinia* spp. and pathogenic *E. coli*. Here we will describe the different approaches used to identify AV compounds, along with their respective targets, and the various methods of target validation, with particular emphasis on the experience we have gained from working on a class of T3SS inhibitors, the salicylidene acylhydrazides.

THE TYPE THREE SECRETION SYSTEM

The T3SS is a key virulence determinant for a diverse range of Gram-negative pathogens. Species as distinct as *Yersinia* and *Erwinia* use the T3SS to secrete and inject pathogenicity proteins into the cytosol of eukaryotic host cell (Hueck, 1998). Whilst the core apparatus proteins of the T3SS are relatively conserved, the functions of the secreted effector proteins are highly species-specific. EHEC and enteropathogenic *E. coli* (EPEC) induce gross reorganization of the actin cytoskeleton of host-epithelial cells leading to the formation of attaching and effacing (A/E) lesions that act as "pedestals" allowing intimate attachment of the bacteria to the host. Attachment is largely achieved by the translocation of effector proteins such as Tir (the translocated initimin receptor). In both EHEC and EPEC, the entire T3SS is chromosomally encoded by a pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). This T3SS is genetically quite distinct from that of *Yersinia* species, the "Ysc-Yop"

system, which is plasmid encoded and regulated by different environmental signals (Lindler, 2004).

THE SEARCH FOR T3SS INHIBITORS

Deletion of the T3SS has a profound effect on the virulence potential of Gram-negative pathogens *in vivo*, making its inhibition an attractive prospect. Initial screens for a T3SS inhibitor made use of a high throughput (HTP) approach that tested large chemical libraries, consisting of both synthetic and natural compounds, against whole bacteria (Linington et al., 2002; Kauppi et al., 2003; Nordfelth et al., 2005). The use of a bacterial screening model overcomes several problems associated with drug discovery, for example cell-permeability or drug-efflux. Several of these screens employ the use of a transcriptional-reporter assay, which couples the expression of virulence genes into a fluorescent or luminescent read-out that can be easily quantified in a HTP manner.

The first reported chemical inhibitor of the T3SS was identified in 2002 by Linington et al. who screened chemical extracts from the marine sponge *Caminus sphaeroconia* against EPEC (Linington et al., 2002). The screen looked for compounds that decreased the secretion of EspB, a T3SS protein, and displayed no antibacterial activity. The product caminoside (**Table 1**; depicts key compounds described in this review) was found to have these properties with an IC_{50} of 5.1 μg/ml. Despite the promise of this caminoside, its cellular targets were not identified due to the difficulty of synthesizing this natural compound (Zhang et al., 2010).

Several other natural products have been shown to decrease the expression of the T3SS. Aurodox, produced by *Streptomyces goldiniensis,* was recently shown to inhibit EPEC T3SS mediated hemolysis *in vitro,* with an IC_{50} of 1.8 μM (Kimura et al., 2011). Aurodox was also shown to be effective *in vivo* when tested in a mouse model of infection using the natural mouse pathogen *Citrobacter rodentium*, where mice treated with Aurodox survived a lethal bacterial load (Kimura et al., 2011). Treatment of Gram-negative species with Aurodox resulted in a specific decrease in expression of the T3SS suggesting that it may be interacting with a T3SS transcriptional regulator. Another class of compounds produced by a *Streptomyces* species (K01-0509) are the guadinomines, which were shown to inhibit T3SS in EPEC *in vitro* with IC_{50} values of lower than 0.01 μg/ml (Iwatsuki et al., 2008). Since the guadinomines appear to be highly potent with no antibacterial activity they are attractive lead compounds, however their efficacy *in vivo* has yet to be confirmed.

One of the most extensively studied group of AV compounds are the salicylidene acylhydrazides (SA), a class of inhibitors that were identified from a chemical screen of 9400 compounds carried out by Kauppi et al. at the University of Umeå (Kauppi et al., 2003). The screen was performed on *Y. pseudotuberculosis* expressing a *yopE*-luciferase transcriptional fusion, where the *yopE* promoter was fused to the *luxAB* cassette. YopE is a secreted effector protein; therefore a decrease in luciferase activity from the *yopE* promoter was correlated to reduced expression of the T3SS. This assay provided a rapid system to monitor processes regulating secretion-specific transcription. However, like all transcriptional reporters, it is rather indirect and does not provide data on whether the T3SS is functional and secreting effectors. Compounds that showed no antibacterial activity were characterized further, leaving four lead compounds from the initial screen. These were all demonstrated to decrease the secretion of effector proteins (YopE, YopD and YopH) in a dose dependent manner with an IC_{50} of less than 50 μM. Owing to the structural similarity between the Ysc T3SS apparatus and the flagellum, the compounds were tested for inhibition of motility. Only one of the four compounds, INP0010/ME0052, was shown to have an effect on motility, which was interpreted by some groups to indicate that the compounds are binding to a related structural component or to a common regulator of these systems (Kauppi et al., 2003).

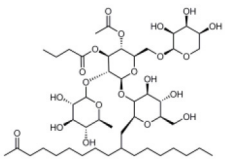
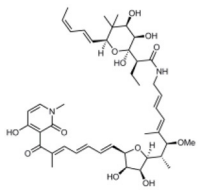
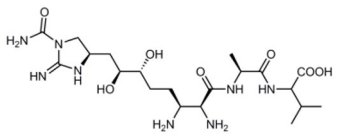
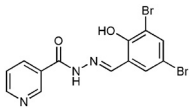
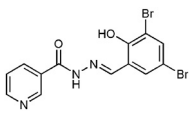
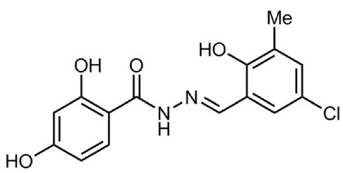
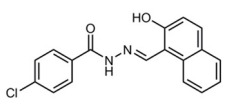
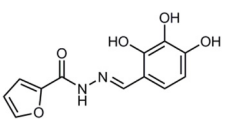
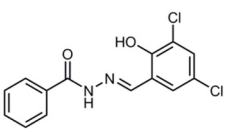
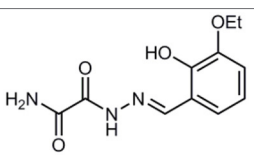
Several groups later tested the SA compounds on a range of Gram-negative pathogens. The obligate intracellular pathogen *Chlamydia trachomatis* was shown to be affected by an SA compound (INP0400), identified in the original screen by Kauppi et al. (2003; Muschiol et al., 2006; Wolf et al., 2006). Treatment with this compound disrupted the normal infection cycle and prevented differentiation and multiplication in mammalian cells (Muschiol et al., 2006; Wolf et al., 2006). At the time of this study relatively little was known about the role played by the T3SS in this pathogen, thus the use of these compounds revealed insights into the importance of the T3SS in the switch between the metabolically inert "elementary body" and the infective vegetative state of the pathogen. At the time, there were only limited genetic tools available for *Chlamydia* therefore INP0400 permitted inhibition of T3SS expression throughout the developmental cycle of this pathogen (Wolf et al., 2006).

Subsequent work showed that *Salmonella enterica* serovar Typhimurium was also susceptible to the SA compounds. Dose dependent inhibition of SPI-1, one of the two T3SSs encoded in *Salmonella* (Hudson et al., 2007) was demonstrated. The compounds (e.g., INP0031/ME0055) had no effect on the growth of the pathogen *in vitro* and reduced SPI-1 mediated invasion of HeLa cells by up to 60%. The study also showed that pre-incubation of the bacteria with the compounds reduced the level of inflammation in an *in vivo* bovine intestinal ligated loop model. These data indicated that the compounds reduced the virulence of *Salmonella in vivo* (Hudson et al., 2007). Further studies in *Salmonella* by Negrea et al. confirmed the ability of the SA compound (INP0400) to inhibit SPI-1 activity (Negrea et al., 2007). They also demonstrated the compounds to be effective inhibitors of SPI-2 mediated secretion, and that treatment with the compounds reduced intracellular replication. Two of the nine compounds tested were shown to significantly reduce the motility of *Salmonella* in soft agar (Negrea et al., 2007).

Veenendaal et al. found that the SA compounds INP0402 and INP0400 were the most effective at reducing T3SS in *Shigella flexneri,* an invasive intracellular Gram-negative pathogen (Veenendaal et al., 2009). Treatment with the compound reduced its ability to invade HeLa cells and its ability to induce macrophage apoptosis, both indicative of T3SS inhibition (Veenendaal et al., 2009).

The activity of the SA compounds against EHEC was shown by our group to be effective at decreasing LEE T3S in a dose dependent manner (Tree et al., 2009). This study showed INP0031

Table 1 | Anti-virulence compounds discussed in this review.

Compound	Structure	Source	Phenotype tested	Effective against	IC_{50}	References
Caminoside		Marine sponge (*Caminus sphaeroconia*)	Effector protein secretion (EspB)	EPEC	5.1 μg ml^{-1}	Linington et al., 2002
Aurodox		*Streptomyces* sp. extract	Effector protein secretion (EspB) T3SS- mediated haemolysis	EPEC *C. rodentium*	1.8 μM	Kimura et al., 2011
Guadinomines		*Streptomyces* sp. extract	T3SS induced haemolysis	EPEC	<0.01 μg ml^{-1}	Iwatsuki et al., 2008
INP0010 / ME0052		Synthetic compound library (ChemBridge)	Effector protein secretion (Yop, EspB)	*Yersinia* *Salmonella* EHEC	25 μM	Nordfelth et al., 2005
INP0031 / ME0055		Synthetic compound library (ChemBridge)	Effector protein secretion (EspB, Tir)	EHEC	25 μM	Hudson et al., 2007
INP0341		Synthetic compound library (ChemBridge)	Intracellular invasion assay	*Chlamydia*	<50 μM	Slepenkin et al., 2007
INP0400		Synthetic compound library (ChemBridge)	Effector protein secretion (Yop, EspB), Intracellular invasion assay	*Yersinia* *Chlamydia* *Salmonella* *Shigella*	25 μM	Muschiol et al., 2006; Negrea et al., 2007; Slepenkin et al., 2007; Veenendaal et al., 2009
INP0402		Synthetic compound library (ChemBridge)	Intracellular invasion assay	*Shigella*		Veenendaal et al., 2009
INP0403 / ME0053		Synthetic compound library (ChemBridge)	Effector protein secretion (EspB), SpI1 expression	*Salmonella* EHEC	25 μM	Tree et al., 2009; Layton et al., 2010; Wang et al., 2011
INP0406		Synthetic compound library (ChemBridge)	Intracellular invasion assay	*Chlamydia*		Slepenkin et al., 2007

to be the most effective compound at inhibiting LEE T3S and A/E lesion formation. All of the compounds tested (INP0010, INP0103, INP0401 and INP0031) increased the production of flagella (Tree et al., 2009). The observation that the SA compounds decreased expression of the T3SS but increased flagella expression indicated that the mechanism of action might be through a regulatory mechanism.

In summary, the SA compounds have been shown to be effective inhibitors of T3S in several species of Gram-negative pathogens. In all studies the authors observed no antibacterial activity, which is key as AV compounds should not decrease the survival of the pathogen. Several studies showed that in addition to affecting the T3SS, the expression of motility genes was also affected, however the effects between species were not consistent. Although it is known that the compounds inhibit the T3SS, the precise mechanisms underlying their mechanism of action (MOA) is yet to be elucidated.

PROPOSED MECHANISM OF ACTION OF THE SA COMPOUNDS

There are three main schools of thought about how the SA compounds function. Firstly, by disrupting cellular iron stores. Secondly, by directly interacting with a component of the T3SS apparatus, and thirdly, by causing dis-regulation of T3SS expression. The finding that the activity of the compounds could be reversed following the addition of iron to the cell culture media was first reported by Slepenkin et al. (2007). This study showed that the addition of iron to HeLa cells infected with *C. trachomatis* reversed the effects of the inhibitors. This effect was not seen when other divalent metal ions were added. However, these results were somewhat inconclusive since INP compounds that did not affect the T3SS in *Chlamydia* (INP0406) chelated iron to the same extent as INP0341, a potent inhibitor (Slepenkin et al., 2007). Indeed, the most promising clinical application of the SA compounds is for protection against *Chlamydia.* For this strict intracellular pathogen the SA compounds affect not only the T3SS but also growth and replication of the bacteria, almost invariably through iron sequestration (Ur-Rehman et al., 2012). When used as a vaginal biocide, SA compounds were able to significantly protect mice from a vaginal infection of *C. trachomatis* (Slepenkin et al., 2011). A similar study by Layton et al. indicated that the effect of the SA compounds could be partially reversed by the addition of iron (Layton et al., 2010). Transcriptomic analysis of *Salmonella* treated with INP0403 showed a significant increase in several genes involved in iron regulation (Layton et al., 2010). However, the addition of iron did not fully reverse the anti-SPI1 T3SS activity of INP0403. Microarray studies carried out on EHEC grown in the presence of iron found that SA compounds (INP0010 and INP0031) lead to a significant decrease in the expression of the LEE (Tree et al., 2009), thus indicating that in this case iron is not inhibiting the action of the SA compounds. Therefore, it remains unclear how iron affects the activity of the SA compounds and further work is required to clarify the effects of iron on the T3SS.

Veenendaal et al. proposed that the compounds were acting directly on a component of the T3SS. The reports of motility also being affected by the compounds led to the conclusion that the component being targeted may be one that is homologous between the T3SS and flagellar systems (Veenendaal et al., 2009). The evidence for this proposed mechanism was that following SA compound treatment of *Shigella*, the needle filaments of the T3SS were significantly shorter than for untreated cells indicating that the compounds were affecting needle assembly (Veenendaal et al., 2009). A further study by the same group sought to determine this common component in *Salmonella*. By using strains deficient in three soluble components of the flagella apparatus they aimed to identify which of these were responsible for the change in motility seen following compound treatment (Martinez-Argudo et al., 2013). However, this study was unable to show that the SA compounds directly affected flagellar components. The authors concluded that the SA compounds were not directly inhibiting T3SS or flagellar components and were most likely interacting with other targets within the cell and indirectly affecting the expression of these virulence factors (Martinez-Argudo et al., 2013).

Transcriptomic profiling of EHEC treated with 20 μM ME0052 or ME0055 resulted in the decreased expression of the five operons that comprise the LEE as well as an increase in the expression of flagellar associated genes (Tree et al., 2009). These data provided important clues as to how the SA compounds might be working. Firstly, the reduction in transcription of the entire LEE suggested that the compounds either affected the master regulator of the system (Ler) or an upstream regulator that affected Ler itself. Indirect support for this hypothesis comes from the observation that deletion mutants for LEE genes encoding proteins of the secretion system itself do not result in regulatory feedback and a reduction in LEE transcription (Deng et al., 2004). This suggests that the SA compounds are unlikely to simply bind to basal apparatus proteins, as this would not be consistent with the transcriptional changes observed. Further evidence to explain the MOA of the SA compounds comes from work investigating the type four secretion system (T4SS) of *Brucella*. T4SS have a completely different protein structure compared to the T3SS but are also important virulence factors for many Gram-negative pathogens (Baron, 2006). Based on the knowledge that dimerization of the assembly factor VirB8 is a prerequisite for VirB8 function, a bacterial two-hybrid assay was established (Smith et al., 2012). This allowed several inhibitors of VirB8 dimerization to be identified including B8I-2, a salicyidene acylhydrazide. Co-crystals of B8I-2 and VirB8 were obtained, allowing the residues critical for the inhibitory activity to be mapped. Interestingly, when seven SAs that inhibited the T3SS were tested, all were found to be inactive in the VirB8 interaction assay (Smith et al., 2012). This raises the possibility that either the SA compounds are rather promiscuous, and bind numerous proteins, or that despite the absence of obvious protein sequence similarities, both of the T3 and T4 secretion systems may be inhibited by a similar mechanism.

In summary, it is easy to assume that because one has established a screen for compounds that affect the expression of the T3SS, the compounds are directly targeting the secretion system itself. This assumption is dangerous and it is wise to determine the global effects of any compound using either transcriptomic or proteomic approaches before focusing on a subset of targets.

DEFINING THE MECHANISM OF ACTION FOR AV COMPOUNDS

From the outline above, it is clear that whilst it is relatively easy to develop a robust screen and identify novel lead compounds, elucidating their specific mode of action is much more problematic. The first step toward unraveling the MOA of a novel compound is to identify its cellular target or targets. There are several different approaches that can be taken to investigate targets such as genetic or biochemical screening and affinity chromatography. However, when used in isolation these approaches will not always give a clear answer and in our experience we have found that using a combination of these approaches is most beneficial.

STRENGTHS AND LIMITATIONS OF APPROACHES TO TARGET IDENTIFICATION

Most groups performing screening projects have a background in bacteriology and therefore adopt classical genetics approaches to try and understand MOA. For traditional antibiotics, simple screens are often employed to identify mutants that are resistant to the effects of the agent. Exposure of a large bacterial population to a high dose of the antibiotic will often yield "escape mutants" that are resistant to the compound (Bergstrom and Feldgarden, 2008). Alternatively, saturated transposon mutagenesis can be employed in which a bank of mutants is created and then screened for escape mutants. Genome sequencing is now routine and affordable such that mutants can be readily analyzed and the mutation identified. Ideally, the transposon would be in a gene encoding the target, directly revealing the likely MOA. However, resistant mutants can be more obscure, for example in a porin or membrane transporter that results in a lower intracellular concentration of the antibiotic (Fernández and Hancock, 2012). For AV compounds, an inherent limitation is often that the screening of mutants is far more time-consuming than for bactericidal antibiotics. By their very design, AV agents do not affect bacterial growth or survival so simply "plating out" a large population on a high concentration is not likely to produce resistant mutants that will inform the MOA.

We have utilized two different approaches to identify escape mutants. Firstly, generation of a transposon insertion library in which each mutant is screened for expression of the T3SS and secondly by screening "wild-type" isolates with a view to finding variants that are less sensitive to the AV compound. In the former, the process has proved to be time-consuming and is absolutely dependent on the quality of the screen, in our case a GFP transcriptional-reporter assay that can be run in 96-well plate format. Given that the EHEC genome carries approximately 5500 genes, there is no overcoming the large volume of work involved in screening thousands of mutants. Moreover, for each mutant that exhibits insensitivity to an AV compound, further screens must be undertaken to verify that growth is unaffected. One inherent limitation of such a screen is that, if the protein target were directly involved in the T3SS itself (such as a structural protein), then insertion of a transposon into corresponding gene would inhibit T3SS function entirely. Overall, the simplicity of the approach needs to be balanced against the time needed to be invested and should only be adopted if the screen is extremely robust.

Our second approach has been to screen a bank of clinical isolates. Selection of 18 clinical *E. coli* O157 isolates with a diverse range of phage types revealed a strain that was completely insensitive to the SA compounds. Next generation sequencing provided rapid and accurate identification of genetic differences when compared to that of reference strains. The success of this approach is largely dependent on having access to a diverse strain collection, as clonal isolates are likely to display little phenotypic variation. However, greater diversity results in more genetic differences. In our insensitive mutant there were over 1300 single nucleotide polymorphisms (Wang et al., 2011). These data were a useful comparator to other target identification approaches but, in isolation, would not be sufficient to inform of a specific target with confidence.

As discussed above, transcriptomic profiling, historically using microarrays and more recently RNA-seq, is a powerful and unbiased method to reveal insights into the global effects of any compound. Although transcriptomic profiling reveals no direct data as to the likely target protein or underlying mechanism it does provide valuable data. In particular, it provides a clear indication as to the specificity of the compound: does it affect a single operon, a network of genes or a substantial proportion of the genome? As already described, for the SA compounds it was clear that several operons, not just the LEE, were affected. These data suggested that numerous target proteins were bound or that the compounds interfered with core aspects of bacterial physiology.

Alternatives to transcriptomic studies are the well-established methods of proteomics and the relatively recent addition, metabolomics. Using two-dimensional difference gel electrophoresis (2D-DIGE) provides a sensitive and robust approach to detecting changes in protein expression (Kondo and Hirohashi, 2006). The most clear advantage being that it is possible to detect possible post-transcriptional effects caused by any compound. The major limitation is that only a proportion of the proteome can be resolved on a single gel.

Metabolomics is widely used in the pharmaceutical industry to test the effects of drugs on host-cell processes. For example, metabolomic studies have demonstrated that D-cycloserine, a second-line treatment for *Mycobacterium tuberculosis*, is rather non-specific and causes inhibition of numerous enzymes (Halouska et al., 2007), a result that might explain some of the less-desirable side effects. However, metabolomics has barely been applied to AV development. Targeted metabolomics follows changes to a specific metabolite based on some prior information, so it is unlikely to be used at an early stage or to reveal information regarding a possible MOA. However, untargeted metabolomics is discovery based and aims to monitor the entirety of the metabolome in order to identify the affected metabolites and pathways. It is feasible that metabolomics might reveal discrete changes in pathways providing insights into the global effects of an AV compound but it is likely to be employed at a later stage to investigate potential toxicity issues when MOA has been established.

Affinity chromatography is a powerful technique that enables identification of compound binding partners from whole-cell lysates. An overview of the basic steps is provided in **Figure 1**.

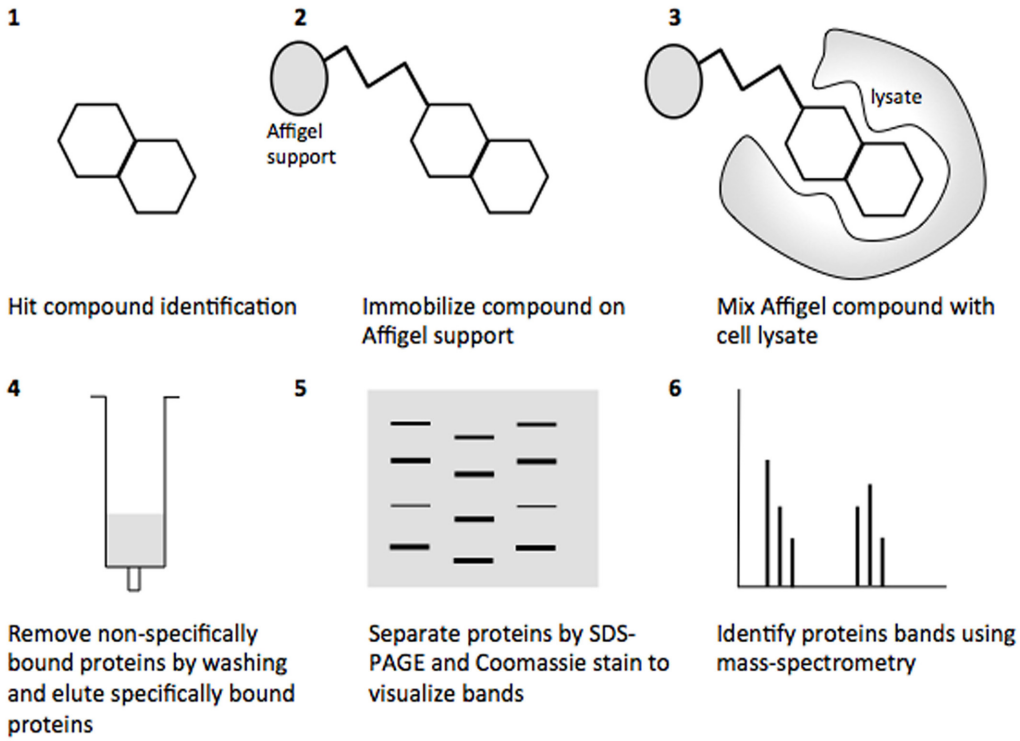


FIGURE 1 | Overview of the affinity pull-down assay to determine the binding proteins of the salicylidene acylhydrazides. (1) The hit compound was identified from reporter assays screening for decreased T3SS expression. **(2)** The hit compound was attached to an Affigel support. **(3)** The Affigel labeled compound was mixed with cell lysate, allowing the putative targets to bind. **(4)** Non-specifically bound proteins were removed by washing and specifically bound proteins were eluted with high concentrations of the free compound. **(5)** Eluted proteins were resolved by SDS-PAGE and protein bands visualized by Colloidal Blue Stain and excised. **(6)** Protein bands were identified by mass-spectrometry.

A successful pull-down first requires that the compound can be attached to an immobile matrix without disrupting the activity of the compound. This is not without complications as not all of the chemical groups on the compound may be suitable for attaching a linker to immobilize the compound. Therefore, it is often valuable to conduct a structure-activity relationship (SAR) where different chemical variants are screened against whole cells in order to identify the regions of the molecule associated with the desired phenotype. In the case of the SA compounds it was found that the active groups of the compounds were located on the right hand phenol group (**Table 1**) (Wang et al., 2011). This knowledge allowed the design and synthesis of ME0055-Aff, an Affigel labeled derivate of the SA inhibitor. Using the Affigel labeled derivative an affinity pull-down assay of *E. coli* O157:H7 cell lystates identified 19 putative protein binding targets (Wang et al., 2011). A combination of phenotypic analyses and biophysical studies on purified proteins were used to critically assess the contribution of these putative targets to the phenotype associated with SA addition.

TARGET VALIDATION

The identification of putative target proteins is a great step forward in understanding the MOA of a compound. However, it is important to confirm any interaction and, more importantly, that the target is associated with the expected phenotype. Our affinity chromatography experiments revealed multiple targets for the SA compounds (Wang et al., 2011). These were likely a mix of genuine targets, false positives but also proteins bound by the compound that did not contribute to the overall phenotype. To test this, we employed a variety of different biophysical methods. These included chemical shift nuclear magnetic resonance (NMR), X-ray crystallography, and analytical ultracentrifugation. Further approaches including surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) would also be applicable but the low solubility of the SA compounds in biologically relevant solutions made these problematic. Chemical shift NMR allowed us to demonstrate that one target protein, a thiol peroxidase called Tpx was indeed a target of the SA compounds. A discrete binding site at the dimer interface was mapped and input from collaborators helped build a model of the SA compounds bound to the oxidized form of Tpx (Gabrielsen et al., 2012), validating the affinity chromatography result. The finding that the SA compound bound at the dimer interface is consistent with the aforementioned work in *Brucella*, which showed a different SA compound inhibited dimerization of VirB8 (Smith et al., 2012). However, deletion of the gene encoding Tpx did not cause a dramatic effect on the expression of the T3SS. Some regulatory changes were seen, but not the stark reduction in expression one might expect if the target protein was central to the phenotype. Our conclusion from

this work was that inhibition of T3SS activity is due to a polypharmacological effect on proteins involved in metabolism and that there was no single clear target that we could attribute to the phenotype. This highlights the importance of generating deletion mutants for all putative targets at the earliest opportunity. Indeed, following this approach we systematically deleted more of the genes encoding putative target proteins including AdhE, a bi-functional acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase involved in central metabolism. Deletion of the gene encoding AdhE in EHEC caused a marked reduction in T3S and an elevation of flagella production (Beckham et al., 2014), both of which are phenotypes seen when the SA compounds are added to EHEC. However, there were some clear regulatory disparities when comparing the deletion of the gene encoding AdhE and when the SA compounds are added. Specifically, deletion of AdhE caused a post-transcriptional regulation of the LEE, whereas addition of the SA compound showed transcriptional repression. This difference might be attributed to a number of factors. The generation of the defined deletion results in no AdhE protein being produced. In comparison, if the SA compounds affect AdhE activity, they are unlikely to completely block both enzymatic functions. The prediction is that the metabolic flux through the pathways associated with AdhE will be different in the two cases, the deletion compared with enzymatic inhibition. However, by systematically analyzing each putative target of the SA compounds, we have found a metabolic enzyme that is clearly linked to virulence gene expression. Our working model, in agreement with other studies (Martinez-Argudo et al., 2013) suggests that the SA compounds bind several bacterial proteins and affect virulence by disrupting several core metabolic proteins.

CONCLUSIONS

The urgent need for need anti-infective agents is one of the most pressing challenges facing the scientific community. AV agents provide one route to new classes of drugs that are targeted to specific pathogens. The availability of small compound and natural product libraries makes screening for leads relatively simple but the largest challenge remains elucidating the precise MOA. An integrated approach using both classical genetics and biochemical methodologies is most likely to reveal this valuable information and allow researchers to make the jump toward structure based drug design and ultimately, clinically-relevant drugs.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 July 2014; paper pending published: 13 August 2014; accepted: 11 September 2014; published online: 30 September 2014.

*Citation: Beckham KSH and Roe AJ (2014) From screen to target: insights and approaches for the development of anti-virulence compounds. Front. Cell. Infect. Microbiol. **4**:139. doi: 10.3389/fcimb.2014.00139*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 03 November 2014
doi: 10.3389/fcimb.2014.00156

Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism

Stephanie Grubmüller[1†], Kristina Schauer[2†‡], Werner Goebel[3], Thilo M. Fuchs[2] and Wolfgang Eisenreich[1]*

[1] Lehrstuhl für Biochemie, Technische Universität München, Garching, Germany
[2] Abteilung Mikrobiologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL), Technische Universität München, Freising, Germany
[3] Department for Bacteriology, Max von Pettenkofer Institute, Ludwig-Maximilians-Universität, München, Germany

Edited by:
Victor J. Torres, New York University School of Medicine, USA

Reviewed by:
Stephanie M. Seveau, The Ohio State University, USA
John-Demian Sauer, University of Wisconsin-Madison, USA

***Correspondence:**
Wolfgang Eisenreich, Lehrstuhl für Biochemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany
e-mail: wolfgang.eisenreich@ch.tum.de

†Present address:
Lehrstuhl für Hygiene und Technologie der Milch, Tiermedizinische Fakultät, Ludwig-Maximilians-Universität, München, Germany

‡*These authors have contributed equally to this work.*

Intracellular bacterial pathogens (IBPs) are dependent on various nutrients provided by the host cells. Different strategies may therefore be necessary to adapt the intracellular metabolism of IBPs to the host cells. The specific carbon sources, the catabolic pathways participating in their degradation, and the biosynthetic performances of IBPs are still poorly understood. In this report, we have exploited the technique of ^{13}C-isotopologue profiling to further study the carbon metabolism of *Listeria monocytogenes* by using the EGDe wild-type strain and mutants (defective in the uptake and/or catabolism of various carbon compounds) replicating in J774A.1 macrophages. For this goal, the infected macrophages were cultivated in the presence of [1,2-$^{13}C_2$]glucose, [U-$^{13}C_3$]glycerol, [U-$^{13}C_3$]pyruvate, [U-$^{13}C_3$]lactate, or a mix of [U-^{13}C]amino acids. GC/MS-based isotopologue profiling showed efficient utilization of amino acids, glucose 6-phosphate, glycerol, and (at a low extent) also of lactate but not of pyruvate by the IBPs. Most amino acids imported from the host cells were directly used for bacterial protein biosynthesis and hardly catabolized. However, Asp was *de novo* synthesized by the IBPs and not imported from the host cell. As expected, glycerol was catabolized via the ATP-generating lower part of the glycolytic pathway, but apparently not used for gluconeogenesis. The intermediates generated from glucose 6-phosphate in the upper part of the glycolytic pathway and the pentose phosphate shunt likely serve primarily for anabolic purposes (probably for the biosynthesis of cell wall components and nucleotides). This bipartite bacterial metabolism which involves at least two major carbon substrates—glycerol mainly for energy supply and glucose 6-phosphate mainly for indispensible anabolic performances—may put less nutritional stress on the infected host cells, thereby extending the lifespan of the host cells to the benefit of the IBPs.

Keywords: bacterial metabolism, bacterial pathogensis, intracellular bacteria, isotopic tracers, isotopologue profiling, *Listeria monocytogenes*

INTRODUCTION

Listeria monocytogenes is a Gram-positive, food-borne pathogen that can cause systemic infections in immune compromised, pregnant or elder persons (for recent reviews, see Velge and Roche, 2010; Camejo et al., 2011; Fuchs et al., 2012; Mostowy and Cossart, 2012; Cossart and Lebreton, 2014). Typical symptoms of listeriosis are septicaemia, (encephalo)-meningitis, placentitis, and stillbirth. The facultative intracellular pathogen is taken up by professional phagocytes, like macrophages and dendritic cells. It can also actively invade (with the help of the internalins A and/or B) non-phagocytic cells, such as epithelial cells, fibroblasts or endothelial cells (Dussurget et al., 2004; Lecuit, 2005; Hamon et al., 2006). The subsequent escape of the bacteria from the enclosing vacuole depends on listeriolysin and two phospholipases (PlcA and PlcB). Within the cytosol of the host cell, *L. monocytogenes* efficiently multiplies with a generation time of approximately 1 h and spreads into neighboring host cells (Hamon et al., 2012).

The growth of intracellular bacterial pathogens (IBPs) depends on the efficient usage of carbon and nitrogen nutrients from the host. The metabolism of mammalian host cells involves hundreds if not thousands of metabolites that could be used by intracellular bacteria as potential nutrients. The major catabolic reactions of the host cells occur in the cytosol (e.g., glycolysis, pentose-phosphate pathway) or in the mitochondria (e.g., citrate cycle, β-oxidation of fatty acids, glutaminolysis), but metabolites can also be exchanged between these compartments. The anabolic pathways (formation of glucose, amino acids, nucleotides, and fatty acids) mainly take place in the cytosol. Therefore, intracellular bacteria living in the cytosolic compartment of host cells

could, in principle, efficiently recruit carbohydrates, amino acids, glycerol, lactate, fatty acids and many other metabolites for their purposes.

Nevertheless, the complex life style of IBPs requires specific metabolic adaptations aimed to optimize survival and proliferation of the pathogen within the different compartments of the host cells. Most features of this complex metabolic interplay between the IBPs and the host cells are still unknown. Even the basic nutrients and their pathways used by the IBPs have not yet been completely elucidated.

Based on the genome sequence, *L. monocytogenes* possesses complete glycolytic and pentose-phosphate pathways (Glaser et al., 2001). Hence, glucose and glucose-6P can in principle be easily catabolized to pyruvate by either of the two pathways. The citrate cycle lacks oxoglutarate dehydrogenase and malate dehydrogenase (Eisenreich et al., 2006). Therefore, and because external Asp can obviously not be imported by *L. monocytogenes*, the formation of oxaloacetate by intracellular *L. monocytogenes* depends fully on the carboxylation of pyruvate catalyzed by pyruvate carboxylase (PycA) (Schär et al., 2010). C_3- and C_4-substrates, deriving from glycolytic and TCA cycle intermediates of the host cell could also be taken up by *L. monocytogenes* and may serve as energy source and could be used for gluconeogenesis. Not surprisingly, *L. monocytogenes* therefore multiplies in defined minimal media (Premaratne et al., 1991; Tsai and Hodgson, 2003; Stoll et al., 2008) either containing a PTS-carbohydrate (e.g., glucose, mannose, cellobiose), or glycerol as sole carbon source (Schneebeli and Egli, 2013). Moreover, *L. monocytogenes* is able to utilize glucose 6-phosphate (glucose-6P) which is transported by the phosphate antiporter UhpT. The encoding *uhpT* gene is under the control of PrfA, the central transcriptional activator for most listerial virulence genes (Chico-Calero et al., 2002; de las Heras et al., 2011).

In defined media, *L. monocytogenes* requires Leu, Ile, Val, Met, Cys, and Arg for growth (Premaratne et al., 1991), although all genes encoding the enzymes for the biosynthesis of these amino acids are present on the genome (Glaser et al., 2001) and expressed under suitable conditions (Joseph et al., 2006; Lobel et al., 2012). The requirement for these amino acids may therefore reflect a severe shortage of necessary precursors, especially pyruvate and sulfide, respectively, when growing in minimal media.

The current knowledge about the metabolism of *L. monocytogenes* growing within host cells is still fragmentary and mainly based on ^{13}C-isotopologue, transcriptome, and mutant analyses using the established macrophage cell line J774A.1 as host cell (Chatterjee et al., 2006; Joseph et al., 2006; Eylert et al., 2008; Schauer et al., 2010; Donaldson et al., 2011; Lobel et al., 2012). Recently, metabolic studies were also performed with primary murine bone marrow-derived macrophages (BMM) as host cells for infection with *L. monocytogenes* (Gillmaier et al., 2012). Intracellular *L. monocytogenes* replicating in both types of host cells yielded—in the presence of [U-$^{13}C_6$]glucose—^{13}C-isotopologue profiles in the protein-derived amino acids that indicated the usage of glucose-6P and/or glycerol as preferred carbon sources (Eylert et al., 2008; Gillmaier et al., 2012). Indeed, mutants defective in the uptake or catabolism of these carbon substrates displayed reduced growth rates under intracellular conditions. Moreover, genes encoding the transport of glucose-6P (i.e., *uhpT*), as well as the uptake and catabolism of glycerol (i.e., *glpF*, *glpK*, and *glpD*) were found to be up-regulated under intracellular conditions compared to *L. monocytogenes* growing in a defined medium (Joseph et al., 2006; Lobel et al., 2012).

However, uptake and the possible catabolism of amino acids, glycerol and other C_3 compounds by intracellular *L. monocytogenes* were not in the focus of the earlier studies. We therefore approached in the present study these questions in considerable detail using ^{13}C-glycerol, ^{13}C-lactate, ^{13}C-pyruvate, or ^{13}C-amino acids as tracer substrates in infection assays with *L. monocytogenes* and J774.1 macrophages as host cells. Results from ^{13}C-isotopologue profiling underline the important role of glycerol and glucose-6P as major carbon substrates for energy generation and anabolic performances, respectively, and show that most amino acids provided by the host cell are directly used for protein biosynthesis and hardly catabolized.

MATERIALS AND METHODS

MATERIALS

[U-$^{13}C_6$]glucose, [1,2-$^{13}C_2$]glucose, [U-$^{13}C_3$]glycerol, [U-$^{13}C_3$]pyruvate, [U-$^{13}C_3$]lactate, and a mixture of [U-^{13}C]amino acids (ISOGRO ^{13}C-Powder Growth Medium) were purchased from Sigma-Aldrich (Steinheim, Germany).

BACTERIAL STRAINS AND GROWTH CONDITIONS

Strains used in this study are listed in **Table 1**. *Escherichia coli* strain DH5α was used for cloning, and pLSV101 as construction vector for mutagenesis. *E. coli* strains were cultivated in Luria-Bertani (LB) medium at 37°C. *L. monocytogenes* wild type strain EGDe and mutant strains were grown under aerobic conditions in brain heart infusion (BHI) broth or defined minimal medium (MM). If appropriate, MM was supplemented with 10 mM [1,2-$^{13}C_2$]glucose. When necessary, media were supplemented with erythromycin to final concentrations of 5 μg/ml for *L. monocytogenes* or 300 μg/ml for *E. coli*. To determine growth curves, aliquots were retrieved at regular intervals, and the optical density at 600 nm (OD_{600}) was determined using a spectrophotometer. For infection of cells, *L. monocytogenes* strains were grown to the late exponential phase (OD_{600} of 1.0) at 37°C in BHI medium, washed twice with sterile phosphate buffered saline (PBS), re-suspended in 20% (v/v) glycerol in PBS, and stored at −80°C.

GENERAL TECHNIQUES

Polymerase chain reaction (PCR) amplifications, cloning procedures, isolation of chromosomal DNA and DNA manipulations were carried out according to standard protocols (Sambrook and Russell, 2001). The *Listeria* homepage of the Pasteur Institute (http://genolist.pasteur.fr/ListiList/) and the NCBI database (http://www.ncbi.nlm.nih.gov/) were used for sequence comparison.

CONSTRUCTION OF DELETION MUTANTS

Deletion mutants of *L. monocytogenes* EGDe were constructed as described previously (Joseph et al., 2006). Briefly, pLSV101

Table 1 | Strains and plasmids used in this study.

Name	Characterization	References
EGDe	*L. monocytogenes* Sv 1/2a, wild type, derivative of EGD	
DH5α	*E. coli: deoR endA1 gyrA96 hsdR17*(r_k-m_{k+}) *recA1 relA1 supE44* λ*thi-1* Δ(*lacZYA-argFV169*)	Hanahan, 1983
EGDe ΔC3	In-frame deletion of *glpD* (EGDe Δlmo1293) and *dhaK-1*/*dhaK-2* encoding DHA kinases (EGDe Δlmo0347/lmo0348/ Δlmo2695/Δlmo2696)	Mertins, unpublished; Eylert et al., 2008
EGDe ΔC3Δ*uhpT*	EGDeΔC3 with in-frame deletion of *hpt*	Mertins, unpublished; this study
EGDe Δ*uhpT*	In-frame deletion of *hpt*	Chico-Calero et al., 2002
GDe Δ*ldhD*	EGDe with in-frame deletion of *ldh* (lmo0210)	This study
pLSV101	Temperature-sensitive shuttle vector; Em^R	Joseph et al., 2006
pLSV101-*hpt*del	Deletion plasmid for *uhpT*	Joseph et al., 2006
pLSV101-*ldh*del	Deletion plasmid for *ldh*	This study

vector constructs were cloned which carried approximately 0.8–1 kb *L. monocytogenes* EGDe DNA fragments, representing the upstream and downstream sequences of the gene to be deleted. These plasmids were transformed into *L. monocytogenes* EGDe by electroporation, and the bacteria were incubated on erythromycin containing BHI plates at 30°C for 2 day. One single erythromycin-resistant colony was resuspended in 1 ml BHI and 20 µl of this suspension were plated on pre-warmed BHI agar plates containing 5 µg/ml erythromycin, and incubated at 42°C for 2 days. Erythromycin-resistant bacteria growing at 42°C harboring the chromosomally integrated plasmid were selected and subcultivated a few times in BHI without erythromycin at a permissive temperature of 30°C. Erythromycin-sensitive bacteria were screened by PCR for gene deletion from a double-crossover recombination event.

CELL INFECTION ASSAYS

Mouse monocyte macrophages (J774A.1; ACC 170) were received from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultured at 37°C and 5% CO_2 in RPMI 1640 medium with glutamine (Biochrom KG, Berlin, Germany) and 10% heat-inactivated fetal calf serum (FCS; Perbio Science, Bonn, Germany). Cells were replated twice a week using a dilution rate of 1:3.

REPLICATION ASSAYS

Mouse monocyte macrophages (J774A.1 cells) were seeded at a density of 1×10^5 cells per well in 24-well tissue culture plates (Biochrom) 24 h prior to infection. The cells were washed twice with 0.5 ml of pre-warmed PBS containing 100 mg/ml $MgCl_2$ and 100 mg/ml $CaSO_4$(PBS-$Mg^{2+}Ca^{2+}$), respectively, and infected at a multiplicity of infection (MOI) of 1 bacterium per cell for 45 min. Then, cells were washed with pre-warmed PBS-$Mg^{2+}Ca^{2+}$ ($t = 0$ h) before they were overlaid with 0.5 ml of RPMI 1640 containing 50 µg/ml gentamicin and incubated for 1 h at 37°C in the presence of 5% CO_2. Subsequently, the medium was replaced with fresh medium containing 10 µg/ml gentamicin. At intervals, cells were washed with cold PBS-$Mg^{2+}Ca^{2+}$, before the monolayer was lysed with 1 ml of cold Triton X-100 (0.1%). Cell lysates were first vortexed for 30 s, and viable bacterial counts of intracellular bacteria were determined by plating serial dilutions on BHI agar plates.

ISOTOPOLOGUE PROFILING OF INTRACELLULAR BACTERIA

Feeding of living organisms with ^{13}C-labeled glucose or other tracers, followed by the determination of the resulting isotopologue patterns in key metabolites (e.g., amino acids) from the bacteria and the host cell fraction, helps to identify substrates and metabolic pathways of intracellular bacteria (Eylert et al., 2008; Gillmaier et al., 2012; Heuner and Eisenreich, 2013; Schunder et al., 2014). Briefly, using this method, ^{13}C-labeled substrates (e.g., glucose) are supplied to host cells infected by intracellular bacteria. After uptake of the labeled supplement into the host cell, the tracer is further shuffled into the bacteria where it is utilized for catabolic or anabolic reactions. By these reactions, the label is distributed through the bacterial metabolic network and gives rise to specific isotopologue mixtures in products. Notably, however, with this experimental setting the original labeled precursor could also by first converted into an intermediate or product by the host metabolism that is then incorporated and utilized by the intracellular bacteria. For example, starting from a ^{13}C-glucose supplement, labeled pyruvate, lactate, glycerol, alanine and more metabolic products could be generated by the host cell finally serving as substrate(s) for the intracellular bacteria. Nevertheless, the careful comparison of the labeling patterns in multiple bacterial and corresponding host metabolites typically suggests the nature of the preferred bacterial growth substrate and its pathways under intracellular conditions. Frequently, however, these hypotheses must be verified by e.g., corresponding labeling experiments using bacterial mutants defective in the uptake and utilization of the potential substrate.

LABELING OF J774A.1 CELLS WITHOUT INFECTION

Cells were seeded in 6 flasks (690 ml/150 cm^2) and grown to semiconfluence (2×10^7 cells per flask) at 37°C in the presence of 5% CO_2. Cells were then washed once with pre-warmed PBS-$Mg^{2+}Ca^{2+}$, before they were overlaid with RPMI 1640 (Invitrogen, Darmstadt, Germany) without unlabeled glucose, but containing 10 mM [U-$^{13}C_6$]glucose or 20–40 mM [U-$^{13}C_3$]glycerol. After incubation with the labeled tracer for 6 h, the cells were washed with 10 ml cold PBS-$Mg^{2+}Ca^{2+}$, overlaid per flask with 10 ml PBS containing 50 µg/ml chloramphenicol,

5 μg/ml tetracycline and 20 mM NaN_3, and shock-frozen for 20 min at −80°C. The frozen suspension was then thawed to room temperature. Cells were harvested by centrifugation at 1000 rpm for 10 min at 4°C. Prior to protein hydrolysis, both supernatant and pelleted cell debris were stored at −80°C.

LABELING OF J774A.1 INFECTED BY *L. MONOCYTOGENES* EGDe

Bacterial infection was performed in 6 flasks (690 ml/150 cm^2) per cell line when the cells were semiconfluent (2×10^7 cells per flask). One day prior to the labeling experiment, the colony-forming units per milliliter (cfu/ml) of *L. monocytogenes* EGDe stock solutions were determined in order to exactly adjust the multiplicity of infection. Before infection, the host cells were washed with 10 ml pre-warmed PBS-$Mg^{2+}Ca^{2+}$ and then overlaid for 1 h with 20 ml of inoculum per flask composed of FCS-free RPMI 1640 with unlabeled glucose and *L. monocytogenes* (MOI = 25). In order to eliminate extracellular bacteria, the infected cells were then washed with 10 ml of pre-warmed PBS-$Mg^{2+}Ca^{2+}$ and overlaid with 20 ml of FCS-free RPMI 1640 with unlabeled glucose containing 50 μg/ml gentamicin and incubated for 15 min at 37°C in the presence of 5% CO_2. After this time, 20 ml of FCS-free RPMI 1640 containing the ^{13}C-tracer source and 50 μg/ml gentamicin to kill non-invaded bacteria were added and incubated for 45 min. Then, the medium was replaced with RPMI 1640 containing 10% FCS, 10 μg/ml gentamicin and one of the ^{13}C-tracers specified below. The final concentrations were 10 mM [1,2-$^{13}C_2$]glucose, 20 mM [U-$^{13}C_3$]glycerol, 20 mM [U-$^{13}C_3$]pyruvate, 20 mM [U-$^{13}C_3$]lactate, or 2g/l of [U-^{13}C]amino acid mix (ISOGRO ^{13}C-Powder Growth Medium).

After incubation for 5 h at 37°C in the presence of 5% CO_2, the cells were washed with 10 ml of cold PBS-$Mg^{2+}Ca^{2+}$, overlaid per flask with 10 ml of PBS containing 50 μg/ml chloramphenicol, 5 μg/ml tetracycline and 20 mM NaN_3, and shock-frozen for 20 min at −80°C. The frozen suspension was then thawed to room temperature. In order to remove eukaryotic cell debris, the suspension was centrifuged at 1000 rpm for 10 min at 4°C. For separation of bacteria from soluble eukaryotic protein, the supernatant was centrifuged again at 6000 rpm for 10 min at 4°C. In order to wash the bacterial cells, the pellet was resuspended in 5 ml of RIPA-buffer containing 10 mM Tris (pH = 7.2), 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% deoxycholic acid and 0.1% SDS and centrifuged at 6000 rpm for 10 min at 4°C. Prior to protein hydrolysis, the supernatant containing soluble eukaryotic proteins (in the following "J774A.1 protein fraction"), and the bacterial pellet were stored at −80°C. Owing to the fact that certain amino acids were only labeled in the bacterial fraction, but not in the J774A.1 protein fraction, cross contamination appeared to be <10%.

PROTEIN HYDROLYSIS AND AMINO ACID DERIVATIZATION

Bacterial cells (approximately 10^9 cells) or ca. 1 mg of the freeze-dried host protein fraction were hydrolyzed in 0.5 ml of 6 M hydrochloric acid. The mixture was heated at 105°C for 24 h under an inert atmosphere. Under these conditions, protein-derived Gln and Asn were converted into Glu and Asp, respectively. Trp and Cys were destroyed by this treatment. The hydrolyzate was placed on a column of Dowex 50 W × 8 (H^+ form, 200–400 mesh, 5 × 10 mm). The column was washed twice with 500 μl of water and was developed with 1 ml of 4 M ammonium hydroxide. The eluate was dried under a stream of nitrogen, and the residue was dissolved in 50 μl of dry acetonitrile. A total of 50 μl of N-(tert-butyldimethyl-silyl)-N-methyl-trifluoroacetamide containing 1% tert-butyl-dimethyl-silylchlorid (Sigma) was added. The mixture was kept at 70°C for 30 min. The resulting mixture of tert-butyl-dimethylsilyl derivatives (TBDMS) of amino acids was used for GC/MS analysis without further work-up. The yields of TBDMS-Arg, -Met, -His, -Lys, and -Tyr were low. Therefore, isotopologue data of theses amino acids are only listed when applicable.

GAS CHROMATOGRAPHY/MASS SPECTROMETRY

GC/MS-analysis was performed on a QP2010 Plus Gas Chromatograph/Mass Spectrometer (Shimadzu, Duisburg, Germany) equipped with a fused silica capillary column (Equity TM-5; 30 m × 0.25 mm, 0.25 μm film thickness; SUPELCO, Bellafonte, PA) and a quadrupol detector working with electron impact ionization at 70 eV. One μl of the solution containing TBDMS amino acids was injected in 1:10 split mode at an interface temperature of 260°C and a helium inlet pressure of 70 kPa. The column was developed at 150°C for 3 min and then with a temperature gradient of 10°C min^{-1} to a final temperature of 260°C that was held for 3 min. With a sampling rate of 0.5 s, selected ion monitoring was used. Data were collected using the GC/MS solution software (Shimadzu). All samples were measured three times. ^{13}C-Excess and isotopologue abundances were calculated as described before (Lee et al., 1991; Eylert et al., 2008) including: (i) determination of the TBDMS-derivate spectrum of unlabeled amino acids, (ii) determination of mass isotopologue distributions of labeled TBDMS-amino acids, and (iii) correction of ^{13}C-incorporation concerning the heavy isotope contributions due to the natural abundances in the TBDMS-moiety and the amino acid atoms.

RESULTS

CHARACTERIZATION OF *L. MONOCYTOGENES* MUTANTS DEFECTIVE IN THE UPTAKE AND CATABOLISM OF GLYCEROL, GLUCOSE 6-PHOSPHATE OR LACTATE

Previous studies (Eylert et al., 2008; Joseph et al., 2008) had shown that glucose-6P and glycerol are important carbon sources for intracellularly replicating *L. monocytogenes*. However, these studies did not rule out the use of amino acids or other C_3-carbon sources, such as lactate or pyruvate, as additional catabolic carbon sources, nor did these studies show whether glycerol is used for gluconeogenesis. To fill these important information gaps, we made use in the present study of the ^{13}C-isotopologue technique to determine the fate of externally added ^{13}C-labeled amino acids, ^{13}C-glycerol,^{13}C-lactate and ^{13}C-pyruvate in *L. monocytogenes* EGDe growing within J774A.1 macrophages. In addition to the *L. monocytogenes* EGDe wild-type strain, we included in this study a previously constructed ΔC3 mutant, incapable of utilizing glycerol and dihydroxyacetone due to the loss of glycerol 3-phosphate dehydrogenase and dihydroxyacetone kinases, the Δ*uhpT* mutant, deficient in glucose-6P uptake, and the newly constructed Δ*ldh* mutant, unable to convert lactate into pyruvate

due to the lack of lactate dehydrogenase (**Table 1**). The growth rates of these mutants were similar to that of the wild-type strain when cultured in brain heart infusion medium (BHI) indicating that these mutations did not affect bacterial growth in rich medium (data not shown). These *L. monocytogenes* strains were then infected (at a MOI of 10 bacteria per cell) in J774A.1 cells, cultured in RPMI medium containing 2 mM glutamine and 10 mM glucose or 20 mM glycerol. At the given time points, intracellular bacteria were isolated and counted.

In the presence of 10 mM glucose, growth of the ΔC3, Δ*uhpT*, and ΔC3Δ*uhpT* mutants was inhibited by 20–50% (**Figure 1A**) which is in line with previous results for the Δ*uhpT* and ΔC3 mutants (Chico-Calero et al., 2002; Joseph et al., 2008). In contrast, growth of the Δ*ldh* mutant was unaffected or even slightly enhanced as compared to the wild-type strain. When glucose was replaced by 20 mM glycerol in the RPMI infection medium, the inhibition of intracellular growth of the ΔC3 mutant was significantly higher (up to 60%) and even the Δ*uhpT* mutant showed reduced growth compared to the wild-type strain (**Figure 1B**).

These data confirmed the role of glycerol and glucose-6P as major carbon sources for intracellularly growing *L. monocytogenes*, but also showed that, in the absence of these carbon substrates, the listeriae are still able to replicate within the host cells suggesting a switch to alternative carbon sources. Possible candidates are amino acids which can be efficiently taken up by intracellular listeriae from the host cells (Eylert et al., 2008), glucose (or other glycolytic carbohydrates) and some of their catabolic intermediates (e.g., pyruvate or lactate).

AMINO ACIDS IMPORTED FROM THE HOST CELLS ARE DIRECTLY USED FOR LISTERIAL PROTEIN BIOSYNTHESIS BUT HARDLY CATABOLIZED

To better understand the role of host amino acids in the intracellular metabolism of *L. monocytogenes*, we supplied the infected J774A.1 macrophages (in the presence of unlabeled glucose and Gln) with a mixture of uniformly ^{13}C-labeled amino acids (containing all amino acids, except for Asn, Gln, Cys, and Trp) (see Materials and Methods). After 6 h of growth, Ala, Asp, Glu, Gly, Ile, Leu, Phe, Pro, Ser, Thr, and Val were isolated from the acidic hydrolysates of the intracellular bacteria, silylated and analyzed by GC/MS. With the exception of Asp and Glu, the amino acids had acquired substantial amounts of ^{13}C-label (about 10% ^{13}C-excess, boxes in red or orange) (**Figure 2A**). Asp and Glu showed only weak ^{13}C-enrichments (about 1% ^{13}C-excess, boxes in green). Some of the amino acids could in principle also be metabolized by *L. monocytogenes* (e.g., Ala, Asp, Glu, Gly, Ile, Leu, Val, Ser, and Thr). However, all of these amino acids showed, again with the exception of Asp and Glu, the virtually same ^{13}C-isotopologue profiles as the respective amino acids in the applied tracer mix (**Figure 2B**). The unaltered ^{13}C-isotopologue patterns of the labeled amino acids indicate that these amino acids were directly incorporated into listerial protein and not significantly catabolized or *de novo* synthesized by the intracellular bacteria.

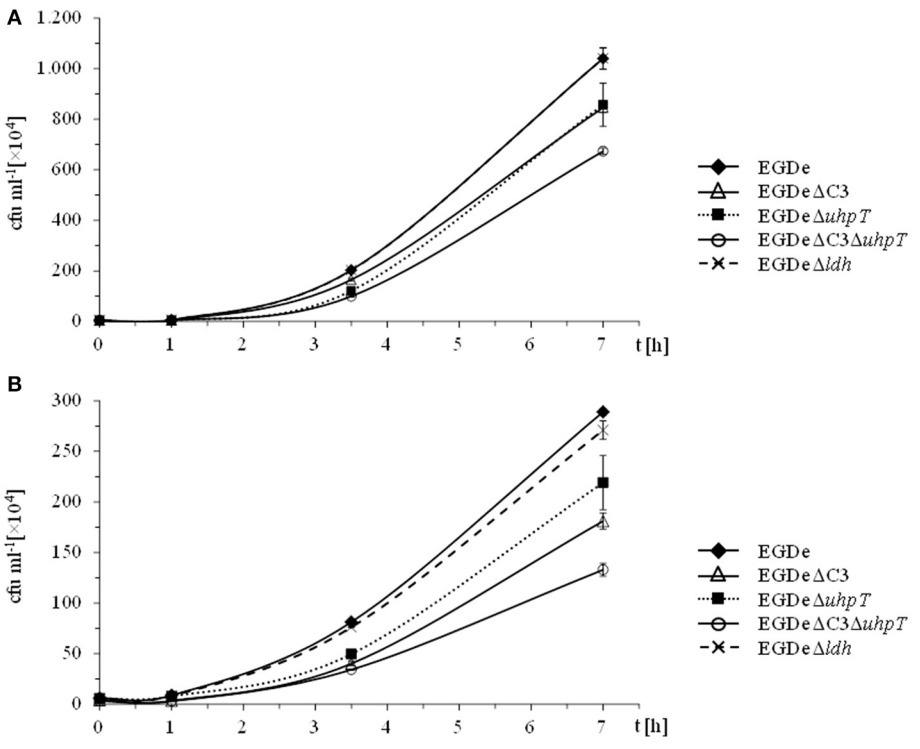

FIGURE 1 | Intracellular replication behavior of *L. monocytogenes*. *L. monocytogenes* EGDe and its mutants were used to infect J774A.1 macrophages in the presence of 10 mM glucose **(A)** or 20 mM glycerol **(B)**. 45 min after infection, extracellular bacteria were removed by washing with PBS and adding gentamycin. At the indicated intervals, intracellular *L. monocytogenes* were counted by disruption of the monolayer, cell lysis and plating of the supernatant on BHI.

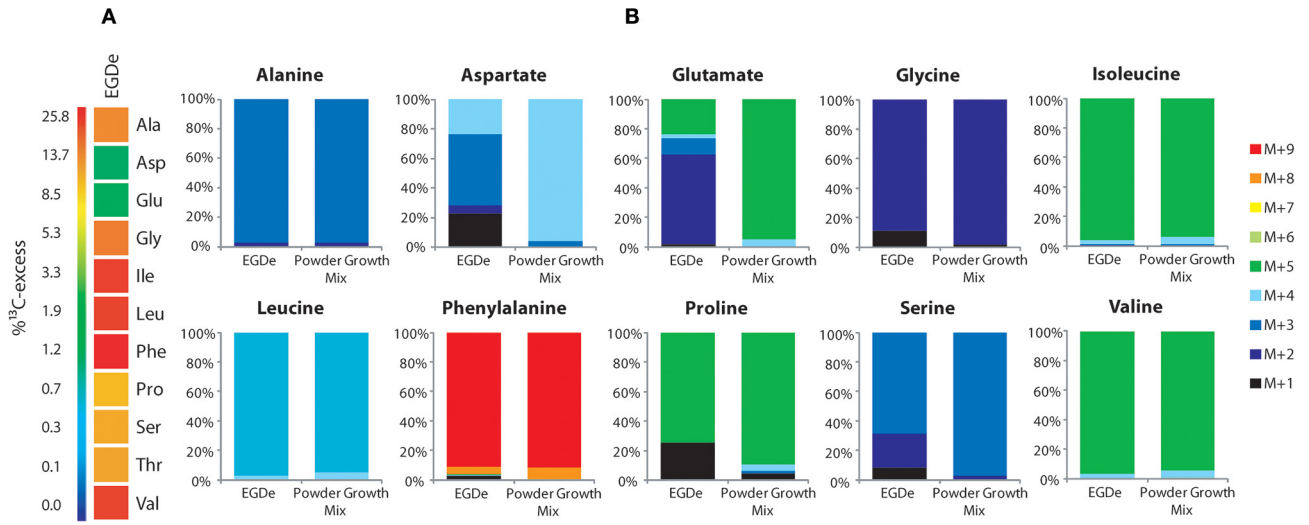

FIGURE 2 | Isotopologue profiling of protein-derived amino acids from *L. monocytogenes* grown in J774A.1 macrophages labeled with a mix of [U-^{13}C]-amino acids. (A) ^{13}C-Excess (in %) of amino acids from *L. monocytogenes* EGDe wild-type. The color map indicates ^{13}C excess from 0 to 25% in quasi-logarithmic form. **(B)** Isotopologue profiles in listerial amino acids from the same experiment with EGDe wild-type. For comparison, the isotopologue profiles of the [U-^{13}C]-amino acid mix (^{13}C-Powder Growth Mix) which was supplied to the infected host cells are also shown. The colored columns indicate the relative fractions (in %) of the ^{13}C-isotopologues carrying one to nine ^{13}C-atoms (M+1 to M+9). For the numerical values, see Supplemental Material.

The fraction of the fully ^{13}C-labeled isotopologue in listerial Asp (i.e., M+4 which was introduced by the externally supplied Asp) was very low, and most of the listerial Asp consisted of the $^{13}C_3$-Asp isotopologue (**Figure 2B**). This suggests that intracellular *de novo* synthesis of Asp occurred from $^{13}C_3$-oxaloacetate generated by pyruvate carboxylase-mediated carboxylation of $^{13}C_3$-pyruvate (possibly deriving from the supplied $^{13}C_3$-Ala). These results were not unexpected as previous studies showed that *L. monocytogenes* is unable to take up external Asp (Schär et al., 2010).

The small ^{13}C-labeled amount of listerial Glu carried as a major fraction the $^{13}C_2$-Glu isotopologue (**Figure 2B**) reflecting its *de novo* production from$^{13}C_2$-oxoglutarate probably deriving from $^{13}C_3$-oxaloacetate generated in the TCA cycle. The lack of a larger fraction of uniformly ^{13}C-labeled Glu (corresponding to the externally added ^{13}C-labeled Glu) is probably due to the large excess of unlabeled Gln in the applied Gln-containing RPMI medium (see Materials and Methods).

Together, the data show that most amino acids imported from the host cell were directly incorporated into bacterial protein and hardly catabolized. Only Asp and (to a minor extent) Glu were synthesized *de novo* by intracellular *L. monocytogenes*, but mainly via intermediates deriving from unlabeled carbon substrates (probably via glycerol and/or glucose-6P) and only to a minor extent from degradation products (e.g., $^{13}C_3$-pyruvate) of the supplied ^{13}C-labeled amino acids (possibly from $^{13}C_3$-Ala).

GLYCEROL IS EFFICIENTLY CATABOLIZED BY INTRACELLULAR *L. MONOCYTOGENES* BUT NOT USED FOR GLUCONEOGENESIS

Glycerol is an important carbon substrate for intracellular *L. monocytogenes* as shown by several reports (Eylert et al., 2008; Joseph et al., 2008; Lobel et al., 2012). However, none of these previous studies addressed the question whether glycerol is also used for gluconeogenesis. To answer this crucial item, we applied [U-$^{13}C_3$]glycerol as a tracer in assays with *L. monocytogenes*-infected J774A.1 cells and followed the ^{13}C-incorporation into amino acids. In control experiments, we noticed that the complete replacement of glucose by glycerol in the RPMI medium was detrimental to J774A.1 macrophages. But J774A.1 cultures could be kept without damage for several days in RPMI/glutamine medium containing 15 mM glycerol and 2.5 mM glucose. However, infected macrophages cultured in this medium yielded only unlabeled listerial and host cell amino acids possibly due to inhibition of glycerol uptake by the host cells in the presence of glucose. Therefore, we used a modified protocol where the J774A.1 macrophages (pre-grown in RPMI medium with unlabeled glucose for three days) were transferred into fresh RPMI medium containing 20 mM [U-$^{13}C_3$]glycerol, but no glucose. The J774A.1 cells were then immediately infected with the EGDe wild-type and appropriate mutant strains, and cultivated for 6 h; the infected host cells were then harvested and processed as described.

Under these conditions, ^{13}C label deriving from [U-$^{13}C_3$]glycerol was found in the following listerial amino acids in different quantities: Ala (12% ^{13}C-excess) > Asp (8%) > Glu (5%) > Ser (4%) > Pro (1%) > Gly (0.7%) > Val (0.5%) (**Figure 3A**, column 2). Leu, Ile, Phe, Tyr, and His were detected in unlabeled form. Incorporation of ^{13}C into Ala, Asp, Glu, and Ser from the corresponding host cells was observed in much lower amounts (2.5 − 1% ^{13}C-excess). This suggests that external glycerol was taken up by the host cells and shuffled into the intracellular listeriae without being catabolized in the host cells in an appreciable amount.

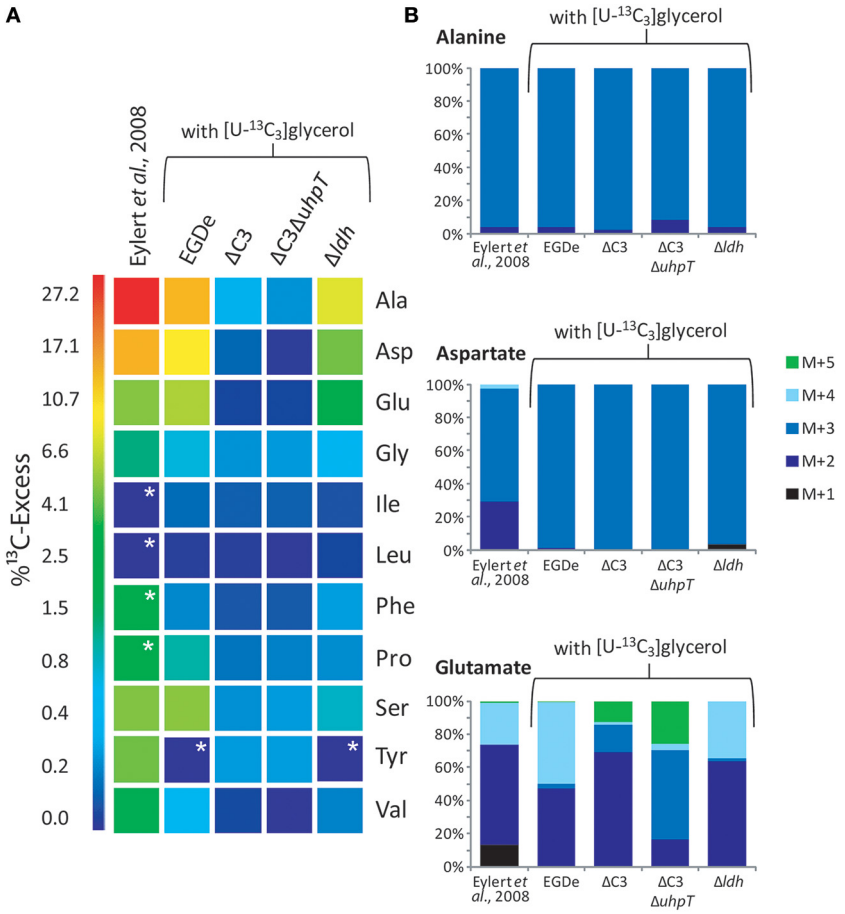

FIGURE 3 | Analysis of protein-derived amino acids from *L. monocytogenes* grown in J774A.1 macrophages labeled with 10 mM [U-$^{13}C_6$]glucose (Eylert et al., 2008) or 10 mM [U-$^{13}C_3$]glycerol. (A) ^{13}C-Excess (in % as a color map) of amino acids from *L. monocytogenes* EGDe. Column 1: from the labeling experiment with [U-$^{13}C_6$]glucose and EGDe wild-type (Eylert et al., 2008), column 2: from the labeling experiment with [U-$^{13}C_3$]glycerol and EGDe wild-type (mean value of three replicates), column 3: from the labeling experiment with [U-$^{13}C_3$]glycerol and the EGDe ΔC3 mutant, column 4: from the labeling experiment with [U-$^{13}C_3$]glycerol and the EGDe ΔC3Δ*uhpT* mutant, and column 5: from the labeling experiment with [U-$^{13}C_3$]glycerol and the EGDe Δ*ldH* mutant. Boxes with white asterisks indicate high standard deviations in the measurement of the overall ^{13}C-enrichments. **(B)** Isotopologue profiles in listerial Ala, Asp, and Glu from the same experiments. The colored columns indicate the relative fractions (in %) of the ^{13}C-isotopologues (M+1 to M+5). For the numerical values, see Supplemental Material.

Notably, a similar set of bacterial amino acids acquired ^{13}C-label in the infection experiments with [U-$^{13}C_6$]glucose (Eylert et al., 2008) (**Figure 3A**, column 1), and the ^{13}C-isotopologue patterns were similar in corresponding amino acids from the experiments with [U-$^{13}C_6$]glucose (Eylert et al., 2008) or [U-$^{13}C_3$]glycerol, as shown in **Figure 3B** for Ala, Asp, and Glu. From [U-$^{13}C_3$]glycerol, Ala was again $^{13}C_3$-labeled at high abundance, suggesting that [U-$^{13}C_3$]glycerol was efficiently converted to [U-$^{13}C_3$]pyruvate that acted as precursor for $^{13}C_3$-alanine. Listerial Asp consisted almost exclusively of the $^{13}C_3$-isotopologue obviously via [1,2,3-$^{13}C_3$]oxaloacetate derived from [U-$^{13}C_3$]pyruvate and unlabeled CO_2 and catalyzed by pyruvate carboxylase (PycA). ^{13}C-Labeled Glu showed high abundance for $^{13}C_2$- and $^{13}C_4$-isotopologues, the formation of which can be explained by the assembly of unlabeled or [1,2,3-$^{13}C_3$]-labeled oxaloacetate with unlabeled or [1,2-$^{13}C_2$]-labeled acetyl-CoA (obtained from [U-$^{13}C_3$]pyruvate) in the incomplete TCA cycle resulting in the formation of [4,5-$^{13}C_2$]-, [2,3-$^{13}C_2$]-, and [2,3,4,5-$^{13}C_4$]α-oxoglutarate isotopologues, respectively, which are finally transaminated to the corresponding Glu isotopologues.

In summary, external [U-$^{13}C_6$]glucose and [U-$^{13}C_3$]glycerol yielded in the intracellular *L. monocytogenes* a similar set of ^{13}C-labeled amino acids with the same ^{13}C-isotopologue patterns. In the case of the experiments with [U-$^{13}C_3$]glycerol, all of the ^{13}C-labeled amino acids derive from intermediates generated in the lower part of the glycolytic pathway or the TCA cycle while amino acids requiring intermediates from the pentose phosphate shunt (e.g., His, Phe, and Tyr) were not ^{13}C-labeled. These data suggest that both external carbon substrates converge at the level of the same C_3-glycolytic intermediate (most likely glyceraldehyde 3-phosphate/dihydroxyacetone 3-phosphate) feeding the lower part of the glycolytic pathway and the TCA cycle. These catabolic pathways include all reactions leading to the intermediates required for the observed *de novo* synthesized amino acids

and to the generation of ATP by substrate phosphorylation, as well as to $NADH/H^+$ necessary for ATP production by oxidative phosphorylation via the electron transfer chain.

Infections of J774A.1 cells with the ΔC3 mutants under these conditions further confirmed the usage of glycerol as major carbon source for intracellular *L. monocytogenes* as the rate of ^{13}C-incorporation into Ala, Asp, and Glu dropped in these mutants by a factor of more than 10 compared to the wild-type strain (**Figure 3A**, columns 3 and 4). This result also showed that ^{13}C-labeled glycerol was channeled into the listerial metabolism mainly by glycerol phosphate dehydrogenase which is defective in the ΔC3 mutants.

There still remained, however, a low but reproducible ^{13}C-incorporation into Ala, Gly, Pro, and Ser in the ΔC3 mutants (approximately 0.3% ^{13}C-enrichments) in the presence of external [U-$^{13}C_3$]glycerol. These ^{13}C-labeled amino acids could be produced in the host cells and transported into the intracellular bacteria. Alternatively, a fraction of [U-$^{13}C_3$]glycerol could be metabolized in the host cells to other C_3-components (e.g., [U-$^{13}C_3$]pyruvate, [U-$^{13}C_3$]lactate) which are then transported into the intracellular listeriae and subsequently converted into these amino acids.

PYRUVATE AND LACTATE ARE INEFFICIENT SUBSTRATES FOR INTRACELLULAR *L. MONOCYTOGENES*

In a defined minimal medium, *L. monocytogenes* EGDe is unable to grow in the RPMI medium supplemented with pyruvate or lactate as carbon and energy source (data not shown). To determine whether these two carbon substrates can nevertheless be used by *L. monocytogenes* as supportive carbon substrates under intracellular conditions, *L. monocytogenes* EGDe-infected J774A.1 cells were supplied with 20 mM [U-$^{13}C_3$]pyruvate or 20 mM [U-$^{13}C_3$]lactate in addition to equimolar amounts of glucose in the RPMI medium. In the presence of [U-$^{13}C_3$]pyruvate, Ala, Asp, and Glu from the *L. monocytogenes*-infected J774A.1 macrophages, showed high ^{13}C-enrichments ranging from 16% (mostly in form of $^{13}C_3$-Ala) to 4% (mostly in form of $^{13}C_2$- and $^{13}C_3$-Asp, and $^{13}C_2$-Glu) (**Figures 4C,D**). This demonstrated that [U-$^{13}C_3$]pyruvate was taken up by the macrophages and converted via $^{13}C_2$-acetyl-CoA into [$^{13}C_2$]α-oxoglutarate and [$^{13}C_2$]oxaloacetate in the TCA cycle and by carboxylation of $^{13}C_3$-phosphoenol pyruvate into [$^{13}C_3$]oxaloacetate.

The amount of ^{13}C-label in the same amino acids from the listerial fraction was considerably lower, i.e., Ala (10.5%) > Asp (2%) > Glu (1.5%) (**Figures 4A,B**). All other amino acids remained unlabeled (<1%) which is different as compared to the infection experiments with uniformly ^{13}C-labeled glucose or glycerol. The ^{13}C-isotopologue patterns of listerial Asp and Glu were also different than those of the host cells, showing in particular a higher amount of [$^{13}C_3$]Asp (**Figures 4C,D**). These data are in line with the assumption that a small amount of [$^{13}C_3$]pyruvate was either directly taken up by the bacteria or converted by the host cell to other C_3-compounds, e.g., [$^{13}C_3$]glycerol, -lactate or -alanine, which were subsequently transported into the intracellular listeriae giving rise to [$^{13}C_3$]pyruvate/Ala and by PycA-mediated carboxylation to [$^{13}C_3$]oxaloacetate/Asp.

Indeed, addition of [U-$^{13}C_3$]lactate to a similar infection assay showed ^{13}C-incorporation into bacterial Ala (8.6 − 1.4%), Asp (5.9 − 0.5%), and Glu (4.2 − 0.6%) (**Figure 4A**). As indicated by the high deviations of the experimental values from several independent experiments (see error bars in **Figure 4B**), the presence of lactate in the medium might lead to harmful effects for the host cells which can hardly be controlled. We also noticed that the ^{13}C-excess values of the bacterial amino acids were higher than those of the corresponding amino acids of the host cells (**Figure 4D**). Based on this observation, it is likely that lactate provided by the host cells may serve as a direct, but rather inefficient C_3-substrate for intracellular *L. monocytogenes*. This assumption was further supported by the finding that the Δldh mutant showed ^{13}C-label only in Ala (9.5%), but not in any other amino acid (**Figure 4A**). This $^{13}C_3$-Ala was probably generated in the host cell and transported into the intracellular listeriae.

GLUCOSE 6-PHOSPHATE IS USED BY INTRACELLULAR *L. MONOCYTOGENES* MAINLY FOR ANABOLIC FUNCTIONS

Besides glycerol (and, as outlined above, to a minor extent possibly also other C_3-compounds), glucose-6P was identified in previous studies as an important carbon substrate for intracellularly replicating *L. monocytogenes* (Chico-Calero et al., 2002; Eylert et al., 2008). However, the question remained unanswered whether (i) glucose-6P is catabolized via glycolysis and/or the pentose phosphate pathway, serves as source for energy and the production for intermediates in anabolic pathways or (ii) whether it is mainly required for anabolic functions by its conversion via the pentose phosphate pathway to the sugar components necessary for the biosynthesis of cell wall structures, aromatic amino acids and nucleotides. For this goal, we compared the ^{13}C-labeled amino acids of *L. monocytogenes* grown in minimal medium (*in vitro*) and in J774A.1 cells in the presence of [1,2-$^{13}C_2$]glucose. Although the ^{13}C-enrichments in Ala, Asp, and Glu were approximately 3-fold higher from *in vitro* growing *L. monocytogenes* than from intracellular bacteria, the ^{13}C-isotopologue patterns of these amino acids were highly similar in both cases (**Figure 5A**).

More specifically, the MS analysis of the silylated Ala (Ala-260) showed the presence of [$^{13}C_2$]Ala. To determine the positions of the ^{13}C-atoms, a mass fragment (Ala-232) that had lost C-1 during ionization and therefore represents C-2 and C-3 of Ala was analyzed. Ala-232 still showed the presence of two ^{13}C-atoms at the same abundance as in Ala-260. It can therefore be concluded that listerial Ala acquired ^{13}C-label from [1,2-$^{13}C_2$]glucose at positions C-2 and C-3. On this basis, pyruvate, the precursor of Ala, was characterized by the [2,3-$^{13}C_2$]-isotopologue (**Figure 5B**).

Moreover, the ^{13}C-isotopologue pattern in Asp-418 (comprising all carbon atoms of the original amino acids) and Asp-390 (after loss of a carboxylic atom) showed the formation of [2,3-$^{13}C_2$]oxaloacetate/Asp by pyruvate carboxylase-mediated carboxylation of [2,3-$^{13}C_2$]pyruvate (**Figure 5B**). The ^{13}C-isotopologue pattern of Glu showing predominantly $^{13}C_2$- and $^{13}C_4$-species can be explained by the incomplete citrate cycle: [2,3-$^{13}C_2$]oxaloacetate reacted with unlabeled acetyl-CoA finally yielding [2,3-$^{13}C_2$]α-ketoglutarate/Glu or with [$^{13}C_2$]acetyl-CoA (obtained from [2,3-$^{13}C_2$]pyruvate through

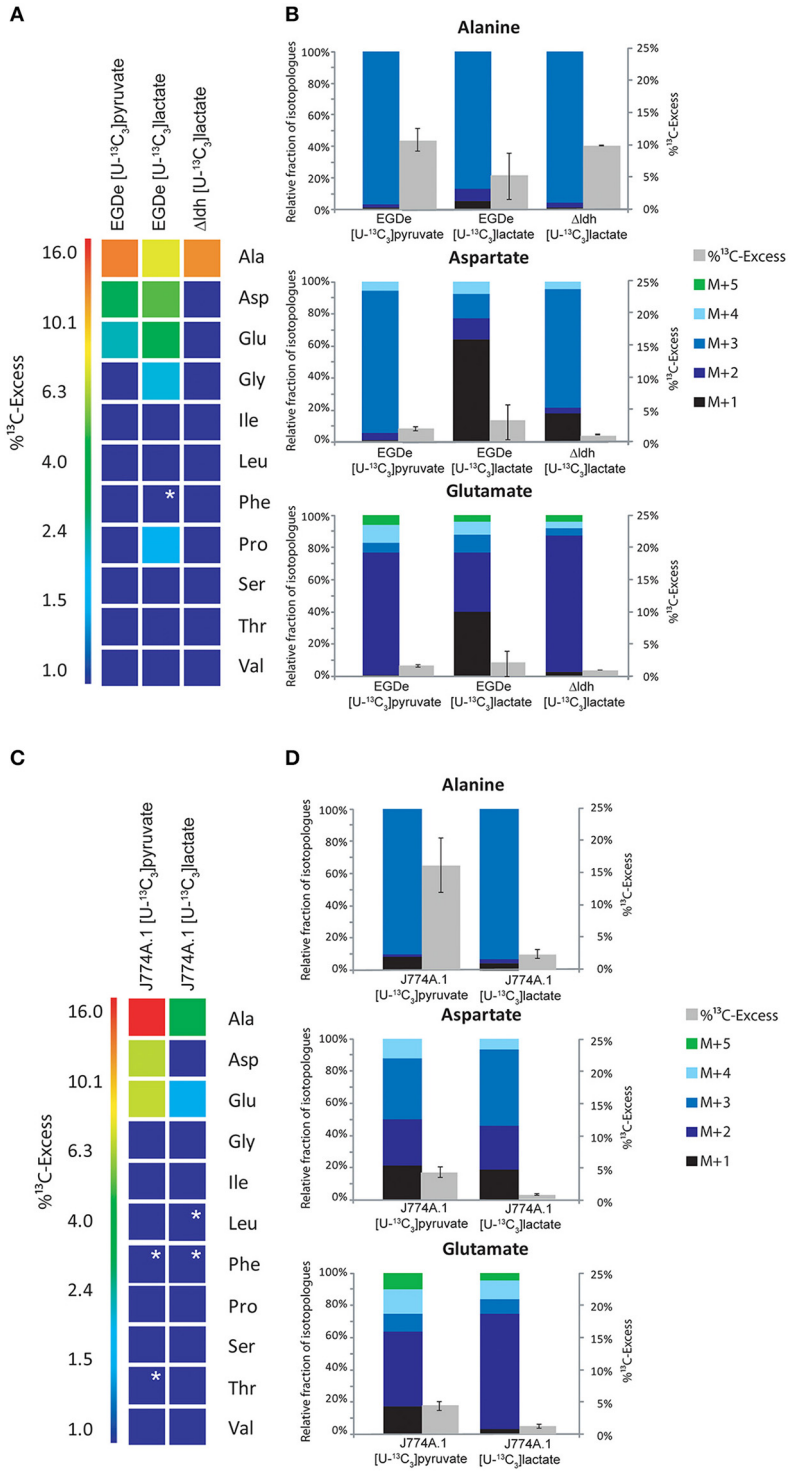

FIGURE 4 | Analysis of protein-derived amino acids from *L. monocytogenes* grown in J774A.1 macrophages labeled with 20 mM [U-$^{13}C_3$]pyruvate or 20 mM [U-$^{13}C_3$]lactate, respectively. (A) ^{13}C-Excess (in %) of amino acids from *L. monocytogenes* wild-type (EGDe) and the Δ*ldh* mutant in the experiment with [U-$^{13}C_3$]lactate. The color map again indicates ^{13}C excess in quasi-logarithmic form in order to also visualize small values. Boxes with white asterisks indicate high standard deviations in the measurement of the overall ^{13}C-enrichments. **(B)** Isotopologue profiles in listerial Ala, Asp, and Glu, respectively, from the same experiments. The colored columns indicate the relative fractions (in %) of the ^{13}C-isotopologues (M+1 to M+5) in the labeled amino acids (left scales). For comparison, the gray bars indicate the ^{13}C-excess values with the standard deviations from three technical replicates (right scales). **(C)**, ^{13}C-Excess (in %) of amino acids from J774A.1 proteins in the infection experiments with [U-$^{13}C_3$]pyruvate or [U-$^{13}C_3$]lactate. **(D)**, Isotopologue profiles of J774A.1 Ala, Asp, and Glu, respectively, from the same experiments. For the numerical values, see Supplemental Material.

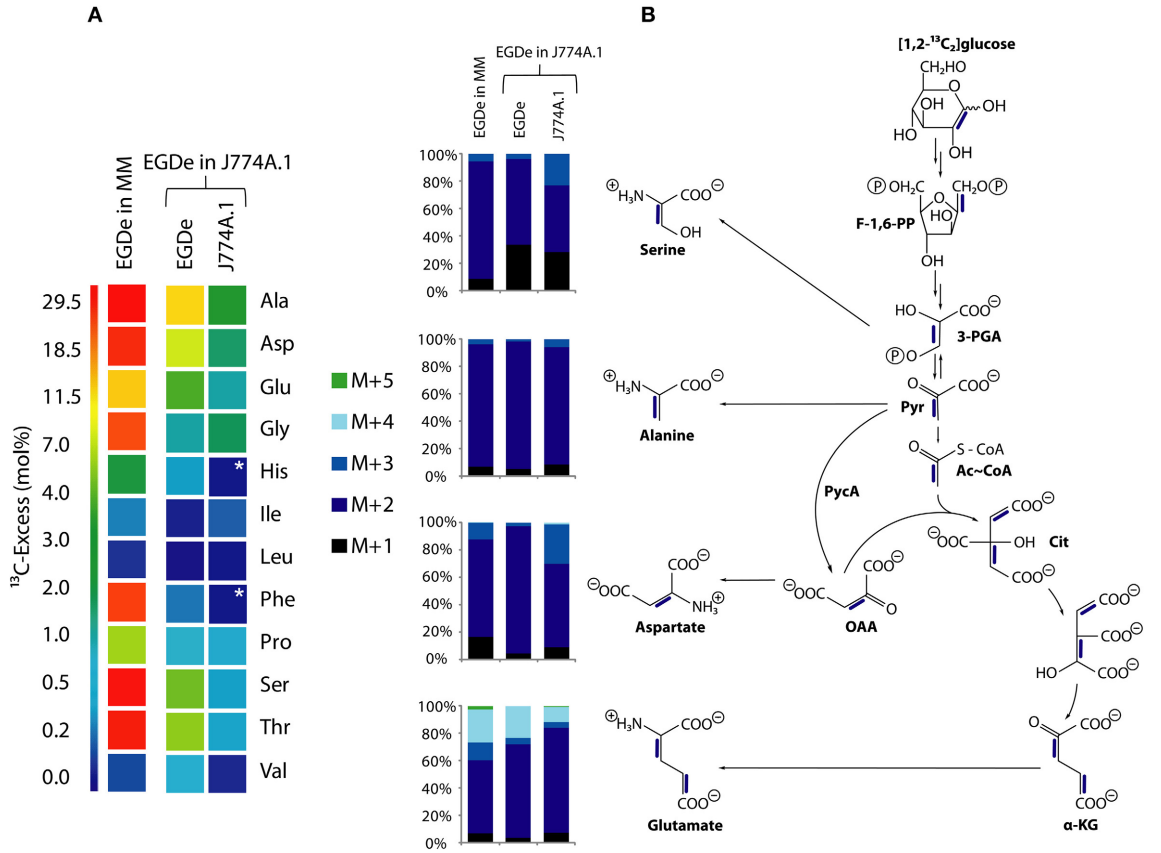

FIGURE 5 | Analysis of protein-derived amino acids from *L. monocytogenes* EGDe in the experiments with [1,2-^{13}C$_2$]glucose. **(A)** ^{13}C-excess (in % as a color map) of *L. monocytogenes* EGDe grown in minimal medium (MM) in the presence of 10 mM [1,2-^{13}C$_2$]glucose (column 1) of from *L. monocytogenes* EGDe grown in J774A.1 cells in the presence of 10 mM [1,2-^{13}C$_2$]glucose. Boxes with white asterisks indicate high standard deviations in the measurement of the overall ^{13}C-enrichments. Isotopologue profiles in listerial Ser, Ala, Asp, and Glu, respectively, from the same experiments. The colored columns indicate the relative fractions (in %) of the ^{13}C-isotopologues (M+1 to M+5) in the labeled amino acids. **(B)** Reaction scheme displaying the conversion of [1,2-^{13}C$_2$]glucose into the detected isotopologues of Ala, Asp, and Glu. ^{13}C-Label is indicated by blue bars. For the numerical values, see Supplemental Material.

decarboxylation by pyruvate dehydrogenase) yielding [2,3,4,5-^{13}C$_4$]α-ketoglutarate/Glu (**Figure 5B**). These data are in accord with assumption that the formation of [2,3-^{13}C$_2$]pyruvate occurs by glycolytic degradation of [1,2-^{13}C$_2$]glucose, and not via the pentose phosphate pathway, which would result in the formation of [^{13}C$_1$]- or unlabeled pyruvate.

While the data obtained from *L. monocytogenes* grown in the minimal medium (MM) clearly indicate that the formation of [2,3-^{13}C$_2$]pyruvate arises from glycolytic degradation of [1,2-^{13}C$_2$]glucose by the listeriae, this is less obvious in case of the bacteria grown in J774A.1 cells. Here, [2,3-^{13}C$_2$]pyruvate could be generated by glycolytic degradation of [1,2-^{13}C$_2$]glucose within the host cell where it is further converted into [2,3-^{13}C$_2$]glycerol. This compound could be subsequently transported into the intracellular listeriae and further metabolized leading to the observed isotopologues in Ala, Ser, Asp, and Glu. Indeed, the latter explanation appears to be more likely when we compare the other ^{13}C-labeled, i.e., *de novo* synthesized amino acids, in MM-grown and in J774A.1 grown listeriae. Thus, MM-grown listeriae showed ^{13}C-label (in addition to the above described Ala, Ser, Asp, and Glu) in Gly, Thr, Pro, His, and Phe (**Figure 5A**). The two latter amino acids require as precursors intermediates from the pentose phosphate pathway, i.e., erythrose-4P and ribose-5P, respectively.

In contrast, *L. monocytogenes* grown within J774A.1 did not show ^{13}C-label in these two amino acids. In this case, all ^{13}C-labeled amino acids require as precursors for their biosynthesis intermediates from the lower part of glycolysis (Ser, Gly, Ala, Val) or the TCA cycle (Asp, Thr, Glu, Pro) which could be generated from host cell-imported [2,3-^{13}C$_2$]glycerol.

These data indicate that MM-grown and J774A.1-grown *L. monocytogenes* use externally supplied glucose in different ways. While in the MM-grown listeriae glucose feeds the glycolytic pathway and the pentose phosphate shunt, the J774A.1-grown *L. monocytogenes* consume glucose in a dual manner with the participation of the host cell which converts glucose to glycerol and glucose-6P which are subsequently taken up as separate carbon substrates by the intracellular listeriae. Glucose-derived glycerol (generated in the host cell) is used as carbon substrate for the supply of energy and intermediates for the biosynthesis of some amino acids and (probably) fatty acids but not for

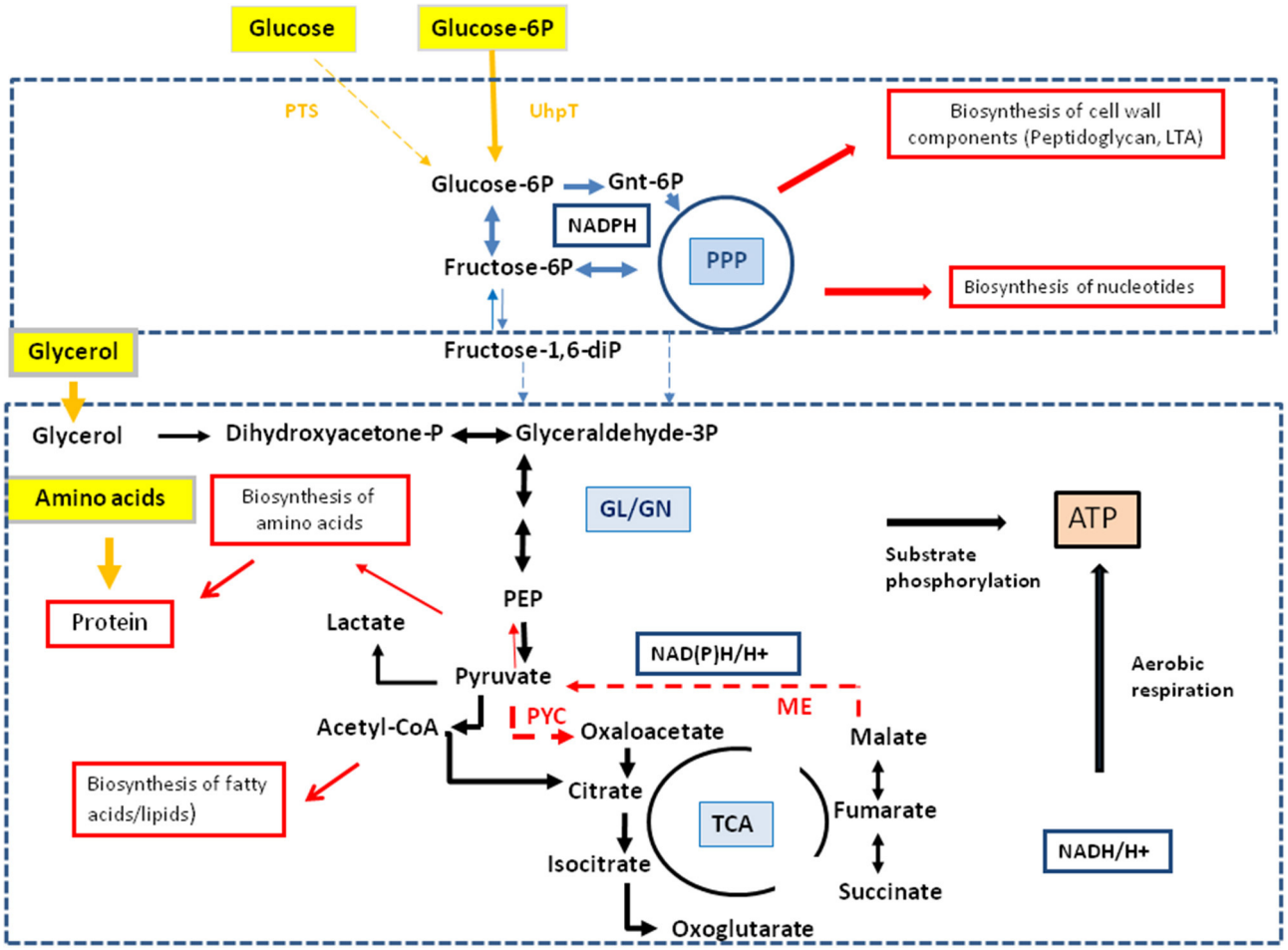

FIGURE 6 | Model for a bipartite metabolism for intracellular *L. monocytogenes*. Gray-framed yellow boxes and yellow arrows mark (by color intensity) the major carbon compounds taken up by the intracellular listeriae from the host cell. Catabolic reactions fed by glucose-6P and glycerol are indicated by blue and black arrows, respectively. Reactions leading to anabolic performances (in red-framed boxes) are indicated by red arrows. Abbreviations: PPP, pentose phosphate pathway; GL, glycolysis; GN, gluconeogenesis; TCA, tricarboxylic acid pathway; PYC, pyruvate carboxylase; ME, malic enzyme (decarboxylating malate dehydrogenase).

gluconeogenesis. Vice versa, glucose-6P (also generated in the host cell) may not be catabolized to pyruvate to an appreciable extent, but rather converted in the pentose phosphate shunt into sugar components essential for the biosynthesis of the cell envelope and nucleotides. The aromatic amino acids which require for their biosynthesis erythrose-4P also generated in the pentose phosphate shunt seem to be mainly imported from the host cell. A model for this bipartite intracellular metabolism of *L. monocytogenes* is outlined in **Figure 6**.

THE CARBON METABOLISM OF J774A.1 HOST CELLS IS DIFFERENTLY AFFECTED BY THE WILD-TYPE AND THE ΔC3 AND Δ*uhp*T MUTANT STRAINS OF *L. MONOCYTOGENES*

This bipartite carbon metabolism carried out by the intracellular listeriae might put less nutrient stress on the host cell thereby extending the lifespan of the host cell—in favor of the intracellular bacteria. Some indirect experimental evidence for this assumption is provided by the different ^{13}C-incorporation rates into Ala, Asp, and Glu from the J774A.1 host cells infected with the wild-type, the ΔC3, or the Δ*uhpT* strains. The balanced bipartite metabolism is expected to be disturbed in these two mutants due to their inability of utilize glycerol and glucose-6P, respectively.

The J774A.1 host cell line derives from a mouse tumor and expresses c-Myc constitutively (Fan et al., 1993). This leads to enhanced aerobic glycolysis and increased glutaminolysis (Fan et al., 1993; Wise et al., 2008; Dang et al., 2009). In the presence of [U-$^{13}C_6$]glucose, the most efficiently ^{13}C-labeled amino acids of J774A.1 cells were therefore Ala, Glu, and Asp. However, the ^{13}C-incorporation rates into these amino acids were slightly but reproducibly increased when the same cells were infected with *L. monocytogenes* EGDe wild-type (WT) (**Figure 7**) which is in line with earlier observations (Gillmaier et al., 2012). Interestingly, however, ^{13}C-incorporation into these amino acids decreased in the J774A.1 cells upon infection with the ΔC3 mutant strains, but increased upon infection with the Δ*uhpT* strain in comparison to the reference experiment with the

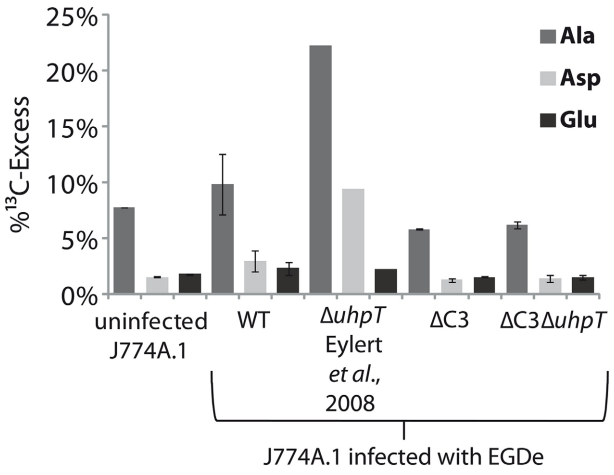

FIGURE 7 | Analysis of protein-derived amino acids from J774A.1 cells labeled with [U-$^{13}C_6$]glucose. The columns indicate ^{13}C-excess (in %) of Ala, Asp, and Glu in experiments with uninfected cells, or cells infected *L. monocytogenes* EGDe wild-type, ΔC3, Δ*uhpT*, and ΔC3Δ*uhpT* mutants, respectively.

wild-type strain (Eylert et al., 2008). This effect was less obvious in Glu which is probably caused by the abundant availability of Glu in the J774A.1 host cells due to the enhanced glutaminolysis (Gillmaier et al., 2012). The relatively low ^{13}C-enrichment in Glu deriving from of [U-$^{13}C_6$]glucose (an indication for Glu *de novo* synthesis) is in line with this assumption.

These changes of ^{13}C-incorporation into host cell amino acids upon infection by the mutant strains compared to the wild type strain cannot be caused by a different efficiencies of intracellular replication since the number of intracellular wild-type bacteria was similar to that of the mutant strains. The data rather suggest that—as compensation for the defective glycerol or glucose-6P consumption—the resulting increased withdrawal of glucose-6P or glycerol, respectively, from the host cells obviously causes severe changes in the central carbon fluxes of the host cells.

DISCUSSION

In order to further elucidate the intracellular metabolism of *L. monocytogenes* and in particular for answering the question which carbon substrates provided by the host cells are essential for driving the intracellular listerial metabolism, we performed infection studies with the EGDe wild-type strain and isogenic mutants defective in the transport or catabolism of anticipated carbon substrates and J774A.1 macrophages as host cells. For this goal, we developed experimental protocols for the application of [U-^{13}C]amino acids, [U-$^{13}C_3$]glycerol, [U-$^{13}C_3$]pyruvate, and [U-$^{13}C_3$]lactate as tracer carbon substrates to the RPMI culture medium. The efficiency of utilization of these carbon sources and the resulting metabolic fluxes were determined from the ^{13}C-isotopologue profiles of the ^{13}C-labeled amino acids, a method which we successfully applied before for studying the intracellular metabolism of bacterial pathogens in mammalian cells (Eylert et al., 2008; Götz et al., 2010; Gillmaier et al., 2012).

The results confirm the essential role of glycerol and glucose-6P as carbon substrates for the intracellular listerial metabolism as already suggested by previous studies that were based on transcriptome analyses (Chatterjee et al., 2006; Joseph et al., 2006; Lobel et al., 2012) and on ^{13}C-isotopologue profiling studies (Eylert et al., 2008; Gillmaier et al., 2012). The present data also show that other C_3-carbon substrates, like pyruvate or lactate proposed as possible alternative or supplemental carbon substrates, play only minor roles, if any. It should be noted, however, that even a *L. monocytogenes* ΔC3Δ*uhpT* mutant which is apparently unable to utilize glycerol and glucose-6P can still replicate in the J774A.1 host cells albeit at a reduced rate. This suggests that there are alternative carbon sources which are able to at least partially replace the two major carbon substrates, glycerol and glucose-6P. Possible candidates are glucose, amino acids, glycerol 3-phosphate, or intermediates of the TCA (especially succinate and malate) that could also be provided by the host cells.

The possible participation of glucose and/or mannose in intracellular listerial metabolism is difficult to demonstrate experimentally, since *L. monocytogenes* possess a large number of PEP-dependent phosphotransferase systems (PTS) and even non-PTS transporters for glucose and mannose (Glaser et al., 2001; Stoll and Goebel, 2010; Ake et al., 2011). Deletions of the major glucose/mannose PTS transporters do not seem to affect the intracellular listerial replication. However, in the *pts* deletion mutants, other transporters for these carbohydrates seem to be activated (Stoll and Goebel, 2010).

There is still another reason which makes the participation of glucose or mannose as major carbon source for intracellular listerial metabolism unlikely. The expression of the genes encoding the virulence factors essential for the intracellular listerial life cycle and also the glucose-6P transporter UhpT depends on the central virulence gene activator PrfA (Chico-Calero et al., 2002). However, the activity of PrfA is strongly inhibited when glucose or other glycolytic carbohydrates are used as the major carbon source for listerial growth while PrfA activity is high with glycerol as carbon source (Joseph et al., 2008; Stoll et al., 2008; Götz, unpublished results).

The data presented here also rule out amino acids as important catabolic carbon substrates for the intracellular metabolism of *L. monocytogenes*. An externally added mix of ^{13}C-labeled amino acids (containing all amino acids except Trp, Cys, Met, and Arg) is efficiently taken up by the bacteria (with the exception of Asp). However, according to the ^{13}C-isotopologue profiles the major glycogenic and ketogenic amino acids are mainly incorporated into listerial protein but hardly catabolized by the intracellular listeriae. This is not surprising for the two most important glycogenic amino acids, Asp and Glu, since Asp cannot be taken up by *L. monocytogenes* (Schär et al., 2010) and the missing oxoglutarate dehydrogenase prevents Asp and Glu degradation in the interrupted listerial TCA cycle (Eisenreich et al., 2006). These results are in line with previous studies (Eylert et al., 2008; Gillmaier et al., 2012) showing limited *de novo* synthesis of most amino acids by intracellular listeriae and import of most amino acids from the host cell in particular of the branched chain and aromatic amino acids. The failure to biosynthesize these amino acids during intracellular growth despite the presence of the

biosynthesis capacity on the basis of the genome sequence (Glaser et al., 2001) and transcriptome analyses (Chatterjee et al., 2006; Joseph et al., 2006; Lobel et al., 2012) suggests a shortage of essential catabolic intermediates and/or co-factors required for the biosynthesis of these amino acids under intracellular conditions.

Those amino acids that are *de novo* synthesized by the intracellular listeriae at significant rates derive from intermediates generated in the lower part of the glycolytic pathway (Ser, Gly, Ala) or in the TCA pathway (Asp, Thr, Glu, Pro). These catabolic pathways can be fed by glycerol as major carbon source. Indeed, the nature of ^{13}C-labeled amino acids as well as their ^{13}C-isotopologue profiles are similar if not identical, irrespective of whether ^{13}C-glucose or ^{13}C-glycerol were added to the culture medium of the infected host cells. This suggests that ^{13}C-glucose is converted in the host cell into ^{13}C-glycerol that enters, after being taken up by the intracellular listeriae, the glycolytic pathway at the same position (most likely at the level of glyceraldehyde-3P/dihydroxyacetone-3P) as the externally added ^{13}C-glycerol. These glycolytic intermediates are then further catabolized into pyruvate, oxaloacetate and α-ketoglutarate and their downstream amino acids (see **Figure 6**), but not used anabolically in gluconeogenesis as indicated by the apparent absence of ^{13}C-label in Phe, Tyr and His from exogenous ^{13}C-glycerol. *De novo* synthesis of these amino acids would require sugar components, such as erythrose-4P and ribose-5P, respectively, which are generated in the glucose-6P-driven pentose phosphate pathway. Indeed, these amino acids are synthesized and ^{13}C-labeled by *L. monocytogenes* growing in an *in vitro* culture medium in the presence of ^{13}C-glucose as major carbon source (Eisenreich et al., 2006) as well as by intracellular (also cytosolically) replicating enteroinvasive *E. coli* (Götz et al., 2010). Notably, the latter bacterial pathogen uses mainly glucose as carbon substrate for its intracellular metabolism.

This glycerol-driven metabolism of intracellular listeriae will lead to active PrfA (Stoll et al., 2008) and hence to the expression of the glucose-6P transporter UhpT resulting in the uptake of glucose-6P by the intracellular listeriae. We postulate that this additional carbon substrate may feed the pentose phosphate shunt allowing the generation of the intermediates needed for the biosynthesis of essential cell envelope components and nucleotides. Our presently applied analytical ^{13}C-isotopologue approach does not allow the direct detection of these compounds and the experimental prove for this assumption therefore awaits further studies with improved analytical tools.

Together, the data suggest that the intracellular metabolism of *L. monocytogenes* relies on two major carbon substrates: (i) glycerol (generated in the host cell from glucose or other precursors) which is used as carbon substrate for the supply of energy (ATP by substrate-level phosphorylation and aerobic respiration) and of intermediates for the biosynthesis of some amino acids (e.g., Ser, Gly, Ala, Asp, Thr, Glu, and Pro) and (probably) fatty acids, but not for gluconeogenesis, and (ii) glucose-6P (also generated in the host cell from e.g., glucose) which may not be catabolized to pyruvate at significant rates, but rather converted in the pentose phosphate shunt to sugar components essential for the biosynthesis of the cell envelope and nucleotides. The aromatic amino acids which also require for their biosynthesis erythrose-4P, also generated in the pentose phosphate shunt, seem to be mainly imported from the host cell. A model for this "bipartite metabolism" of intracellular *L. monocytogenes* is outlined in **Figure 6**.

This kind of bipartite metabolism of an IBP could in general have a selective advantage for its survival within the host cell, since it may impose less nutrient stress on the infected host cell than a bacterial metabolism based mainly on the use of glucose. Indeed, *L. monocytogenes* ΔC3 and Δ*uhpT* mutants which are impaired in the balanced utilization of the two carbon substrates seem to impose a greater metabolic burden on the host cells than the wild-type strain as shown by the changes of the isotopologue profiles of host amino acids in infection experiments with *L. monocytogenes* ΔC3 or Δ*uhpT* in comparison to the wild-type strain. Furthermore, enteroinvasive *E. coli* strains which also replicate—similar to *L. monocytogenes*—in the host cells' cytosol, but mainly rely on glucose as a preferred carbon source for the intracellular metabolism (Götz et al., 2010), kill the same host cells much faster than *L. monocytogenes*.

AUTHOR CONTRIBUTIONS

Wolfgang Eisenreich, Thilo M. Fuchs, and Werner Goebel designed the experiments; Kristina Schauer and Thilo M. Fuchs characterized the mutants, Stephanie Grubmüller and Kristina Schauer performed the labeling experiments, SG performed the GC/MS analysis; Stephanie Grubmüller, Kristina Schauer, Wolfgang Eisenreich, Thilo M. Fuchs, and Werner Goebel analyzed and interpreted the data; and Werner Goebel, Wolfgang Eisenreich, and Thilo M. Fuchs wrote the paper.

ACKNOWLEDGMENTS

This work was supported by the priority program SPP1316 of the Deutsche Forschungsgemeinschaft (EI-384/6 and FU-375/5). We thank Birgit Lange and Christine Schwarz for their expert help in sample preparation, and Erika Kutzner for help with the graphics.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00156/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 August 2014; accepted: 14 October 2014; published online: 03 November 2014.

*Citation: Grubmüller S, Schauer K, Goebel W, Fuchs TM and Eisenreich W (2014) Analysis of carbon substrates used by Listeria monocytogenes during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism. Front. Cell. Infect. Microbiol. **4**:156. doi: 10.3389/fcimb.2014.00156*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

OPINION ARTICLE
published: 26 August 2014
doi: 10.3389/fcimb.2014.00111

Nutrient generation and retrieval from the host cell cytosol by intra-vacuolar *Legionella pneumophila*

Christopher T. D. Price, Ashley M. Richards and Yousef Abu Kwaik*

Department of Microbiology and Immunology and Center for Predictive Medicine, College of Medicine, University of Louisville, Louisville, KY, USA
**Correspondence: abukwaik@louisville.edu*

Edited by:
Wolfgang Eisenreich, Technische Universität München, Germany

Reviewed by:
Klaus Heuner, Robert Koch-Institute, Germany

Keywords: Legionnaires' disease, proteasomes, ubiquitin, slc transporter, AnkB, F-box, farnesylation

Microbial acquisition of nutrients *in vivo* is a fundamental aspect of infectious diseases, and is a potential target for antimicrobial therapy. Part of the innate host defense against microbial infection is nutritional restriction of access to sources of host nutrients (Abu Kwaik and Bumann, 2013; Eisenreich et al., 2013). Despite this host nutritional restriction, there has been a long held presumption that the host cell cytosol has sufficient nutrients for any intracellular pathogen, although many bacteria fail to grow in the host cytosol if they are microinjected (Goetz et al., 2001). However, recent studies on the two intra-vacuolar pathogens *Anaplasma phagocytophilum* (Niu et al., 2012) and *Legionella pneumophila* (Price et al., 2011) and the cytosolic pathogen *Francisella tularensis* (Steele et al., 2013) have clearly shown that the levels of amino acids in the host cell cytosol are below the threshold sufficient to meet the tremendous demands for carbon, nitrogen and energy to power the robust intracellular proliferation of these pathogens (Abu Kwaik and Bumann, 2013). Therefore, these intracellular pathogens have evolved with efficient strategies to boost the levels of host amino acids to meet their demands for higher levels of carbon, nitrogen and energy sources (Abu Kwaik and Bumann, 2013; Fonseca and Swanson, 2014). There is an emerging paradigm of specific microbial strategies that directly trigger the host cell to boost the cellular levels of essential microbial nutrients, and this paradigm has been designated as "nutritional virulence" (Abu Kwaik and Bumann, 2013). This opinion article is focused on nutritional virulence of *L. pneumophila*.

In the aquatic environment, *L. pneumophila* proliferates within protozoa, which impact bacterial ecology and pathogenicity (Al-Quadan et al., 2012). Upon transmission to humans, *L. pneumophila* proliferates in alveolar macrophages within the *Legionella*-containing vacuole (LCV) that is ER-derived and evades lysosomal fusion (**Figure 1**). Within both evolutionarily distant host cells, the Dot/Icm type IV secretion system of *L. pneumophila* injects ∼300 protein effectors (Zhu et al., 2011; Luo, 2011a) that govern biogenesis of the LCV and modulate a myriad of cellular processes to enable intra-vacuolar proliferation (**Figure 1**) (Luo, 2011b; Richards et al., 2013).

Amino acids are the main sources of carbon, nitrogen and energy for *L. pneumophila*, which metabolizes them through the TCA cycle (Pine et al., 1979), but also metabolizes minor amounts of glucose *in vitro* using the Entner-Doudoroff pathway (Eylert et al., 2010; Price et al., 2011). Although *L. pneumophila* utilizes amino acids as the main sources of carbon and energy, the pathogen is auxotrophic for seven amino acids (Cys, Met, Arg, Thr, Val, Ile, and Leu) (Eylert et al., 2010; Price et al., 2014). Remarkably, there is a high level of synchronization in amino acids auxotrophy between *L. pneumophila* and its host cells, which has likely played a factor in nutritional evolution of *L. pneumophila* as an intra-vacuolar pathogen (Price et al., 2014).

Interestingly, intra-vacuolar *L. pneumophila* up-regulates its own amino acids transporters, indicating increased demands for amino acids in the intra-vacuolar environment (Bruggemann et al., 2006; Faucher et al., 2011; Eisenreich et al., 2013). Since the generation time of intra-vacuolar *L. pneumophila* is ∼40 min, this organism requires high levels of amino acids to be imported from the host cytosol into the LCV lumen (Schunder et al., 2014). A long-held presumption has been that the host cell cytosol is rich in nutrients for invading pathogens.

However, recent studies clearly indicate that the basal levels of host cellular amino acids are below the threshold sufficient for the robust intra-vacuolar proliferation of *L. pneumophila* (Sauer et al., 2005; Wieland et al., 2005) To achieve that needed threshold, *L. pneumophila* promotes host proteasomal degradation (Price et al., 2011) of LCV-decorated polyubiquitinated proteins (Dorer et al., 2006; Price et al., 2009, 2011; Lomma et al., 2010) mediated by the AnkB effector.

Within human macrophages and amoeba, the AnkB translocated effector of *L. pneumophila* strain AA100/130b is localized to the cytosolic face of the LCV membrane through host-mediated farnesylation of its C-terminal CaaX motif (**Figure 1**) (Price et al., 2010; Al-Quadan et al., 2011; Al-Quadan and Kwaik, 2011). On the LCV membrane, AnkB interacts with the host SCF1 ubiquitin ligase (**Figure 1**) (Bruckert et al., 2014). As a *bona fide* F-box effector (Ensminger and Isberg, 2010; Lomma et al., 2010; Price and Abu Kwaik, 2010), AnkB triggers decoration of the LCV with Lys^{48}-linked polyubiquitinated proteins that are targeted for proteasomal degradation (**Figure 1**) (Price et al., 2011). The metabolomic profile

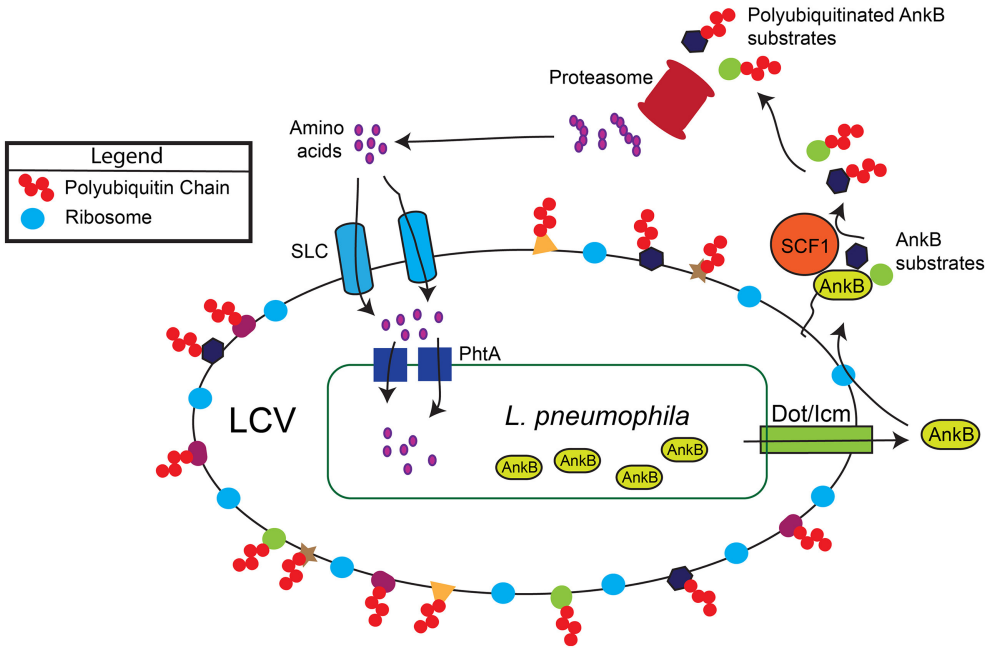

FIGURE 1 | Generation of a surplus of host amino acids by *L. pneumophila* and their import into the LCV. The AnkB effector is translocated into macrophages and amoebae by the Dot/Icm type IV secretion system of *L. pneumophila*. AnkB is immediately farnesylated and anchored into the cytosolic face of the LCV membrane where it interacts with the eukaryotic SCF1 ubiquitin ligase complex. The AnkB effector functions as a platform for the docking of Lys^{48}-linked polyubiquitinated proteins to the LCV that are subsequently degraded by the proteasome, which generates a surplus of cellular amino acids above the threshold necessary for intra-vacuolar proliferation. The amino acids are likely imported into the LCV through various host SLC amino acid transporters in the LCV membrane, but the identity of the transporters is still to be determined. The amino acids are acquired by *L. pneumophila* through numerous ABC transporters and amino acid permeases such as the threonine transporter PhtA.

of *L. pneumophila*-infected amoeba and human cells have shown an AnkB-dependent dramatic rise in the levels of all cellular amino acids (Price et al., 2011), and this is initiated rapidly upon bacterial attachment to the macrophage plasma membrane (Bruckert et al., 2014). Importantly, inhibition of host proteasomal degradation abolishes intracellular proliferation of *L. pneumophila* strains AA100/130b and Philadelphia (Dorer et al., 2006; Price et al., 2011). The *L. pneumophila*-generated surplus of host cell amino acids may explain the lack of an intracellular defect for the lysine and tryptophan auxotrophic mutants of *L. pneumophila* (Mintz et al., 1988; Ensminger et al., 2012).

Loss of AnkB in two independent isolates (AA100 and Paris) results in varying degrees of failure in intra-vacuolar proliferation and attenuation in the mouse model of Legionnaires' disease (Al-Khodor et al., 2008; Lomma et al., 2010). These defects for the AA100 strain are totally overcome upon supplementation of a mixture of amino acids in macrophages, amoeba and in the mouse model, similar to genetic complementation (Price et al., 2011). Importantly, silencing of the host SCF1 ubiquitin ligase, interference with Lys^{48}-linked polyubiquitination, or inhibition of the host proteasomes block intra-vacuolar proliferation of *L. pneumophila*, but the block is relieved upon supplementation of an excess mixture of amino acids (Price et al., 2009, 2011). Surprisingly, the intra-vacuolar proliferation defect of the *ankB* mutant is rescued by many individual amino acids, such as Cys, Ala or Ser, which are essential or metabolically favorable for *L. pneumophila* (Pine et al., 1979). Interestingly, although Gln is the most abundant amino acid in human cells, supplementation of infected hMDMs with excess Gln alone efficiently rescues the *ankB* mutant (Price et al., 2011), while Glu is a major source of carbon and energy *in vitro* (Pine et al., 1979). These findings indicate that the basal levels of cellular amino acids are below the threshold sufficient for intra-vacuolar proliferation of *L. pneumophila*.

Remarkably, pyruvate or citrate supplementation is as effective as amino acids in rescuing the intra-vacuolar growth defect of the *ankB* mutant, which indicates that the LCV is capable of importing these two substrates that can feed the TCA cycle, in addition to the documented reliance of intra-vacuolar *L. pneumophila* on amino acids (Schunder et al., 2014). In addition, *L. pneumophila* utilizes glucose through the Entner-Doudoroff pathway, which is required for proliferation within the amoeba host (Eylert et al., 2010). During inflammation, macrophages undergo up-regulation of glucose uptake and anaerobic glycolysis (Warburg-effect), which generates additional pyruvate (Eisenreich et al., 2013), and it is likely that both glucose and pyruvate are imported by the LCV. In addition, *L. pneumophila*-infected macrophages exhibit a pro-inflammatory phenotype. Taken together, it is likely that a multi-prong nutritional virulence strategy is utilized by *L. pneumophila* to

generate and retrieve a diversified portfolio of sources of carbon and energy from the host cell. The host solute carrier (SLC) family of membrane proteins (Cedernaes et al., 2011; Schioth et al., 2013) that transport various compounds, including amino acids, TCA intermediates, glucose, lipids, and drugs are likely to be involved in import of various compounds by the LCV membrane (**Figure 1**) (Wieland et al., 2005). Future studies should unravel the host metabolites and the mechanism of their import into the LCV lumen, and subsequently by the bacterial membrane. Deciphering microbial nutrition and metabolism *in vivo* is essential for our understanding of host-microbe interaction, and nutrient retrieval strategies by intracellular pathogens are potential targets for therapy.

ACKNOWLEDGMENTS

Yousef Abu Kwaik is supported by Public Health Service Awards R01AI069321 and R21AI107978 from NIAID and by the commonwealth of Kentucky Research Challenge Trust Fund.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 July 2014; paper pending published: 24 July 2014; accepted: 27 July 2014; published online: 26 August 2014.

Citation: Price CTD, Richards AM and Abu Kwaik Y (2014) Nutrient generation and retrieval from the host cell cytosol by intra-vacuolar Legionella pneumophila. Front. Cell. Infect. Microbiol. ***4****:111. doi: 10.3389/fcimb.2014.00111*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 29 January 2015
doi: 10.3389/fcimb.2014.00191

Salmonella—how a metabolic generalist adopts an intracellular lifestyle during infection

Thomas Dandekar[1]*, Astrid Fieselmann[1], Eva Fischer[1], Jasmin Popp[2], Michael Hensel[2] and Janina Noster[2]

[1] Department of Bioinformatics, Biocenter, University of Würzburg, Würzburg, Germany
[2] Division of Microbiology, Biology/Chemistry, University of Osnabrück, Osnabrück, Germany

Edited by:
Alfredo G. Torres, University of Texas Medical Branch, USA

Reviewed by:
Giovanna Suzzi, Università degli Studi di Teramo, Italy
Manuela Raffatellu, University of California, Irvine, USA

***Correspondence:**
Thomas Dandekar, Biocenter, Am Hubland, 97074 Würzburg, Germany
e-mail: dandekar@biozentrum.uni-wuerzburg.de

The human-pathogenic bacterium *Salmonella enterica* adjusts and adapts to different environments while attempting colonization. In the course of infection nutrient availabilities change drastically. New techniques, "-omics" data and subsequent integration by systems biology improve our understanding of these changes. We review changes in metabolism focusing on amino acid and carbohydrate metabolism. Furthermore, the adaptation process is associated with the activation of genes of the *Salmonella* pathogenicity islands (SPIs). Anti-infective strategies have to take these insights into account and include metabolic and other strategies. *Salmonella* infections will remain a challenge for infection biology.

Keywords: metabolism, *Salmonella*-containing vacuole (SCV), regulation, virulence, "-omics"

INTRODUCTION

Salmonella enterica is a Gram-negative enterobacterium closely related to *Escherichia coli* (Neidhardt, 1996). *Salmonellae* reside in humans, a range of animals as well as in the environment and hence are facultative pathogens, often taken up by contaminated food and causing self-limited gastrointestinal disease. In weakened conditions the non-typhoidal serovars may lead to severe bloodstream infections, with high fatality rates in developing countries (Feasey et al., 2012) while typhoidal forms (*S. enterica* serovars Typhi, Paratyphi) strike with endotoxins, typhoid fever, and severe systemic illness. The millions of infections and thousands of fatal cases every year are an important reason for a better understanding and control of *Salmonella* infection (Feasey et al., 2012). To capture the diversity of the *Salmonella* lifestyle in infection is a challenging task. In this review, we will focus on metabolic aspects as well as on insights from "-omics" data, systems biology, and new technologies studying *Salmonella* infection. *Salmonella*, like several other Gamma-proteobacteria, are found in various environments including soils, water systems, and sewage, as well as in the gut flora of various animals. To survive and multiply in this large variety of environments, their metabolism has to adapt well (Rosenkrantz et al., 2013). The large genome of *Salmonella* contains more than 4000 genes encoding a large range of metabolic pathways, for instance an *S.* Typhi chromosome comprises 4,809,037 bp corresponding to 4599 ORFs (including 204 pseudogenes; Parkhill et al., 2001). The pseudogene complement of *S.* Typhi is involved in the tight host restriction of this important human pathogen. There is no zoonotic reservoire. The *S.* Typhi genome reveals an unexpectedly large diversity compared to its relatives *E. coli* and non-typhoidal *Salmonella*.

The lifestyle of *Salmonella*, featuring intestinal colonization, environmental survival, and transmission is reflected in unique gene clusters for adaptation to environmental niches and pathogenicity such as inside the host cell the *Salmonella*-containing vacuole (SCV). Adaptations include multiple abilities for oxygen and nitrate respiration (Rowley et al., 2012). Many further substrates can be used in multiple pathways, depending on environmental conditions. As a food-borne pathogen, various sugars such as D-glucosaminate can be used, supported by suitable permeases (Miller et al., 2013). A vivid picture emerges from data gained by recently established methodologies. Still, not enough is known about regulatory networks around the *Salmonella* Pathogenicity Island, the impact of effector proteins and transport processes and their role in shaping the conditions in the SCV.

In the following we will present established and new approaches of studying *Salmonella* infections, after which we address new perspectives on systems biology including postgenomic modeling techniques and functional genomics. We next discuss stress conditions and specific nutrient supplies and their impact on *Salmonella* metabolism, in particular amino acids and carbohydrate metabolism. Furthermore, connections between metabolism and virulence are discussed. These include SPI1 and SPI2 inducing conditions and their interplay with metabolism. New anti-infective *Salmonella* strategies take these aspects into account. In particular, one has to refine metabolic targeting and drug strategies accordingly. *Salmonella* infection is a particular challenging aspect of its versatile, highly adaptive life style.

TECHNIQUES FOR STUDYING THE INTRACELLULAR LIFESTYLE OF *SALMONELLA*

Systems biology provides a new technological perspective on *Salmonella* metabolism and virulence: this includes scarless mutation techniques, metabolic flux measurements by isotopologs and sophisticated -omics techniques allowing to study all aspects

of the intracellular lifestyle of *Salmonella* in unprecedented detail.

Genetics

The very first step in the analysis of the importance of different metabolic enzymes is the generation of mutant strains. For *Salmonella,* the preferred method to rapidly delete chromosomal genes is the phage λ Red deletion technique (Datsenko and Wanner, 2000). Defined single or multiple gene deletion collections for *S.* Typhimurium have recently been published, covering deletions of 3517 genes (Porwollik et al., 2014). Double or multiple mutations, often needed to delete all isoenzymes of a given metabolic pathway, are commonly generated by repeated rounds of Red deletion, combined with phage P22 transduction (Zinder and Lederberg, 1952) and curing of antibiotic resistance. Since the sequential mutagenesis may lead to accumulation of recombination scars and generation of genomic chimera, newer approaches are based on scarless Red recombinase-mediated deletion (Blank et al., 2011).

Phenotyping

Before testing the influence of a deactivated metabolic enzyme on *Salmonella* virulence, a primary phenotypic characterization is often performed via determination of growth kinetics. By using minimal medium with different C-sources, Paterson et al. could reveal the ability of a Tpi (triosephosphate-isomerase) deficient strain to utilize gluconate, but not other sources such as glucose (Paterson et al., 2009). Additionally, one can perform growth kinetics with media which mimic different *in vivo* conditions. For instance Wallrodt et al. studied the role of the sulfurtransferases GlpE and PspE for resistance against NO radicals via growth kinetics in minimal medium with S-nitrosoglutathion supplementation (Wallrodt et al., 2013). To investigate the adaptation of *Salmonella* to life within the SCV, conditions inducing SPI2 genes are frequently used, such as minimal medium with low phosphate concentrations (Deiwick et al., 1999).

After these first phenotypic characterizations, the impact of defined gene deletions on *Salmonella* virulence is tested most commonly in cell culture experiments, such as gentamicin protection assays, which provide first clues about the role of metabolic enzymes, transporters, etc., on virulence. In this kind of assays the inability of gentamicin to penetrate into eukaryotic cells is used to kill extracellular bacteria, whereas internalized bacteria do not come into contact with the antibiotic substance (Lobo, 1973). With this method not only *Salmonella's* ability to enter host cells by invasion or phagocytosis but also the intracellular replication ability can be examined (Hölzer and Hensel, 2012).

Animal models

Comprehensive *Salmonella* infection models are animals and specific mouse strains are often used. In mice, *Salmonella enterica* serovars pathogenic for humans have been reported (Mathur et al., 2012) not to cause any disease due to an additional Toll-like receptor in mice (TLR11) but further studies have to further confirm this. However, *S. enterica* serovar Typhimurium, which can cause human diarrhea, causes a systemic infection in mice with pathology and disease progression similar to human typhoid fever in mice defective in *Slc11a1* (or *NRAMP*) encoding a $Fe^{2+}/Mn^{2+}/Zn^{2+}$transporter. Thus, to study the mechanisms of systemic disease caused by *Salmonella,* infection models using *Salmonella*-susceptible inbred mouse strains such as BALB/c or C57BL/6 with defective *Slc11a1* allele are frequently used (Steeb et al., 2013). To understand gastroenteritis caused by *Salmonella*, a major breakthrough was the advent of the Streptomycin-pretreated mouse model. Application of Streptomycin reduces the intestinal microbiota and renders mice susceptible to *Salmonella*-induced intestinal inflammation. For this, C57BL/6 or similar mouse laboratory strains can be used and the *Salmonella* have to be Streptomycin resistant, e.g., *S. enterica* serovar Typhimurium SL1344 (reviewed in Kaiser et al., 2012).

Genomics

Methods useful in analyzing the global impact of gene deletions on *Salmonella* and "-omics" techniques (genomics, proteomics, transcriptomics, metabolomics) facilitate studies on virulence mechanisms and metabolic activities on a molecular level and allow a detailed picture of host-pathogen interactions. Comparative genomics was used for example to identify the presence of different metabolic pathways for non-typhoidal and typhoidal pathovars of *Salmonella* (Nuccio et al., 2014). Several recent studies use next generation sequencing (NGS) to understand non-typhoidal *Salmonella* genomes (reviewed by Wain et al., 2013). A broad collection of African isolates showed that they share a common ancestry with *S.* Typhimurium ST313. The study furthermore implies antibiotic resistances were acquired independently in two lineages of *S.* Typhimurium. These data are complemented by phage typing and pulse field gel electrophoresis (PFGE) for additional high resolution typing of *Salmonella* isolates by phage types and different PFGE patterns. This allows investigation in unprecedented detail of virulent strains as well as their correlation with metabolic resistance features such as pathways for degradation of antibiotics.

Transcriptomics

The second "-omics" level, namely transcriptomics including microarrays and high throughput sequencing approaches, gives insights into how *Salmonella* regulates its metabolic pathways in response to changing nutritional environments. A study performed by Blair et al. focused on changes in transcriptomic profiles when using LB or various minimal media for growth. Transcription profiles were established and the article instructively starts from microarray experiments (pan-*Salmonella* generation IV microarray) and verifies putative differences by quantitative real-time PCR (Blair et al., 2013). RNA sequencing was applied by Shah (2014) in a recent comparative study of global transcriptomes of high and low pathogenicity (LP) *S. enterica* serovar Enteritidis strains. This technique reveals important links between metabolism and virulence: in LP strains, reduced expression of virulence genes in SPI1 and SPI5 and defensive virulence factors were observed. Interestingly, this was combined with down regulation of metabolic defense pathways, in particular osmotic (glycine betaine/choline transport), oxidative (*katE*, *sodC*), and iron-limiting metabolic protection. In the four

ferritins, bacterioferritin (Bfr) was found to be down-regulated in LP strains.

Proteomics and metabolomics

Mass spectroscopy (MS)-based proteomics is a method of choice when analyzing gene products: with this approach protein expression is directly measured. Typically only several matching peptides from a protein are identified applying the knowledge of the genome sequence and identified reading frames. This only partial peptide coverage for a given *Salmonella* protein is a challenge for MS analyses. Nevertheless, with more effort even quantification of proteins is possible applying different labeling techniques and standards. A good example for the application of the technique to *Salmonella* is the enzyme quantifications of *ex vivo* purified *Salmonella* performed by Steeb et al. (2013), also illustrating that many proteins can be fast analyzed in this way.

Metabolomics is an upcoming technique as it provides at the same time a global as well as direct view on *Salmonella* metabolism. In particular, isotopolog profiling (IP) allows analysis of current metabolic fluxes under defined conditions. For a detailed method explanation see the study by Härtel et al. (2012), demonstrating the technique on the central carbon metabolism and how individual fluxes are deduced by isotopolog patterns. Furthermore, Götz et al. used this technique to analyze the carbon metabolism of enterobacteria infecting CaCo cells and analyzed which carbon sources are used during intracellular growth (Götz et al., 2010). Metabolic measurements have also been improved by other new techniques such as engineering genetically encoded nanosensors from citrate binding proteins such as the histidine sensor kinase CitA to achieve *in vivo* measurements of changing citrate concentrations in *E. coli* by FRET. This system is readily applicable to *Salmonella* (Ewald et al., 2011).

In general, imaging techniques promote and complement the above approaches to studying the intracellular lifestyle of *Salmonella*. Non-invasive imaging techniques like radioisotope-labeled nucleosides, bioluminescence or the use of microscopy (e.g., advanced light microscopy such as with polarized light) coupled to different cell culture techniques (including establishing tissue infection models) offer here a wealth of information. A nice example including bioluminescent *Salmonella*, the Streptomycin mouse model and bioimaging is Pontier-Bres et al. (2014). Here metabolism and virulence are investigated on possibly the highest level: the protective effect of a pro-biotic food, *Saccharomyces boulardii* and its effect on *Salmonella* clearance in mice.

"-OMICS" DATA INTEGRATION AND SYSTEMS BIOLOGY FOR STUDYING *SALMONELLA* DURING INFECTION

Data repositories

The combination of the various "-omics" approaches provides an integrated view on the adaptation of a pathogen to its host, ranging of from understanding of the genetic basis of virulence to the control of metabolic functions within a host organism or host cell. To describe infection processes on a holistic level, multi-omics strategies are required. Large "-omics" datasets on pathogens have become more readily available and have until now shaped the vivid picture of *Salmonella* infection. We present resources of "-omics" data which can be used to integrate and study different levels of systems biology of *Salmonella* infection (**Table 1**). This list compiles several useful resources but it is of course not exhaustive. Many "-omics" studies rely on large-scale sequence analysis using next-generation sequencing techniques on the genome or on RNA (RNAseq). This includes genome information from the Venter institute, different transcriptome data on gene expression and miRNAs from the Gene Expression Omnibus databank (GEO), proteomics data on membrane proteins from TU Munich, a *Salmonella* wiki on genome information as well as links for veterinary and medical resources on *Salmonella* infection.

Integrated analysis

Integration of high dimensional "-omics" datasets improves genome annotations, discovers novel virulence-related factors, and models *Salmonella* growth under infectious states (Ansong et al., 2012).

A multi-omics view on *Salmonella* in intestinal infection helps to better understand the interdependence of regulation and virulence vs. metabolic change, specific techniques and examples are given in **Table 2**. Thus, proteome, metabolome, glycome, and metagenome all change during the murine infection by *S. enterica* serovar Typhimurium. After multiplication in the mouse gut inflammation occurs and the whole microbiome changes: Bacteroidetes and Firmicutes are suppressed, *Salmonella* and *Enterococcus* grow (Deatherage Kaiser et al., 2013). In response to *S. enterica* serovar Typhimurium infection, potential novel innate immune factors can be discovered, there is transmigration and activation of neutrophils and up-regulation of cell surface molecules. Coordinate murine immune responses include complement activation and inflammatory antibacterial response. *Salmonella* metabolism reacts by induction of stress response proteins, synthesis of outer membrane proteins and lipoproteins.

The combination of integrated analysis of the different data sets shows that *Salmonella* reshapes its metabolism for its adaptation to different host environments. Virulence-associated remodeling adapts *Salmonella* to new niches and locations in the host, there nutrient-poor conditions are encountered and a strong protection against hostile environments of the host is mounted (**Figure 1**).

Metabolic modeling

Metabolic modeling of *Salmonella* in infection reveals an integrated picture of *Salmonella* adaptation processes. Furthermore, in the past few years, several groups established extensive, well-curated, models of *Salmonella* metabolism (Raghunathan et al., 2009; Thiele et al., 2011). Metabolic models are refined by considering additional energy required for stress defense mechanisms and adaptation during infection (Steeb et al., 2013) or considering metabolic bottlenecks (**Table 2**).

Modeling regulation of Salmonella metabolism

Several studies analyzed *Salmonella* regulatory networks of genes in various SPI by means of mathematical models (Temme et al., 2008; Bailly-Bechet et al., 2011). Current results allow to model close to observation the sequential activation of virulence gene clusters in adaptation to distinct host environments (**Table 2**).

In the analysis of *Salmonella*-human interactions, large-scale cellular networks can already be described by looking at their

Table 1 | Useful WEB resources for *Salmonella* -omics.

http://gsc.jcvi.org/projects/msc/salmonella/index.shtml	Genomic sequencing center for infectious disease (J. Craig Venter institute) *Salmonella* genome project (many serovar genome sequences, good resource)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3032673/	*Salmonella* community effort metabolic model (Thiele et al., 2011) (down load model)
GSE32995	GEO genome array data sets, examples: GSE27703 Analysis of the host microRNA response to *Salmonella* uncovers the control of major cytokines by the let-7 family (Schulte et al., 2011) Transcriptional profiling of four growth phases *S.* Typhimurium comparing immobilized growth with planktonic growth
http://patricbrc.org/portal/portal/patric/GenomeList?cType=taxon&cId=590&dataSource=&displayMode=genome	Pathosystems Resource Integration Center (PATRIC) *Salmonella* genomes and large collection of sequences
Salmonella—Cbcb—umiacs https://wiki.umiacs.umd.edu/cbcb/index.php/Salmonella	wiki on *Salmonella* genome reads
http://www.poultryhub.org/production/food-safety/salmonella/	Poultry Hub (professional resource on *Salmonella* infections in veterinary medicine)
http://microbes.ucsc.edu/cgi-bin/hgGateway?hgsid=555757&clade=eukaryota-protista&org=Salmonella+typhimurium+LT2&db=0	Complete browsable genome viewer and genome sequence of *Salmonella enterica* serovar Typhimurium LT2 at UC Southern California
http://webclu.bio.wzw.tum.de/binfo/proj/proamp/Target_organisms/target_organisms.html	Integral membrane protein analysis of *Salmonella* (and other bacteria) at TU Munich
http://www.about-salmonella.com/	*Salmonella* food poisoning and outbreaks

structure, without attempting a dynamical simulation. Such graph-based methods mainly focusing on the topology to predict the chain of events in signaling or estimate metabolic capabilities. Here, cellular modules for different functions are identified as sub-graphs (sub-networks) with proteins mediating only this function in the complete network. Furthermore, hubs, central nodes in the network receiving many connections and indicating strongly connected genes or proteins, are of interest. For instance, interactome networks describing protein-protein interactions are built up and serve as scaffolds for further analysis (Schleker et al., 2012).

Rosenkrantz et al. (2013) compared two types of networks for *S.* Typhimurium strain LT2 regarding stress response and metabolic adaptation: a transcriptional data network using transcriptional data for 425 selected genes under different growth and stress conditions identifying the significantly and strongly regulated genes (transcriptional network) for each condition. This was compared to a genome-scale network connecting genes with metabolic pathways and cellular functions. Looking at the top five connecting hub proteins from the transcriptional network (*wraB, ygaU, uspA, cbpA, and osmC*) as well as the hubs in the genome scale metabolic pathway and cellular function network (*ychN, siiF, yajD, ybeB, and dcoC*), all these hubs were found to be dispensable for virulence in mutation studies. However, double mutants of these two sets of regulatory proteins showed clear effects on virulence in mouse infection experiments (Rosenkrantz et al., 2013). This is a particular strong example confirming the robust and well-buffered *Salmonella* regulation of metabolism and cellular function with virulence factors having partly redundant, overlapping functions.

METABOLIC ADAPTATION OF *SALMONELLA* DURING STRESS CONDITIONS

Stress factors linking virulence and metabolism

When *Salmonella* enters into an intestinal epithelial cell, environmental factors such as high osmolarity and neutral pH lead to an activation of *HilD*, which in turn induces *HilA* and *invF* gene expression (Altier, 2005). *HilA* as transcriptional regulator in turn activates all SPI1 genes necessary for assembly of the T3SS (Ellermeier and Slauch, 2007) and translocation of various SPI1 effector and host interaction proteins (Sop proteins, SipA) as well as DksA to coordinate NAD(P)H/NAD(P)(+) redox balance under nutrient limitation (Henard et al., 2010). For instance, SopB protein changes host cell exocytosis (Perret and Zhou, 2013). SPI1 gene expression is dependent on the growth phase (e.g., there is highest SPI1 induction after 3.5 h of growth in rich medium, Cossart and Sansonetti, 2004). Effector protein activity leads to reorganization of the host cell actin cytoskeleton, followed by membrane ruffling and internalization of *Salmonella* (Haraga et al., 2008). Next key factors influencing SPI2 expression (Haraga et al., 2008) such as detection of low osmolarity, low calcium concentrations and acidic pH by the two-component systems Envz/OmpR and SsrAB lead to activation of SPI2 gene expression (Garmendia et al., 2003) with factors such as SifA, SseJ, PipB2, and SseG (Núñez-Hernández et al., 2014) and result in a SCV containing multiplying *Salmonella* and inducing filaments.

Table 2 | Techniques to model *Salmonella* metabolism and its regulation.

Model	Insight	Author, weblink
TECHNIQUES TO STUDY METABOLIC ADAPTATION IN *SALMONELLA*		
S. Typhimurium metabolite profiling for different (nutrient poor, virulence induced) environments and genome-scale metabolite model	→Central carbon metabolism strongly altered (depletion in glycerol catabolism (glycerol, glycerol 3-phosphate, dihydroxyacetone phosphate, and pyruvate), increased glucose. Synthesis and uptake of polyamines (may protect *Salmonella* against osmotic stress inside host cells).	Kim et al., 2013 doi: 10.1039/C3MB25598K
Metabolic model and microbiology, metabolite measurements; for survival (unrelated to growth): "maintenance requirements" and "costs" (to resist host) are calculated as ATP expenditure.	→ Accumulation of metabolites in the infected gut (lactose, galactinol, melibiose, and raffinose) *Salmonella* and murine host lack necessary enzymes → used by Bacteroidetes and other commensals (glycosidases). → Model predicted hundreds of virulence phenotypes with 90% accuracy. → Costs become very high under excess nutrient availability	Deatherage Kaiser et al., 2013 PMID: 22168414
Model	**Result**	**Author, weblink**
METABOLIC MODELING TECHNIQUES		
Hypothesis of nutrient-limitation during infection	→ Inactivation of *Salmonella* enzyme → metabolic bottleneck → overexpression of another enzyme.	Steeb et al., 2013 PMID: 23633950
Deletion effects, e.g., calculations for Δppc	→ No compensatory flux via the glyoxylate shunt.	Fong et al., 2013 PMID: 23432746
Model	**Result**	**Author, weblink**
MODELING REGULATION OF *SALMONELLA* METABOLISM		
Boolean modeling of genes in SPI1, SPI2, and T6SS Integrated: osmolarity, glucose, iron, calcium and magnesium concentrations, growth phase-dependent stationary phase factors.	→ Description of pathogenicity island cross-talk (e.g., SPI2-secreted proteins low → activation of T6SS). → Antagonistic cross-talk (e.g., *SsrAB* to *SciS; MviA* to *RcsB*).	Das et al. (2013) doi: 10.1186/1757-4749-5-28
Spatiotemporal distribution of ROS in neutrophils, macrophages carrying Salmonella and in vivo expression of ROS defense enzymes (KatG, SodAB, and host NADPH oxidase).	→ (a) Neutrophils: lethal concentrations of hydrogen peroxide → (b) Macrophages: only sub-lethal ROS concentration during infection	Burton et al. (2014) PMID: 24439899

The combined action of these regulatory mechanisms ensures that sufficient nutrients are available for *Salmonella* during the infection (**Figure 2**).

Metabolic defense pathways

Salmonella has to adapt its metabolism to different environmental stresses and niches when entering the human host, starting with the challenging acidic environment of the stomach (**Table 3**). Furthermore, immune defense reactions from the host involve free radicals, complement reaction, enzymatic degradation and autophagy reactions. Individual examples for these biochemical assaults on *Salmonella* have been studied in detail. Nitric oxide (NO) produced by the NO synthase of several immune cells of the host has a severe impact on central carbon metabolism of *Salmonella*. NO targets the pyruvate and α-ketoglutarate dehydrogenase complexes (Richardson et al., 2011).

Carbohydrate metabolism

Citrate is a TCA cycle intermediate (**Figure 1**) and is an important regulatory molecule in the control of glycolysis and lipid metabolism (Neidhardt, 1996). Furthermore, acetylation and deacetylation regulate the amount of glycolysis vs. gluconeogenesis as well as branching between citrate cycle and glyoxylate (Wang et al., 2010; **Table 3**). Moreover, citrate is a crucial iron-chelator which is involved in the homeostasis of iron in the pathogen, as well as the host. Iron is an essential component for several enzymes, but in high concentrations, it may cause damage. Citrate is consumed during NO exposure and other stress conditions because the export pump IctE (iron citrate efflux transporter, former called MdtD) transports iron chelated with citrate out of the cell. Export of citrate leads to growth arrest (Frawley et al., 2013), a status that allows it to survive antibiotic challenges as observed in persister bacteria. This function decreases harmful cellular iron content and reduces growth of *Salmonella* making it more stress resistant (**Figure 1**).

The broad influence of amino acids on metabolic adaptation during infection. The work on acetylation regulation in *Salmonella* by Wang et al. (2010) also underlines also that the central carbon as well as connected amino acid metabolism, including the TCA cycle, can directly be linked to stress response (**Figure 1**).

In particular, the bacterial arginine permease *ArgT* is an essential virulence determinant which decreases the host's cellular

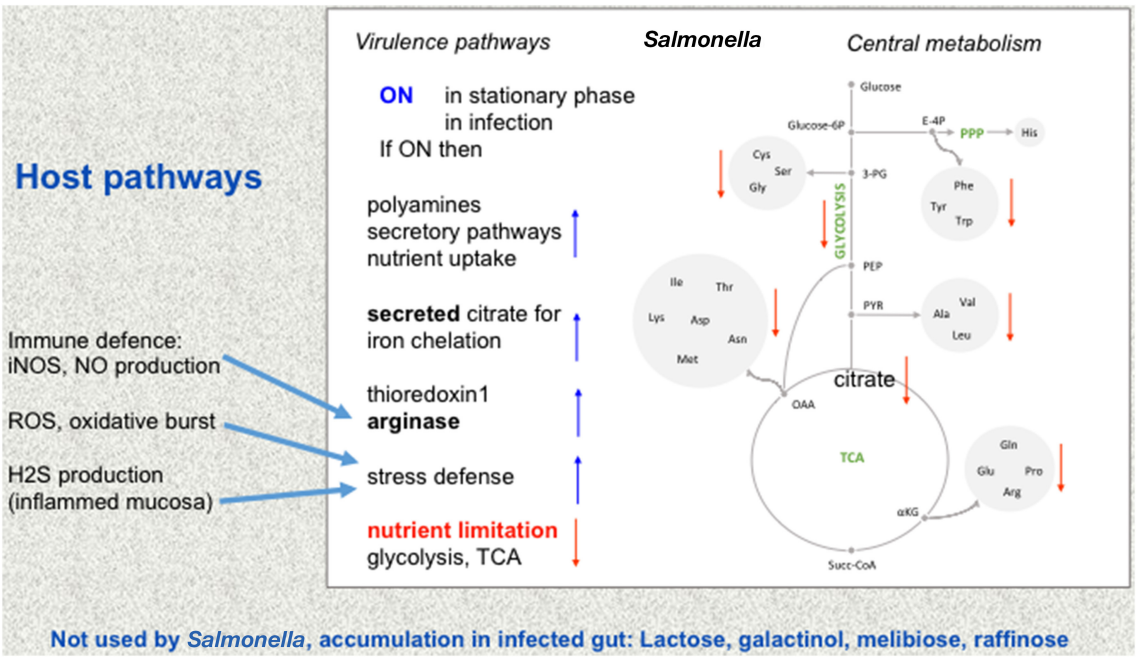

FIGURE 1 | Metabolic adaptation of *Salmonella*. Changes from intestinal to intracellular lifestyle in the mucosa and the resulting adaptations are depicted. The central metabolism of *Salmonella* is shown in the mid panel, while virulence pathways are shown on the left. Environments may change from rather nutrient-rich conditions to nutrient-restricted conditions (red letters) such as in infection, for instance when *Salmonella* is ingested with contaminated food. Amino acids are abbreviated by their three letter code. Other abbreviations: PPP, pentose phosphate cycle; E-4P, erythrose 4-phosphate; Glucose-6P, glucose 6-phosphate; TCA, tricarbonic acid cycle (citric acid cycle). Some of the ensuing changes in *Salmonella* pathways are indicated (right, blue arrows up or red arrows down compared to rich nutrient environment, e.g., TCA goes down while some of the now less used citrate is used to chelate iron). Some metabolic changes from the host that influence *Salmonella* metabolism (Winter et al., 2010; blue arrows) are given on the left. Bottom: these sugars which are not used by *Salmonella* nor by the host accumulate in the infected gut.

arginine content and reduces by this way the NO production of the host (Das et al., 2010; **Table 3**). In contrast, arginine degradation by *Salmonella* appears to be without influence on NO production. Although arginine degradation pathways are up-regulated in *Salmonella* during infection of macrophage and essential for virulence, this is due to other mechanisms but not related to substrate degradation of iNOS (Choi et al., 2012).

Cysteine is a key amino acid during oxidative stress response in *Salmonella*. In a study on cysteine biosynthesis during oxidative stress, cysteine biosynthesis regulation was blocked in Δ*cysB* and Δ*cysE* mutants and oxidative defense pathways encoded by *katG* and *soxS* were up-regulated compared to the wild-type strain (Turnbull and Surette, 2010). Consequently, the cysteine biosynthesis and cysteine-derived molecules such as thioredoxin play an important role for intracellular *Salmonella* survival and replication (Bjur et al., 2006). In this regard, the oxidoreductase thioredoxin 1 (TrxA) was found to be co-induced and essential for SPI2-T3SS activity under conditions that mimic life in the SCV (Negrea et al., 2009).

THE RICHNESS OF *SALMONELLA* METABOLISM AND ITS INFLUENCE ON VIRULENCE

The *SsrAB* virulon controlling SPI2 gene expression is induced under nutrient-poor conditions (e.g., presence in the phagosome, Kuhle and Hensel, 2004).

The interplay of Salmonella pathogenicity islands and metabolism

Various metabolic pathways which have an impact on the SPI1 activity of *Salmonella enterica* (**Table 3**). One example is the interaction between the invasion acyl carrier protein (IacP; Viala et al., 2013) and secretion of SPI1 effector proteins into the host cell to achieve rearrangement of the host cytoskeleton and engulfment of the bacterium (reviewed in Cossart and Sansonetti, 2004).

There are also indications for the influence of SPI1 functions on the host's metabolism in order to facilitate survival in the intestine and subsequently intracellular to promote the infection process. Thus, the SPI1-T3SS effector protein SopE is known to increase *Salmonella* invasiveness and to induce strong inflammatory host responses (Humphreys et al., 2012).

Although SPI1 and SPI2 are induced under very distinct nutritional environments (SPI1 in a nutrient rich environment, SPI2 by nutrient starvation, Kuhle and Hensel, 2004), there are some bacterial metabolites which effect SPI1 as well as SPI2 activity and have a general impact on *Salmonella* virulence (**Table 3**). One example are polyamines, short cationic amines, of which spermidine and putrescine are mostly common in bacteria (Jelsbak et al., 2012).

Intracellular adaptation and metabolism of Salmonella. While conditions in the intestinal lumen are nutrient rich, the situation changes after *Salmonella* invades into the epithelial cells and is phagocytosed at the basolateral cell side by macrophages or

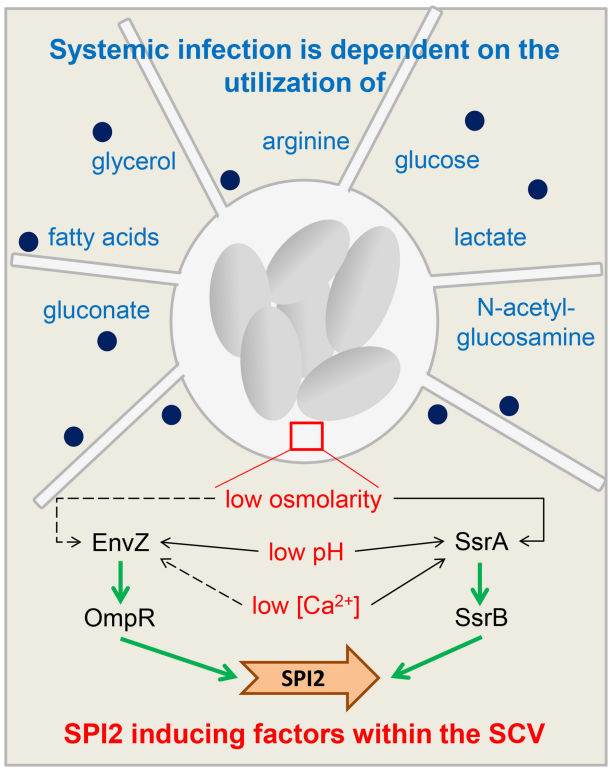

FIGURE 2 | Regulatory adaptations and nutritional requirements of *Salmonella*. A schematic overview of functions of SPI2 during host cell infection and nutritional requirements to manifest a systemic infection. The *Salmonella*-containing vacuole (SCV) with replicating *Salmonella* (gray ovals) is connected to *Salmonella*-induced filaments. Blue writing: substrates *Salmonella* depends on to manifest systemic infections (Steeb et al., 2013). Dark blue spots: SPI2 effectors. The insert (brown rectangle) shows key factors influencing SPI2 expression (Haraga et al., 2008). Detection of low osmolarity, low calcium concentrations and acidic pH by the two-component systems Envz/OmpR and SsrAB inside the SCV leads to activation of SPI2 gene expression (Garmendia et al., 2003). Dotted arrow: minor effect on EnvZ/OmpR activity and SsrAB activity. Non-dotted arrow: strong effect on EnvZ/OmpR activity and SsrAB activity.

dendritic cells. Staying inside the SCV, the pathogen has to deal with nutrient limitations. To investigate which metabolites could interact with expression of genes in SPI1 or mainly SPI2, one issue is to define the nutritional situation of *Salmonella* gain inside the SCV and to figure out which metabolites *Salmonella* has access to. Mouse infection experiments showed on the one hand that intracellular *Salmonella* get access to a wide range of nutrients, including nearly all amino acids except proline. On the other hand, it was shown that the ability to manifest a full systemic infection is dependent on the utilization of "glycerol, fatty acids, N-acetylglucosamine, gluconate, glucose, lactate, and arginine" (Steeb et al., 2013). However, *Salmonella* is able to counteract various defense mechanisms in order to facilitate growth or reduce immune responses (**Table 3**). Invasion of pathogens into epithelial cells is followed by cytosolic amino acid starvation in host cells, which seems to be explained by membrane damage during the invasion process (Tattoli et al., 2012).

However, in contrast to *Shigella*-infected cells, amino acid levels of epithelial cells invaded by *Salmonella* normalized 3 h after infection, which leads to relocalization of mTor invasion sustaining pathway to the SCV, phosphorylation of ATG protein 13, leading to a low ATG protein 1 activity and thus reduced autophagy (Ganley et al., 2009). By this, *Salmonella* is able to avoid autophagy in epithelial cells. Further investigations are required to clarify if normalization of amino acid levels is directly induced by *Salmonella*. At least the invasion-induced membrane disturbance is only severe in the first hour of infection and somehow repaired faster than in cases of invasion by other intracellular pathogens (Tattoli et al., 2012).

ANTI-INFECTIVE STRATEGIES IN THE FACE OF ROBUST *SALMONELLA* METABOLISM

As *Salmonella* adapts rapidly and successfully to changing conditions including intracellular survival in macrophages, in epithelia and in the gut, we will now examine which antibiotic strategies are nevertheless available for *Salmonella* infections. A seminal work by Becker and co-workers showed that the robust metabolism of *Salmonella* limits possibilities for new antibiotics (Becker et al., 2006) and Bumann stressed this point asking "has nature already identified all useful antibacterial targets?" (Bumann, 2008). It is of course important to mention the billions of years sampling time to test and select bacteria and bacterial metabolism during evolution. Furthermore, the parallel exploitation of diverse host nutrients often enhances often *Salmonella* virulence (Steeb et al., 2013) and persistent *Salmonella* are highly resilient (Barat et al., 2012). On the other hand, as many medical areas such as cancer research or aging research also make clear, any medical intervention happened only very recently in evolutionary times. Hence, additional medical interventions are not limited by evolutionary constraints such as positive epistatic selection or direct metabolic energy costs. There are many potential targets still in stock, both by targeting metabolic pathways in pathogenic bacteria and *Salmonella* in particular, as well as by exploring novel ways of anti-infectives. One inspiring example is metabolic engineering of *Salmonella* vaccine bacteria in the mevalonate pathway to boost human Vγ2Vδ2 T cell immunity (Workalemahu et al., 2014). As reviewed earlier (Dandekar and Dandekar, 2010), anti-infective action starts furthermore from typical hygienic measures such as isolation of patients with multiresistant strains including silent clinical carriers, but also includes targeted disturbance of metabolic pathways for example by sulfonamides. In particular, both targeted therapy (direct delivery of an antibiotic to only the location it should act, e.g., in the intestine) as well as targeted modification of standard drugs (so that they are more detrimental to the pathogen even if the host shares similar proteins) are options which have high potential and are not much explored. Our own research highlights the interconnectivity of metabolism. This renders *Salmonella* also vulnerable also in conserved and well investigated pathways, such as TCA cycle and its anaplerotic reactions. Thus, *Salmonella* Typhimurium is controlled by host NO production as shown in mice experiments *in vivo*. Methionine or lysine auxotrophy results from reduced succinyl-CoA availability as the lipoamide dehydrogenase activity is targeted by NO while compensatory

Table 3 | Studies on different metabolic conditions for *Salmonella*.

Condition	Result	Author, weblink
METABOLIC DEFENSE, NO		
NO (murine host) → lipoamide dehydrogenase (Salmonella) reduced activity	→ Methionine and lysine precursor succinate low → transporters (e.g., for succinate) important under nitrosative stress	Richardson et al., 2011 PMID: 21767810
NO (murine host) → reduction of aerobic energy by nitrosylating terminal quinol cytochrome oxidases.	→ Diminishes energy-dependent aminoglycoside uptake → protects antibiotic challenges during host nitric oxide generation	Husain et al., 2008 PMID: 18198179 McCollister et al., 2011 doi: 10.1128/AAC.01203-10
NO (murine host) → decrease in NADH dehydrogenase activity → NADH high in cytoplasm hydrogen peroxide protection	→ Direct detoxification of NO by the NADH dehydrogenase (RNS defense by acid-induced regulator Fur regulates NADH dehydrogenase)	Husain et al., 2008 PMID: 18198179 Husain et al., 2014 PMID: 24166960
Condition	**Result**	**Author, weblink**
CARBOHYDRATE METABOLISM		
Carbohydrate metabolism adaptations of Salmonella during infection	→ Aconitase isoenzymes: acoA for oxidative stress → Repair of oxidized aconitase by bacterial frataxin ortholog proteins CyaY and YggX	Baothman et al., 2013 PMID: 23637460 Velayudhan et al., 2014 PMID: 24421039
S. Typhimurium TCA cycle mutations (gltA, mdh, sdhCDAB, sucAB, and sucCD	→ Incomplete TCA helps survival and replication in resting and activated murine macrophages compared to wt → Epithelial cell infection: Δ*sucCD* and Δ*gltA* replicate less than wt → *S.* Typhimurium Δ*sucAB* and Δ*sucCD* attenuated in murine infection	Bowden et al., 2010 doi: 10.1371/journal.pone.0013871
Influence	**Result**	**Author, weblink**
THE BROAD INFLUENCE OF AMINO ACIDS ON METABOLIC ADAPTATION DURING INFECTION		
Amino acid decarboxylase systems consume protons, raise cytosolic pH	→ *Salmonella* decarboxylases for lysine (*CadA*), arginine (*AdiA*), and ornithine (*SpeF*), not glutamate → acid tolerance but not essential for virulence in mice	Alvarez-Ordóñez et al., 2010 PMID: 19864032 Viala et al., 2011 PMID: 21799843
Arginine has no decarboxylase, but key immune modulator from Salmonella	→ Substrate competition *Salmonella* arginase II and iNOS of the host → *Salmonell*a up-regulates arginase II activity in RAW264.7 macrophages → down regulates host iNOS → by this in intestinal lumen beneficial increase of electron acceptor nitrate	Das et al., 2010 doi: 10.1371/journal.pone.0015466 Lahiri et al., 2008 PMID: 18625332 Humphreys et al., 2012 PMID: 22341462
Feature	**Result**	**Author, weblink**
THE INTERPLAY OF *SALMONELLA* PATHOGENICITY ISLANDS AND METABOLISM		
lacP downstream of sipA for effector protein within SPI1	→ Facilitates *Salmonella* invasion to HeLa cells by secretion of SPI1 effectors *SopA, SopB,* and *SopD* → *lacP* activated by 4′-phosphopantetheine transferase AcpS	Kaniga et al., 1995 PMCID: PMC177584 Kim et al., 2011 PMID: 21263021 Viala et al., 2013 PMID: 23893113
The transferase is a link between bacterial fatty acid metabolism and SPI1 virulence (Hung et al., 2013)	→ Propionyl-CoA represses *AcpS*, *HilD, Salmonella* invasion → Low SopB secretion → May be priming of fatty acid metabolism inside the SCV.	Hung et al., 2013 PMID: 23289537 Viala et al., 2013 PMID: 23893113
Virulence → high iNOS, and NO levels → radical chain reaction, isomeri-zation → nitrate increase	→ Growth advantages for nitrate respiring strains such as SL1344	Lopez et al., 2012 PMID: 22691391
In SCV nitrate respiration tries to avoid host cell damage	→ *NapA* respiration instead of *NarG* pathway (Rowley et al., 2012)	Rowley et al., 2012 PMID: 22039967

(Continued)

Table 3 | Continued

Condition	Result	Author, weblink
INTRACELLULAR ADAPTATION AND METABOLISM OF *SALMONELLA*		
polyamines required for replication in epithelial cells (Jelsbak et al., 2012)	Δ*spe* polyamine synthesis mutant → no polyamines → decreased invasion ability (*hilA* lower → *invF* and *sipB* virulence factor down)	Jelsbak et al., 2012 PMID: 24602405
Glycerol and glucose → major carbon sources in systemic infections (Eisenreich et al., 2013)	→ Δ*tpi* triose phosphate isomerase mutant attenuated in mouse infection → Δ*glpE* mutant strain, too	Eisenreich et al., 2013 PMID: 23847769 Paterson et al., 2009 PMID: 19493007
Amino acid starvation in host → xenophagy, autophagy dependent targeting and degradation of intracellular bacteria	→ Requires host mTOR pathway triggered by autophagy-related gene (ATG) protein 13	Tattoli et al., 2012 PMID: 22704617 Ganley et al., 2009 PMID: 19258318 Kamada et al., 2000 PMID: 10995454

Salmonella pathways to achieve more succinyl-CoA are again blocked by NO (Richardson et al., 2011). Here it also becomes obvious why the therapeutic strategies are not easy exhausted, for instance by direct delivery of NO-increasing drugs to the severely infected gut. Anesthetic drugs are membrane modifiers yielding even multi-resistant pathogens again vulnerable to additional antibiotics, just to cite another possibility (Dandekar and Dandekar, 2010). Furthermore, novel vaccination strategies may proof successful. Hence, the task is more to implement some of the many open alleys for novel antibiotic therapies in clinic. This includes targeting of the metabolism. Furthermore, clinical studies are required for each novel antibiotic strategy, these are currently too expensive for high patient numbers and the prize margin for antibiotics is low so antibiotic development pipelines dry out. However, the prices for such clinical studies could be drastically lowered by modern patient hospital information systems, and furthermore, public awareness and willingness to have better protection against infections is currently increasing.

CONCLUSIONS: *SALMONELLA* GENERAL METABOLIC LIFESTYLE DURING INFECTION

We saw that multiple "-omics" and especially metabolomic data are currently used to determine the needs for *Salmonella* to facilitate intracellular survival within the SCV in host cells and its nutrient supply.

Salmonella's generalist metabolic lifestyle meets all types of environmental challenges, be it ROS or nutrient limitation by its broad metabolic capabilities. The broad metabolism suggests nevertheless potential for novel anti-infective strategies. However, under severe conditions *Salmonella* regulation and metabolism are spiked up by input from SPI1, SPI2, T3SS and T6SS, modified invasion abilities, redox protection and central metabolism to turn the neutral environmental lifestyle of *Salmonella* into a pathogenic lifestyle for its host. On top of this such genetic modules catalyze rapid genetic exchange between *Salmonella* strains showing that only an integrated picture will help to sustain antibiotic efficiency against *Salmonella* infections.

ACKNOWLEDGMENTS

This work was supported by the German Research Foundation (DFG), grants Da 208/13-1 and He 1964/14-2. We thank Jennifer Heilig for critical revision of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 July 2014; accepted: 21 December 2014; published online: 29 January 2015.

*Citation: Dandekar T, Fieselmann A, Fischer E, Popp J, Hensel M and Noster J (2015) Salmonella—how a metabolic generalist adopts an intracellular lifestyle during infection. Front. Cell. Infect. Microbiol. **4**:191. doi: 10.3389/fcimb.2014.00191*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 09 September 2014
doi: 10.3389/fcimb.2014.00125

Metabolism of the vacuolar pathogen *Legionella* and implications for virulence

Christian Manske[1] and Hubert Hilbi[1,2]*

[1] Max von Pettenkofer Institute, Faculty of Medicine, Ludwig-Maximilians University, Munich, Germany
[2] Institute of Medical Microbiology, Faculty of Medicine, University of Zürich, Zürich, Switzerland

Edited by:
Wolfgang Eisenreich, Technische Universität München, Germany

Reviewed by:
Marina Santic', University of Rijeka, Croatia
Joseph Vogel, Washington University School of Medicine, USA

***Correspondence:**
Hubert Hilbi, Institute of Medical Microbiology, Faculty of Medicine, University of Zürich, Gloriastrasse 30/32, 8006 Zürich, Switzerland
e-mail: hilbi@imm.uzh.ch

Legionella pneumophila is a ubiquitous environmental bacterium that thrives in fresh water habitats, either as planktonic form or as part of biofilms. The bacteria also grow intracellularly in free-living protozoa as well as in mammalian alveolar macrophages, thus triggering a potentially fatal pneumonia called "Legionnaires' disease." To establish its intracellular niche termed the "*Legionella*-containing vacuole" (LCV), *L. pneumophila* employs a type IV secretion system and translocates ~300 different "effector" proteins into host cells. The pathogen switches between two distinct forms to grow in its extra- or intracellular niches: transmissive bacteria are virulent for phagocytes, and replicative bacteria multiply within their hosts. The switch between these forms is regulated by different metabolic cues that signal conditions favorable for replication or transmission, respectively, causing a tight link between metabolism and virulence of the bacteria. Amino acids represent the prime carbon and energy source of extra- or intracellularly growing *L. pneumophila*. Yet, the genome sequences of several *Legionella* spp. as well as transcriptome and proteome data and metabolism studies indicate that the bacteria possess broad catabolic capacities and also utilize carbohydrates such as glucose. Accordingly, *L. pneumophila* mutant strains lacking catabolic genes show intracellular growth defects, and thus, intracellular metabolism and virulence of the pathogen are intimately connected. In this review we will summarize recent findings on the extra- and intracellular metabolism of *L. pneumophila* using genetic, biochemical and cellular microbial approaches. Recent progress in this field sheds light on the complex interplay between metabolism, differentiation and virulence of the pathogen.

Keywords: amoeba, *Dictyostelium*, *Legionella*, macrophage, metabolism, nutrition, pathogen vacuole, type IV secretion

INTRODUCTION

Legionella pneumophila is an environmental bacterium ubiquitously found in freshwater, where it is associated with biofilm communities (Lau and Ashbolt, 2009; Hilbi et al., 2011). Protozoan predators like amoebae are part of these communities and feed on bacteria residing within these biofilms. *L. pneumophila* has developed a way to survive and replicate within these free-living protozoa by forming a unique compartment called the *Legionella*-containing vacuole (LCV). The LCV is a pathogen vacuole, wherein *L. pneumophila* dodges lysosomal degradation by acquiring components of early and late endosomes, mitochondria, the endoplasmic reticulum and ribosomes (Isberg et al., 2009; Urwyler et al., 2009a; Hilbi and Haas, 2012). To establish this intracellular niche, the bacterial Icm/Dot type IV secretion system (T4SS) is essential, as it translocates around 300 different "effector" proteins into the host cell, many of which target central eukaryotic pathways like endocytic, secretory or retrograde vesicle trafficking by exploiting small GTPases, phosphoinositide lipids and other host factors (Hubber and Roy, 2010; Finsel et al., 2013; Haneburger and Hilbi, 2013; Rothmeier et al., 2013; Hoffmann et al., 2014a). Besides its natural protozoan hosts, *L. pneumophila* also replicates within human alveolar macrophages and epithelial cells, thus causing a severe pneumonia called Legionnaires' disease. Most processes involved in survival in protozoa or macrophages are very similar and appear to be evolutionarily conserved (Gao et al., 1997; Greub and Raoult, 2004; Hoffmann et al., 2014b). In addition to biofilm and protozoan niches, *Legionella* spp. are also naturally found in physically more challenging habitats, such as extremely acidic environments, antarctic freshwater lakes and water sources with temperatures over 60°C (Hilbi et al., 2011). Accordingly, these facultative intracellular bacteria are an example of a microorganism colonizing many different environmental niches.

To survive within its extra- and intracellular niches, *L. pneumophila* employs a biphasic life cycle, where it alternates between two different forms in response to environmental and metabolic stimuli (Molofsky and Swanson, 2004). In its transmissive form the pathogen is motile, resistant to environmental stress like nutrient starvation and infectious to host cells. In its replicative form the bacteria lack these traits but are able to replicate intracellularly (Rowbotham, 1986; Brüggemann et al., 2006). Further manifestations of *L. pneumophila* differentiation include

a mature intracellular form (MIF) that develops late during infection (Garduno et al., 2002). MIFs are motile, metabolically inert, highly infectious and loaded with cytoplasmic inclusions of poly-3-hydroxybutyrate. Moreover, under harsh conditions, *L. pneumophila* appears to adopt a viable but non-culturable (VBNC) state (Steinert et al., 1997; Garcia et al., 2007; Al-Bana et al., 2014). To ensure bacterial survival in different environments, the biphasic life cycle of *L. pneumophila* is strictly regulated. Consequently, *L. pneumophila* employs a multitude of regulatory systems devoted to the control of gene expression, including transcriptional regulators and two-component systems (Molofsky and Swanson, 2004).

As *L. pneumophila* survives in various environmental niches, it is likely that the bacterium exploits numerous different carbon and energy sources. Furthermore, the intracellular milieu might represent a richly set table for pathogens, as eukaryotic host cells contain many different nutrients, which are potentially accessible to intracellular pathogens (Eisenreich et al., 2010; Rohmer et al., 2011; Abu Kwaik and Bumann, 2013). An intriguing aspect of intracellular metabolism is its compartmentalization into processes that occur within the host cytoplasm, the LCV lumen or the bacteria (**Figure 1**).

Early metabolic studies suggested that amino acids are the major if not only source of carbon and energy for *L. pneumophila* (Pine et al., 1979; Tesh and Miller, 1981; Tesh et al., 1983). However, the subsequent availability of genome sequences, transcriptome, proteome and metabolism data indicated that *L. pneumophila* possess much broader metabolic capacities (Cazalet et al., 2004; Chien et al., 2004; Urwyler et al., 2009b; Eylert et al., 2010; Faucher et al., 2011; Hoffmann et al., 2014a; Schunder et al., 2014). In this review we will summarize the metabolic capacities of *L. pneumophila* regarding amino acid and carbohydrate degradation. Moreover, we will highlight further nutrient requirements of the bacteria and assess the regulation of their life cycle by metabolites.

AMINO ACID METABOLISM

Initial studies of the nutrient requirements of *L. pneumophila* in chemically defined minimal media showed a preference for amino acids as main source of carbon and energy (Pine et al., 1979; Ristroph et al., 1981; Tesh and Miller, 1981; Tesh et al., 1983). A preference for amino acid utilization is also illustrated in the genome sequence of *L. pneumophila*, where around 12 classes of ATP binding cassette transporters, amino acid permeases and

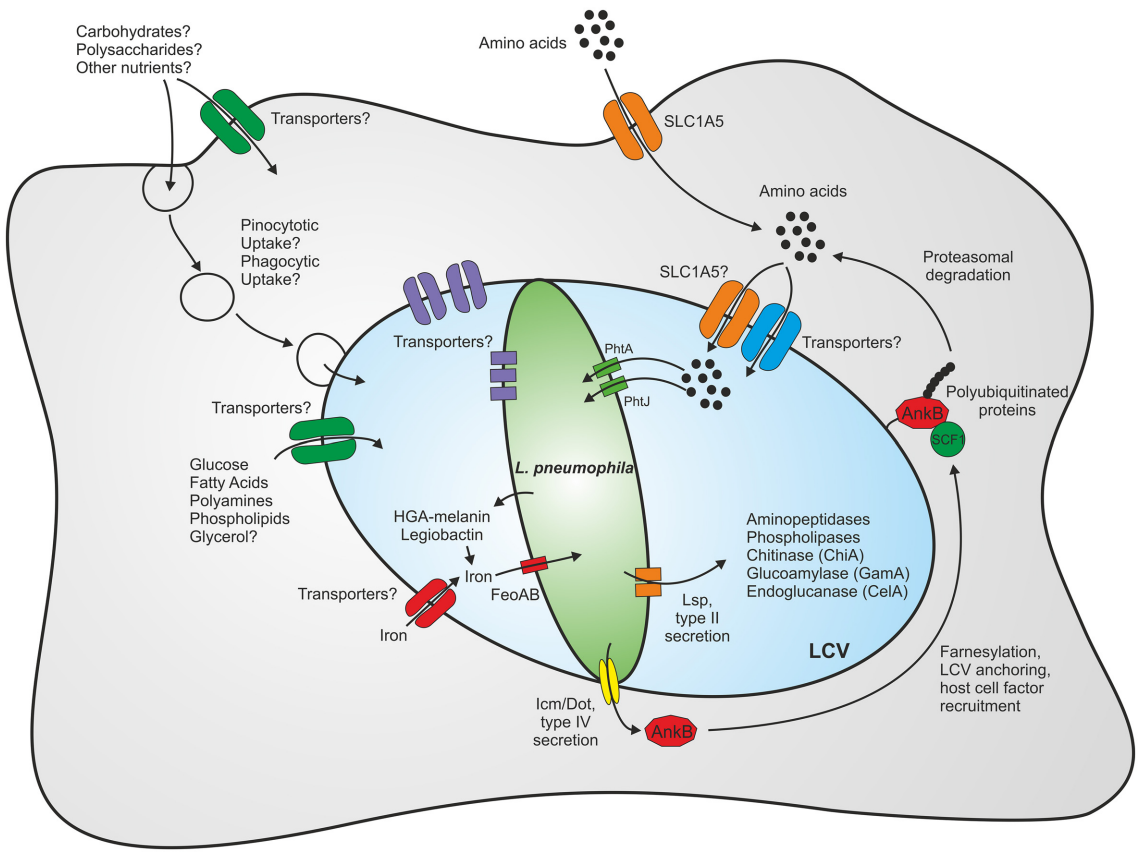

FIGURE 1 | Compartmentalization of the metabolism of *L. pneumophila*. Within eukaryotic host cells *L. pneumophila* forms a membrane-bound replication-permissive compartment, the *Legionella*-containing vacuole. The bacteria gain access to nutrients through intrinsic membrane transporters localizing to the plasma membrane or the pathogen vacuole membrane, respectively, or through fusion with host vesicles and compartments such as endosomes/macropinosomes or the endoplasmic reticulum. Amino acids represent the main carbon and energy source of *L. pneumophila*, yet carbohydrates and complex polysaccharides are also catabolized. For details see text.

proteases can be found (Cazalet et al., 2004; Chien et al., 2004). Furthermore, genes involved in synthesis and transport of amino acids are highly induced during growth inside macrophages (Faucher et al., 2011). *L. pneumophila* employs transport systems to take up and utilize amino acids (Sauer et al., 2005), but also exploits host cell transporters (Wieland et al., 2005) and host proteolytic processes (Price et al., 2011).

L. pneumophila is an obligate aerobe organism and auxotroph for several amino acids including cysteine, arginine, isoleucine, leucine, threonine, valine, and methionine. The observed auxotrophy corresponds to the notion that cysteine biosynthetic genes and other anabolic genes are absent in the genomes of *L. pneumophila* (Cazalet et al., 2004; Chien et al., 2004; Glöckner et al., 2007; D'Auria et al., 2010; Schroeder et al., 2010) and *L. longbeachae* (Cazalet et al., 2010; Kozak et al., 2010). Compared to chemically defined media, the complex ACES-buffered yeast extract (AYE) broth routinely used to grow *L. pneumophila* contains several additional amino acids: alanine, asparagine, glutamine and glycine. The common solid growth medium for *Legionella* species is buffered charcoal-yeast extract (BCYE) agar, supplemented with L-cysteine and ferric pyrophosphate. *L. pneumophila* growth depends on excess cysteine in the medium (Feeley et al., 1979; George et al., 1980). Yet, the amount of cysteine added to the BCYE medium is much higher than what is required to support growth. The major part of cysteine in the *Legionella* growth medium is rapidly oxidized to cystine and becomes unavailable to the bacteria, as *L. pneumophila* is not able to utilize this compound (Ewann and Hoffman, 2006). The remaining concentration of cysteine is around 0.5 mM, which is enough to support *Legionella* growth. Furthermore, using radio-labeled cysteine and mutant strains, it was found that cysteine is not only imported by specific transporters but also consumed during *L. pneumophila* growth (Ewann and Hoffman, 2006).

L. pneumophila is also auxotroph for arginine, as the bacteria lack enzymes that allow synthesis of arginine from glutamate. However, the bacteria produce arginine in chemically defined medium supplemented with ornithine or citrulline, which are precursors of arginine emerging in the later steps of the synthesis from glutamate (Tesh and Miller, 1983; Hovel-Miner et al., 2010). Furthermore, *L. pneumophila* mutants lacking the arginine repressor ArgR fail to replicate within host cells. ArgR might sense the availability of arginine within the host. This leads to the expression of genes (many of them not involved in arginine metabolism), which are required for intracellular growth (Hovel-Miner et al., 2010).

The identification of the phagosomal transporter A (PhtA) revealed a major role of threonine not only for replication but also for differentiation of *L. pneumophila* (Sauer et al., 2005) (**Figure 1**). A mutant strain lacking *phtA* does not grow in a chemically defined medium, but is rescued by excess tryptone or dipeptides containing threonine, indicating that PhtA is not the only threonine uptake system. Intriguingly, *phtA* mutant bacteria are defective for intracellular replication in macrophages due to their inability to differentiate from the transmissive to the replicative state. Analogously, PhtJ was identified as a valine transporter also required for differentiation and replication within macrophages (Sauer et al., 2005; Chen et al., 2008). These findings highlight the role of the Pht transporters as means for *L. pneumophila* to scavenge amino acids from host cells.

Further evidence for the importance of the Pht transporter family for nutrient acquisition of *L. pneumophila* was obtained by investigating the *phtC-phtD* locus (Chen et al., 2008). The *phtC* and *phtD* genes are paralogs in an operon containing genes involved in nucleotide metabolism. The transporter genes are required for successful replication within macrophages and survival of thymidine deprivation. Expression of *phtC* and *phtD* in *E. coli* bestowed pyrimidine transport activity upon strains lacking all known nucleoside transporters, identifying PhtC and PhtD as thymidine transporters (Fonseca et al., 2014).

To take up and utilize amino acids, *L. pneumophila* does not only produce many own systems, but also exploits host metabolic functions (**Figure 1**). The eukaryotic neutral amino acid transporter SLC1A5 was found to be upregulated in *L. pneumophila*-infected cells, and blocking the transporter with the competitive inhibitor BCH (2-amino-2-bornonane-carboxylic acid) or depletion by RNA interference impaired intracellular growth of *L. pneumophila* (Wieland et al., 2005). This study demonstrated the requirement of a single host cell transporter for intracellular replication and also indicated that SLC1A5 may be recruited to the LCV, thus enabling *L. pneumophila* to import amino acids from the cytoplasm into the LCV lumen. Other host-cell transporters might be utilized in a similar manner. Notably, similar to *L. pneumophila*, *Francisella tularensis* modulates the expression of SLC1A5 upon infection of THP-1 human monocytes and is also impaired for intracellular replication when this transporter is downregulated (Barel et al., 2012).

L. pneumophila uses the Icm/Dot T4SS to translocate effector proteins across the LCV membrane to interfere with central host cell processes (**Figure 1**). The Icm/Dot substrate AnkB subverts amino acid metabolism and protein degradation by hijacking the host cell ubiquitination machinery and the proteasome to create nutrients for bacterial growth (Al-Khodor et al., 2010). AnkB harbors several eukaryotic domains: an F-box domain that allows interaction with the host SCF1 ubiquitin ligase complex, two ANK domains, which mediate protein-protein interactions in eukaryotes and a CaaX motif that is modified by farnesylation (Price et al., 2009, 2010a,b; Ensminger and Isberg, 2010; Ivanov et al., 2010; Lomma et al., 2010). Farnesylation of AnkB leads to localization of the effector to the LCV membrane, and intracellular replication of *L. pneumophila* fails when farnesylation is blocked. Anchoring of the effector to the LCV membrane recruits polyubiquitinated host cell proteins, which are degraded by the host proteasome generating a pool of amino acids utilized for intracellular bacterial replication (Price et al., 2011).

Isotopolog profiling is a powerful approach to study metabolic pathways. The method is based on the incorporation of carbon isotopes from stable isotope-labeled precursors such as [U-$^{13}C_3$]serine or [U-$^{13}C_6$]glucose. To elucidate the metabolic pathways and fluxes used, key metabolites such as protein-derived amino acids or storage compounds are then analyzed for the presence of labeled carbon atoms (Zamboni et al., 2009). Metabolomic flux analysis and isotopolog profiling have recently provided detailed insights into the metabolism of the pathogenic bacteria *Listeria monocytogenes* (Gillmaier et al.,

2012) or *Streptococcus pneumonia* (Hartel et al., 2012), and the metabolic responses of infected host cells to the pathogens have also been investigated (Eisenreich et al., 2013).

Upon growth of *L. pneumophila* in AYE medium supplemented with [U-$^{13}C_3$]serine, incorporation of the ^{13}C-label indicated that the amino acid was not only used for protein biosynthesis but also to synthesize other amino acids and poly-3-hydroxybutyrate (Eylert et al., 2010). Thus, in agreement with earlier studies (Pine et al., 1979; George et al., 1980; Ristroph et al., 1981) serine can serve as a major carbon source during growth of *L. pneumophila* in broth. Yet, no ^{13}C-label was detected in isoleucine, leucine, phenylalanine, tyrosine, histidine, proline or valine, confirming the auxotrophy of *L. pneumophila* regarding these amino acids (Eylert et al., 2010). Finally, isotopolog profiling also revealed that *L. pneumophila* growing intracellularly in *Acanthamoeba castellanii* previously fed with [U-$^{13}C_6$]glucose utilizes amoebae-derived amino acids (e.g., phenylalanine, tyrosine) for protein biosynthesis (Schunder et al., 2014).

CARBOHYDRATE AND POLYSACCHARIDE METABOLISM

While amino acids seem to represent the preferred carbon source of *L. pneumophila*, the bacteria can also metabolize carbohydrates, other small organic compounds and complex nutrients (**Figure 1**). Early studies using ^{14}C-radio-labeled substrates indicated that glucose, α-ketoglutarate, pyruvate, glycerol and acetate are metabolized by *L. pneumophila*, yet only some of these compounds stimulated extracellular bacterial growth under the conditions used (Pine et al., 1979; Weiss et al., 1980; Tesh et al., 1983). Moreover, during infection of macrophages *L. pneumophila* genes required for glycerol catabolism—namely *lpg1414* and *glpD*—were highly upregulated compared to growth in rich broth (Faucher et al., 2011). Therefore, glycerol likely plays a role during intracellular growth of *L. pneumophila*, similar to other intracellular bacteria such as *L. monocytogenes* (Eylert et al., 2008; Joseph et al., 2008) and *Salmonella enterica* (Steeb et al., 2013).

Glucose was not found to stimulate growth of *Legionella* spp.; however, the genomes of *L. pneumophila* (Cazalet et al., 2004; Chien et al., 2004; Glöckner et al., 2007; D'Auria et al., 2010; Schroeder et al., 2010) as well as *L. longbeachae* (Cazalet et al., 2010; Kozak et al., 2010) encode complete pathways required for metabolism of carbohydrates, including the Emden-Meyerhof-Parnas (EMP) pathway, the Entner-Doudoroff (ED) pathway, as well as an incomplete pentose phosphate (PP) pathway. In support of the notion that carbohydrate metabolism is crucial during infection, genes associated with the ED pathway, as well as a glucokinase and a glucoamylase, were upregulated upon intracellular growth of *L. pneumophila* in *A. castellanii* (Brüggemann et al., 2006). Another intracellular pathogen that depends on sugar assimilation via the ED pathway during intracellular growth is *S. enterica*. Yet, in this case the parallel exploitation of several different host nutrients enhances bacterial virulence (Steeb et al., 2013).

Using isotopolog profiling, it was recently shown that *L. pneumophila* indeed catabolizes glucose via the ED pathway (Eylert et al., 2010). Upon growth in a chemically defined medium containing [U-$^{13}C_6$]glucose, followed by analysis of the isotopolog pattern by mass spectrometry and NMR spectroscopy, the ^{13}C-label was recovered with high efficiency in alanine and also in poly-3-hydroxybutyrate. In contrast, an *L. pneumophila* mutant lacking the glucose-6-phosphate dehydrogenase gene (*zwf*), the first gene of an operon comprising the genes of the ED pathway (*zwf-pgl-edd-glk-eda-ywtG*), did not incorporate label from glucose and was outcompeted by the wild-type strain in co-infection experiments using *A. castellanii* (Eylert et al., 2010). In line with these observations, *L. pneumophila* lacking other components of the ED pathway, either glucokinase (*glk*), phosphogluconate dehydratase (*edd*), 2-keto-3-deoxy-phosphogluconate aldolase (*eda*) or the putative sugar transporter (*ywtG*), was no longer able to metabolize glucose and was defective for growth in *Acanthamoeba culbertsoni* or mammalian cells (Harada et al., 2010). Together, these findings strongly support the notion that the ED pathway is essential for glucose metabolism and intracellular growth of *L. pneumophila*. The results also implicate that under the conditions prevailing within LCVs in host cells *L. pneumophila* does not solely grow on amino acids as carbon and energy sources, but rather, carbohydrates are also utilized (at least as co-metabolites). Yet, the relative contribution of amino acids and carbohydrates to intracellular growth is difficult to assess, and many carbohydrates do not support extracellular growth as sole source of carbon and energy.

The transporters promoting the uptake of sugars have not been studied in molecular detail at present. The gene *ywtG* (lpg0421) is conserved among *L. pneumophila* and *L. longbeachae*, and annotated as a putative D-xylose (galactose, arabinose)-proton symporter (Cazalet et al., 2004, 2010). However, arabinose appears to be barely taken up by *L. pneumophila* (excluding genetic approaches based on the arabinose promoter, P_{bad}). Moreover, glucose-1-phosphate is metabolized much faster than glucose-6-phosphate or glucose, suggesting that the former compound is transported efficiently into the cells (Weiss et al., 1980).

In addition to simple carbohydrates and small organic compounds, polymeric compounds also likely serve as carbon sources for *L. pneumophila*. The exogenous supply of polyamines during infection moderately favored intracellular replication of *L. pneumophila* (Nasrallah et al., 2011). Moreover, similar to other bacteria (Khosravi-Darani et al., 2013), *L. pneumophila* might use the intracellular "energy reserve" poly-3-hydroxybutyrate as an endogenous source of carbon and energy, which is synthesized via pyruvate and acetyl-coenzyme A (James et al., 1999; Eylert et al., 2010). Further support for the notion that *Legionella* spp. degrade complex polysaccharides stems from the genome sequences. *L. longbeachae* harbors a number of genes likely involved in cellulose degradation (Cazalet et al., 2010), and *L. pneumophila* contains genes putatively involved in the degradation of cellulose, chitin, starch and glycogen (Cazalet et al., 2004).

The Lsp type II secretion system (T2SS) is essential for intracellular growth of *L. pneumophila* in amoebae and macrophages (Hales and Shuman, 1999a; Liles et al., 1999) (**Figure 1**). Proteome studies on the type II "secretome" of *L. pneumophila* revealed that the bacteria secrete a chitinase (ChiA), as well as an endoglucanase, which metabolizes carboxymethyl cellulose (Debroy et al., 2006). An endoglucanase (CelA) was indeed found to degrade cellulose (Pearce and Cianciotto, 2009), and a eukaryotic-like glycoamylase (GamA) degraded carboxymethyl

cellulose, glycogen and starch (Herrmann et al., 2010). Yet, neither CelA nor GamA was required for growth of *L. pneumophila* in amoebae. In summary, insights from genomics, transcriptomics, metabolomics, as well as biochemical experiments indicate that *L. pneumophila* utilizes simple and also complex carbohydrates as important sources of carbon and energy during extra- and intracellular growth.

MICRONUTRIENT REQUIREMENTS

Iron is essential for growth of most if not all bacteria, as it is a co-factor for many enzymes of the central metabolism as part of prosthetic groups like heme or iron-sulfur clusters (Ratledge and Dover, 2000). Moreover, the availability of iron is especially important for pathogens, as iron limitation plays an important role in host defense against infections. For *L. pneumophila* iron represents an essential nutrient and has to be supplemented in high concentrations to growth media (Reeves et al., 1981; Ewann and Hoffman, 2006). The major iron-containing protein of *L. pneumophila* is aconitase of the tricarboxylic acid cycle (Mengaud and Horwitz, 1993). *L. pneumophila* grown under iron-limited conditions showed reduced virulence and was impaired for survival in host cells (James et al., 1995). Furthermore, host cells treated with iron chelators did not support growth of *L. pneumophila*, presumably due to iron limitation, as the addition of iron as iron-transferrin or ferric iron-nitrilotriacetate reversed growth inhibition (Gebran et al., 1994; Byrd and Horwitz, 2000; Viswanathan et al., 2000). Notably, patients with iron overload or smokers are at increased risk for Legionnaires' disease, probably because their lungs contain increased levels of iron (Fields et al., 2002; Vikram and Bia, 2002).

Iron exists in equilibrium between a ferrous (Fe^{2+}) and a ferric (Fe^{3+}) form, depending mostly on the pH and availability of oxygen (Williams, 2012). In *L. pneumophila*, many systems are devoted to iron metabolism and involved in iron reduction, complexation and transport (**Figure 1**). Iron reductase enzymes may promote iron assimilation in the periplasm and cytoplasm (Johnson et al., 1991; Poch and Johnson, 1993). Iron reduction is also catalyzed by the secreted compound homogentisic acid (HGA) and its polymerized derivative HGA-melanin (Chatfield and Cianciotto, 2007; Zheng et al., 2013). HGA is a product of the phenylalanine and tyrosine catabolism of *L. pneumophila* and was identified as the brown pigment secreted by *L. pneumophila*, which is produced from oxidative polymerization of HGA to HGA-melanin (Steinert et al., 2001). HGA and HGA-melanin stimulate growth of *L. pneumophila* under iron-limiting conditions, enhance the uptake of iron and can release ferrous iron from transferrin and ferritin, two major protein iron chelators of mammalian cells (Zheng et al., 2013).

L. pneumophila chelates and transports iron with the secreted high-affinity iron siderophore legiobactin (Liles et al., 2000; Starkenburg et al., 2004). The gene *lbtA*, which has homology with siderophore synthetases, and *lbtB* that encodes a homolog of a multidrug efflux pump, were identified as key players in the synthesis of legiobactin (Allard et al., 2006). Moreover, an *L. pneumophila lbtA* mutant strain showed reduced ability to infect lungs of A/J mice, demonstrating the importance of legiobactin *in vivo* (Allard et al., 2009). Iron is also transported in *L. pneumophila* via the *FeoAB* system (Robey and Cianciotto, 2002). The *L. pneumophila feoAB* operon bears homology to the *E. coli* system, a well-characterized ATP-driven ferrous iron transporter. An *L. pneumophila feoB* mutant was outcompeted by wild-type bacteria during infection of A/J mice, highlighting an important role of the transporter *in vivo*.

Finally, in addition to iron, the extracellular growth of *L. pneumophila* is also stimulated by calcium, magnesium and zinc. Calcium and magnesium might also play a role in biofilm formation, as the two metal ions enhance the adherence of *Legionella* to surfaces (Reeves et al., 1981; Koubar et al., 2013).

METABOLIC REGULATION OF DIFFERENTIATION AND VIRULENCE

The biphasic life cycle of *L. pneumophila* is regulated by a variety of environmental and metabolic stimuli (Molofsky and Swanson, 2004). As long as nutrients are not limiting, the post-transcriptional regulator CsrA suppresses transmission traits and promotes replication (Molofsky and Swanson, 2003). Given the importance of amino acids as carbon source for *L. pneumophila*, it is not surprising that these compounds are also main regulatory factors of the phenotypic switch (Byrne and Swanson, 1998; Sauer et al., 2005). Amino acid starvation or otherwise nutrient-limiting conditions trigger the shift from the replicative/non-motile to the virulent/motile form of *L. pneumophila*, which is mediated through the second messenger guanosine 3′,5′-bispyrophosphate (ppGpp) (Hammer and Swanson, 1999; Dalebroux et al., 2010) (**Figure 2**). The "alarmone" ppGpp is synthesized by the synthase RelA as part of the "stringent response" that senses the accumulation of uncharged tRNAs at the ribosome. A second stringent response enzyme called SpoT also synthesizes ppGpp (Dalebroux et al., 2009). However, rather than sensing amino acid shortage, SpoT monitors fatty acid biosynthesis by interacting with the acyl-carrier protein ACP (Edwards et al., 2009). In addition, SpoT hydrolyzes ppGpp during exponential growth to ensure that transmissive traits are not expressed during replication.

The alternative sigma factor RpoS (σ^{38}/σ^{S}) represents the pivotal transcriptional regulator of the *L. pneumophila* life cycle (Hales and Shuman, 1999b; Bachman and Swanson, 2001; Zusman et al., 2002). An *L. pneumophila rpoS* mutant is not affected regarding extracellular growth in broth and retains significant stress resistance, but is not able to replicate in amoebae. This severe defect in intracellular replication is not due to impaired Icm/Dot function or *icm/dot* gene expression, but because of major transcriptional changes affecting basic cellular processes and other central regulatory networks (Hovel-Miner et al., 2009). The transcription of more than 70 genes required for central metabolism, 40 of these associated with amino acid metabolism, was negatively regulated in the *rpoS* mutant. Furthermore, small regulatory RNAs (*rsmY* and *rsmZ*) (Rasis and Segal, 2009; Sahr et al., 2009), two component systems (CpxRA, PmrAB), the transcriptional regulator ArgR and the quorum sensing response regulator LqsR (Tiaden et al., 2007) are regulated by RpoS (Bachman and Swanson, 2004; Hovel-Miner et al., 2009) (**Figure 2**).

At least three two component systems and one quorum sensing system influence the virulence of *L. pneumophila:* CpxRA (Gal-Mor and Segal, 2003a; Altman and Segal, 2008), PmrAB

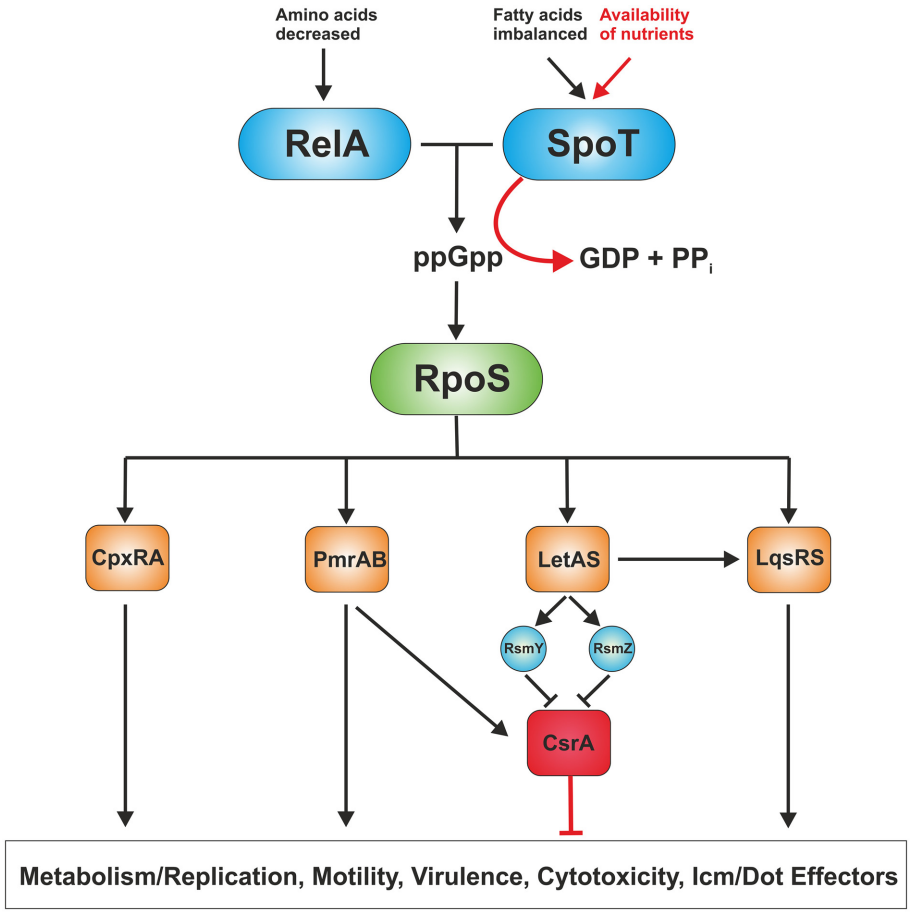

FIGURE 2 | Regulation of replicative and transmissive traits of *L. pneumophila*. *L. pneumophila* senses its metabolic state by means of the two ppGpp synthases RelA and SpoT. RelA detects amino acid starvation, whereas SpoT monitors disturbances in fatty acid synthesis. When nutrients become limiting, the "alarmone" ppGpp acumulates in the bacteria leading to production of the alternative sigma factor RpoS. In turn, RpoS regulates the two component or quorum sensing systems CpxRA, PmrAB, LetAB, and LqsRS, which control metabolism/replication, motility as well as virulence traits, and hence, govern the transition from the replicative to the transmissive form. The RNA-binding global regulator CrsA controls the biphasic switch as an antagonist of the two component and quorum sensing systems.

(Zusman et al., 2007; Al-Khodor et al., 2009; Rasis and Segal, 2009), LetAS (GacAS) (Hammer et al., 2002; Gal-Mor and Segal, 2003b; Lynch et al., 2003) and the *Legionella* quorum sensing (*lqs*) gene cluster (Tiaden et al., 2010) (**Figure 2**). The *lqs* system of *L. pneumophila* comprises the autoinducer synthase LqsA, the sensor kinases LqsS and LqsT (Kessler et al., 2013), and the response regulator LqsR (Tiaden et al., 2007). LqsA produces the compound LAI-1 (*Legionella* autoinducer-1, 3-hydroxypentadecane-4-one) (Spirig et al., 2008), which presumably binds to the cognate sensor kinases. The kinase-mediated phosphorylation signal converges on LqsR (Schell et al., 2014), which among many other processes also controls the switch from the stationary phase to the replicative phase (Tiaden et al., 2007). Lqs-regulated processes include pathogen-phagocyte interactions, production of extracellular filaments, natural competence for DNA uptake and the expression of a 133 kb genomic "fitness island" (Tiaden and Hilbi, 2012). Furthermore, transcriptome analysis of *L. pneumophila* strains lacking *lqsR*, *lqsS* or *lqsT* or the entire *lqs* cluster indicates that the Lqs system also regulates a number of metabolic pathways (Tiaden et al., 2007, 2008; Kessler et al., 2013).

CONCLUSIONS AND PERSPECTIVES

The amoebae-resistant bacterium *L. pneumophila* colonizes a variety of extra- and intracellular niches in the environment. Upon reaching the human lung, *L. pneumophila* grows in mammalian macrophages and possibly also in epithelial cells. Accordingly, the bacteria are equipped to utilize a broad range of compounds as carbon and energy sources. In addition to amino acids, which initially have been regarded as the main if not sole nutrients, carbohydrates have recently been shown to be catabolized by extra- and intracellularly growing *L. pneumophila*. Novel technological approaches such as isotopolog profiling allow analyzing metabolic fluxes with unprecedented resolution and sensitivity. Transcriptome and genome studies indicate that a number of other compounds, including complex polysaccharides, are also metabolized by *L. pneumophila*. Further studies will unravel the manifold and robust metabolic pathways that the bacteria employ

to thrive in diverse environmental niches. Importantly, future investigations will also shed light on the intricate relationship between the physiology and pathogenesis of *L. pneumophila*, and thus might contribute to control Legionnaires' disease.

ACKNOWLEDGMENTS

We would like to thank Ina Haneburger and Bernhard Steiner for critically reading the manuscript. Research in our laboratory was funded by the Max von Pettenkofer Institute, Ludwig-Maximilians University Munich, the Deutsche Forschungsgemeinschaft (DFG; SPP 1316, SPP 1617) and the Swiss National Science Foundation (31003A-125369).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 June 2014; paper pending published: 24 July 2014; accepted: 20 August 2014; published online: 09 September 2014.

*Citation: Manske C and Hilbi H (2014) Metabolism of the vacuolar pathogen Legionella and implications for virulence. Front. Cell. Infect. Microbiol. **4**:125. doi: 10.3389/fcimb.2014.00125*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 August 2014
doi: 10.3389/fcimb.2014.00110

The *Mycobacterium avium* ssp. *paratuberculosis* specific *mpt*D gene is required for maintenance of the metabolic homeostasis necessary for full virulence in mouse infections

Thorsten Meißner[1], Elke Eckelt[1], Tina Basler[1], Jochen Meens[1], Julia Heinzmann[1†], Abdulhadi Suwandi[2], Walter M. R. Oelemann[3,4], Sandra Trenkamp[5], Otto Holst[4], Siegfried Weiss[2], Boyke Bunk[6,7], Cathrin Spröer[6,7], Gerald-F. Gerlach[1†] and Ralph Goethe[1]*

[1] Department of Infectious Diseases, Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany
[2] Helmholtz Centre for Infection Research, Molecular Immunology, Braunschweig, Germany
[3] Departamento de Imunologia, Instituto de Microbiologia Paulo Góes, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil
[4] Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany
[5] Metabolomic Discoveries GmbH, Potsdam-Golm, Germany
[6] Bioinformatics, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany
[7] German Centre of Infection Research, Partner Site Hannover-Braunschweig, Braunschweig, Germany

Edited by:
Wolfgang Eisenreich, Technische Universität München, Germany

Reviewed by:
Maria Júlia Manso Alves, University of São Paulo, Brazil
Adel M. Talaat, University of Wisconsin Madison, USA

***Correspondence:**
Ralph Goethe, Zentrum für Infektionsmedizin, Institut für Mikrobiologie, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany
e-mail: ralph.goethe@tiho-hannover.de

†Present address:
Julia Heinzmann, Institute of Farm Animal Genetics, Federal Research Institute of Animal Health, Friedrich-Loeffler-Institut, Neustadt, Germany;
Gerald-F. Gerlach, Innovative Veterinary Diagnostics Laboratory GmbH, Hannover, Germany

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease, a chronic granulomatous enteritis in ruminants. Furthermore, infections of humans with MAP have been reported and a possible association with Crohn's disease and diabetes type I is currently discussed. MAP owns large sequence polymorphisms (LSPs) that were exclusively found in this mycobacteria species. The relevance of these LSPs in the pathobiology of MAP is still unclear. The *mpt*D gene (MAP3733c) of MAP belongs to a small group of functionally uncharacterized genes, which are not present in any other sequenced mycobacteria species. *mpt*D is part of a predicted operon (*mpt*ABCDEF), encoding a putative ATP binding cassette-transporter, located on the MAP-specific LSP14. In the present study, we generated an *mpt*D knockout strain (MAPΔ*mpt*D) by specialized transduction. In order to investigate the potential role of *mpt*D in the host, we performed infection experiments with macrophages. By this, we observed a significantly reduced cell number of MAPΔ*mpt*D early after infection, indicating that the mutant was hampered with respect to adaptation to the early macrophage environment. This important role of *mpt*D was supported in mouse infection experiments where MAPΔ*mpt*D was significantly attenuated after peritoneal challenge. Metabolic profiling was performed to determine the cause for the reduced virulence and identified profound metabolic disorders especially in the lipid metabolism of MAPΔ*mpt*D. Overall our data revealed the *mpt*D gene to be an important factor for the metabolic adaptation of MAP required for persistence in the host.

Keywords: Mycobacterium infections, metabolism, lipids, macrophages, survival

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) belongs to the *M. avium* complex (MAC), which comprises subspecies of *M. avium* with different extents of host adaptation and virulence. For instance *M. avium* subsp. *hominissuis* is found ubiquitously in the environment and causes opportunistic infections in humans, pigs and ruminants, whereas *M. avium* subsp. *avium* is the causative agent of tuberculosis in birds (Thorel et al., 1990; Mijs et al., 2002; Dvorska et al., 2003; Dhama et al., 2011; Ignatov et al., 2012). In contrast, MAP is an obligate pathogen for ruminants causing Johne's disease (JD, paratuberculosis) - a chronic granulomatous enteritis (Kreeger, 1991; Harris and Barletta, 2001). Moreover, infections of humans with MAP have been reported and a possible association with Crohn's disease and more recently with diabetes type I has been discussed (Greenstein, 2003; Feller et al., 2007; Mendoza et al., 2009; Rani et al., 2010; Rosenfeld and Bressler, 2010; Cossu et al., 2011; Chiodini et al., 2012; Naser et al., 2014).

JD is characterized by an extending transmural inflammation of the intestine without caesification of the granulomas. Noteworthy, lesions in other areas are less common, indicating a specific tropism of MAP for the intestine which is not seen in other mycobacteria (Buergelt et al., 1978; Clarke, 1997; Burrells et al., 1998). The intestinal tropism of MAP is particularly evident in ruminants, but it has become apparent that the organism has a much broader host range including monogastric species such

as carnivore (fox, stoats), aves (crow and jackdaw), lagomorpha (rabbits) and recently miniature donkeys (Greig et al., 1999; Beard et al., 2001; Glanemann et al., 2008; Stief et al., 2012; Carta et al., 2013).

The pathobiology of MAP infection including its tropism to the gut is still unresolved. After crossing the intestinal barrier, MAP is taken up by intestinal macrophages and there is common consensus that the persistence in these macrophages is the key step in MAP infection (Ryan et al., 2014). Nevertheless, the persistence in macrophages, characterized by inhibition of the phagosomal maturation process, altered antigen processing and presentation is a common feature of any pathogenic mycobacterial species and might not explain the characteristics of intestinal MAP infection (Kuehnel et al., 2001; Hostetter et al., 2002; Vergne et al., 2004). However, MAP additionally inhibits T cell and Dendritic cell (DC) activation and restricts the macrophage inflammatory cytokine response in cell culture systems (zur Lage et al., 2003; Basler et al., 2010, 2013). Thus, it seems that MAP persists and survives in its intestinal niche by subverting the immune defense mechanisms of the host and therefore might remain locally restricted to the intestine for long time (Atreya et al., 2014). In addition, on the bacterial side consequences of infection are characterized by a strong metabolic adaptation to the intestinal environment of the host (Weigoldt et al., 2011, 2013), which suggest that this capacity considerably adds to MAP pathobiology.

Within the MAC, MAP exhibits particular phenotypic features in culture. Thus, MAP growth in culture is dependent on mycobactin supplementation, extremely slow with the average doubling time of 22–26 h (other MAC ssp. 10–12 h), and shows a strong tendency to clump formation (Merkal and Curran, 1974). Differences in the phenotype and virulence of MAP might be linked to MAP specific genes (Li et al., 2005) and/or the acquisition, loss and rearrangement of specific genetic elements (Alexander et al., 2009). Sixteen large sequence polymorphisms (LSPs) were exclusively found in MAP. Six out of these 16 LSPs are common in all MAP strains and considered to be genomic insertions. These insertions comprise ∼125 kb of DNA with 82 open reading frames (ORFs), with most of them being not of mycobacterial origin but exhibiting similarities to genes from environmental actinomycetes (Marri et al., 2006). Yet it is not clear whether the genes encoded on MAP-specific LSPs contribute to the phenotype and pathogenicity of MAP.

Previously, we had identified a MAP-specific putative 38 kb pathogenicity island which is located on LSP14, the largest LSP found in MAP. LSP14 harbors mostly genes which encode for proteins with predicted functions in metal metabolism (Stratmann et al., 2004). Within the 38 kb DNA region, a predicted operon *mpt*ABCDEF encodes for two putative ATP binding cassette transporters. The *mpt*ABCDEF operon (MAP3736c-31c) exhibits the highest similarity to other genera of the order Actinomycetales such as *Salinispora* or *Bifidobacter* and to a lesser extent to other mycobacteria. In addition, these genes have no other orthologs in MAP (data not shown). Among the operon genes, *mpt*D is unique in MAP within the genus of *Mycobacterium* since it is present in all sequenced cattle- and sheep-type MAP strains, but it is not found in any other so far sequenced mycobacterial species, even not in other subspecies of the MAC. Interestingly, *mpt*D was found to be surface-exposed and expressed during infection (Stratmann et al., 2004; Shin et al., 2006). In addition, Cossu and colleagues found antibodies against *mpt*D in sera of type I diabetes mellitus patients (Cossu et al., 2011), suggesting a possible role of *mpt*D in MAP pathogenicity in humans.

In the present study, we used specialized transduction to generate a *mpt*D knockout strain. By different comparative analyses we found that the gene is important for the metabolic homeostasis of MAP, which appears to be necessary for adaptation to the macrophage environment and survival in a mouse infection model.

MATERIALS AND METHODS

BACTERIAL STRAINS, CHEMICALS, AND GROWTH CONDITIONS

All chemicals were purchased from Sigma-Aldrich (Munich, Germany) if not stated otherwise. All bacterial strains, phages, and plasmids are listed in Table S1. The *Escherichia coli* strains DH5α and HB101 and the *M. smegmatis* mc^2 155 strain were cultured in Luria-Bertani (LB) broth or on LB agar plates (Roth, Karlsruhe, Germany) containing appropriate antibiotics with a concentration of 100 μg/ml. The *E. coli* strains DH5α and HB101 were used for cloning of homologous regions and for construction of the allelic exchange substrate (AES) in pYUB854. Additionally, *E. coli* HB101 was used for the *in vitro* λ-packaging reaction (GIGApack® II plus kit, Stratagene, La Jolla, CA, USA). For transformation experiments, competent *E. coli* HB101 were prepared following standard procedures (Sambrook and Russell, 2001). Liquid cultures of *E. coli* and *M. smegmatis* were grown at 37°C in a shaking incubator. *M. smegmatis* mc^2 155 was used for the generation of high titer phage lysates and phage construction as described (Carriere et al., 1997). *M. avium* subsp. *paratuberculosis* strain DSM 44135 (MAPwt) (Stratmann et al., 2004) was grown in Middlebrook 7H9 broth (MB7H9) or on Middlebrook 7H10 agar supplemented with 10% OADC and mycobactin J (2 mg/l; Allied Monitor, Fayette, USA). MB7H9 medium was further supplemented with Tween® 80 (0.05% final concentration) unless stated otherwise. For growth experiments, MAPwt and the mutant strain MAPΔ*mpt*D were cultured in supplemented MB7H9 medium until an OD_{600} of 3.0 was reached. The bacteria were then harvested, washed two times with phosphate-buffered saline (PBS) and diluted in supplemented MB7H9 without Tween® 80 to an OD_{600} of 0.2. Bacteria were cultivated with stirring at 120 rpm at 37°C and OD_{600} was measured every 2–3 days until entry of stationary phase.

DNA TECHNIQUES

Chromosomal mycobacterial DNA was prepared according to standard procedures (Belisle and Sonnenberg, 1998). Plasmids of *E. coli* were isolated using the NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacture's protocol. Southern blot analyses were performed with *Eco*RV-restricted chromosomal DNA according to standard protocols (Sambrook and Russell, 2001), using a α^{32}-P-dCTP labeled PCR fragment obtained with primers omptD3a and omptD4a as a DNA probe. After hybridization, membranes were exposed to X-ray film (Kodak X-OMAT® Biomax, Sigma Aldrich GmbH, Deisenhofen,

Germany). Polymerase-chain-reactions (PCR) were run on a Mastercycler system (Eppendorf, Hamburg, Germany), using HotStart HiFidelity Polymerase (Qiagen, Hilden, Germany) and the following conditions: 95°C for 10 min, 95°C for 45 s, 58°C for 60 s, and 72°C for 60 s. The oligonucleotides are listed in Table S1.

CONSTRUCTION OF MAPΔ*mpt*D

The construction of the specialized transducing mycobacteriophage containing the AES was performed according to Park and colleagues (Park et al., 2008) with slight modifications. A detailed protocol is given in the supplementary methods file. Briefly, in order to generate an *mpt*D (MAP3733c) deletion in MAP, two oligonucleotide primer pairs (Table S1) were generated to amplify the up- and downstream flanking regions of the *mpt*D gene resulting in an deletion of 518 bps of the *mpt*D ORF upon restriction digest and ligation of the amplified products into the cosmid vector pYUB854 (Bardarov et al., 2002). The resulting construct pMP1310 was introduced into the mycobacterial phage vector phAE87 (Bardarov et al., 1997), resulting in phage phAE111. To construct a MAPΔ*mpt*D mutant, MAP was cultured in MB7H9 medium to OD_{600} of 1.0. Bacterial suspensions of MAP, free of clumps, were transduced and plated on MB7H10 agar plates containing 50 µg/ml hygromycin B and incubated at 37°C for up to 12 weeks. Successful recombination was confirmed by PCR, Southern blot and quantitative real-time PCR (qRT-PCR) analyses (**Figures 1A–C**).

PacBio *RSII* RESEQUENCING STUDY

SMRTbell™ template library was prepared according to the instructions from PacificBiosciences, Menlo Park, CA, USA, following the Procedure and Checklist Greater than 10 kb Template Preparation and Sequencing. Briefly, for preparation of 10 kb libraries, ∼10 µg genomic DNA were end-repaired and ligated overnight to hairpin adapters applying components from the DNA/Polymerase Binding Kit P4 from Pacific Biosciences, Menlo Park, CA, USA. Reactions were carried out according to the manufacturer's instructions. SMRTbell™ template was Exonuclease treated for removal of incompletely formed reaction products. Conditions for annealing of sequencing primers and binding of polymerase to purified SMRTbell™ template were assessed with the Calculator in RS Remote, PacificBiosciences, Menlo Park, CA, USA. SMRT sequencing was carried out on the PacBio *RSII* (PacificBiosciences, Menlo Park, CA, USA) taking one 180-min movie for each SMRT cell. In total 1 SMRT cell was run.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from MAPwt and MAPΔ*mpt*D grown to mid log phase (OD_{600} of 1.0) using a TRIzol®protocol according to Rustad et al. (2009). RNA was additionally purified using

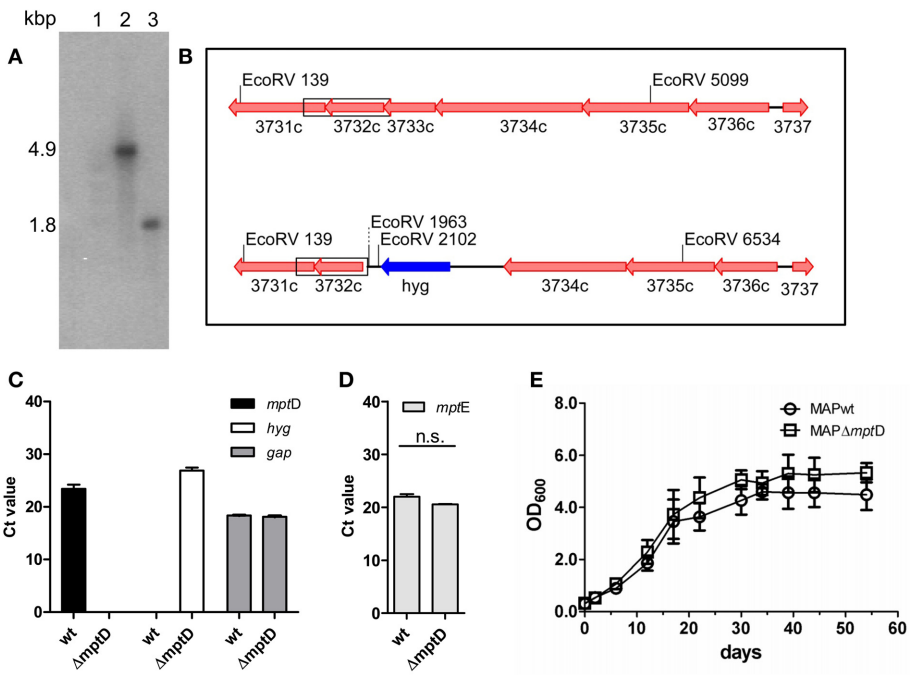

FIGURE 1 | Confirmation and characterization of MAPΔ*mpt*D mutant by Southern blot analysis (A,B), qRT-PCR (C,D) and growth experiments (E). **(A)** EcoRV restricted genomic DNA of MAPwt (lane 2) and MAPΔ*mpt*D mutant strain (lane 3), Southern blotted and hybridized with a probe against *mpt*E (MAP3732c). In the wild type a 4.9 kbp fragment is labeled, the mutant strain shows a 1.8 kbp fragment. Lane 1 DNA marker. **(B)** Genomic sketch of the *mpt* region spanning genes *mpt*F (MAP3731c) to *mpt*A (MAP3736c) in MAPwt (top) and Δ*mpt*D mutant strains (bottom). Position of EcoRV restriction sites are indicated, the α^{32}-P-dCTP labeled probe fragment is boxed. **(C)** Transcription of *mpt*D (black bars) and hygromycin (hyg) (white bars) in MAP wild type (wt) and MAPΔ*mpt*D were analyzed by qRT-PCR. As a control, cDNA samples were tested for the housekeeping gene *gap* (gray bars). **(D)** Analysis of *mpt*E (MAP3732c) transcription in MAPwt and MAPΔ*mpt*D by qRT-PCR (n.s., no significant difference). **(E)** Growth of MAPwt and MAPΔ*mpt*D in Middlebrook 7H9 medium. Cultures were inoculated with an initial OD_{600} of 0.2 and growth was monitored at OD_{600} (ordinates) at different time points (abscissa) until stationary phase. The results of **(C,D)** represent the mean ± standard error (s.e.m.) of three replicates.

an RNeasyMini kit (Qiagen, Düsseldorf, Germany) with DNase I (50 U) in tube treatment according to the manufacturer's manual. DNAse digested RNA was used for double strand cDNA-synthesis. Briefly, in a total volume of 20 µl, 4 µg of RNA were mixed with 0.4 µg random primers (Promega, Madison, WI, USA), incubated for 10 min at 70°C in a thermal cycler and subsequently cooled on ice for 5 min. Aliquots (10 µl) were mixed with 5 µl 5× reaction buffer and 2 µl 10 mM dNTP's in a total volume of 25 µl. Reverse transcription was performed by adding either 200 U MMLV-superscript transcriptase (Promega, Madison, WI, USA) or RNase free water as a negative control. Reactions were incubated for 1 h at 42°C, followed by an incubation for 5 min at 85°C. Samples were diluted with 90 µl ddH_2O and stored at −20°C until further analysis.

For real-time PCR experiments, 2.5 µl of cDNA were mixed with 0.4 µM primer each and 10 µl SYBR-Green Mix (Qiagen, Hilden, Germany) in a total volume of 20 µl and subsequently analyzed using a Mx3005P qPCR system (Stratagene, Agilent Technologies, La Jolla, CA, USA) with a thermal cycling profile as follows: segment 1, 20 min at 95°C, 1 cycle; segment 2, 45 s at 95°C, 1 min at 58°C, 1 min at 72°C, 45 cycles; segment 3, 1 min at 95°C, 30 s at 55°C, 30 s at 95°C, 1 cycle. Results were normalized to the housekeeping gene *gap* (MAP1164) and expressed as fold-change to the untreated control.

METABOLOMIC SCREEN

For metabolomic screening, three independent biological replicates of MAPwt and MAP∆*mpt*D were grown to mid log phase (OD_{600} of 1.0) in supplemented MB7H9 medium. To stop metabolic activity, 15 ml of each culture were transferred to a quenching solution (60% methanol kept at −20°C) in a dilution of 1:3 (vol/vol). Bacteria were harvested by centrifugation at 4°C at 8000 × g for 3 min, pellets were washed with 1 ml quenching solution and bacteria were pelleted again. Supernatants were removed and pellets were resuspended in 1 ml 80% methanol (−20°C) for metabolite extraction. Ten microliter of an internal standard (0.2 mg of $^{13}C6$ labeled sorbitol in 1 ml methanol) were added to the samples. Samples were transferred to a Fastprep tube and disrupted in a bead beater using four cycles at intensity setting 6.5 for 40 s with intermediate cooling on ice. Samples were incubated at 70°C for 15 min, centrifuged at 4°C at 4000 × g for 15 min, and 10 µl were transferred to an appropriate glass tube for freeze-drying overnight. Dried samples and 300 µl of undried samples were used for gas and liquid chromatography (GC/LC) analyses, respectively. All subsequent steps were carried out at Metabolomic Discoveries GmbH (Potsdam, Germany; www.metabolomicdiscoveries.com). Derivatization and analysis of metabolites in a GC-MS 7890A mass spectrometer (Agilent, Santa Clara, USA) were carried out as described (Lisec et al., 2006). Metabolites were identified in comparison to Metabolomic Discoveries' database entries of authentic standards. The LC separation was performed using hydrophilic interaction chromatography with a ZIC-HILIC 3.5 µm, 200 A column (Merck Sequant, Umeå, Sweden), operated by an Agilent 1290 UPLC system (Agilent, Santa Clara, CA, USA). The LC mobile phase was a linear gradient from 90 to 70% acetonitrile over 15 min, followed by a linear gradient from 70 to 10% acetonitrile over 1 min, 3 min wash with 10% and 3 min reequilibration with 90% acetonitrile. The flow rate was 400 µl/min, the injection volume was 1 µl. The mass spectrometry was performed using a 6540 QTOF/MS Detector (Agilent, Santa Clara, CA, USA). The measured metabolite concentration was normalized to the internal standard. Significant concentration changes of metabolites in different samples were analyzed by normal distribution (Shapiro–Wilk-Test) and variance homogeneity testing (*F*-Test) using appropriate statistical test procedures (Students test, Welch test, Mann–Whitney test) (see Tables S2A,B). A *p*-value of $p < 0.05$ was considered as significant.

LIPID PROFILING

MAPwt and MAP∆*mpt*D (400 and 600 mg lyophilized cells, respectively) were suspended in 20 ml of a mixture of chloroform/methanol (2:1, v/v) and extracted by sonication on ice (Branson sonifier, output 4, duty cycle 40, 2 × 15 min, pulsed). After centrifugation at 3000 × *g* for 30 min at 4°C, the supernatant was transferred and dried using a rotary evaporator yielding 100 and 170 mg of crude lipids, respectively. The crude lipids were fractionated according to their polarity on 10 × 1.5 cm columns filled with Silica gel K60 (0.04–0.063 mm mesh size, Merck, Darmstadt, Germany) sequentially using chloroform, acetone and methanol (100 ml each), followed by a final wash with 10 ml of a mix containing equal volumes of chloroform and methanol.

All fractions were evaporated and dry masses determined. The lipid fractions were analyzed on high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany). Briefly, 10 µg of each lipid fraction were applied and resolved either in chloroform/methanol (9:1) or chloroform/methanol/water (65:25:4). Commercial trehalose dimycolate (TDM; Bioclot GmbH, Aidenbach, Germany), and a mixture of phosphoinositol mannosides (PIMs) extracted from *M. tuberculosis* were included as marker. After evaporation of the solvent, lipid bands were visualized by dipping the plates in a solution of Hanessian's stain [2.5 mM cerium (IV) sulfate, 40 mM ammonium hepta-molybdate in 5.8% H_2SO_4] followed by heating to 150°C for 5 min.

MACROPHAGE CELL CULTURE AND VIABILITY ASSESSMENT OF INTRACELLULAR MYCOBACTERIA

The mouse macrophage cell lines J774A.1 (Ralph et al., 1975) and RAW264.7 (Raschke et al., 1978) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 1% glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin at 37°C and 8% CO_2. For infection experiments, cells were maintained in antibiotic-free DMEM for 48 h. To determine mycobacterial viability during infections, macrophages were infected as described (Kuehnel et al., 2001) with MAPwt and MAP∆*mptD* in a multiplicity of infection (MOI) of 10:1. After infection at the indicated time points, monolayers were washed twice with PBS and scraped off the plates in 1 ml of 1% Nonidet P40 in PBS. Cells were disrupted by 10 passages through a 24-gauge needle. A 10-fold serial dilution of the homogenates was prepared in PBS and 100 µl of each dilution level were plated on

supplemented Middlebrook 7H10 agar plates. After incubation for up to 12 weeks at 37°C, the colony-forming units (cfu) were counted.

BACTERIAL ADHESION ASSAY AND FLOW CYTOMETRY

Adhesion of mycobacteria was assayed by flow cytometry as described previously (Pott et al., 2009; Basler et al., 2010). Briefly, mycobacteria grown to an OD_{600} of 1.0 were fluorescently labeled using carboxyfluorescein succinimidyl ester (CFSE, Life Technologies GmbH, Darmstadt, Germany) at 10 μM final concentration in PBS for 20 min at 37°C. After two washes in PBS, labeled bacteria were resuspended in DMEM and used for infection of macrophages. To quantify mycobacterial adhesion to J774.A1 macrophages, confluent grown cells were pretreated with latrunculin (1 μg/ml) for 30 min to inhibit phagocytosis and subsequently incubated for 1 h with CFSE labeled MAPwt and MAP Δ*mptD* in a MOI of 10:1. Then, bacteria-containing medium was removed, and cells were scraped off in PBS, washed for 5 min with PBS by overhead shaking at 4°C, and pelleted by centrifugation at 250 × g for 5 min. Washing was repeated twice. Cells were analyzed with a FACSCalibur (BD biosciences, San Jose, CA, USA). Results were expressed as means ± standard error of the mean (SEM) fluorescence intensities.

ANIMAL EXPERIMENT

The mouse infection experiments were approved by the Lower Saxony Federal State Office for Consumer Protection and Food Safety, Germany (reference number 08/1504). Female C57BL/6 mice aged 8 weeks (Charles River, Erkrath, Germany) were used. Mice were infected intraperitonially (i.p.) with an infection dose of 1×10^8 or 2×10^8 pathogens of each strain in 200 μl Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies GmbH, Darmstadt, Germany; 10 mice per group). Application of PBS was used as negative control. Two days postinfection the body mass monitoring started and was repeated three times per week. After 4 weeks mice were sacrificed, liver, spleen and mesentery tissue were weighted, and MAP (MAPwt and MAPΔ*mpt*D) were quantified by plating of the homogenized tissue on MB7H10 agar plates supplemented with 10% OADC and mycobactin.

Histology was performed in the Mouse Pathology platform at HZI Braunschweig. Briefly, organs were fixed in 10% formaldehyde (v/v), dehydrated with ethanol, and embedded in paraffin. Paraffin sections (0.5 μm) were stained with hematoxylin-eosin (HE) according to standard laboratory procedures. HE stained slices of liver were investigated at 400 × magnification and granuloma counts per area were determined manually using AxioVision Le software (Carl Zeiss AG, Jena, Germany) based on the contrast between granulomas (blue) and other cells of the liver (pink). To minimize false positive results, only granuloma with a size of $>2000\,\mu m^2$ were counted and compared to the liver slices of uninfected mice, where no granuloma have been detected.

STATISTICS

Data are expressed as mean ± s.e.m. By using GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA) a parametric *t*-test or One-Way ANOVA tests were used for statistical analyses of macrophage and the animal experiment. Differences between treated samples and controls were considered statistically significant at a level of $p < 0.05$.

RESULTS

CONSTRUCTION OF MAPΔ*mpt*D

In order to gain insight into the role of *mpt*D in MAP pathogenicity we generated a Δ*mpt*D mutant strain by specialized transduction (Braunstein et al., 2002; Park et al., 2008). For this, the *mpt*D gene (MAP3733c) in MAP strain DSM44135 was replaced by a hygromycin cassette. Hyg-resistant colonies were picked and deletion of *mpt*D was confirmed by Southern blot hybridization and qRT-PCR analyses (**Figures 1A–C**). A positive clone designated MAPΔ*mpt*D was further propagated. To exclude polar effects by the mutation on downstream genes, RNA extracted from OD_{600} of 1.0 cultures of MAPΔ*mpt*D and MAPwt was analyzed by qRT-PCR. As shown in **Figure 1D**, the mRNA expression level of the downstream gene *mpt*E (MAP3732c) was not affected in MAPΔ*mpt*D, excluding polar effects. On the other hand, this implied that the following genes *mpt*E and *mpt*F were not part of the predicted *mpt*ABCDEF.

Any illegitimate recombination in MAPΔ*mpt*D could be excluded by PacBio resequencing study applying the RefSeq genome of strain MAP-K10 (NC_002944.1, Li et al., 2005) in the RS_Resequencing.1 protocol of SMRTPortal 2.1.1. Hereby, coverage analysis using samtools mpileup (PMID 19505943) on the resulting BAM-file confirmed no further large-scale deletions in MAPΔ*mpt*D. Alignment analyses of PacBio long reads by BLAST confirmed that the hygromycin cassette was inserted nonrecurring and at exactly that position as expected.

Following, we analyzed growth of MAPΔ*mpt*D and MAPwt in supplemented MB7H9 broth (**Figure 1E**). Both wild type and mutant strains grew with similar kinetics and entered stationary phase after 34 days. This indicated that *mpt*D is not necessary for growth of MAP in MB7H9.

REDUCED INTRACELLULAR SURVIVAL OF MAPΔ*mpt*D IN MACROPHAGES

To investigate the potential role of *mpt*D in pathogenicity, we performed survival experiments in murine RAW264.7 macrophages and also included J774A.1 macrophages of which we had detailed information of the phagosomal acidification process (Kuehnel et al., 2001). Exponentially grown bacteria of MAPΔ*mpt*D and MAPwt were harvested at an OD_{600} of 1.0, and single cell suspensions of an OD_{600} of 0.1 in DMEM were used to infect macrophages as described in Materials and Methods. CFU were counted at 2 h, 8 and 14 days of macrophage infection. Intracellular survival rates were calculated by relating the CFU after 8 and 14 days to those at 2 h. By this, we observed similar intracellular survival rates for MAPwt and MAPΔ*mpt*D representatively shown for RAW264.7 macrophages in **Figure 2A**. An interesting observation of these experiments was, however, that the number of cultivable MAPΔ*mpt*D after 2 h of infection was significantly lower as compared to MAPwt (**Figure 2B**, filled bars). This was true for MAP infection of RAW264.7 and J774A.1 macrophages (**Figure 2B**). However, incubating MAPwt and MAPΔ*mpt*D in macrophage whole cell lysates for 2 h led to similar bacterial CFU counts indicating that exponentially

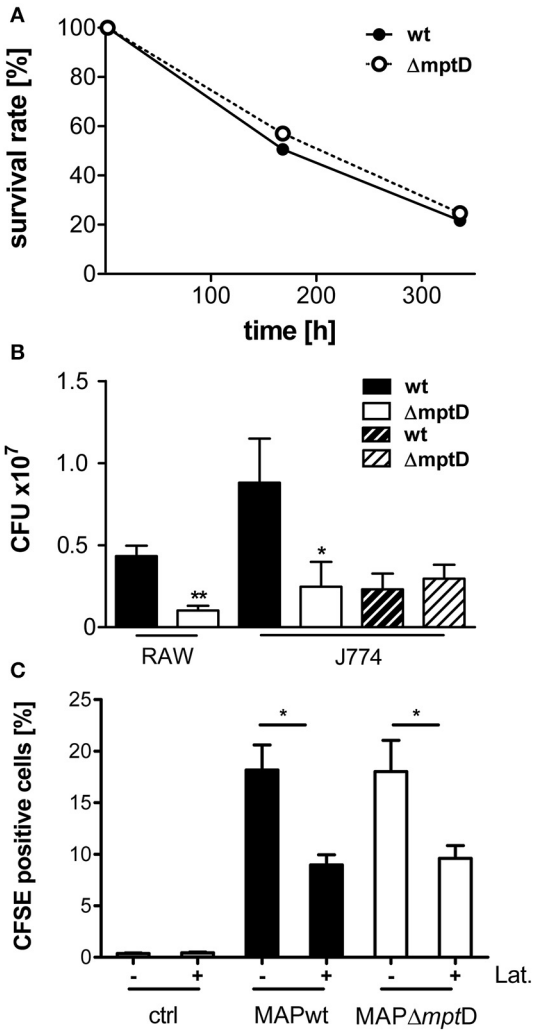

FIGURE 2 | Survival and association of MAPwt and MAPΔ*mpt*D strain in mouse macrophages. (A) Survival rates of MAPwt and MAPΔ*mpt*D in RAW264.7 macrophages. Exponentially growing bacteria of MAPΔ*mpt*D and MAPwt were harvested at OD_{600} of 1.0, and single cell suspensions of an OD_{600} of 0.1 in DMEM were used to infect murine RAW264.7 macrophages as described in Materials and Methods. CFU were counted at 2 h, 8 and 14 days of macrophage infection. Intracellular survival rates were calculated by relating the CFU after 8 and 14 days to those of 2 h. **(B)** CFU numbers of bacteria after infection of mouse macrophages (RAW264.7/J774.A1) with MAPwt and MAPΔ*mpt*D for 2 h (filled bars) and after incubation of the strains in macrophage cell lysates for 2 h (dashed bars). The results represent the mean ± standard error (s.e.m.) of at least three independent replicates for the macrophage infections and two replicates for the incubation in macrophage lysates. **(C)** Association and invasion of CFSE labeled MAP wild type and MAPΔ*mpt*D strains in J774.A1 macrophages after 2 h incubation with (+) and without (−) latrunculin (Lat.). The statistical analysis was performed using a parametric *t*-test (CFU) or a One-Way ANOVA combined with Dunnett's multiple comparison test (CFSE experiments). A *p*-value ≤ 0.05 was defined as statistically significant (** $p < 0.005$; * $p < 0.05$).

growing mutant and wild type strain had similar capability to survive in this environment (**Figure 2B**, dashed bars).

Next we analyzed whether the lower levels of cultivable MAPΔ*mpt*D after macrophage infection resulted from lower adhesion of MAPΔ*mpt*D to macrophages and/or decreased uptake of MAPΔ*mpt*D by the macrophages. For this, we performed FACS analyses of untreated and latrunculin-treated J774A.1 macrophages incubated with CFSE-labeled MAPwt and MAPΔ*mpt*D. Non-infected macrophages were included as negative control. As shown in **Figure 2C**, no difference between MAPwt and MAPΔ*mpt*D bacteria at the level of general association with macrophages (adherent and intracellular mycobacteria) was observed. Also the level of adhesion was similar (**Figure 2C**, latrunculin treated macrophages). Together these results suggested that the reduced cell number of MAPΔ*mpt*D in macrophage compartments was due to a hampered adaptation of MAPΔ*mpt*D to the early phagosomal environment of macrophages.

MAPΔ*mpt*D IS ATTENUATED AFTER PERITONEAL CHALLENGE OF MICE

The infection experiments of macrophage cultures revealed that MAPΔ*mpt*D is hampered in intracellular survival and indicated that *mpt*D might be important for survival in the host. Therefore, to analyze survival of MAPΔ*mpt*D and MAPwt in a more complex system, we infected C57BL/6 mice intraperitoneally with 1×10^8 or 2×10^8 bacteria of exponentially growing cultures of OD_{600} of 1.0 and animals were sacrified 4 weeks post infection. As shown in **Figure 3**, compared to the untreated controls, mycobacteria infected mice exhibited increased liver and spleen weight which was more pronounced in the animals infected with the higher infection dose (**Figures 3A,D**). In lower dose infections, a significant increase in spleen weight was observed in wild type-infected mice but not in mice infected with the mutant (**Figure 3D**). The CFUs in liver tissue were higher in wild type-infected than in mutant-infected mice (**Figure 3B**). These differences became significant when the higher infection dose was used. Correspondingly, a higher number of granuloma was counted in HE-stained histological sections (**Figure 3C**, Suppl. Figure S1), however, no significant difference in granuloma sizes was detected (data not shown). We have recently observed that after i.p. infection of mice, MAP persists in large numbers in mesenteric macrophages (Suwandi et al., in press). Therefore, we also analyzed CFU in the mesentery. As shown in **Figure 3E**, also in this tissue MAPΔ*mpt*D was isolated in significantly lower numbers than MAPwt. Overall, our animal experiments indicate a significantly lower biological fitness of MAPΔ*mpt*D in the mouse infection model.

MAPΔ*mpt*D EXHIBITS AN ALTERED LIPID METABOLISM

In order to determine the functional cause for the decreased survival of MAPΔ*mpt*D in macrophages and its attenuation in a mouse infection model, comparative metabolomic profiling was performed to detect differences in metabolite concentrations between MAPΔ*mpt*D and its parental strain. The concentrations of 175 metabolites in MAPwt and the MAPΔ*mpt*D were compared (Suppl. Table S2). Among the 175 metabolites, the abundances of 32 were significantly different (**Figure 4A**). Means and standard deviation for the three experiments are shown in **Table 1**. Different relative abundances for 13 lipids and lipid-intermediates, 5 carbohydrates (mannose, xylose, xylite, tagatose, threitol), 4 amino acids (lysine, arginine,

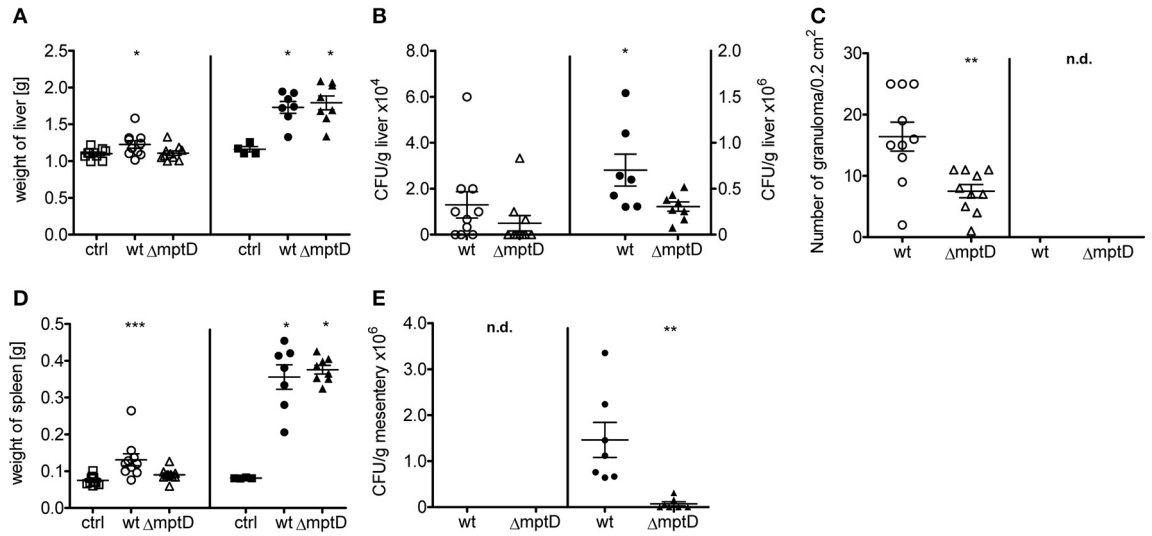

FIGURE 3 | Biological fitness of MAP wild type (MAPwt) and Δ*mpt*D mutant strain (MAPΔ*mpt*D) in infected mice. C57BL/6 8 week old female mice were infected with either $1*10^8$ (open symbols) or $2*10^8$ cells (black symbols) of MAPwt (circle) and MAPΔ*mpt*D (triangle) for 4 weeks. DPBS buffer was used for the control group (ctrl, squares). The read out parameters for this experiment were the weight of liver **(A)** and spleen **(D)**, CFU detection of bacteria from liver **(B)** and mesentery **(E)** as well as the amount of granuloma in the liver **(C)**. The results represent the mean ± standard error (s.e.m.) of animal experiments with 7–10 mice of each group. The statistical analysis was performed using One-Way ANOVA analysis (Kruskal–Wallis test). A p-value ≤ 0.05 was defined as statistically significant (***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$).

pyroglutamine, tyrosine), 2 nucleobases (guanine, adenine), 4 nucleotides (adenosine, deoxycytidine, guanosine-5-phosphate, methyladenosine), as well as the abundance of hippurate, citric acid, FAD and the CoA precursor panthetheine were found in MAPΔ*mpt*D. Twelve metabolites had a reduced abundance and 20 an increased abundance in MAPΔ*mpt*D (**Table 1**).

To examine the consequences of the marked differences in lipid metabolites, crude lipids were extracted from MAPwt and MAPΔ*mpt*D, fractionated on silica gel columns and analyzed by thin-layer chromatography. In almost all fractions, we found differential band pattern of lipids in MAPΔ*mpt*D compared to its parental strain (**Figures 4B,C**). These analyses confirmed the severe changes in lipid metabolism suggested by our metabolic analyses. Overall, these data clearly show the presence of a deregulated lipid metabolism with severe alteration of central metabolic processes in MAPΔ*mpt*D.

DISCUSSION

The pathogenesis of JD is still only partially resolved. In contrast to other pathogenic mycobacteria, MAP is very slow growing and mycobactin-dependent in culture (Merkal and Curran, 1974). Furthermore, unlike the related *M. avium* ssp. *hominissuis*, MAP exhibits a strong tissue tropism to the gut which is not seen in other mycobacteria. These phenotypical features might be attributed to MAP-specific genotypical features. Thus, MAP possesses eight common MAP-specific LSPs (Alexander et al., 2009). Thirty-four MAP genes are not present in other mycobacteria species; 13 of these genes are most probably acquired from other Actinomycetales and 21 genes without a homology to other bacteria have been sequenced so far. Although MAP-specific elements are promising candidates for explaining the peculiarities of MAP, the knowledge concerning their relevance in pathogenicity is poor.

The *mpt*D gene is one of the MAP-specific genes not present in any other yet sequenced mycobacterial species. It is expressed from the 38 kb pathogenicity island within the LSP14. LSP14 contains mainly genes assigning to metal acquisition (Stratmann et al., 2004; Alexander et al., 2009). This predicted function refers a particular importance to LSP14 as it might contain a putative, alternative iron uptake locus, supplementing for MAP mycobactin deficiency caused by a truncated *mbt*A gene (Li et al., 2005). In addition to its location on LSP14, the fact that *mpt*D was shown to be expressed during infection (Stratmann et al., 2004) suggests a possible role of *mpt*D in metabolism in the host environment and in pathogenicity.

Our studies with the Δ*mpt*D mutant strain clearly denote the important standing of *mpt*D for MAP metabolism and pathogenicity. We were not able to restore the MAPΔ*mpt*D phenotype by complementation of the MAPΔ*mpt*D with wild-type *mpt*D or the complete operon (data not shown), most likely due to the complex organization of the predicted transporter proteins and their yet unknown regulation. Yet we found no evidence for alterations on the genome level except the deletion of the gene itself which might be responsible for the MAPΔ*mpt*D phenotype.

Overall, MAPΔ*mpt*D was considerably hampered with respect to survival in macrophages and in mice. Most probably MAPΔ*mpt*D is not fully able to resist phagosomal factors to which it is exposed during the first 2 h after infection. This is strongly suggested since the logarithmically grown bacteria showed similar survival after 2 h of incubation in the macrophage cytosol but the survival of MAPΔ*mpt*D was strongly reduced after

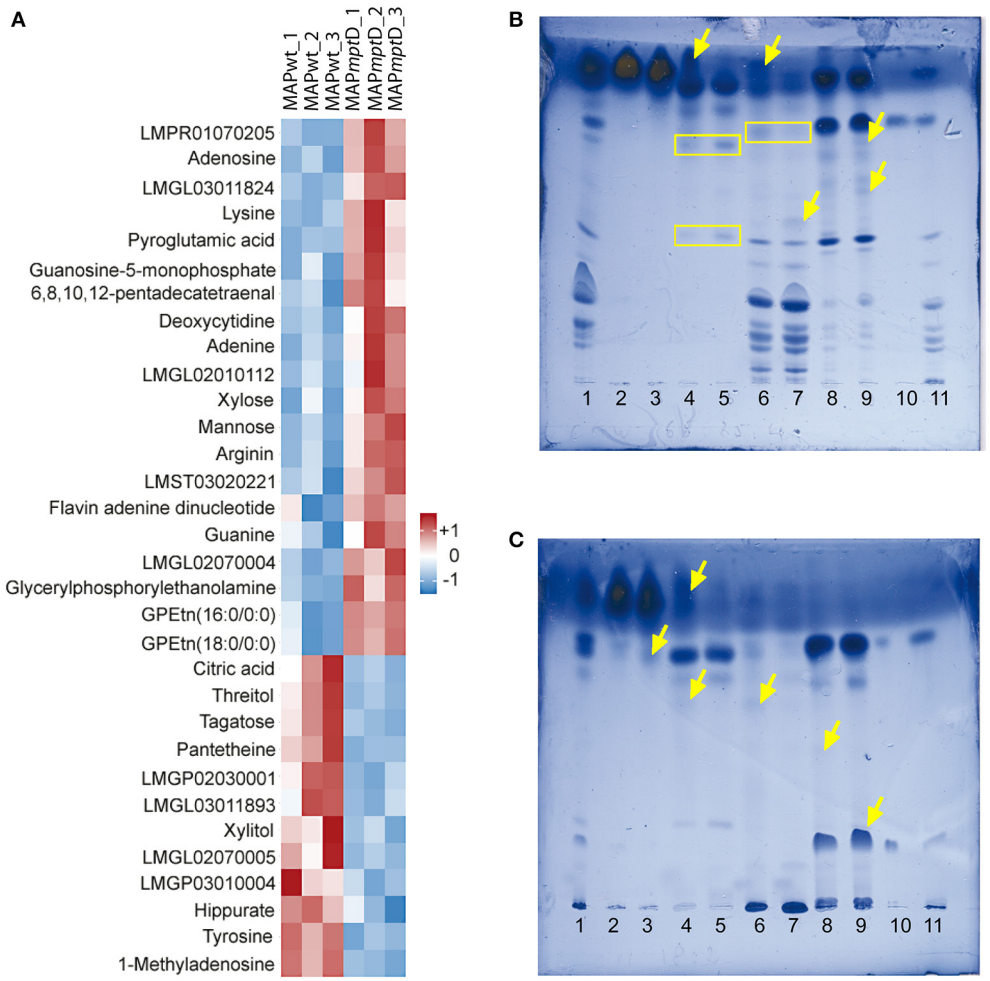

FIGURE 4 | GC/LC-MS analysis of metabolites (A) and HPTLC analysis of lipids (B,C) from MAPwt and MAPΔ*mpt*D. (A) The heat map illustrates the significant concentration changes (overall results of three biological replicates). The color intensity and tones represent magnitude and direction of metabolic changes, respectively, with the magnitude of change ranging from white (reference) to red (positive deviation to reference) or blue (negative deviation to reference). **(B,C)** HPTLC analysis of polarity-fractionated lipids. Mobile phase in **(B)** chloroform/methanol 18:2 (v/v); in **(C)** chloroform/methanol/water 65:25:4 (v/v/v). Lane 1: crude lipids from MAPΔ*mpt*D; lanes 2, 4, 6 and 8: fractions of MAPwt; lanes 3, 5, 7, and 9: fractions of MAPΔ*mpt*D; lanes 2 and 3: chloroform fraction; lanes 4 and 5: acetone fraction; lanes 6 and 7: methanol fraction; lanes 8 and 9: chloroform/methanol 1:1 (v/v) column wash; lane 10: TDM standard; Lane 11: PIM standard (see text) yellow arrows = additional bands; yellow boxes = same bands with different intensities.

2 h macrophage infection. The observed long-term survival of MAPΔ*mpt*D after infection of macrophages and in mice, however, indicates that the absence of *mpt*D does not lead to a general attenuation thereby indicating an important role of *mpt*D in early infection immediately after encountering the hostile environment of the early phagocytic vacuole.

Our metabolic analyses clearly show that reduced survival is correlated with an altered metabolism of MAPΔ*mpt*D. Metabolic analyses revealed a reduced activity of the TCA cycle in MAPΔ*mpt*D which was indicated by the negative deviation of citric acid. This might be caused by a lower availability of acetyl-CoA induced by an enhanced acetyl-CoA consuming fatty acid synthesis, which is suggested by the accumulation of nine fatty acid metabolites. In addition, the activity of the TCA cycle appears to be stressed by carbon efflux used for the generation of arginine and lysine. Increased fatty acid synthesis is also reflected by a higher activity of the pentose phosphate pathway which is deducible from the accumulation of nucleosides and FAD. The three fatty acid metabolites with negative deviation and the accumulation of FAD indicate an enhanced β-oxidation of fatty acids to generate acetyl-CoA in MAPΔ*mpt*D which, however, seems not to satisfy the demand. This metabolic derailment in MAPΔ*mpt*D might be enforced by a diminished CoA availability as indicated by significantly diminished levels of pantetheine, an intermediate for CoA formation. Furthermore differential carbohydrate conversion in MAPΔ*mpt*D is indicated by the accumulation of mannose and xylose, a precursor of arabinose (Wolucka, 2008). Mannose and arabinose are principal

Table 1 | Relative abundance of significantly differential metabolites (MAP Δ*mpt*D vs. MAPwt).

Metabolite name	Technique	Fold change	Metabolic classification
LMGL03011824*	LC	11.8	Lipid transport and metabolism
Lysine	GC	8.0	Amino acid metabolism
Glycerylphosphorylethanolamine	LC	7.1	Lipid transport and metabolism
LMPR01070205*	LC	6.5	Lipid transport and metabolism
LMGL02010112*	LC	5.0	Lipid transport and metabolism
Deoxycytidine	LC	4.2	Nucleic acid metabolism
LMST03020221*	LC	3.6	Lipid transport and metabolism
LMFA06000087*	LC	3.1	Lipid transport and metabolism
Adenosine	LC	3.0	Nucleic acid metabolism
Arginine	LC	2.8	Amino acid metabolism
GPEtn(18:0/0:0)	LC	2.7	Lipid transport and metabolism
Pyroglutamic acid	GC	2.3	Amino acid metabolism
Adenine	LC	2.1	Nucleic acid metabolism
Guanosine-5-monophosphate	GC	2.1	Nucleic acid metabolism
LMGL02070004*	LC	2.0	Lipid transport and metabolism
GPEtn(16:0/0:0)	LC	1.9	Lipid transport and metabolism
Guanine	LC	1.8	Nucleic acid metabolism
Mannose	GC	1.7	Carbohydrate metabolism
Xylose	GC	1.7	Carbohydrate metabolism
Flavin adenine dinucleotide (FAD)	LC	1.4	Co-factor metabolism
LMGL02070005*	LC	−1.4	Lipid transport and metabolism
Hippuric acid	LC	−1.5	Amino acid metabolism
Xylitol	GC	−1.6	Carbohydrate metabolism
LMGP02030001*	LC	−1.6	Lipid transport and metabolism
Tyrosine	GC	−2.0	Amino acid metabolism
LMGP03010004*	LC	−2.4	Lipid transport and metabolism
1-Methyladenosine	LC	−2.5	Nucleic acid metabolism
Citric acid	GC	−2.7	Carbohydrate metabolism
Threitol	GC	−3.1	Carbohydrate metabolism
Tagatose	GC	−3.6	Carbohydrate metabolism
Pantetheine	LC	−7.6	Co-factor metabolism
LMGL03011893*	LC	−7.7	Lipid transport and metabolism

**LIPID MAPS ID according to the LIPID MAPS Structure Database (http://www.lipidmaps.org/data/structure/index.html).*

components of important cell envelope constituents such as lipoamannan (LM), lipoarabinomannan (LAM), and arabinogalactan (Brennan, 2003).

MAP possesses ∼300 genes involved in lipid metabolism and the highest number of redundant genes for this metabolic extent among pathogenic mycobacteria (Li et al., 2005; Marri et al., 2006). The most obvious changes in MAP Δ*mpt*D were seen in the lipid metabolism (**Figures 4B,C**). This is in accordance with the reduced survival in macrophages and in mice since fatty acid metabolism in pathogenic mycobacteria has been shown to be essential for the survival of the bacterium in the host (Russell et al., 2010). On the one hand, the solid cell wall with mycolic acids and lipoglycans such as LAM and LM, produced from elongated fatty acids, provides a protective lipid layer (Rowe and Grant, 2006; Hett and Rubin, 2008), on the other hand lipids are implicated as major nutrient sources of pathogenic mycobacteria in the host. Thus, mRNA expression analysis of *M. tuberculosis* (Mtb) obtained from macrophages *in vitro* and from the lungs of mice and humans implied that Mtb changes its intermediary metabolism *in vivo* by using host-derived lipids such as cholesterol during the course of infection rather than using glucose and glycerol, the primary carbon sources metabolized *in vitro* (Schnappinger et al., 2003; Timm et al., 2003; Talaat et al., 2004). More recently, it was shown that cholesterol degradation appears to be important for feeding Mtb during chronic infection (Miner et al., 2009). Accordingly, we have recently shown that, in the host, MAP increases the activity of the TCA cycle by enhancing β-oxidation of lipids, most probably cholesterol (Weigoldt et al., 2013).

Our data indicate that a dysfunction of the balanced lipid metabolism necessary for survival of MAP in the host might be responsible for the attenuation of MAP Δ*mpt*D in macrophages and in mice. The exceptional role of the mycobacterial lipid metabolism for mycobacterial survival has been emphasized in many studies (Russell et al., 2010). Disorders in the lipid homeostasis can dramatically influence mycobacterial viability. For

example, exhaustive degradation of cholesterol or other lipids may result in the accumulation of stable catabolic intermediates such as propionate which, if not detoxified, may reduce biological fitness (Chang et al., 2009). In addition, within the phagosome, MAP has to resist other microbicidal defense mechanism, e.g., changing levels of bivalent cations such as Fe, Cu, Zn (Soldati and Neyrolles, 2012). The predicted function of *mpt*D as part of an ABC transporter systems and its location on LSP14 suggest *mpt*D to be involved in ion homeostasis and imply that bivalent cations may serve as important regulators for balancing lipid metabolism of MAP during adaptation to the intracellular environment. Even though the precise function of *mpt*D is not yet known, our study exemplified that MAP-specific elements have a considerable role in MAP metabolism and give novel insights into their importance for metabolic adaptation of MAP to the host environment.

AUTHOR CONTRIBUTIONS

Ralph Goethe, Gerald-F. Gerlach, and Jochen Meens designed the experiments; Gerald-F. Gerlach and Julia Heinzmann constructed the mutant, Boyke Bunk and Cathrin Spröer performed the mutant sequencing and alignments, Thorsten Meißner and Elke Eckelt characterized the mutant, Thorsten Meißner and Tina Basler performed the macrophage infections, Ralph Goethe and Siegfried Weiss designed the mouse infection experiments, Thorsten Meißner and Abdulhadi Suwandi performed the mouse infections, Sandra Trenkamp performed LC/MS-MS analysis; Walter M. R. Oelemann and Otto Holst performed the lipid profiling, Thorsten Meißner, Elke Eckelt, Jochen Meens, Ralph Goethe analyzed data; and Thorsten Meißner, Elke Eckelt, Gerald-F. Gerlach, and Ralph Goethe wrote the paper.

ACKNOWLEDGMENTS

This work was supported by a grant from the German Research Foundation (DFG, Ge522/6-1). Ralph Goethe and Gerald-F. Gerlach were additionally supported by the German Federal Ministry of Education and Research (BMBF, ZooMAPII: 01KI1003A, 01KI1003B). We thank Regina Engel (RCB) for providing highly purified mycobacterial phosphoinositolmannosides. We are grateful to Sabine Goebel and Kristin Laarmann (TiHo) and Simone Severitt und Nicole Mrotzek (DSMZ) for excellent technical assistance. Walter M. R. Oelemann received a Postdoctoral fellowship of CAPES Foundation, Ministry of Education of Brazil (process no. BEX 1438/11-5).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/journal/10.3389/fcimb.2014.00110/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 May 2014; accepted: 25 July 2014; published online: 14 August 2014.
Citation: Meißner T, Eckelt E, Basler T, Meens J, Heinzmann J, Suwandi A, Oelemann WMR, Trenkamp S, Holst O, Weiss S, Bunk B, Spröer C, Gerlach G-F and Goethe R (2014) The Mycobacterium avium ssp. paratuberculosis specific mptD gene is required for maintenance of the metabolic homeostasis necessary for full virulence in mouse infections. Front. Cell. Infect. Microbiol. ***4****:110. doi: 10.3389/fcimb.2014.00110*
This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 20 October 2014
doi: 10.3389/fcimb.2014.00146

Coregulation of host-adapted metabolism and virulence by pathogenic *yersiniae*

Ann Kathrin Heroven and Petra Dersch*

Department of Molecular Infection Biology, Helmholtz Centre for Infection Research, Institut für Mikrobiology, Technische Universität Braunschweig, Braunschweig, Germany

Edited by:
Thomas Dandekar, University of Würzburg, Germany

Reviewed by:
Dongsheng Zhou, Beijing Institute of Microbiology and Epidemiology, China
Astrid Fieselmann, University of Würzburg, Germany
Friedrich Götz, University of Tübingen, Germany

****Correspondence:***
Petra Dersch, Department of Molecular Infection Biology, Helmholtz Centre for Infection Research, Institut für Mikrobiology, Technische Universität Braunschweig, Inhoffenstrasse 7, 38106 Braunschweig, Germany
e-mail: petra.dersch@helmholtz-hzi.de

Deciphering the principles how pathogenic bacteria adapt their metabolism to a specific host microenvironment is critical for understanding bacterial pathogenesis. The enteric pathogenic *Yersinia* species *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* and the causative agent of plague, *Yersinia pestis*, are able to survive in a large variety of environmental reservoirs (e.g., soil, plants, insects) as well as warm-blooded animals (e.g., rodents, pigs, humans) with a particular preference for lymphatic tissues. In order to manage rapidly changing environmental conditions and interbacterial competition, *Yersinia* senses the nutritional composition during the course of an infection by special molecular devices, integrates this information and adapts its metabolism accordingly. In addition, nutrient availability has an impact on expression of virulence genes in response to C-sources, demonstrating a tight link between the pathogenicity of *yersiniae* and utilization of nutrients. Recent studies revealed that global regulatory factors such as the cAMP receptor protein (Crp) and the carbon storage regulator (Csr) system are part of a large network of transcriptional and posttranscriptional control strategies adjusting metabolic changes and virulence in response to temperature, ion and nutrient availability. Gained knowledge about the specific metabolic requirements and the correlation between metabolic and virulence gene expression that enable efficient host colonization led to the identification of new potential antimicrobial targets.

Keywords: *Yersinia*, host-adapted metabolism, virulence, gene regulation, Csr, Crp

INTRODUCTION

Intensive work on the virulence strategies of all three human pathogenic *Yersinia* species (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) as well as studies on the molecular and cellular aspects of *Yersinia* pathogenesis have considerably increased our knowledge of how *yersiniae* establish infections and cause diseases. Over the last decades a large set of specific pathogenicity factors has been identified, which mediate efficient resistance against the host defense systems, manipulate host cell processes and enable the bacteria to colonize, invade and multiply in host tissues. The structure, function and regulation of many of those classical virulence factors have been characterized and their role in pathogenesis has been analyzed using different animal models. However, a simple additional premise for their fitness and success is that they are able to obtain nutrients (C-/N-/energy sources) and ions at the infection site. As a consequence, bacterial pathogens have evolved efficient host-adapted nutrient retrieval strategies to optimize their metabolism and maximize the harvest of essential ions, energy sources and biomass building blocks from tissues encountered during infection. Here, we will discuss recent advances in our knowledge of (i) nutritional adaptation strategies of human pathogenic *yersiniae* and (ii) molecular mechanisms dedicated to control and adjust metabolic processes with virulence functions.

YERSINIA LIFE STYLES AND PATHOGENESIS

The genus *Yersinia* belongs to the family of *Enterobacteriaceae* and encompasses 17 species, whereby only *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis* are known to cause diseases in mammals (Koornhof et al., 1999; Smego et al., 1999). All human pathogenic yersiniae are zoonotic, Gram-negative, facultative anaerobes that are well adapted for survival in a variety of external environments and persistence in various host animals. *Y. pseudotuberculosis* and *Y. enterocolitica* are both enteric pathogens which have emerged within the last 200 million years, whereas *Y. pestis* has evolved as a separate clone from *Y. pseudotuberculosis* about 2000–20,000 years ago (Achtman et al., 1999; Wren, 2003). A recent comprehensive study analyzing over 200 genomes of different *Yersinia* species further demonstrated that human pathogenic *Yersinia* species have evolved by following parallel evolutionary paths: (i) acquisition of similar virulence determinants, e.g., the virulence plasmid pYV that encodes the main virulence genes of *Yersinia* and the chromosomally-encoded cell adhesion and invasion protein Ail, and (ii) functional gene loss and reduced metabolic flexibility (Reuter et al., 2014).

Both enteric *Yersinia* species cause various gut-associated symptoms (e.g., enteritis, ileitis, diarrhea, and mesenteric lymphadenitis) commonly called yersiniosis. Only in very rare cases they can lead to systemic infections and induce extra-intestinal

sequelae such as erythema nodosum and reactive arthritis (Koornhof et al., 1999). *Y. pseudotuberculosis* and *Y. enterocolitica* can occupy many different environmental habitats and have been isolated from ground water, soil, plants, and insects. In addition, certain domestic and wild animals were shown to be reservoirs for enteropathogenic *Yersinia* species (Fredriksson-Ahomaa et al., 2006; Fredriksson-Ahomaa, 2012). Both enteric *Yersinia* species are transmitted via the fecal-oral route. Undercooked pork meat is considered to be the major infection source of *Y. enterocolitica* (Bottone, 1997), and vegetables and lettuce for *Y. pseudotuberculosis* (**Figure 1**).

Y. pestis is the causative agent of plague. It is unique in its choice of host habitats and primary mode of transmission. *Y. pestis* generally resides within the lymphatic system, blood, or tissues of rodents and is transmitted to other mammals through direct contact or the bite of an infected flea, when the bacteria are regurgitated from the proventriculus into the dermis during the flea blood meal (Perry and Fetherston, 1997). During this early stages of the infection, *Y. pestis* replicates within macrophages at peripheral host sites (Perry and Fetherston, 1997). From there, they spread into the draining lymph nodes where they replicate and lead to the formation of buboes (hemorrhagic, swollen lymph nodes), which is the characteristic clinical feature of bubonic plague. Subsequently, *Y. pestis* can disseminate into the blood stream leading to a fulminant systemic infection and fatal septicemia. In rare occasions the infection can progress to pneumonia (pneumonic plague) which enables the bacteria to be transmitted from person-to-person via contaminated droplets (Perry and Fetherston, 1997) (**Figure 1**).

HOST-PATHOGEN INTERACTIONS

Both enteric *Yersinia* species are armed with a set of pathogenicity factors that enable the pathogens to efficiently colonize the intestinal tract. To survive the acidic environment of the stomach they both induce the expression of urease, an enzyme that counteracts the gastric acidity by neutralizing low pH with ammonia (Young et al., 1996; Hu et al., 2009). Furthermore, they express diverse membrane-anchored surface adhesins and invasins that contribute to interactions with the intestinal epithelium and promote bacterial passage of the intestinal barrier into deeper tissues. During the early stages of the infection, the bacteria attach to and invade into the specialized microfold epithelium (M cells), overlaying the Peyer's patches (PPs) in the most distal part of the small intestine, the ileum (Grutzkau et al., 1990; Isberg and

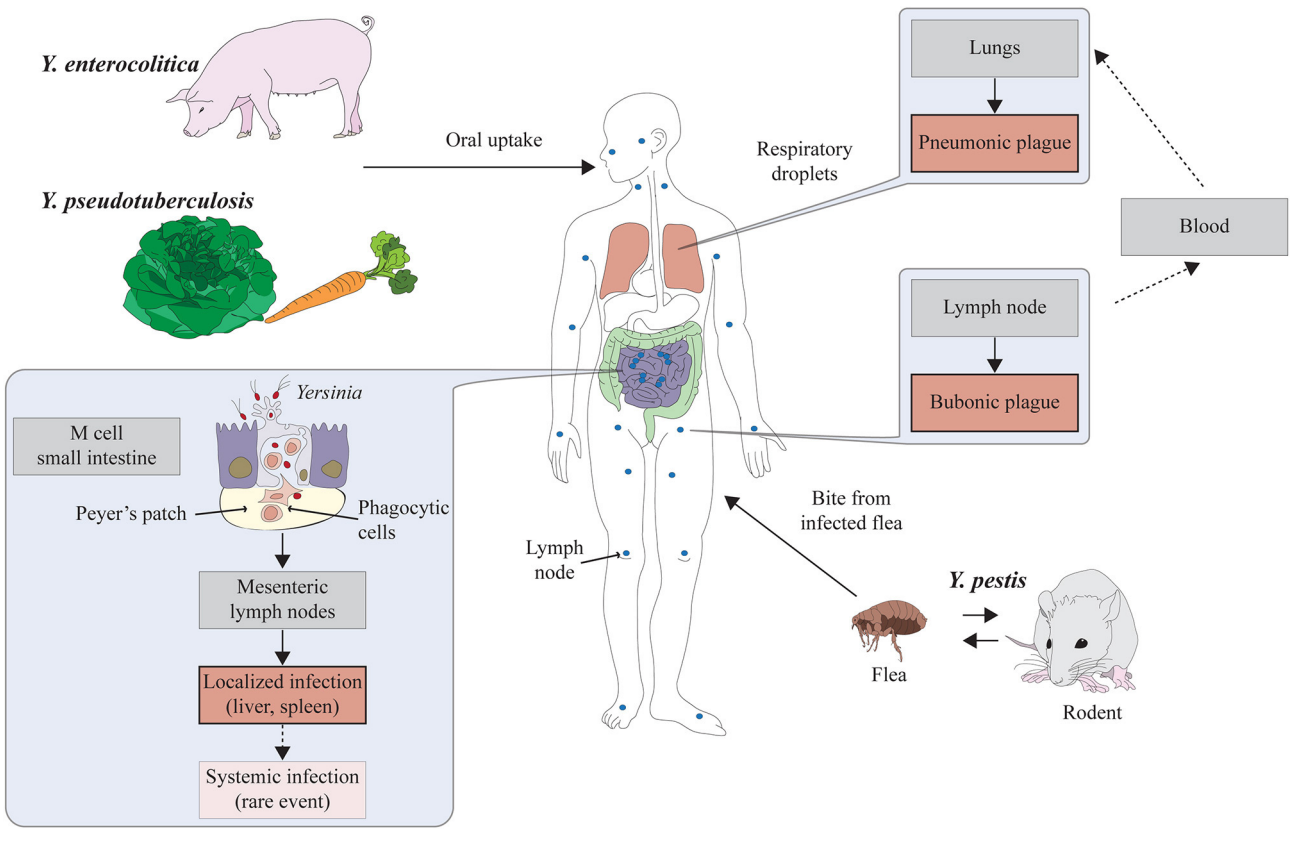

FIGURE 1 | Lifestyles and pathogenesis of the human pathogenic *Yersinia* species. The enteropathogenic *Yersinia* species *Y. enterocolitica* and *Y. pseudotuberculosis* are associated with meat (mainly pork) and lettuce/vegetables. They are ingested via contaminated food and enter the lymphatic system through the M cells in the small intestine. The main reservoirs of *Y. pestis* are rodents. Transmission of the bacteria to humans occurs through the bite of an infected flea resulting in bubonic plague. Pneumonic plague is developed when *Y. pestis* reaches the lung and is transmitted via respiratory droplets.

Barnes, 2001). From the PPs, the bacteria disseminate to the mesenteric lymph nodes (MLNs), or other extra-intestinal tissues such as liver and spleen (Cornelis and Wolf-Watz, 1997; Plano and Schesser, 2013) (**Figure 1**). The outer membrane protein invasin is the most efficient adhesion and internalization factor of enteropathogenic *yersiniae*. Other homologous Inv-type adhesins (InvB/Ifp, InvC), Ail, the homotrimeric autotransporter adhesin YadA, and the PsaA/Myf fimbriae seem to contribute to the dissemination process (Marra and Isberg, 1997; El Tahir and Skurnik, 2001; Grassl et al., 2003). Certain adhesins are also likely to promote colonization of liver and spleen during later stages of the infection, which in case of *Y. pseudotuberculosis* was shown to occur directly without previous passage of the PPs and the MLNs (Handley et al., 2005; Barnes, 2006). *Y. pestis* carries non-functional copies of the adhesin/invasin genes *invA* and *yadA*, and has lost other genes required for intestinal pathogenesis. Instead, it acquired the pMT1 (pFra) plasmid that enables *Y. pestis* to replicate in and be transmitted by the flea (Achtman et al., 1999). The adhesins Ail and PsaA are also present and appear to contribute to host-pathogen interactions (Lindler et al., 1990; El Tahir and Skurnik, 2001).

RESISTANCE AGAINST IMMUNE RESPONSES

Yersinia pathogenesis is also tightly correlated with its ability to resist or evade host immune responses. Besides their role in host-pathogen interactions, Ail and YadA were shown to provide resistance against complement killing by binding of the regulator factor H and the C4b-binding protein (Bliska and Falkow, 1992; Pierson and Falkow, 1993; Kirjavainen et al., 2008). PsaA was shown to inhibit phagocytosis most likely by binding lipoproteins that prevent recognition by host cells (Payne et al., 1998; Makoveichuk et al., 2003). In addition, all three pathogenic *Yersinia* species possess a type three secretion system (T3SS) to deliver anti-phagocytic Yop effector proteins into host cells, mainly professional phagocytes (Cornelis and Wolf-Watz, 1997; Plano and Schesser, 2013). The T3SS-Yop apparatus is encoded on the 70 Mb virulence plasmid pYV (pCD1 in *Y. pestis*) and is absolutely required for virulence of all three pathogenic *Yersinia* species. This is based on the fact that the translocated Yop effectors are exotoxins that disable the phagocytic machinery by (i) destabilizing the actin cytoskeleton, (ii) suppression of cytokine production and (iii) induction of apoptosis of phagocytic cells (Viboud and Bliska, 2005; Plano and Schesser, 2013). Another crucial anti-phagocytic factor is the F1 capsule protein (Caf1), which is solely produced by *Y. pestis*. Caf1 promotes transmission by flea bite (Du et al., 2002) and is required for pathogenesis during bubonic and pneumonic plague (Sebbane et al., 2009; Weening et al., 2011). Anti-phagocytic capabilities are acquired by *Y. pestis* during early stages of infection, when the bacteria are internalized by macrophages and neutrophils, and enable extracellular survival of the bacteria later on (Lukaszewski et al., 2005). Acquisition of the *Y. pestis* plasmid pMT1 and pPCP1, encoding the *Yersinia* murine toxin (Ymt) that supports survival in the flea (Hinnebusch et al., 2002), and the omptin-like plasminogen activator protease Pla was critical in the evolution of *Y. pestis* (Kukkonen and Korhonen, 2004; Haiko et al., 2009).

LINK BETWEEN METABOLISM AND VIRULENCE OF *YERSINIA*

It is striking that both enteropathogenic *Yersinia* species, which are more distantly related (<60% nucleotide identity), induce similar gastrointestinal disease symptoms, whereas *Y. pestis* and *Y. pseudotuberculosis* which are very similar genetically (>97% nt identity) exhibit markedly different pathogenicities (Chain et al., 2004; Derbise et al., 2010). The underlying molecular mechanism(s) are still unknown, but in addition to the small differences concerning classical virulence factors, also variations in metabolic functions and/or differences in the regulatory mechanisms implicated in metabolic adaptation could contribute to observed differences in pathogenicity and clinical outcome.

NUTRIENT SENSING AND RETRIEVAL

The mammalian gastrointestinal tract can be considered an excellent source of nutrients for enteropathogenic *yersiniae*. Nevertheless, nutrient supply can vary considerably in the different gut sections (Rohmer et al., 2011). Simple sugars such as glucose and galactose, resulting from the degradation of disaccharides (lactose, maltose), and starch are readily absorbed in the proximal part of the mammalian small intestine, whereas polysaccharides from plant cell walls (e.g., cellulose, xylan, and pectin) pass into the distal portion of the gastrointestinal tract. Inflammation and hypoxic conditions induced by the immune response can dramatically change the availability of nutrients (Nizet and Johnson, 2009). Furthermore, the host restricts the pathogen's access to essential ions such as magnesium, manganese, zinc, and iron (Brown et al., 2008; Abu Kwaik and Bumann, 2013; Zhang and Rubin, 2013). Within the digestive tract, *Yersinia* also has to compete with the resident microbiota for resources. The intestinal flora comprises about 10^{14} well-adapted bacteria from more than 400 species, which form a special cross-feeding ecosystem, in which anaerobes degrade complex polysaccharides. Other bacteria such as *Escherichia coli* utilize the simple sugars released as breakdown products (Hooper et al., 2002; Chang et al., 2004; Le Bouguenec and Schouler, 2011). In order to successfully colonize the intestinal tract, *Yersinia* must either grow on non-utilized energy/C-sources or process nutrients much more efficiently to outgrow others. A similar situation will be envisioned by *Y. pestis* during replication in the intestinal tract of fleas.

One prerequisite to succeed in the their hosts is the ability of *yersinia*e to sense available C-sources. In particular carbon catabolite repression (CCR) triggered in response of the availability of readily digestible sugars (e.g., glucose) was shown to modulate metabolism and is used to coordinately control the expression of virulence factors via the cAMP receptor protein (Crp) (see below; Görke and Stülke, 2008; Poncet et al., 2009; Heroven et al., 2012b). Moreover, changes in the supply of amino acids are used to adapt metabolism and regulate virulence via the stringent response through (p)ppGpp (Sun et al., 2009; Dalebroux et al., 2010). Usually other environmental cues, such as temperature, oxygen and ion concentration, and pH are used in combination with nutrient sensing to determine the location of *Yersinia* within the host to properly adjust metabolism and pathogenicity (see below).

METABOLIC FUNCTIONS IMPORTANT FOR *YERSINIA* TO COLONIZE HOST TERRITORIES

All pathogenic *Yersinia* species are chemoheterotrophs, consuming organic molecules for energy and carbon. They possess a complex metabolic system with a full complement of often redundant or alternative catabolic and biosynthetic pathways that render them very flexible and robust against changing nutrient concentrations and genetic manipulations. Early studies revealed that all pathogenic *yersiniae* are able to catabolize glucose by the Emden–Meyerhof and Entner–Doudoroff pathway with a complete tricarboxylic acid (TCA) cycle and a functional glyoxylate bypass (Santer and Ajl, 1955; Mortlock, 1962; Brubaker, 1968; Motin et al., 2004). However, in contrast to the enteropathogenic *Yersinia* species, the glyoxylate shunt is constitutive in *Y. pestis*. Moreover, *Y. pestis* is unable to metabolize hexoses via the pentose phosphate pathway due to the absence of glucose 6-phosphate dehydrogenase (Mortlock, 1962). Furthermore, the bacteria lack the methionine salvage pathway and aspartase (AspA) activity. AspA is essential for the complete catabolism of L-aspartate, which undergoes reductive deamination by AspA to yield fumerate that is recycled into the TCA cycle (Dreyfus and Brubaker, 1978). As a consequence L-aspartate accumulates in the bacteria and is excreted, e.g., during expression of the low calcium response (LCR). This causes a loss of metabolic carbon that otherwise would be conserved as oxaloacetate (Brubaker, 2007; Viola et al., 2008). Other differences are that *Y. pestis* is meiotrophic in the biosynthesis of glycine/threonine, L-valine and L-isoleucine, L-phenylalanine, and L-methionine, as well as in the fermentation of melibiose and rhamnose, and in the urease pathway (Burrows and Gillett, 1966; Brubaker and Sulen, 1971; Sebbane et al., 2001; Brubaker, 2006, 2012). *Y. pseudotuberculosis* and *Y. enterocolitica* depend only on the presence of the aspartic family of amino acids (Brubaker, 1991). As a consequence, *Y. pestis* is more dependent on its host to obtain required nutrients. Interestingly, *Y. enterocolitica*, but not *Y. pestis* and *Y. pseudotuberculosis*, is able to metabolize cellobiose, sucrose, and inositol. It further produces cobalamine (vitamin B_{12}) under anaerobiosis and can degrade 1,2-propanediol and ethanolamine by cobalamine-dependent enzymes using tetrathionate as terminal electron acceptor (Reuter et al., 2014). Tetrathionate is produced in the inflamed mammalian intestine, e.g., during a *Salmonella enterica* serovar Typhimurium infection (Winter et al., 2010). All these additional metabolic activities could provide a species-specific competitive growth advantage over the largely fermentative intestinal microbiota.

Adaptation of Yersinia to virulence-relevant conditions

To provide a more comprehensive view, multiple "omic" approaches were performed with pathogenic *yersiniae in vitro* grown under virulence-relevant conditions to identify metabolic pathways and virulence genes that might contribute to pathogenesis. The first of these studies addressed temporal changes in gene expression during a temperature transition from 26 to 37°C mimicking transmission of *Y. pestis* from the flea vector to its mammalian host (Motin et al., 2004). Transcriptional profiling revealed that about 10% of the chromosomal genes were influenced by temperature of which the majority encodes important metabolic functions. The thermal induced global changes caused an inhibition of glycolysis while terminal oxidation of a variety of carbohydrates, amino acids, and fatty acids known to exist in the host was favored. This suggested that plague bacilli might favor fermentative pathways during slow growth within the flea, whereas they prefer oxidative catabolism during rapid proliferation in mammals (Motin et al., 2004). Another analysis addressing differential protein expression in *Y. pestis* following a thermal upshift also demonstrated that several enzymes involved in sugar metabolism (e.g., α-enolase, phosphoglycerate kinase, glyceraldehyde-3-phosphate (G3P) dehydrogenase) are under thermal control (Chromy et al., 2005). Differential expression of these enzymes suggests that different types/concentrations of carbohydrates are metabolized after temperature transition. The metabolic switch in the utilization of specific sugars in different milieus appears to be crucial to trigger virulence.

A transcriptomic analysis of *Y. pestis* was also performed in human plasma in order to identify genes which are required during septicaemic plague in humans (Chauvaux et al., 2007). The most marked plasma-triggered virulence factors are the pYV-encoded T3SS/Yop apparatus, whereas PsaA fimbriae were downregulated. In addition, several genes related to purine/pyrimidine metabolism were upregulated in plasma at 37°C and support a previous observation that purine metabolism is necessary for *Y. pestis* virulence (Munier-Lehmann et al., 2003). An equivalent study analyzing the transcriptome of *Y. pseudotuberculosis* during growth in human plasma showed that this closely related pathogen switches to consumption of glucose, which is readily available in blood/plasma (about 7 mM). Phosphotransferase system (PTS)-encoding genes, glycolysis and phosphoenolpyruvate (PEP)-dependent systems were found to be upregulated (Rosso et al., 2008). This is reminiscent to the "glucose overflow metabolism" channeling the carbon flow toward acetate formation instead of citrate formation to prevent accumulation of NADH. In other *Enterobacteriaceae* such as *E. coli*, acetate accumulation is supported by a simultaneous repression of the glyoxylate shunt, but this is not the case for *Y. pseudotuberculosis*. In the opposite, the *aceBAK* genes encoding the key enzymes of the glyoxylate shunt are upregulated (Rosso et al., 2008), suggesting a need for this species to limit acetate overloads. The constitutive expression of the *aceBAK* operon in *Y. pestis* indicates that derepression of the glyoxylate shunt might also be important for the plague bacilli.

Metabolic pathways that contribute to pathogenicity

In the last few years, several studies were published analyzing the *in vivo* transcriptome of *Y. pestis* in the flea vector (Vadyvaloo et al., 2010) and in the mammalian host (Lathem et al., 2005; Sebbane et al., 2006; Liu et al., 2009). Several genes involved in the metabolic adaptation to the different niches as well as classical virulence genes known or predicted to be important for *Yersinia* colonization in the respective host or for resistance against the innate immune response were found to be upregulated.

The *in vivo* transcriptome of *Y. pestis* in the proventriculus of infected fleas revealed numerous metabolic genes involved in the adaptation to the flea gut (Vadyvaloo et al., 2010). Flea meals appear to consist primarily of proteins and lipids with low

amounts of carbohydrates. Thus, it is not surprising that mainly amino acids, in particular the L-glutamate group (e.g., glutamine, histidine, arginine, proline) are catabolized by *Y. pestis* in the flea vector (**Figure 2**). Degradation of these amino acids results in an increased flux of the amino acid carbon through the TCA cycle, the enzymatic genes for which are highly induced in the flea (Vadyvaloo et al., 2010). In contrast, catabolism of hexoses seems not to be important. The glucose PTS is only slightly increased and most other sugar uptake systems are repressed. An exception is the PTS uptake and utilization system for chitobiose, a C-source that is present in the flea's proventriculus spines (**Figure 2**). During growth in the digestive system of the flea synthesis of most important virulence factors, e.g., the T3SS/Yop apparatus, the iron sequestration systems Ybt, and Yfe, the virulence regulator RovA, and PsaA fimbriae are repressed. However, other crucial pathogenicity genes (e.g., *pla*, *yadBC*) and insecticidal-like toxin genes are upregulated (**Figure 2**). Expression of these genes is critical for dissemination from the extravascular tissue at the fleabite site and seems to preadapt *Y. pestis* to resist mammalian innate immunity by acquisition of a phagocytosis-resistant phenotype. This may enhance plague pathogenesis in the very early stages while the full set of thermal controlled virulence factors is still not produced (Vadyvaloo et al., 2010). Also genes of the *Y. pestis hms* operon which are required for the formation of the poly-*N*-acetylglucosamine (PNAG) surface carbohydrate, a major component of biofilms, were found to be induced at moderate temperature and within fleas. Thus, *hms*-dependent biofilms were assumed to support colonization of the proventriculus and facilitate transmission of plague bacteria (Hinnebusch et al., 1996; Vadyvaloo et al., 2010). However, a recent report showed that in two other fully virulent *Y. pestis* strains PNAG synthesis is maximal at 37°C, indicating that this factor may also have a role during mammalian infection (Yoong et al., 2012).

To better understand host-pathogen interactions, adaptation of *Y. pestis* to its mammalian host was further investigated by *in vivo* gene expression profiling in the bubo in a rat model as well as in the lung of a murine pneumonic infection model (Lathem et al., 2005; Sebbane et al., 2006; Liu et al., 2009). Adaptation of the bacterium to the environments encountered within infected mouse lungs includes the induction of genes involved in amino acid biosynthesis (e.g., histidine, pyruvate, glutamate, and aspartate). Of note is also down-regulation of the TCA cycle and the ATP-proton motive force. Simultaneously, iron acquisition systems, the hemin uptake operon, the antiphagocytic F1 protein capsule (Caf1), as well as the T3SS/Yop apparatus are strongly induced, indicating a role in pneumonic plague development (Lathem et al., 2005; Liu et al., 2009) (**Figure 2**). Iron (Fe), zinc (Zn), and manganese (Mn) ions are structural or catalytic

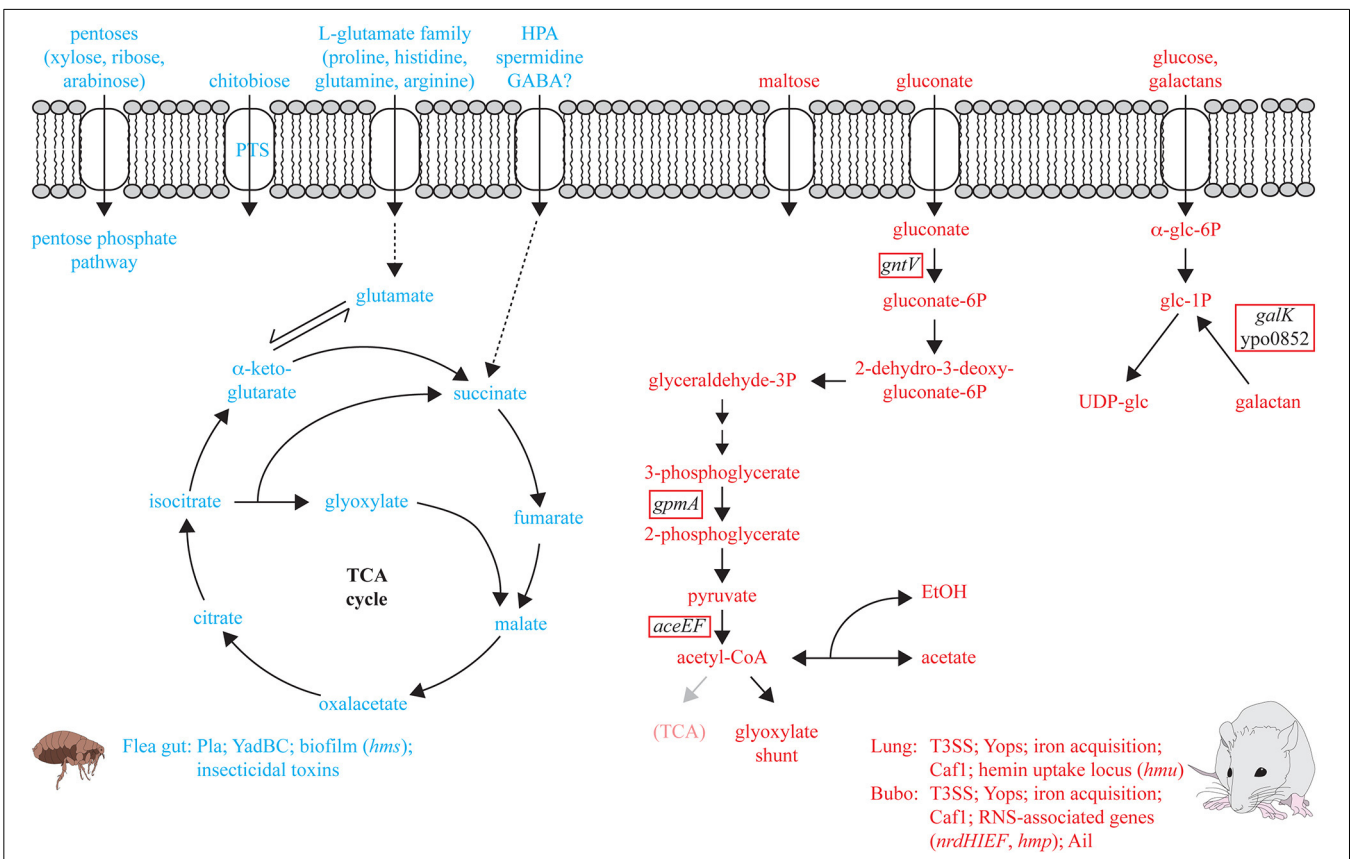

FIGURE 2 | Metabolic pathways and virulence factors of *Y. pestis* induced in the flea and the mammalian host. Metabolic functions and pathogenicity traits upregulated *in vivo* are illustrated which are considered to be important for colonization of the flea gut (in blue) and the lung or bubo of the mammalian host (in red). The red box marks genes shown to be required for full virulence of *Y. pestis* in the bubo of infected rats (Pradel et al., 2014).

cofactors in many proteins involved in several crucial processes including regulation of gene expression, oxidative stress resistance, and production of virulence genes (Scrutton et al., 1971; Campbell et al., 2007; Ammendola et al., 2008; Ortiz de Orué Lucana et al., 2012). The ability of the host to limit access to these divalent cations has been recognized as a crucial component of the host defense against invading pathogens, and it is well known that high-affinity Fe, but also of Zn and Mn uptake systems are crucial for the establishment of a successful infection by pathogenic yersiniae. In particular acquisition of Fe has been studied for quite some time and the role of metal divalent cations for *Yersinia* pathogenesis was topic of several reviews in recent years (e.g., Carniel, 2001; Rakin et al., 2012; Perry et al., 2012a). In addition to the yersiniabactin (Ybt) biosynthetic or transport genes, several other Fe transporters (e.g., YfeABCD, FeoABC) were shown to contribute to virulence (Heesemann et al., 1993; Bearden et al., 1997; Perry et al., 2012a). Systems known to be involved in Mn uptake also include the Yfe transporter and MntH, and the ZnuABC transporter constitutes a high-affinity Zn uptake system (Desrosiers et al., 2010; Perry et al., 2012a,b; Bobrov et al., 2014). Expression of the transport systems were shown to be upregulated *in vivo* in *Y. pestis* and this supports their role in pathogenesis, but modest effects of (multiple) transporter mutants further suggest that there are additional not yet identified transport systems that also contribute to this process (Sebbane et al., 2006; Desrosiers et al., 2010; Perry et al., 2012a,b; Pradel et al., 2014).

The Caf1 capsule, Ail, and the T3SS/Yop machinery belong also to the most highly expressed known virulence factors of *Y. pestis* in the rat bubo (Sebbane et al., 2006). In contrast to the mouse lung, *Y. pestis* in the bubo is exposed to reactive nitrogen species (RNS) released by polymorphonuclear neutrophils (PMNs). Expression of the *nrdHIEF* operon encoding for the ribonucleotide reductase and *hmp* which encodes a flavohemoglobin that detoxifies RNS was highly increased and required for full virulence of *Y. pestis* (Sebbane et al., 2006; Pradel et al., 2014). In contrast, *Y. pestis* is not or to a lesser extent exposed to reactive oxygen species (ROS) stress in the buboes (Sebbane et al., 2006). In a recent study, a *Y. pestis* mutant library of genes upregulated during bubonic plague in the bubo of rats was constructed and their importance for virulence in a rodent model of bubonic plague investigated (Sebbane et al., 2006; Pradel et al., 2014). About 40% of the mutants that were affected in virulence encoded for metabolic genes. Attenuation most likely reflects reduction of *in vivo* growth due to the loss of a specific metabolic pathway. The results further suggest that *Y. pestis* depends mainly on carbohydrates as C-source (i.e., glucose, galactans, gluconate, and maybe maltose) (Pradel et al., 2014) (**Figure 2**). The galactans and glucose are most likely channeled toward UDP-glucose synthesis and not to glycolysis, as deletion of the first two upper genes in the glycolysis pathway (*pgi*, *pfkA*) did not impact *Y. pestis* virulence (Pradel et al., 2014). Virulence mutant testing also supported previous results suggesting that gluconate is an important C-source of *Y. pestis* in its mammalian host (Motin et al., 2004; Pradel et al., 2014). It is likely that gluconate is metabolized to glyceraldehyde-3-phosphate (G3P), pyruvate, acetyl-CoA and acetate, as enzymes of the terminal part of the glycolysis (*gpmA, aceEF*) were essential for competition with the wildtype *in vivo* (**Figure 2**). Additional observations that (i) deletions of TCA cycle genes such as *gltA* (encoding citrate synthase), *acnA* (acotinase A), and *fumC* (fumarase C) did not affect virulence, that (ii) most of the genes of the TCA were downregulated *in vivo* (Sebbane et al., 2006), and that (iii) the glyoxylate shunt is constitutively expressed, strongly suggest that plague bacilli shift to anaerobic respiration or fermentation during colonization of rodents. Another recent study used transposon mutagenesis and high-throughput sequencing (Tn-seq) to probe the *Y. pestis* genome to detect genes contributing to virulence in mice following intravenous injection (Palace et al., 2014). More than 30 genes with roles in nutrient acquisition and metabolism (e.g., purine biosynthesis, aromatic amino acid biosynthesis) were found to be required for fitness of *Y. pestis in vivo.* Several candidates were also identified by Tn-seq probing of *Y. pseudotuberculosis* (Crimmins et al., 2012). Some identified genes were further shown to be important for *Y. pestis* fitness in the *in vivo* competition experiment by Pradel et al. However, also considerable differences were observed between both *Y. pestis* studies which may be explained by a higher infection dose used by Palace et al.

COREGULATION OF METABOLISM AND VIRULENCE

Transmission from an environmental/vector-associated lifestyle into the intestine/lymphatic tissues in mammals demands rapid adaptation not only of virulence gene expression but also of metabolic pathways to ensure maximal fitness and competeveness required for pathogen colonization. Sensing of surrounding nutrients/metabolites is an important mechanism signaling the arrival of the pathogen in a certain location within the host and is used to regulate metabolic functions in tight coordination with virulence traits. To endure frequent variations in the nutrient composition *yersiniae* possess a large variety of sophisticated sensing, signal transduction and regulatory strategies to react to abrupt and pronounced changes of the C-source composition. Over the last decades mainly regulatory proteins have been characterized with respect to metabolic control, but lately more and more post-transcriptional control mechanisms implicating small non-coding RNAs have been identified as additional elements controlling virulence and metabolism.

Transcriptional control by global regulators

Bacterial two component systems (TCS) are able to sense external stimuli and convert them into a cellular response, typically by controlling expression of multiple metabolic, but also virulence-associated genes. Some TCS have been reported to influence metabolism and virulence in *Yersinia*; among them is the PhoP/PhoQ system.

The pleiotropic TCS PhoP/PhoQ constitutes one of the most crucial signal transduction systems controlling bacterial virulence. It is composed of the membrane-bound sensor kinase PhoQ that responds to low magnesium, low pH environments and host-secreted cationic antimicrobial peptides (CAMPs) and phosphorylates the cytoplasmic response regulator PhoP (Groisman, 2001). Recent studies have shown that PhoP controls the global carbon storage regulator (Csr) system in *Y. pseudotuberculosis* (see below), and Crp in *Y. pestis* (see below) (Zhang

et al., 2013b; Nuss et al., 2014). The *phoP* gene was found to be upregulated in the flea and shown to be required for a normal foregut-blocking infection (Vadyvaloo et al., 2010; Rebeil et al., 2013). It is likely that the system is activated by CAMPs which are secreted into the flea gut during blood meal (Lehane et al., 1997). Furthermore, the *phoP* gene was found to be induced in the lung in an intranasally challenged plague model in mice (Liu et al., 2009). It has been clearly shown that the PhoP/PhoQ system promotes survival and proliferation in macrophages and neutrophils (Miller et al., 1989; Oyston et al., 2000; Grabenstein et al., 2004, 2006; Groisman and Mouslim, 2006). Furthermore, PhoP of *Y. pestis* was shown to repress synthesis of the pH6 antigen (Zhang et al., 2013a). However, the role of the PhoP/PhoQ system for pathogenesis of the different *Yersinia* species is less clear as conflicting results were obtained with different strains and infection models. A strong attenuation was reported for a *phoP* mutant of *Y. pestis* GB and the *Y. pseudotuberculosis* derivative 32777, but only modest defects have been observed with a *phoP* mutant of *Y. pestis* CO92 and the *phoP*-deficient *Y. pseudotuberculosis* strain YPIII (Oyston et al., 2000; Grabenstein et al., 2004; Bozue et al., 2011; Pisano et al., 2014). This strongly indicated that the impact of *phoP* depends on strain-specific differences that remodel regulation and/or composition of the regulon with different outcomes on the virulence phenotype. In fact, in a recent study we could demonstrate strain-specific variations in the PhoP-mediated influence on the Csr system in *Y. pseudotuberculosis* (Nuss et al., 2014).

Global regulators that govern complex networks and cascades of control elements in a concerted manner achieve coordination of metabolic pathways with pathogenicity mechanisms. One important global transcriptional factor known to control metabolism and pathogenicity in all three human pathogenic *Yersinia* species is Crp. Crp binds the signal metabolite cAMP produced by the adenylate cyclase in the absence of glucose or other efficiently utilizable sugars (Hanamura and Aiba, 1991; Ishizuka et al., 1994). Crp also represses expression of the adenylate cyclase gene *cyaA* (Qu et al., 2013). The cAMP-Crp complex controls at least 6% of the genes in *Y. pestis* and *Y. pseudotuberculosis*, including genes required for growth on different C-sources, survival under carbon, nitrogen, and phosphate limitation as well as virulence (Gosset et al., 2004; Heroven et al., 2012b; Zhan et al., 2008, 2009). In *Y. pestis*, expression of *crp* is crucial for the development of bubonic and pneumonic plague. Most likely this is based on the function of Crp as regulator of the T3SS/Yop machinery and the plasminogen activator protease Pla (Kim et al., 2007; Liu et al., 2009; Zhan et al., 2008, 2009; Lathem et al., 2014). In *Y. enterocolitica*, a *crp* mutant strain was shown to be strongly attenuated in an oral infection model, and Crp-mediated influence on the expression of the flagellar, Ysc/Yop, and the Ysa T3SS is anticipated to contribute to loss of virulence (Petersen and Young, 2002). Similarly, Crp is required for colonization and/or persistence of *Y. pseudotuberculosis* in the MLNs and organs later during infection (Heroven et al., 2012b). In our recent study using comparative metabolomics, transcriptomics and a phenotypic microarray analysis, we could demonstrate that Crp of *Y. pseudotuberculosis* promotes oxidative catabolism of many different C-sources, whereas it represses fermentative patterns. Furthermore, it links carbon metabolism to the regulation of virulence factors via the control of the virulence-associated small RNAs CsrC and CsrB of the Csr system (Heroven et al., 2012b) (**Figure 3**).

Posttranscriptional regulation of adaptation processes

The Csr system constitutes an important global posttranscriptional regulator system. It regulates stability and translation of various mRNAs involved in metabolism and virulence in *Yersinia* and many other pathogens (Timmermans and Van Melderen, 2010; Heroven et al., 2012a). It is composed of the RNA-binding protein CsrA and of two Csr-type sRNAs (CsrB and CsrC in *Y. pseudotuberculosis*) that counteract the activity of CsrA. CsrA acts by binding to conserved (N)GGA motifs in the loop of hairpin structures found close to the ribosomal binding site of the target mRNA thereby affecting translation and/or stability of the transcript. The Csr-RNAs contain multiple CsrA-binding sites and can sequester and inactivate CsrA (Romeo et al., 2012; Heroven et al., 2012a). CsrA has a global influence on the transcriptome of *Yersinia* (Heroven et al., 2012b). The Csr system of *Y. pseudotuberculosis* was first identified to be crucial for the initiation of the infection process. CsrA was found to repress expression of the global virulence regulator RovA that is required for the activation of the primary entry factor invasin and the PsaA fimbriae (Nagel et al., 2001; Cathelyn et al., 2006; Heroven et al., 2008) (**Figure 3**). In total, about 20% of the CsrA-dependent genes are involved in metabolic processes (Heroven et al., 2012a). The Csr-RNAs seem to be controlled by different regulatory mechanisms in response to ions and availability of C-sources. This is achieved through (i) Crp which positively affects expression of CsrC, and represses CsrB (Heroven et al., 2012b), (ii) the Mg^{2+}-dependent TCS PhoP/PhoQ controlling *csrC* transcription (Nuss et al., 2014), and (iii) the TCS BarA/UvrY shown to induce *csrB* (Heroven et al., 2008). The UvrY/BarA system is activated by metabolic end products (format, acetate) in *E. coli* and by an imbalance of the TCA cycle in *Pseudomonas* (Takeuchi et al., 2009; Chavez et al., 2010). The signals to which the *Yersinia* BarA/UvrY system responds are unknown. However, expression of *uvrY* from *Y. pestis* in the lung, but not in the liver and spleen of infected mice, indicate that metabolites/ions present in a certain host niche are able to induce this TCS during infection (Liu et al., 2009).

Another important regulator of post-transcriptional processes in many bacterial species is the small RNA-binding chaperone Hfq. It forms a hexameric ring complex that enables Hfq to simultaneously bind more than one RNA molecule and facilitates binding of sRNAs to their cognate mRNA to strengthen interactions. Binding of Hfq can either stabilize or promote degradation of mRNA transcripts (De Lay et al., 2013). A recent study comparing the global transcriptome and proteome response of *Y. pseudotuberculosis* and *Y. pestis* grown under physiologically relevant temperatures demonstrated that gene and protein expression of conserved virulence factors such as the Yop effector proteins is higher in *Y. pestis* than in *Y. pseudotuberculosis*. In contrast, regulation of metabolism and of the translational machinery seems to underlie a conserved posttranscriptional control. Among these are proteins of the purine and pyrimidine metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, the TCA cycle,

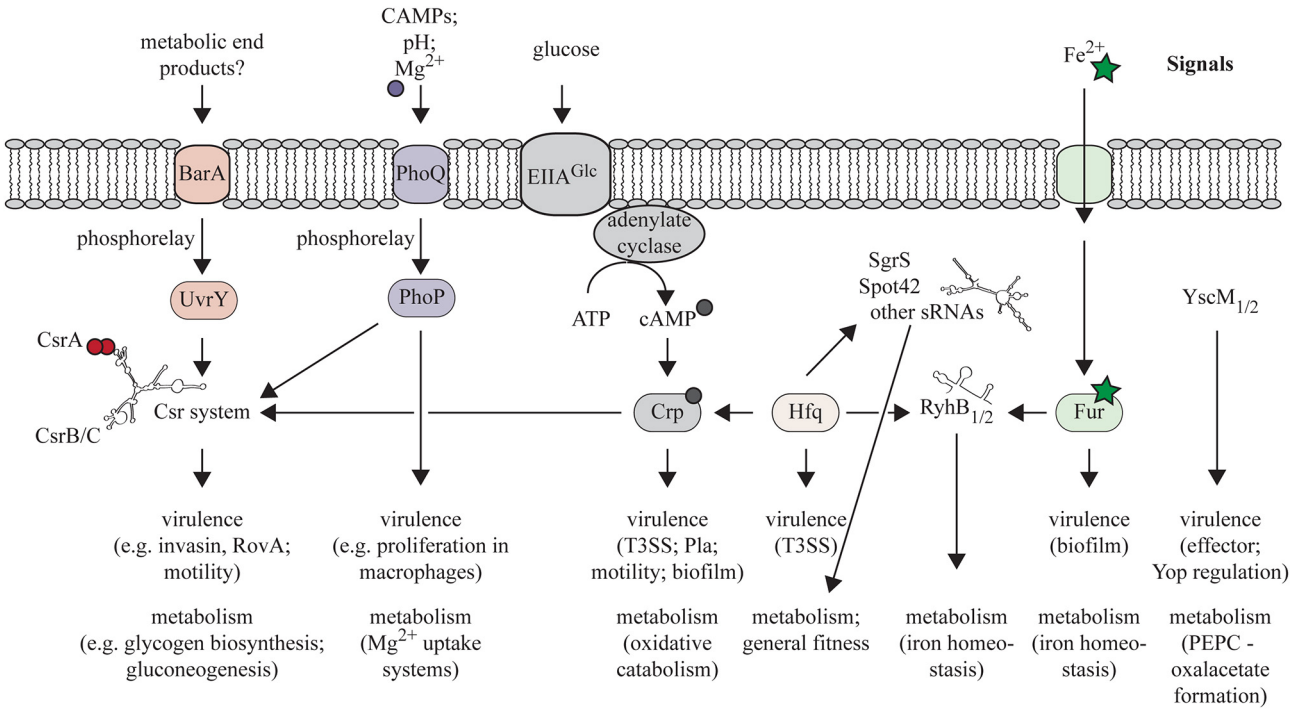

FIGURE 3 | Schematic overview of regulatory factors that are known to coordinate expression of metabolic functions but also virulence-associated traits in pathogenic *Yersinia* species. It should be noted that not all regulatory networks have been experimentally verified in all pathogenic *yersiniae*.

and amino-acyl tRNA biosynthesis (Ansong et al., 2013). All these pathways are controlled by the RNA chaperone Hfq in *S.* Typhimurium (Sittka et al., 2007, 2008; Ansong et al., 2009). As Hfq of *Yersinia* has also been shown to play an important role in the regulation of metabolism and general fitness, it is very likely that Hfq controls similar metabolic functions in *Yersinia* (Geng et al., 2009; Bai et al., 2010; Schiano et al., 2010; Kakoschke et al., 2014). Hfq and sRNAs also contribute to virulence of all pathogenic *Yersinia* species, and one important fact is that they are implicated in the posttranscriptional regulation of T3SS/Yop machinery in *Y. pestis* and *Y. pseudotuberculosis* (Geng et al., 2009; Schiano et al., 2010, 2014). Hfq influence seems to occur through Crp. Hfq was recently shown to be required for efficient synthesis of Crp via an unknown positive posttranscriptional mechanism that involves the 5′ untranslated region (UTR) of the *crp* mRNA (Lathem et al., 2014) (**Figure 3**). Furthermore, the *Y. pestis hfq* mutant is unable to form biofilms in the proventriculus of infected fleas and thus contributes to flea transmission (Rempe et al., 2012).

The implication of sRNAs in the regulation of cellular metabolism has become increasingly recognized (Michaux et al., 2014). Several sRNAs conserved between *Enterobacteriaceae* have been characterized over the last years and their molecular function and targets have been identified mainly by studies performed with *Salmonella* and *E. coli*. Multiple conserved sRNAs were also recently discovered in *Y. pestis* and *Y. pseudotuberculosis* using deep sequencing approaches; among them are SgrS, Spot42, GcvB, and RyhB. The SgrS RNA is involved in a phenomenon called "phosphosugar stress" (Morita et al., 2004). Glucose uptake via the PTS system resulting in accumulation of high levels of generated glucose 6-phosphate is toxic for many *Enterobacteriaceae*. Under these phosphosugar stress conditions, the transcriptional activator SgrR is activated and induces among others the synthesis of the sRNA SgrS. Base pairing of SgrS with the *ptsG* mRNA results in the degradation of *ptsG* and therefore in less uptake of glucose through this glucose-specific permease (Papenfort et al., 2013). Furthermore, the SgrS RNA encodes a small peptide (SgrT), which also helps to rescue *E. coli* from phosphosugar stress by inhibiting glucose transporter activity at the posttranscriptional level. The base-pairing function of SgrS is conserved in *Y. pestis* and *Y. pseudotuberculosis*. However, the sRNA does not produce SgrT as the 5′ end is truncated, indicating differences in the control of phosphosugar stress in *Yersinia* (Wadler and Vanderpool, 2007, 2009; Horler and Vanderpool, 2009). Another conserved sRNA identified in *Y. pestis* and *Y. pseudotuberculosis* is Spot42 (Koo et al., 2011; Beauregard et al., 2013), which is also involved in the regulation of sugar metabolism in *E. coli*. The synthesis of galactokinase (GalK), a protein encoded by the *galETKM* operon important for the conversion of galactose to glucose 1-phosphate is repressed in the presence of glucose. Repression is mediated by binding of the Crp-dependent Spot42 sRNA to the leader of the *galK* mRNA which prevents its translation (Beisel and Storz, 2011). The sRNA GcvB in *S. enterica* serovar Typhimurium and *E. coli*, is primarily expressed under high glycine concentrations and prevents translation of transcripts encoding peptide and amino acid transport systems (Urbanowski et al., 2000; Sharma et al., 2007, 2011; Pulvermacher et al.,

2009). In *Y. pestis* KIM6, GcvB possesses two different termination sites leading to two distinct sRNAs. They repress *dppA*, a periplasmic binding component of a major peptide transport system. Deletion of *gcvB* has pleiotropic effects resulting in reduced growth rates and altered colony morphology (McArthur et al., 2006). Furthermore, GcvB was one of the most abundant sRNAs identified in *Y. pestis* under *in vitro* conditions, although the implications of this are still unknown (Koo et al., 2011). In addition, *Yersinia* encodes two RyhB homologs. In *E. coli* and other *Enterobacteriaceae*, RyhB is a key player for adaptation to iron-limiting conditions. It prevents the synthesis of non-essential iron-containing proteins and leads the induction of iron-scavenging siderophores (Massé and Gottesman, 2002; Massé et al., 2007). The RyhB RNAs are highly expressed in *Y. pestis* within infected lungs (but not in the spleen). However, loss of both RyhB variants had no obvious effect on the dissemination capacity and survival of the bacteria after subcutaneous and intranasal infection. It has been assumed that this is due to redundant iron uptake systems (Deng et al., 2012, 2014; Yan et al., 2013).

Posttranslational regulation of adaptation processes and virulence regulation

A striking observation of the multi-omic approach by Ansong et al. (2013) was the difference of certain metabolites, in particular glutamate, between *Y. pestis* and *Y. pseudotuberculosis*. As this difference was not reflected at the level of transcription nor on the level of protein synthesis, these results implicate that also post-translational mechanisms are involved in modulating the metabolism *in vivo*. In fact, a recent study investigating the regulation of the T3SS in *Y. enterocolitica* showed that components of the secretion machinery are able to directly interfere with metabolic enzymes. The two homologous proteins YscM1 (homolog of LcrG in *Y. pestis* and *Y. pseudotuberculosis*) and YscM2 regulate expression of the Yop effector proteins. Under non-secretion conditions YscM1/YscM2 repress the synthesis of the Yops. This suppression is relieved when both proteins are secreted upon cell contact (Pettersson et al., 1996; Stainier et al., 1997). Furthermore, they are able to bind and inhibit the function of the *Yersinia* phosphoenolpyruvate carboxylase (PEPC) (Schmid et al., 2009). Under virulence-relevant conditions, PEPC replenishes the oxaloacetate pool in the TCA cycle. Mutants in *yscM1* and *yscM2* displayed increased rates of (i) pyruvate formation via glycolysis or the Entner–Doudoroff pathway, (ii) oxaloacetate formation via the TCA and (iii) amino acid biosynthesis. This indicates that both Yops are involved in the repletion of central carbon metabolism. Modulation of PEPC activity might be important for the metabolic adaptation process of *Yersinia* during the infection. The authors proposed a "load-and-shoot" cycle: In order to prepare the bacteria against the phagocytic attack, PEPC is active and replenishes the TCA cycle, as amino acid synthesis is required (loading). During cell contact, the pre-synthesized Yops are rapidly secreted (shooting). As maintaining the energy charge is more needed than biosynthesis during this process, PEPC is inhibited resulting in a shut down of anaplerosis (Schmid et al., 2009). This cross-talk between T3S and metabolism is further supported by the observation that secretion of the Yop effectors is activated by the amino acids glutamate, glutamine, aspartate and asparagine, feeding into the TCA cycle (Lee et al., 2001).

FUTURE ASPECTS

The analysis of bacterial metabolism specific to infection is most important to fully understand bacterial pathogenesis to design more effective therapies against pathogens. Use of novel technologies (e.g., deep sequencing, ^{13}C isotopologue profiling and phenotype microarrays) has significantly increased our knowledge about the complex crosstalk between the primary metabolism and virulence in bacteria. However, current knowledge of host-adapted metabolic functions is still limited since most results are derived from "omic" data obtained *in vitro* under different virulence-relevant conditions, but not during infection. Thus, future efforts are needed to define growth conditions in the infections sites, tackle utilized C/N sources and identify essential metabolic pathways in the different stages of the infection, e.g., by establishment of *in vivo* "omics" and fluxome analysis. Furthermore, high-throughput screens aimed to identify metabolic genes essential for infection were often performed with a single strain isolate and a particular animal model. Based on observed strain-specific variations and different types, routes, and animals used for the infection obtained results cannot easily be generalized and requests a comparative analysis of multiple strains per species. Other important issues that need to be addressed are: (i) how certain metabolic traits confer a fitness advantage for enteric *Yersinia* when faced with different gut commensals or certain host defense mechanisms, (ii) how do *yersiniae* interfere with the carbon and nitrogen metabolism when they are internalized in host cells, and (iii) how gained information can be exploited to develop novel antimicrobial therapies based on the interference with host-adapted metabolic pathways. Interference with global regulators (e.g., CsrA, Crp, Hfq) or blockage of essential metabolic pathways is an attractive but still unexploited way of controlling plague and other fatale diseases of related pathogens.

ACKNOWLEDGMENTS

We thank Aaron Nuss for comments on the manuscript. This work was funded by support from the DFG Priority Program SPP1316 (DE/5-2). PD is supported by the German Centre for Infection Research.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 July 2014; paper pending published: 12 August 2014; accepted: 30 September 2014; published online: 20 October 2014.

Citation: Heroven AK and Dersch P (2014) Coregulation of host-adapted metabolism and virulence by pathogenic yersiniae. Front. Cell. Infect. Microbiol. ***4****:146. doi: 10.3389/fcimb.2014.00146*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 29 September 2014
doi: 10.3389/fcimb.2014.00137

Defining the metabolic requirements for the growth and colonization capacity of *Campylobacter jejuni*

Dirk Hofreuter*

Hannover Medical School, Institute for Medical Microbiology and Hospital Epidemiology, Hannover, Germany

Edited by:
Wolfgang Eisenreich, Technische Universität München, Germany

Reviewed by:
Odile Tresse, French National Institute for Agricultural Research, France
David Kelly, The University of Sheffield, UK

Correspondence:
Dirk Hofreuter, Hannover Medical School, Institute for Medical Microbiology and Hospital Epidemiology, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
e-mail: hofreuter.dirk@mh-hannover.de

During the last decade *Campylobacter jejuni* has been recognized as the leading cause of bacterial gastroenteritis worldwide. This facultative intracellular pathogen is a member of the Epsilonproteobacteria and requires microaerobic atmosphere and nutrient rich media for efficient proliferation *in vitro*. Its catabolic capacity is highly restricted in contrast to *Salmonella* Typhimurium and other enteropathogenic bacteria because several common pathways for carbohydrate utilization are either missing or incomplete. Despite these metabolic limitations, *C. jejuni* efficiently colonizes various animal hosts as a commensal intestinal inhabitant. Moreover, *C. jejuni* is tremendously successful in competing with the human intestinal microbiota; an infectious dose of few hundreds bacteria is sufficient to overcome the colonization resistance of humans and can lead to campylobacteriosis. Besides the importance and clear clinical manifestation of this disease, the pathogenesis mechanisms of *C. jejuni* infections are still poorly understood. In recent years comparative genome sequence, transcriptome and metabolome analyses as well as mutagenesis studies combined with animal infection models have provided a new understanding of how the specific metabolic capacity of *C. jejuni* drives its persistence in the intestinal habitat of various hosts. Furthermore, new insights into the metabolic requirements that support the intracellular survival of *C. jejuni* were obtained. Because *C. jejuni* harbors distinct properties in establishing an infection in comparison to pathogenic Enterobacteriaceae, it represents an excellent organism for elucidating new aspects of the dynamic interaction and metabolic cross talk between a bacterial pathogen, the microbiota and the host.

Keywords: *Campylobacter jejuni*, intermediary metabolism, amino acid catabolism, peptide catabolism, respiration, colonization, intracellular survival

INTRODUCTION

Campylobacteriosis has emerged as major bacterial food-borne disease in industrialized countries in recent years (Epps et al., 2013). Mainly *C. jejuni* is associated with acute *Campylobacter* enteritis in humans causing more than 80% of the registered *Campylobacter* infections. Predominant sources of infections are contaminated meat (especially chicken), raw milk and water. The clinical manifestations of *Campylobacter* enteritis are indistinguishable from *Salmonellosis* and range from mild watery to severe, inflammatory and bloody diarrhea accompanied with abdominal pain and fever (Allos, 2001). Such variations in the disease outcome might correlate with the well documented different virulence potential of individual *C. jejuni* isolates and are possibly linked to dissimilarities in motility and surface structures involved in the direct interaction with the host. These variable structures include the lipooligosaccharide, the capsule and the glycosylation pattern of the flagellin (Wilson et al., 2010). Interestingly, not only variable surface structures but also metabolic traits are highly variable between *C. jejuni* isolates: Genes supporting the oxygen-independent respiration and the catabolism of amino acids and peptides are particular over-represented in robust-colonizing strains compared to poor-colonizing strains (Ahmed et al., 2002; Hofreuter et al., 2006; Hepworth et al., 2007; Seal et al., 2007; Hiett et al., 2008). Such observations suggest that the physiological properties of *C. jejuni* play a crucial role for its pathogenesis. While some aspects of *C. jejuni* pathogenesis have been described in detail previously (Young et al., 2007; Janssen et al., 2008; van Putten et al., 2009; Gilbreath et al., 2011; Szymanski and Gaynor, 2012), this review specifically summarizes our increasing knowledge about the *in vitro* and *in vivo* metabolism of *C. jejuni* and its impact on the virulence and colonization process of this important pathogen.

GENERAL PHYSIOLOGICAL PROPERTIES OF *C. JEJUNI*

Though *C. jejuni* shows fastidious growth characteristics *in vitro* and easily looses viability as well as culturability, it resides as a commensal in a wide range of diverse animal hosts (e.g., chicken, cattle, sheep, goat, dog, duck and pig) and can be isolated from various environmental sources or refrigerated foods. This life style implies that *C. jejuni* is able to resist varying temperatures, oxygen concentrations, pH values, osmotic environments and nutrient availabilities as reviewed comprehensively elsewhere (Park, 2002; Murphy et al., 2006). *C. jejuni* belongs to the group of thermophilic *Campylobacter* species that grow preferentially between 42°C and 37°C, but do not proliferate below 30°C (Penner, 1988). It was suggested that the absence of cold-shock proteins might be

responsible for the inability of this pathogen to grow at lower temperatures (Hazeleger et al., 1998). Nonetheless, *C. jejuni* shows respiration and ATP generation at temperatures as low as 4°C, maintaining its metabolic activities at low temperatures for an extended time period (Hazeleger et al., 1998). *C. jejuni* survives at 4°C even better than at 25°C (Blaser et al., 1980), which leads to the common problem that refrigerated meat contaminated with *C. jejuni* during the slaughter process represents a particular frequent source of *C. jejuni* infections (Bhaduri and Cottrell, 2004). While *C. jejuni* is able to withstand low temperature, atmospheric oxygen concentration affects its viability dramatically. The microaerophilic nature of *C. jejuni* requires an atmosphere with reduced oxygen and elevated carbon dioxide concentrations for its efficient cultivation *in vitro*: Variable oxygen tolerance has been described for *C. jejuni* isolates, but gas mixtures of 5% oxygen, 10% carbon dioxide and 85% nitrogen provide optimal cultivation conditions for most *C. jejuni* isolates (Bolton and Coates, 1983). *C. jejuni* has with about 800 bacteria a lower infective dose than *Salmonella* Typhimurium or pathogenic *Escherichia coli* strains (Black et al., 1988; Kothary and Babu, 2001), indicating that *C. jejuni* is well adapted to survive the harsh, acidic environment of the human stomach. Moreover, it demonstrates the proficiency with which *C. jejuni* is able to multiply in the gastrointestinal tract of humans and to consume the nutritional sources present in the host intestine in order to overcome the microbiota-mediated colonization resistance. Therefore, characterizing the metabolic properties of *C. jejuni* that allows this successful human pathogen and widespread animal commensal to thrive in its diverse hosts has gained increasing attention in recent years.

FINDING A NUTRITIONAL NICHE: THE LOW-CARB, HIGH-PROTEIN DIET OF *C. JEJUNI*

THE NON-GLYCOLYTIC NATURE OF *C. JEJUNI*

C. jejuni is a chemoheterotrophic bacterium with metabolic properties that clearly distinguish it from other enteropathogenic bacteria. Most striking is its restricted carbohydrate catabolism: Early physiological studies examining the substrate utilization of *Campylobacter* identified its incapability to use glucose and other carbohydrates as growth substrates, and since then *Campylobacter* is generally considered to be a non-saccharolytic bacterium. These observations were supported by genome sequence analysis (Parkhill et al., 2000; Velayudhan and Kelly, 2002; Gundogdu et al., 2007) and recent growth-independent BIOLOG phenotype microarray analyses based on the tetrazolium redox dye chemistry that allows monitoring the respiratory activity of metabolically active cells (Bochner, 2009). This approach confirmed that pentoses and hexoses like glucose, fructose, galactose, rhamnose and the disaccharides lactose, maltose, trehalose and sucrose do not enhance the respiratory activity of *C. jejuni* (Line et al., 2010; Gripp et al., 2011; Muraoka and Zhang, 2011). However, these studies did reveal that certain strains like *C. jejuni* NCTC 11168 are capable to catabolize fucose in contrast to other isolates. The observed metabolic diversity is explained by the occurrence of a 9 kb genomic island, comprised in *C. jejuni* NCTC 11168 of the open reading frames Cj0480 to Cj0490, which are absent in *C. jejuni* 81–176 (Hofreuter et al., 2006; Muraoka and Zhang, 2011; Stahl et al., 2011). This gene region encodes for a putative fucose permease FucP (Cj0486) with homology to major facilitator superfamily (MFS) transporters. It was demonstrated that FucP enhanced the growth of *C. jejuni* NCTC 11168 when cultivated in chemically defined media containing 25 or 50 mM fucose as an additional carbon and energy source (Muraoka and Zhang, 2011; Stahl et al., 2011). The catabolic pathway of fucose in *fucP*-positive *C. jejuni* strains is not clear yet. Preliminary data suggest that the fucose utilization does not occur as observed for other intestinal bacteria like *E. coli* or *Bacteroides* species but as described in *Xanthomonas campestris* generating pyruvate and lactate (Stahl et al., 2011). Both end products are utilized by *C. jejuni* and efficiently promote its *in vitro* growth (Mendz et al., 1997; Velayudhan and Kelly, 2002; Thomas et al., 2011). Interestingly, no secreted fucosidase enzymes that cleave fucose residues from glycosylated host proteins have been described for the fucose-catabolizing *C. jejuni* strains. This suggests that *fucP* positive *C. jejuni* isolates might rely on the fucose released from intestinal mucins and host glycans by commensal bacteria like *Bacteroides thetaiotaomicron* as demonstrated for enterohaemorrhagic *Escherichia coli* (Pacheco et al., 2012). Alternatively, the activity of an induced host fucosidase could make free fucose available for *C. jejuni* similar as described for the human fucosidase FUCA2 of cultured human gastric and pancreatic adenocarcinoma cells upon infection with *Helicobacter pylori* (Liu et al., 2009).

The incapability to catabolize glucose distinguishes *C. jejuni* from many other gastrointestinal pathogens like *Salmonella* Typhimurium, enteropathogenic *E. coli* or *Listeria monocytogenes* (Dandekar et al., 2012; Fuchs et al., 2012) but also from its close relative *H. pylori* (Mendz et al., 1993). *C. jejuni* and *H. pylori* have an interrupted Embden–Meyerhof–Parnas (EMP) pathway because they lack the glycolytic enzyme phosphofructokinase, which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. However, *H. pylori* possesses a complete pentose phosphate pathway and an Entner–Doudoroff pathway (Doig et al., 1999) that enable the catabolism of glucose to pyruvate (Mendz et al., 1994), while *C. jejuni* does not encode for the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and the 6-phosphogluconolactonase (EC 3.1.1.31) of the oxidative pentose phosphate pathway branch but harbors all enzymes of the reductive branch of the pentose phosphate pathway (Parkhill et al., 2000; Fouts et al., 2005). Consequently, gluconeogenesis seems to be required for the biosynthesis of glucose and its derivatives that are precursor substrates for the LPS and capsule biosynthesis as well as the N- and O-glycosylation of various secreted proteins (Karlyshev et al., 2005). The genome of *C. jejuni* harbors all enzymes necessary for gluconeogenic synthesis of glucose from phosphoenolpyruvate (PEP) (Parkhill et al., 2000), but gluconeogenesis has not been experimentally proven yet. Only few studies have characterized the intermediary metabolism of *C. jejuni* and the anaplerotic reactions fueling gluconeogenesis, but it was demonstrated that the PEP carboxykinase PckA (Cj0932c), the pyruvate kinase Pyk (Cj0392c) and the pyruvate carboxylase (PycA, Cj1037c; PycB, Cj0933c) comprise a crucial metabolic junction between catabolism and anabolism in *C. jejuni* (Velayudhan and Kelly, 2002). These enzymes are

required for the oxaloacetate—PEP—pyruvate conversion that comprises a metabolic triangle, which plays the central role in controlling the carbon flow by connecting the tricarboxylic acid (TCA) cycle with the lower portion of the EMP pathway (Sauer and Eikmanns, 2005). Interestingly, *C. jejuni* does not encode for a PEP carboxylase catalyzing the generation of oxaloacetate from PEP and is lacking a PEP synthase to mediate the first ATP-consuming gluconeogenic reaction that synthesizes PEP from pyruvate. Consequently, the PEP carboxykinase PckA, catalyzing the synthesis of PEP by the decarboxylation of oxaloacetate, plays an important role in the intermediary metabolism of *C. jejuni* (Velayudhan and Kelly, 2002). Further studies are needed to identify the preferred substrates fueling the lower portion of the EMP pathway and to investigate how the intermediary metabolism is regulated and fine-tuned in *C. jejuni* in order to establish a balance between anabolism and catabolism.

ORGANIC ACIDS AND AMINO ACIDS FUEL THE GROWTH OF *C. JEJUNI*

Because the gluconeogenesis seems to play a crucial role in the physiology of *C. jejuni*, it raises the question of which substrates are efficiently utilized by this pathogen to fuel its intermediary metabolism and cope with its necessities for carbohydrate, lipid and protein biosynthesis. Various studies using the API test system (Elharrif and Megraud, 1986), the detection of CO_2 release upon incubation of *C. jejuni* with ^{14}C-labeled substrates (Westfall et al., 1986), substrate oxidation experiments with an oxygen electrode system (Mohammed et al., 2004) and *in vitro* growth experiments (Velayudhan and Kelly, 2002; Hinton, 2006; Guccione et al., 2008; Wright et al., 2009) revealed that *C. jejuni* catabolizes organic acids like lactate, pyruvate, acetate and intermediates of the TCA cycle as well as a restricted number of amino acids. The importance of organic acids for the proliferation of *C. jejuni* was further demonstrated by studies that identified pyruvate, 2-oxoglutarate, fumarate, succinate, malate and lactate as chemoattractants of *C. jejuni* (Hugdahl et al., 1988; Vegge et al., 2009).

The L- and D-lactate catabolism of *C. jejuni* has been described in detail: L-lactate is taken up by *C. jejuni* NCTC 11168 with the symport of protons by the transporter protein Cj0076c that belongs to the lactate permease LctP family (TC 2.A.14). In addition, other not yet identified transporter proteins are probably involved in the uptake of L- and D-lactate (Thomas et al., 2011). The imported L-lactate is subsequently oxidized to pyruvate, which itself is a growth substrate of *C. jejuni* (Mendz et al., 1997; Velayudhan et al., 2004) though no pyruvate carrier has been described yet. *C. jejuni* does not harbor a pyruvate dehydrogenase found in other enteropathogenic bacteria but catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA through a pyruvate:acceptor oxidoreductase (POR; Cj1476c) similar as described for *H. pylori* (St Maurice et al., 2007). However, the POR enzyme of *H. pylori* is composed of the four subunits PorABCD (Hughes et al., 1998), whereas the pyruvate-flavodoxin oxidoreductase of *C. jejuni* is comprised of one polypeptide with four functional domains. It is conserved in other *Campylobacter* species and has homologs in host-associated bacteria like *Fusobacterium nucleatum*, *Fusobacterium necrophorum*, *Cetobacterium somerae* or *Sebaldella termitidis*, as well as environmental bacteria such as *Psychrilyobacter atlanticus* and *Orenia marismortui*. Interestingly, the pyruvate-flavodoxin oxidoreductase of *C. jejuni* is responsible for its sensitivity against nitazoxanide, an antiprotozoal drug that inhibits PORs but not pyruvate dehydrogenases (Hoffman et al., 2007). The POR catalyzed generation of acetyl-CoA both fuels the TCA cycle of *C. jejuni* and is used for fatty acid biosynthesis (Leach et al., 1997; Gundogdu et al., 2007; Kirkpatrick et al., 2009). Furthermore, acetyl-CoA can be converted to acetyl-P and subsequently to acetate by the consecutive activities of the phosphate acetyltransferase Pta (Cj0688) and the acetate kinase AckA (Cj0689). The generated acetate is secreted by a yet unknown transporter during the logarithmic growth phase (Wright et al., 2009). In the late logarithmic growth phase an "acetate switch," which is well characterized for *E. coli* (Wolfe, 2005), could be observed for *C. jejuni*: The previously secreted acetate is taken up and can be used as a growth substrate through the conversion to acetyl-CoA by the acetyl-CoA synthetase ACS (Cj1537c) (Wright et al., 2009).

It is well established that the utilization of amino acids plays an important role in fueling the central metabolism of *C. jejuni*. Strikingly, however, only few glucogenic amino acids are degraded by this pathogen and support its proliferation, and some amino acids such as arginine and lysine have been described as chemorepellents (Rahman et al., 2014). For most *C. jejuni* isolates the growth-promoting amino acids are aspartate, glutamate, proline and serine (Leach et al., 1997; Guccione et al., 2008; Hofreuter et al., 2008). This finding is in agreement with the metabolic profiling of supernatants from *C. jejuni* liquid cultures demonstrating a significant depletion of these four amino acids from nutrient-rich brain heart infusion (BHI) or Mueller-Hinton (MH) medium (Guccione et al., 2008; Wright et al., 2009). The utilization of the growth-promoting amino acids in liquid cultures occurs in sequential phases: Aspartate and serine are first catabolized and facilitate the rapid growth of *C. jejuni* followed by the usage of glutamate. Proline seems to be a less-preferred growth substrate of *C. jejuni* because its consumption from the culture medium occurred less rapidly in comparison to the depletion of aspartate, serine and glutamate (Leach et al., 1997; Weingarten et al., 2009; Wright et al., 2009). Accordingly, L-aspartate, L-glutmate and L-serine but not L-proline are effective chemoattractants for *C. jejuni* (Hugdahl et al., 1988; Vegge et al., 2009).

Serine is imported by the high-capacity, low affinity serine transport protein SdaC (Velayudhan et al., 2004), a serine/H^+ symporter of the hydroxy/aromatic amino acid permease (HAAAP) family TC 2.A.42.2. The *sdaC* gene (*cj1625c*) is organized in an operon with *sdaA* (*cj1624c*) encoding for a serine dehydratase that catalyzes the deamination of serine to pyruvate. SdaA harbors, like various respiratory enzymes of *C. jejuni*, an oxygen-labile [4Fe–4S] cluster that might contribute to the oxygen sensitivity of this pathogen (Velayudhan et al., 2004). Serine utilization seems to be a variable catabolic trait of *C. jejuni* because not all tested isolates were able to grow with this amino acid a sole carbon source (Hofreuter et al., 2008). The molecular basis for this metabolic diversity is not known yet, as *C. jejuni* strains unable to utilize serine have no mutations in the *sdaA*

and *sdaC* genes though the serine dehydratase activity was fairly reduced (Hofreuter et al., 2012).

In contrast to serine catabolism, proline utilization seems to be a more conserved metabolic trait of *C. jejuni* as also shown for the usage of aspartate and glutamate (Hofreuter et al., 2008). A directed mutagenesis approach demonstrated that the growth of *C. jejuni* 81–176 with proline is mediated by the PutP transporter and the enzyme PutA (Hofreuter et al., 2012). PutP (CJJ81176_1494) belongs to the Na^+/solute symporter family TC 2A.21, which is widespread in Gram-positive and Gram-negative bacteria (Jung et al., 2012). It is highly conserved in *C. jejuni* and shows about 80% amino acid identity to respective transporter proteins of *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus*, whereas no homologs are present in other *Campylobacter* species. In addition, the proline symporter protein ($PutP_{Cj}$) of *C. jejuni* shows 75% identity to the PutP transporter ($PutP_{Hp}$) of the closely related *H. pylori* (Hofreuter et al., 2012). So the $PutP_{Cj}$-mediated proline import might have comparable properties as the $PutP_{Hp}$-catalyzed uptake of proline, which depends entirely on Na^+ as a coupling ion and binds specifically to L-proline with high affinity (Rivera-Ordaz et al., 2013). The PutA (CJJ81176_1495) enzyme of *C. jejuni* is predicted to possess both proline dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase activities and to use FAD and NADH as cofactors, respectively. It catalyzes the oxidation of the imported proline to glutamate. The *putA* and *putP* genes of *C. jejuni* and other Epsilonproteobacteria compromise an operon structure, whereas the adjacent located *putA* and *putP* genes of Enterobacteria have an inverse orientation (Hofreuter et al., 2012). Furthermore, the PutA protein of *C. jejuni* does not contain the N-terminal DNA-binding domain, which is involved in the repression of *putAP* gene cluster in *Salmonella* Typhimurium during the absence of proline (Ostrovsky de Spicer and Maloy, 1993).

C. jejuni takes up glutamtate through an ABC transporter system encoded by the *peb* locus (*cj0919c-cj0922c*) harboring genes for two permeases, one ATP-binding protein and one periplasmic substrate-binding protein Peb1A (Cj0921c). The latter has been first described as surface-bound antigen of *C. jejuni* with homology to amino acid-binding proteins like GlnH and HisJ (Pei and Blaser, 1993). Further studies characterized Peb1A as a glutamate- and aspartate-binding protein (Leon-Kempis Mdel et al., 2006; Müller et al., 2007), and an isogenic *C. jejuni peb1A* mutant showed impaired growth with aspartate or glutamate as sole carbon sources (Leon-Kempis Mdel et al., 2006). This phenotype correlated with an abolished glutamate uptake. In addition to the Peb ABC transporter, mutations of the permease PaqP (Cj0467) and the ATPase PaqQ (Cj0469) of the Paq (pathogenesis-associated glutamine) ABC transporter system negatively affected the uptake of glutamate in *C. jejuni* 81–176 (Lin et al., 2009). Imported glutamate is unlikely to be converted directly to 2-oxoglutarate through deamination since *C. jejuni* lacks a glutamate dehydrogenase (EC 1.4.1.2, EC 1.4.1.3, EC 1.4.1.4), which is present in other representatives of the *Campylobacteriaceae*. Instead, glutamate might either be converted to glutamine by the type I glutamine synthetase GlnA (Cj0699c) or is substrate of the aspartate:glutamate transaminase AspB (Cj0762c) catalyzing the generation of aspartate and 2-oxoglutarate from oxaloacetate and glutamate (Guccione et al., 2008). AspB plays an important role in the intermediary metabolism of *C. jejuni* and its efficient proliferation, since the inactivation of *aspB* results in an severe growth defect of the respective mutant in nutrient rich MH or BHI media as well as in defined medium with 20 mM glutamate or serine as sole energy sources (Guccione et al., 2008; Novik et al., 2010).

Besides serine, proline and glutamate catabolism, the utilization of aspartate is crucial for the metabolic fitness of *C. jejuni*. This is reflected by the identification of two chemoreceptors, Tlp1 (Hartley-Tassell et al., 2010) and CcmL (Tlp3) (Rahman et al., 2014), that interact with aspartate. The uptake of aspartate is convoluted and involves various transporters: While the periplasmic binding protein Peb1A interacts with aspartate and participates in its uptake, the *peb1A* mutant still possesses capacity to import aspartate although with a 20-fold reduction (Leon-Kempis Mdel et al., 2006). The direct involvement of the other Peb ABC transporter components like the two Peb permeases and the Peb ATP-binding protein has not been experimentally proven yet. In addition to the Peb1A-mediated aspartate uptake, the C4-dicarboxylate antiporters DcuA (Cj0088) and DcuB (Cj0671) have been shown to transport aspartate into *C. jejuni* under oxygen-limited conditions. Their activities were redundant so only a *dcuA*/*dcuB* double mutant showed reduced growth with aspartate (Guccione et al., 2008). It was suggested that aspartate uptake through the C4-dicarboxylate transport protein DctA (Cj1192) was responsible for the remaining growth of the *dcuA*/*dcuB* double mutant (Guccione et al., 2008). Interestingly, also a *paqP* mutant but not a *paqQ* mutant of the Paq ABC transporter showed a reduced level of aspartate uptake (Lin et al., 2009). The fate of imported aspartate is multifaceted involving central anabolic as well as catabolic pathways of *C. jejuni*: Aspartate represents an efficient carbon and energy source for *C. jejuni* as it directly fuels the TCA cycle by the aspartate ammonia lyse AspA (Cj0087) catalyzing the deamination to fumarate (Guccione et al., 2008; Novik et al., 2010). This AspA-catalyzed reaction of aspartate to fumarate also plays a role in the response of *C. jejuni* 81–176 to high pressure and its recovery from cell injury (Bieche et al., 2012). Fumarate might be converted to oxaloacetate, which can be used as substrate for the gluconeogenesis and synthesis of essential carbohydrates. Alternatively, fumarate represents an important electron acceptor for the oxygen-independent respiration of *C. jejuni* (Sellars et al., 2002), generating a significant amount of succinate, which is secreted during the growth by *C. jejuni* (Guccione et al., 2008). In addition, aspartate is the precursor for the biosynthesis of several proteinogenic amino acids (lysine, methionine, threonine, isoleucine) as well as β-alanine. The latter is generated from aspartate by the activity of an aspartate alpha-decarboxylase PanD (Cj0296c) and serves as precursor for the synthesis of pantothenate and coenzyme A.

Genome sequence analysis showed that *C. jejuni* encodes for an asparaginase (AnsB) facilitating the conversion of asparagine to aspartate and for a glutamate synthase (GltBD) catalyzing the generation of glutamate from glutamine and 2-oxoglutarate (Gundogdu et al., 2007). These enzymes seem functional since asparaginase activity was detected in cell extracts of *C. jejuni*

(Guccione et al., 2008) and ^{14}C-labeled glutamine was catabolized under CO_2 production (Westfall et al., 1986). These studies indicated that *C. jejuni* is able to catabolize asparagine as well as glutamine and both amino acids are chemoattractants for *C. jejuni* (Vegge et al., 2009). Although glutamine uptake in *C. jejuni* is facilitated by the conserved Paq transporter system (Lin et al., 2009), growth experiments revealed that just a subset of the tested *C. jejuni* isolates could use glutamine as sole carbon and energy source (Hofreuter et al., 2008). These results imply that the direct uptake of glutamine by the Paq and any additional transporter systems is not sufficient to promote pronounced proliferation of *C. jejuni* under the tested *in vitro* conditions. Only *C. jejuni* strains encoding for a secreted γ-glutamyltranspeptidase (GGT) were able to utilize glutamine efficiently for growth (Hofreuter et al., 2008). This periplasmic GGT enzyme facilitates the hydrolysis of glutamine to glutamate and ammonia similar to what has been described for *H. pylori* (Shibayama et al., 2007). Whereas the occurrence of GGT seems to be a conserved genetic trait in *H. pylori* (Leduc et al., 2010), not more than a third of the analyzed *C. jejuni* isolates harbored the GGT gene: Significant variations in the *ggt* frequency (between 8 and 30%) resulted from differences in the sequence type (ST) / clonal complex (CC) composition of the analyzed *C. jejuni* collections as determined by multilocus sequence typing (MLST): The *ggt* gene was strongly associated with ST-45 CC and ST-22 strains (Revez et al., 2011; de Haan et al., 2012) but typically absent in the common ST-21 isolates (Gripp et al., 2011; Zautner et al., 2011; de Haan et al., 2012). Surprisingly, GGT-activity provides *C. jejuni* strains not only an expanded amino catabolism but also a survival advantage in the presence of bactericidal isothiocyanates as shown for *C. jejuni* 81–176 (Dufour et al., 2012).

Most of the analyzed *C. jejuni* strains, including the reference strain NCTC 11168, were not able to grow with asparagine as the primary source of carbon and energy although all possess an L-asparaginase gene that encodes for a cytoplasmic AnsB enzyme (Hofreuter et al., 2008). This suggests that efficient asparagine import does not occur in *C. jejuni*. In contrast to *C. jejuni* NCTC 11168, certain isolates like *C. jejuni* 81–176 harbor an *ansB* allele variation that encodes for an additional sec-dependent secretion signal. Such a modified AnsB enzyme (AnsB^s) is translocated to the periplasm where it catalyzes the deamination of asparagine to aspartate. Consequently, the growth of *ansB*^{s}- and *ggt*-positive *C. jejuni* isolates with asparagine and glutamine relies on the conversion of both amino acids to aspartate and glutamate in the periplasm and the subsequent uptake of the deaminated amino acids (Hofreuter et al., 2008) through the DcuAB, DctA and Peb transporters (Leon-Kempis Mdel et al., 2006; Guccione et al., 2008). Interestingly, many *ggt*-positive *C. jejuni* strains encode also for a secreted asparaginase but lack the *fucP*-gene cluster, while *C. jejuni* isolates without the GGT- and AnsB^s-mediated expanded amino acid catabolism harbor the genes required for fucose utilization (Gripp et al., 2011; Zautner et al., 2011; de Haan et al., 2012). This correlation may reflect the selection pressure to compensate the restricted amino acid catabolism with enhanced carbohydrate utilization. Future studies are required to elucidate how these differences in substrate utilization might correlate with a variable fine-tuning of the intermediary metabolism of GGT/AnsB^s-positive and -negative *C. jejuni* strains and how these metabolic variations could reflect the physiological adaptations of certain *C. jejuni* strains to different hosts.

Besides the transport systems for the growth-promoting amino acids serine, proline, aspartate and glutamate, *C. jejuni* harbors homologs of the LIV (leucine, isoleucine, valine) branched-chain amino acid ABC transporter system of *E. coli* (Ribardo and Hendrixson, 2011). Though none of the branched-chain amino acids directly fuels the growth of *C. jejuni* (Guccione et al., 2008; Hofreuter et al., 2008), isoleucine interacts with the transducer-like protein Tlp3 of *C. jejuni* NCTC 11168 and induces a positive chemotactic response (Rahman et al., 2014). The LIV-locus in *C. jejuni* is comprised of six genes encoding for the periplasmic binding proteins LivJ and LivK, the two permeases LivH and LivM, and the cytoplasmic ATPases LivG and LivF. Targeted mutagenesis confirmed that the individual LIV ABC transporter components are responsible for the uptake of the branched amino acids, particularly of leucine (Ribardo and Hendrixson, 2011). Expression of the *liv* genes in *C. jejuni* is not repressed in the presence of leucine (Ribardo and Hendrixson, 2011) as shown for *E. coli* (Quay and Oxender, 1976), further exemplifying the metabolic specificity of *C. jejuni*.

Several additional putative amino acid transporters are present within the sequenced genomes of *C. jejuni*. For example, Cj0903c with homology to members of the sodium:alanine symporter family and SstT (Cj1097), the putative sodium/serine-threonine symporter, are conserved in *C. jejuni* and future studies are needed to elucidate their substrate specificity. Besides the aspartate/glutamate-binding protein Peb1A, *C. jejuni* harbors CjaA, CjaC and GlnH, three additional periplasmic proteins that belong to the family 3 of solute-binding proteins, which might also facilitate the uptake of amino acids. CjaA (Cj0982c) is an N-glycosylated 30 kDa lipoprotein attached to the inner membrane (Pawelec et al., 1997; Wyszynska et al., 2008) that binds cysteine (Müller et al., 2005). Its direct involvement in the import of cysteine has not been experimentally proven so far, and growth experiments suggested that CjaA might not be solely involved in the uptake of cysteine (Vorwerk et al., 2014). CjaC (Cj0734c) is a N-glycosylated 28 kDa protein with unknown substrate specificity that is anchored in the cytoplasmic membrane of *C. jejuni* and shows best homologies to various periplasmic binding proteins of ABC-type amino acid transporters (Pawelec et al., 1998; Wyszynska et al., 2007). Interestingly, a *hisJ* mutant of *S.* Typhimurium could be complemented by heterologous expression of the *C. jejuni cjaC* gene indicating that CjaC participates in the uptake of histidine in *C. jejuni* as well (Garvis et al., 1996). The third family 3 solute-binding protein, GlnH (Cj0817), remains uncharacterized. Taken together, though much progress has been made in recent years to characterize the amino acid catabolism of *C. jejuni*, future work is required to identify the transport systems involved in the uptake of amino acids that do not directly promote the growth of *C. jejuni* as energy or carbon sources.

Whereas the importance of amino acid uptake and utilization for the growth of *C. jejuni* is well documented, only few studies have investigated the *de novo* biosynthesis of amino acids in this pathogen, like e.g., the involvement of *ilvE* and *cysM* in the synthesis of leucine and cysteine, respectively (Ribardo

and Hendrixson, 2011; Vorwerk et al., 2014). Other studies have examined the amino acid biosynthesis capability of *C. jejuni* by heterologous expression of certain genes of arginine (Hani and Chan, 1994; Hani et al., 1999), cysteine (Garvis et al., 1997), leucine (Labigne et al., 1992) and aromatic amino acid (Wösten et al., 1996) biosynthesis pathways in respective auxotrophic *Escherichia coli* mutants. Therefore, information about the complete biosynthesis pathways of amino acids are primarily obtained through the *in silico* analysis of available genome sequences of *C. jejuni* isolates: In contrast to *H. pylori*, which has limited capacity for the synthesis of amino acids and requires arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine for growth (Doig et al., 1999), *C. jejuni* NCTC 11168 encodes for enzymes enabling the synthesis of all amino acids (Gundogdu et al., 2007). Future studies are required to clarify how conserved the functionality of the predicted amino acid biosynthesis pathways are among *C. jejuni* strains as several amino acid auxotrophies, e.g., for methionine, proline or the branched amino acids isoleucine, leucine and valine, have been previously described in various *C. jejuni* isolates (Tenover et al., 1985; Blaser et al., 1986; Tenover and Patton, 1987).

PEPTIDASES AND PEPTIDE CATABOLISM OF *C. JEJUNI*

The central role of amino acid catabolism for the proliferation of *C. jejuni* suggests that peptides may also be important growth-promoting substrates for this pathogen, especially, since the digestion of proteins in the gastrointestinal tract of its hosts generates a variety of peptides besides free amino acids (Adibi and Mercer, 1973). Several putative peptidases and proteases are encoded by the *C. jejuni* genome (Hofreuter et al., 2006; Gundogdu et al., 2007), and some, like ClpP (Cj0192c), HtrA (Cj1228c), CJJ81176_1086 (Cj1068), CJJ81176_1228 (Cj1215), Cj0511 or Pgp1 (Cj1345c; CJJ81176_1344), have been associated with the virulence of *C. jejuni* (Brondsted et al., 2005; Cohn et al., 2007; Novik et al., 2010; Boehm et al., 2012; Frirdich et al., 2012; Karlyshev et al., 2014).

The role of peptidases in the catabolism and nutrient acquisition of *C. jejuni* has not been characterized in detail so far. BIOLOG phenotype microarray analysis suggested that dipeptides like glycyl-glutamine and glycyl-proline enhance the respiratory activity of *C. jejuni* and can be used as carbon sources though strain specific differences exist (Gripp et al., 2011; Muraoka and Zhang, 2011). This variability in peptide catabolism might be the consequence of *C. jejuni* isolates having variable number of peptidases: GGT, the putative S15 family dipeptidyl-peptidase CJJ81176_1680 or the putative subtilisin-like serine peptidases Cj1365 / CJJ81176_1367 and CJJ81176_1371 occur in a subset of *C. jejuni* strains (Champion et al., 2005; Hofreuter et al., 2006; Hepworth et al., 2007; Gonzalez et al., 2009; Zautner et al., 2011). Only *ggt*-positive *C. jejuni* strains can efficiently use the tripeptide glutathione as carbon/energy (Hofreuter et al., 2008) and cysteine source (Vorwerk et al., 2014). A recent study using the BIOLOG phenotype microarray technology suggested that the *C. jejuni* NCTC 11168 transporter protein Cj0917c, which has homology to the carbon starvation protein A (CstA) of *E. coli*, is involved in the catabolism of several tri- and dipeptides (Rasmussen et al., 2013). Additional experiments are necessary to demonstrate the growth-promoting effect of respective peptides. Moreover, the *Campylobacter* peptide transporter A (CptA; CJJ81176_0236), a member of the Proton-dependent Oligopeptide Transporter (POT) family, has been described to promote the growth of *C. jejuni* 81–176 with the dipeptides Cys-Gly, Arg-Trp and Arg-Ile (Vorwerk et al., 2014).

Taken together, *C. jejuni* shows an intriguing metabolic diversity. The diverse growth properties of *C. jejuni* isolates result from the variable presence or absence of metabolic genes involved in the strain-specific utilization of particular substrates such as fucose, asparagine or glutamine and peptides. In addition, *C. jejuni* isolates are equipped with different sets of group A chemoreceptor *tlp* genes that response to a variety of potential nutrients (Day et al., 2012; Rahman et al., 2014). Such a variable presence of chemosensory receptor genes in *C. jejuni* suggests that different strains may not respond equivalently to certain nutrients and consequently cannot utilize and benefit from the same growth substrates.

FINDING THE OPTIMAL OXYGEN LEVEL: HOW DOES A MICROAEROPHILIC PATHOGEN PERSISTS IN THE ANAEROBIC ENVIRONMENT OF THE INTESTINE?

In contrast to facultative anaerobe pathogens like *S.* Typhimurium or enteropathogenic *E. coli*, *C. jejuni* faces the challenging situation to proliferate as obligate microaerophilic bacterium in the intestine of its hosts where lower levels than the preferred 5% oxygen exist. It has been suggested that *C. jejuni* is adapted to this disadvantageous circumstance by its colonization pattern and distinct respiratory capability: (i) *C. jejuni* colonizes preferentially the mucus layer and the intestinal crypt close to the epithelium (Lee et al., 1986; Beery et al., 1988) where the oxygen tension is higher than in the intestinal lumen. In addition, the region between the mid small intestine and the mid colon, which is the preferred colonization site of *C. jejuni*, harbors higher oxygen tensions than the distal colon and rectum (He et al., 1999). (ii) Early biochemical characterizations of *C. jejuni* demonstrated the presence of a surprisingly complex and highly branched respiratory chain, allowing this pathogen to use a variety of electron donors and several other electron acceptors besides oxygen (Carlone and Lascelles, 1982; Hoffman and Goodman, 1982; Hitchcock et al., 2010). Menaquinone-6 and methyl-substituted menaquinone-6 mediate in *C. jejuni* the electron transfer along the respiration chain between electron donors and receptors (Carlone and Anet, 1983; Moss et al., 1984). The *menBCDEF* menaquinone biosynthesis genes found in enterobacteria to catalyze the transformation of chorismate to menaquinone are absent in *C. jejuni*. Instead *C. jejuni* harbors enzymes of an alternative menaquinone biosynthesis pathway similar to the futalosine pathway described for *Streptomyces coelicolor* (Hiratsuka et al., 2008; Li et al., 2011). This modified futalosine pathway of *C. jejuni* is also employed by *H. pylori* (Arakawa et al., 2011) and uses 6-amino-6-deoxyfutalosine instead of futalosine as an intermediate for the synthesis of menaquinone. Consequently, orthologs to enzymes MqnA and MqnB of *S. coelicolor* are not required by *C. jejuni* and replaced by the menaquinone biosynthetic enzyme A2 (MqnA2; Cj1285c) and the 5′-methylthioadenosine nucleosidase MTAN (Cj0117)

(Li et al., 2011). MTAN might be a promising new drug target for *C. jejuni* as demonstrated for *H. pylori* (Wang et al., 2012).

Genome sequence analysis of *C. jejuni* (Parkhill et al., 2000; Sellars et al., 2002) revealed that the respiratory electron chain of *C. jejuni* is comprised of two terminal, membrane-bound oxidases. Both enzymes mediate the oxygen-dependent respiration of *C. jejuni* but harbor strikingly different oxygen affinities: the cyanide-insensitive oxidase CioAB (initially named CydAB; Cj0081, Cj0082) exhibits low affinity for oxygen, whereas the cyanide-sensitive, *cb*-type cytochrome c oxidase CcoNOQP (Cj1490c-Cj1487c) shows high affinity to oxygen and might be crucial for respiration under oxygen-limited conditions (Jackson et al., 2007). The CioAB oxidase receives electrons directly from the oxidized menaquinone pool, whereas oxygen reduction by the CcoNOQP oxidase involves electron transfer from the oxidized menaquinone pool via the proton-translocating cytochrome *bc* complex (PetABC: Cj1186c-Cj1184c) and a periplasmic *c-type* cytochrome (Cj1153).

Though *C. jejuni* is equipped with various enzymes facilitating oxygen-independent respiration, no growth can be observed under strictly anaerobic conditions (Veron et al., 1981). It was suggested that the class I ribonucleotide reductase (NrdAB-type RNR) of *C. jejuni* is responsible for its inability to grow anaerobically because this enzyme requires low amounts of oxygen for the DNA synthesis (Sellars et al., 2002). This prerequisite for oxygen brings the disadvantage that non-specific electron transfer from the respiratory chain to oxygen occurs (Cabiscol et al., 2000), leading to the generation of toxic reactive oxygen species (ROS) like hydroxyl ($^{\cdot}OH$) and superoxide (O_2^-) radicals as well as hydrogen peroxide (H_2O_2). *C. jejuni* harbors a variety of ROS-detoxifying enzymes including the superoxide dismutase SodB (Pesci et al., 1994), the alkyl hydroxide reductase AhpC (Baillon et al., 1999), the catalase KatA (Cj1385) (Day et al., 2000) as well as the thiolperoxidases Tpx and Bcp (Atack et al., 2008). Surprisingly, these enzymes are unable to provide sufficient oxygen tolerance in aerobic conditions for *C. jejuni*. A recent study linked the oxygen-labile, iron-sulfur (4Fe-4S)-containing metabolic enzymes pyruvate:acceptor oxidoreductase POR and the *oorDABC* (*cj0535-cj0538*) encoded 2-oxoglutarate:acceptor oxidoreductase (OOR) to the oxygen sensitivity of *C. jejuni* (Kendall et al., 2014). The enzymatic activity of POR and OOR are partially protected by the hemerythrin proteins HerA (Cj0241c) and HerB (Cj1224), but *C. jejuni* lacks sufficient mechanisms to repair POR and OOR once damaged through exposure to atmospheric oxygen concentrations (Kendall et al., 2014).

A VARIETY OF ELECTRON DONORS FUEL THE RESPIRATORY ACTIVITY OF *C. JEJUNI*

The reduction equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) serve in many bacteria as major electron sources for the respiratory electron transport chain, which generates through the proton-translocating NADH:quinone oxidoreductase (Nuo/NDH-1) complex a proton gradient that drives the oxidative phosphorylation (Haddock and Jones, 1977). Strikingly, NADH is a poor respiratory electron donor in *C. jejuni* in contrast to FADH (Hoffman and Goodman, 1982) as consequence of the specific NDH-1 complex property encoded by the *nuo* gene cluster *cj1566c* to *cj1579c* in *C. jejuni* NCTC 11168: The genes *nuoE* and *nuoF* encoding for the NADH dehydrogenase subunits of the NDH-1 complex are replaced in *C. jejuni* NCTC 11168 by the genes *cj1575c* and *cj1574c* (Smith et al., 2000). It was shown that Cj1574c, probably in conjunction with Cj1575c, mediates the electron transfer from the reduced, flavin monoculeotide containing flavodoxin FldA (Cj1382c) to the NDH-1 complex (Weerakoon and Olson, 2008). Consequently, the NDH-1 complex of *C. jejuni* seems to participate rather in the oxidation of flavin mononucleotides than of NADH. It was further demonstrated that reduced FldA is generated by the oxidation of 2-oxoglutarate to succinyl-CoA catalyzed by the OOR, whereas the putative ferredoxins FdxA (Cj0333c) and Cj0369c are no electron acceptors for OOR (Weerakoon and Olson, 2008).

Several other electron donors besides FADH have been described to fuel the respiration chain of *C. jejuni*: some of them, like hydrogen, formate, lactate or succinate, are generated through the catabolic activity of the host gut microbiota (Bernalier-Donadille, 2010), which suggests that *C. jejuni* might benefit from metabolic cross-feeding. Hydrogen can be used as an electron donor by *C. jejuni* through the activity of the membrane-bound NiFe-type hydrogenase HydABCD (Cj1267c-Cj1264c) (Hoffman and Goodman, 1982; Weerakoon et al., 2009). The accessory factors encoded by the *hypFBCDEA* operon (*cj0622-cj0627*) are required for assembling of the hydrogenase enzyme complex and insertion of the nickel cofactor. An ABC-transporter system (Cj1584c-Cj1580c) of *C. jejuni* NCTC 11168 has recently been identified as a high-affinity nickel uptake system and was named NikZYXWV (Howlett et al., 2012). Under low nickel concentrations the inactivation of *nikZ* (*cj1584c*), encoding for a periplasmic binding protein, led to an abolished hydrogenase activity of the mutant strain. This result demonstrated the importance of the Nik-transporter system for the acquisition of nickel as a cofactor for the enzyme. Yet hydrogenase activity of the *nik*Z mutant was observed in the presence of high nickel concentrations, indicating the presence of additional nickel transporters (Howlett et al., 2012). In contrast to studies with *E. coli*, showing the importance of the nickel chaperone SlyD for the hydrogenase activity, a mutation in the *slyD* ortholog gene *cj0115* of *C. jejuni* NCTC 11168 did not abolish the mutants nickel uptake capacity and hydrogenase activity (Howlett et al., 2012).

Formate is mainly generated by the mixed-acid fermentation of the intestinal microbiota and is sensed as chemoattractant by *C. jejuni* through the Tlp7 chemoreceptor (Vegge et al., 2009; Tareen et al., 2010). It can be oxidized by *C. jejuni* to CO_2, protons and electrons through a membrane-bound formate dehydrogenase (FDH) complex comprised of the selenocysteine-containing subunit FdhH (Cj1511c), the iron-sulfur subunit FdhB (Cj1510c) and the formate dehydrogenase cytochrome-b subunit FdhC (Cj1509c), which requires FdhD (Cj1508c) for its activity. The released electrons are directly transferred from the FDH complex to the menaquinone pool (Weerakoon et al., 2009). The FDH activity of *C. jejuni* is controlled by the accessory proteins FdhT (Cj1500), FdhU (Cj1501) and a high-affinity TupABC-like tungstate transporter (Cj1538-Cj1540) indicating that tungstate

might be incorporated into the FDH complex (Smart et al., 2009; Pryjma et al., 2012; Shaw et al., 2012).

Lactate is oxidized by *C. jejuni* to pyruvate by the membrane-associated NAD-independent respiratory lactate dehydrogenase complex (L-iLDH; Cj0075c, Cj0074c; Cj0073c) though inactivation of these genes in *C. jejuni* NCTC 11168 did not abolish the growth of respective mutants with lactate (Thomas et al., 2011). However, a second L-iLDH, the oxidoreductase Cj1585c, was identified and demonstrated to be responsible for the observed redundancy in the catabolism of L-lactate. Only a double mutation inactivating *cj0075c* and *cj1585c* abolished the growth of *C. jejuni* NCTC 11168 with 20 mM L-lactate as a carbon source but it did not affect in the utilization of D-lactate. The gene locus of *cj1585c* is not conserved in *C. jejuni* (Hofreuter et al., 2006) and is replaced by a dimethyl sulfoxide reductase (*dmsABC*) gene cluster encoding for an anaerobic dimethyl sulfoxide reductase complex in strains like *C. jejuni* 81–176 (Hofreuter et al., 2006), 81116 (Pearson et al., 2007), M1 (Friis et al., 2010) or 327 (Takamiya et al., 2011). This finding is verified by the observation that a mutant in the Cj0075c homolog of wild-type strain *C. jejuni* 81116, which naturally lacks the L-iLDH Cj1585c, was unable to grow with L-lactate (Thomas et al., 2011).

The TCA cycle intermediate succinate is not only a carbon and energy source for *C. jejuni* but serves also as an electron donor. Consequently, succinate is oxidized to fumarate accompanied with the generation of $FADH_2$ and the subsequent electron transfer to the menaquinone pool. One predicted succinate dehydrogenase (succinate:quinone oxidoreductase) SdhABC (Cj0437-Cj0439) of *C. jejuni* was misannotated and not involved in the conversion of succinate to fumarate (Weingarten et al., 2009). Instead, the putative FrdABC fumarate reductase complex comprised of a membrane-associated diheme cytochrome B (FrdC, Cj0408), the flavoprotein FrdA (Cj0409) and the Fe-S protein FrdB (Cj0410) showed properties of a succinate:quinone reductase and was solely responsible for the oxidation of succinate to fumarate (Weingarten et al., 2009).

C. jejuni is unable to catabolize gluconate due to the absence of an Entner-Doudoroff pathway, but it can use gluconate as electron donor through a temperature-regulated flavin-containing gluconate dehydrogenase (Pajaniappan et al., 2008). Proteomic analysis demonstrated that the gluconate dehydrogenase (GADH) expression increased in *C. jejuni* upon a temperature shift from 37 to 42°C correlating with an elevated GADH activity. The co-transcribed genes *cj0414* and *cj0415* encode for the two components of the gluconate-oxidizing oxidoreductase, which is predicted to be localized in the periplasm peripherally associated with the cytoplasmic membrane and to transfer electrons to the periplasmic cytochrome *c* (Pajaniappan et al., 2008). Interestingly, both GADH subunits are conserved in *C. jejuni* but absent from other *Campylobacter* species.

Another example of the unique respiratory capacity of *C. jejuni* is its sulfite respiration system, which uses sulfite and metabisulfite as electron donors. This sulfite:cytochrome c oxidoreductase (SOR) system is conserved in *C. jejuni* and can also be found in *C. lari* but not in any other examined *Campylobacter* and *Helicobacter* species. The periplasmic proteins SorA (Cj0005c) and SorB (Cj0004c) with properties of a molybdopterin oxidoreductase and a monoheme cytochrome c_{552}, respectively, catalyze the oxidation of sulfite to sulfate accompanied by an electron transfer to cytochrome *c* (Myers and Kelly, 2005).

In summary, the highly branched electron transport chain of *C. jejuni* enables respiration with a variety of electron donors that are secreted catabolic end products of the surrounding microbiota of the intestinal habitat. This cross-feeding might provide an important advantage for *C. jejuni* to overcome the colonization resistance of its host and to establish its nutritional niche.

THE VERSATILITY OF OXYGEN-INDEPENDENT RESPIRATION IN *C. JEJUNI*

C. jejuni is unable to grow under anaerobic conditions but is well adapted to the oxygen-limited conditions of its intestinal habitat by harboring a variety of respiration systems using alternative electron acceptors to oxygen, like nitrate, nitrite, S- and N-oxides or fumarate (Sellars et al., 2002).

Under oxygen-limited cultivation conditions of *C. jejuni*, fumarate is reduced to succinate by the bifunctional FrdABC complex and the methlymenaquinol:fumarate reductase complex comprised of MrfA (Cj0437), MrfB (Cj0438) and MrfE (Cj0439), initially described as SdhABC complex (Weingarten et al., 2009; Guccione et al., 2010). While the fumarate reductase FrdABC complex acts in the cytoplasm of *C. jejuni*, the MfrABE-mediated fumarate reduction occurs in the periplasm where MfrA is exported by the twin arginine translocase (TAT) system (Hitchcock et al., 2010). The periplasmic MrfABE complex of *C. jejuni* also participates in the reduction of the C4-dicarboxylate mesaconate and the C4-monocarboxylate crotonate (Guccione et al., 2010). Interestingly, both substances are fumarate analogs, which are produced as metabolic intermediates by anaerobic bacteria of the intestinal microbiota, suggesting a metabolic interaction between *C. jejuni* and species of *Clostridium* and *Fusobacterium* (Guccione et al., 2010).

Dimethyl sulfoxide (DMSO) and trimethylamine *N*-oxide (TMAO) are additional electron acceptors of *C. jejuni in vitro* (Sellars et al., 2002): The gene *cj0264c* of *C. jejuni* NCTC 11168 is conserved in *C. jejuni* and encodes for a molybdopterin-containing oxidoreductase, which is responsible for its DMSO and TMAO reductase activity. The TMAO- and DMSO-dependent respiration improved the *in vitro* growth of *C. jejuni* NCTC 11168 under oxygen-limited but not under microaerobic conditions. Future studies have to clarify if the putative cytochrome C-type heme-binding periplasmic protein, encoded by *cj0265c*, participates in the elector transfer to DMSO, TMAO and other *N*- or *S*- oxides (Sellars et al., 2002). Beside Cj0264c, *C. jejuni* 81–176 harbors a putative oxidoreductase with homology to the anaerobic dimethylsulfoxide reductase DmsA (Hofreuter et al., 2006). This oxidoreductase has 28% protein sequence identity with Cj0264c and may catalyze the reduction of DMSO / TMAO as well. The *dmsA* gene (*cju34* / *cjj81176_1570*) of *C. jejuni* 81–176 is organized in a gene cluster together with three other gene genes encoding for the iron-sulfur containing DMSO reductase subunit DmsB (CJJ81176_1571), the DMSO reductase anchor subunit DmsC (CJJ81176_1572) and chaperon protein TorD (CJJ81176_1572) that are predicted to participate

with DmsA at the electron transfer to DMSO / TMAO or other S- and N-oxides (Hofreuter et al., 2006).

Several human pathogens harbor nitrate and nitrite respiration systems (Sparacino-Watkins et al., 2014). *C. jejuni* encodes for the periplasmic located Nap-type nitrate reductase (Cj0780-Cj0785) catalyzing the reduction of nitrate to nitrite (Pittman and Kelly, 2005). Nitrite can subsequently be used as electron acceptor and reduced to ammonia by *C. jejuni* through the concerted activity of the pentaheme nitrite reductase NrfA (Cj1357c) and the electron donor protein NrfH (Cj1358c) of the tetraheme NapC/NirT cytochrome C family (Pittman et al., 2007). Exposure to nitrite leads to a nitrosative stress response in *C. jejuni* and the nitrite reductase NrfA catalyzes not only the reduction of nitrite to ammonia but participates with the single domain globin Cgb (Cj1586) in the detoxification of nitric oxide (Pittman et al., 2007).

S. Typhimurium uses the TtrRSBCA system for the oxygen-independent respiration with the alternative electron acceptor tetrathionate (Hensel et al., 1999). The recently described thiosulfate/tetrathionate-dependent respiration of *C. jejuni* is mediated by the bifunctional tetrathionate reductase/thiosulfate dehydrogenase TsdA (Liu et al., 2013) and seems to be a variable property in *C. jejuni* that promotes growth only of certain isolates in oxygen-limited conditions. Some *C. jejuni* strains like 81116 (Pearson et al., 2007) or M1 (Friis et al., 2010) harbor an active TsdA (C8j_0815) enzyme that catalyzes the reduction of tetrathionate to thiosulfate in the presence of additional electron donors like formate (Liu et al., 2013). In addition, these strains can use thiosulfate as an electron donor that is converted by TsdA to tetrathionate under microaerobic conditions. The *C. jejuni* isolates NCTC 11168 (Parkhill et al., 2000), RM1221 (Fouts et al., 2005) or 81–176 (Hofreuter et al., 2006) harbor truncated alleles of *tsdA* (*c8j_0815*) but possess like *C. jejuni* 81116 a less-active TsdA homolog (C8j_0040), which catalyzes the conversions between thiosulfate and tetrathionate at low levels (Liu et al., 2013). Future studies are needed to examine if the minimal thiosulfate oxidation and tetrathionate reduction driven by C8j_0040 can contribute to the growth of the *C. jejuni* strains harboring truncated *tsdA* genes. Furthermore, animal infection studies are required to clarify if tetrathionate respiration in *C. jejuni* provides a similar benefit for the colonization process as described for *S.* Typhimurium (Winter et al., 2010).

PHYSIOLOGICAL FACTORS PROMOTING THE COLONIZATION PROCESS OF *C. JEJUNI*

Transposon mutagenesis projects and *in silico* analysis of metabolic pathways led to the identification of genes essential for the proliferation of *C. jejuni in vitro* (Metris et al., 2011; Stahl and Stintzi, 2011). In addition, recent studies provided comprehensive data of genes expressed during the *in vitro* growth of *C. jejuni* when cultivated in nutrient rich media like BHI or Brucella broth by high-throughput deep sequencing of messenger RNA transcripts (Chaudhuri et al., 2011; Dugar et al., 2013; Porcelli et al., 2013). However, the knowledge of the nutritional requirements and metabolic traits that facilitate the growth of *C. jejuni* during the colonization of various hosts is limited. Still, our understanding about the metabolic necessities of *C. jejuni* required for a successful host infection has significantly improved in recent years by *in vivo* transcriptome analyses and screening of isogenic *C. jejuni* mutants in colonization models using 1 day old chickens, neonatal piglets and immunodeficient, antibiotic treated or germfree mice.

Taveirne et al. (2013) applied the RNAseq approach to study the *in vivo* transcriptome of *C. jejuni* after infection of 1-day-old chicks. Comparing the gene expression profile of *C. jejuni* isolated from the caecum of the chicks with the transcriptome during *in vitro* cultivation identified several gene groups that were more highly expressed *in vivo* including, for example, genes involved in the oxidative stress response (*katA*), iron (*chuABCD*; *cjj81176_1601-cjj81176_1604*) and phosphate (*pstSCAB*; *cjj81176_0642-cjj81176_0645*) transport. Elevated expression of the single-domain globin gene *cbg* (Elvers et al., 2004) indicated that *C. jejuni* was also exposed to nitrosative stress during colonization of the chicken caecum (Taveirne et al., 2013). One of the most increased transcripts *in vivo* was *katA* (Taveirne et al., 2013), the product of which is essential to counteract the damaging effect of H_2O_2 (Grant and Park, 1995). Such oxidative stress might occur through the production of H_2O_2 by the intestinal epithelial cells at the colonization site of *C. jejuni* as indicated by *ex vivo* experiments with biopsy samples and infection of cultured HCT-8 cells (Corcionivoschi et al., 2012). In addition, the production of reactive oxygen species like superoxide anions and H_2O_2 are central for the innate immune defense (Rada and Leto, 2008). The KatA-mediated detoxification of H_2O_2 is crucial for *C. jejuni in vivo*, as the inactivation of *katA* reduced the colonization of the chicken caecum by the respective *C. jejuni* mutant (Bingham-Ramos and Hendrixson, 2008; Palyada et al., 2009). The severity of the observed colonization defects differed between these two studies, and this variability is likely due to dissimilar experimental settings or different *C. jejuni* and chicken strains used in the experiments. The ankyrin repeat-containing protein Cj1386 of *C. jejuni* NCTC 11168 is required for its full catalase activity by facilitating the correct heme trafficking to KatA (Flint et al., 2012). Similar to the *katA* mutant, a *cj1386* mutant exhibited a reduced capability to colonize the caecum of chicken and to compete with the wild-type strain in the intestinal colonization of neonate piglets (Flint et al., 2012), further illustrating the importance of H_2O_2 detoxification for the pathogenesis of *C. jejuni* (**Figure 1**). The periplasmic c-type cytochrome Cj1153 has been suggested to participate in the defense against H_2O_2 through the reduction and detoxification of exogenous H_2O_2 to water and oxygen via electron transfer to the potential *cytC* peroxidases DocA (Cj0020c) and Cj0358 (Sellars et al., 2002; Hendrixson and DiRita, 2004). But in contrast to KatA, both peroxidases play no role in the resistance of *C. jejuni* against H_2O_2; nonetheless *docA* and *cj0358* mutants were defective in the colonization of chicken (Hendrixson and DiRita, 2004; Bingham-Ramos and Hendrixson, 2008). The up-regulation of *katA* and additional oxidative stress response genes like *sodB*, *ahpC* and *tpx* was also observed in a microarray study analyzing the gene expression profile of *C. jejuni* in a rabbit ileal loop infection model (Stintzi et al., 2005). This observation hinted once more to the importance to counteract oxidative stress for a successful colonization process of *C. jejuni* and was confirmed by

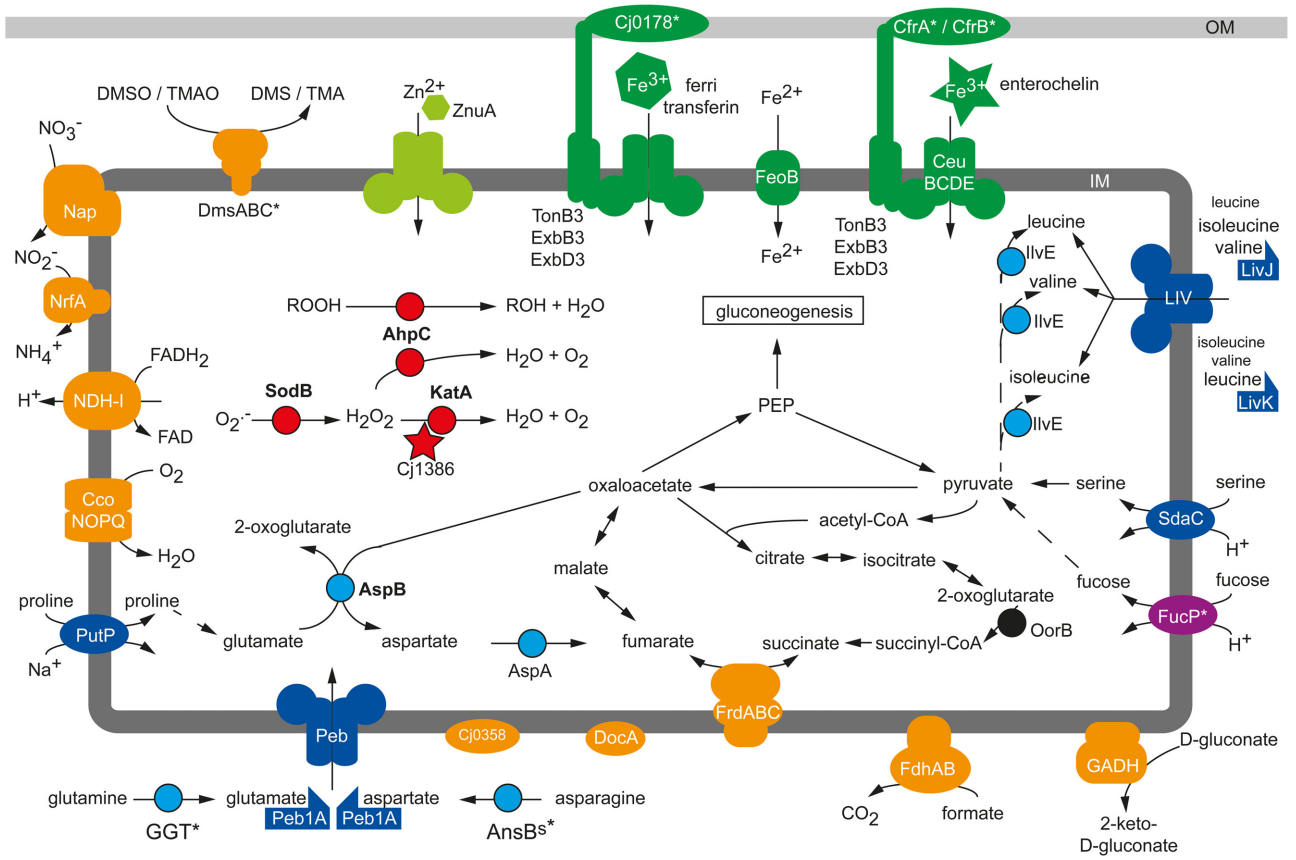

FIGURE 1 | Physiological colonization factors of *C. jejuni*. Schematic presentation of metabolic factors being required for the efficient colonization process of *C. jejuni* in chicken, murine and porcine infection experiments. Blue, amino acid metabolism; red, reactive oxygen defense; orange, respiration; green, micronutrient utilization; IM, inner membrane; OM, outer membrane, *, Strain-specific gene; not conserved in all *C. jejuni* isolates.

infection experiments demonstrating that AhpC and SodB activities promote the colonization of *C. jejuni* in chickens and mice (Palyada et al., 2009; Novik et al., 2010).

Microarray-based gene expression analysis of *C. jejuni* isolated from the caecum of infected chickens suggested that an optimal respiratory adaptation of *C. jejuni* to its intestinal habitat is central for the colonization process (Woodall et al., 2005). This is reflected by the upregulation of the *petABC* and *ccoNOQP* operons *in vivo*, both of which facilitate the enhanced respiration of *C. jejuni* with the *cb*-type cytochrome c oxidase, which harbors high affinity to oxygen. Mutation of *ccoNOQP* abolished the ability of *C. jejuni* to colonize chicken, illustrating the crucial role of the terminal oxidoreductase for this microaerophilic pathogen to maintain its oxygen-dependent respiratory activity in the avian gastrointestinal tract (Weingarten et al., 2008). In contrast, inactivation of the terminal cyanide-insensitive oxidase CioA did not affect the colonization process (Weingarten et al., 2008), though the *cioA* expression was significantly higher *in vivo* than *in vitro* (Stintzi et al., 2005). Infection experiments with isogenic mutants of the highly branched respiratory chain revealed that especially the usage of alternative electron acceptors is crucial for the persistence of *C. jejuni* in the oxygen-limited environment of the intestine (**Figure 1**): The *nap* and *nrf* genes, facilitating the oxygen-independent respiration with nitrate and nitrite, were up-regulated *in vivo* (Woodall et al., 2005) and the NapA-mediated nitrate respiration of *C. jejuni* supported colonization in chickens (Weingarten et al., 2008). Interestingly, an increased intestinal nitrate level produced through an inflammatory host response also promoted the anaerobic respiration and luminal growth of *E. coli* in a murine infection model (Winter et al., 2013). Though the nitrite reductase activity of *C. jejuni* participates in nitrite respiration and protects against nitrosative stress *in vitro*, a *nrfA* mutant showed no general chicken colonization defect of the respective mutant in two independent studies (Pittman et al., 2007; Weingarten et al., 2008). Such discrepancy might be the result of different *C. jejuni* and chicken strains used in the studies as well as dissimilar infection doses and protocols. DMSO and TMAO have been proposed as electron acceptors that could be used by *C. jejuni* in aquatic environments since neither substances is found in significant amounts in the gastrointestinal tract of animals (Sellars et al., 2002). However, TMAO might eventually be present in the gastrointestinal tract under certain circumstances, as its precursor TMA is commonly produced by the human microbiota through the catabolism of carnitine and oxidized to TMAO in the host liver (Koeth et al., 2013; Zhu et al., 2014). This could explain why the inactivation of the *dmsA*

gene in *C. jejuni* 81–176 resulted in a mutant with diminished persistence capability in immunodeficient mice when co-infected with the wild-type strain (Hofreuter et al., 2006). In contrast, the TMAO/DMSO reductase Cj0264c of *C. jejuni* NCTC 11168 was in single-infection experiments negligible for the colonization of chicken (Weingarten et al., 2008). Further work is needed to clarify if these different observations are the consequences of co- vs. single-infections, distinctive functions of DmsA and Cj0264c, or result from the dissimilarities in the *C. jejuni* strains and animal infection models used. Additional studies are required to identify the definite electron acceptors for the DmsA and Cj0264c oxidoreductases during the persistence of *C. jejuni* in the host intestine.

Increased *in vivo* gene expression levels were found for the C4-dicarboxylate transporter genes *ducA* and *dcuB*, the aspartase gene *aspA*, the fumarate reductase *frdABC* genes and the methylmenaquinol:fumarate reductase *mfr* genes (Woodall et al., 2005) participating in the fumarate respiration of *C. jejuni* under microaerobic and oxygen-limited conditions (Guccione et al., 2008, 2010). Higher *in vitro* expression of these genes was also observed under microaerobic and oxygen-limited growth conditions for the robust colonizer *C. jejuni* NCTC11168-O in comparison to its poor-colonizing variant *C. jejuni* NCTC11168-GS, likewise hinting toward an important role of oxygen-independent respiration for the persistence of *C. jejuni* in its host (Gaynor et al., 2004). Unexpectedly, the MrfA activity was dispensable for the *C. jejuni* chicken colonization (Weingarten et al., 2008), besides the impaired fumarate respiration under oxygen-limited conditions (Guccione et al., 2010). In contrast, the bifunctional fumarate reductase FrdA was required for optimal colonization (Weingarten et al., 2009). It is not yet clear to what extent the abolished utilization of growth substrates like the TCA cycle intermediates succinate or 2-oxoglutarate is responsible for the colonization defect of the *frdA* mutant in comparison to its inability to perform fumarate respiration. The importance of a functional TCA cycle for a *C. jejuni* infection was shown by the inactivation of the 2-oxoglutarate:acceptor oxidoreductase (*oorB*) resulting in a mutant with significantly reduced colonization capability in chicken compared to the wild-type strain as well (Weerakoon et al., 2009).

Intestinal pathogens have to overcome the gut microbiota-mediated colonization resistance of the host in order to establish a stable infection (Stecher et al., 2013). This might involve the successful competition with the intestinal microflora for common nutrients or, more often, the occupation of a specific metabolic niche in order to circumvent a direct competition with the commensal gut bacteria (Kamada et al., 2013). Murine infection models demonstrated that microbiota-derived molecular hydrogen promotes the growth of *H. pylori* (Olson and Maier, 2002) and *S.* Typhimurium (Maier et al., 2013) in a hydrogenase dependent manner, but hydrogenase activity of *C. jejuni* was not required for the persistence in chicken (Weerakoon et al., 2009). Other defects in systems fueling the respiration chain with electrons, like mutation in the formate dehydrogenase Fdh and the NDH-I complex, reduced the colonization capability of *C. jejuni* in chicken (Weerakoon et al., 2009). The gluconate dehydrogenase GADH, mainly active at 42°C, provided a host-specific benefit and was required by *C. jejuni* to persist in the chicken but not in the murine intestine (Pajaniappan et al., 2008), probably reflecting the different body temperatures of the avian and murine host. Whereas *C. jejuni* has to compete with commensal *E. coli* for gluconate (Chang et al., 2004), it generally does not catabolize carbohydrates that are utilized by commensal *E. coli*. *C. jejuni* strains harboring the FucP transporter are able to catabolize fucose (Muraoka and Zhang, 2011; Stahl et al., 2011), which is one of the less preferred growth substrates used by commensal and pathogenic *E. coli in vivo* (Fabich et al., 2008). The utilization of fucose is not essential for *C. jejuni in vivo*, as *fucP* mutants could be recovered from mono-infected chickens in similar amounts as the wild-type and had no disadvantage in chicken co-infection experiments with high infectious doses (Muraoka and Zhang, 2011). In low dose co-infection experiments with chicken the wild-type strain either outcompeted the *fucP* mutant (Muraoka and Zhang, 2011) or had no advantage (Stahl et al., 2011). In latter study, the additional administration of fucose allowed the wild-type strain to outcompete the mutant (Stahl et al., 2011), suggesting that sufficient amount of free fucose is not always available for *C. jejuni* under normal circumstances. Still, fucose catabolism seems to support the colonization of *fucP*-positive *C. jejuni* isolates in certain settings since a *C. jejuni fucP* mutant was recovered in lower numbers than the wild-type strain from co-infection experiments with neonatal piglets (Stahl et al., 2011).

The catabolism of free amino acids, derived through proteolytic protein degradation by the host and its intestinal microbiota, plays a central role for the colonization process of *C. jejuni* (**Figure 1**). Microarray experiments showed the upregulation of the serine dehydratase *sdaA* gene in *C. jejuni* colonizing the chicken caecum (Woodall et al., 2005), and an *sdaA* mutant with abolished serine utilization exhibited a severe colonization defect in chicken (Velayudhan et al., 2004) and mice (Hofreuter et al., 2012). Similarly, the GGT-dependent utilization of glutamine/glutathione contributed to the colonization of *C. jejuni* in the murine and avian intestine (Hofreuter et al., 2006; Barnes et al., 2007). Furthermore, a *C. jejuni peb1A* mutant, abolished in the growth with glutamate and aspartate *in vitro*, showed a colonization defect in mice, as did a *putP* mutant unable to catabolize proline (Pei et al., 1998; Hofreuter et al., 2012). With the exception of serine, the usage of amino acids provided no general but rather a tissue-specific benefit for *C. jejuni* during infection experiments with immune deficient Myd88^{-}/$^{-}$ mice: Glutamine / glutathione and proline catabolism were required for the efficient colonization of the murine intestine, but had no supporting effect during the persistence of *C. jejuni* in the murine liver after intraperitoneal infection (Hofreuter et al., 2006, 2008, 2012). In contrast, asparagine utilization through the secreted asparaginase AnsB^{s} did not promote the intestinal colonization of *C. jejuni* 81–176 but rather enhanced its colonization in the liver of mice (Hofreuter et al., 2008). Future studies have to clarify if the observed colonization defect of the *C. jejuni ansB*^{s} mutant in the murine liver is solely the consequence of a growth disadvantage or if the secreted asparaginase of *C. jejuni* has an additional immune modulatory effect as described for *S.* Typhimurium (Kullas et al., 2012). The asparaginase-catalyzed deamination of asparagine leads to aspartate, which represents a growth substrate

but also a precursor of the alternative electron acceptor fumarate for *C. jejuni*, as describe above. Thus, the colonization defect in chicken and mice of an *aspA* mutant (Guccione et al., 2008; Novik et al., 2010) might be the combined effect of a reduced carbon and energy source availability as well as a diminished capability to perform fumarate respiration in the oxygen-limited intestinal environment. Moreover, the *C. jejuni* aspartate aminotransferase *aspB* mutant was defective in mouse colonization (Novik et al., 2010). Amino acids that cannot be used as energy sources by *C. jejuni* promote its infection process as well. This observation was made in chicken infection experiments with a *C. jejuni* transposon mutant library that identified the putative amino acid transporter Cj0903c and the periplasmic binding protein LivJ of the LIV branched-chain amino acid ABC transporter as colonization factors of *C. jejuni* (Hendrixson and DiRita, 2004). Additionally, inactivation of the periplasmic binding proteins LivK, which facilitates primarily the high level uptake of leucine, showed a reduced colonization capacity of *C. jejuni* in chicken when a low inoculum dose was used (Ribardo and Hendrixson, 2011). The mutation of the branched-chain amino acid aminotransferase *ilvE* gene resulted in a similar colonization phenotype as observed for the *livJ* and *livK* mutants. Unexpectedly, inactivation of other components of the LIV ABC transporter system did not affect the colonization levels of *C. jejuni* in chicken, indicating that other permeases might be involved in the uptake of the branched amino acids as well (Ribardo and Hendrixson, 2011).

C. jejuni requires not only energy sources but also micronutrients like iron, nickel, molybdate, tungsten, cobalt and zinc (Stahl et al., 2012) for the successful competition with the host microbiota. The growth-supporting effect of zinc has been demonstrated *in vivo* (**Figure 1**): A *C. jejuni* mutant with an inactivated periplasmic binding protein ZnuA (Cj0143c) of a high-affinity zinc ABC transporter system (Cj0143c-Cj0141c) showed a caecum colonization defect in chickens with normal microbiota (Davis et al., 2009) but not in chickens harboring a limited intestinal flora (Gielda and DiRita, 2012). Interestingly, an increased amount of zinc, magnesium and iron was measured in the caecum of chickens with limited flora whereas the concentrations of copper and manganese were reduced (Gielda and DiRita, 2012).

In addition to zinc, the availability of iron plays a central role in the pathogenesis of pathogens (Braun, 2001). Iron occurs in the host organism generally in its oxidized form as ferric iron (Fe^{3+}). To reduce the availability of Fe^{3+} for pathogens, specific iron-binding proteins like lactoferrin and transferrin sequester ferric iron in host organisms. To overcome this iron limitation commensal and pathogenic bacteria secret siderophores, specific iron chelators with higher affinity to Fe^{3+} than the iron-binding proteins of the host (Skaar, 2010). Several studies have demonstrated that the acquisition of iron is important for its infection process. However, *C. jejuni* does not produce and encode Fe^{3+}-binding siderophores (Baig et al., 1986; Gundogdu et al., 2007). Instead, it benefits from the host and its microbiota by encoding for several iron uptake systems that bind and import the iron-chelating siderophores of other bacteria such as ferric-enterobactin and salmochelin, the fungal ferrichrome or the host-derived iron-binding substances transferrin/lactoferrin, hemin and hemoglobin (Miller et al., 2009; Naikare et al., 2013). The outer membrane ferric-enterobactin receptor CfrA (Cj0755) of *C. jejuni* NCTC 11168 is expressed under iron-restricted growth conditions and required for colonization of chicken (Palyada et al., 2004). Interestingly, the robust colonizer *C. jejuni* 81–176 lacks the *cfrA* gene (Hofreuter et al., 2006), as do various other *C. jejuni* strains (Zeng et al., 2013a). These strains harbor the alternative ferric-enterobactin receptor *cfrB* (*cjj81176_0471*), which occurs in *C. jejuni* NCTC 11168 and other *cfrA*-positive strains as a pseudogene (Xu et al., 2010). Inactivation of *cfrB* in the bovine *C. jejuni* isolate JL11, which lacks the *cfrA* gene, clearly demonstrated that CfrB is required by this strain for the colonization of chicken (Xu et al., 2010). The CeuBCDE (Cj1352-Cj1355) ABC transporter system mediates, in cooperation with CfrA, the uptake of enterobactin/enterochelin into *C. jejuni*, and a *C. jejuni* NCTC 11168 *ceuE* mutant showed a similar chicken colonization defect as the *cfrA* mutant (Palyada et al., 2004). A further putative siderophore receptor is encoded by *cj0178* in *C. jejuni* NCTC 11168 and RM1221 but is absent in the isolate *C. jejuni* 81–176 (Hofreuter et al., 2006). The outer membrane protein Cj0178 (CtuA) was characterized as a receptor for the iron-binding host glycoproteins ferri-transferrin, ferri-lactoferrin and ferri-ovotransferrin (Miller et al., 2008). A *cj0178* mutant strain of *C. jejuni* NCTC 11168 showed a severe colonization defect in chicken infection experiments (Palyada et al., 2004) and a slightly attenuated phenotype in the rabbit ileal loop model (Stintzi et al., 2005). The *chu*ABCDZ (*cj1613c-cj1617*) gene cluster is widespread in *C. jejuni* and encodes for an iron uptake system that facilitates the utilization of the host compounds like hemoglobin and hemin as iron sources (Pickett et al., 1992; Ridley et al., 2006). Microarray and RNAseq analysis revealed the upregulation of *chu* genes in *C. jejuni* NCTC 11168 and 81–176 isolated from the caecum of infected chicken (Woodall et al., 2005; Taveirne et al., 2013), suggesting that the utilization of the host-derived heme is required for the colonization process of *C. jejuni*. However, a *C. jejuni* NCTC 11168 *chuA* mutant showed the same ability to colonize the chicken intestine as the wild-type strain (Naikare et al., 2013).

The uptake of bacterial or host-derived siderophores by *C. jejuni* requires, in addition to specific receptor and ABC transporter proteins, a periplasma-bridging TonB-ExbB-ExbD system, which energizes the translocation of the siderophores across the outer membrane (Braun, 2001). *C. jejuni* isolates are equipped with different numbers of TonB-ExbBD systems: While *C. jejuni* NCTC 11168 encodes for three TonB-ExbB-ExbD systems, *C. jejuni* 81–176 harbors only the TonB2-ExbB2-ExbD2 system, which seems to be conserved in *C. jejuni* (Hofreuter et al., 2006; Zeng et al., 2013b). It was recently demonstrated that TonB3 of *C. jejuni* NCTC 11168 mediates the uptake of a wide range of siderophores from bacteria and vertebrates, including enterobactin and salmochelin, and a *tonB3* mutant showed an abolished colonization capability in chicken infection experiments (Naikare et al., 2013). Moreover, a *C. jejuni* NCTC 11168 *tonB1* mutant exhibited like the *tonB3* mutant, an attenuated colonization phenotype in chicken, whereas the inactivation of *tonB2* in *C. jejuni* NCTC 11168 did not dramatically affect the enterobactin utilization as well as colonization capability (Naikare et al., 2013). *C. jejuni* relies not only on the utilization of siderophore-bound

ferric iron but can also use ferrous (Fe^{2+}) iron. Some *C. jejuni* strains take up Fe^{2+} through the FeoB transport protein (Naikare et al., 2006). Though a *feoB* mutant of *C. jejuni* NCTC 11168 had no growth disadvantage compared the wild-type strain during *in vitro* co-cultivation experiments, it showed a significant colonization defect in chickens and was outcompeted by the parental strain during a co-infection experiment with newborn piglets (Naikare et al., 2006). Future studies have to clarify how the puzzling diversity in the iron uptake systems affects the virulence and colonization fitness as well as the oxygen detoxification of different *C. jejuni* strains.

A DISCREET TENANT: THE PERSISTENCE OF *C. JEJUNI* INSIDE INVADED EPITHELIAL CELLS

C. jejuni colonizes efficiently the gut of its diverse hosts where the majority of the population proliferates in the extracellular space of the mucus-filled intestinal crypts (Lee et al., 1986; Beery et al., 1988). Only a small fraction of the *C. jejuni* population can also be found inside intestinal epithelial cells when biopsies of infected humans (van Spreeuwel et al., 1985) or piglets (Babakhani et al., 1993) are analyzed. Similar to other facultative intracellular pathogens, like e.g., *S.* Typhimurium or *Legionella pneumophila*, *C. jejuni* resides during its intracellular stage inside a specific membrane-bound compartment, the so-called *Campylobacter*-containing-vacuole (CCV) (Watson and Galan, 2008). Though significant progress has been made in characterizing the metabolism of various facultative intracellular pathogens and in identifying bacterial factors facilitating efficient growth inside the host cells (Eisenreich et al., 2010), our knowledge about the metabolic properties that allow *C. jejuni* to persist in its intracellular compartment is still limited. In contrast to *S.* Typhimurium and *L. pneumophila*, *C. jejuni* does not multiply intracellularly (Watson and Galan, 2008). The absence of intracellular growth indicates that reduced metabolic activity might be sufficient for *C. jejuni* to maintain its membrane potential and viability inside its intracellular compartment. Accordingly, a metabolic downshift was recently described by quantitative proteome analysis of *C. jejuni* cells isolated from the CCVs of infected T84 colonic epithelial cells (Liu et al., 2012). This study showed that 20 h after infection 225 of the over 1400 detected *C. jejuni* proteins exhibit different levels compared to the proteome of *C. jejuni* cells isolated 2 h post-infection. Interestingly, 211 of the 225 proteins were found at lower levels, whereas only 14 proteins showed a 2- to 11-fold higher level, most prominently the acetyl-CoA synthetase ACS. The levels of the catalase KatA and the thiol peroxidase Tpx showed slightly but significant higher levels at 20 h compared to 2 h after infection. Surprisingly, KatA activity was not required by *C. jejuni* to survive within Hep-2 cells (Day et al., 2000), whereas the superoxide dismutase *sodB* mutant of *C. jejuni* showed abolished intracellular survival (Pesci et al., 1994; Novik et al., 2010). Interestingly, other proteins involved in oxidative stress response like AhpC, SodB, Trx and TrxB showed unaltered or slightly decreased levels, similar to proteins involved in the protection of *C. jejuni* against environmental stress (GroEL, DnaK, HtrA, GroES, GrpE, ClpB, DnaJ) (Liu et al., 2012).

The quiescent status of this pathogen inside the CCV is further enforced by the observation that many *C. jejuni* proteins with lower levels during the extended intracellular persistence are required for protein synthesis (Rps and Rpl proteins), the biosynthesis of amino acids (HisA, DapA, LysC, PheA), purines (PurH, PurL, PurM) and fatty acids (FabH, FabZ). In addition, several protein- and peptide-degrading proteins (ClpB, HslU, M16 and M24/M37 peptidase) and transporters involved in the uptake of amino acids (LivFJK, SdaC), phosphate (PstS) and metals like iron (Cj0175c), tungsten (TupA) or nickel (NikZ) showed lower levels 20 h post-infection (Liu et al., 2012). The metabolic downshift observed for intracellular *C. jejuni* is further reflected by decreased levels of proteins involved in the intermediary metabolism and gluconeogenesis like Tal, TpiA, GapA, FbaA and Pyk (Liu et al., 2012).

Most strikingly, *C. jejuni* undergoes a remarkable respiratory reprogramming when residing in its intracellular compartment for an extended time period: Proteins involved in the aerobic respiration like the cbb3-type cytochrome c oxidase subunits CcoO and CcoP and the ubiquinol-cytochrome c reductase (PetA, PetC) exhibited decreased protein levels after 20 h inside cultured epithelial cells. Such a decreased level was also seen for the formate dehydrogenase (FdhAB), the nitrate (NapAB) and nitrite (NrfA) reductases. *C. jejuni* seems to adapt its respiration mode to the oxygen-restricted conditions inside the CCV (Watson and Galan, 2008; Liu et al., 2012; Pryjma et al., 2012) by fumarate respiration: The fumarate reductase *frdA* mutant of *C. jejuni* 81–176 showed a significant intracellular survival defect inside COS-1 cells (Liu et al., 2012) and the importance of fumarate respiration was further supported by the reduced intracellular survival rate of an aspartate ammonia-lyase *aspA* mutant (Novik et al., 2010). In contrast to the *frdA* mutant, an *mrfA* mutant showed no decreased viability in the CCV after 20 h (Liu et al., 2012). *C. jejuni* 81–176 mutants with abolished formate (*fdhB*; *cjj81176_1502*), nitrate (*napG*; *cjj81176_0802*) and TMAO or other N- and S-oxide (*torA*; *cjj81176_0291*) respiration showed also no intracellular survival defect (Liu et al., 2012). Moreover, the cyanide-insensitive oxidase subunit CioA (CJJ81176_0118) with low affinity to oxygen is not required for the intracellular survival of *C. jejuni* 81–176 (Liu et al., 2012).

Additional insights into the intracellular lifestyle of *C. jejuni* provided microarray experiments analyzing the expression of *C. jejuni* 81–176 genes during the adhesion to and invasion into the human INT 407 cell line. This approach identified the stringent response regulator protein SpoT (Cj1272c) as being upregulated during the first 6 h post-infection of the cultured epithelial cells (Gaynor et al., 2005). SpoT modulates the guanosine tetraphosphate (ppGpp) metabolism in *C. jejuni* and inactivation of *spoT* in *C. jejuni* 81–176 resulted in a decreased synthesis of ppGpp similar as described for other bacterial pathogens (Dalebroux et al., 2010). The *spoT* mutant of *C. jejuni* 81–176 showed only a slight defect in adherence to and invasion into INT 407 cells, but a more pronounced defect in its intracellular survival over 20 h in comparison to the wild-type strain (Gaynor et al., 2005). Moreover, inactivation of *spoT* resulted in a decreased level of poly-phosphate (poly-P) in *C. jejuni* 81–176 comparable to a mutant strain lacking the polyphosphate kinase 1 (*ppk1*) mutant. PPK1 (Cj1359; CJJ81176_1361) uses ATP for the generation of poly-P, which participates in the stress response

of *C. jejuni* and enables the survival during osmotic shock and low-nutrient stress. It was demonstrated that PPK1 is required for extended survival in infected INT407 cells (Candon et al., 2007), but as the *ppk1* mutation results, similar to the mutation of *spoT*, in pleiotropic effects, it does not allow the identification of mechanisms solely required for the intracellular survival. Poly-P might not only be important as a metabolic regulator and protector against various stresses like hydrogen peroxide and osmotic stress, it might also serve as an energy and phosphate source (Kornberg, 1995) that enhances the viability of *C. jejuni* while residing in the nutrient restricted environment of the CCV.

Though progress has been made in recent years to describe the nutrient requirements that allow *C. jejuni* to thrive in the gastrointestinal lumen of its hosts, how *C. jejuni* maintains its viability inside invaded epithelial cells and energizes its intracellular persistence remains ill-defined.

OUTLOOK

Campylobacteriosis of humans in industrialized countries is assumed to be an accidental and transitory event in the life style of *C. jejuni*. Such short-term interactions prevent co-evolution and potential fine-tuning of *Campylobacter* virulence factors. Consequently, the evolutionary forces that shape the metabolic and pathogenic properties of *C. jejuni* occur during the interactions with the microbiota of its primary hosts rather than within the human gastrointestinal ecosystem. Still, the metabolic framework of *C. jejuni* is sufficient to overcome the colonization resistance in the human gastrointestinal tract and to efficiently promote its proliferation, leading consequently to the development of diarrhea. These findings illustrate the necessity for future comprehensive studies to investigate the extent to which metabolic factors mediate the long-term persistence of *C. jejuni* in its natural hosts like chicken and cattle. Specifically targeting such growth-promoting metabolic pathways for the development of drugs might not only lower the colonization burden in animal hosts with *C. jejuni* but will consequently lower the exposure doses for humans. Moreover, the restricted carbohydrate catabolism of *C. jejuni* in comparison with other enteropathogenic pathogens like *S.* Typhimurium (Becker et al., 2006; Steeb et al., 2013) should facilitate the discovery of "metabolic Achilles heels" for the successful design of drugs against *Campylobacter*.

ACKNOWLEDGMENTS

The author would like to thank Alyssa Ingmundson, Juliane Mohr and Hanne Vorwerk for critical review of the manuscript. The research is supported by the Deutsche Forschungsgemeinschaft with the DFG grants HO 4553/1-1 and HO 4553/2-1 as part of the priority program 1316 "Host-Adapted Metabolism of Bacterial Pathogens" as well as the FBI-Zoo grant from the German Ministry for Education and Research (BMBF).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2014; accepted: 11 September 2014; published online: 29 September 2014.

*Citation: Hofreuter D (2014) Defining the metabolic requirements for the growth and colonization capacity of Campylobacter jejuni. Front. Cell. Infect. Microbiol. **4**:137. doi: 10.3389/fcimb.2014.00137*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 21 October 2014
doi: 10.3389/fcimb.2014.00141

Staphylococcus aureus small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism

André Kriegeskorte[1], Stephanie Grubmüller[2], Claudia Huber[2], Barbara C. Kahl[1], Christof von Eiff[1†], Richard A. Proctor[3], Georg Peters[1], Wolfgang Eisenreich[2] and Karsten Becker[1]*

[1] Institute of Medical Microbiology, University of Münster, Münster, Germany
[2] Lehrstuhl für Biochemie, Technische Universität München, Garching, Germany
[3] Department of Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
Matthew J. Wargo, University of Vermont College of Medicine, USA
Christiane Wolz, University Tübingen, Germany
Agnes Fekete, University Wuerzburg, Germany

***Correspondence:**
Karsten Becker, Institute of Medical Microbiology, University of Münster, Domagkstraße 10, 48143 Münster, Germany
e-mail: kbecker@uni-muenster.de

†Present address:
Christof von Eiff, Pfizer Pharma GmbH, Berlin, Germany

In addition to the classical phenotype, *Staphylococcus aureus* may exhibit the small colony-variant (SCV) phenotype, which has been associated with chronic, persistent and/or relapsing infections. SCVs are characterized by common phenotypic features such as slow growth, altered susceptibility to antibiotic agents and pathogenic traits based on increased internalization and intracellular persistence. They show frequently auxotrophies mainly based on two different mechanisms: (i) deficiencies in electron transport as shown for menadione- and/or hemin-auxotrophs and (ii) thymidylate biosynthetic-defective SCVs. To get a comprehensive overview of the metabolic differences between both phenotypes, we compared sets of clinically derived menadione-, hemin- and thymidine-auxotrophic SCVs and stable site directed mutants exhibiting the SCV phenotype with their corresponding isogenic parental strains displaying the normal phenotype. Isotopologue profiling and transcriptional analysis of central genes involved in carbon metabolism, revealed large differences between both phenotypes. Labeling experiments with [U-$^{13}C_6$]glucose showed reduced ^{13}C incorporation into aspartate and glutamate from all SCVs irrespective of the underlying auxotrophism. More specifically, these SCVs showed decreased fractions of $^{13}C_2$-aspartate and glutamate; $^{13}C_3$-glutamate was not detected at all in the SCVs. In comparison to the patterns in the corresponding experiment with the classical *S. aureus* phenotype, this indicated a reduced carbon flux via the citric acid cycle in all SCV phenotypes. Indeed, the aconitase-encoding gene (*acnA*) was found down-regulated in all SCV phenotypes under study. In conclusion, all SCV phenotypes including clinical isolates and site-directed mutants displaying the SCV phenotype were characterized by down-regulation of citric acid cycle activity. The common metabolic features in central carbon metabolism found in all SCVs may explain similar characteristics of the *S. aureus* SCVs irrespective of their auxotrophism as well as the specific genetic and/or regulatory backgrounds.

Keywords: *S. aureus*, SCV, metabolism, TCA

INTRODUCTION

Staphylococcus aureus (*S. aureus*) has been recognized as one of the most important human pathogens world-wide causing a wide range of mild to serious infections within and outside the hospital aggravated by the dissemination of different methicillin-resistant *S. aureus* (MRSA) lineages (Lowy, 1998; David and Daum, 2010). Besides its capability to cause acute infections, *S. aureus* can cause chronic courses of infection despite adequate antimicrobial therapy that are often associated with a defined *S. aureus* phenotype, designated as small-colony variants (SCVs) (Proctor et al., 2006). SCVs represent a sub-population with distinct phenotypic and pathogenic traits adapted to an intracellular lifestyle (von Eiff et al., 2001, 2006; Sachse et al., 2010; Tuchscherr et al., 2010). As main feature, they show frequently auxotrophies (auxotrophism) for menadione, hemin and/or thymidine, however, strains without any detectable auxotrophy or with other auxotrophies including those for CO_2 and thiamin have been described (Thomas, 1955; von Eiff et al., 1997; Kahl et al., 2003; Chatterjee et al., 2008; Lannergård et al., 2008; Gómez-González et al., 2010). The best-investigated and most prevalent SCV phenotypes, the menadione and/or hemin autotrophic SCVs as well as thymidine autotrophic SCVs, are characterized by deficiencies in the electron transport and in the thymidylate biosynthetic pathway, respectively (von Eiff et al., 1997; Chatterjee et al., 2008). It has been shown for hemin and menadione auxotrophic SCVs, based on mutations in *hemB* and *menD* (von Eiff et al., 1997; Kohler et al., 2003, 2008), that genes involved in the central metabolic processes were affected. Transcriptomic and proteomic approaches revealed

Table 1 | Bacterial strains used in this study.

Strain designation in this study	Description	Source
CLINICALLY DERIVED STRAIN PAIRS		
I^{WT}	Clinical wild-type (strain 1549I)	This study
I^{SCV}	Clinical SCV; heme auxotroph (strain 1549III)	This study
III^{WT}	Clinical wild-type (strain F2418II)	Chatterjee et al., 2008
III^{SCV}	Clinical SCV; thymidine auxotroph (strain F2418)	Chatterjee et al., 2008
V^{WT}	Clinical wild-type (strain 22616/1)	Lannergård et al., 2008
V^{SCV}	Clinical SCV; menadione auxotroph (strain 22616/3)	Lannergård et al., 2008
WT^{3878}	Clinical wild-type (strain 3878I)	Kriegeskorte et al., 2011
$cSCV^{3878}$	Clinical SCV (strain3878III)	Kriegeskorte et al., 2011
STABLE *IN VITRO* GENERATED MUTANTS		
II^{WT}	Wild type (strain 8325-4)	O'Neill, 2010
II^{SCV}	Δ*hemB* SCV (strain 8325-4)	This study
IV^{WT}	Wild type (strain SH1000)	O'Neill, 2010
IV^{SCV}	Δ*thyA* SCV (strain SH1000)	Kriegeskorte et al., 2012
VI^{WT}	Wild type (strain 6850)	Fraunholz et al., 2013
VI^{SCV}	Menadione auxotroph SCV (strain JB1; generated from strain 6850)	Balwit et al., 1994
$\Delta hemB^{3878}$	Site directed *hemB* mutant SCV (generated from wild-type strain 3878I)	Kriegeskorte et al., 2011
KM^{3878}	Complementation of $\Delta hemB^{3878}$ with pCX19 *hemB*	Kriegeskorte et al., 2011
REV^{3878}	Spontaneous revertant strain of $cSCV^{3878}$	Kriegeskorte et al., 2011
G^{3878}	Gentamicin -induced SCV of WT^{3878} (menadione auxotroph)	Kriegeskorte et al., 2011

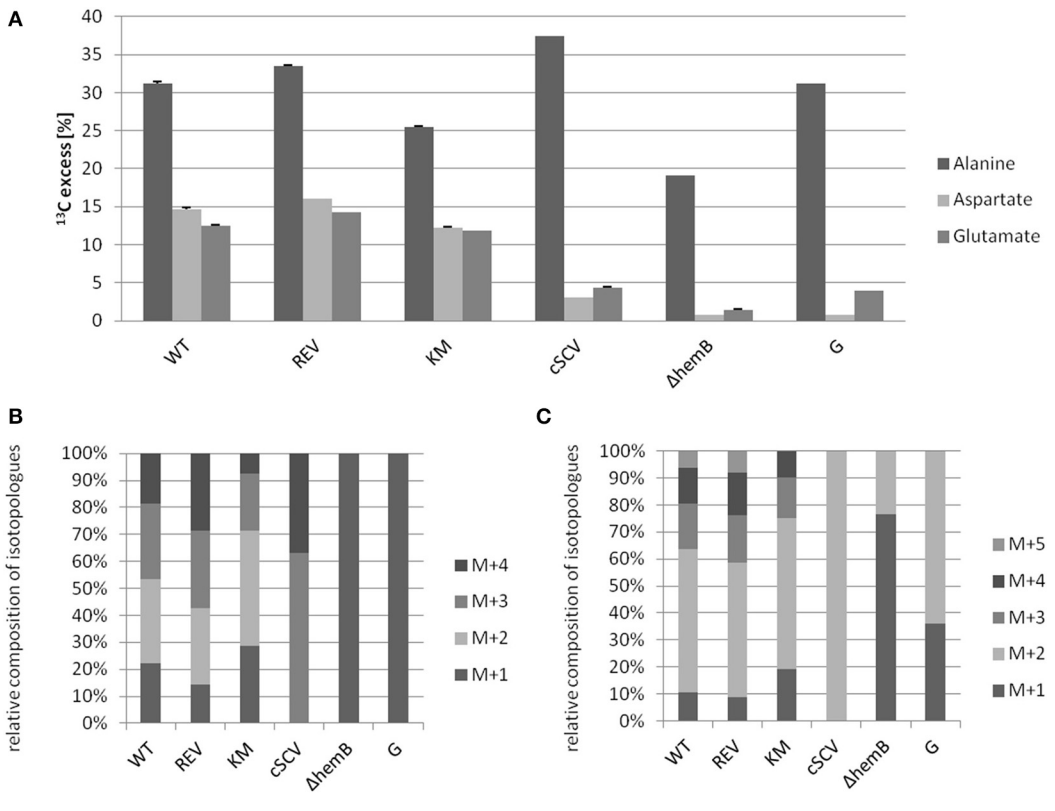

FIGURE 1 | ^{13}C-Excess in alanine, aspartate and glutamate from *S. aureus* strains grown with [U-$^{13}C_6$]glucose (A). Error bars indicate the standard deviation of three technical replicates. WT (clinically isolated normal phenotype), REV (spontanous revertant of the clinical SCV) and KM (complemented Δ*hemB* mutant with an intact *hemB* gene in trans) display an *S. aureus* wild-type phenotype. cSCV (clinically isolated SCV phenotype), Δ*hemB* (site-directed *hemB* mutant of WT) and G (gentamicin induced SCV phenotype) display a *S. aureus* SCV phenotype. Relative contribution of ^{13}C-isotopologs carrying one to five ^{13}C-atoms (M+1–M+5) in aspartate **(B)** and glutamate **(C)**. The group of M+X comprises all isotopologs of aspartate and glutamate with only X labeled carbon units irrespective of the location within the molecule. Isotopologue patterns of amino acids with <1% ^{13}C-excess are not shown due to the high standard deviations.

considerable differences between the wild type and SCV phenotypes especially in the fermentative pathways (Kohler et al., 2003, 2008; Seggewiss et al., 2006). However, because clinically derived SCVs tend to revert quickly back into the wild type phenotype, most of SCV studies were performed with genetically defined, stable mutants. A recent proteomic study comparing a clinically derived SCV with a corresponding *hemB* mutant SCV and a gentamicin-induced SCV revealed common, but also distinct features between naturally occurring and genetically generated SCVs apart from changes triggered by the mutational inactivation of the electron transport chain (Kriegeskorte et al., 2011). Nevertheless, the complex metabolic and physiological changes along with the SCV phenotype are still not fully understood and more multifaceted than revealed from studies with genetically defined mutants displaying the SCV phenotype.

The aim of this study was to get additional insights into the metabolic properties of *S. aureus* SCVs as compared to their corresponding isogenic normal phenotype. For this purpose, we investigated a comprehensive set of SCVs including both clinically derived strains and stable site directed mutants by ^{13}C-isotopologue profiling and transcriptional analysis.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

Clinical *S. aureus* wild types and SCVs were recovered in parallel from patients with chronic infections (e.g., osteomyelitis and cystic fibrosis). Clonality was verified by *Sma*I macrorestriction analyses by pulsed-field gel electrophoresis (PFGE). Strains used in this study were summarized in **Table 1**. The *S. aureus* isolates were grown on Columbia sheep blood agar and tryptic soy agar at 37°C for 24–48 h. Liquid cultures were grown aerobically in 50 ml tryptic soy broth (TSB) in 500 ml flasks at 37°C and 160 rpm. For labeling experiments (isotopolog profiling) TSB without dextrose (Bacto Tryptic Soy without dextrose, BD, New Jersey, USA) including 17 g of pancreatic digest of casein, 3 g of enzymatic digest of sojabean meal, 5 g of sodium chloride and 2.5 g of dipotassium phosphate was used. The medium was supplemented with 2.5 g of [U-$^{13}C_6$]glucose.

CELL ISOLATION AND GROWTH CURVE ANALYSIS

For isotopolog profiling, 50 ml cultures were inoculated to an optical density of 0.05 (578 nm) from overnight cultures. Cells were harvested after 540 min by centrifugation (10 min, 5000 × g,

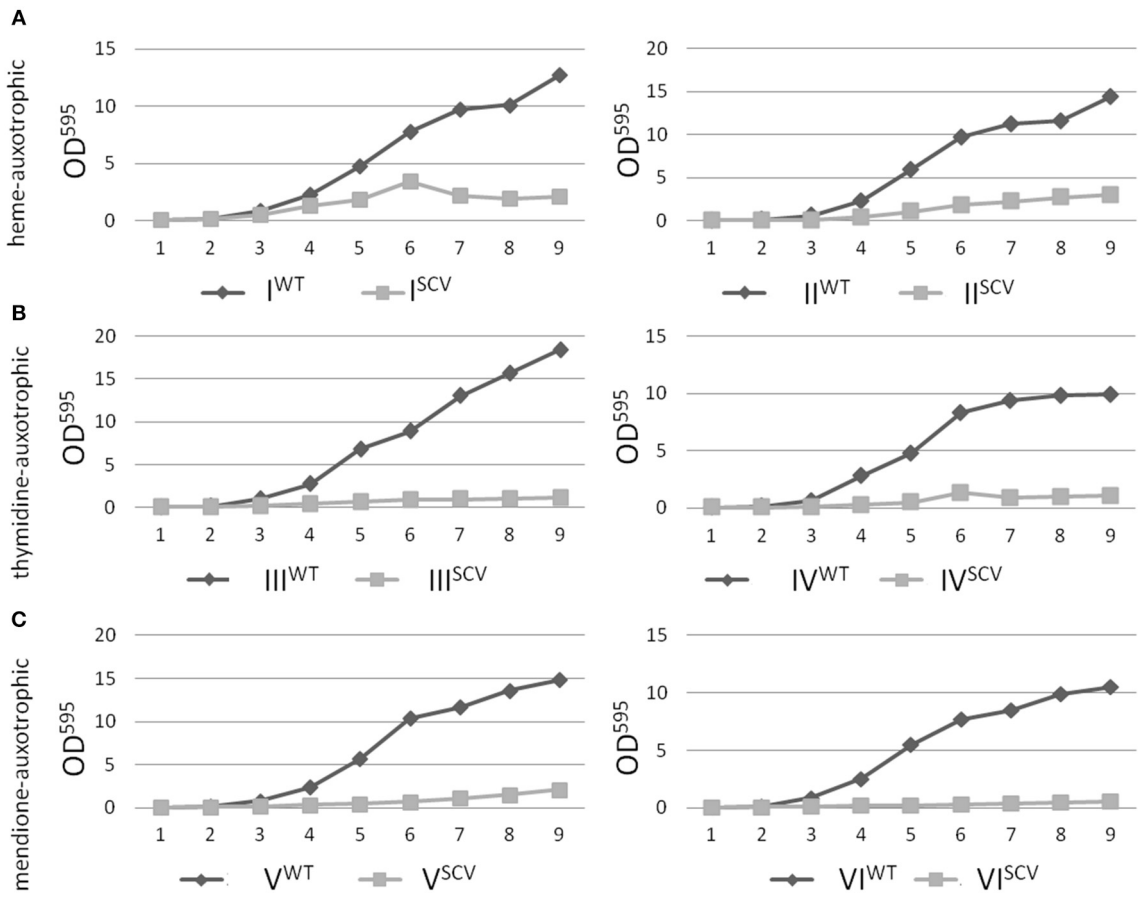

FIGURE 2 | Growth curve analysis of *S. aureus* isolates with normal and SCV phenotype. Heme-auxotrophic **(A)**, thymidine-auxotrophic **(B)** and menadione-auxotrophic **(C)**. Strain pairs I, III, and V represent clinical isolates consisting of a normal phenotype (WT) and a SCV phenotype (SCV). Strain pairs II, IV, and VI consist of a normal phenotype and an *in vitro* generated SCV phenotype. The optical density of the cultures was measured at 595 nm and values were plotted against the time.

4°C) and washed three times with 10 ml PBS. Pellets were stored at −80°C. Cells were resuspended in 10 ml PBS and autoclaved (20 min, 121°C). For the growth curve analysis, cultures were grown in 50 ml TSB in 500 ml flasks at 37°C on a rotary shaker at 160 rpm. The optical density was measured every hour at 578 nm using Ultraspec 1100 pro spectrophotometer (Amersham Biosciences, Freiburg, Germany).

CONSTRUCTION OF A Δ*HemB*-MUTANT IN *S. AUREUS* 8325-4

The *hemB* knockout mutant of *S. aureus* 8325-4 was constructed by allelic replacement of the *hemB* gene with *ermB* cassette (mediating erythromycin resistance) using the vector pCE8 as described before (von Eiff et al., 1997). The mutant was verified by restriction analysis and sequencing.

ISOTOPOLOGUE PROFILING

Bacterial cells (approximately 5 mg) were suspended in 0.5 ml of 6 M hydrochloric acid and incubated at 105°C for 24 h. The amino acids were purified on a Dowex 50W×8 column (washing 2 × 750 µl H_20; developing 1 ml 2 M ammonium hydroxide). The eluate was dried under a steam of nitrogen and resuspended in 50 µl dry acetonitrile. 50 µl of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide containing 1 % of tert-butyldimethylsilylchlorid were added and the mixture was incubated at 70°C for 30 min. The tert-butyl-dimethylsilyl derivatives of amino acids were then used for gas chromatography–mass spectrometry (GC/MS) and isotopolog analysis as described elsewhere (Eylert et al., 2008).

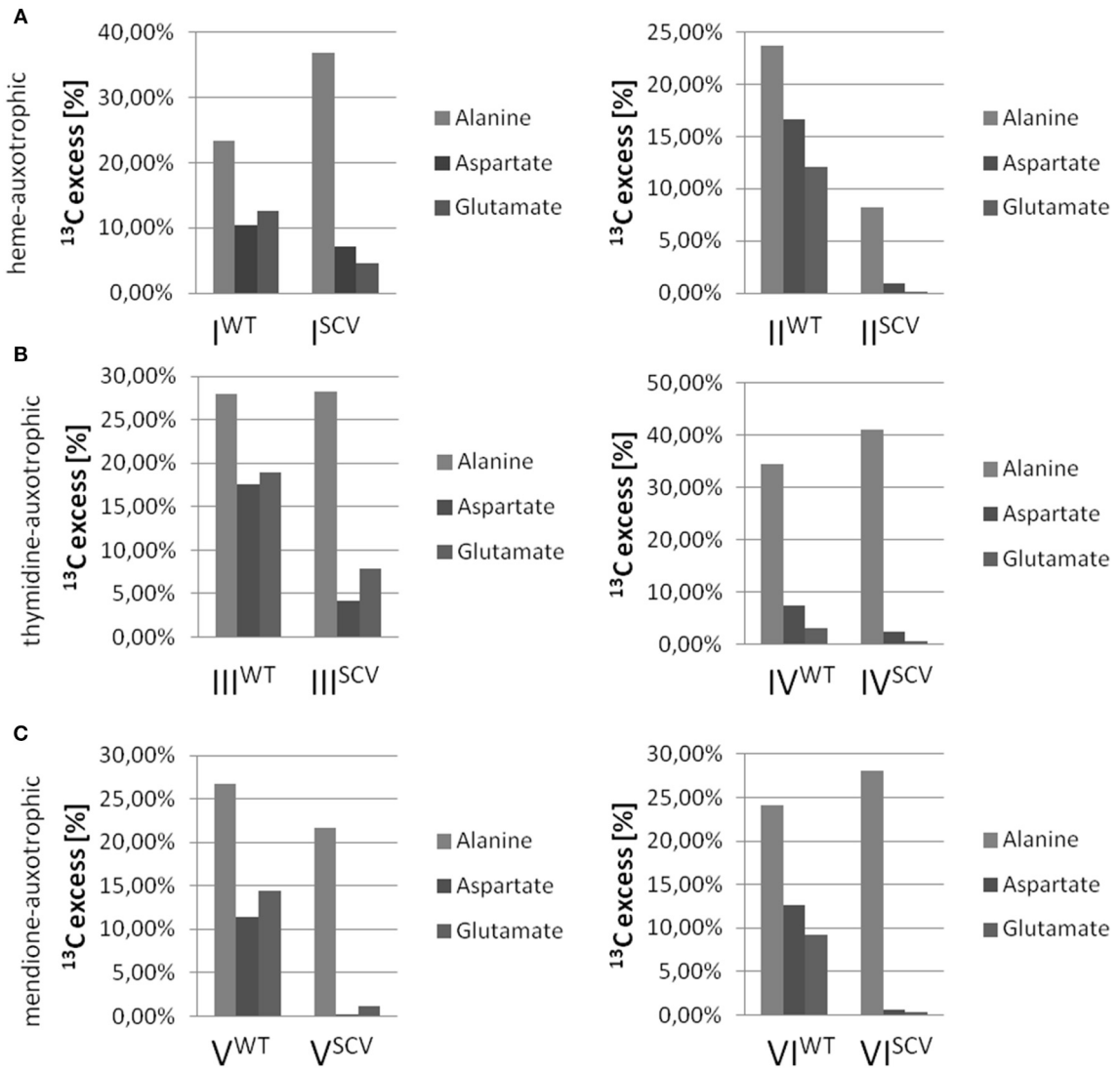

FIGURE 3 | ^{13}C-Excess in alanine, aspartate and glutamate from *S. aureus* strains grown with [U-$^{13}C_6$]glucose. Columns represent the mean values of three technical replicates; heme-auxotrophic **(A)**, thymidine-auxotrophic **(B)** and menadione-auxotrophic **(C)**. Strain pairs I, III, and V represent clinical isolates consisting of a normal phenotype (WT) and a SCV phenotype (SCV). Strain pairs II, IV, and VI consist of a normal phenotype and an *in vitro* generated SCV phenotype.

SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted from bacteria grown in TSB medium to late exponential growth phase using QuantiTect reverse transcription kit (QIAGEN) according to the manufacturer's recommendations. PCR reaction were performed with the CFX96 system (Bio-Rad Laboratories, München, Germany) under the following conditions: 95°C for 15 min, 50 cycles (95°C for 10 s, 10 s at 55°C for 10 s, 72°C for 30 s) using the EvaGreen Kit (Segentic, Borken, Germany). Three independent biological replicates were tested in duplicate. N-fold expression values relative to the house-keeping genes *gyrB*, *gmk* and *aroE* (normalized by the geometric mean of the relative quantities of all three reference genes) and for each strain set normalized to expression of the wild type isolate were calculated using CFX Manager v3.1 (Bio-Rad).

RESULTS AND DISCUSSION

S. aureus SCVs show many common features, such as slow growth, reduced pigmentation and changed expression of virulence determinants, independent of their underlying auxotrophic phenotype, molecular mechanism of SCV phenotype generation or genetic strain background. A similar phenotypic appearance may reflect analogous metabolic properties or a similar metabolic status. To investigate the metabolic differences (i) between *S. aureus* normal and SCV phenotypes and (ii) between different kinds of SCVs by isotopolog profiling, respective isogenic strains sets were analyzed (**Table 1**) and regulatory differences in central metabolic and virulence related genes were determined.

Using a *S. aureus* strain "sextet," consisting of three wild type isolates and three isolates displaying different SCV phenotypes including a clinically derived SCV, a site-directed *hemB* mutant and a gentamicin induced SCV, we identified significant phenotypic specific differences in the labeling patterns of amino acids (**Figures 1A–C**) (Kriegeskorte et al., 2011). In experiments with 2.5 g/l [U-$^{13}C_6$]glucose as a supplement to the TSB medium, all isolates, independent of their phenotype, showed high ^{13}C incorporation of about 20–40% into alanin (**Figures 1A**, **7A**), reflecting a high glycolytic activity in both phenotypes. As expected, under *in vitro* nutrient rich conditions, glucose served as the major energy source for growth of *S. aureus*. Nevertheless, the fraction of unlabeled amino acids in the experiment with [U-$^{13}C_6$]glucose reflected the pronounced capacity of *S. aureus* to uptake and to use external (unlabeled) amino acids or peptides from the medium. In comparison to alanine, more pronounced differences were noticed in the labeling patterns of glutamate and aspartate (**Figures 1B,C**). While all normal phenotypes showed a ^{13}C-excess between 11.8 and 16.0% in aspartate and glutamate, all SCV phenotypes were characterized by a substantial reduced ^{13}C-excess between 0.7 and 4.4% (**Figure 1A**). As glutamate and aspartate are directly linked to the citric acid cycle intermediates α-ketoglutarate and oxalacetate, respectively, via transamination, the reduced ^{13}C-label of these amino acids could indicate a reduced citric acid cycle activity in all SCV phenotypes (**Figure 5**). The comparison of the averaged ^{13}C-excess values between the three normal and the three SCV phenotypes revealed no significant differences in the ^{13}C-excess of alanine, but a significant reduction of the ^{13}C-excess of aspartate and glutamate in the SCV phenotypes (**Figure 7A**). Moreover, the isotopolog distributions in aspartate and glutamate from the SCVs were clearly different from the corresponding patterns in aspartate and glutamate from the wild-type phenotype and the revertant or complemented strains (**Figures 1B,C**). Whereas the later group was characterized by multiple ^{13}C-isotopologs also comprising **three** and more ^{13}C-atoms, the amino acids from the SCVs displayed higher fractions of $^{13}C_1$-isotopologs. Again, this could reflect that the carbon flux via the citrate cycle producing oxaloacetate/Asp and **α**-ketoglutarate/Glu carrying multiple ^{13}C-atoms in the

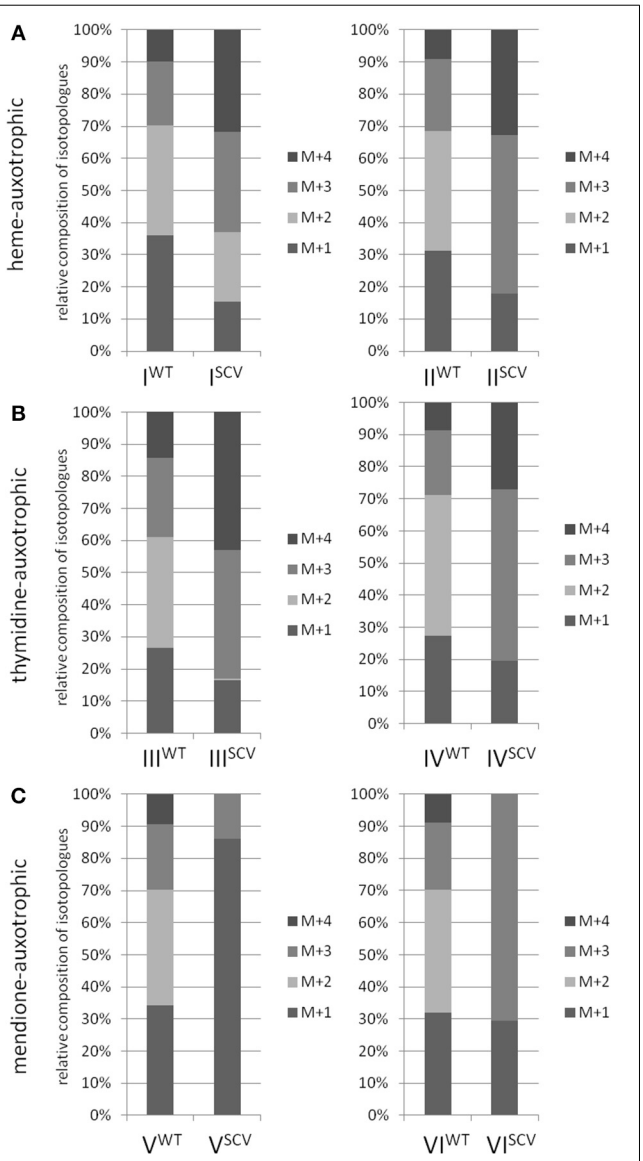

FIGURE 4 | Isotopologue composition of aspartate. Comparison between normal and SCV phenotypes of *S. aureus* displaying different auxotrophisms. Heme-auxotrophic **(A)**, thymidine-auxotrophic **(B)** and mendione-auxotrophic **(C)**. Strain pairs I, III, and V represent clinical isolates. Strain pairs II, IV, and VI consist of a normal phenotype and an *in vitro* generated SCV phenotype. Patterned columns represent the mean values of three technical replicates. The relative contributions of each ^{13}C-isotopolog (M+1–M+4) with regard to the overall enrichment were shown.

experiments with [U-$^{13}C_6$]glucose is substantially reduced in the *hemB* mutant and the gentamicin induced SCVs. This hypothesis is in line with earlier conclusions made on the basis of proteome and transcriptome studies (Kohler et al., 2003, 2008; Seggewiss et al., 2006).

Interestingly, the clinical SCV showed similar patterns of the ^{13}C-excess in aspartate and glutamate, in comparison to the profiles from the site-directed *hemB* mutant and to the gentamicin induced SCV, respectively (**Figure 1A**). However, the relative isotopolog distributions differed between the SCV phenotypes (**Figures 1B,C**). While in the *hemB* mutant and in the gentamicin induced SCV, the M+1 species were dominant, the $13C_2$-species were more abundant indicating that single runs, but no multiple runs, via the citric acid cycle were still operative in the clinically derived SCV. To investigate whether reduced carbon flux via the citrate cycle is a general feature of *S. aureus* SCVs that also includes the major auxotrophic phenotypes (hemin, menadione, and thymidine), we analyzed a comprehensive set of six isogenic strain sets, each comprising the normal wild type and different SCV phenotypes including clinical derived SCVs and genetically defined mutants displaying the SCV phenotype. The growth properties of the strain pairs are shown in **Figures 2A–C, 7B**. All SCVs showed a pronounced growth defect compared to their normal phenotypes, irrespective of the underlying auxotrophism and reached considerably lower optical densities under aerobic conditions. Again, ^{13}C incorporation into alanine was highly efficient in all phenotypes and strains and no significant difference between the normal and the SCV phenotypes could be observed (**Figures 3A–C, 7B**). However, irrespective of the underlying auxotrophism, all SCV phenotypes again showed reduced ^{13}C-incorporation into aspartate and glutamate, revealing the assumed reduced activity of carbon flux via the citric acid cycle (**Figures 3A–C, 7B**). This conclusion was verified by the isotopolog distribution as shown in **Figures 4A–C**. While all of the normal phenotypes showed in aspartate high fractions of the M+2 species, all SCVs were devoid of this species. In contrast, the SCV phenotypes showed increased relative fractions of M+1 and M+3 species indicating the reduced flux via the citric acid cycle with $^{13}C_2$-isotopologs, but higher contributions of oxaloacetate/Asp formation by anaplerotic reactions [i.e., giving rise to the $^{13}C_3$-isotopologs by carboxylation of [U-$^{13}C_3$]pyruvate or PEP in the SCVs (**Figure 5**)].

Previously, we could show that the expression of *acnA* (aconitase) which catalyzes the first step of the TCA cycle (**Figure 5**), was reduced in a clinical SCV as well as in a site-directed *hemB* mutant (Seggewiss et al., 2006; Al Laham et al., 2007; Kriegeskorte et al., 2011). With the recent isotopolog data, down-regulation of *acnA* resulting in reduced carbon flux via the citric acid cycle seems to be a common feature of the SCV metabolism irrespective of the underlying molecular mechanism leading to this phenotype. Not surprisingly, all of the investigated clinical SCVs comprising heme, menadione and thymidine auxotrophic phenotypes, respectively, displayed a transcriptional down-regulation of *acnA* compared to their corresponding normal phenotype (**Figures 6A–C, 7A,B**). This is in line with previous studies on transcriptomic or proteomic level (Kohler et al., 2003; Seggewiss et al., 2006). Along with the decreased metabolic activity, all SCVs showed markedly reduced expression of the major virulence regulators *hld* and *sigB*. Hld is the effector molecule (a regulatory RNA) of the agr system which regulates virulence determinants in

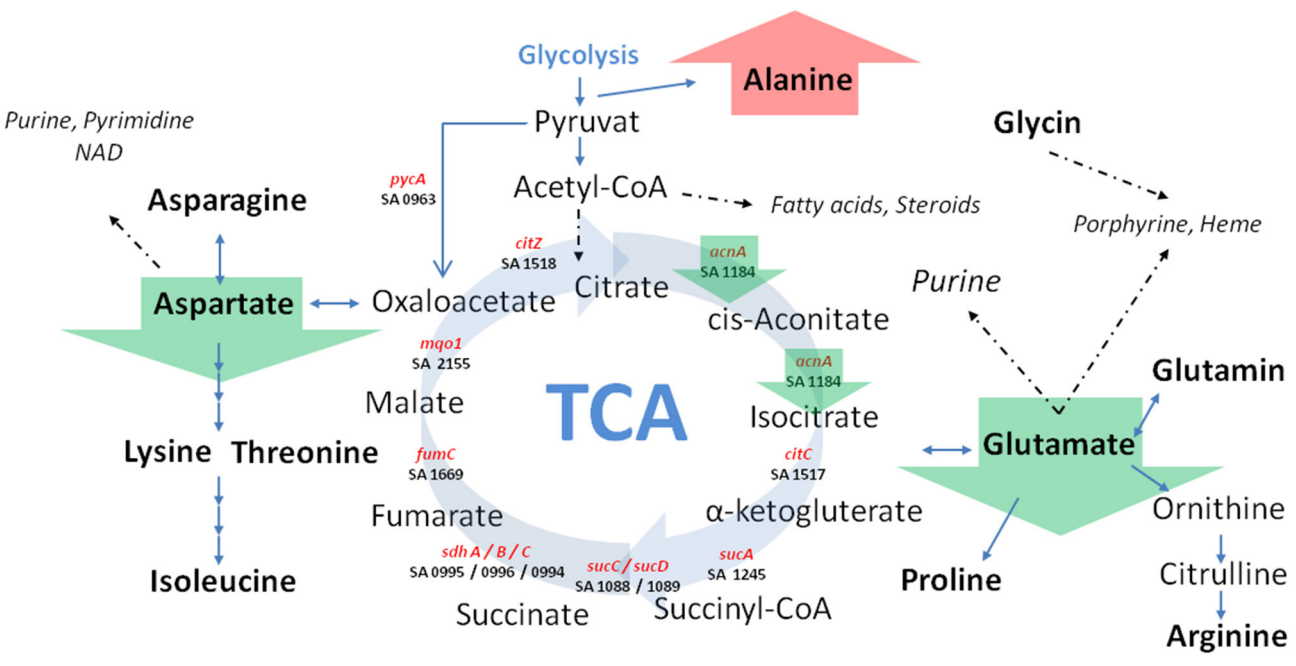

FIGURE 5 | Schematic view of central carbon metabolism in *S. aureus*. Green arrows marked SCV specific differences irrespective of the underlying auxotrophism compared to their corresponding normal phenotypes. TCA: tricarboxylic acid cycle; Involved genes (marked in red) and corresponding KEGG gene identifier were shown.

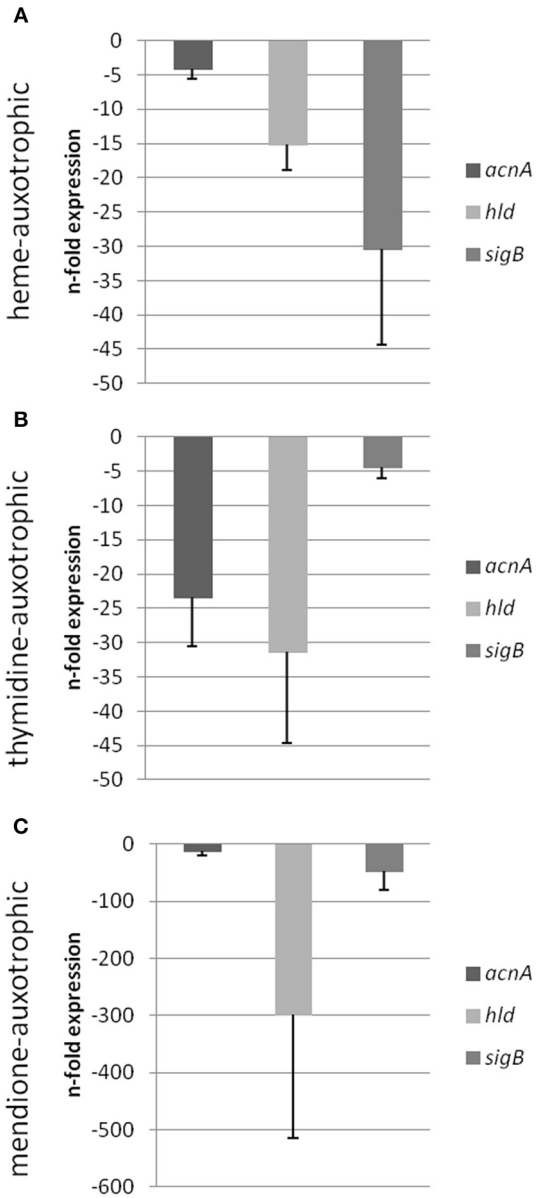

FIGURE 6 | Semi-quantitative rt-PCR. Quantification of the expression of *acnA*, *hld* and *sigB* of different *S. aureus* strains displaying the SCV phenotype compared to its respective isogenic wild-type isolate. Expression data is normalized using three internal control genes (*gmk*, *aroE*, and *gyrB*) and displayed relative to the corresponding wild-type isolates (I^{WT}, III^{WT}, and V^{WT} were set as 1). Isogenic strain pair I^{WT} and I^{SCV} [heme auxotrophic **(A)**], isogenic strain pair III^{WT} and III^{SCV} [thymidine auxotrophic **(B)**] and isogenic strain pair V^{WT} and V^{SCV} [menadione auxotrophic **(C)**] were shown.

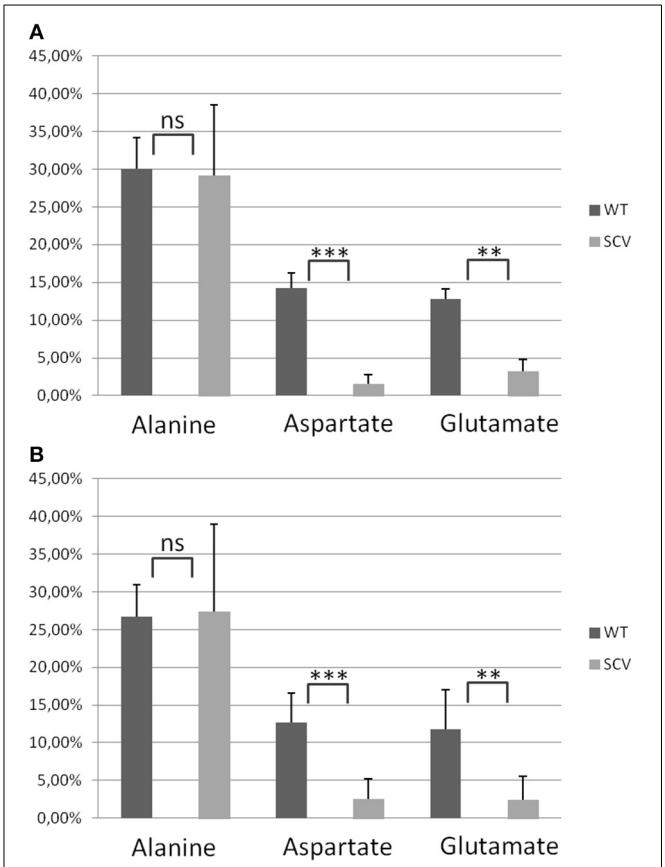

FIGURE 7 | Averaged ^{13}C-Excess in alanine, aspartate and glutamate from *S.aureus* normal and SCV phenotypes. (A) Columns represent the averaged values ± standard deviation (SD) of the normal phenotypes (WT) including *S. aureus* 3878 WT, REV and KM and the SCV phenotypes (SCV) including *S. aureus* cSCV, Δ*hemB* and G. **(B)** Columns represent the averaged values ± standard deviation (SD) of the normal phenotypes (WT) including *S. aureus* I-VI^{WT} and the SCV phenotypes (SCV) including *S. aureus* I-VI^{SCV}. Statistical significance was assessed in pairwise comparison between WT and SCV for each amino acid with the two-tailed Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

S. aureus such as the major toxins *hla* (α-hemolysin) and *hlb* (β-hemolysin). Corresponding to the reduced expression of *hld*, the investigated SCV phenotypes showed a clearly reduced hemolytic activity on Columbia blood agar plates (data not shown).

In conclusion, all SCV phenotypes irrespective of their auxotrophism and genetic background revealed down-regulation of citric cycle activity as shown by the reduced ^{13}C-incorporation into aspartate and glutamate with modified isotopolog profiles, as well as by down-regulation of *acnA* on the transcriptional level. A reduced metabolic status of all kinds of SCVs may explain the concordant major characteristics of the *S. aureus* SCV phenotypes regardless of the mechanism of their formation.

ACKNOWLEDGMENT

We thank D. Kuhn, E. Leidig and M. Bach for excellent technical assistance. This work was supported by grants to Karsten Becker (BE 2546/1-2) and Wolfgang Eisenreich (EI 384/5-2) from the Deutsche Forschungsgemeinschaft (DFG) within the SPP1316.

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Conflict of Interest Statement: The Guest Associate Editor Thomas Dandekar declares that, despite having collaborated with authors Claudia Huber (Eisenreich group) and Wolfgang Eisenreich (Co-Topic Editor of this issue), the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 July 2014; accepted: 17 September 2014; published online: 21 October 2014.

Citation: Kriegeskorte A, Grubmüller S, Huber C, Kahl BC, von Eiff C, Proctor RA, Peters G, Eisenreich W and Becker K (2014) Staphylococcus aureus small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism. Front. Cell. Infect. Microbiol. ***4****:141. doi: 10.3389/fcimb.2014.00141*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 20 August 2014
doi: 10.3389/fcimb.2014.00114

Metabolism and virulence in *Neisseria meningitidis*

***Christoph Schoen*[1,2]*, *Laura Kischkies*[1], *Johannes Elias*[1,3] *and Biju Joseph Ampattu*[1]**

[1] *Institute for Hygiene and Microbiology, University of Würzburg, Würzburg, Germany*
[2] *Research Center for Infectious Diseases (ZINF), University of Würzburg, Würzburg, Germany*
[3] *National Reference Centre for Meningococci and Haemophilus influenzae (NRZMHi), University of Würzburg, Würzburg, Germany*

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
Charlene Kahler, University of Western Australia, Australia
Odile Tresse, French National Institute for Agricultural Research/Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering, France

****Correspondence:***
Christoph Schoen, Institute for Hygiene and Microbiology, Research Center for Infectious Diseases, University of Würzburg, Josef-Schneider-Str. 2, Bldg. E1, 97080 Würzburg, Germany
e-mail: cschoen@hygiene.uni-wuerzburg.de

A longstanding question in infection biology addresses the genetic basis for invasive behavior in commensal pathogens. A prime example for such a pathogen is *Neisseria meningitidis*. On the one hand it is a harmless commensal bacterium exquisitely adapted to humans, and on the other hand it sometimes behaves like a ferocious pathogen causing potentially lethal disease such as sepsis and acute bacterial meningitis. Despite the lack of a classical repertoire of virulence genes in *N. meningitidis* separating commensal from invasive strains, molecular epidemiology suggests that carriage and invasive strains belong to genetically distinct populations. In recent years, it has become increasingly clear that metabolic adaptation enables meningococci to exploit host resources, supporting the concept of nutritional virulence as a crucial determinant of invasive capability. Here, we discuss the contribution of core metabolic pathways in the context of colonization and invasion with special emphasis on results from genome-wide surveys. The metabolism of lactate, the oxidative stress response, and, in particular, glutathione metabolism as well as the denitrification pathway provide examples of how meningococcal metabolism is intimately linked to pathogenesis. We further discuss evidence from genome-wide approaches regarding potential metabolic differences between strains from hyperinvasive and carriage lineages and present new data assessing *in vitro* growth differences of strains from these two populations. We hypothesize that strains from carriage and hyperinvasive lineages differ in the expression of regulatory genes involved particularly in stress responses and amino acid metabolism under infection conditions.

Keywords: *Neisseria meningitidis*, virulence, pathometabolism, oxidative stress, glutathione, γ-glutamyl cycle, glutamate dehydrogenase, nitrite respiration

INTRODUCTION

The Gram-negative species *Neisseria meningitidis* (the meningococcus) belongs to the β-subgroup of proteobacteria. They are facultative commensals, and their only habitat are humans with no other known reservoirs. Meningococci colonize the nasopharynx of up to 35% of healthy individuals at any given time, and direct person-to-person spread of meningococci occurs by large droplet transmission (Caugant et al., 2007; Caugant and Maiden, 2009). Due to reasons not fully understood so far, they can occasionally traverse the mucosal barrier and enter the bloodstream, often resulting in life-threatening septicaemia (Coureuil et al., 2013). After crossing the blood-brain barrier, invading bacteria can multiply in the cerebrospinal fluid (CSF) and cause fulminant meningitis with potentially high lethality (Rosenstein et al., 2001; Stephens et al., 2007). However, the invasive behavior is not part of the normal meningococcal life cycle since once they have entered the bloodstream or the central nervous system they cannot be easily transmitted to other hosts (Levin and Bull, 1994; Lipsitch and Moxon, 1997). Invasive meningococcal disease (IMD) is therefore an evolutionary dead end for this "accidental" pathogen (Moxon and Jansen, 2005).

All attempts to identify genes that code for *bona fide* virulence factors in *N. meningitidis* such as a polysaccharide capsule (Frosch and Vogel, 2006), adhesins (Virji, 2009) or certain lipooligosaccharide (LOS) types (Wright et al., 2006) and that are common to and at the same time restricted only to hyperinvasive strains have failed so far (Stabler et al., 2005; Hotopp et al., 2006; Schoen et al., 2008). In fact, many of the so called meningococcal "virulence genes" have also been found in purely commensal neisserial species (Snyder and Saunders, 2006; Marri et al., 2010). Likewise, although statistically significant associations between some mobile genetic elements and hyperinvasive lineages have been found in genome-wide analyses the potential mechanistic contribution if any of these elements to virulence still remains elusive (Bille et al., 2008; Joseph et al., 2011). The conundrum of meningococcal virulence thus challenges general concepts in infection biology such as, e.g., the association between a pathogen and disease (Fredericks and Relman, 1996), the definition and meaning of virulence factors (Falkow, 1988; Casadevall and Pirofski, 2001; Wassenaar and Gaastra, 2001), the relation between transmission and virulence (Lipsitch and Moxon, 1997), the distinction between commensal and pathogenic bacteria (Merrell and Falkow, 2004), or the mode of bacterial virulence evolution (Levin and Bull, 1994; Fraser et al., 2005).

Studies in a number of bacterial pathogens in recent years have made it increasingly clear that the ability of a pathogen to

successfully adapt to and survive within the niche in which it resides in terms of nutrient assimilation is crucial for pathogenesis (Brown et al., 2008; Eisenreich et al., 2010). For example, many potential pathogens have to scavenge amino acids from their hosts in order to make proteins, and they have evolved a diversity of means to subvert the mechanism mammalian hosts employ to starve bacteria from these critical nutrients (Zhang and Rubin, 2013). The term "nutritional virulence" consequently describes specific mechanisms that target major host biosynthetic pathways or nutrient rich sources to enhance host supply of limiting nutrients (Abu Kwaik and Bumann, 2013).

As in other bacterial pathogens, invasive disease caused by *N. meningitidis* can be regarded as a multistep process (Finlay and Falkow, 1989, 1997). As in colonization, it starts with the adhesion of meningococci to the epithelial cell layer of the human nasopharynx (Rosenstein et al., 2001; Stephens et al., 2007). Meningococci have to further cross the epithelial cell layer of the nasopharynx and invade the bloodstream, evade the defenses of the human immune system, adhere to the endothelial cell layer of the brain vessels, cross the blood brain barrier and eventually replicate in the CSF of the subarachnoidal space (Coureuil et al., 2013). It is obvious that the host environments that meningococci consecutively encounter in the course of an invasive infection each pose a specific metabolic challenge to the bacterium in terms of nutrient availability and host immune effectors. With the notable exception of iron (Perkins-Balding et al., 2004) and lactate metabolism (Chen et al., 1989; Smith et al., 2007), the contribution of central metabolic pathways to meningococcal infection biology has deserved less attention yet.

Since dedicated metabolic measurements such as isotopolog profiling under infection condition have not been carried out in meningococci so far, most information on the relation between metabolism and virulence is thus indirect and stems mostly from "omic" technologies such as (comparative) genomics (Dunning Hotopp et al., 2006; Rusniok et al., 2009; Hao et al., 2011; Joseph et al., 2011), transcriptomics (Grifantini et al., 2002a,b; Dietrich et al., 2003; Joseph et al., 2010; Echenique-Rivera et al., 2011; Hedman et al., 2012; Hey et al., 2013), proteomics (Bernardini et al., 2007; Van Alen et al., 2010) and genome-wide signature-tagged mutagenesis (STM) (Sun et al., 2000; Mendum et al., 2011) in conjunction with metabolic modeling (Baart et al., 2007). Therefore, after providing a very short overview of the metabolic capabilities of *N. meningitidis* derived in large part from genome-based approaches, we will discuss data also derived mostly from different "omic" approaches addressing the role of meningococcal core metabolism in the context of meningococcal colonization and invasion of host tissues. The presentation will follow the multi-step course of an invasive infection, i.e., starting with metabolic adaptations to colonization of the host nasopharynx, continuing with the contribution of core metabolism to successful replication in the bloodstream and immune evasion and ending with the invasion of the subarachnoidal space with subsequent replication in human CSF. Finally, we will discuss potential metabolic differences between strains from so called hyperinvasive and carriage lineages with particular emphasis on the potential role of amino acid metabolism and oxidative stress response for meningococcal virulence.

A GENOME-DERIVED BLUEPRINT OF MENINGOCOCCAL CORE METABOLISM

Based on the premise that genomic regions coding for proteins with a role in pathogenicity exhibit high rates of recombination (Didelot and Maiden, 2010), comparative genomics revealed that in meningococci the set of recombinant genes is in fact enriched for core genes coding for metabolic functions, with over 75% of all metabolic genes being affected by recombination (Hao et al., 2011; Joseph et al., 2011). Of the 459 recombinant core genes identified by Joseph and co-workers in a test set of 8 meningococcal genomes (Joseph et al., 2011), amino acid metabolism with 89 genes constituted the single largest functional class among the recombinant genes. Another 68 recombinant genes are involved in carbohydrate metabolism, 40 in the metabolism of cofactors and vitamins, 27 in nucleotide metabolism, 26 in energy metabolism, and 19 in lipid metabolism. Meningococcal metabolism is thus very likely a key player in colonization as well as in IMD, and a proper understanding of the metabolic capabilities of this species will help better understanding of the virulence differences observed among different meningococcal lineages.

The first comprehensive blueprint of meningococcal metabolism was provided by the genome sequence of strain MC58 (Tettelin et al., 2000; Dunning Hotopp et al., 2006), and Leighton and co-workers used ^{13}C- and ^{1}H-NMR in combination with conventional enzyme assays to investigate the central metabolic pathways predicted by the genome sequence (Leighton et al., 2001). Combining flux balance analysis (FBA) and metabolic modeling Baart and co-workers later modeled the core metabolism of *N. meningitidis* comprising at that time 555 gene products and over 496 associated reactions (Baart et al., 2007). By further combining FBA with STM, Mendum and co-workers extended and partially corrected this first genome-scale metabolic network now comprising 1255 reactions which are encoded by 586 genes and 59 orphan genes with no annotated function (Mendum et al., 2011). Here, we will present only a general description of the central metabolic pathways of *N. meningitidis*.

In agreement with experimental findings the genome-based model of meningococcal central metabolism indicates that *N. meningitidis* is able to grow on minimal media with a range of carbon sources, including glucose, lactate, pyruvate, and some amino acids such as glutamate, but not on acetate as sole carbon source, and that it catabolizes glucose primarily via the Entner-Douderoff (ED) and to a lesser extent via the pentose phosphate (PP) pathway. The Embden-Meyerhof-Parnas (EMP) glycolytic pathway does not contribute to pyruvate synthesis due to a lack of the phosphofructokinase gene in *N. meningitidis*. With the exception of the malate dehydrogenase gene, the complete tricarboxylic acid (TCA) cycle is encoded on the meningococcal genome, and the oxidation of malate to oxalacetate is established by a membrane-bound malate: quinoneoxidoreductase (Leighton et al., 2001). It is noteworthy that the TCA cycle is required in *N. meningitidis* for the synthesis of metabolic precursors rather than for catabolism. In the absence of external glutamate the anaplerotic replenishment of the TCA cycle is via phosphoenolpyruvate carboxylase operating in the carboxylating direction, as *N. meningitidis* has no glyoxalate shunt.

The meningococcal genome encodes the respiratory complexes I, II, and III, and oxygen is utilized by cytochrome cbb_3 oxidase which is the only respiratory oxidase in meningococci. This type of oxidase is typically found in proteobacteria that can grow also under microaerobic conditions, permitting also colonization of oxygen-limited environments. Under oxygen limitation, nitrite can replace oxygen as an alternative respiratory substrate since *N. meningitidis* is able to express a truncated denitrification pathway. Nitrite (NO_2^-) is first reduced to nitric oxide (NO) by the copper nitrite reductase AniA, and NO is then further reduced to nitrous oxide (N_2O) by the quinol-oxidizing nitric oxide reductase NorB (Rock et al., 2005; Rock and Moir, 2005). The expression of *aniA* is subject to complex regulation in response to oxygen depletion and nitrite availability (Bartolini et al., 2006; Huis in 't Veld, 2011). An evolutionarily interesting finding is the fact that while *norB* appears to be intact, *aniA* is frequently observed to be truncated in *N. meningitidis*, but not in other neisserial species (Barth et al., 2009). Also in contrast to the other closely related neisserial species such as *N. gonorrhoeae* in which electrons can be transferred to AniA via either the membrane-associated di-haem protein cytochrome c_5 or the tri-haem CcoP protein component of cytochrome cbb_3, cytochrome c_5 appears to be responsible for all electron flow to AniA in *N. meningitidis* strains competent for nitrite reduction (Aspholm et al., 2010). This is due to a single nucleotide polymorphism (SNP) resulting in CcoP truncation which consequently acts as a molecular signature for the species *N. meningitidis*.

Although numerous alternatives can be used and a wide range of sulfur-acquisition routes are available, meningococci preferably use cysteine or cystine as sulfur sources. In line with experimental results, genomic analyses suggest that meningococci can also use sulfate as sole sulfur source, and that the five proteins encoded by *cysD*, *cysH*, *cysI*, *cysJ*, and *cysN* are expected to give this species the ability to reduce sulfate (SO_4^{2-}) into hydrogen sulfide (H_2S) (Rusniok et al., 2009). However, sulfate reduction might differ slightly from the classical pathway since adenosine phosphosulfate (APS) might be directly reduced into sulfite by the APS reductase CysH. Cysteine can be converted into glutathione (GSH) which is further oxidized to glutathione disulfide (GSSG) thereby controlling the cellular hydrogen peroxide level, and meningococci have a functional γ-glutamyl cycle which helps to maintain redox balance. In addition, they are also able to process reactive oxygen by superoxide dismutases and catalase (Seib et al., 2004).

According to the genome-based model, glutamate or, after adaptation to glutamate-free medium, also ammonium can further serve as nitrogen sources. Since the meningococcal genome lacks a functional glutamate synthase gene, L-glutamate has to be either taken up from the environment (*gltS*, *gltT*) or synthesized by the NADPH-specific glutamate dehydrogenase GdhA in the presence of high external NH_4^+ from 2-oxoglutarate. Glutamate dehydrogenases are key enzymes that link carbohydrate (energy) and nitrogen metabolism, and the NADPH-specific glutamate dehydrogenase catalyses the reversible reaction: 2-oxoglutarate + NH_4^+ + NADPH ↔ L-glutamate + H_2O + $NADP^+$ suggesting a major role for NADP-GdhA in ammonia and thus nitrogen assimilation (Pagliarulo et al., 2004). The meningococcal genome further encodes several amino acid transporters, aminotransferases and all biochemical pathways for amino-acid biosynthesis (Leighton et al., 2001).

Like many other bacteria, meningococci are in need for trace elements, in particular iron, which is essential for the production of proteins involved in numerous key metabolic processes such as DNA replication, electron transfer in the respiratory chain, and the metabolism of oxygen, peroxide and superoxide. Since there is little free iron in the host (also called "nutritional immunity" by Stork et al., 2013), meningococci possess several iron uptake systems that rely on high-affinity receptors for iron-bound host proteins, including transferrin, lactoferrin, and hemoglobin (Perkins-Balding et al., 2004). In addition, they might also be able to use heterologous siderophors secreted by other bacteria. As these host iron-binding proteins are differentially distributed within the human body, with, e.g., the mucosal surface being rich in lactoferrin while the blood-stream contains high amounts of hemoglobin, these proteins were suggested to serve as niche indicators for *N. meningitidis*, leading to specific changes in gene expression (Jordan and Saunders, 2009). Since iron acquisitions systems in *N. meningitidis* have been the subject of an excellent review (Perkins-Balding et al., 2004) this topic will not be dealt here in any more detail. In addition, dedicated transporters for the acquisition and uptake of zinc (Stork et al., 2010, 2013) and manganese (Veyrier et al., 2011) have also been described in *N. meningitidis*. Of note, the manganese transporter MntX was found to be conserved and functional within *N. meningitidis* but mutated in a majority of *N. gonorrhoeae* strains and commonly absent in non-pathogenic species, adding another metabolic phenotype that differentiates *N. meningitidis* from other neisserial species (Veyrier et al., 2011). Transporters for yet other transition metals essential for meningococcal growth such as Cu^{2+}, Co^{2+}, Ca^{2+} have also been annotated in the meningococcal genome(s) but still remain to be functionally characterized.

Meningococci are thus metabolically quite versatile organisms that are able to successfully meet the metabolic challenges posed by the different environments they encounter during colonization and invasive disease, respectively.

METABOLIC SIGNATURE OF NASOPHARYNGEAL COLONIZATION AND CARRIAGE

Since successful colonization of the nasopharyngeal epithelium is an essential first step in the normal commensal life style as well as in accidentally causing IMD, several studies have addressed meningococcal traits that might be involved in successful adhesion to and colonization of human nasopharyngeal cells (Trivedi et al., 2011).

A prime example of how metabolic adaptation renders this bacterium with features that support in long-term colonization and immune evasion is the selective use of lactate as carbon source which is catabolized at a faster rate compared to glucose (Smith et al., 2007). Colonization experiments using human nasopharyngeal mucosa explants from resected adenoids consequently showed that the colony forming units (CFU) of a strain deficient for lactate transport (Δ*lctP*) were 10 times lower than that of the wild type strain while there was no detectable change in

the expression of known adhesins like type IV pili, Opa, and Opc (Exley et al., 2005a).

In a similar study addressing transcriptional changes in meningococci during long-term colonization of nasopharyngeal cells, Hey et al. (2013) showed that the transcriptome at 4 h was markedly different from those at prolonged co-cultivation times at 21 days. Of the 2062 genes whose expression was compared 382 and 552 genes were differently expressed in meningococci at 4 h and 21 days, respectively. In both cases, more than 200 differently expressed genes encoded proteins with metabolic function. Among the differently expressed metabolic genes, genes involved in amino acid metabolism and inorganic ion transport and metabolism constituted the first and second largest groups, respectively.

Jamet and co-workers used STM in an epithelial cell-culture system to screen at a genome-wide scale for genes involved in colonization (Jamet et al., 2013). They identified five mutants with a decreased colonization ability 18 h post infection that all had mutations in genes apparently involved in adaptation to hypoxic conditions and stress resistance. None of the mutants exhibited initial adhesion defect to human epithelial cells at 3 h post infection, indicating that meningococci utilize different genes and metabolic pathways in initial adhesion and long-term colonization. Of the five mutant strains, one mutant harbored a transposon insertion in a gene which encodes a putative membrane-associated thioredoxin. This compound belongs to a class of small redox proteins known to be present in all organisms and which are involved in redox signaling. Another two mutants had transposon insertions in genes involved in the metabolism of nitrogen oxides: *narP* encoding the NarP regulator of the two-component system NarP/NarQ, which is involved in the denitrification process, and *nnrS* encoding a heme- and copper-containing membrane protein (Honisch and Zumft, 2003) that plays a role in the metabolism of nitrogen oxides. Nitrite is present in human tissues as a result of oxygenation of nitric oxide which is produced by various human cells types, and/or through the dietary intake of nitrate which is further reduced to nitrite by nitrate reducing bacteria in the human oral microbiota (Lundberg et al., 2004).

Further evidence toward the potential importance of sulfur acquisition as well as amino acid metabolism for successful nasopharyngeal colonization was provided by the finding that the pathway required to reduce sulfate to hydrogen sulfide is complete only in the nasopharynx colonizers *N. meningitidis* and *N. lactamica* but absent in the urogenital colonizer *N. gonorrhoeae* (Rusniok et al., 2009). Several studies analyzing early changes (<4 h) in the transcriptomes of meningococci upon adhesion to human epithelial cell lines consistently found an induction of the sulfate ABC transporter genes *cysW* (permease), *cysA* (ATP-binding protein) and *cysT* (permease) during adhesion (Grifantini et al., 2002a; Dietrich et al., 2003; Joseph et al., 2010). Along with the sulfate uptake genes the expression activation also of some of the genes involved in the synthesis of histidine, methionine, cysteine, and their seleno derivatives, as well as the genes for the synthesis of adenosylmethionine and N-formylmethionly-tRNA were found to be particularly pronounced (Grifantini et al., 2002a).

Via the cysteine biosynthesis pathway the uptake of sulfate is also required for the synthesis of glutathione. As with thioredoxin mentioned above glutathione acts as an antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange thus reducing oxidative stress (Carmel-Harel and Storz, 2000). Accordingly, the gene expression profile in meningococci after cysteine depletion was found to resemble oxidative stress (van de Waterbeemd et al., 2013), and of the 149 cysteine regulated genes, 36 enriched gene ontologies were identified from 11 functional groups, comprising in particular redox functions, iron-sulfur cluster, sulfur metabolism and amino acid biosynthesis. In addition to its role in glutathione biosynthesis, it was hypothesized that the resulting oxidative stress after cysteine depletion was due to impaired sulfur supply for iron-sulfur protein biogenesis.

In the human nasopharynx meningococci persist in a biofilm-like state as suggested by previous observations in human tonsillar tissues (Sim et al., 2000). This sparked interest in the analysis of transcriptomic and proteomic profiles of meningococci grown in biofilms which are used as *in vitro* model system reflecting asymptomatic carriage (O'Dwyer et al., 2009; Van Alen et al., 2010). O'Dwyer et al. (2009) compared the transcriptomes of *N. meningitidis* MC58 grown on plate and in biofilm and showed that of the top 50 genes up-regulated in biofilms 26 coded for proteins with metabolic functions comprising in particular energy production and conversion (13 genes) and amino acid synthesis (5 genes). Proteomic analysis of meningococcal biofilms further revealed a response to reactive oxygen species (ROS, see below) and to nutrient and oxygen limitation (Van Alen et al., 2010). The periplasmic Cu–Zn superoxide dismutase SodC and the periplasmic substrate-binding protein MntC were more abundantly expressed in biofilms than in planktonic culture. In addition to oxidative stress and oxygen limitation, the bacteria have to cope with nutrient limitation, especially in the deeper layers of the biofilm. In meningococci, the global regulator NMB0573 controls the response to nutrient availability through indicators of general amino acid abundance (leucine and methionine) (Ren et al., 2007). In support of a central role of this regulator and consequently of amino acid metabolism in biofilm formation, proteins less expressed in meningococcal biofilms, i.e., Opa, Opc, SdhA (succinate dehydrogenase), SucD (succinyl-CoA synthetase), AldA (aldehyde dehydrogenase A), as well as the cell division protein FtsZ all belonged to the NMB0573 regulon. Speculation about leucine limitation in meningococcal biofilm is also supported by the increased expression of LeuA (2-isopropylmalate synthase). In addition to LeuA, another enzyme involved in amino acid synthesis, the aspartate aminotransferase AspC, was upregulated in the biofilm.

Together, these data suggest that the ability to cope with changing oxygen concentrations and limitations in key nutrients such as amino acids and sulfur might be metabolic adaptations of meningococci which allow them to thrive in the human nasopharynx as their sole ecological niche. The metabolic state of colonizing meningococci might further enable them to also invade deeper host tissues and to finally reach the bloodstream. Of course, one important caveat of these *in vitro* studies is that the previous studies did not take account of potential host

factors other than the epithelial cell itself as well as the influence of the colonizing microbiota and their metabolic products on meningococcal growth and adhesion.

METABOLIC SIGNATURE OF MENINGOCOCCI IN INVASIVE DISEASE

First experimental evidence for the importance of the core metabolism also in meningococcal virulence came from functional genomic studies. Using STM of a *N. meningitidis* serogroup B strain Sun and co-workers identified 73 genes that were essential for bacteraemia in an infant rat model (Sun et al., 2000). Remarkably, about half the 73 genes encode enzymes that are involved in metabolism and transport of nutrients. Eleven are involved in amino acid biosynthesis, including five (*aroB*, *aroC*, *aroD*, *aroE*, *aroG*) in the shikimate pathway, two (*metF*, *metH*) in methionine biosynthesis, two (*ilvD*, *ilvI*) in the synthesis of isoleucine and valine, one (*fhs*) in the synthesis of 10-formyltetrahydrofolate, which is used directly in purine biosynthesis and formylation of Met-tRNA, and one, *gdhA*, in glutamate metabolism (**Figure 1**).

In line with these *in vivo* findings and reflecting large scale metabolic adaptations that *N. meningitidis* makes after entering the bloodstream, *ex vivo* transcriptomic analyses showed that a large proportion of genes involved in nutrient transport and different core metabolic pathways were differentially expressed upon incubation of bacterial cells in human blood (Echenique-Rivera et al., 2011; Hedman et al., 2012). Along with numerous iron uptake systems such as for the transferrin binding proteins (*tbpA*, *tbpB*), the lactoferrin binding proteins (*lbpA*, *lbpB*) and for the hemoglobin receptor (*hmbR*), also transporters for glucose (*gluP*) and lactose (*lctP*) as well as genes encoding enzymes involved in glycolysis (*pgi-1, fbp, pgm, tpiA*), the TCA cycle (*pprC, acnA, icd, sdhC, sdhD, sdhB, gltA, sucC, sucD, fumC, acnB, fumB, yojH*) and 12 out of the 14 genes of the *nuo* operon encoding subunits for the NADH dehydrogenase complex I were up-regulated. Genes involved in pyruvate metabolism, which is part of the protein synthesis pathway, were also induced during growth in blood as well as numerous genes involved in the biosynthesis of amino acids. The latter comprised in particular genes involved in glutamate metabolism like the ABC-type L-glutamate transporter gene *gltT* or *gdhA* encoding NADP-specific glutamate dehydrogenase.

Based on the assumption that metabolic requirements for *N. meningitidis* growth in human serum are likely to be similar to those for growth in blood, Mendum et al. (2011) used STM to screen for genes essential for growth in human serum, supporting in large parts the findings of transcriptomic analyses. Since aromatic amino acid synthesis has been associated with virulence in *N. meningitidis* as described above, a particularly interesting finding of this study was the conditional essentiality in serum of a number of genes associated again with amino acid biosynthesis, particularly those involved in the synthesis of aromatic amino acids (*trpBCE, aroCDGK*), leucine (*leuBC, ilvC*), histidine (*hisCG*), glycine (*glyA*) and proline (*proC, putA*), and of a number of genes for amino acid transporters (NMB0787, NMB0788, NMB2031). Other major groups of metabolic genes essential for growth in serum were those involved in the synthesis of folic acid, pantothenate and pyridine, purines as well as genes required for iron acquisition (*exbBD, tonB, fetC, fbpB, lbpA*) and *lctP*. Several genes of central carbon metabolism (parts of TCA and pyruvate metabolism) were also reported to be conditionally essential. Mutations that lost fitness in minimal medium but not in sera included those involved in sulfur acquisition, suggesting that sulfur can be acquired from organic sources in sera.

In addition to successful replication within the bloodstream, meningococci have to concomitantly evade the humoral and cellular effector mechanisms of the innate and acquired immune defenses (Lo et al., 2009). Complement mediated lysis by deposition of the complement molecule C3 is an important mechanism for pathogen elimination, and eukaryotic cells have sialic acid deposits on their outer surface which is used by the host immune system to identify such cells as "self." Amongst others, *N. meningitidis* uses molecular mimicry to evade human immune system by preferentially using lactate as a carbon and energy source, as intermediates of lactate catabolism feed directly into the sialylation pathway increasing sialic acid biosynthesis (Exley et al., 2005b). Increased coating of the outer membrane with sialic acid results in decreased deposition of the complement molecule C3 and consequently reduced complement mediated killing. Mutant strains deficient for sialic acid modification of the outer membrane by the inability to transport lactate into the cell (Δ*lctP*) were found to be more susceptible to complement-mediated killing (Lo et al., 2009). LOS sialylation was also shown to reduce phagocytosis of meningococci by dendritic cells which are key antigen presenting cells linking innate and adaptive immune responses (Unkmeir et al., 2002). Together with the complement system blood phagocytes such as macrophages and polymorphonuclear neutrophil leucocytes (PMNs) constitute the major line of defense against invasive neisserial infections (Criss and Seifert, 2012). Amongst other antibacterial compounds, both produce reactive oxygen species (ROS) like superoxide anion ($.O_2^-$), hydroxyl radical (.OH) or H_2O_2 and the reactive nitrogen species like nitric oxide (NO) which can react with a plethora of nitrogen and oxygen radicals collectively known as reactive oxygen and nitrogen species (RONS) (Kozlov et al., 2003). ROS and RNS have potent effects on bacterial proteins, lipids, and DNA (Imlay, 2013). For example, ROS damage proteins like the iron sulfur ([Fe-S]) clusters of dehydratases in the respiratory chain which are especially vulnerable to oxidation by ROS, and NO can inhibit or damage by reacting with [Fe-S] clusters. Consequently, PMNs and macrophages exert oxidative and nitrosative stresses ultimately resulting in the destruction of the phagocytosed pathogen (Storz and Spiro, 2011).

Once phagocytosed, the synthesis of the antioxidant glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) from L-glutamate taken up from the host is of central importance for meningococcal immune evasion as suggested by the following observations. Firstly, the transport of L-glutamate by the ABC-Type L-glutamate transporter GltT and its subsequent conversion into GSH by glutamate-cysteine ligase (GshA) and glutathione synthetase (GshB) was shown to prevent killing of meningococci by PMNs by providing defense against ROS (Tala et al., 2011). Secondly, *gdhA* expression was induced upon incubation of *N. meningitidis* with human whole blood

		"colonization"			"IMD"						
Infant rat model (Sun *et al.* (2000)	**Recombination** (Joseph *et al.* 2011)	**16HBE14** (3h, Grifantini *et al.* 2002)	**16HBE14** (4h, Hey *et al.* 2013)	**FaDu** (3h, Joseph *et al.* 2011)	**Blood** (1h, Echenique-Rivera *et al.*2011)	**Blood** (1h, Hedman *et al.* 2012)	**HBMEC** (6h, Dietrich *et al.* 2006))	**Biofilm** (O'Dwyer *et al.* 2009)	**Gene**	**Product Name**	**COG(s)**	**COG number**
INFORMATION STORAGE AND PROCESSING												
NMB1838	■			■				■	-	translation-associated GTPase	J	COG0012J
NMB0030									*metG*	methionyl-tRNA synthetase	J	COG0143J
NMB0139						■			*tufA*	elongation factor Tu	J	COG0050J
NMB0758	■			■					*pnp*	polynucleotide phosphorylase/polyadenylase	J	COG1185J
NMB0112	■								*rsmB*	ribosomal RNA small subunit methyltransferase B	J	COG0144J
NMB1348									-	putative tRNA/rRNA methyltransferase	J	COG0565J
NMB0686				■	■				*rnc*	ribonuclease III	K	COG0571K
NMB1422	▒			■	■				-	putative ATP-dependent RNA helicase	LKJ	COG0513LKJ
NMB0116	▒			■	■				*dprA*	DNA processing chain A	LU	COG0758LU
METABOLISM												
NMB0536	■		■	■	■			■	-	Na+/H+ antiporter	C	COG1757C
NMB0543			■	■	■			■	-	putative L-lactate permease	C	COG1620C
NMB1814	▒			■					*aroB*	3-dehydroquinate synthase	E	COG0337E
NMB1680	■			■		■			*aroC*	chorismate synthase	E	COG0082E
NMB1446					■				*aroD*	3-dehydroquinate dehydratase	E	COG0710E
NMB0358	▒			■				■	*aroE*	shikimate dehydrogenase	E	COG0169E
NMB0307				■					*aroG*	phospho-2-dehydro-3-deoxyheptonate aldolase	E	COG0722E
NMB1710		■			■	■	■	■	*gdhA*	glutamate dehydrogenase	E	COG0334E
NMB0943	■			■	■				*metF*	5,10-methylenetetrahydrofolate reductase	E	COG0685E
NMB0944					■			■	*metH*	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	E	COG0620E
NMB1188	▒								*ilvD-2*	dihydroxy-acid dehydratase	EG	COG0129EG
NMB1577	■						■		*ilvI*	acetolactate synthase isozyme III large subunit	EH	COG0028EH
NMB1970	▒			■					-	4-amino-4-deoxychorismate lyase	EH	COG0147EH
NMB1612								■	-	amino acid ABC transporter	ET	COG0834ET
NMB1839	■			■					*fhs*	formate--tetrahydrofolate ligase	F	COG2759F
NMB1996	▒			■					*purL*	phosphoribosylformylglycinamidine synthase	F	COG0046F
NMB2044	■		■	■					*ptsI*	phosphoenolpyruvate-protein phosphotransferase (protein I)	G	COG1080G
NMB1869	■							■	*cbbA*	fructose-1,6-bisphosphate aldolase	G	COG0191G
NMB2045			■	■					*ptsH*	phosphocarrier protein HPr (phosphotransferase system, histidine-containing protein)	G	COG1925G
NMB2006				■					-	chloride channel protein-related protein	P	COG0038P
NMB0792	▒		■						-	putative transporter	P	COG0471P
CELLULAR PROCESSES AND SIGNALING												
NMB0342	■					■			-	putative intracellular septation protein	D	COG2917D
NMB0083	▒			■					*lipB*	capsule polysaccharide modification protein	M	COG3562M
NMB1929	■			■		■			*lgtA*	lacto-N-neotetraose biosynthesis glycosyl tranferase	M	COG0463M
NMB1527	■			■	■		■		*rfaF*	ADP-heptose:LPS heptosyltransferase II	M	COG0859M
NMB1705									*rfaK*	alpha 1,2 N-acetylglucosamine transferase	M	COG0438M
NMB1730	▒				■		■	■	*tonB*	TonB protein	M	COG0810M
NMB1558									*dgk*	diacylglycerol kinase	M	COG0818M
NMB1145	■			■					*mpl-1*	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	M	COG0773M
NMB1279	▒	■		■	■	■		■	*mltB*	putative membrane-bound lytic murein transglycosylase B	M	COG2951M
NMB0825	■				■			■	*rfaE*	putative ADP-heptose synthase	M	COG2870M
NMB0713				■	■				*lnt*	apolipoprotein N-acyltransferase	M	COG0815M
NMB0638				■					*galU*	UTP--glucose-1-phosphate uridylyltransferase	M	COG1210M
NMB1797			■						*dacB*	D-alanyl-D-alanine carboxypeptidase (DD-peptidase; DD-carboxypeptidase)	M	COG2027M
NMB0548	■								-	putative membrane fusion protein	M	COG0845M
NMB0345					■			■	-	putative cell-binding factor	O	COG0760O
NMB1790	▒		■						*grxC*	glutaredoxin 3	O	COG0695O
NMB0114	▒				■				-	putative nitrogen regulation protein NtrY	T	COG5000T
NMB1728						■		■	*exbD*	biopolymer transport protein	U	COG0848U
NMB1729			■					■	*exbB*	biopolymer transport protein	U	COG0811U
NMB0668				■				■	*ampD*	N-acetyl-anhydromuranmyl-L-alanine amidase	V	COG3023V
POORLY CHARACTERIZED												
NMB1240				■					-	ABC transporter, ATP-binding protein	R	COG0488R
NMB1671	▒			■					*pqiB*	pqiB protein	R	COG3008R
NMB0748			■		■				*hfq*	Hfq protein	R	COG1923R
NMB1689									-	putative DedA-family integral membrane protein	S	COG0586S
NOT IN COG												
NMB1362	■			■	■				-	putative oxalate/formate antiporter	-	-
NMB1515	▒		■						-	putative transporter	-	-
NMB0067	▒					■			*siaD*	polysialic acid capsule biosynthesis protein SiaD	-	-

FIGURE 1 | Results from omic approaches for genes found to be essential for bacteremic disease in an infant rat model. Based on the gene set identified to be essential for bacteremic disease in an infant rat model by Sun et al. (2000) (first column), for each gene the results are depicted (from left to right) for detection of intragenic recombination (Joseph et al., 2011), expression changes in strain MC58 upon adhesion to 16HBE14 human respiratory epithelial cells after 3 h (Grifantini et al., 2002b) and 4 h (Hey et al., 2013), to FaDu nasopharyngeal cells after 3 h (Joseph et al., 2010), in human whole blood after 1 h taken from Echenique-Rivera et al. (2011) and from Hedman et al. (2012), respectively, upon adhesion to human brain microvascular endothelial cells after 6 h (Dietrich et al., 2003) and in cells grown in biofilm vs. planctonic culture (O'Dwyer et al., 2009). Black boxes indicate that the respective gene was identified in the corresponding study, and gray boxes indicate that the gene was not included in the study considered. The last column gives the COG functional assignment for each gene (Tatusov et al., 2001) (for COG abbreviations see legend to **Figure 2**).

(Echenique-Rivera et al., 2011), and a mutation in *gdhA* rendered meningococci attenuated in infant rat model (Sun et al., 2000). Finally, glutathione peroxidase (GpxA) mutants were much more sensitive to the oxidative stress caused by paraquat and slightly more sensitive to H_2O_2 (Moore and Sparling, 1996).

Besides GSH, superoxide anion can also be processed by superoxide dismutases SodC present in the periplasm or SodB present in the cytosol, followed by catalase to regenerate oxygen. Also in line with an important role for oxidative stress resistance in meningococcal bloodstream survival was the finding that a *sodC* mutant was less virulent in an intraperitoneal mouse infection model (Wilks et al., 1998), and *sodC* mutant bacteria were endocytosed in significantly higher numbers than wild-type organisms by human monocytes/macrophages (Dunn et al., 2003).

Blood not only constitutes an immunologically challenging compartment but also an oxygen-limiting environment as oxygen is linked to hemoglobin, and the denitrification pathway enables meningococcal survival via anaerobic respiration. In addition the denitrification pathway provides a RNS detoxification system since NorB and to a lesser extent the *cycP* gene product cytochrome c' were also found to provide protection against RNS accumulation due to exogenous NO (Anjum et al., 2002; Laver et al., 2010) and to enhance survival of *N. meningitidis* within primary human macrophages (Stevanin et al., 2005). NO is an important physiological platelet inhibitor and cardiovascular signaling molecule and is also known as endothelium-derived relaxing factor. The finding that *N. meningitidis*-derived NO inhibits platelet aggregation and significantly increases endothelial monolayer permeability in humans consequently provides a direct link between meningococcal metabolism and the pathogenesis of IMD (Kobsar et al., 2011), and the pathological inhibition of platelet function with massive hemorrhage into the adrenal glands and widespread petechial bleeding is a hallmark clinical feature of the fulminant septicemic course of IMD, named Waterhouse-Friderichsen syndrome (Rosenstein et al., 2001; Stephens et al., 2007).

In the case of causing acute bacterial meningitis, meningococci must finally be able to cross the blood-brain barrier and to multiply in the CSF of the subarachnoideal space, an environment that is quite different in its composition from human whole blood. Although our knowledge about these adaptations is still very limited, one intriguing finding is that lactate was found to stimulate growth also in CSF (Exley et al., 2005b). Apart from lactate utilization also the meningococcal ABC-type L-glutamate transporter GltT was found to be necessary for the development of experimental meningitis in mice (Colicchio et al., 2009), and a mutant strain deficient for γ-glutamyltranspeptidase (Ggt) which catalyzes the hydrolysis of γ-glutamyl compounds to yield cysteine did also not grow in rat CSF (Takahashi et al., 2004). These findings indicate that cysteine may be essential for meningococcal survival in CSF and that meningococci may use L-glutamate also in CSF as a nutrient source as well as a precursor to synthesize the antioxidant glutathione.

DIFFERENCES IN THE METABOLIC SIGNATURES BETWEEN COLONIZATION AND INVASION

To assess whether there might be differences in the functional profiles of regulated genes between colonization and invasive disease, we compared the sets of genes found to be differently expressed in strain MC58 in whole blood (Echenique-Rivera et al., 2011; Hedman et al., 2012) with the sets of genes found to be differently expressed upon adhesion to human airway epithelial cells (Grifantini et al., 2002b; Joseph et al., 2010; Hey et al., 2013). A more robust formal meta-analysis of these transcriptomic studies is unfortunately not possible at the moment due to the comparatively small number of analyses available.

As depicted in **Figure 2A** of the 1627 genes compared 1406 were found to be differently expressed in at least one study. There was substantial variation in the number of significantly differently expressed genes among the studies analyzing gene expression in whole blood as well as in adherent bacteria. For both conditions, we therefore considered only genes that were found to be differently expressed in more than one study for further analysis. Accordingly, we called genes differently expressed in at least two of the three studies analyzing gene expression changes upon adhesion to airway epithelial cells but not in any of the two studies analyzing gene expression changes in strain MC58 in whole blood as colonization-associated genes. In turn, we considered the complementary set comprising genes differently expressed in both studies analyzing gene expression changes in strain MC58 in whole blood but not in more than one of the three studies analyzing gene expression changes upon adhesion to airway epithelial cells as invasion-associated genes. Based on this definition, there were 86 colonization- and 101 invasion-associated genes in the dataset of which, 56 and 76 genes, respectively, had functional annotations according to the COG classification scheme (Tatusov et al., 2001).

As can be seen in **Figure 2B**, there were some significant differences in the functional profiles between the colonization-associated genes and the invasion-associated genes (Fisher's exact test, $p < 0.05$). Compared to the invasion-associated genes, the colonization-associated genes comprised in particular genes involved in cell motility and envelope biogenesis (COG functional categories N and V) such as genes required for type IV pilus biogensis (*pilS* casettes, *pilP*) as well as for LOS (*rfaE*, *kdsA*, NMB1418) or capsule (*ctrB*) biosynthesis. The invasion-associated genes in turn included genes for chaperon proteins like DnaK, GrpE, Lon and HscB and another eight genes involved in protein synthesis and turnover (*thrS*, *pheS*, *efp*, *rplL*, *rpsJ*, *rpsF, infB,* and *rnpA*) (COG category O).

The metabolic profile differed between colonization- and invasion-associated genes. For example, of the 21 metabolic genes among the colonization-associated genes three are involved in sulfur metabolism (*sbp*, *cysJ,* and *cysN*). Of note, the two genes *cysJ* and *cysN* were present in the genomes of seven nasopharynx colonizers but missing in the two gonococcal genital tract colonizers compared by Rusniok et al. (2009). The result of the transcriptome comparison is thus in line with the supposedly important role of sulfur metabolism for nasopharyngeal colonization by *N. meningitidis* (Rusniok et al., 2009). Among the colonization-associated genes, there was also a slightly higher percentage of genes involved in carbohydrate transport and metabolism like the gene for the phosphoenolpyruvate-protein phosphotransferase PtsI.

In turn, the 35 invasion-associated genes involved in metabolic processes comprised relatively more genes for inorganic ion

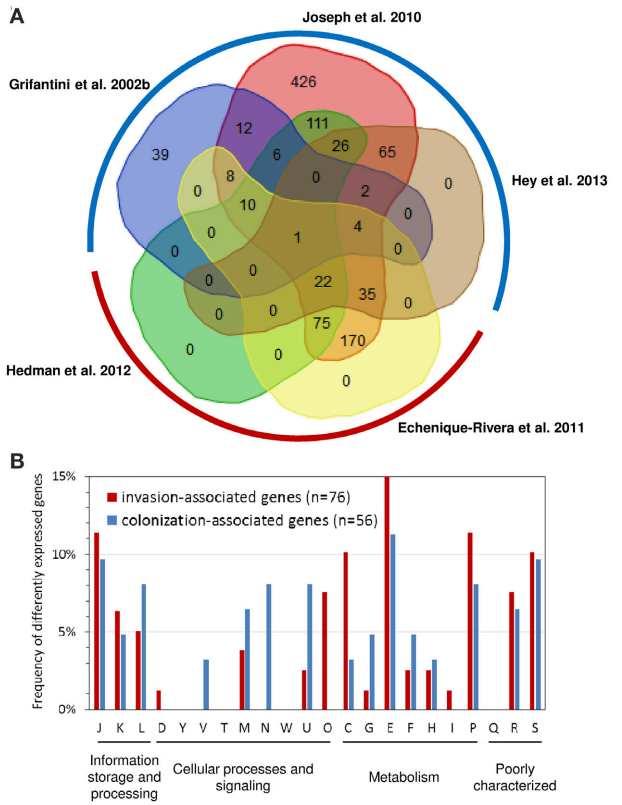

FIGURE 2 | Comparison of transcriptome studies in strain MC58. (A) Venn diagram comparing the sets of genes found to be differently expressed in strain MC58 either upon adhesion to human airway epithelial cells or upon contact with human whole blood. The transcriptomic data describing the colonizing state were taken from Grifantini et al. (2002b); Joseph et al. (2010) and Hey et al. (2013), respectively, and are framed by a blue hemicircle. The data corresponding to the invasive disease state were taken from Echenique-Rivera et al. (2011) and Hedman et al. (2012) and are framed by a red hemicircle. The histogram in panel **(B)** compares the functional profiles of colonization-associated genes with the functional profile of the invasion-associated genes as defined in the text. The functional classification is based on the COG classification scheme (Tatusov et al., 2001). Abbreviations: C, energy production and conversion; D, cell cycle control, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover, and chaperones; P; inorganic ion transport and metabolism; secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanism; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; W, extracellular structures; Y, nuclear structure.

transport and metabolism (COG P), energy production and conversion (COG C), and for amino acid transport and metabolism (COG E) (**Figure 2B**). The genes for inorganic ion transport and metabolism comprised genes required for iron-uptake like *hmbR*, *lbpA*, and *fbpA* which is consistent with the well-established dependence of *N. meningitidis* growth on iron availability (Perkins-Balding et al., 2004). This group also included the catalase gene *cat* as well as *laz* (NMB1533) which are both known to be involved in defense against ROSs. Among the genes involved in energy metabolism were the genes for methylcitrate synthase (*pprC*), L-lactate dehydrogenase (*lldA*), succinate semialdehyde dehydrogenase (*gabD*), cytochrome c and the cytochrome c oxidase subunit III (*fixP*) and in particular for fumaratehydratase (*fumC*) which is part of TCA cycle. It is important to note in this respect that the *fumC* gene sequence is used in multilocus sequence typing (MLST) of *N. meningitidis* and differs between strains from hyperinvasive and carriage lineages as described in more detail in the next section. The last group of genes comprised *aspC* encoding an aromatic amino acid aminotransferase, a gene for a putative sodium/alanine symporter (NMB0177), the aspartate kinase genes *lysC* as well as genes involved in glycine metabolism (*gcvT*, *gcvH* and *metF*) and the glutamate dehydrogenase (*gdhA*). Of note, differences in the nucleotide sequence and expression of *gdhA* have been observed in clinical isolates of *N. meningitidis* (Pagliarulo et al., 2004) as outlined below. The reliance on their hosts for amino acids has been shown in a number of other bacterial pathogens (Zhang and Rubin, 2013), and this finding is also in accordance with previous experimental observations described above indicating a close link between amino acid metabolism and virulence (Sun et al., 2000).

Further experimental work is needed to clearly assess differences in the meningococcal transcriptomes and metabolomes between the carriage and the invasive state.

METABOLIC DIVERSITY AND VIRULENCE DIFFERENCES AMONG MENINGOCOCCAL STRAINS

The genetic analysis of meningococcal population structure by MLST over the past decades provided clear evidence that the propensity to cause IMD is associated with particular lineages that coexist with less invasive carriage lineages (Maiden et al., 1998; Yazdankhah et al., 2004). In MLST analysis, approximately 500 bp-sized fragments of seven housekeeping genes are sequenced, and the alleles present at each of these seven loci for a given isolate are combined into an allelic profile and assigned a sequence type (ST). Groups of related STs are termed clonal complexes (CCs) (Maiden, 2006), and disease-causing meningococci were found to belong predominantly to certain clonal complexes such as, e.g., ST-4/5, ST-11, ST-32, ST-41/44, or ST-269, which were consequently termed hyperinvasive lineages, while lineages that were typically found to be associated with asymptomatic carriage comprise, amongst others, ST-23 or ST-53 complexes (Yazdankhah et al., 2004). With the exception of *abcZ* which encodes an ABC transporter, all genes used for meningococcal MLST encode enzymes involved in key metabolic pathways (Maiden, 2006): *adk* encodes adenylate kinase which catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP; *aroE* encodes shikimate dehydrogenase required for chorismate biosynthesis from 3-dehydroquinate; *fumC* which is part of the TCA cycle as described above; *gdh* encodes the glucose-6-phosphate 1-dehydrogenase which is part of the PP pathway; *pdhC* (*aceE*) encodes the pyruvate dehydrogenase subunit E1 which has a central role in energy generation via the TCA cycle; and *pgm* encoding phosphoglucomutase, which, like *pdhC*,

participates in both the breakdown and synthesis of glucose and glucose-1-phosphate degradation.

It has been a matter of some debate whether the genetic variation in these loci is neutral as initially proposed (Maiden et al., 1998; Fraser et al., 2005). However, analyses of epidemiological data gathered over a time span of almost 30 years combined with mathematical modeling suggested that combinations of alleles at these loci might be subject to selection and that certain co-adapted combinations of housekeeping gene alleles that define hyperinvasive lineages are associated with small differences in meningococcal transmission fitness (Buckee et al., 2008). Such small differences in transmission fitness were previously accounted for increases in disease incidence by corresponding strains (Stollenwerk et al., 2004; Moxon and Jansen, 2005). Via differences in the combinations of housekeeping gene alleles this model consequently imparts a central role for differences in the metabolic efficiency of housekeeping proteins for the emergence of virulence in meningococci.

An additional indication that differences in metabolic adaptation might contribute to virulence differences in *N. meningitidis* derives from *in vitro* transcriptome comparisons of two related serogoup B strains upon adhesion to human nasopharyngeal cells (Joseph et al., 2010) which showed differences in the expression of metabolic genes but, surprisingly, not for genes coding for outer membrane proteins. Of the 1731 orthologous genes present in both strains, the 55 genes that were higher expressed in the invasive strain MC58 comprised, amongst others, genes coding for proteins required for amino acid transport and metabolism (*argH*, *aroA*, *aroB, ilvC,* and *gdhA*), genes for ATP synthase subunits (*atpA*, *atpD*, *atpG*), and an operon coding for subunits of the Na^+-translocating NADH-quinone reductase (*nqrB, nqrC, nqrD*). The 81 genes that were higher expressed in the carriage strain α710 were enriched for genes involved in inorganic ion transport and metabolism and included two sigma factor encoding genes (*rpoD* and *rpoE*).

Pagliarulo et al. (2004) likewise focused on gene expression differences among different meningococcal strains and analyzed the regulation and differential expression of *gdhA* in 59 *N. meningitidis* clinical isolates. They found that strains belonging to the hypervirulent ET-5 and IV-I lineages exhibited levels of *gdhA* mRNA about fourfold higher than most of the other strains, and there was a strong correlation observed between the *gdhA* alleles and *gdhA* expression. As described above, GdhA is involved in the mobilization of nitrogen from ammonia to amino acids, and these data therefore indicate that nitrogen assimilation and/or glutamate biosynthesis might be differently regulated among different meningococcal lineages.

COMPARISON OF THE *IN VITRO* GROWTH PROPERTIES OF MENINGOCOCCAL STRAINS FROM CARRIAGE AND HYPERINVASIVE LINEAGES

Genetic differences in metabolic genes of *in vitro* evolved strains of *Escherichia coli* have been shown to phenotypically result in growth rate differences (Herring et al., 2006; Conrad et al., 2009). Consequently, to assess whether meningococcal strains with genetic differences in metabolic genes as indicated by MLST also differed in their *in vitro* growth properties we performed growth experiments using the compositionally defined medium RPMI 1640 (Biochrom AG, Germany) as well as the complex proteose peptone medium supplemented with 1% Polyvitex (Biomereux) (PPM+). The test panel consisted of 29 strains that were previously characterized genetically by comparative genome hybridization (mCGH) as well as MLST and comprised 15 strains from hyperinvasive CCs and 14 strains from CCs that are mostly associated with asymptomatic carriage (Joseph et al., 2011). For each strain and growth condition we fitted a logistic growth model to the data to estimate the growth rate r [1/min] and the capacity K (OD_{600nm} in the stationary phase) (shown for two exemplary strains in **Figure 3**). Biologically, the growth rate r relates to the exponential growth phase and reflects to some extent the metabolic efficiency of a bacterial strain in a given environment to optimize its reproduction (Edwards et al., 2001). In population genetics, a higher relative growth rate of a bacterial strain relative to another is often equated to a higher relative fitness (Hartl and Clark, 2007). The carrying capacity K of a biological species in an environment is by definition the maximum population size of a (bacterial) species that the environment can sustain and relates to the stationary growth phase (Goo et al., 2012). In stationary phase, bacterial cells are exposed to a number of environmental stresses such as nutrient limitation or the accumulation of toxic metabolic waste products. Therefore, the higher the carrying capacity for a bacterial strain relative to another the better it is probably able to cope with these environmental stresses.

As can be seen in **Figure 4**, there was no correlation between a strains' median capacity and its median growth rate, neither for growth in RPMI nor for growth in PPM medium (Spearman rank correlation test, $p > 0.05$), indicating that these two parameters indeed reflect independent biological properties for each strain. Likewise, there was no significant correlation between the median growth rates or the median capacities between growth in RPMI and PPM, suggesting the activation of (partially) independent metabolic pathways in these two media. Surprisingly, while the median values for r and K were normally distributed for growth in RPMI (Shapiro-Wilk normality test, $p > 0.5$) they were not normally distributed for growth in PPM ($p < 0.0001$). Normal distributions are usually the rule when the phenotype is determined by the cumulative effect of individually small independent contributions from many loci. The observed growth differences in PPM might thus be due to differences at only a few genetic loci among the strains. As can further be seen in **Figure 4**, the variation in growth rates as well as in capacities were significantly larger between the strains than within each strain irrespective of the growth medium (Kruskal-Wallis rank sum test, $p < 10^{-5}$). These differences were, however, not reflected by their assignments to different clonal complexes or genomic groups reflecting their gene repertoire. In both growth media, the differences in the median growth parameters r and K between the different clonal complexes were not significantly larger than within the clonal complexes (Kruskal-wallis rank sum test, $p > 0.05$).

With respect to potential growth differences between strains from hyperinvasive and carriage lineages, there was however a surprisingly large and significant difference in the median

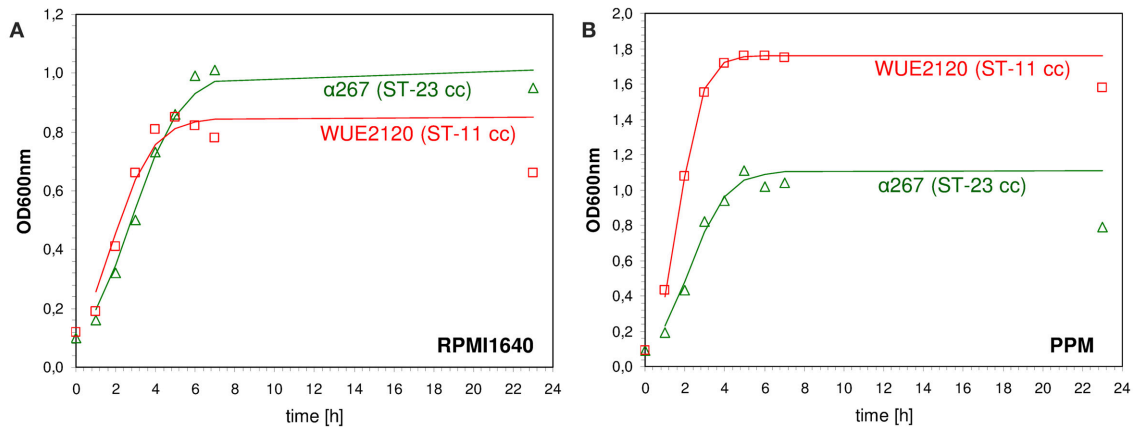

FIGURE 3 | Measured and fitted growth kinetics for two different meningococcal strains. The cultures were incubated at 37.0°C at 200 rpm either in defined medium (RPMI 1640, Biochrom AG, Germany) **(A)** or in rich medium [proteose peptone medium supplemented with 1% Polyvitex (Biomereux) (PPM+)] **(B)**. The optical density at 600 nm (OD_{600nm}) was determined at eight different time points over a period of 24 h starting with an initial optical density [$OD_{600nm}(t_0)$] of 0.1. A logistic growth model according to $OD_{600nm}(t) = \frac{K \cdot OD_{600nm}(t_0) \cdot e^{r \cdot t}}{K + OD_{600nm}(t_0) \cdot (e^{r \cdot t} - 1)}$ was fitted to the data by non-linear regression analysis using R version 2.7.0 and the nls package (R Development Core Team, 2008) to estimate the growth rate r [1/min] and the capacity K ($OD_{600\,nm}$ in the stationary phase). Red squares: ST-11 clonal complex strain WUE2120, green triangles: ST-23 clonal complex strain α267.

capacity between carriage strains and strains from hyperinvasive lineages when grown in PPM (Wilcoxon rank sum test with continuity correction, $p < 0.05$), which was higher in the hyperinvasive than in the carriage strains (**Figure 4**). To test for a potential correlation between epidemiological and experimental parameters we further compared for each strain the median *in vitro* growth rates and capacities, respectively, with the corresponding disease-carriage ratios based on its clonal assignment as taken from Caugant and Maiden (2009). As can be seen in **Figure 5**, there was indeed a slight correlation between the median carrying capacity in PPM and the carriage/disease ratio (Spearman's rank correlation $\rho = 0.31$, $p = 0.14$).

From these observations we conclude that compared to carriage strains the hyperinavisve strains are better equipped to cope with stress conditions prevailing in the stationary growth phase in PPM, and that this property is likely linked to their higher propensity to cause invasive meningococcal disease. In this respect it is particularly noteworthy that a correlation between the stages of a typical growth curve and virulence gene expression has been demonstrated in other bacterial pathogens like *Streptococcus pyogenes*, emphasizing an ordered progression of bacterial gene expression with genes required for colonization expressed during the exponential growth phase and genes involved for spread during the stationary phase, respectively (Kreikemeyer et al., 2003). We therefore, hypothesize that strains from carriage and hyperinvasive lineages might have a similar ability for colonization as reflected by their almost identical growth rates but differ in the expression of genes required for spread within the host as reflected by their different carrying capacities in PPM. Based on our results and the published literature reviewed above, we further hypothesize that strains from carriage and hyperinvasive lineages differ in the expression and regulation of genes involved particularly in oxidative stress responses and GSH metabolism under infection conditions.

GLUTAMATE METABOLISM PROVIDES A POTENTIAL LINK BETWEEN CARBON SOURCE AND OXIDATIVE STRESS RESPONSE, AND THUS MENINGOCOCCAL VIRULENCE

Pagliarulo et al. (2004) were the first to provide a mechanistic hypothesis how differences in the host environment might lead to growth and/or virulence differences among different meningococcal strains (**Figure 6**). They noted that glucose and lactate are present at very different ratios in microenvironments relevant to meningococcal infection. Accordingly, glucose is the predominant carbon source in blood as well as in CSF, whereas lactate is the major carbon source in saliva and in mucosal environments that are colonized by lactic bacteria, such as the nasopharynx. Lactate and pyruvate also tend to be used as major carbon and energy sources within phagocytic cells (Smith et al., 2001). They further noted that, in pathogenic *Neisseria*, there is evidence that the availability of different carbon sources affect the activity of the TCA cycle and consequently the intracellular pool of 2-oxoglutarate. Via two electron transport-linked lactic dehydrogenases, lactate provides energy by being immediate substrate for electron transport when it is oxidized to pyruvate (Smith et al., 2001). Pyruvate then provides energy and constituents of the TCA cycle like 2-oxoglutarate. As outlined above, glucose is metabolized largely via the ED and PP pathways, which generate relatively small amounts of energy (Baart et al., 2007). As a consequence, the intracellular pool of 2-oxoglutarate depends on the carbon source and is expected to be lower in glucose than in lactate-growing meningococci. They further showed that via the positive regulatory protein GdhR, 2-oxoglutarate indirectly represses the expression of the NADPH-specific glutamate dehydrogenases GdhA. As noted above, glutamate dehydrogenases are key enzymes that link energy and carbon metabolism, respectively, with nitrogen assimilation. The NADPH-specific enzymes like GdhA are primarily involved in ammonia assimilation and glutamate biosynthesis from the TCA cycle intermediate

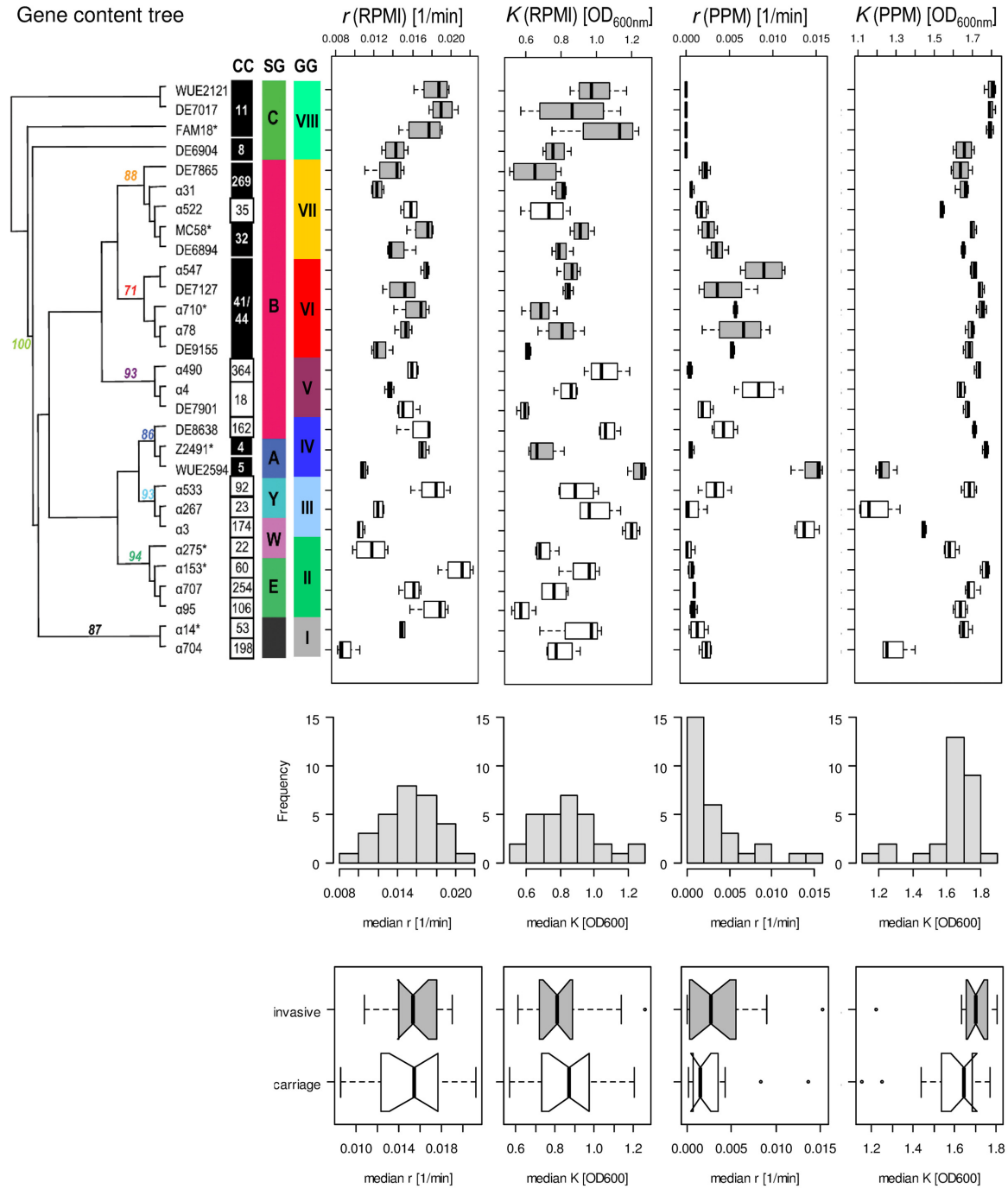

FIGURE 4 | Comparison of the fitted growth parameters for 29 strains from 20 clonal complexes. On the left in the upper panel, a maximum parsimony tree taken from Joseph et al. (2011) is shown with bootstrap values at nodes for grouping of the strains based on gene content. Strains with an asterisk right to their name have been used for computational analyses of intragenic recombination and pathway mapping as depicted in **Figure 1**. The clonal complexes (CC), serogroups (SG) and genome groups (GG) for each strain are given next to each strain. Consecutively and from left to right, box-and-whiskers-plots summarize for each strain the estimated growth rates and capacities in RPMI and PPM medium, respectively. The boxes indicate the upper and lower quartiles, respectively, and the lines in the middle of the boxes the corresponding median values. The whiskers show the largest and smallest observations, respectively, and outliers are plotted as individual points. Boxes for strains belonging to hyperinvasive clonal complexes are shaded in dark gray. In the middle panel, histograms with the marginal distributions for the median growth rates and capacities in RPMI and in PPM, respectively, are given. In the bottom panel, box-and-whiskers-plots comparing the distributions of the respective median values for strains from carriage and hyperinvasive lineages are shown. For each strain and condition, growth measurements were repeated four times (technical replicates) and from the fitted curves the two parameters *r* and *K* were determined for each growth experiment independently.

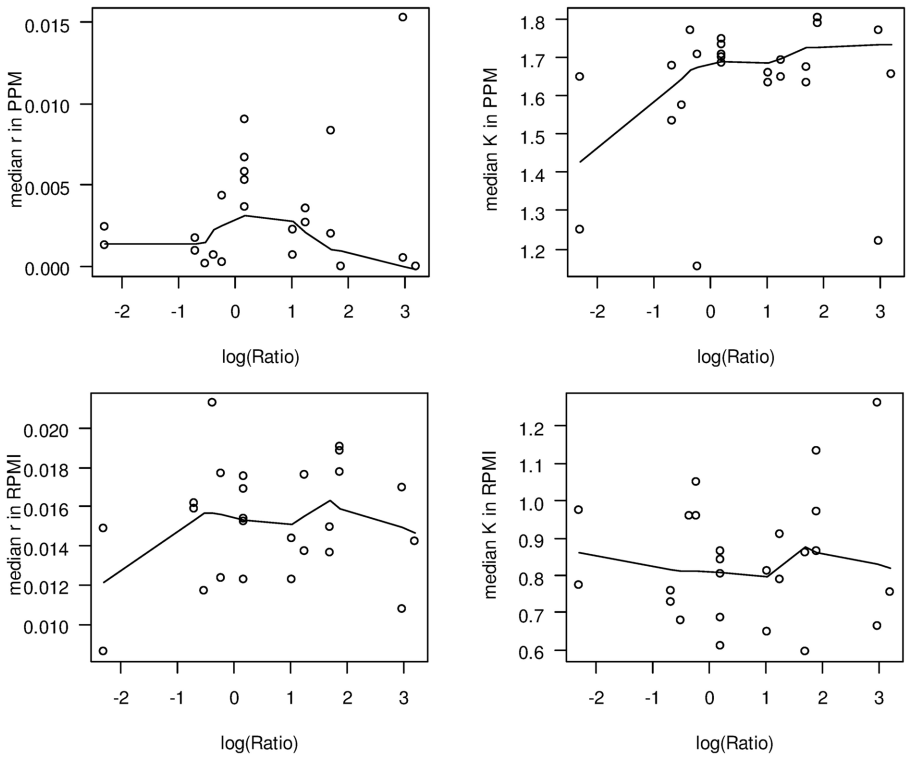

FIGURE 5 | Scatter plots comparing *in vitro* growth parameters and carriage/disease ratios. In each panel, the logarithm of the corresponding carriage/disease ratio is depicted for each clonal complex on the abscissa. The solid lines in each plot give a locally weighted scatter plot smoothing of the respective data. The ratios have been taken from Caugant and Maiden (2009) and are based on data deposited in the PubMLST database, which contains only those isolates submitted to it by members of the Neisseria research community. Although it represents the most comprehensive overview of the diversity observed to date, it is yet not a coherent population sample. **Upper left panel:** median growth rates in PPM; **upper right panel:** median capacities in PPM; **lower left panel:** median growth rates in RPMI; **lower right panel:** median capacities in RPMI.

2-oxoglutarate, which occurs most efficiently at high external ammonia concentrations. Via the level of 2-oxoglutarate affecting GdhA expression, the synthesis of L-glutamate is consequently linked to the available carbon source (Pagliarulo et al., 2004).

GdhA also links central carbon metabolism with the synthesis of GSH, which is synthesized from the amino acids L-glutamate, L-cysteine and L-glycine and prevents damage to important cellular components caused by ROS (Ritz and Beckwith, 2001) (**Figure 6**). L-Glycine and L-cysteine are both derived from L-serine, which is generated from 3-phosphoglycerate. GSH can further be converted to L-cysteine via Ggt and aminopeptidase N (PepN). In turn, L-cysteine can be converted into GSH, via GshA and GshB, yielding a functional γ-glutamyl cycle, and the finding that L-cysteine depletion causes oxidative stress (van de Waterbeemd et al., 2013) underscores the potential importance of this cycle in maintaining the redox balance (Ritz and Beckwith, 2001). GSH can be oxidized to glutathione disulfide (GSSG) by GpxA, thereby controlling the cellular hydrogen peroxide level, and GpxA mutants were much more sensitive to the oxidative stress caused by paraquat and slightly more sensitive to H_2O_2 (Seib et al., 2004). In further support of an important role of GSH and the γ-glutamyl cycle for meningococcal survival within the host and during invasive disease, *gdhA* was found to be essential for meningococcal survival in the infant rat model (Sun et al., 2000), and its expression was associated with invasive disease (**Figure 1**). In addition, a mutant strain deficient in Ggt did not grow in rat CSF (Takahashi et al., 2004), and Ggt, GshA and GdhA (but not the NADH-specific enzyme GluD) involved in L-glutamate biosynthesis were found to be up-regulated in blood (Echenique-Rivera et al., 2011; Hedman et al., 2012).

The γ-glutamyl cycle and GSH/glutamate metabolism might also have an important role once meningococci enter the stationary phase being subject to nutritional as well as oxidative stress. In line with this hypothesis, upon onset of stationary growth in *N. meningitidis* L-cysteine was found to be depleted and to constitute the growth-limiting component in chemically defined media (van de Waterbeemd et al., 2013). In addition, a decrease in the intracellular pool of 2-oxoglutarate was shown to be responsible for the induction of *gdhA* upon reaching the stationary (Pagliarulo et al., 2004).

The potential of several strains to express high levels of GdhA as demonstrated by Pagliarulo et al. (2004) may therefore result in growth advantages in the host in sites where glucose concentration is higher than that of lactate, and glutamate is present as a nitrogen (and carbon) source. Via the γ-glutamyl cycle it probably contributes to the enhanced stress resistance of hyperinvasive strains as also demonstrated by their higher growth capacities *in vitro* (**Figure 6**). Together, these two properties might

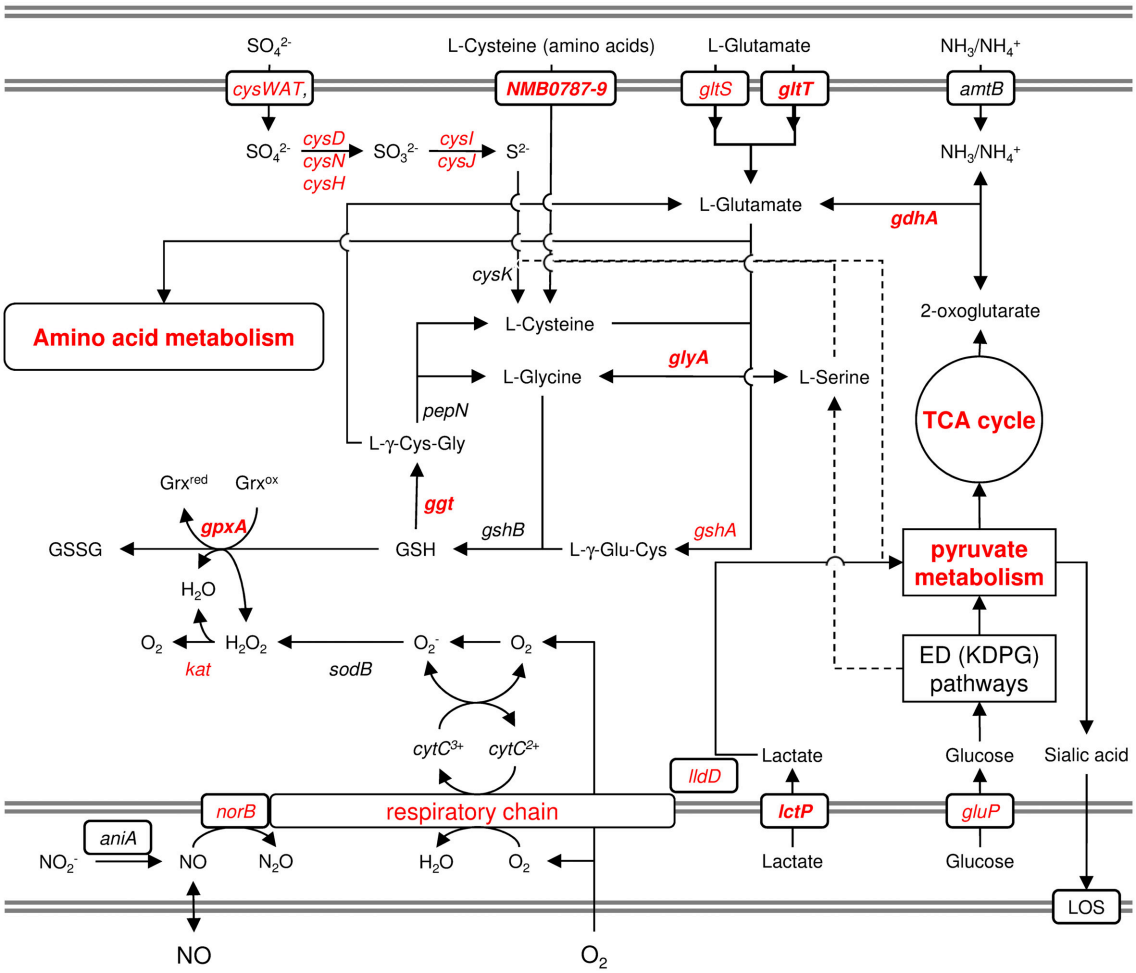

FIGURE 6 | Schematic graphical representation of the links between the oxidative stress response and the metabolism of lactate, cysteine and glutamine. This figure illustrates just the genes and pathways described in the text and does not give a comprehensive overview of the entire metabolism and stress responses of *N. meningitidis*. The gray double-lines indicate the inner and outer membrane, respectively. Genes and pathways that were found to be differently expressed under infection-mimicking conditions (blood) are depicted in red. Printed in bold are those genes whose deletion resulted in attenuation either in virulence assays *in vitro* or in the infant-rat model *in vivo* (Sun et al., 2000). For more details, see text.

conspire to allow a higher bacterial load in blood and CSF, which were the major determinants of clinical presentation and outcome (Ovstebo et al., 2004), and the highest *gdhA* expression levels were indeed found in strains belonging to hypervirulent lineages ST-32 (electrophoretic type (ET)-5, serogroup B) and IV-1 (serogroup A).

CONCLUSION AND OUTLOOK

It is very clear that it is not only the encoded repertoire of adhesins and invasins that allows the bacteria to adhere to host cells and to evade the innate and acquired immunity of the host, respectively, but foremost metabolic adaptation that enables the bacteria to exploit host resources to their advantage that plays a central role in its interaction with the host (Abu Kwaik and Bumann, 2013). Much of the lack of knowledge about nutritional virulence in meningococci may be attributed to inadequate animal models for this pathogen so much adapted to the human host (Vogel and Frosch, 1999), and better model systems that mimic the conditions prevailing inside the host will be needed to asses any phenotypic differences between carriage strains and strains from hyperinvasive lineages. Over 10 years ago Arthur Kornberg pointed out that more attention should be given to the study of the adaptations to the stationary phase, as the survival of any microbial species depends on being able to manage in the stationary phase (Kornberg et al., 1999). Therefore, we anticipate that the analysis of the transcriptomic and metabolic adaptations under stationary growth conditions will supplement existing experimental approaches in the study of virulence-associated mechanisms in meningococci, and with the ever increasing possibilities of high throughput technologies (Brochado and Typas, 2013) we are now prepared to enter the era of experimental population biology. Accordingly, comparative transcriptomics and metabolomics in combination with genome-wide mutation studies and systems modeling in a careful selection of strains from carriage as well as hyperinvasive lineages will allow identifying genes affecting meningococcal pathosystem.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Christoph Schoen. Performed the experiments: Biju Joseph Ampattu and Laura Kischkies. Analyzed the data: Christoph Schoen and Johannes Elias. Wrote the paper: Biju Joseph Ampattu and Christoph Schoen.

ACKNOWLEDGMENTS

We would like to thank Barabara Conrad and Kathrin Bevilacqua for their excellent technical assistance, Roland F. Schwarz (EMBL-EBI, Hinxton, Cambridge, UK) for help with growth data analysis and Gabriele Gerlach (IHM, Würzburg, Germany) for careful reading of the manuscript. We are also grateful to Ming-Shi Li (Imperial College, London) for the sharing microarray data associated with the study described in Hey et al. (2013). This work was supported by the German Research Foundation (DFG) grant SCHO 1322/1-1. The publication was funded by the DFG and the University of Wuerzburg in the funding programme Open Access Publishing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 June 2014; paper pending published: 08 July 2014; accepted: 31 July 2014; published online: 20 August 2014.

*Citation: Schoen C, Kischkies L, Elias J and Ampattu BJ (2014) Metabolism and virulence in Neisseria meningitidis. Front. Cell. Infect. Microbiol. **4**:114. doi: 10.3389/fcimb.2014.00114*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 12 August 2014
doi: 10.3389/fcimb.2014.00107

The arginine-ornithine antiporter ArcD contributes to biological fitness of *Streptococcus suis*

Marcus Fulde[1,2†‡], Joerg Willenborg[1‡], Claudia Huber[3], Angela Hitzmann[2], Daniela Willms[1], Maren Seitz[1], Wolfgang Eisenreich[3], Peter Valentin-Weigand[1]* and Ralph Goethe[1]

[1] Department of Infectious Diseases, Institute for Microbiology, University of Veterinary Medicine, Hannover, Germany
[2] Department of Medical Microbiology, Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany
[3] Lehrstuhl für Biochemie, Technische Universität München, Garching, Germany

Edited by:
Thomas Dandekar, University of Wuerzburg, Germany

Reviewed by:
Sven Hammerschmidt, Universität Greifswald, Germany
Stephan Fuchs, University Greifswald, Germany

****Correspondence:***
Peter Valentin-Weigand, Zentrum für Infektionsmedizin, Institut für Mikrobiologie, Stiftung Tieraerztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany
e-mail: peter.valentin@tiho-hannover.de

†Present address:
Marcus Fulde, Institute for Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany

‡These authors have contributed equally to this work.

The arginine-ornithine antiporter (ArcD) is part of the Arginine Deiminase System (ADS), a catabolic, energy-providing pathway found in a variety of different bacterial species, including the porcine zoonotic pathogen *Streptococcus suis*. The ADS has recently been shown to play a role in the pathogenicity of *S. suis*, in particular in its survival in host cells. The contribution of arginine and arginine transport mediated by ArcD, however, has yet to be clarified. In the present study, we showed by experiments using [U-$^{13}C_6$]arginine as a tracer molecule that *S. suis* is auxotrophic for arginine and that bacterial growth depends on the uptake of extracellular arginine. To further study the role of ArcD in arginine metabolism, we generated an *arc*D-specific mutant strain and characterized its growth compared to the wild-type (WT) strain, a virulent serotype 2 strain. The mutant strain showed a markedly reduced growth in chemically defined media supplemented with arginine when compared to the WT strain, suggesting that ArcD promotes arginine uptake. To further evaluate the *in vivo* relevance of ArcD, we studied the intracellular bacterial survival of the *arc*D mutant strain in an epithelial cell culture infection model. The mutant strain was substantially attenuated, and its reduced intracellular survival rate correlated with a lower ability to neutralize the acidified environment. Based on these results, we propose that ArcD, by its function as an arginine-ornithine antiporter, is important for supplying arginine as substrate of the ADS and, thereby, contributes to biological fitness and virulence of *S. suis* in the host.

Keywords: *Streptococcus suis*, zoonosis, arginine-ornithine antiporter, biological fitness, arginine deiminase system

INTRODUCTION

Streptococcus (*S.*) *suis* is a frequent colonizer of mucosal surfaces of the upper respiratory and the gastrointestinal tract in pigs. As a facultative pathogen, *S. suis* is able to cross epithelial barriers and induce a variety of fatal diseases, such as meningitis, septicaemia, arthritis, and bronchopneumonia. Thus, high economic losses characterize *S. suis* as one of the most important agents in pig breeding and pork processing industries (Clifton-Hadley and Alexander, 1980; Arends and Zanen, 1988; Chanter et al., 1993; Staats et al., 1997; Swildens et al., 2004; Fulde and Valentin-Weigand, 2013).

S. suis is receiving increasing attention as a zoonotic agent due to outbreaks in China in 1998 and 2005. Noteably, *S. suis* is currently considered as the most frequent cause of adult bacterial meningitis in Vietnam (Tang et al., 2006; Yu et al., 2006; Mai et al., 2008; Wertheim et al., 2009). Furthermore, recent reports from different countries of human infections indicate a growing awareness of *S. suis*' zoonotic potential (Lun et al., 2007). However, despite of its increasing importance, pathogenesis of *S. suis* infections in humans and pigs including knowledge on bacterial virulence factors and host responses is far from being understood (Fulde and Valentin-Weigand, 2013).

One of first virulence-associated traits discovered for *S. suis* was the arginine deiminase system (ADS). The ADS comprises an enzymatic pathway converting arginine to citrulline with the concomitant production of ornithine, ammonia, carbon dioxide, and ATP (Cunin et al., 1986; Barcelona-Andrés et al., 2002). The wide distribution of the ADS among all kingdoms of life and the high conservation of genes and their arrangement supports its outstanding role as a secondary, energy providing pathway (Gruening et al., 2006). The ADS has a pivotal role in the pathogenicity of many bacteria, such as *Streptococcus* spp., *Listeria monocytogenes*, *Staphylococcus* spp. and parasites, such as *Giardia lamblia*. Under acidic conditions, e.g., in the phagolysosome of host cells, the ammonium produced by the ADS-dependent arginine catabolism is sufficient to significantly prolong the intracellular survival of the bacteria (Casiano-Colon and Marquis, 1988; Curran et al., 1995; Degnan et al., 1998; Benga et al., 2004; Ryan et al., 2009; Fulde et al., 2011; Cheng et al., 2013; Lindgren et al., 2014). In addition, the arginine deiminase ArcA and the antimicrobial host protein inducible NO-synthase (iNOS) compete for the

same substrate as shown for the intestinal pathogen *G. lamblia* (Ringqvist et al., 2008).

The important role of the ADS in metabolism and pathogenesis suggests a tight regulation by a complex regulatory network which responds to a variety of different environmental stimuli. Indeed, type and amount of carbon sources, oxygen tension, substrate (arginine) availability and temperature have been shown to directly or indirectly influence ADS expression (Winterhoff et al., 2002; Dong et al., 2004; Gruening et al., 2006; Zeng et al., 2006; Makhlin et al., 2007; Liu et al., 2008; Ryan et al., 2009; Fulde et al., 2011; Willenborg et al., 2011, 2014; Hitzmann et al., 2013). In *S. suis*, the transcriptional regulator ArgR is highly specific for the regulation of ADS, underlining a particular relevance of arginine and its catabolism by the ADS for the metabolism of *S. suis* (Fulde et al., 2011). Two genes with significant homologies to an arginine-ornithine antiporter (*arc*D) and a putative Xaa-His dipeptidase (*arc*T) are associated with the ADS of some bacteria including streptococci. In *Pseudomonas aeruginosa*, ArcD is a transmembranal protein composed of 13 helices (Lüthi et al., 1990; Verhoogt et al., 1992; Bourdineaud et al., 1993). Similar to ArcD of *Lactococcus* (*Streptococcus*) *lactis*, ArcD facilitates an ATP-independent, electro-neutral exchange of arginine and ornithine across the bacterial membrane, thereby providing a substrate for ADS mediated arginine catabolism (Driessen et al., 1987; Verhoogt et al., 1992; Bourdineaud et al., 1993). The putative function of ArcD as an arginine-ornithine antiporter was also shown by Wimmer et al. for the archaeon *Halobacterium salinarum* (Wimmer et al., 2008). On the other hand, an involvement of ArcD in virulence is yet speculative. A recent publication by Gupta et al. (2013) showed that an *arc*D-deficient *S. pneumoniae* mutant was attenuated in murine models of pneumonia and bacteraemia. However, whether or not the *arc*D gene of *S. pneumoniae* functions as an arginine-ornithine antiporter remained unanswered.

The present study focused on ArcD of *S. suis*. Determination of extracellular arginine and intracellular ornithine confirmed an involvement of ArcD in arginine uptake. Subsequent phenotypic characterization of an isogenic *arc*D-deficient mutant strain revealed a significant attenuation in terms of biological fitness and survival under acidic conditions.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

S. suis strain 10 (Smith et al., 1999), a highly virulent serotype 2 strain, was used in this study. Bacteria were routinely grown on blood agar plates (BD) at 37°C with 5% CO_2, or cultivated in liquid TSB (BD, Heidelberg, Germany) medium under the same conditions. Following day, bacteria were adjusted to an optical density at 600 nm of 0.05 in a tryptone-yeast-based medium supplemented with 10 mM glucose or galactose, respectively. If indicated, 50 mM arginine was supplemented (Burne et al., 1987; Zeng et al., 2006). Auxotrophy studies were performed in a chemically defined medium (CDM) in the presence of absence of arginine essentially as described elsewhere (van de Rijn and Kessler, 1980; Hitzmann et al., 2013). Growth was monitored every hour using a Nova Spec II Photometer (Pharmacia, Freiburg, Germany). Assays were performed in triplicates and repeated at least four times.

To determine the transcriptional organization of the ADS, bacteria were grown in TY medium supplemented with 50 mM arginine and 10 mM glucose or galactose, respectively, to an OD_{600} of 0.2. Then, 10 ml of bacterial culture were harvested by centrifugation. Pellets were resuspended in 1 ml of Trizol (Invitrogen/Life Technologies, Carlsbad, California, USA) and immediately snap-frozen in liquid nitrogen.

DNA AND RNA TECHNIQUES, cDNA SYNTHESIS AND REVERSE TRANSCRIPTASE PCR

If not stated otherwise, all enzymes and reagents were purchased from Invitrogen (Life Technologies, Carlsbad, California, USA) and NEB (New England Biolabs, Frankfurt am Main, Germany). Chromosomal DNA was prepared using the Qiagen's DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Plasmid DNA was purified with the NucleoSpin® Plasmid Kit (Macherey-Nagel, Dueren, Germany) according to manufacturer's instructions. RNA was prepared as described by Hitzmann et al. (2013) using the Ambion's RiboPure™-Bacteria Kit. Residual DNA was digested with the Ambion TURBO DNA-free™-kit. Complementary DNA (cDNA) was synthesized from 1 µg total RNA using random primers (3 µg). Primers and RNA were heated for 10 min at 70°C in 12 µl of dH2O and then chilled on ice. Eight µl of master mix, consisting of 4 µl 5x first strand buffer, 2 µl 10 mM dNTP mix, 1 µl RNase Inhibitor and 1 µl 100 mM DTT, were added and incubated for 5 min at 25°C. Then, 1 µl of Reverse Transcriptase (SuperScript II) was added and another 10 min incubation-step at 25°C followed. Then the reaction was incubated at 42°C for 1 h and the Reverse Transcriptase was inactivated. cDNA was purified using the Qiagen PCR-purification kit. To analyse the transcriptional organization of the ADS, RT-PCR was performed using following intergenic primer pairs: *flp*S-*arc*A (CGA TGG TCT TGT TTG AAA CCT/ACA CCA GCC ATC GTT TTC TC), *arc*D-*arc*T (CTC CAC ATG GGT GAA GAA GG/CGC CAT CGA AGG ACC TTT A), *arc*T-*arc*H (CTG CGG ATA AAG AAG CCC TA/CTG ATG CTG GCT GTT GGT TA).

MUTAGENESIS

ArcD was inactivated by insertion mutagenesis as described earlier (Fulde et al., 2011). Briefly, the gene *arc*D was amplified from the streptococcal genome with the primers *arc*DKOfor (CCG TTA CTG TGG CTG AAT TGG) and *arc*DKOrev (CCT TGC AAT CCT TCT TCA CC) and subsequently introduced into the cloning vector *p*GEM®-T Easy (Promega, Mannheim, Germany). The resulting plasmid *p*GEM-*arc*D was linearized by *Hpa*I. Then, the *Pvu*II released erythromycin resistance cassette derived from vector *p*IC*erm* (kindly provided by Christoph Baums, Institute for Microbiology, University of Veterinary Medicine, Hannover) was introduced to disrupt *arc*D. Electroporation was essentially performed as previously described (Smith et al., 1995). Mutants were tested for integrity by PCR using the primer pair *arc*DKOfor/*arc*DKOrev.

QUANTIFICATION OF ARGININE

A method to determine arginine in the supernatant of bacterial cultures was developed based on the Sakaguchi reaction (Sakaguchi, 1925). Briefly, bacteria were grown in TY medium in the presence of 10 mM arginine. At an OD_{600} of 0.2, streptococci were harvested by centrifugation and the resulting supernatant was filtrated using the Millex® Syringe Filters with pore size of 0.22 μm (Merck Millipore, Schwalbach, Germany). Then, 100 μl of bacterial supernatant was mixed with 100 μl reagent A (0.05% (w/v) chloronaphthol, 5% urea (m/v) in 95% EtOH). After extensive shaking, 200 μl reagent B (0.7% Brom (v/v), 5% NaOH (w/v) in H_2O) was added. A change in color was determined spectrophotometrically by an OD of 500 nm. Quantification was done along a calibration curve with different concentrations of arginine diluted in TY medium. Non-inoculated TY medium served as control.

LABELING EXPERIMENTS USING [U-$^{13}C_6$]ARGININE

In all labeling experiments, a CDM overnight culture of the indicated *S. suis* strain was harvested by centrifugation, washed twice in PBS, and then inoculated in fresh CDM to an OD_{600} of 0.002. In experiments determining the ^{13}C-label in proteinogenic amino acids, bacteria were grown in CDM containing 2.5 mM [U-$^{13}C_6$]arginine (Campro Scientific) at 37°C and harvested at an OD_{600} of 0.2 by centrifugation at 4000 × g at 4°C for 5 min. The bacterial cells were washed twice in ice-cold PBS, immediately autoclaved at 120°C for 15 min, and lyophilized. Samples were hydrolyzed under acidic conditions. The resulting amino acids were purified using a cation exchange column, converted into TBDMS derivatives (except for arginine, see below), and analyzed by GC/MS as described earlier (Eylert et al., 2008). For the [U-$^{13}C_6$]arginine uptake experiments, bacteria were first grown in CDM with ^{12}C-arginine to an OD_{600} of 0.2, then harvested by centrifugation, washed twice in PBS, and afterwards transferred to CDM without any arginine to induce arginine starvation for 15 min at 37°C. The bacteria were concentrated by centrifugation and then incubated in CDM containing 2.5 mM [U-$^{13}C_6$]arginine at 37°C for 30 min. Bacteria were then pelleted by centrifugation, washed twice in ice-cold double-distilled water, and immediately disrupted by ultrasonic disintegration in a Branson Sonifier with continuous water cooling for 15 min at 4°C, and output control of 8. The lysates were cleared by centrifugation at 10,000 × g at 4°C for 30 min and the remaining supernatant lyophilized for further analysis.

ARGININE AND ORNITHINE DETERMINATION

Arginine does not form trimethylsilyl (TMS) and tert-butyldimethylsilyl (TBDMS) derivatives (Halket et al., 2005) and can be analyzed as arginine- trifluoroacetic acid (TFA)-methylester (Darbre and Islam, 1968). An aliquot of the cation exchange eluate described above was dried under a stream of nitrogen and dissolved in 200 μl of methanolic HCl (3N). The mixture was heated to 70°C for 30 min and then dried under a stream of nitrogen. The residue was dissolved in 50 μl of TFA and heated to 140°C for 10 min. The mixture was dried again, dissolved in 100 μl of anhydrous ethylacetate and subjected to GC/MS analysis. General GC/MS conditions were the same as described for amino acid TBDMS derivatives (Eylert et al., 2008). For TFA-methylester derivatives the column was kept at 70°C for 3 min and then developed with a temperature gradient of 10°C min^{-1} to a final temperature of 200°C that was kept for 3 min. The retention time for the arginine-TFA-methylester was 17.2 min. The molecular mass of the arginine derivative was 476. ^{13}C-excess calculations were performed with m/z 407 $[M\text{-}CF_3]^{+\cdot}$, a fragment still containing all C atoms of arginine.

Ornithine was determined from the freeze dried supernatant and derivatized as described for arginine. Under identical GC/MS conditions, ornithine was analyzed as ornithine-TFA-methylester (M: 338) at R_t 14.0 min. The observed fragment m/z 306 corresponds to $[M\text{-}CH_3OH]$. The $^{13}C/^{12}C$ ratio was calculated with the relative intensities of m/z 306 (^{12}C-ornithine) and m/z 311 ([U-$^{13}C_5$]ornithine).

DETERMINATION OF pH AND AMMONIA IN THE CULTURE SUPERNATANT

Determination of ammonia in the culture supernatant of WT strain 10 and its *arc*D-deficient mutant strain was performed using the ammonia assay kit (Sigma, Munich, Germany) as described previously (Fulde et al., 2011). pH values in the bacterial culture supernatant were determined using a specific electrode (pH 197, WTW, Weilheim, Germany).

DETERMINATION OF ARGININE DEIMINASE (AD) ACTIVITY

AD activity was determined according to the protocol of Oginsky (1957) and Degnan et al. (1998) as described previously (Gruening et al., 2006; Winterhoff et al., 2002). Briefly, bacteria were grown in CDM medium as in the [U−$^{13}C_6$]arginine uptake assays and harvested by centrifugation. Then, bacteria were lysed and the respective lysates were incubated for 2 h in a 0.1 M potassium phosphate buffer containing 10 mM L-arginine at 37°C. The supplementation of 250 μl of an acidic solution (1:3, 96% sulfuric acid and 85% orthophosphoric acid) stopped enzymatic reactions. After addition of 31.3 μl of a 3% diacetyl monoxime solution, the suspension was incubated for 15 min at 100°C. Production of citrulline was determined colorimetrically at an OD_{450}. Results are given in nmol citrulline produced in 1 h per mg whole cell protein

BACTERIAL SURVIVAL UNDER ACIDIC CONDITIONS

Experiments were performed essentially as described earlier (Benga et al., 2004; Gruening et al., 2006). Briefly, WT strain 10 and its isogenic mutant strain 10Δ*arc*D were grown overnight in TSB. Then, bacteria were harvested by centrifugation and resuspended in a buffer containing 20 mM Na_2HPO_4, 1 mM $MgCl_2$, 25 mM arginine-HCl adjusted to pH 5, 6, or 7, respectively. Bacteria were incubated at 37°C for the indicated intervals and survival was monitored by plating. Results represent means and standard deviations of one experiment performed in triplicates. Experiments were repeated at least three times.

INTRACELLULAR SURVIVAL OF *S. SUIS* IN HEp-2 CELLS

The ability of the wild-type strain 10 and the *arc*D deficient mutant strain to survive in HEp-2 epithelial cells was determined as described previously with some modifications (Benga et al., 2004; Fulde et al., 2011). Briefly, in addition to untreated HEp-2 cells, parallel assays were done with HEp-2 cells that had been pretreated with bafilomycin (200 nM) for 1 h to inhibit endosomal

acidification. HEp-2 cells were then infected with 100 bacteria per cell (MOI 100:1) for 2 h and afterwards washed thrice with PBS. In parallel, cells were incubated in DMEM containing 31.25 μg ml^{-1} Daptomycin (Cubicin®) for 90 and 210 min, respectively, at 37°C with 8% CO_2 to kill extracellular bacteria. The monolayers were washed three times with PBS and 100 μl trypsin-EDTA solution was added to each well. After 5 min, 900 μl of 1% sterile saponin was added and the lysates were plated in triplicates on blood agar and incubated at 37°C for 24 h. The number of CFU was determined at 90 and 210 min post-infection of the cells and expressed as percentage of intracellular bacterial survival after 2 h. Thus, one hundred percent indicates that no difference in intracellular CFU was detected after two hours. The experiments were repeated three times.

COMPUTATIONAL ANALYSIS

Prediction of localization and topology of ArcD was performed using the SignalP 4.1 Server and the TMHMM Server v. 2.0 available at: http://www.cbs.dtu.dk.

RESULTS

THE *S. SUIS* ADS IS TRANSCRIBED IN FIVE TRANSCRIPTIONAL UNITS

The ADS is a highly conserved cluster of seven genes encoding the most important arginine-catabolizing pathway in *S. suis* and two flanking genes encoding for transcriptional regulators (**Figure 1A**, Gruening et al., 2006). The core ADS, facilitating the degradation of arginine to ATP, is composed of three genes: *arc*A, encoding for an arginine deiminase; *arc*B, an ornithine-carbamoyltransferase, and *arc*C, a carbamate kinase. These genes are in close proximity to the putative arginine-ornithine antiporter gene *arc*D and a potential Xaa-His dipeptidase gene *arc*T, as well as *arc*H, a putative endo-β-galactosidase C. Thus, we performed RT-PCR analysis using intergenic primer pairs from RNA of bacteria grown under inducing (TY medium supplemented with 50 mM arginine and 10 mM galactose) and repressive (50 mM arginine and 10 mM glucose) conditions. As depicted in **Figure 1B**, a positive PCR signal indicating polycistronic transcription was only detected for the *arc*D-*arc*T intergenic region (primer pair C/D) indicating expression of *arc*D and *arc*T from an operon. Interestingly, similar to what is known for the *arc*ABC operon (Gruening et al., 2006), the transcription of *arc*DT was significantly increased when galactose was present as the sole carbon source. In contrast, the regulatory gene *flp*S (intergenic primer pair A/B) located upstream of *arc*A as well as the accessory gene *arc*H (primer pair E/F) were transcribed monocistronically.

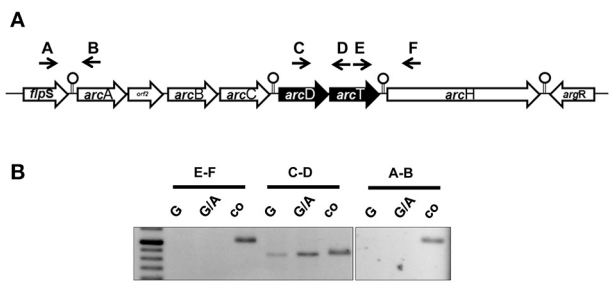

FIGURE 1 | The *S. suis* ADS is transcribed in five transcriptional units. **(A)** Schematic representation of the ADS of *S. suis*. Genes are depicted as arrows pointing toward their transcriptional orientation. Intergenic primers were enumerated with capitals. Stemloop structures separate single transcriptional units. **(B)** Semi-quantitative RT-PCR analysis of *S. suis* WT strain 10 grown in a tryptone-based medium supplemented with 10 mM glucose (G) or 10 mM galactose (G/A) and 50 mM arginine, respectively. Chromosomal DNA was used as positive control. Capitals indicate the intergenic primers depicted in **(A)**.

ArcD CONTRIBUTES TO GROWTH OF *S. SUIS* AND ENVIRONMENTAL pH HOMEOSTASIS

In silico analysis of the *S. suis* ArcD revealed significant homologies to transmembranal proteins with arginine-ornithine antiporter function of other streptococci and other arginine-fermenting bacteria (**Table 1**). However, functional studies on this topic are rare. Therefore, we inactivated *arc*D by insertion mutagenesis and characterized the phenotype of the mutant by growth kinetics. As depicted in **Figure 2A**, a comparable growth of WT strain 10 and its isogenic mutant strain 10Δ*arc*D was observed in the first hours of growth with a mean OD_{600} ranging between 0.0279 ± 0.008 (WT, with arginine supplementation) and 0.0315 ± 0.0055 (10Δ*arc*D, without arginine supplementation). After 4 h, the growth of WT strain 10 (red lines) was higher as compared to the *arc*D-deficient mutant strain (blue lines). Interestingly, supplementation of arginine (solid lines) did not lead to a higher growth, neither of WT strain 10 nor of the mutant until 5 h. After 6 h, WT strain 10 reached an OD_{600} of 0.2253 ± 0.047 (broken red line) without arginine supplementation, and an OD_{600} of 0.3983 ± 0.12 (solid red line) when arginine had been supplemented. In contrast, significantly lower OD values were detected for the *arc*D-deficient mutant strain, with (0.1343 ± 0.025, solid blue line) and without arginine supplementation (0.093 ± 0.001, broken blue line). Nevertheless, though less prominent the mutant strain 10Δ*arc*D showed an arginine-dependent phenotype similar to the WT strain. Overall, differences in bacterial numbers and arginine availability increased over time between both strains.

Next we monitored changes in the pH of the medium during growth of WT strain 10 and its *arc*D-deficient mutant. As depicted in **Figure 2B**, pH values of the culture medium decreased similarly for strain 10 and 10Δ*arc*D without arginine supplementation. Nevertheless, a slight difference between strain 10 (6.855 ± 0.065) and 10Δ*arc*D (6.655 ± 0.005) was detected at 8 h of growth. This difference was even more prominent when external arginine was supplemented to the growth medium (solid lines). WT strain 10 was able to antagonize growth-dependent acidification of the culture medium resulting in an increase in pH from 6.905 ± 0.059 at the time of inoculation to 8.063 ± 0.2 (solid red line) after 24 h, whereas the pH values detected for the *arc*D-deficient strain (blue lines) dropped similarly to those monitored without arginine supplementation from 6.8425 ± 0.03 (0 h) to 5.54 ± 0.07 (24 h).

Our previous studies showed that ADS-dependent ammonia production as a by-product of arginine catabolism is essentially involved in environmental pH homeostasis (Fulde et al., 2011). To investigate whether similar effects hold true for the ArcD-deficient mutant strain, we determined ammonia production

Table 1 | Comparison of putative arginine-ornithine transporters of different bacterial species.

Species	Protein name	Identities[a]	Transmembranal helices[b]	Accession number
S. suis	ArcD	–	13	AAY78938
S. gordonii	ArcD	71	12	ABV10292
S. pneumoniae	Arginine-ornithine antiporter	68	12	ACA36359
S. uberis	C4-dicarboxylate anaerobic carrier protein	64	12	CAR42879
S. pyogenes	Arginine-ornithine antiporter	64	13	AAT87428
S. equi sub. *zooepidemicus*	ArcD	63	13	ACG91640
Enterococcus faecium	C4-dicarboxylate anaerobic carrier, arginine transporter	57	12	EEI60191
Vibrio parahaemolyticus	Arginine-ornithine antiporter	44	10	EED27648
Escherichia. coli	ArcD	44	13	YP_001816563
Pseudomonas aeruginosa	ArcD	25	13	AAA25719

[a] *Based on ArcD of S. suis (accession number: AAY78938).*

[b] *Prediction, as evaluated by TMHMM.*

of WT strain 10 and its *arc*D-deficient mutant strain grown under conditions with and without supplementation of arginine (**Figure 2C**). As expected, supplementation led to a more than 10-fold increase of ammonia production (0.11 ± 0.08 mg ml^{-1} vs. 1.37 ± 0.36 mg ml^{-1}) for WT strain 10. Interestingly, although strain 10Δ*arc*D was also able to increase the amount of ammonia in the presence of arginine (0.084 ± 0.04 mg ml^{-1} vs. 0.026 ± 0.001 mg ml^{-1}), this effect was comparable to that seen in growth of WT strain without arginine supplementation.

Since ArcD is predicted to be an arginine-ornithine antiporter, we wondered whether a deletion in the respective gene would lead to deficiencies in arginine uptake. For this, we adapted the method described by Sakaguchi (1925) to determine extracellular arginine concentrations. As depicted in **Figure 2D**, WT strain 10 was able to completely deplete free arginine from the bacterial culture medium (red bar). In contrast, the medium inoculated with strain 10Δ*arc*D (blue bar) still contained significantly higher amounts of arginine (10.371 ± 0.18 mM) at the same OD.

In summary, these results indicate that ArcD is involved in the arginine uptake which is necessary to support the central functions of the ADS. Furthermore, they show that extracellular arginine is important for bacterial growth and a substrate for the arginine deiminase system in *S. suis*.

S. SUIS IS AUXOTROPHIC FOR ARGININE PROVIDED BY ArcD

In order to demonstrate the contribution of ArcD to arginine uptake we performed growth experiments in a chemically defined medium (CDM) containing all amino acids including or excluding arginine. These experiments revealed that *S. suis* strain 10 and strain 10Δ*arc*D were not able to grow in CDM medium containing all amino acids except arginine (**Figure 3A**). Supplementation of arginine restored growth of both strains, even though the growth of strain 10Δ*arc*D was remarkably diminished when compared to that of the parental strain. These data indicate that *S. suis* strain 10 is auxotrophic for arginine and that ArcD contributes to arginine uptake.

In order to verify that *S. suis* is capable to take up arginine by ArcD and incorporate arginine in newly synthesized proteins, we performed labeling experiments in CDM supplemented with [U-$^{13}C_6$]arginine that were followed by the detection of the ^{13}C-label in protein derived amino acids by GC/MS analysis. As depicted in **Figure 3B**, [U-$^{13}C_6$]arginine was taken up and used for protein biosynthesis in both strains. It is important to note that in this experiment the ^{13}C excess in arginine is no quantitative value for uptake, because [U−$^{13}C_6$]arginine is the sole arginine source which is also taken up by the *arc*D-deficient strain (**Figure 3A**). However, high levels of ^{13}C-enrichment were not detected in any other proteinogenic amino acid excluding *de novo* biosynthesis of these amino acids from [U-$^{13}C_6$]arginine as a precursor. Nevertheless, ^{13}C-excess below 1 mol% was found as a $^{13}C_1$-labeled isotopolog in aspartate. This indicates that $^{13}CO_2$, formed as a by-product of ADS mediated [U-$^{13}C_6$]arginine catabolization, is used as a precursor in a carboxylation reaction required for aspartate biosynthesis. However, the overall ^{13}C excess in arginine did not differ between the WT strain and strain 10Δ*arc*D. Since bacteria were harvested at the same optical density this may be an explanation for that, and these results emphasize that arginine uptake is the growth limiting step for strain 10Δ*arc*D in CDM.

To further elucidate if 10Δ*arc*D has a reduced capacity to take up arginine, we reduced the [U-$^{13}C_6$]arginine labeling time to 30 min and determined the $^{13}C/^{12}C$ ratio of free intracellular ornithine, a product of the arginine deiminase pathway, since the free arginine levels were under the detection limit. The efficiency of [U-$^{13}C_6$]arginine derived ^{13}C incorporation in intracellular [U-$^{13}C_5$]ornithine was approximately 15-fold higher in the WT strain when compared to strain 10Δ*arc*D (**Figure 3C**, left panel). The arginine deiminase activity did not differ between both strains under these conditions (**Figure 3C**, right panel) which excluded a different arginine consumption of the strain. Taken together, these results indicate that ArcD is an arginine transporter.

ArcD FACILITATES SURVIVAL UNDER ACIDIC CONDITIONS

The above data indicate a central role of arginine and arginine uptake for the metabolism of *S. suis.* Therefore, we next analyzed the relevance of ArcD for bacterial survival. As shown in **Figure 4A** survival of WT strain 10 (black bars) and its isogenic, *arc*D-deficient mutant strain 10Δ*arc*D (white bars) differed when incubated in an arginine-containing buffer with pH values adjusted to 5.0, 6.0, and 7.0, respectively. Bacteria were replica-plated after 4 h to monitor survival. No significant differences were observed at pH values of 7.0 and 6.0. However,

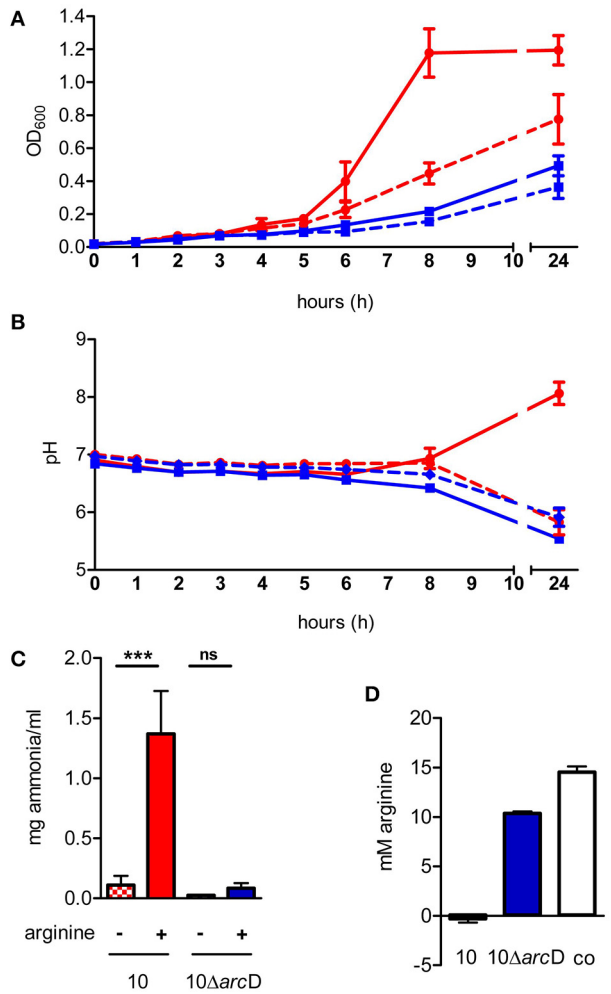

FIGURE 2 | The *S. suis* ArcD contributes to environmental pH homeostasis and biological fitness in an arginine-dependent manner. **(A)** Growth kinetics of *S. suis* WT strain 10 (red) and its isogenic *arc*D-deficient mutant strain 10Δ*arc*D (blue) in a tryptone-based medium containing 10 mM galactose (dotted lines) and 50 mM arginine (solid lines) if indicated. The optical density at 600 nm OD_{600} was measured every hour. Data represent means and standard deviations of a representative experiment performed in triplicates. Experiments were repeated at least two times. **(B)** Bacteria were grown as described in **(A)**, the course of pH values were measured every hour. Data represent means and standard deviations of a representative experiment performed in triplicates. Statistics were performed in a one-tailed *t*-test with $p < 0.01$. **(C)** Ammonia production of the culture supernatant was measured after 24 h of growth. Results are given as mg ammonia per ml of medium (ml^{-1}). Statistical significance is indicated for a one-sided *t*-test (***$p < 0.001$; ns, not significant). **(D)** The amount of arginine in the culture supernatant of WT strain 10 (red bar) and 10Δ*arc*D (blue) was detected by a method adapted from Sakaguchi (1925). Results are given in mM arginine. TY medium alone (white bar) served as negative control.

at pH 5.0, the survival rate of the WT strain was 65.5% ± 6.5, whereas the *arc*D-deficient mutant strain was almost completely killed (0.5% ± 0.5). As a control, bacteria were incubated in buffer adjusted to pH 5.0 without the supplementation of arginine. Under these conditions, strain 10 and 10Δ*arc*D were similarly affected in survival emphasizing the important role of ArcD as an arginine supplier for ADS-mediated resistance under acidic conditions.

In order to elucidate the relevance of ArcD for biological fitness of *S. suis* in a biological model, we performed infection experiments with the epithelial cell line HEp-2. As shown in **Figure 4B**, the WT strain 10 was able to survive intracellularly at a rate of about 70%, whereas significantly lower survival rates (approximately 35%) were determined for the *arc*D mutant strain. To analyse whether reduced survival correlated with acidification and, thus, the inability of strain 10Δ*arc*D for efficient arginine supply to generate ammonia via the ADS and prevent acidification, HEp-2 cells were treated with bafilomycin to inhibit endosomal acidification before infection. Compared with the infection of untreated cells, the pretreatment of the cells with bafilomycin significantly increased the survival rate of strain 10Δ*arc*D. These data suggest that ArcD substantially contributes to efficient arginine uptake in *S. suis* and, thereby, to its resistance against endosomal acidification in HEp-2 cells.

DISCUSSION

Streptococci are characterized by a small genome size of approximately 2 Mbp and a homofermentative metabolism with the glycolysis as the primary energy providing pathway (Hoskins et al., 2001; Tettelin et al., 2002; Yamamoto et al., 2005). It is well established that the ADS and the catabolism of arginine is important in metabolism and virulence. The impact of substrate uptake and supply, however, remains mostly elusive. This study focussed on the characterization of ArcD, a putative arginine-ornithine antiporter, located in the gene cluster of *S. suis* ADS. An association of *arc*D to the genes of the ADS is common among different bacterial species, but the genetic organization varies substantially. For example, in *S. suis*, *arc*D is located downstream of *arc*C and closely associated to the putative Xaa-His-dipeptidase *arc*T (Zuniga et al., 2002; Gruening et al., 2006; Hitzmann et al., 2013). Such an intimate and conserved occurrence of genes often indicates a functional relation of the respective proteins. Indeed, our RT-PCR analysis using intergenic primers revealed that *arc*D and *arc*T are transcribed from a single RNA, the *arc*DT operon, which is separated from *arc*ABC but co-regulated. Yet, the function of *arc*T has not been proven experimentally, though ArcT is a predicted dipeptidase which might provide arginine from oligopeptides.

In order to get more insights into the role of ArcD in *S. suis* metabolism and virulence, we inactivated the respective gene by insertion mutagenesis. Phenotypic characterization was done by growth experiments under ADS inducing conditions. Thus, we used a tryptone-based medium with galactose as the sole carbon source. By this, in contrast to glucose, the ADS is relieved from carbon catabolite repression (CCR). We hypothesized that if ArcD is an antiporter facilitating arginine uptake, phenotypic differences between the WT and the *arc*D negative mutant strain might be more pronounced under ADS inducing conditions, since a substantial contribution of the *arc*ABC operon to the biological fitness of *S. suis* was indicated from our previous studies (Gruening et al., 2006; Fulde et al., 2011). Indeed, strain 10Δ*arc*D was markedly hampered in growth and this effect occurred at very

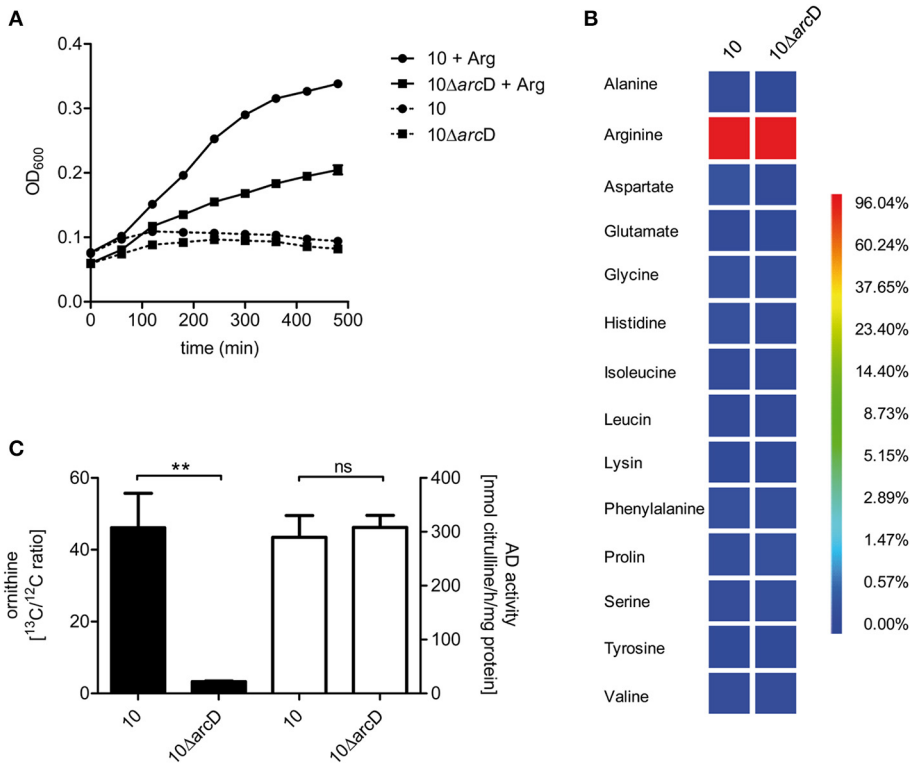

FIGURE 3 | *S. suis* is auxotrophic for arginine and *arc*D contributes to the uptake of extracellular arginine. (A) Growth of *S. suis* strain 10 and strain 10Δ*arc*D in chemically defined medium (CDM) in which the amino acid arginine was omitted if required. Streptococcal growth was monitored hourly by measuring the optical density at 600 nm (OD_{600}). Results are given as mean and standard deviation of one representative experiment performed in triplicates. Assays were repeated at least four times. **(B)** Isotopolog profiles of proteinogenic amino acids after growth of *S. suis* in CDM supplemented with 2.5 mM [U-$^{13}C_6$]arginine. Multiple ^{13}C-labeled isotopologs were determined by GC/MS spectroscopy and the overall ^{13}C excess (%) of labeled isotopologs is shown in the color map. Results are shown for one representative experiment. **(C)** Measurement of ^{13}C incorporation in ornithine after [U-$^{13}C_6$]arginine uptake of *S. suis*. After growth in CDM with ^{12}C-arginine, *S. suis* strain 10 and strain 10Δ*arc*D were incubated in CDM supplemented with 2.5 mM [U-$^{13}C_6$]arginine for 30 min. The $^{13}C/^{12}C$ ratio of intracellular ornithine was determined as an indirect measure of arginine uptake (black bars, left y-axis). No difference in arginine deiminase (AD) enzymatic activity was observed for both strains under these conditions (white bars, right y-axis). Results are given as mean and standard deviation of three independent experiments. Statistical significance is indicated for a two-sided *t*-test (**$p < 0.01$; ns, not significant).

early growth times. Supplementation of the growth medium with arginine led to an increase in growth in both strains but could not compensate the growth defect of the *arc*D mutant. These results suggested a particular role of arginine for growth of *S. suis* and a contribution of ArcD to arginine uptake. Thus, arginine supplied by ArcD seemed to be responsible for the enhanced growth of WT strain 10 under ADS inducing conditions. To further confirm these observations, we repeated the growth experiments in a standardized chemically defined medium to exclude effects mediated by the use of tryptone (**Figure 3A**). Interestingly, without arginine supplementation, neither WT strain 10 nor the *arc*D deficient mutant strain were able to grow, demonstrating the essentiality of arginine. In turn, supplementation of arginine enabled both the WT strain 10 and its mutant 10Δ*arc*D to grow in CDM, although the growth of the mutant strain was significantly attenuated. This further indicated that ArcD has a considerable impact on the uptake of arginine. Indeed, by using labeled [U-$^{13}C_6$]arginine, the $^{13}C/^{12}C$ ratio of intracellular ornithine was determined to be higher in the WT strain than in its *arc*D deficient derivative. Importantly, this phenotype is not due to a reduced AD activity, as demonstrated in **Figure 3C**, but to a diminished arginine uptake. This is also shown by an additional independent technique. The comparison of the amount of arginine in the supernatant of bacterial cultures was monitored colorimetrically and revealed significant lower levels in those of the WT strain (**Figure 2D**).

Arginine auxotrophy was also shown for other streptococci such as *S. canis* and *S. pneumoniae*, respectively (Kloosterman and Kuipers, 2011; Hitzmann et al., 2013). It is well known that streptococci must acquire many nutrients since *de novo* synthesis of metabolic intermediates is restricted due to the small genome size. Thus, closely related *S. pneumoniae* and *S. agalactiae* strains express up to four different arginine uptake systems under starvation conditions (Bryan et al., 2008; Kloosterman and Kuipers, 2011). It is, therefore, conceivable that in *S. suis* alternative arginine providing systems exist which enable the pathogen to multiply even without ArcD. Nevertheless, *arc*DT is closely localized to the *arc*ABC operon and the considerable impact for the growth of *S. suis* substantiates its importance under arginine limited conditions which might be present in different host

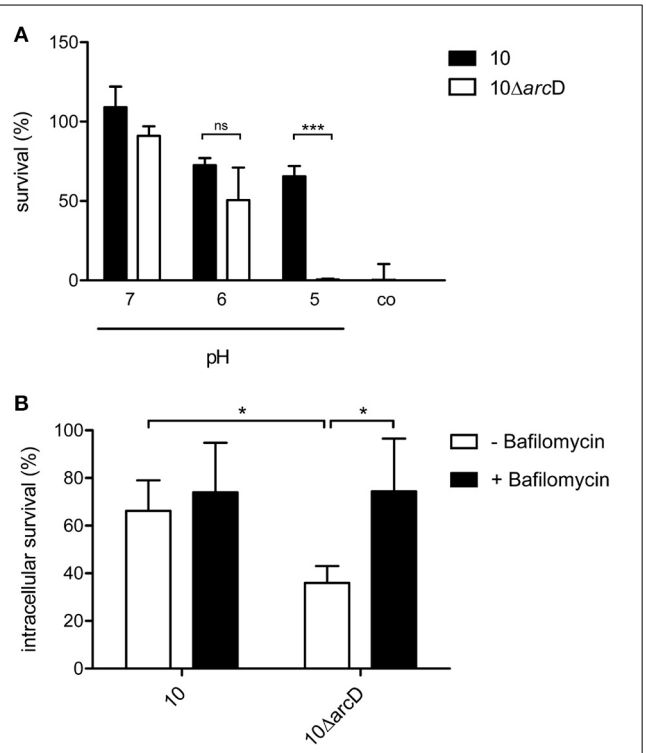

FIGURE 4 | ArcD facilitates survival under acidic conditions. (A) *S. suis* strain 10 (black bars) and 10Δ*arc*D (white bars) were incubated for 4 h in a phosphate buffer supplemented with arginine and adjusted to the depicted pH values. Acidic resistance was monitored by replica-plating. Results are given as percentage of the inoculum. Phosphate buffer adjusted to pH 5 without arginine supplementation served as negative control. Data represent means and standard deviations of a representative experiment performed in triplicates. Experiments were repeated at least four times. **(B)** Intracellular survival of the wild-type strain 10 and strain 10Δ*arc*D in HEp-2 cells that were treated with bafilomycin (200 nM) for 1 h before infection (black bars) and in untreated cells (white bars). Results are given as percentage of intracellular bacterial survival after 2 h. Data represent means and standard deviation of three independent experiments. Results were considered statistically significant with $p < 0.05$ in a one-tailed *t*-test, as indicated by asterisks.

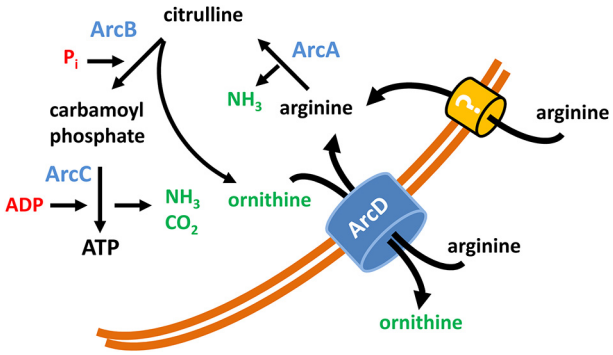

FIGURE 5 | Model of the function of ArcD as part of the *S. suis* ADS. The core ADS enzymes (ArcA, ArcB, ArcC) facilitating the conversion from arginine to ornithine are depicted in blue. Metabolic intermediates are indicated in black. The input of energy in terms of phosphate derivatives (P_i, ADP) are marked in red, non-catabolized and excreted products have a green color. ArcD, located in the bacterial membrane, facilitates an arginine/ornithine antiport. The occurrence of an alternative arginine transporter is illustrated by a question mark.

comportments such as the phagosomal vacuole. We have previously shown that *S. suis* resides in acidified phagolysosome-like compartments after uptake into HEp-2 cells and that ammonia production by ADS-mediated arginine catabolism significantly contributes to intracellular survival of *S. suis* (Benga et al., 2004; Fulde et al., 2011). In agreement, our present data clearly show that a lack in *arc*D leads to a significant reduction in the biological fitness of *S. suis*. The mutant strain was hampered in counteracting environmental acidification and to survive in epithelial cells, which are target host cells during infection.

In conclusion our data clearly denote an important role of arginine and arginine uptake executed by ArcD for the metabolism and survival of *S. suis* (summarized in a model as depicted in **Figure 5**). Furthermore, they emphasize the outstanding importance of the ADS for biological fitness and pathogenic potential for zoonotic *S. suis*.

AUTHOR CONTRIBUTIONS

Peter Valentin-Weigand, Marcus Fulde, Joerg Willenborg, and Ralph Goethe designed research; Marcus Fulde, Joerg Willenborg, Daniela Willms, Angela Hitzmann, and Maren Seitz performed the experiments and analyses; Claudia Huber and Wolfgang Eisenreich performed the isotopolog profiling experiments and analyses; and Marcus Fulde, Joerg Willenborg, Ralph Goethe and Peter Valentin-Weigand wrote the paper.

ACKNOWLEDGMENTS

We gratefully acknowledge Nina Janze, Franziska Voigt, and Birgit Lange for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (SFB 587 and SPP1316 grants EI-384/5-2 and GO-983/3-1).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 May 2014; paper pending published: 29 June 2014; accepted: 21 July 2014; published online: 12 August 2014.

Citation: Fulde M, Willenborg J, Huber C, Hitzmann A, Willms D, Seitz M, Eisenreich W, Valentin-Weigand P and Goethe R (2014) The arginine-ornithine antiporter ArcD contributes to biological fitness of Streptococcus suis. Front. Cell. Infect. Microbiol. ***4****:107. doi: 10.3389/fcimb.2014.00107*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 28 July 2014
doi: 10.3389/fcimb.2014.00099

Staphylococcus aureus Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections

Richard A. Proctor[1]*, André Kriegeskorte[2], Barbara C. Kahl[2], Karsten Becker[2], Bettina Löffler[2] and Georg Peters[2]

[1] Departments of Medical Microbiology/Immunology and Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA
[2] Institute of Medical Microbiology, University Hospital, Münster, Münster, Germany

Edited by:
Wolfgang Eisenreich, Technische Universität München, Germany

Reviewed by:
Martin I. Voskuil, University of Colorado Denver, USA
Alain Charbit, University Paris Descartes, France

***Correspondence:**
Richard A. Proctor, Departments of Medical Microbiology/Immunology and Medicine, University of Wisconsin School of Medicine and Public Health, 835 Asa Gray, Ann Arbor, MI 48105-2565, USA
e-mail: rap@wisc.edu

Persistent and relapsing infections, despite apparently adequate antibiotic therapy, occur frequently with many pathogens, but it is an especially prominent problem with *Staphylococcus aureus* infections. For the purposes of this review, persistence will encompass both of the concepts of long term survival within the host, including colonization, and the concept of resisting antibiotic therapy even when susceptible in the clinical microbiology laboratory. Over the past two decades, the mechanisms whereby bacteria achieve persistence are slowly being unraveled. *S. aureus* small colony variants (SCVs) are linked to chronic, recurrent, and antibiotic-resistant infections, and the study of SCVs has contributed significantly to understanding of persistence. In our earlier work, defects in electron transport and thymidylate biosynthesis were linked to the development of the SCV phenotype (reviewed in 2006), thus this work will be discussed only briefly. Since 2006, it has been found that persistent organisms including SCVs are part of the normal life cycle of bacteria, and often they arise in response to harsh conditions, e.g., antibiotics, starvation, host cationic peptides. Many of the changes found in these early SCVs have provided a map for the discovery mechanisms (pathways) for the development of persistent organisms. For example, changes in RNA processing, stringent response, toxin-antitoxin, ribosome protein L6 (RplF), and cold shock protein B (CspB) found in SCVs are also found in other persisters. In addition, many classic persister organisms also show slow growth, hence SCVs. Recent work on *S. aureus USA300* has elucidated the impact of aerobic expression of arginine deiminase genes on its ability to chronically colonize the skin and survive in abscesses. *S. aureus* SCVs also express arginine deiminase genes aerobically as well. Thus, many pathways found activated in electron transport type of SCVs are also increased in persisters that have intact electron transport. Many of these changes in metabolism result in slow growth; hence, small colonies are formed. Another common theme is that slow growth is also associated with reduced expression of virulence factors and enhanced uptake/survival within host cells. These adaptations to survive within the host are rooted in responses that were required for organisms to survive in a harsh environment long before they were mammals on the earth.

Keywords: *Staphylococcus aureus*, small colony variants, metabolism, RNA processing, post-transcriptional, persistence

INTRODUCTION

Persistent and relapsing infections, despite organism susceptibility and apparently adequate antibiotic therapy, occur frequently with many pathogens, but it is an especially prominent problem with *Staphylococcus aureus* infections (Lowy, 1998). The basis for persistence has been slowly unraveled over the past two decades, and many of the pathways involve changes in metabolism. One phenotype of microorganisms has helped to pave the way to understand persistence, and this is represented by small colony variants (SCVs) (Proctor et al., 2006). The linkage between *S. aureus* SCVs and persistent infection was first reported in a small clinical series in 1995 (Proctor et al., 1995). Because *S. aureus* SCVs were able to establish an intracellular infection in cultured cells, it was hypothesized that this might form the basis for the development of persistent infections (Balwit et al., 1994). Moreover, the instability of these SCVs wherein they could revert to the parental normal phenotype would also provide a mechanism for relapsing, virulent infections. An important part of the ability to persist was associated with the quiescent metabolic state (Proctor et al., 1995). *S. aureus* SCVs were found to produce fewer lytic enzymes, thereby allowing them to persist within the host cells (Proctor et al., 2006). Since then, further work

has demonstrated enhanced uptake by *S. aureus* SCVs due to a high expression of adhesins that facilitate host cell uptake (Sendi and Proctor, 2009; Tuchscherr et al., 2010). Early studies suggested that the intracellular milieu could select for SCVs (Vesga et al., 1996), perhaps because cationic antimicrobial peptides, e.g., lactoferrin, provide the selective pressure within host cells (Samuelsen et al., 2005; Gläser et al., 2014). While early studies suggested that the intracellular milieu could select for SCVs (Vesga et al., 1996), more recent and detailed studies demonstrated that a high percentage of the initial inoculum (as much as 25%) of *S. aureus* could undergo phenotypic switching to produce SCVs (Tuchscherr et al., 2011). This was shown to not only occur in tissue culture, but also within the kidneys and bones of these intravenously challenged mice (Horst et al., 2012). The SCVs that persisted stimulated a reduced immune response, expressed increased adhesins, and reduced toxins (Tuchscherr et al., 2010). Moreover, the failure of *S. aureus* SCVs to stimulate host cells to produce hypoxia-inducible factor, which would normally alert the host to the presence of intracellular pathogens, also is important for promoting persistence (Werth et al., 2010). Finally, persistence of *S. aureus* SCVs may also relate to the relative inefficiency of antibiotics to clear the organisms with host cells (Garcia et al., 2013). Thus, *S. aureus* SCVs have been established as intracellular pathogens that persist within the host. This review will largely concentrate upon information that has been published since the 2006 review of SCVs (Proctor et al., 2006) and will emphasize metabolic mechanisms involved in persistence. *S. aureus* SCVs as a model persister organism

How do *S, aureus* SCVs relate to the broader concept of persistence? Many other species of bacteria that persist within the host also form SCVs (Proctor et al., 2006). Hence, the specific example of *S. aureus* SCVs is also found within many other pathogenic species. In addition, persisters share many characteristics, and these are also part of the SCV life cycle. Persisters and *S. aureus* SCVs grow slowly due to decreased metabolic activity (Amato et al., 2014). A subset of organisms within many bacterial populations can be part of the normal growth cycle (Lechner et al., 2012), and this is certainly true for *S. aureus* SCVs, which are part of the normal growth cycle that occurs without any external stresses (Massey et al., 2001; Edwards, 2012). However, persisters also form as a response to harsh conditions such as antibiotics, acid, and starvation stresses (Morikawa et al., 2010). Biofilm formation can be thought of as a persister strategy (Abdallah et al., 2014). Naturally, persistence is inter-related to chronic colonization and chronic infections as these may be considered types of persistence.

ELECTRON TRANSPORT TYPE OF *S. AUREUS* SCVs

The initial description of *S. aureus* SCV persisters identified alterations in electron transport due to mutations in hemin and menadione biosynthetic genes, which resulted in a loss of menaquinone and the heme prosthetic group in cytochromes (Proctor et al., 2006). Others found that mutations in the cytochrome c assembly protein (CtaA) also produced SCVs (Clements et al., 1999). Thymidine auxotrophic SCVs were soon added (Proctor et al., 2006) to the list of metabolic changes in SCVs. Mutations in *thyA* were found to produce SCVs that were phenotypically very similar to the electron transport deficient (Chatterjee et al., 2008). The reason that thymidine-dependent SCVs behaved like electron transport defective organisms came through decreased Krebs cycle activity (Chatterjee et al., 2005, 2007). Of course, when Krebs cycle is decreased, electron transport is down regulated. It was found that *thyA* mutation was associated with decreased aconitase (CitB) abundance. A further connection became apparent wherein CitB expression requires ClpC, which is reduced in *thyA* mutants (Chatterjee et al., 2005, 2007, 2008). Hence, *thyA* mutants are grouped with organisms defective in electron transport. All of the electron transport types of SCVs are able to persist within the host and/or cultured mammalian cells.

CHANGES IN RNAIII METABOLISM IN ELECTRON TRANSPORT GROUP OF SCVs

As noted above, slow growth is found in many, but not all, organisms that persist. When examining clinical and tissue-cultured induced *S. aureus* SCVs, only ∼20% can be assigned to a defined auxotrophy (Vesga et al., 1996; Proctor et al., 2006; Tuchscherr et al., 2011). Therefore, the underlying mechanism for forming a persistent SCV is unknown. Publications in the past six years have revealed a number of mutations that result in SCVs, but most of these have not been examined in clinical SCVs. Surprisingly, many of these newer mutations can be grouped as being involved with the ribosome and RNA processing. This will be discussed in the balance of this article.

One of the first indications that electron transport type of *S. aureus* SCVs were atypical for RNA processing came from the observation that there was a complete absence of RNAIII (Vaudaux et al., 2002; Kohler et al., 2003, 2008; Kahl et al., 2005), which is the effector molecule in the quorum sensing apparatus, *agr* (accessory gene regulator), in *S. aureus* (Novick and Geisinger, 2008). RNAIII arises from nontranslated portion of the *hld* (hemolysin D) gene in the *agr* operon. RNAIII positively regulates production of toxins and proteases but negatively regulates adhesins.

Because *agr* is a quorum-sensing system, one might postulate that the decreased growth of electron-transport types of SCVs simply do not reach sufficient density to activate the quorum-sensing pathway. This seems unlikely for several reasons. The first is that SCV colonies on blood agar, wherein the local density is very high, fail to produce hemolytic colonies, and the hemolysins are controlled by *agr*/RNAIII (Proctor et al., 2006). The second is that RNAIII is not found in any detectable amount in SCVs (Vaudaux et al., 2002; Kohler et al., 2003, 2008; Kahl et al., 2005), which is distinctly unusual in that a basal level of RNAIII is produced in *S. aureus* during all phases of growth (Novick and Geisinger, 2008). Surprisingly, mutation in *citB* produces a major growth defect, yet a 14-fold increase in RNAIII was found in the *citB* mutant as compared to the parent strain *S. aureus* strain. This work was performed in a clinical isolate, SA564, in TSB supplemented with 0.25% glucose (Somerville et al., 2002). The differences in RNAIII production were found in the post-exponential phase of growth, with no differences in the exponential phase of growth. Finally, RNAIII is reduced under anaerobic conditions in *S. aureus* MN8 along with the production of toxic

shock syndrome toxin–1 (TSST-1) (Pragman et al., 2004), which is regulated by RNAIII. However, the anaerobic suppression of TSST-1 production can be reversed if excess quantities of pyruvate are added to the growth medium, resulting in TSST-1 production during late stationary phase (**Table 1**). This shows that the anaerobic conditions alone do not block RNAIII-regulated production of TSST-1, which is consistent with an earlier report where both pyruvate and uracil were added to the culture medium (Sarafian and Morse, 1987). Taken together, these data strongly suggest an altered RNAIII metabolism when electron transport is reduced, which is not simply due to reduced growth/concentration of the quorum-sensing peptide.

There is a strong inverse connection between *agr,* hence RNAIII, and persistent infection. The loss of the major virulence factor regulator in *S. aureus*, *agr*, has been associated with chronic infections. These *agr* mutants have been obtained from clinical isolates from patients with long-term lung infections (cystic fibrosis) (Kahl et al., 2005; Hirschhausen et al., 2013), chronic catheter infections (Rothfork et al., 2003; Yarwood and Schlievert, 2003), and chronic soft tissue infections (Schwan et al., 2003; Beenken et al., 2004). Of interest, the development of skin abscesses requires the presence of *agr* to establish the initial infection, but persistence within the abscess is associated with decreased RNAIII (Wright et al., 2005). Thus, these infections are strongly linked to situations wherein the organism persists within the host and resists antibiotic therapy. This suggests that a complete absence of the RNAIII effector molecule from the *agr* operon is associated with persistence.

THE RNA DEGRASOME AND SCVs

Of interest, small colonies and changes in RNA metabolism are seen in mutants in *cshA, cspB, rsaE, rplF, mazF*, and *rsh* (see **Table 2**). The role of these mutations in persistent infection is discussed in the balance of this manuscript.

Low levels of RNAIII can occur by two mechanisms. The *agr* quorum-sensing operon may produce less RNAIII or there may be increased RNAIII degradation to account for low levels of RNAIII. As noted above, the low RNAIII levels in electron transport type of SCVs are more likely related to increased RNAIII degradation. The RNA degrasome in *S. aureus* has recently been elucidated (Roux et al., 2011; Marincola et al., 2012; Redder and Linder, 2012; Oun et al., 2013), and a number of degrasome proteins are increased in SCVs as suggested by transcriptomics and proteomics data from *hemB* and *menD* mutant SCVs (Seggewiss et al., 2006; Kriegeskorte et al., 2011). A model of the RNA degrasome is provided in **Figure 1**.

The *hemB* mutant SCV has increased enolase, helicase (CshA), RsaA (a small noncoding RNA involved in stress responses and biofilm formation), *cspB* (cold shock protein B), SigB, SarA, and FruR (Seggewiss et al., 2006; Kriegeskorte et al., 2011). Of note, metabolism is embedded in the degrasome with enolase and phosphofructokinase (Pfk) directly interacting with CshA, but the effects that they have on CshA has yet to be characterized. Some of the targets of the degrasome are small nonprotein coding RNAs (sRNAs) that control central carbon metabolism (Geissmann et al., 2009; Bohn et al., 2010; Felden et al., 2011; Guillet et al., 2013; Romilly et al., 2014; Xue et al., 2014), and SCVs show changes in the levels of these sRNAs (Abu-Qatousch et al., 2010). This is not surprising as SCVs showed markedly increased levels of SigB (Mitchell et al., 2013), which positively regulates RsaA (Felden et al., 2011), and decreased levels of RsaE that regulates central carbon metabolism. The low levels of RsaE in SCVs are likely due to low levels of RNAIII, which is positively RsaE (Donegan and Cheung, 2009; Donegan et al., 2010; Guillet et al., 2013). In *Bacillus subtilis*, CspB associates with CshA (Hunger et al., 2006). CspB is included in the degrasome, and it is increased in *hemB* mutant SCVs (Seggewiss et al., 2006; Kriegeskorte et al., 2011). RNase Y processes *saeRS* mRNA so that it is active. (Marincola et al., 2012). Thus, as changes in RNA processing impact virulence factor production, TCA cycle, and biofilms, a number of the phenotypic characteristics of SCVs may be attributable to RNA processing.

A mutation in the ATP-dependent hellicase, *cshA*, in *S. aureus* results in a temperature-dependent SCV, with reduced growth at 30°C and complete growth inhibition at 22°C (Oun et al., 2013). Disruption of *cshA* results in higher RNAIII stability with increased hemolysis and reduced biofilm formation. Knocking *agrA* reversed the phenotype, indicating that *cshA* is genetically upstream of *agr* (Oun et al., 2013). In electron transport deficient SCVs, the higher levels of CshA might be expected to have a role in the lower levels of RNAIII.

As noted above, another potential element of the degradosome may be CspB. Of interest, a mutation in *cspB* in *S. aureus* also produces an SCV with reduced pigment production and increased resistance to aminoglycosides as found in electron transport SCVs (Duval et al., 2010). Other characteristics of SCVs such as RNAIII levels and expression of adhesins has not been studied. Similarly, the ability of the *cspB* mutant to persist has also not been reported.

Table 1 | Anaerobic production of toxic shock syndrome toxin – 1.

mM Pyruvate added	ng TSST-1/10^6 cfu
0	0.7
40	0.05
200	24.6
400	63

Exogenous pyruvate and anaerobic toxin production in S. aureus MN8 grown in tryptic soy broth for 18 h under anaerobic conditions, and supernatants collected for TSST-1 measurements. TSST-1 was measured by an ELISA as previously described (McNamara et al., 2009).

SMALL RNAs AND SCVs

Based on investigations of cDNA libraries, phenotype-specific expression of sRNAs have been described for *S. aureus* (Abu-Qatousch et al., 2010). Selected sRNAs have also been found to in the *hemB* mutant SCV as compared to the wild type strain (Abu-Qatousch et al., 2010). Over expression of RsaE give SCVs that have decreased TCA cycle activity (Geissmann et al., 2009; Bohn et al., 2010). RsaE pairs with mRNAs of *citB*, *citZ*, and *sucCD*, thereby inhibiting their translation and reducing TCA cycle. While detailed metabolic studies are not available, one would anticipate that these organisms would have reduced electron transport and behave like electron transport deficient

Table 2 | Comparison of phenotypes in SCVs and persisters.

Phenotype	SCV (*men/hem/ctaA*) Also, unsaturated fatty acids	SCV (*thyA*)	Δ*cshA*	Δ*cspB*	RsaE over production	*rplF* mutant (Ribosome protein L6)	Increased MazF (Toxin –AT)	Rsh constitutively active(RelA)
Biochemical	**Electron transport**	**Thymidy-late synthesis**	**Hellicase (ATP dependent)**	**Cold shock protein**	**Regulatory small RNA**	**Fusidic acid resistance**	**Endoribo-nuclease**	**ppGpp synthesis**
Colony size	Small	Small	Small(22°C)	Small	Small	Small	Small	Small
Persistence	Yes	Yes					Yes	Yes (↑PSMs)
Hla	Decrease	Decrease	Increase				Decreased	Increase
Hld	Decrease	Decrease	Increase					Increase
Spa	Decrease	Increase	Decrease				Decrease	Decrease
FnBP	Increase	Increase						Increase
RNAIII	Decrease	Decrease	Increase		Decreased			Increase(↓RnpA)
CPS	Increase	Decrease	Increase					Increase
Biofilm	Increase	Increase	Decrease					Increase capsule
TCA	Decrease	Decrease	Decrease		↓*sucD*/TCA			Increase
ClpC/ClpP		Decreased	Decrease					Decrease
Ilv pathway	Increase							Increase
ppGpp								Increase
Cell wall thick	Increased	Increased						
Pigment	Decreased	Decreased		Decreased		Decreased	Decreased	
Cold growth	Increased CspB		Decreased	Decreased				
Antibiotic	AG^r, βL^r, $Dapto^s$	$SXT^{r,}$				AG^r		

AG^r, aminoglycoside resistant; βL^r, betalactam resistant; $Dapto^s$, daptomycin susceptible; ClpC, ATP-dependent protease C; ClpP, ATP-dependent protease P; CSP, capsular polysaccharide; CspB, cold shock protein B; FnBP, fibronectin binding protein; Hla, α-toxin; Hld, δ-toxin; Ilv, Isoleucin, leucine, valine; Spa, Protein A; $SXT^{r,}$, sulfamethoxazole-trimethoprim resistant; sucD, succinyl-CoA synthetase subunit alpha; TCA, tricarboxylic acid cycle.

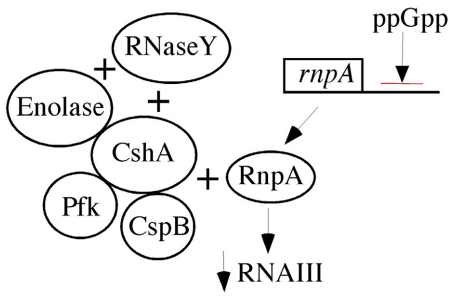

FIGURE 1 | The RNA degrasome is shown with respect to its impact on RNAIII as low levels of RNAIII are associated with persistent *S. aureus infections*. An ATP-dependent *S. aureus* hellicase (CshA) is shown at the center with three proteins associated with it: Enolase, phosphofructokinase (Pfk), and cold shock protein B (CspB). Two RNase are shown, RNaseY and RnpA. RnpA has activity against RNAIII and its gene is negatively regulated by the alarmone, ppGpp.

variants. Mutation of a sRNA, *rsaA*, has been show to alter virulence and cause persistent infection (Romilly et al., 2014). RsaA binds to MgrA, a global transcriptional regulator, and attenuates the severity of infections while increasing biofilm formation. Hence, it contributes to chronic infections (Romilly et al., 2014).

The higher levels of RsaA seen in electron transport SCVs might help to explain the increased biofilm formation in these variants as RsaA represses the synthesis of MgrA thereby increasing biofilm (Seggewiss et al., 2006). Also, SCVs have been reported to be readily killed by phagocytes (Quie, 1969), and this is also true of strains over-expressing RsaA (Seggewiss et al., 2006). Hence, increased amounts of RsaA may be contributing to the SCV phenotype. (A more detailed review of sRNAs in *S. aureus* is in Felden et al. (2011).

TOXIN-ANTITOXIN, MazEF, AND SCVs

Another system that is involved in mRNA stability in *S. aureus* is the MazEF toxin-antitoxin system (Fu et al., 2007, 2009; Donegan and Cheung, 2009; Donegan et al., 2010; **Figure 2**). Toxin-antitoxin systems are produced by many bacteria (Magnuson, 2007) and transcribed as an operon so that the toxin (MazF) is readily bond and neutralized by the antitoxin (MazE) (Fu et al., 2007, 2009; Donegan and Cheung, 2009; Donegan et al., 2010). MazE is an RNase that acts upon selected mRNAs. In *S. aureus*, MazE targets *sigB, hla, spa* mRNAs, but it avoids mRNAs from *recA, gyrB, sarA* (Fu et al., 2007, 2009). In most bacteria, the MazEF system is regulated by feedback inhibition by its own gene products, but in *S. aureus*, the negative regulation comes via SigB (Donegan and Cheung, 2009). The *mazEF* promoter allows transcription of not only *mazEF* but also the *sigB* operon, and activation of the *mazEF* promoter is needed for full SigB activity.

High levels of free MazF result in slow growth (SCVs), decreased pigment, and persistence, which can be achieved by overexpression of MazF or increased activity of ATP-dependent

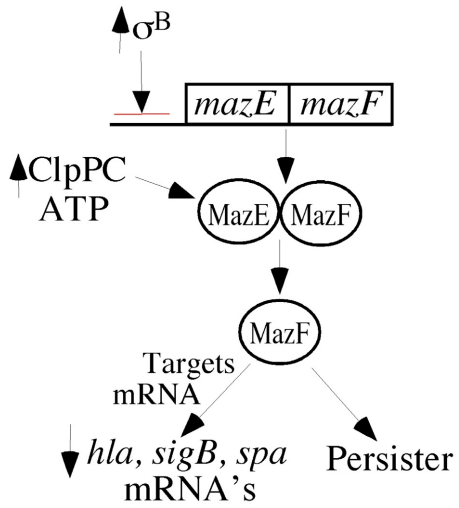

FIGURE 2 | MazEF are transcripbed as a pair of proteins, and their transcription is negatively regulated by SigmaB. MazE is an anti-toxin protein that binds to MazF, thereby preventing its activity. MazF has RNase activity, which targets selected mRNAs in *S. aureus*. The ATP-dependent protease pair, ClpPC, digests the MazE antitoxin, thereby releasing MazF. Increased MazF has been associated with persistence.

protease system, ClpCP, which proteolytically targets the anti-toxin, MazE (Fu et al., 2007, 2009; Donegan and Cheung, 2009; Donegan et al., 2010). This is compatible with the concept that toxin-antitoxin systems are involved in persistence (Magnuson, 2007). On the other hand, the low levels of ATP and ClpP and the higher levels σ^B in electron transport SCVs suggest that the MazEF system is probably not contributing to persistence in electron transport SCVs as electron transport SCVs have high levels of SigB (Mitchell et al., 2013).

MUTATION IN *rplF* AND SCVs

The mutations in *rlf*, which encodes ribosomal protein L6, cause SCVs that have decreased pigmentation, like electron transport type of SCVs, and that allow for persistence (Norström et al., 2007; Lannergård et al., 2011). L6 is required for efficient protein synthesis, thus it is not surprising that cells with defective L6 would grow slowly. Rfl is also called FusE, which comes from the fact that these mutants are resistant to fusidic acid because of changes in ribosomal protein L6. In addition, *rfl* mutation in *E. coli* results in increased membrane fluidity and aminoglycoside resistance (Bosl and Böck, 1981), which is also seen with electron transport deficient SCVs. Aminoglycosides can also select for simultaneous fusidic acid resistance (Norström et al., 2007; Lannergård et al., 2011). Of note, in *S. aureus*, L6 is reduced in *hemB* mutants and under anaerobic growth (Fuchs et al., 2007; Kriegeskorte et al., 2011), suggesting that part of the phenotype seen in electron transport SCVs may relate to changes in L6. Little more in terms of the phenotype is known except that many, but not all of the *rfl* mutants, also carry mutations in *hem* or *men (gerC)*. These would be double SCVs as a mutation in *rlf* alone is sufficient to produce an SCV phenotype. Again, we see the ribosome, hence RNA, being involved in persistence and the SCV phenotype.

STRINGENT RESPONSE, PERSISTENCE, AND SCVs

When investigating a *S. aureus* strain from a patient with persistent and recurrent infection, a constitutively active mutation in *rsh*, a RelA homolog in *S. aureus*, was recovered (Gao et al., 2010). The mutation resulted defective ppGpp hypdrolase activity, thereby giving high levels of ppGpp and a persistent stringent response (Gao et al., 2010). Resistance to linezolid was also found. As ppGpp is the effector molecule in the stringent response (Geiger et al., 2012, 2014; Geiger and Wolz, 2014), which limits protein synthesis, it is not surprising that there is a growth defect, hence small colonies, i.e., an SCV (Geiger et al., 2012, 2014; Geiger and Wolz, 2014). Mutations in *rsh* result in reduced virulence (Geiger et al., 2012, 2014; Geiger and Wolz, 2014). Aside from the small colony size, the *rsh* mutant does not resemble the electron transport SCVs because *rsh* mutants show increased RNAIII and all of the characteristics seen when the Agr regulon is active.

ARGININE DEIMINASE AND PERSISTENCE

In electron transport deficient SCVs, the *agrABDC* pathway, which is responsible for arginine deiminase activity, is markedly increased aerobically, whereas in the parent strains it is expressed only anaerobically (Kohler et al., 2003, 2008; Seggewiss et al., 2006; Makhlin et al., 2007). The anaerobic regulator of this operon, ArgR, is also significantly increased in the *hemB* mutant (Seggewiss et al., 2006), and it also regulates other normally anaerobic genes (Makhlin et al., 2007). This is of interest because the arginine catabolic mobile element (ACME) in USA300 is found along with the SCC*mec* element and is expressed aerobically whereas the other arginine deiminase operon are expressed only anaerobically (Thurlow et al., 2013). *S. aureus* USA300 is the hypervirulent epidemic strain in the USA. Arginine metabolism by ACME allows *S. aureus* to survive on the acidic environment of skin and within skin abscesses because it neutralizes acid with ammonia released during arginine catabolism (Thurlow et al., 2013). This provides a mechanism for the epidemiological observation that USA300 has an increased propensity to colonize the acidic environment of the skin chronically, which can be considered a type of persistence (Miko et al., 2012). Within ACME is another gene, *speG*, which encodes, spermidine acetyltransferase, a polyamine-resistance enzyme, thereby allowing *S. aureus* to survive spermidine exposure on the skin and in abscesses (Thurlow et al., 2013). Spermidine is otherwise a toxic product of arginine metabolism (Thurlow et al., 2013). The elucidation of the role of ACME in USA300 helps in the understanding how electron transport SCVs are able to survive for longer periods of time than other strains of *S. aureus* by using similar metabolic mechanisms.

METABOLISM AND ANTIBIOTIC-PERSISTERS

A large literature is available linking slow growth and resistance to antibiotics (Wood et al., 2013), and this is also found in *S. aureus* (Lechner et al., 2012). Discussing this type of persistence is beyond the scope of this review; however, one specific gene will be covered as it has a special link between phosphate metabolism and slow growth, hence an SCV, specifically *phoU*.

PhoU is a global negative regulator that regulates genes involved in central carbon metabolism and cytochrome expression, thus it provides strong links to the electron transport type of SCVs (Li and Zhang, 2007; Gardner et al., 2014). In *S. aureus*, *phoU* can be found to be important for resistance to cationic antimicrobial peptides (Overton et al., 2011), which may relate to expression of the *dlt* and *snoD* operons that have been related to cationic antimicrobial peptide resistance (Peschel et al., 1999; Bayer et al., 2006). Both the *dlt* and *snoD* operons are regulated by PhoU. We have found that the electron transport type of SCVs are also persist in the presence of antibiotics and more resistant to cationic peptides (Koo et al., 1996; Gläser et al., 2014). Studies of *S. aureus phoU* mutants ability to persist within the host are not available, but one would surmise that this may be the case.

CONCLUSIONS

Persistence and metabolism are intimately intertwined (Kahl, 2014), and it can be achieved via altering many pathways. Slow growth is common theme for persisters. The electron transport deficient SCVs show changes in many of the other pathways used for persistence, hence, the studies of these SCVs have a broader relevance for persistence. Of interest, altered RNA processing is suggested in the electron transport SCVs by the exceptionally low levels of RNAIII. A number of other persister types also show changes in RNA metabolism either directly through the RNA degrasome or at the ribosome. While the loss of *agr* is associated strongly with persistent and chronic infections, it is not associated with a growth defect. Naturally, there is a clear RNA effect in *agr* mutants in that the effector molecule, RNAIII, is not produced. Thus, there are many connections between the SCV phenotype and altered mRNA levels. There are also a number of shared phenotypic characteristics PhoU mediated changes and SCVs, but direct connections have yet to be studied. Finally, a growth defect is seen with high ppGpp levels, but these strains need to be tested for persistence.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 May 2014; accepted: 07 July 2014; published online: 28 July 2014.
*Citation: Proctor RA, Kriegeskorte A, Kahl BC, Becker K, Löffler B and Peters G (2014) Staphylococcus aureus Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections. Front. Cell. Infect. Microbiol. **4**:99. doi: 10.3389/fcimb.2014.00099*
This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY

REVIEW ARTICLE
published: 24 December 2013
doi: 10.3389/fcimb.2013.00106

Yersinia pestis: mechanisms of entry into and resistance to the host cell

Yuehua Ke[1,2], Zeliang Chen[1]* and Ruifu Yang[2]*

[1] *Institute of Disease Control and Prevention, Academy of Military Medical Sciences, Beijing, China*
[2] *Laboratory of Analytical Microbiology, State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, China*

Edited by:
Thomas Dandekar, University of Würzburg, Germany

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Stacey Gilk, Indiana University School of Medicine, USA

****Correspondence:***
Zeliang Chen, Department of Infectious Disease Control, Institute of Disease Control and Prevention, Academy of Military Medical Sciences, Beijing 100071, China
e-mail: zeliangchen@yahoo.com;
Ruifu Yang, Laboratory of Analytical Microbiology, State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Beijing 100071, China
e-mail: ruifuyang@gmail.com

During infection, *Yersinia*, a facultative intracellular bacterial species, exhibits the ability to first invade host cells and then counteract phagocytosis by the host cells. During these two distinct stages, invasion or antiphagocytic factors assist bacteria in manipulating host cells to accomplish each of these functions; however, the mechanism through which *Yersinia* regulates these functions during each step remains unclear. Here, we discuss those factors that seem to function reversely and give some hypothesis about how bacteria switch between the two distinct status.

Keywords: invasion, anti-phagocytosis, intracellular survival, T3SS

INTRODUCTION

Three *Yersinia* species are known to be pathogenic to humans: *Yersinia enterocolitis*, *Y. pseudotuberculosis*, and *Y. pestis*. *Y. enterocolitis* and *Y. pseudotuberculosis* are enteropathogenic bacteria causing enteritis, ileitis, and mesenteric lymphadenitis, whereas *Y. pestis* is the causative agent of bubonic plague, among the most deadly human infectious disease in history. All three species harbor a virulence plasmid, which encodes a type III secretion system (T3SS) for secreting Yop protein substrates, to establish a successful infection. Six Yops, including YopE, YopH, YopM, YopO/YpkA, YopP/J, and YopT, are delivered by T3SS into host cells and these then inhibit phagocytosis and block pro-inflammatory signals (Shao, 2008). In cases involving infection with enteropathogenic *Yersinia* species other than *Y. pestis*, two adhesins, invasin and YadA, have been shown to be important for mediating contact with host cells (Isberg et al., 1987; Paerregaard et al., 1991). However, although both adhesins are inactive in *Y. pestis* due to an IS1541 element insertion within *inv* and a frameshift mutation in *yadA* (Parkhill et al., 2001; Song et al., 2004; Chain et al., 2006), *Y. pestis* maintains the ability to attach to and enter into host cells (Davis et al., 1996; Perry and Fetherston, 1997; Cowan et al., 2000), indicating that these adhesins are not necessary for the virulence of *Y. pestis* and that other adhesins and invasins are required for mediating the association with host cells. *Y. pestis* is also known for its ability to survive in macrophages during its early invasion process. After arming itself in the macrophage, *Y. pestis* becomes resistant to phagocytosis and is then capable of surviving outside the cell, which is critical for its pathogenesis. In this review, we will summarize what is known regarding the mechanisms through which *Y. pestis* survives in a host, inside or outside the cell.

LIFE CYCLE OF *Y. PESTIS* DURING INFECTION

Y. pestis is a facultative intracellular gram-negative bacterium. During the early stages of infection, *Y. pestis* can enter both macrophages and neutrophils through mechanisms of active or passive entry (Lukaszewski et al., 2005). However, *Y. pestis* is typically killed in neutrophils, whereas in macrophages, it can survive and acquire antiphagocytic capabilities, which enables its extracellular survival *in vivo* (Lukaszewski et al., 2005). Interestingly, *Y. pestis* can also enter into non-professional phagocytes, such as epithelial cells (Cowan et al., 2000; Leigh et al., 2005), which indicates that *Y. pestis* can not only be passively phagocytized by the professional phagocytes, but can also evolve a mechanism allowing it to invade host cells that generally do not possess phagocytic ability. The invasion of *Y. pestis* into host cells, including phagocytes and non-professional phagocytes, may be mediated by binding of adhesive factors present on their surface, including Ail, Pla, and Psa, to receptors on the membranes of host cells (Lahteenmaki et al., 2001; Miller et al., 2001; Benedek et al., 2004; Liu et al., 2006; Galvan et al., 2007; Felek et al., 2010).

After arming itself inside the macrophages, *Y. pestis* escapes from the cell and develops resistance to phagocytosis by both macrophages and neutrophils. Therefore, during the late stage of infection, phagocytes cannot ingest *Y. pestis*, and the bacteria exists mainly in an extracellular environment, which has been confirmed through autopsies of human pneumonic plague victims, wherein the samples examined exhibited abundant extracellular bacteria but little evidence of phagocytosis. The mechanism of release from macrophages is largely unknown but may be associated with apoptosis or necrosis observed through *in vitro* cell models. Although the mechanism through which *Y. pestis* relocates from an intracellular compartment to an extracellular environment is unclear, the antiphagocytic ability of *Yersinia* has been attributed to Caf1, F1 antigen, and Yops (e.g., YopH, YpkA, YopE, and YopT) (**Figure 1**). Many *in vitro* studies have demonstrated that these virulence factors act synergistically to promote evasion or inhibit ingestion by host cells, including professional phagocytes and some non-professional phagocytes.

Y. pestis carries both invasive factors, which promote contact with and entry into host cells, and antiphagocytic factors that inhibit uptake by host cells. The mechanism through which this bacterium balances these contradictory factors and utilizes them in the different stages of infection to manipulate host cells remains an interesting topic. Here, we have discussed all of the known factors used by *Y. pestis* to enter into or escape from host cells.

PHAGOCYTIC RECEPTORS OF HOST CELLS AND INVASIVE LIGANDS OF BACTERIA

The process of bacterial entry into host cells can be divided into two types. The first type, bacterial invasion, involves active bacterial entry, during which bacteria are not welcomed and grudgingly accepted by the host cells. Many bacteria initially depend more on their own invasive factors to enter into the host cells in order to escape the harsh extracellular environment conditions, including low pH and shear stress, and host defensive mechanisms, such as recognition and killing by macrophages and cytotoxic T cells, thereby allowing them to survive in the relatively mild intracellular milieu. The second type, phagocytosis of bacteria, involves passive entry during which bacteria are resistant to internalization and are initially ingested by the host cells through a surface mechanism. Phagocytosis plays important roles in reducing the threat from bacterial pathogens; therefore, it is used by host cells to degrade and clear hazardous exogenous material, avoid disruption of normal functions by bacteria, and present signals for activation of the innate and adaptive immune systems. In some cases, these two types of entry are indistinguishable, as both modes of entry involve initial binding of bacterial ligands to phagocytic receptors (Greenberg, 1999). Although these two types of entry mechanisms are very popular in most bacterial pathogens and are easy to differentiate, some bacterial pathogens, such as *Mycobacteria* and *Coxiella*, utilize phagocytic signaling pathways to enter into the host cells, which may be hard to define their types of entry (Méresse et al., 1999; Friedrich et al., 2012).

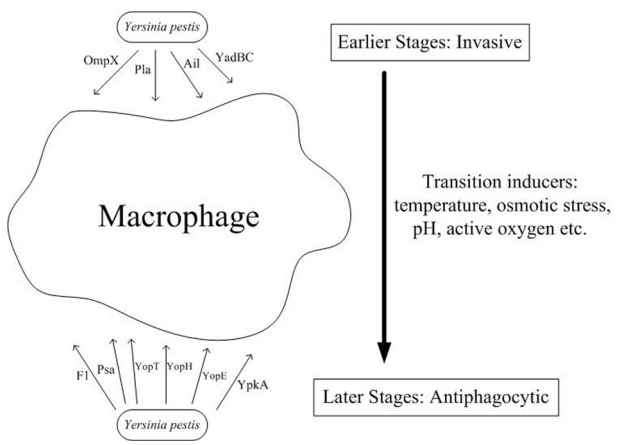

FIGURE 1 | Both invasive and antiphagocytic factors are involved in *Y. pestis* pathogenesis. During the initial encounter with macrophages during infection, *Y. pestis* enters the macrophage through binding of its surface proteins, such as Pla, Ail, YadBC, and OmpX, to undetermined receptors present on the macrophage surface. However, following release from the macrophage through another undefined mechanism, *Y. pestis* expresses several virulence factors, including F1 antigen, Psa, and four Yops (YopT, YopH, YopE, and YpkA) which are employed to resist phagocytosis by the surrounding professional phagocytes. An additional mechanism subsequently arms *Y. pestis* against host phagocytes of the innate immune system, thereby allowing it to invade more tissues and organs and cause more severe impairment.

Phagocytosis, classically defined as the cellular engulfment of particles larger than 0.5 μm in diameter (Jaumouille and Grinstein, 2011), is a highly conserved process that has evolved to counteract the threat of altered "self" molecular or "non-self" molecular. Phagocytosis encompasses an orchestrated cascade of events that involves particle recognition, signal transduction, cytoskeleton rearrangement, membrane remodeling, and phagosome maturation (Groves et al., 2008). In higher organisms, phagocytosis is mainly performed by professional phagocytes, such as macrophages, neutrophils, and dendritic cells (DCs), but several other cell types, including epithelial cells, endothelial cells, and fibroblasts, collectively identified as non-professional phagocytes, can also perform phagocytosis under certain conditions (Cannon and Swanson, 1992; Lowry et al., 1998; Sousa et al., 2005; Kim et al., 2010). Although non-professional phagocytes do not express FcRs and CRs, which are unique feature of professional phagocytes, the downstream molecular pathways of phagocytic receptors are generally expressed and once surface receptors are acquired, they can initiate the same phagocytic signaling pathway as professional phagocytes (Ezekowitz et al., 1991; Odin et al., 1991; van Zon et al., 2009).

The phagocytosis process begins with binding of a phagocytic receptor, directly or indirectly through opsonins, to their ligands on the surface of pathogens, which mediates the adherence of bacteria to the host cells (Pizarro-Cerda and Cossart, 2006; Huang et al., 2011). The best characterized phagocytic receptors are immunoglobulin receptors (FcRs) and complement receptors (CRs), which bind to bacteria, opsonized by antibody and complement respectively, and which activate downstream signaling pathways in a similar manner (May and Machesky, 2001; Groves et al., 2008; Nimmerjahn and Ravetch, 2008). A growing number of other cell surface receptors mediating phagocytic uptake of particles have been identified, such as scavenger receptors (e.g.,

SR-A, MARCO, CD36, and SR-B) and C-type lectins (e.g., mannose receptor, DC-SIGN, and dectin-1) (East and Isacke, 2002; Peiser et al., 2002; Cambi and Figdor, 2003; McGreal et al., 2005; Pluddemann et al., 2006; Vachon et al., 2006; Areschoug and Gordon, 2009). Additionally, members of the integrin family, excluding CR3, which belongs to the β2 subfamily, can also mediate entry of bacteria into host cells (Cambi and Figdor, 2003; Schraven and Reth, 2007). In particular, some receptors such as CD14 participate in the uptake of bacteria, but act only to tether the particles and cannot initiate phagocytic signaling alone, as this requires accessory receptors to deliver the phagocytic signal and co-start the phagocytic process (Wright et al., 1990).

A number of bacterial components, specifically ligands presented on their surface, are engaged during entry into host cells. Various components found on the plasma membrane of most gram-positive and gram-negative bacteria are likely involved in entry into host cells, including lipopolysaccharides, peptidoglycans, lipoteichoic acid, capsules, pili, and even CpG DNA (Pluddemann et al., 2006). In the past few years, a number of bacterial surface proteins have been shown to bind to the extracellular or surface receptors of the host cells or to be delivered into the cytosol of host cells, where they then assist in the invasive progress (Pluddemann et al., 2006; Areschoug and Gordon, 2008). The active entry of bacteria can be classified into two different mechanisms: "zippering" and "triggering" (Swanson and Baer, 1995; Cossart and Sansonetti, 2004). Zippering means that bacteria present ligands on their surface allowing them to bind to host cells and initiate the entry process (Cossart and Sansonetti, 2004). This zippering mechanism is exemplified by FcR- and CR3-mediated phagocytosis, which is characterized by the formation of inclusion shaped by the bacteria they ingest. Triggering is a mechanism through which bacteria inject effectors into host cells via T3SS to regulate phagocytosis (Swanson and Baer, 1995; Cossart and Sansonetti, 2004). The triggering mechanism enables host cells to internalize bacteria and fluids together (i.e., macropinocytosis) (Cossart and Sansonetti, 2004). For *Yersinia*, the zippering mechanism is the primary mode of entry since surface molecules of *Yersinia* mediate contact with, and adhesion of, host cells (Cossart and Sansonetti, 2004).

INVASION FACTORS

Bacterial pathogens can utilize many kinds of receptors present on the host cell surface, including the following, to facilitate invasion into host cells: FcγR; CR3; β1, β3, and β5 integrins; Toll-like receptors; mannose receptor; galactose receptor; and scavenger receptors (Taylor et al., 2005; Pluddemann et al., 2006). In addition to common strategies used by many other bacterial pathogens, *Yersinia* also employs several kinds of specific molecules on its outer membrane through which it can bind to and invade various host cells, including epithelial cells, endothelial cells, and fibroblasts, in addition to professional phagocytes. Indeed, *Y. pestis* has three identified adhesins: Ail (attachment-invasion locus), Pla, and Psa, in which Ail and Pla acts as invasive factors, but results of mediating adherence by Psa to host cells is not invasion, but prevention of invasion(as discussed below).

AIL

Ail in *Y. pestis* is a 17.5 kDa outer membrane protein, encoded chromosomally, which is predicted to have eight transmembrane domains and four short extracellular loops extending from the surface of the bacterial outer membrane (Miller et al., 1990; Beer and Miller, 1992). Ail from all three species demonstrates cell adhesion and serum-resistance activities (Miller and Falkow, 1988; Miller et al., 2001; Kolodziejek et al., 2007; Bartra et al., 2008; Felek and Krukonis, 2009; Kolodziejek et al., 2010). In a mouse model of infection, Ail mutant *Y. pestis* exhibited a >3000-fold increase in the 50% lethal dose and a lower rate of colonization to the host tissue than the wild type (Felek and Krukonis, 2009).

In *in vitro* cell cultures, Ail-deficient strains have demonstrated reduced epithelial cell association and internalization, that is, at approximately 90 and 98%, respectively (Kolodziejek et al., 2007). *Escherichia coli* carrying Ail is highly invasive to CHO cells (Peiser et al., 2002) and moderately invasive to Hep-2 cells (Groves et al., 2008). Similar to YadA, Ail was also recently found to bind to fibronectin (Tsang et al., 2010), an extracellular matrix component. Additionally, treatment of cultivated cells with antifibronectin antibody decreased Ail-mediated adherence and inhibited KIM5-mediated cytotoxicity of host cells in an Ail-dependent manner (Tsang et al., 2010). Therefore, fibronectin, which has many integrin-binding sites, may act as a bridging molecular to engage host cells with this pathogen (Tsang et al., 2010). Moreover, because biochemical data and genetic analysis suggest that the C-terminal half of extracellular loop 2 of Ail mediates interaction with host cell surface components (Kirjavainen et al., 2008), it is possible that this domain interacts with fibronectin and is subsequently involved in attachment and entry into host cells.

Interestingly, despite sharing 100% homology with *Y. pestis*, Ail from *Y. pseudotuberculosis* fails to confer the same adhesion and invasion functions, which may be due to two amino acid substitutions in extracellular loop 3 (Yang et al., 1996; Miller et al., 2001; Tsang et al., 2010). However, Ail from *Y. enterocolitis* exhibits the same activity as that from *Y. pestis*, although they share only 70% sequence identity (Miller et al., 2001). These data provide strong evidence that some other factors contribute to Ail-mediated phagocytosis.

PLASMINOGEN ACTIVATOR

Plasminogen activator (Pla), encoded by the *Y. pestis*-specific plasmid pPCP1, is a membrane protein of the omptin family of bacterial outer membrane proteases (Kukkonen and Korhonen, 2004). Pla exhibits different phenotypes in inducing plague. In comparison with the wild type, *Y. pestis* lacking Pla has been reported to have greatly reduced virulence and was found to be one of the highly expressed genes when inoculated subcutaneously, but produced equivalent or nearly equivalent virulence when introduced by aerosols or directly into the blood stream (Sodeinde et al., 1992; Welkos et al., 1997). In models of pneumonic plague, dissemination of Pla-deficient *Y. pestis* to the circulation system was found to be unaffected, but restricted outgrowth was observed in lungs (Sebbane et al., 2006; Lathem et al., 2007). However, *Y. pestis* lacking Pla in mouse models of bubonic plague was observed

to grow normally at the subcutaneous sites of inoculation but was not disseminated to the lymphatic system and deeper tissues (Sebbane et al., 2006; Lathem et al., 2007).

Pla is a 10-strand antiparallel β-barrel with four short periplasmic turns and has five surface-exposed loops where catalytic residues are located (Kukkonen and Korhonen, 2004). A striking feature of Pla is its ability to activate plasminogen to plasmin, which then dissolves fibrin clots and digests laminin that further impairs tissue barriers (Degen et al., 2007). In addition to proteolytic functions, Pla also plays a role in adhesion and invasion to epithelial cells, probably by binding to extracellular matrix components such as laminin and reconstituted basement membrane (Lobo, 2006). *Escherichia coli* expressing Pla is capable of invading HUVECs (Kukkonen et al., 2004), ECV304 (Lahteenmaki et al., 2001; Kukkonen et al., 2004), HeLa cells (Benedek et al., 2004), and macrophages (Zhang et al., 2008), and this process appears to be independent of residues S99 and D206, which are required for proteolytic activity of Plas (Lahteenmaki et al., 2001), thus providing evidence that the adhesion activity of Pla is independent of its protease activity. Pla-mediating internalization of bacteria by HeLa cells can be inhibited by phagocytic signaling inhibitors, such as wortmannin, staurosprin, genistein, C3 exoenzymes, and NDGA, and actin polymerization inhibitors, such as cytochalasin D, although bacterial association was not affected (Benedek et al., 2004). Additionally, the adhesion of Pla in *Y. pestis* is more efficient than that of its counterparts in other bacteria, such as PgtE of *Samonella*, OmpT of *Escherichia coli*, and Epo of *Erwinia* (Kukkonen et al., 2004).

Recently, Zhang et al. found that Pla is a ligand for a macrophage and DC surface receptor DEC-205, which is usually thought to be related to antigen presentation (Zhang et al., 2008). Using alveolar macrophage and CHO cells stably expressing DEC-205, the authors determined that Pla interacted with DEC-205 and that this interaction mediated adherence of *Y. pestis* to host cells and promoted early *Yersinia* dissemination from lungs to spleens during pneumonic plague (Zhang et al., 2008). These results suggested a new target of Pla activity, which may help elucidate the rapid progression of primary pneumonic plague.

OTHER

YadBC, representing two surface proteins, is thought to be able to help bacteria to invade into WI-26 type1 pneumocytes and HeLa epithelioid cells (Forman et al., 2008). Although the YadBC double mutant of *Y. pestis* exhibited similar attachment to host cells, the invasion defect was small but significant (Forman et al., 2008).

ANTIPHAGOCYTIC FACTOR

Psa

Psa, a homopolymer macromolecular complex known as an adhesion pilus, is assembled into a molecular mass of 15 kDa subunits, forming a capsule-like structure on the surface of *Y. pestis* (Lindler et al., 1990; Lindler and Tall, 1993). It is ideally expressed in an environment where the temperature remains at 37°C and pH ranges from 5.8 to 6.0 (Bichowsky-Slomnicki and Ben-Efraim, 1963). The expression of Psa is positively regulated by a global transcription regulator, RovA (Cathelyn et al., 2006) and is negatively regulated by Fur (Zhou et al., 2006). Mutated Psa is slightly attenuated by the intravenous mode of infection but exhibits a significant dissemination defect (Lathem et al., 2007). Recent studies have reported that the virulence of *Y. pestis* KIM5 lacking Psa was either unaffected or only slightly affected during subcutaneous challenge of Swiss Webster mice (Bearden et al., 1997) and was unaltered in native or immunized BALB/C mice (Anisimov et al., 2009).

Psa is known to bind to β1-linked galactosyl residues in glycosphingolipids (Payne et al., 1998) and apoB-containing LDL in human plasma (Makoveichuk et al., 2003), and these interactions could prevent binding of purified Psa to macrophages or fibroblasts (Payne et al., 1998; Makoveichuk et al., 2003). Indeed, Psa acts as a antiphagocytic factor independent of Yops (Huang and Lindler, 2004). Although Psa mediates the association of *Y. pestis* with human epithelial cells or mouse macrophages, it cannot resist uptake by host cells (Huang and Lindler, 2004; Liu et al., 2006; Grabenstein et al., 2006). In contrast to wild-type strains, Psa-mutated *Y. pestis* binding to human respiratory tract epithelial cells has been found to be significant, although the number of internalized bacteria was equivalent for both (Grabenstein et al., 2006). Additionally, Psa-modified *Escherichia coli* or polystyrene beads were observed to bind to host cells to a greater extent than either the control bacteria or beads, respectively, and their internalization by host cells was similarly poor (Huang and Lindler, 2004; Liu et al., 2006). These results indicated that Psa confers adhesive but not invasive activity to bacteria. Psa-mediating inhibition of phagocytosis may be caused by binding to lipoprotein, which could prevent recognition of bacterial pathogens by host cells.

F1

Another fimbrial structure expressed by *Y. pestis* is fraction 1 (F1) antigen, which is composed of linear fibers of the Caf1 subunit (Zavialov et al., 2003). F1 is produced at high yields at 35–37°C to cover the bacterial surface. After the initial intracellular stage during infection, *Y. pestis* is released from macrophages and expresses large amounts of F1 (Du et al., 2002). Together with other anti-phagocytic factors, F1 efficiently limits phagocytosis of *Y. pestis* by host cells and contributes to the extracellular survival of *Y. pestis in vivo*, although F1-negative strains exhibit similar virulence as the wild type (Du et al., 2002).

A recent study provided direct evidence of the inhibiting effect of F1 on phagocytosis by epithelial cells (Liu et al., 2006). Using human respiratory tract epithelial cells as models, binding of F1-coated latex beads to A549 cells was reduced when compared with that to BSA-coated latex beads (Benedek et al., 2004). *Y. pestis* deficient in Caf1 was better internalized by host cells, and complementing this strain with Caf-containing plasmid significantly reduced internalization by host cells, confirming the F1-mediated inhibition of uptake of *Y. pestis* by respiratory tract epithelial cells (Benedek et al., 2004). Additionally, the authors of this study reported that the Caf-Psa double mutant was internalized more efficiently than the single mutant, thereby indicating that both F1 and Psa contribute to inhibition of phagocytosis (Benedek et al., 2004).

YopH

YopH, a 468-amino-acid protein secreted by T3SS, exhibits the most potent activity of all PTPase(Protein Tyrosine Phosphatase) enzymes isolated to date (Zhang et al., 1992). The crystal structure of YopH indicates that its N- and C-terminal domains are linked by a proline-rich sequence. The N-terminal domain contains a type III secretion signal, a chaperon-binding region and a substrate-binding domain (Khandelwal et al., 2002), while the C-terminal domain includes a PTPase catalytic domain and an additional substrate-binding domain (Phan et al., 2003; Bahta and Burke, 2012). These two substrate recognition domains cooperate to reinforce binding of YopH to its substrate and enhance its activity and virulence.

The main function of YopH is anti-phagocytosis, which is executed by dephosphorylating its substrates, including $p130^{Cas}$, FAk, and paxillin in epithelial cells and $p130^{Cas}$, SKAP-HOM and Fyb in macrophages (Hamid et al., 1999; Deleuil et al., 2003; Aepfelbacher, 2004). These form the so-called focal adhesion complexes, which play pivotal roles in β1 integrin-mediating phagocytosis. By targeting and dephosphorylating these proteins, YopH may antagonize β1 integrin-mediated uptake of bacteria by host cells at an early stage. Antiphagocytosis activity is essential for the virulence of *Yersinia* since strains lacking YopH are efficiently ingested by host cell phagocytosis (Kerschen et al., 2004).

YopE

YopE is a 219-amino-acid protein that exhibits eukaryotic GAP activity (Rosqvist et al., 1990; Black and Bliska, 2002; Pawel-Rammingen et al., 2002). YopE is comprised of three domains: an N terminal type III secretion signal followed by an intracellular membrane targeting domain and a C terminal Rho GAP domain (Black and Bliska, 2002; Pawel-Rammingen et al., 2002). In infected cells, translocated YopE has been found to cause cell rounding up and detachment through its ability to disrupt actin microfilaments (Black and Bliska, 2002; Pawel-Rammingen et al., 2002).

YopE inactivates small RhoA-like G proteins by inducing their GDPase activity, which results in conversion of the active GTP-bound state to an inactive GDP-bound state, and blocking of downstream signaling cascades (Aili et al., 2006). Given the importance of Rho GTPase in regulating actin polymerization, which is an important event in phagocytosis, YopE can antagonize phagocytosis of *Yersinia* by host cells (Viboud et al., 2006). Indeed, research has shown that YopE-deficient mutants can be efficiently internalized by macrophages and neutrophils (Songsungthong et al., 2010).

A recent study also demonstrated that YopE from *Y. enterocolitis* could bind to and inactivate RhoG, an upstream regulator of Rac1 and other Rho GTPases, both *in vivo* and *in vitro* (Roppenser et al., 2009). YopE colocalized with RhoG in the ER and Golgi, and this localization determined its substrate specificity and activity (Roppenser et al., 2009). As RhoG can be activated by the β1 integrin-mediating pathway of host cells when challenged by bacteria, inhibition of RhoG by YopE may contribute to the overall antiphagocytic activity in a new manner (Mohammadi and Isberg, 2009; Roppenser et al., 2009).

YopT

YopT (322 amino acids) was identified as the second Yop, in addition to YopE, that can disrupt the actin cytoskeleton (Iriarte and Cornelis, 1998). YopT is similar to YopE in that its N-terminal region includes a type III secretion signal and a chaperon-binding domain (Sorg et al., 2003), although its C-terminus comprises a novel cysteine protease belonging to clan CE (Shao et al., 2003). YopT is expressed in *Y. pestis*, but in more than 50% cases, *Y. pseudotuberculosis* does not carry a functional YopT due to deletions in the region of the *yop*T gene (Schmidt, 2011).

YopT specifically recognizes and cleaves the phenyl groups of lipid-modified RhoA, Rac, and Cdc42, which results in detachment of Rho GTPase from the plasma membrane (Zumbihl et al., 1999; Shao et al., 2003). Mislocalization of Rho GTPase impairs its function and paralyzes various downstream signaling pathways (Iriarte and Cornelis, 1998). In cultured cells, ectopic expression of YopT leads to disruption of the actin cytoskeleton, cell rounding up, and inhibition of phagocytosis (Iriarte and Cornelis, 1998). Phagocytosis by macrophages and neutrophils of *Yersinia* mutants lacking YopT has been reported to be significantly greater than that by the wild type (Viboud et al., 2006).

However, the virulence of YopT-mutated *Yersinia* is not altered in mouse oral infection, and bacterial dissemination to the liver is not affected, suggesting that YopT is not required for virulence (Trülzsch et al., 2004; Mohammadi and Isberg, 2009). Additional studies have reported that YopT enhances virulence only in the absence of YopE (Viboud et al., 2006). Considering their similar activity, YopT may be a redundancy due to the presence of YopE, and this may partially explain why YopT is inactivated in certain strains.

YpkA/YopO

YpkA (732 amino acids) is a multifunctional protein containing an N-terminal Ser/Thr kinase catalytic domain and C-terminal GDI domain (Navarro et al., 2007). The first 150 amino acids in the sequence comprise a type III secretion signal and membrane localization domain, while the last 20 amino acids comprise an actin-binding domain (Juris et al., 2000), which localizes to the inner face of the plasma membrane. YpkA is produced in bacteria as an inactive kinase and, when translated into host cells, is activated by binding to actin (Juris et al., 2000; Trasak et al., 2007). Upon activation, YpkA undergoes autophosphorylation, and then subsequently phosphorylates both native and artificial substrates, such as MBP and histone (Juris et al., 2000; Trasak et al., 2007).

Surprisingly, a recent study showed that the C-terminal domain of YpkA mimics the host GDI function to inhibit GDP-GTP exchange of small G proteins, like RhoA and Rac1, and to keep them in their inactive states (Prehna et al., 2006). This activity enables YpkA to repress Rho activity and to limit functions of its downstream signaling pathway, such as actin stress fiber formation (Barz et al., 2000; Dukuzumuremyi et al., 2000; Prehna et al., 2006). On the other hand, the kinase domain of YpkA can bind to and phosphorylate a G protein subunit $G_{\alpha}q$, thereby efficiently impairing guanine nucleotide binding and preventing activation of $G_{\alpha}q$ (Navarro et al., 2007). Because both Rho proteins and $G_{\alpha}q$ control actin stress fiber formation, simultaneous inactivation of these two targets enables YpkA to more efficiently

usurp downstream signaling pathways of the host cells (Navarro et al., 2007).

Considering its obvious effect on the actin cytoskeleton, YpkA is believed to act as another important virulent factor, in addition to YopH, YopE, and YopT, in inhibiting phagocytosis of *Y. pestis* by host cells during infection (Wiley et al., 2006), as YpkA can sequester Rac and block Rac-dependent Fcγ receptor-mediated phagocytosis at the plasma membrane; however, this is not observed with RhoA and RhoA-dependent complement-mediated phagocytosis (Groves et al., 2010) as RhoA is trapped in the cytosol by endogenous RhoGDIα (Groves et al., 2010).

SWITCH FROM INVASIVE TO ANTIPHAGOCYTIC STATUS

How does *Y. pestis* switch from the earlier invasive status to the later antiphagocytic status? After migrating to the inside of macrophages, *Y. pestis* bacteria would encounter harsher microenvironments, including higher temperature, low pH values, osmotic pressure, and reactive oxygen species (as shown in **Figure 1**). Consequently, *Y. pestis* may have evolved the ability to adapt to these challenges. Triggering by various kinds of environmental signals may have up- or down-regulated their survival- or virulence-associated genes, thereby changing surface components and presenting heterogeneous phenotypes. This transition may enhance their survival within host cells and their virulence once escaped.

First, and most importantly, the temperature shift from 28°C in vitro to 37°C in vivo could induce the expression of F1, Psa, and Yops, all of which work in coordination to block phagocytosis by host cells (Lawson et al., 2006; Fukuto et al., 2010). Second, the low pH value of the phagosome inside the macrophage allows for upregulation of the expression of PsA (Lawson et al., 2006; Fukuto et al., 2010). Although Psa mediates adhesion to host cells, this binding does not initiate the progress of uptake, and in contrast, inhibits the internalization of bacteria through an unknown mechanism(as discussed above). In addition, the *Y. pestis*-Containing Vacuole (YCV) may provide a niche for bacteria to make this transition. Around 8 h postinfection, the YCV acquired markers of late endosomes or lysosomes and had a spacious morphology, but it could prevent vacuole acidification and avoid bactericidal autophagy (Grabenstein et al., 2006; Pujol et al., 2009). Residence in the YCV and responses to environmental stress may promote bacteria to initiate the transcription regulation of invasive or anti-phagocytic factors, resulting into the final anti-phagocytic status.

Although the adhesive factors Ail and Pla are still present on the surface of bacteria, owing to their relatively short outer membrane regions, they are unable to escape the masking effects of the F1 capsule, which would block contact between Ail and Pla and their respective receptors, leading to further activation of the uptake signaling pathway. Conversely, the Psa is long enough to overcome the masking effects of the F1 capsule, but binding to its receptor does not prime the internalization progress and produces the opposite effect, which inhibits phagocytosis by host cells. Furthermore, adhesion of Psa to the surface of host cells facilitates the delivery of Yops into host cells, which further blocks the phagocytic ability of the host cells.

CONCLUSIONS

Deficiency in invasin and YadA does not hamper the invasion of *Y. pestis* into host cells, which is necessary for infection during the earlier stage, which suggests that *Y. pestis* must utilize other invasive factors to aid its entry into host cells. Loss of virulence of these invasive-factor mutants indicates that *Y. pestis* must acquire the ability to tip the balance of entry into host cells and to subsequently have antiphagocytic activities. During the earlier stages of infection, *Y. pestis* requires invasive factors to enter into host cells, and during the later stages, it must produce antiphagocytic factors to escape uptake by host cells. Although the mechanism through which *Y. pestis* fine-tunes the temporal expression of these seemingly contradictory factors remains unknown, the simultaneous presence of these opposing functions in this bacterium represents a unique capability to survive both inside and outside host cells. Further analysis of the regulation of invasion and antiphagocytic associated virulence factors will improve our understanding of the pathogenesis of *Y. pestis* and will provide greater insights into the *in vivo* survival mechanism of facultative intracellular or extracellular bacteria.

ACKNOWLEDGMENTS

Financial support was provided by the National Natural Science Foundation of China (30930001, 81301405) and by the National Basic Research Program of China (2009CB522600, 2012CB518704).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 October 2013; paper pending published: 31 October 2013; accepted: 10 December 2013; published online: 24 December 2013.

*Citation: Ke Y, Chen Z and Yang R (2013) Yersinia pestis: mechanisms of entry into and resistance to the host cell. Front. Cell. Infect. Microbiol. **3**:106. doi: 10.3389/fcimb.2013.00106*

This article was submitted to the journal Frontiers in Cellular and Infection Microbiology.

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