BACTERIAL PATHOGENS IN THE NON-CLINICAL ENVIRONMENT

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BACTERIAL PATHOGENS IN THE NON-CLINICAL ENVIRONMENT

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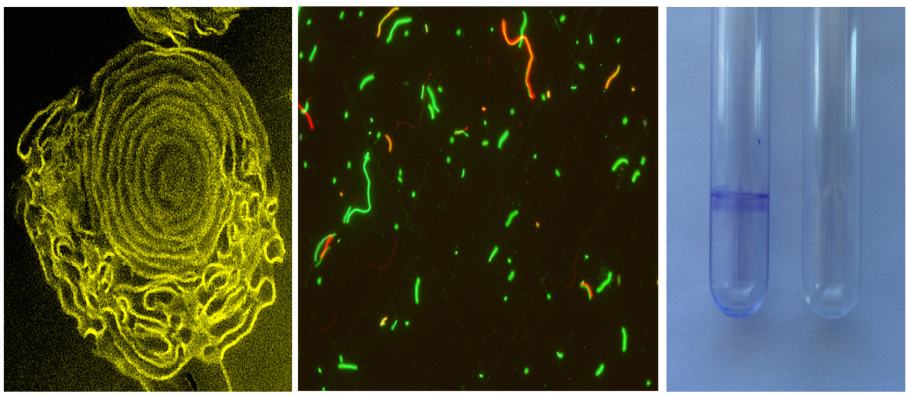

Bacterial pathogens can be found in many forms in the non-clinical environment. Left panel: electron transmission micrograph of a multilamellar body produced and secreted by the amoeba *Dictyostelium discoideum* (false color). Bacteria can be packaged in multilamellar body. Center panel: Live/Dead® staining of viable but non-culturable *Legionella pneumophila* in water. Live bacteria are stained green, while dead bacteria are stained red. Right panel: biofilm produced by *Escherichia coli* grown in LB broth and stained with crystal violet (left tube). The right tube is a negative control consisting of broth only. The left image is from Steve J. Charette. The center and right images are from Sebastien P. Faucher.

The transmission route used by many bacterial pathogens of clinical importance includes a step outside the host; thereafter refer to as the non-clinical environment (NCE). Obvious examples include foodborne and waterborne pathogens and also pathogens that are transmitted by hands or aerosols. In the NCE, pathogens have to cope with the presence of toxic compounds, sub-optimal temperature, starvation, presence of competitors and predators. Adaptation of bacterial pathogens to such stresses affects their interaction with the host. This Research Topic presents important concept to understand the life of bacterial pathogens in the NCE and provides the reader with an overview of the strategies used by bacterial pathogens to survive and replicate outside the host.

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EDITORIAL
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Editorial on: Bacterial pathogens in the non-clinical environment

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When thinking about bacterial pathogens, most will consider their interaction with humans. Nevertheless, many pathogens affecting humans will not be transmitted directly from one individual to another but will rather come from or transit through the environment to infect the human host. Outside their hosts, bacterial pathogens must be able to resist environmental stresses and perhaps grow in order to get to another hosts. The environment outside the host is referred therein as the non-clinical environment (NCE).

In this research topic, a collection of articles is presented that covers some of the strategies and factors that influence the survival and growth of bacterial pathogens in the NCE, and therefore affects transmission to humans, and outbreaks. Such knowledge could be important to limit the transmission during an outbreak. For example, a Legionnaires' disease outbreak in Quebec City (Canada) in 2012 prompted Trudel et al. (2014) to review the effort to find the source. They conclude that better collaboration between government agencies, academia, and the industry could prove beneficial in the fight against bacterial infections.

Importantly, bacterial pathogens will require adapting their genome to persist and grow in the NCE. This may affect the interaction with human hosts, as stated by Dr. Martinez: "Evolution of human pathogens is not exclusively driven by the infection of human" (Martínez, 2014). To support his opinion, the author gives the example of *Yersinia pestis* who has lost genes to kill its insect host, which allowed for a better transmission between animal hosts, including human by using the insect as a vector. Martinez also discusses the concept of short-sighted evolution during infection.

Evolution of transmission properties is an important aspect of pathogens coming from the NCE. To illustrate that, Leong et al. (2014) studied the presence of *Listeria monocytogenes* in several processing plants in Ireland and on the food produced by those plants. They showed that some pulsotypes were commonly found in several different facilities. Some strains are likely better equipped to persist in the facilities and consequently contaminate food more frequently (Leong et al., 2014).

In the NCE, the pathogens will also interact with other microorganisms, invertebrates and plants, which may all shape how the pathogens behave as well as its ability to infect human hosts. Growth of bacterial pathogens in the NCE may allow them to reach concentrations that ensure their transmission to new hosts. The complex relationship between the NCE and outbreak occurrence is very well illustrated by *Clostridium botulinum* (Espelund and Klaveness, 2014). The authors suggest that accumulation of *C. botulinum* spores in carcasses, algal mats and biomass, and further bioaccumulation of the toxin is central in causing diseases.

In the NCE, bacterial pathogens will encounter protozoa. These are ubiquitous unicellular eukaryotes and many feed on bacteria. Therefore, there is an evolutionary pressure on bacteria to resist grazing by protozoa. Some, such as *Legionella pneumophila*, have evolved a strategy to highjack them and grow intracellularly. In their review, Robertson et al. (2014) point out that

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L. pneumophila has a far more complex developmental cycle than normally thought which includes the production of mature infectious forms by amoebae thought to increase the potential of transmission of *L. pneumophila* from water systems to the human host (Robertson et al., 2014).

Some species of protozoa are known to produce and expel vesicles while grazing on bacteria. These vesicles, sometime referred to as pellets, may contain live bacteria (Denoncourt et al., 2014). Packaging of bacteria by protozoa increases their resistance to biocides and other environmental stresses. In addition, pellets may increase infectivity of bacterial pathogens. The authors, based on the literature, thus propose the hypothesis that packaging of bacterial pathogens by protozoa is important for their persistence and for their transmission to human and animal hosts (Denoncourt et al., 2014).

Bacteria exposed to stresses in the NCE have evolved strategies to deal with them. Therefore, bacterial pathogens have learned tricks in the NCEs that prove to be efficient to promote infectious diseases. Biofilm is important for the persistence of bacterial cells in the NCE, since bacterial cells inside biofilm are more resistant to biocides and stressful conditions than planktonic cells. Vogeleer et al. (2014) discuss the role of biofilm for the persistence of Shiga-toxin producing *E. coli* (STEC) in the NCEs, including soil, water systems, meat processing plants, and on fresh produce. They argue that STEC biofilm are likely an important source of contamination of finished products and a concern for public health (Vogeleer et al., 2014).

The viable-but-not-culturable (VBNC) state is characterized by live and metabolically active cells, but unable to grow on standard laboratory medium (Li et al., 2014). This can complicate the detection of bacterial pathogens in water, food, and from infected tissues. This state can be triggered by a variety of stressful conditions frequently encountered in NCE. VBNC cells are notoriously more resistant than culturable cells to physical and chemical stresses. In addition, resuscitation of VBNC cells can occur in conditions permissive for their growth or when exposed to their hosts. Many genetic factors are involved in the induction of the VBNC state and resuscitation from it, but we are still far away from being able to adequately detect cells in this state and develop ways to avoid them (Li et al., 2014).

Persisters are non-growing phenotypic variant of a population that are tolerant to antibiotic (Amato et al., 2014). They are genetically identical to the rest of the population; only their physiological state is different. Persistence has probably evolved in response to antibiotic producing microbes in the NCE. This state has clinical importance for the treatment of infectious diseases: following an antibiotic treatment, a small proportion of the population will survive and when the antibiotic fades away, the survivors resume growth. Efforts are needed to find drugs to block persistence during antibiotic treatment (Amato et al., 2014).

The articles published in this research topic clearly highlight that the behavior of bacterial pathogens and their interaction with other organisms in the NCE influence their transmission and their performance during infection. A complete understanding of virulence and epidemiology and the development of effective countermeasures against bacterial pathogens would be ultimately successful only if their whole life cycle, including their life in the NCE, is taken into account.

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Management of the 2012 *Legionella* crisis in Quebec City: need for a better communication between resources and knowledge transfer

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INTRODUCTION

Legionella pneumophila is one of the few bacteria that can be considered as a genuine environmental pathogen. Whilst most infections of hydric origins result from the faecal pollution of a stream or ground water, it is indeed not the case for *Legionella pneumophila* since this bacterium can be found in an ubiquitous manner in fresh water where it can survive temperature variations from 5.7 to 63°C (Fliermans et al., 1981). Consequently, any machinery or device using a water supply can be colonized with *Legionellae*, especially if the water temperature is high as it favor its growth: cooling towers, plumbing, water-heaters and hot tubs are few examples.

Between July 18th and October 8th, 2012, 181 cases of legionellosis have been reported in the Quebec City area, 14 of which being sadly fatal. The investigation done by the Direction de la santé publique (DSP) (public health management office) assisted by the Ministère du Développement durable, de l'Environnement, de la Faune et des Parcs (MDDEFP) (Ministry of sustainable development, environment, fauna and parks) and the Institut de recherche Robert Sauvé en santé et sécurité du travail du Québec (IRSST) (Robert Sauvé occupational health and safety research institute) has been long and tedious for various reasons that will be discussed later on. This extended delay between first case notification and resolution of crisis has enticed the media to spread messages, sometimes contradictory, and to give the floor to pseudo-experts who, by proposing, for example, source of outbreak that were totally improbable in these circumstances, needlessly alarmed and increase the panic response of the population (Pelchat et al., 2012).

It was people living in or frequently visiting the St-Roch and Limoilou districts (lower part of the city) that were contaminated by this strain of *Legionella* and, from the first notified cases, the cooling towers found in the area were suspected of harboring the pathogenic strain (Isabelle Goupil Sormany, 2012). These towers are essentially heat exchangers between the water and ambient air. The water to be cooled, the temperature of which usually varies between 25 and 40°C, is pulverized upward in the cooling tower using forced ventilation, loading the air released by the tower with steam created by the evaporation stream and tiny droplets which are the preferred conveyers for this pulmonary pathogen (Keller et al., 1996). The factors known to favor the proliferation of legionellae are the temperature (25–40°C), stagnancy, presence of sediments, scale, biofilms and corrosion, as well as the presence of amoebas and ciliate protozoans that could support the *Legionella* intracellular growth, all conditions found in cooling towers during summertime (Buse et al., 2012).

HISTORY OF AN OUTBREAK

The report from the DSP published after the Quebec City 2012 legionellosis outbreak (Isabelle Goupil Sormany, 2012) is a precious information source when trying to explain the extended delay between the first cases and the resolution of the crisis. **Figure 1** shows the evolution of the situation and lists key dates of the outbreak of *Legionella* in Quebec City during summer 2012.

From this report, we can first learn the regulated procedures in case of such outbreaks. Indeed, the Public Health Law allows the DSP to proceed with an epidemiologic investigation in any situation where there is serious motives to believe the public health is or could be threaten.

Therefore the DSP set in motion an epidemiologic investigation.

Whilst an intervention guide on *Legionella* (Décarie et al., 2010) mentioning that the "control intervention on the source should be done as soon as possible" was published in 2010 by the health authorities, it does not provide the following precisions:

1. How the validation of the cooling towers maintenance should be done;
2. At what distance from the location of the clinical case should the samples be taken when no source has been clearly identified;
3. How the water sampling in the cooling towers should be performed;
4. Where should the samples be forwarded (although it is clearly suggested to proceed with a service agreement with the Quebec Public Health Laboratory (LSPQ);
5. How the results should be interpreted.

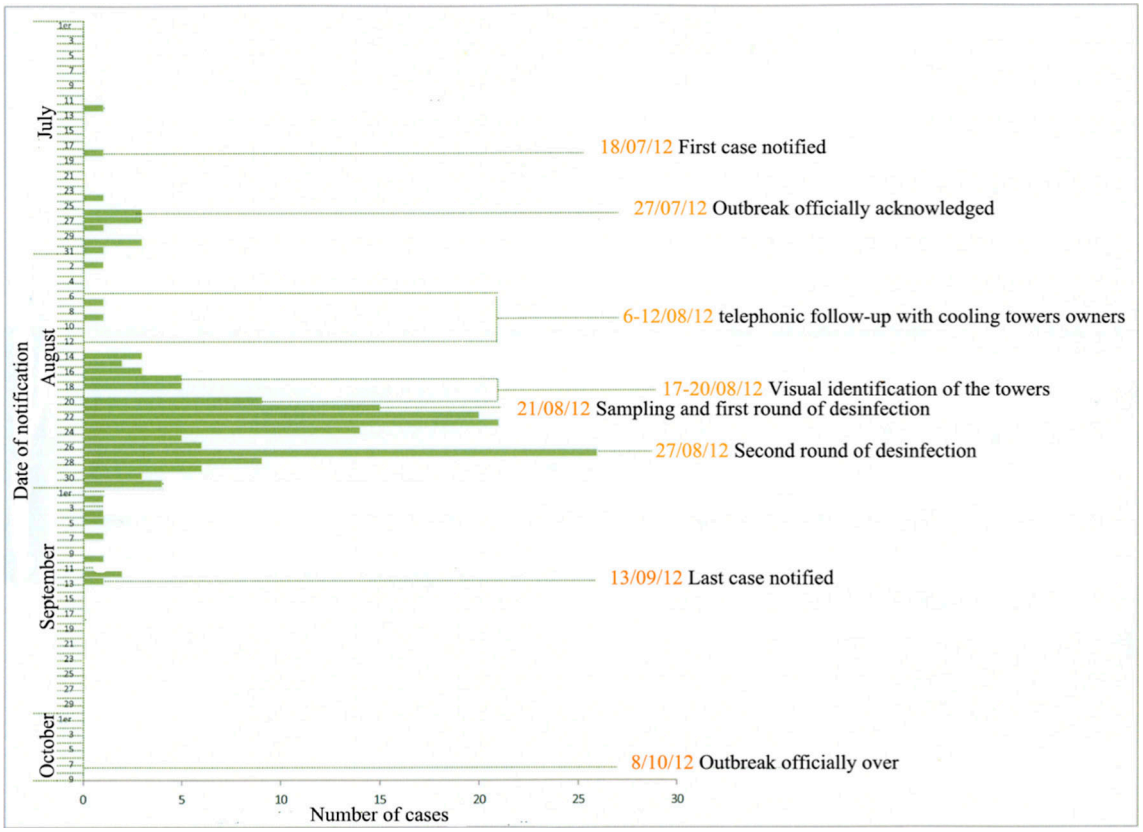

FIGURE 1 | Important dates and actions during the 2012 legionellosis outbreak in Quebec (modified from the "Rapport du directeur de la santé publique, François Desbiens, M.D. Éclosion de légionelles dans la ville de Québec").

Furthermore, this guide provides no detail regarding the treatment that could help control the *Legionella* contamination in a cooling tower during such situation. It is therefore understandable that the people who had to intervene were resource-less due to the lack of information.

Hence, from August 2nd, the DSP proceeded with a first wave of intervention entitled ≪ voluntary measures ≫ during which the building's owners were made aware of the problem, through both a media campaign and individual mail contact, and encouraged to proceed to a water quality control and thorough cleaning of their installations. A second wave of intervention, entitled ≪ mandatory measures ≫ was set off as of August 14th and aimed at:

1. Identify the cooling towers in the area where the highest number of affected people were found;
2. Identify the contamination source using water samples;
3. Perform a visual evaluation of the cooling towers maintenance;
4. Proceed to the sanitization whilst awaiting the analyses results;
5. Prescribe control measures according to the results obtained from the water samples and observations from the cooling towers inspections.

The sampling and sanitization treatments of the cooling towers were initiated on August 21st. It therefore took over a month before proceeding to the first inspections aimed at identifying and sanitizing the source or sources responsible for this outbreak. Why such a delay? Several assumptions can be made:

- Any governmental machine is weighty and complicated to get started;
- The authorities were not ready to face this crisis;
- There were no inventory, nor maintenance registry of the cooling towers even if so recommended by several reports dating from the previous Quebec City *Legionella* outbreak in 1997;
- Legally, it is impossible to proceed with any sampling in private buildings without being mandated to do so.

The analysis of the samples was done by the MDDEFP and the IRSST and both used culture to evaluate the concentration of *Legionella* found in various cooling towers. This approach, according to the Public Health Director, requires 20 days before any information on the quantity and the exact identity of the *Legionella* strains can be obtained. These 20 days added to the 30 or so spent prior to the beginning of the analyses and you end up with more than 50 days from the start of the outbreak to the first experimental results pointing toward a potential source. Even though a first round of disinfection of the towers has been initiated on August 21st, a survey made 1 week later showed that 21% of the towers

still shelter significant concentrations of *Legionella*.

COULD IT HAVE BEEN DONE BETTER. . . OR DIFFERENTLY?

The answer is definitely YES. The delay of almost 2 months could have been noticeably reduced if academic or private research laboratories had been involved from the start. What would have been the advantages of consulting and using the expertise found locally or internationally?

Several research laboratories own the necessary tools to perform rapid molecular analyses for the quantification and identification of legionellae as well as characterization of water microbial flora. These research methods, whilst non-standardized or validated as those routinely used by the Quebec government laboratories or any other reference laboratory, are extremely rapid, accurate and powerful and would have allowed for an answer regarding either the presence of *Legionella* or their concentration. Several reports state that real time PCR and its derivative, viable qPCR, have immense potential for the accurate, rapid and cost-effective detection and enumeration of *Legionella* in environmental samples and can be used as a complementary tool for the detection and monitoring of *Legionella* in different water systems (Dusserre et al., 2008; National Guidelines for the Control of Legionellosis in Ireland, 2009; Qin et al., 2012; Slimani et al., 2012). Obviously, the presence, even in large concentration, of *Legionella* in cooling towers does not guarantee that the strain responsible for the infection is detected. Supplementary tests such as sequence-base typing must be done to assert the link between clinical isolates and environmental strains (Ginevra et al., 2009). However, this step did not delay the process and had no consequences in the *Legionella* outbreak in Quebec in 2012 and is not criticized in the present paper.

Other laboratories are active in the aerosol science research and also possess the equipment required for *Legionella* aerosol measurement and detection. As previously demonstrated (Blatny et al., 2011), air sampling from various distances from the suspected sources could have helped determining the high concentration zones, circumscribing the area where cooling towers were heavily contaminated and released high concentrations of this pathogen. For example, concentration measurements of the aerosolized *Legionella* over an open air biological treatment plant and along the aerosol plume emitted from this plant demonstrated that decreasing but notable concentrations of *Legionella* could be found hundreds of meters from the plant (Blatny et al., 2011). Models, including wind, temperature and geography data, could have been developed to predict the transport, dispersion and dilution of the airborne contaminants.

The combination of these two approaches could have permitted to determine, within a few days or, at worst, weeks, the epicenter of this outbreak and allow for a much quicker intervention. It is estimated that this type of intervention could have accelerated the source-detection process when it is a known fact that each day gained can be critical in order to halt this type of outbreak.

WHY WERE THE ACADEMIC LABORATORIES NOT INVOLVED?

Two hypotheses can be proposed as an answer:

1. The research laboratories might not be sufficiently present and known by the governmental organizations;
2. The governmental administration has the tendency to use its own resources when faced with such situation.

The answer, at least for the Quebec City outbreak, is mitigated and each of these two hypotheses has its own merit. On one side, the governmental administration could have made more efforts to build a complete database of skilled scientists in this field (water research, infectious disease specialists, environmental microbiologists, bioaerosols scientists) and use this expertise even if outside of the government administration regular network. As far as water samples, few limitations exist and large bulk samples were available. Distribution of water samples through other provincial government Public Health labs to research institutions could also be a potential way to seek help and collaboration. The lack of a readily available expert database may have impaired this process. Most likely, the governmental administration was not aware of the research capacity and expertise available. Research laboratories have their own responsibility in being not sufficiently known outside their research network. Standard research activities (conferences, workshops) rarely make their way through to the general public and governmental agencies. Research laboratories should make significant efforts toward knowledge transfer and public communication. Since the medical world, which is in charge of public health, and the world of fundamental and applied research are directed by people trained in different academic contexts, they may not all be readily disposed to spontaneous collaboration.

What could be done to prevent such situation from happening again? A database, identifying the numerous governmental para-governmental, private and public organizations possessing an expertise in the field of *Legionella* and, to an extent, in all other agents susceptible of creating situations such as the one experienced in Quebec City in 2012, should be built. The quick integration of a multi-disciplinary special team with diverse field of expertises would have certainly speed up the process and, maybe, even saved a few lives.

This sad story reinforced the importance of the de-compartmentalization of the research laboratories and, unfortunately, the public health office new action plans do not mention this type of integration. If they want to be consulted during such crises, maybe the research experts should build their own database and make it readily available to the numerous governmental agencies. It should be noted that this crisis led to new regulations amending the safety code incorporating provisions relating to the maintenance of cooling towers' water and that use of qPCR will soon be authorized to quantify *Legionella* in cooling towers.

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Short-sighted evolution of bacterial opportunistic pathogens with an environmental origin

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Evolution is frequently considered as a directional process in which the organisms do not return, after evolving, to a pre-evolved situation. However, there are occassions in which evolution follows a sort of cycling process. Each time an organism is confronted with a given selective situation, it follows a similar evolution path. However, once the selection pressure is resumed, the organism is outcompeted by non-evolved partners and the evolved lineage disappears. This type of process has been dubbed as "short sighted evolution" (Levin and Bull, 1994) and is fundamental for understanding the *in host* adaptation processes of bacterial opportunistic pathogens.

Opportunistic pathogens are a group of microorganisms that do not usually infect healthy hosts but produce infections in hospitals, to immunodepressed persons or those patients presenting underlying diseases as cystic fibrosis, which favors infection (Koch and Hoiby, 1993). Commensal bacteria are among the most prevalent opportunistic pathogens. However, the use of antibiotics, which usually kill commensals besides pathogens, increased the incidence of infections due to environmental microorganisms presenting reduced susceptibility to antibiotics (Bergogne-Berezin et al., 1993).

The evolution of non-pathogenic bacteria towards virulence has been deciphered in occasions. Many "professional" pathogens harbor different sets of genes, dubbed as pathogenicity islands, which have been acquired by horizontal gene transfer, and allow such pathogens to infect the human host (Groisman and Ochman, 1996; Morschhauser et al., 2000; Gal-Mor and Finlay, 2006). The best studied among these evolution processes is that of *Yersinia pestis*, the causal agent of plague (Achtman et al., 2004; Zhou and Yang, 2009). The genus *Yersinia* is formed by 15 different species among which only *Y. pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* are human pathogens. Phylogenetic reconstruction of the evolution of this genus indicates that *Y. pseudotuberculosis* and *Y. enterocolitica* diverged more than 40 million years ago, while *Y. pestis* diverged from *Y. pseudotuberculosis* less than 20,000 years ago (Achtman et al., 1999). The ancestor of these pathogenic *Yersiniae* evolved towards virulence, from a non-pathogenic environmental *Yersiniae*, by acquiring a virulence plasmid named pCD1 (Wren, 2003). This plasmid contains genes coding for a Type III secretion system, which activity allows subversion of the immune system, and is required for the virulence of this pathogen. It is worth mentioning that the acquisition of this plasmid occurred before the divergence between *Y. pseudotuberculosis* and *Y. enterocolitica*, hence more than 40 million years ago; long before the origin of the genus *Homo* (Wood, 1992; McHenry, 1994). This means that the evolution of human pathogens is not exclusively driven by the infection of humans (Martinez, 2013). As stated before, the speciation of *Y. pestis* from *Y. pseudotuberculosis* is a much more recent process that involves both the acquisition of some genes and the loss of other ones. The loss of genes has been frequently associated to genome reduction evolution mostly in the case of endosymbionts (Perez-Brocal et al., 2006). For these microorganisms, several metabolic functions can be covered by the host and un-needed genes are lost just by gene drift. The situation concerning *Yersinia* is not the same. In its process of evolution *Y. pestis* had lost a set of genes encoding insect toxins. Consequently, the evolved organism does not kill insects, a property that allowed its improved transmission by beats of colonized insects (Chouikha and Hinnebusch, 2012). Since this may mean that *Y. pestis* is in the process of evolution toward commensalism in insects, we may conclude that a relevant element for the success of *Y. pestis* as a human pathogen comes from its adaptation to a completely different host, in this case the insects.

As described in this example, evolution towards virulence involves the acquisition of some elements and the loss of other ones. This process leads to speciation from non-virulent towards virulent microorganisms. Whilst this is the situation for several "professional" pathogens, the evolution process of opportunistic bacterial pathogens is rather different. As above stated, these organisms do not infect healthy people and the main factor allowing infection is the patient predisposition for being infected. This may mean that any organism may infect an immunocompromised patient, something that is not fully true.

The human body can be considered as an extreme environment. Actually, when we talk about human microbiota we refer to microorganisms colonizing the surfaces of human body; the body itself is an sterile environment unless an infection occurs. The entrance in this ecosystems requires either the acquisition of virulence determinants to cope with the human defences, as

happens with the aforementioned example of *Y. pestis*, either a decay in these defences, as occurs for opportunistic pathogens. However, even in this last case, to colonize the human host a microorganism requires to survive under the physicochemical conditions of human body: a narrow range of temperature oxygen tension and pH; some specific nutrients and low iron availability. In the case of opportunistic pathogens infecting immunocompetent patients as those suffering cystic fibrosis, mechanisms to cope with the immune response are also needed. In addition, and since patients at hospitals are frequently under antibiotic therapy, these opportunistic pathogens frequently display low susceptibility to antibiotics (Martinez and Baquero, 2002). Indeed, antibiotic treatment is a risk factor for being infected by some environmental opportunistic pathogens presenting low susceptibility to antibiotics as *Stenotrophomonas maltophilia* (Alonso and Martinez, 1997; Sanchez et al., 2009).

One of the best studied bacterial opportunistic pathogens is *Pseudomonas aeruginosa*. This microorganism is currently among the most prevalent causes of infection at hospitals and is the major cause of chronic infections in cystic fibrosis patients. These patients are frequently infected by a single *P. aeruginosa* clone that remains and evolves in the lung of the patient for decades (Yang et al., 2011).

Since *P. aeruginosa* is an environmental bacterium, it might be thought that virulent strains constitute a specific branch in the species, which is in its route to speciation as it has happened with "professional" pathogens. Nevertheless, molecular epidemiology has shown that the same clonal complexes present in non-clinical ecosystems are the ones producing infections (Morales et al., 2004; Wiehlmann et al., 2007; Kidd et al., 2012). Further, the comparison of clinical and non-clinical isolates showed that they were functionally equivalent. All of them extruded the synthetic antibiotics quinolones, presented the genes encoding the elements of the Type III secretion system and of the quorum sensing regulation response and were capable to invade epithelial cells; all these characteristics typical of virulent strains. In addition, all strains were able of use oil hydrocarbons as carbon sources, a feature characteristic of environmental biodegradative microorganisms, which is not required for producing an infection (Alonso et al., 1999). Together with the aforementioned epidemiological evidences, this indicates that *P. aeruginosa* does not present two evolutionary branches, one of them towards virulence. Rather, several if not all of the strains have the capability of infecting patients presenting underlying diseases. The reason for this situation resides likely in the ability of *P. aeruginosa* for infecting several different hosts besides humans, including plants, protozoans, worms or insects (Rahme et al., 1995; Tan et al., 1999; Mahajan-Miklos et al., 2000; Navas et al., 2007; Broderick et al., 2008; Carilla-Latorre et al., 2008). Since the same virulence determinants serve to infect all these host it is worth thinking that such determinants evolved during the interaction with the oldest organisms in this phylogenetic tree. In this respect, it is worth mentioning that *P. aeruginosa* is able to avoid being killed by bacteriovorous nematodes and protozoans (Mahajan-Miklos et al., 1999; Cosson et al., 2002). It is then possible that a major force in the evolution of *P. aeruginosa* (and by extension of other opportunistic pathogens with an environmental origin) resides in ancient prey/predator relationships (Martinez, 2013).

All these studies reinforce the idea *P. aeruginosa* did not evolve toward virulence in humans. Rather, this bacterial species can infect humans by using elements that evolved for its interaction with other hosts (Rahme et al., 1995; Mahajan-Miklos et al., 2000). Nevertheless, once a strain enters inside the human host, it can evolve to improve its adaptation to this new environment (Folkesson et al., 2012). Whereas in the case of "professional" pathogens, these changes are usually fixed and contribute to speciation, the same does not necessarily happen in the case of opportunistic pathogens. The evolution of *P. aeruginosa* during chronic infections has been studied in detail. It has been found that strains producing chronic infection carry mutations in global regulation factors that regulate expression of *P. aeruginosa* social cooperative traits, including secretion of exo-products and quorum sensing. As a consequence, expression of these elements, which are of relevance in *P. aeruginosa* acute infections, is reduced in chronic strains (Martinez-Solano et al., 2008; Damkiaer et al., 2013; Jiricny et al., 2014). This in-host evolution process is favored by the increased prevalence of strains presenting high mutation rates found in chronic infections (Oliver et al., 2000; Mena et al., 2008).

It can be thought that these adaptive mutations could be fixed in the population, hence constituting the beginning of a speciation process towards virulence. However, with the exception of some epidemic clones causing infections in cystic fibrosis patients, mainly at Denmark and England (see below), most patients are infected by independent *P. aeruginosa* isolates (Bragonzi et al., 2009), which do not present, during the first stages of infection, the pathoadaptive mutations displayed by the evolved chronic strains. This evolution process by which an organism follows the same pattern of evolution (that is not fixed afterwards) each time it enters in a given ecosystem has been dubbed as short-sighted evolution (Levin and Bull, 1994). This process is likely fundamental for the adaption of microorganisms producing chronic infections with low inter-patient transmission rates.

Although each bacterial isolate colonizes the lung of an independent patient, all lungs are quite similar habitats. It is then expected that the evolutionary landscapes of each of this chronic strains may be similar. However, once bacteria are released, these adaptations are of no use unless the microorganism colonize another cystic fibrosis patient. This is likely the situation of countries as Denmark in which patients have been usually in contact for treatment and in occasions even on holiday camps (Ojeniyi et al., 2000). Similarly, it has been shown that the acquisition of novel prophage islands is a critical event in increasing the competitiveness of epidemic *P. aeruginosa* clones as the Liverpool strain (Winstanley et al., 2009). It could be predicted that under those circumstances epidemic clones, presenting cystic fibrosis adaptive mutations spread, a situation that has been already described in occasions (Jelsbak et al., 2007; Rau et al., 2010; Dettman et al., 2013). Whether or not these epidemic clones are in the route of speciation remains to be established. However, the fact that the implementation

of strict cohort-based patients segregation policy has halted the epidemic of the Liverpool strain, with an increase in the percentage of patients with unique *P. aeruginosa* strains (Ashish et al., 2013), indicates this adaptation to be still transient.

In other cases in which inter-host transmission is low, the evolved isolates are released into natural ecosystems. The loss of elements that were fixed, long time ago, during the evolution of *P. aeruginosa* in its natural habitats, make these evolved clones to be outcompeted by the bulk of the population. In this regard, it is worth mentioning that *P. aeruginosa* strains adapted for growing in the lungs of cystic fibrosis patients present a decreased resistance to natural phage and protists predators (Friman et al., 2013).

Short-sighted evolution is a common process in the evolution of microorganisms and has been explained in the frame of a source-sink situation (Sokurenko et al., 2006). In the case of opportunistic environmental pathogens, as *P. aeruginosa*, the majority of the population is present at its original, non-clinical habitat, which constitutes the source of this species. Only a few of these microorganisms infect some patients (sink), just by chance. Evolution during chronic infection allows the adaptation required to persist inside the infected host. However, this happens at the cost of de-adapting for growing in the original habitat, including resistance to natural predators (Friman et al., 2013). The only possibility for the fixation of mutations acquired during chronic infections is a high inter-host transmission, in which case the adapted strain can remain in a habitat (the lung of a different host) that is similar to the one where it evolved without affording the costs of returning to its original non-clinical habitat.

We can then conclude that while the acquisition and loss of genes are at the roots of the speciation of "professional" pathogens, speciation toward virulence is not necessarily required for bacterial opportunistic pathogens. This does not mean however these pathogens do not evolve for a better adaptation for growing in the human host. Indeed, the study of *P. aeruginosa* shows that there are common evolution patterns among isolates producing chronic infections. Nevertheless, these mutations are rarely fixed (with the exception of some epidemic clones Yang et al., 2011, see above); those strains infecting for the first time a patient present in most cases the characteristics of a wild-type strain, not of the ones that evolved during chronic infections. This process that has been dubbed as short-sighted evolution because the organism cannot "foresee" the consequences of such evolution is fundamental for the adaptation of environmental bacterial opportunistic pathogens to the human host.

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Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland

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Although rates of listeriosis are low in comparison to other foodborne pathogenic illness, listeriosis poses a significant risk to human health as the invasive form can have a mortality rate as high as 30%. Food processors, especially those who produce ready-to-eat (RTE) products, need to be vigilant against *Listeria monocytogenes*, the causative pathogen of listeriosis, and as such, the occurrence of *L. monocytogenes* in food and in the food processing environment needs to be carefully monitored. To examine the prevalence and patterns of contamination in food processing facilities in Ireland, 48 food processors submitted 8 samples every 2 months from March 2013 to March 2014 to be analyzed for *L. monocytogenes*. No positive samples were detected at 38% of the processing facilities tested. Isolates found at the remaining 62% of facilities were characterized by serotyping and Pulsed Field Gel Electrophoresis (PFGE). A general *L. monocytogenes* prevalence of 4.6% was seen in all samples analyzed with similar rates seen in food and environmental samples. Differences in prevalence were seen across different food processors, food sectors, sampling months etc. and PFGE analysis allowed for the examination of contamination patterns and for the identification of several persistent strains. Seven of the food processing facilities tested showed contamination with persistent strains and evidence of bacterial transfer from the processing environment to food (the same pulsotype found in both) was seen in four of the food processing facilities tested.

Keywords: Listeria monocytogenes, ready-to-eat foods, persistence, food safety, food processing

INTRODUCTION

Mild listeriosis, an infection of the gastrointestinal tract by *Listeria monocytogenes*, generally presents itself with typical "food poisoning" symptoms including abdominal cramps, nausea and diarrhea. However, *L. monocytogenes* has the ability to cross the epithelial barrier of the intestinal tract to cause more serious infection throughout the body including bacteremia. It can also cross the blood-tissue barrier which allows the bacteria to infect organs such as the brain or uterus, where it can cause severe life-threatening infections such as meningitis, encephalitis, spontaneous abortion, or miscarriage. Although the incidence of human listeriosis is comparatively low, at up to 30% it has the third highest mortality rate of all food borne pathogens (EFSA, 2014) and immunocompromised individuals are particularly at risk (Vazquez-Boland et al., 2001; Cartwright et al., 2013). According to the most recent EU data, the mortality rate was 12.1% for the 1642 cases reported in the year 2012 (EFSA, 2014).

Food processors need to be vigilant against *L. monocytogenes* as the bacterium is ubiquitous in the environment, therefore contamination of the food processing environment is highly likely and cross-contamination of *L. monocytogenes* to foods is seen to be a major route of food contamination (Pérez-Rodríguez et al., 2008). *L. monocytogenes* can survive for long periods of time in a seemingly inhospitable environment such as a food processing facility due, in part, to its ability to resist various stresses (Moorhead and Dykes, 2004; Zhang et al., 2011) and its ability to form biofilm (Latorre et al., 2010; Cruz and Fletcher, 2011). Consistent identification of specific *L. monocytogenes* strains in food processing facilities over many years has shown that strains can persist in food processing environments. For example, Holch et al. (2013) used genome sequencing to demonstrate the persistence of two separate strains over 6 years in two different fish processing facilities and Vongkamjan et al. (2013) used ribotyping to show the 11 year persistence of a *L. monocytogenes* strain in a smoked fish processing facility (Vongkamjan et al., 2013).

Ready-to-eat (RTE) foods are in a higher risk category than other foods as the heat step of cooking, which would kill any *L. monocytogenes* present, is missing in these foods (Luber et al., 2011; EFSA, 2013). *L. monocytogenes* can replicate even at refrigeration temperatures so it is of concern especially in products with a long shelf life. In recent years, foodborne listeriosis outbreaks have included several RTE products for example cheese (Choi et al., 2014; Rychli et al., 2014), cantaloupe (Mccollum et al., 2013), and cooked ham (Hachler et al., 2013). A European Union baseline study of *L. monocytogenes* in RTE foods has been conducted in 2010 and 2011 and prevalence rates found were of 2.07% in meat

products, 0.47% in cheese products, and a more concerning rate of 10.4% in seafood products (EFSA, 2013). According to current European Commission (EC) regulations, RTE foods which cannot support the growth of *L. monocytogenes* must contain fewer than 100 CFU/g during their shelf-life while there is a zero tolerance policy in place for *L. monocytogenes* in RTE foods which can support its growth or which are intended for infant consumption or as a medicinal food (EC, 2005).

The aim of this study was to monitor the occurrence and persistence of *L. monocytogenes* in foods and food processing environments of 48 food processing facilities in the Republic of Ireland by regular sampling and characterization of isolates by serotyping and Pulsed Field Gel Electrophoresis (PFGE).

MATERIALS AND METHODS

L. MONOCYTOGENES MONITORING PROGRAMME

From March 2013 to March 2014, a total of 48 food processing facilities from various food sectors, i.e., dairy (18 facilities), meat (12 facilities), seafood (8 facilities), fresh-cut vegetable (6 facilities), miscellaneous (4 facilities), were analyzed bimonthly for the presence of *L. monocytogenes*. Of these food processing facilities, 43 process RTE food products. The selection of food processing facilities allowed coverage of major geographic areas of the Republic of Ireland. Sampling packs, which consisted of a polystyrene box (DS Smith, UK) containing six pre-moistened 3M sponge-stick swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland), two sterile bags (VWR, Ireland), two cable ties, and two ice packs, were sent to all participating food processing facilities.

Food business operators (FBOs) received detailed instructions which included information on how to take swab samples, which areas to sample, the type of food samples required and on the packaging and shipment of the samples to the laboratory. For swab samples, all FBOs were asked to take samples from three specific areas: a drain in the main processing hall, an area of floor (1 m^2) and a storage shelf. Because of the variation in layout of the facilities, the area to swab for the remaining samples was freely chosen by the FBO from anywhere in the food processing environment, although cutting areas, brine (if relevant), walls, other drains and pooled water were suggested as optimum locations. For food samples, FBOs were instructed to send two food samples which were at the stage of being ready to be sent from the processing facility.

Every second month, FBOs took 6 environment samples and sent them to the laboratory at Moorepark by overnight courier along with 2 food samples. Thirty-seven FBOs were initially enrolled in the monitoring programme and 11 further FBOs later showed their interest in joining the collaborative network at different stages during the sampling year. On the other hand, 3 FBOs no longer wished to take part in the analysis or went out of business and several other companies missed one or various sample submissions throughout the year's sampling.

ISOLATION OF *L. MONOCYTOGENES* FROM FOOD AND ENVIRONMENTAL SAMPLES

Samples were analyzed for the presence of *L. monocytogenes* by the ISO 11290-1 method, except that only one agar was used. After the environment swabs arrived at the laboratory, 100 ml of half-Fraser broth (VWR, Ireland), was added to bags containing 3M stick-sponge swabs, after which they were incubated at 30°C for 24 h. Then, a 0.1 ml aliquot was transferred to 10 ml of full-Fraser broth, which was further incubated at 37°C for 48 h. In addition, a 0.02 ml aliquot of the 1st enrichment broth was plated onto Agar Listeria acc. to Ottavani & Agosti (ALOA) agar plates (Biomérieux, UK), which were incubated at 37°C for 48 h. After incubation, 2nd enrichment broths were streaked onto ALOA agar plates, which were again incubated at 37°C for 48 h. For liquid or food samples, 225 ml of half-Fraser broth was added to 25 ml or 25 g of randomly selected analytical units of the food samples. Samples were then homogenized in a stomacher (Colworth Stomacher 400) for 4 min, and incubated at 30°C for 24 h. Subsequently, analysis of samples was continued by following the same approach used for environmental samples. The food samples were analyzed after their "best before date" so that any sample positive for *L. monocytogenes* did not create food recall issues.

After incubation, ALOA agar plates were examined for typical *L. monocytogenes* colonies (blue-green colonies with halo), and, if present, two characteristic *L. monocytogenes* colonies for each positive enrichment were streaked first onto Brilliance *Listeria* Agar (BLA) plates (Fannin, Ireland), which were incubated at 37°C for 48 h, and then onto Brain Heart Infusion (BHI) agar plates, which were incubated at 37°C for 24 h. Cryoinstant tubes (VWR, Ireland) were prepared by resuspending the bacterial mass from BHI agar plates, and were kept at −20°C for bio-conservation.

MOLECULAR CHARACTERIZATION OF *L. MONOCYTOGENES* ISOLATES

All stocked isolates were confirmed as *L. monocytogenes* by multiplex PCR as previously described (Ryu et al., 2013). Isolates were also serogrouped by multiplex PCR and serotyped by antisera testing (Denka Seiken UK Ltd, UK) as described previously (Fox et al., 2009). This allowed differentiation of all serotypes except 4b and 4e which cannot be differentiated with the currently available antisera.

PFGE analysis was performed with the restriction enzymes *Asc*I and *Apa*I, in two separate experiments, according to the International Standard PulseNet protocol (PulseNetUsa, 2009). Isolate similarity dendrograms were generated using Bionumerics version 5.10 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%, as previously described (Fox et al., 2012).

RESULTS

L. MONOCYTOGENES OCCURRENCE IN FOODS AND FOOD PROCESSING ENVIRONMENTS

Overall, 2006 samples were analyzed for the presence of *L. monocytogenes*, which accounted for 1574 environmental samples and 432 food samples. In general, 4.6% prevalence of *L. monocytogenes* was observed with slightly higher incidences in food samples (5.3%) than in environmental samples (4.4%). Positive food samples were obtained from all the food sectors and included cheese, smoked salmon, apple juice, mushrooms, milk, sausages, pudding, gammon, stuffing, and chicken samples. Regarding

environments, although the majority of positive environmental samples were sampled from non-food contact surfaces, 16.0% of positive environmental samples were from food contact surfaces.

In the total samples, slight variability in *L. monocytogenes* prevalence during the sampling year was observed. The lowest prevalences occurred in July 2013, November 2013, and January 2014 (3.9, 3.8, and 2.0%, respectively), while *L. monocytogenes* prevalence ranged between 4.2 and 6.0% for the rest of sampling months (**Figure 1**).

Thirty out of the 48 processing facilities analyzed had at least one positive sample over the course of the study. However, the majority of processing facilities consistently had a low prevalence of *L. monocytogenes*, in the range 0–5%, although some outliers occurred in which very high prevalence rates were found (**Figure 2**). Thus, 5, 4, 3, and 1 facilities presented prevalence rates of 5–10, 10–15, 15–20, and >30%, respectively (**Figure 2**).

Variability in *L. monocytogenes* prevalence rates was also observed among the different industry sectors. The highest rate of prevalence occurred in the vegetable sector in which 9.4% of samples tested positive for *L. monocytogenes*, followed by the meat sector at 4.2%, the dairy sector at 3.9% and the seafood sector at 1.6%. A miscellaneous group of processing facilities that process a range of foods belonging to different industrial sectors had a mean prevalence rate of 7.1%.

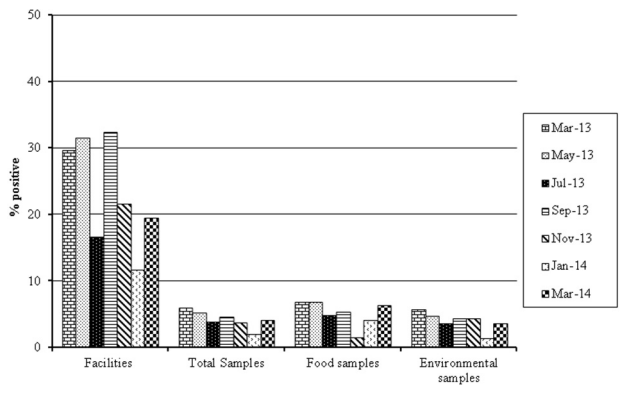

FIGURE 1 | Seasonal variation *L. monocytogenes* prevalence in samples from different processing facilities isolated between March 2013 and March 2014.

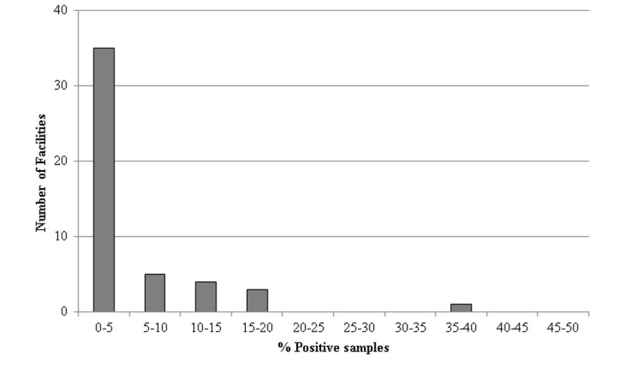

FIGURE 2 | Breakdown of the number of processing facilities positive for *L. monocytogenes* between March 2013 and March 2014.

MOLECULAR CHARACTERIZATION OF *L. MONOCYTOGENES* ISOLATES

When a food or environmental sample was found positive for *L. monocytogenes*, in 48.9% of cases both the first and second enrichment broths were positive, while in 20.4% of occasions the first enrichment was positive and the second was negative (most likely due to overgrowth by other related bacterial species) and in 30.7% of cases the first enrichment was negative but the second enrichment was positive (most likely due to a low contamination level of the sample). In any case, *two* colonies from each positive enrichment broth were isolated and further characterized by serotyping and PFGE analysis. Of the 370 isolates collected, the majority belonged to serotype 1/2a (41%), with 4b/4e (27%), 1/2b (17%), and 1/2c (15%) serotypes also being found but less frequently.

PFGE analysis of *L. monocytogenes* isolates provided information on strain diversity within positive samples. For the majority of samples (91.8%), indistinguishable PFGE pulsotypes were obtained for all strains isolated, while for 8.2% of samples isolates from more than one *L. monocytogenes* PFGE pulsotype were recovered, which suggests the possibility of food or environmental contamination with multiple *L. monocytogenes* strains.

PFGE analysis also allowed the identification of transfer of *L. monocytogenes* from environments to foods, or vice versa, and persistence within food processing facilities (**Table 1**). Indistinguishable pulsotypes in environmental samples and food samples of the same processing facility were observed for facilities 10, 22, 39, and 46. Facility 39 had an indistinguishable pulsotype in food and environmental samples at the same sampling time. Facilities 10, 22, and 46 had an indistinguishable *L. monocytogenes* pulsotype in food and environmental samples across different time points. Both facilities 22 and 46 had more than one type of food positive for *L. monocytogenes:* black pudding and sausages, and, chicken fillet and stuffing, respectively. In facilities 39 and 46, *L. monocytogenes* was isolated from food contact surfaces including a packing bench and shelf in facility 39 and a mincer and shelf in facility 46.

L. monocytogenes strains with indistinguishable PFGE profiles isolated at times 6 months or more apart are considered persistent strains for the purposes of the present study. Therefore, persistent strains occurred in seven separate facilities. Persistent strains were isolated from facilities of all industrial sectors except the seafood sector. In facility 44, a vegetable processor, pulsotype T32 (serotype 1/2b) was consistently isolated for over 6 months from drain and floor swabs and pulsotype T23 (serotype 4b/4e) was isolated for over 8 months from floor swabs. Similarly, at facility 46, a miscellaneous facility, pulsotype T13 (serotype 1/2a) was isolated separately up to a year apart from drain and floor swab samples and pulsotype T5 (serotype 1/2a) was consistently isolated for over 1 year from chicken fillet and stuffing food samples as well as from environmental swabs of drains.

A few persistent pulsotypes were found in more than one food business (**Figure 3**). This was the case for pulsotypes T15 (serotype 1/2c), T17 (serotype 1/2a), and T28 (serotype 4b/4e), which were isolated from several different facilities at various

Table 1 | *L. monocytogenes* positives listed according to facility, sampling month, sample type, and pulsotype, e.g., T23.

Facility no.	% Positives	March 13		May 13		July 13		September 13		November 13		January 14		March 14	
		Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods
DAIRY															
1	9.8		T23	**T17**	T20					**T17**, T25					
2	14.3	T2			**T28**		T30						**T28**		T17, **T28**
3	9.3	**T15**		**T15**				**T15**						**T15**, T30	
4	1.8			T18											
5	0														
6	0														
7	5.4							T27, T28							
8	2.5	T14													
9	0														
10	4.0								T29	T29					
11	0														
12	4.5			T33						T17, T26					
13	1.8				T35										
14	0														
15	3.2	T28													
16	1.8							T1							
17	4.5									T9					
18	4.2									T22					
MEAT															
19	0														
20	0														
21	1.8	T8													
22	12.7			T19			T15		T15	T15	T12	T11			
23	0														
24	0														
25	0														
26	16.7	T1													
27	7.5	**T17**						T6, **T17**							
28	4.3							**T21**						**T21**	
29	12.0					T15		T15							
30	2.1														T15, T36
SEAFOOD															
31	0														
32	2.0				T12										
33	2.9													T25	
34	0														
35	6.1			T3				T4							

(Continued)

Table 1 | Continued

Facility no.	% Positives	March 13		May 13		July 13		September 13		November 13		January 14		March 14	
		Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods
36	0														
37	0														
38	0														
VEGETABLE															
39	18.1	T16						T31	T31	T26		T10		T30	
40	16.7					T28									
41	3.6			T17, T23, T24											
42	4.2									T1, T34		T7			
43	3.9		T28						T22						
44	13.3			T32		**T23**, T32		T32						T22, **T23**	
MISCELLANEOUS															
45	0														
46	35.3	**T13**, T23	**T5**	T17	**T5**	**T5**	**T5**							**T13**	**T5**
47	0														
48	0														

Empty white boxes indicate no L. monocytogenes detected in submitted samples. Shaded boxes indicate non-submission of samples during that sampling month. Pulsotypes which persist in a single facility (isolated at least 6 months apart) are in bold. Persistent pulsotypes which are found in multiple facilities are outlined with a rectangle.

different sampling points during the sampling year (**Figure 3**). Pulsotype T15 was isolated in three separate facilities at several time points, pulsotype T17 in six separate facilities (in two of them it was isolated on more than one occasion) and T28 in five separate facilities (but only in one of them on more than one occasion). These persistent pulsotypes were not restricted to any particular industry sector with pulsotype T15 found in the dairy and meat sectors, pulsotype T17 in the dairy, meat, vegetable and miscellaneous sectors and pulsotype T28 in the dairy and vegetable sectors. All three of these pulsotypes were isolated from food samples at least once during the present study. Pulsotype T17 was isolated from cheese, pulsotype T15 was isolated from sausages and pulsotype T28 was isolated from milk, cheese and apple juice. Pulsotypes T17, T15, and T28 were all isolated from environmental swabs taken in both food contact and non-food contact areas.

Several other pulsotypes were isolated from more than one facility but were only found at one time point and, as such, have not yet been shown to be persistent.

DISCUSSION

L. MONOCYTOGENES OCCURRENCE IN FOOD AND FOOD PROCESSING ENVIRONMENTS

Two thousand and six samples were submitted for analysis. Only 20 FBOs submitted all scheduled samples, therefore the number of samples submitted was less than originally planned. This was not detrimental to the study as a large number of samples were analyzed, allowing the examination of general *L. monocytogenes* prevalence trends across the different food sectors studied. The general *L. monocytogenes* prevalence of 4.6% agrees with the mean occurrence of *L. monocytogenes* in RTE foods across Europe found in the recent EFSA baseline study of approximately 4% (EFSA, 2013). Although the majority of facilities had consistently low or no presence of *L. monocytogenes*, outliers with high prevalence occurred in every sector except the seafood sector. The general, low *L. monocytogenes* prevalence was similar to that seen by Williams et al. (2011), who described an occurrence in small RTE food production facilities ranging from 1.7 to 10.8%. The higher prevalences of 23.68% in RTE seafood in Italy (Gambarin et al., 2012) and approximately 22% in various RTE foods in Spain (Sanchez et al., 2012) were closer to those of the outliers seen in the present study. Previous studies conducted in Ireland in farmhouse cheese processing facilities reported a higher prevalence of 13.1% (Fox et al., 2012) in the food processing environment. Significant contamination rates in food processing facilities greatly increase the chance of causing human listeriosis as was seen in Denmark in 2000 where human listeriosis cases were traced to a turkey processing facility in which *L. monocytogenes* prevalence ranged from 25.9 to 41.4% during production (Ojeniyi et al., 2000).

While both the dairy and meat sectors showed an average prevalence of 3.9 and 4.2%, respectively, previous studies have found a much higher prevalence in seafood samples (Garrido et al., 2009; Chen et al., 2010) than the present study. Chen et al. (2010) found 21.6% of samples positive from food and processing environment, Garrido et al. (2009) found 25% of food samples positive and in the current study, an occurrence of 1.6% was

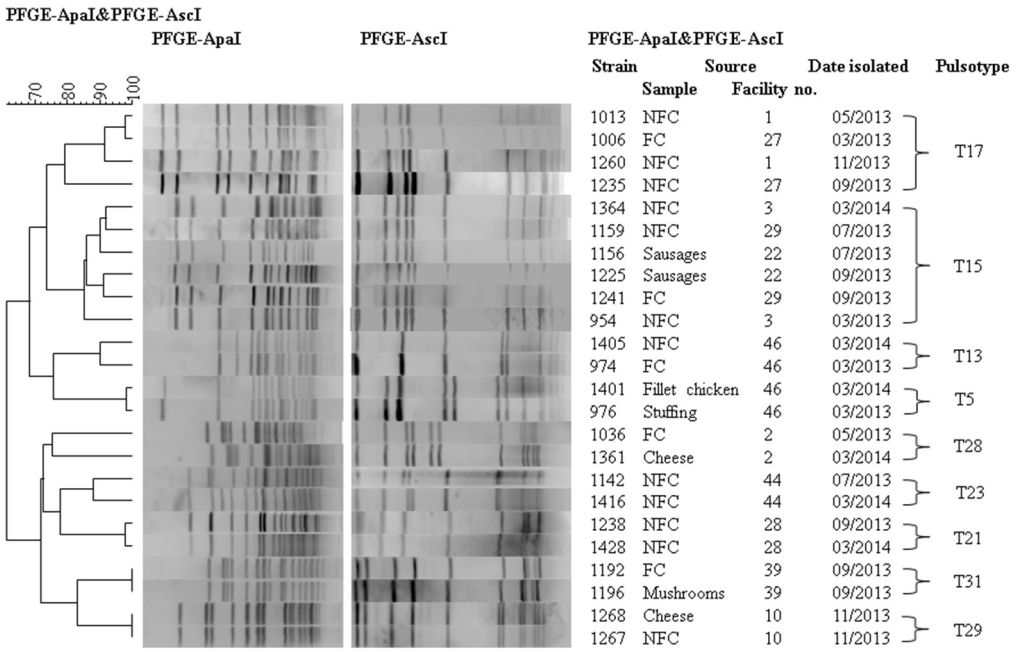

FIGURE 3 | Dendrogram of PFGE pulsotypes which persisted for over 6 months or were isolated from both food and environment samples within the same facility. Examples of interest from each pulsotype are shown. FC, Food contact; NFC, Non-food contact.

detected in food and the processing environment. The EFSA baseline study also showed a *L. monocytogenes* prevalence in smoked fish of 10.4%. The seafood industry is currently seen as a high risk industry for *L. monocytogenes* contamination e.g., an increase in listeriosis cases in Finland in 2010 was found to have been caused by two fishery plants which contained persistent *L. monocytogenes* strains (Nakari et al., 2014). It can be speculated that seafood processors in Ireland may be more aware of their vulnerability to *L. monocytogenes* contamination and have taken more steps to combat contamination in recent years which may have helped to reduce occurrence.

On the other hand, the food sector with the highest occurrence of *L. monocytogenes* was the fresh-cut vegetable sector, with a prevalence of 9.4%. The large variety of pulsotypes isolated from this sector indicates frequent contamination which did not originate at the same source. Therefore, a likely source of this contamination would be soil rather than materials from a common supplier, staff or equipment. To date, little research on *L. monocytogenes* in the vegetable processing industry has been conducted. The occurrence found in the present study is comparable to the *L. monocytogenes* occurrence found in mushrooms in Spain (Venturini et al., 2011). Only six facilities in the fresh-cut vegetable sector took part in the study, therefore it is not possible to determine if this is a general trend in the vegetable industry in Ireland. However, the data indicates that vegetable processors should be more vigilant against *L. monocytogenes*. As uncut vegetables are considered primary production, they are not included in the RTE category in regard to regulations concerning *L. monocytogenes* so are not subject to the same regulatory sampling as RTE foods. Therefore, vegetable processors may not be as aware of *L. monocytogenes* as other food processors from RTE sectors of the industry included in this study.

Regarding the miscellaneous sector, although a high occurrence of 7.1% was observed, only one of the four facilities (facility 46) tested positive for *L. monocytogenes*, with a very high contamination rate (35.3%).

For 37.5% of the facilities tested, *L. monocytogenes* was absent over the course of the present study. This requires further investigation to determine whether particular hygiene practices, equipment types, staff procedures etc. used in these facilities contributed to this absence of *L. monocytogenes.*

Differences in *L. monocytogenes* prevalence were not influenced by season. Not all facilities submitted samples on every occasion so it is possible that seasonal variation may have been observed if all of the samples had been submitted. However, this is unlikely as facility 46, which had the highest overall prevalence, still had a high prevalence of 25% in July 2013. In the literature, some debate exists concerning whether or not Listeria is subject to seasonal variation. Data which has found no seasonal variation (Ho et al., 2007; Esteban et al., 2009; Mohammed et al., 2010) exists alongside data which has found clear seasonal variation, including higher numbers of *Listeria* spp. occurring in both summer (Rivoal et al., 2010) and in winter months (Guerini et al., 2007).

MOLECULAR CHARACTERIZATION OF *L. MONOCYTOGENES* ISOLATES

From the present study, the importance of analyzing isolates from both the 1st and 2nd enrichment can be seen. In only 48.9% of positive samples, both the 1st and 2nd enrichments were positive while in 51.1% of positive samples, either the 1st or the 2nd enrichment only was positive. In some studies, only the

2nd enrichment is plated for *L. monocytogenes* isolation; therefore, from the present data, that method would have missed 20.4% of positive samples. In addition, 8.2% of samples showed some strain diversity (multiple PFGE pulsotypes) indicating multiple colonization of the environment or food by more than one *L. monocytogenes* strain. This finding highlights the importance of isolating several strains from positive samples in monitoring programs in order to account for the possible strain diversity that can exist.

The ratio of serotypes found in the present study was in agreement with a 5 year surveillance report on similar food and food processing environment samples conducted in Italy in 2010 (Nucera et al., 2010) where serotype 1/2a isolates accounted for almost half of *L. monocytogenes* isolates followed by serotypes 4b/4e, 1/2b, and 1/2c in slightly varying lower percentages. Listeriosis outbreaks are most commonly caused by 4b/4e serotypes and to a lesser extent 1/2b serotypes while sporadic cases are commonly caused by 4b/4e, 1/2a, or 1/2c serotypes (Todd and Notermans, 2011). Therefore, all *L. monocytogenes* isolates found in the current study could have the potential to cause illness.

Several strains of *L. monocytogenes* were seen to persist over long periods of time in the food processing environment as their pulsotypes were identified repeatedly by PFGE. Long-term survival of strains in a food processing facility, such as these, confer a higher risk of bacterial transfer to food and therefore a higher risk of human exposure to the pathogen (Lambertz et al., 2013). Seven of the 48 facilities included in the study showed contamination with persistent strains. It is possible that at least some of these strains have adaptions which facilitate long-term survival in a food processing environment. These adaptations could include resistance to sanitizers/disinfectants, adaptation to cold or high salt conditions and ability to form biofilms (Holch et al., 2013). One opposing theory suggests that rather than strains possessing particular characteristics which contribute to their persistence, bacteria simply persist in harborage sites i.e., areas which cannot be effectively disinfected, and proliferate from these sites (Carpentier and Cerf, 2011).

Several pulsotypes were also seen to be common to several different facilities. There is no known epidemiological link between any of the facilities which shared *L. monocytogenes* pulsotypes. For example, T17 was isolated in facilities 1, 2, 12, 27, 41, and 46. These facilities belong to various industry sectors (dairy, meat, vegetable, and miscellaneous), are located throughout the country and are not known to share any suppliers, equipment, or staff. Further characterization, including ongoing genome sequencing, may shed light on whether or not certain strains have adapted to the food processing environment.

CONCLUSION

The prevalence of *L. monocytogenes* in food and food processing environments among 48 FBOs in the Republic of Ireland was 4.6% and is equivalent to the prevalence rates found in RTE foods across the E.U. (EFSA, 2013). PFGE analysis allowed the identification of several strains as persistent in the food processing environment and several strains were also seen to occur in more than one facility. The general prevalence of approximately 5% of *L. monocytogenes* positives found in samples of both food and food processing environments, in addition to the evidence of bacterial transfer and persistence observed, emphasizes the need for food processors to be vigilant against *L. monocytogenes* contamination in order to avoid public health risks.

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Botulism outbreaks in natural environments – an update

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Clostridium botulinum comprises a diverse group of botulinum toxin-producing anaerobic rod-shaped spore-forming bacteria that are ubiquitously distributed in soils and aquatic sediments. Decomposition of plants, algae, and animals creates anaerobic environments that facilitate growth of *C. botulinum*, which may then enter into food webs leading to intoxication of animals. Via saprophytic utilization of nutrients, the bacteria rapidly sporulate, creating a reservoir of highly robust spores. In the present review, we focus on the occurrence of *C. botulinum* in non-clinical environments, and examine factors influencing growth and environmental factors associated with botulism outbreaks. We also outline cases involving specific environments and their biota. In wetlands, it has been found that some *C. botulinum* strains can associate with toxin-unaffected organisms—including algae, plants, and invertebrates—in which the bacteria appear to germinate and stay in the vegetative form for longer periods of time. We suggest the need for future investigations to resolve issues related to the environments in which *C. botulinum* spores may accumulate and germinate, and where the vegetative forms may multiply.

Keywords: *Clostridium botulinum*, botulism, serotype, spore, anaerobe, lakes, wetlands, soil

INTRODUCTION

The species *Clostridium botulinum* comprises multiple highly heterogeneous strains of rod-shaped anaerobic spore-forming bacteria, which are categorized into four groups (Groups I–IV) based on genomic relatedness. All *C. botulinum* strains produce botulinum toxin, which paralyzes animals by inhibiting acetylcholine release from synaptic vesicles at neuromuscular junctions. This toxin is classified into eight serotypes designated A–H (Collins and East, 1998; Barash and Arnon, 2014), of which A, B, E, and F are shown toxic to humans. Botulinum toxin-producing bacteria are divided into six groups: *C. botulinum* Groups I–IV as well as some strains of *C. baratii* and *C. butyricum* (Peck, 2009). Group I includes the proteolytic *C. botulinum* strains that produce botulinum toxin serotypes A, B, and F. Group II comprises non-proteolytic strains that produce toxin serotypes B, E, and F. The strains in Group III produce serotypes C and D, or mosaic C/D toxins. Group VI strains, referred to as *C. argentinense* (Suen et al., 1988), produce toxin serotype G. Among the other species, *C. butyricum* produces botulinum toxin serotype E and *C. baratii* produces serotype F (Hill et al., 2009).

Botulinum toxin genes exhibit remarkably variable organization. They can be chromosomally localized or localized on plasmids or phages (serotypes C and D). Serotype B transcription can occur through both genome-encoded and plasmid-encoded toxin gene clusters (Franciosa et al., 2009). Genome comparisons have revealed evidence of toxin cluster evolution through horizontal gene transfer, site-specific insertion, and recombination, and genomic analysis has supported the historic group classifications (Hill and Smith, 2013; Stringer et al., 2013). Thus, the factors affecting pathogenicity are apparently subjected to a higher evolutionary rate than the core genomes, allowing for fast environmental adaptation of the pathogen.

The ecology and properties are similar enough among Groups I–IV that it remains meaningful to discuss *C. botulinum* in the environment as a single group. *C. botulinum* spores persist in soils and aquatic sediments for decades, and propagate by predator-dependent disease transmission. Upon entering the food webs of animals, *C. botulinum* toxins may intoxicate and kill the animal, or infect and proliferate and kill the prey. Saprophytic utilization of the prey via enzymes, including proteases and chitinases, makes nutrients available for massive spore and toxin production. Neurotoxin gene expression and toxin complex formation reportedly occur in the late exponential growth phase and the early stationary phase (Bradshaw et al., 2004; Kouguchi et al., 2006; Artin et al., 2008; Cooksley et al., 2010), and toxin production and sporulation seem to be co-regulated (Cooksley et al., 2010).

It appears that contaminated soils and sediments are primary environments for spores and serve as an incubation area, from which the pathogens may be mobilized (Long and Tauscher, 2006). *C. botulinum* is detected in, or may be associated with, various organisms that are not affected by the toxins—such as algae, plants, and invertebrates (Quortrup and Holt, 1941; Duncan and Jensen, 1976; Bohnel, 2002). Fish are carriers of *C. botulinum,* but botulism outbreaks in fish populations may lead to death on a large scale (Yule et al., 2006; Hannett et al., 2011). Avian botulism caused by *C. botulinum* type C, mosaic C/D, or E is a common cause of death among waterfowl (Skulberg and Holt, 1987; Friend, 2002; Takeda et al., 2005; Lafrancois et al., 2011; Vidal et al., 2013). Unpredictable outbreaks with variable losses have been reported worldwide (Friend, 2002; Babinszky et al., 2008; Shin et al., 2010; Vidal et al., 2013). In recent years, large outbreaks in the Great Lakes, with high mortalities among fish and birds, have been well documented and analyzed (Perez-Fuentetaja et al., 2006, 2011; Lafrancois et al., 2011; Chun et al., 2013). In this review, we discuss factors related to botulism outbreaks in natural environments.

ENVIRONMENTS AND REGIONS

Clostridium botulinum is ubiquitously present in the environment in soils, dust, and the marine and freshwater sediments of wetlands, rivers, and lakes. Spores in soil may be mobilized by surface waters in heavy rain, or dust carried away by wind (Long and Tauscher, 2006). Botulism has been characterized as a particularly substantial risk to humans in northern climatic regions, due to intoxication from poorly preserved food (Dolman, 1960; Hauschild and Gauvreau, 1985; Austin and Leclair, 2011; Fagan et al., 2011; Leclair et al., 2013b). Serotype E is dominant in sediments of the arctic and subarctic regions, whereas serotype B is most prevalent in soil (Johannsen, 1963; Miller, 1975; Huss, 1980; Hielm et al., 1998; Leclair et al., 2013a). The temperate climate zone of Europe shows the same distribution pattern, in which serotype B is most prevalent in soil and serotype E is found in sediments (Huss, 1980), although serotypes C and D are also commonly found (Woudstra et al., 2012). In the temperate zone of Northern America, serotype A is most common west of the Mississippi river, and serotype B east of the Mississippi river (Shapiro et al., 1998), whereas serotype E is most common in the areas of the Great Lakes and the Pacific Northwest. In China, serotypes A–F have all been detected in the soil (Yamakawa et al., 1988; Gao et al., 1990; Fu and Wang, 2008). In Japan, the presence of botulinum toxin serotypes B, C, and E has been documented (Yamakawa et al., 1988; Yamakawa and Nakamura, 1992; Umeda et al., 2013). In general, environmental botulism outbreaks have been connected to serotypes C, mosaic C/D, and E.

Less documentation is available regarding botulism outbreaks in natural environments within subtropical and tropical climate zones. On the African continent, *C. botulinum* has been detected in the soils of Zambia and Kenya, with identification of serotypes A–D (Nightingale and Ayim, 1980; Yamakawa et al., 1990; Karasawa et al., 2000). In Australia, the serotypes A, B, and D have been detected, either identified from cases of botulism or in soil (Eales and Turner, 1952; Murrell and Stewart, 1983; Koepke et al., 2008). In the tropical region of Indonesian waters, botulinum toxin serotypes A, B, C, D, and F were detected, but not serotype E (Suhadi et al., 1981). In the tropical Indian subcontinent, C and D are the predominant serotypes found in fish and aquatic environments (Lalitha and Gopakumar, 2000), and serotype E has not been detected (Lalitha and Surendran, 2002).

In the field of food safety research, laboratory studies have investigated spore resistance and factors favoring and limiting *C. botulinum* germination and growth—for example, the tolerated ranges for temperature, pH, and salinity (Chea et al., 2000; Hinderink et al., 2009; Derman et al., 2011; Stringer et al., 2011). However, the mechanisms triggering a botulism outbreak in the environment remain poorly understood. Several large-scale factors, such as lower water levels and/or higher summer surface water temperatures, have been correlated with larger outbreaks (Rocke et al., 1999; Perez-Fuentetaja et al., 2006, 2011; Lafrancois et al., 2011). Higher environmental botulism prevalences have also been reported when the sediment has a high organic matter content, the water has a pH of between 7.5 and 9.0, there is an overall negative redox potential, and the water temperature is above 20°C (Rocke and Samuel, 1999).

Pollution supports mass production of algae, followed by decay when packed ashore. In Lake Saint-Pierre, St. Lawrence River in Canada, years with low water levels coincided with eutrophic conditions and higher prevalence of filamentous green algae (Chlorophyceae), especially *Cladophora* (Cattaneo et al., 2013). Floating algae can create spots of strict anoxic conditions (Quortrup and Holt, 1941). In larger clearwater oligotrophic lakes in temperate climates, wind can cause circulation of surface water to a depth of 6–12 m or more. In the summer, this can expose the sediment surfaces within this depth range to temperatures of 10–20°C at an acceptable pH range for bacterial growth. Pollution of the nearshore waters can lead to developments as described for the great American lakes, in which massive shore accumulations of *Cladophora* served as biotic incubators for *C. botulinum* (Chun et al., 2013). Taken together, this observation indicate co-occurence between low water levels, growth of filamentous algae, and an increased risk of botulism.

A large number of samples from coastal waters and lakes have been investigated for the presence of *C. botulinum* strains or their spores. Many of these early studies were of importance for detection but provide superficial characterization of the localities sampled (Johannsen, 1963; Smith et al., 1978). Holomictic lakes in temperate regions may have two annual periods of circulation: immediately after ice-break and during the cooling period in autumn. A meso- to eutrophic lake may exhibit stagnation of the bottom water at a temperature near 4°C, with complete oxygen deficit during the late summer and late winter seasons. During seasonal circulations, this oxygen-deficient water is mixed in with the rest of the lake. In temperate regions where ice is uncommon, the lakes may be mixing and fully aerated through the coldest season. Brown-water forest/bog lakes and meromictic lakes (with permanent anoxic water at the bottom) may accumulate sinking organic particulates, crustacean exuvia, dead fish, etc. Although decomposition may be delayed by pH and/or low temperature, such lakes should be of interest as reservoirs of anaerobic bacteria. It is possible that some degree of vertical transport, upwards from suboxic or anoxic levels, may be mediated by resistant zooplankton with diurnal migrations, e.g., larvae of *Chaoborus* and some *Daphnia*. Overall, the yearly cycles of lakes may be relevant with regards to conservation and distribution of spores and substrates (Wetzel, 2001).

Extensive reed beds are found in shallow lakes in temperate climates, like Lake Balaton in Hungary and Lake Neusiedler See in Austria. Wildlife botulism has rarely been recorded from within the reeds. However, bird botulism has been reported in ponds on the shores of Neusiedler See (Zechmeister et al., 2005). In Spain, inland wetlands are more often troubled by bird botulism (Vidal et al., 2013) than coastal wetlands with a tidal regime (Contreras de Vera et al., 1991). This is probably due to both the water movement and the salt concentration. *C. botulinum* serotype C was less prevalent in seasonally flooded marshes than in permanently flooded marshes (Sandler et al., 1993). Furthermore, higher salinity has a negative effect on *C. botulinum* growth (Segner et al., 1971; Webb et al., 2007), decreasing the risk of botulism outbreaks.

BIOTA AS RESERVOIRS AND VECTORS

Clostridium botulinum spores released into the environment are robust, potentially persisting in soils and sediments for decades (Long and Tauscher, 2006). The bacterium has been found in the intestinal tract of healthy fish, birds, and mammals. *C. botulinum* serotype E does not multiply in the fish gut (Bott et al., 1968), and fish fed 500,000 spores per day (in pellets) did not acquire botulism (Eklund et al., 1984). Thus, the initial proliferation of bacterial germination and vegetative growth must occur somewhere in the environment. Once established, a botulism outbreak is self-perpetuating. During an avian botulism outbreak, the disease spreads through necrophagous flies depositing eggs on dead and toxic animal carcasses. The resulting maggots feed on the carcasses and concentrate the botulinum toxin. When other animals ingest the toxic maggots, they become the next victims (the carcass–maggot cycle). During outbreaks in fish, decomposing invertebrates and decaying fish sink to the lake bottom and are consumed by scavenging fish in an amplifying cycle. A study of channel catfish showed that their lethal dose of botulinum toxin E was less than the median lethal dose for mice (Chatla et al., 2012). Toxin levels may persist and remain lethal over the winter in larvae (Hubalék and Halouzka, 1991). A wide variety of organisms—such as algae, plants, and invertebrates—have been shown to contain botulinum toxin or *C. botulinum* DNA (**Table 1**). These organisms represent a biotic reservoir for *C. botulinum*, and may themselves become toxic upon anaerobic decomposition (Quortrup and Holt, 1941; Heckman, 1986).

For most of the insects listed, it is their submerged instars (e.g., mayflies) or larvae on carcasses (e.g., flies) that are vectors, with the important exception of the Coleoptera (beetles) and possibly the Corixidae (water boatmen).

It is clear that these organisms are involved in botulism outbreaks as part of the food web, and that birds and fish consume toxic decaying organic matter or toxic invertebrates, but little is known about the primary substrate in botulism outbreaks. Animals that die for other reasons but that contain spores in their digestive tract can serve as a substrate for bacterial germination. In the Great Lakes, invasive dreissenid mussels (*Dreissena polymorpha* and *Dreissena rostriformis bugensis*) and round gobies (*Neogobius melanostomus*; a benthic fish) have been suggested to contribute to the increased number of outbreaks by increasing the amount of decaying biomass (Getchell and Bowen, 2006). However, numerous other benthic organisms could potentially be responsible for transmitting *C. botulinum* to vertebrate prey organisms (Perez-Fuentetaja et al., 2011). During a disease outbreak in the Salton sea, PCR was used to test fish for serotype C-producing *C. botulinum*, but no difference in numbers of positives was detected among the groups of healthy, sick, and dead fish (Nol et al., 2004).

The filamentous green macroalgae *Cladophora glomerata* is reportedly associated with *C. botulinum* type E in Lake Michigan and Lake Ontario (Byappanahalli and Whitman, 2009; Chun et al., 2013). *C. glomerata* is globally widespread and can produce dense populations, especially under eutrophic conditions. Their high surface area is covered with organic compounds, which may form an ecological niche to diverse microbiota (Zulkifly et al., 2012). In floating algal mats, *C. botulinum* type E was found in high amounts of up to 15,000 cells (most probable number) per gram of dried algae (Chun et al., 2013). Heat treatment of *Cladophora* mat samples indicated the presence of *C. botulinum* vegetative cells (Chun et al., 2013). Another study analyzed senescent *Cladophora* samples from Lake Erie, and did not detect *C. botulinum* type E (Perez-Fuentetaja et al., 2011). An extensive survey revealed rich epiphytic microbiota on *Cladophora* thalli, but did not identify pathogenic bacteria, such as *C. botulinum*, associated with the alga and its epibionts (Zulkifly et al., 2012). The algae tested in this case were sampled from the attached macroalgae and not from floating decaying mats. In a laboratory experiment, sterilized plants and algae of different species, including *Cladophora*, were found

Table 1 | *Clostridium botulinum* and possible vector organisms.

Environment	Vectors	Taxonomy	Sero-type	Area	Reference
Freshwater	Plants	*Ceratophyllum*	C	Norway (Oslo)	Skulberg and Holt (1987)
Freshwater	Plants	*Phragmites Schoenoplectus*	n.d.	Germany (Elbe estuary)	Heckman (1986)
Freshwater	Invertebrates	*Gammarus* (Crustacea), Oligochaeta (Annelida), Chironomidae (Insectae), Ephemeroptera (Insectae), Dreissenidae (Mollusca)	E	USA (Great Lakes)	Perez-Fuentetaja et al. (2006, 2011)
Freshwater	Algae, invertebrates	*Cladophora* (Chlorophyceae)	E	USA (Great Lakes)	Byappanahalli and Whitman (2009), Chun et al. (2013)
Wetlands	Invertebrates	Calliophoridae (Insectae), Ptychopteridae (Insectae), Hirudineae (Annelida), Isopoda (Crustacea)	C	Czech Republic (Moravia)	Hubalék and Halouzka (1991)
Wetlands	Invertebrates	Chironomidae (Insectae), Corixidae (Insectae), Sarcophagidae (Insectae), Calliophoridae (Insectae)	C/D	Spain (central Spain)	Vidal et al. (2013)
Wetlands	Invertebrates	Calliophoridae (Insectae), Coleoptera (Insectae)	C	USA (Utah)	Duncan and Jensen (1976)

to support anaerobe growth and toxin production of inoculated *C. botulinum* (Quortrup and Holt, 1941). The role of plants and algae as primary substrate for *C. botulinum* in wetland ecosystems must be further elucidated. **Figure 1** presents a schematic view of *C. botulinum* in a freshwater environment. It has been speculated that botulism outbreaks may be triggered by animals dying of other reasons than *C. botulinum* infection. Spores will germinate in the dead body, and after toxin production the carcass in the next turn is food for maggots and other invertebrates and an outbreak cycle starts. It remains to investigate/show if algal- and plant-associated toxin can start a botulism outbreak.

CONDITIONS PREVENTING BOTULISM OUTBREAKS

Under natural conditions, there are a number of factors that can prevent *C. botulinum* growth. One limiting factor is the strong competition or even inhibiting effect by other bacteria (Smith, 1975; Girardin et al., 2002). Studies of marshland sediments have demonstrated inhibition of *C. botulinum* type C by other bacteria, including *Bacillus licheniformis*, *Bacillus mycoides/cereus*, *Streptococcus* spp., and *Clostridium* spp. (Smith, 1975; Sandler et al., 1998). Additionally, degradation of preformed botulinum toxin by aerobic bacteria has been experimentally demonstrated (Quortrup and Holt, 1941). In some environments, salt is a growth-inhibiting factor. Growth can also be reduced by lower temperature and pH, and acidification by fermentation (Quortrup and Holt, 1941). Fermentative processes of plant material in water by facultative anaerobic lactic acid bacteria (e.g., *Leuconostoc*) may initially create CO_2, acetic acid and alcohol. However, as succession proceeds, the homofermentative species (*Lactobacillus* s. str.) take over and produce lactic acid, tolerating a lower pH (Buchanan and Gibbons, 1974; Giraffa et al., 2010).

One control measure that has been proposed to prevent outbreaks is to remove oxygen-deficient environments by raking the floating algae (Quortrup and Holt, 1941). Attempts have also been made to reduce the magnitude of botulism outbreaks by collecting carcasses, which appears to enhance survival compared to in areas with a higher carcass density (Evelsizer et al., 2010). It has been suggested that a functional ecosystems can better resist disease outbreak than dysfunctional ecosystems (Riley et al., 2008). An interesting research focus will be to further elucidate the mechanisms by which *Clostridia* are excluded, prevented, or outcompeted in many complex bacterial communities, in spite of favorable physical conditions, such as pH, salinity, and anoxia.

C. botulinum AND CLIMATE CHANGE

An important question to discuss is whether climate change has or will contribute to increasing outbreaks of botulism. A study of the Salton Sea from 1907 to 1999 showed that avian diseases

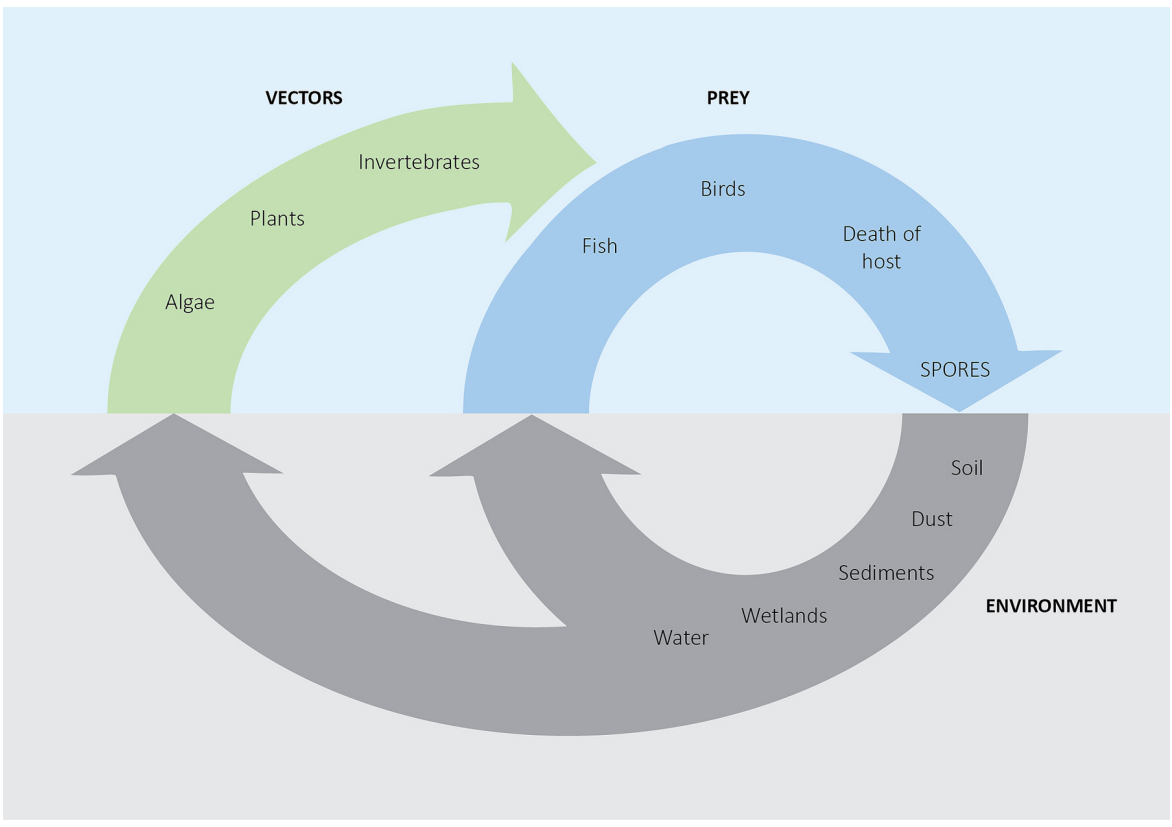

FIGURE 1 | Schematic representation of the role of *C. botulinum* in a freshwater ecosystem. Resistant spores are released from dead hosts into the abiotic environment, and are spread by air and waterways. The spores may directly infect prey hosts or can be taken up by toxin-unaffected organisms, forming a biotic reservoir.

caused by various agents increased over the course of the 1990s (Friend, 2002). A study of Lake Michigan from 1963 to 2008 found a cyclic occurrence of outbreaks, with no increased frequency of outbreaks during the study period (Lafrancois et al., 2011). Since 1998, there have been yearly botulism outbreaks in Lake Erie, which have been spreading to other deeper Great Lakes (Perez-Fuentetaja et al., 2011). During dry periods, lower lake levels and high summer temperatures increase the growth of the filamentous green macroalgae *Cladophora* (Zulkifly et al., 2012), along with the risk of botulism outbreaks (Lafrancois et al., 2011). In the wetlands of central Spain, drought induced by overexploitation of groundwater resources represents an increased risk factor for local botulism outbreaks (Vidal et al., 2013). As climate forecasts predict warmer and wetter weather, in addition to more weather extremes, one may expect more outbreaks due to the warmer climate, especially if combined with prolonged dry periods and polluted water supporting blooms of benthic algae.

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The many forms of a pleomorphic bacterial pathogen—the developmental network of *Legionella pneumophila*

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Legionella pneumophila is a natural intracellular bacterial parasite of free-living freshwater protozoa and an accidental human pathogen that causes Legionnaires' disease. *L. pneumophila* differentiates, and does it in style. Recent experimental data on *L. pneumophila*'s differentiation point at the existence of a complex network that involves many developmental forms. We intend readers to: (i) understand the biological relevance of *L. pneumophila*'s forms found in freshwater and their potential to transmit Legionnaires' disease, and (ii) learn that the common depiction of *L. pneumophila*'s differentiation as a biphasic developmental cycle that alternates between a replicative and a transmissive form is but an oversimplification of the actual process. Our specific objectives are to provide updates on the molecular factors that regulate *L. pneumophila*'s differentiation (Section The Differentiation Process and Its Regulation), and describe the developmental network of *L. pneumophila* (Section Dissecting *Lp*'s Developmental Network), which for clarity's sake we have dissected into five separate developmental cycles. Finally, since each developmental form seems to contribute differently to the human pathogenic process and the transmission of Legionnaires' disease, readers are presented with a challenge to develop novel methods to detect the various *L. pneumophila* forms present in water (Section Practical Implications), as a means to improve our assessment of risk and more effectively prevent legionellosis outbreaks.

Keywords: differentiation, developmental forms, intracellular infection, disease transmission, pathogen detection

BACKGROUND

L. PNEUMOPHILA IS A FACULTATIVE INTRACELLULAR PATHOGEN THAT DIFFERENTIATES INTO NUMEROUS FORMS WITHIN A DEVELOPMENTAL NETWORK

Legionella pneumophila (*Lp*) is an intracellular bacterial pathogen predicted to have co-evolved with freshwater protozoa (Barker and Brown, 1994; Weissenberger et al., 2007; Garduño, 2008) to optimize the acquisition of intracellular nutrients (Price et al., 2014). The fact that *Lp* can grow outside host cells, either in nutrient-rich media *in vitro*, or within microbial communities (reviewed by Declerck, 2010), technically defines it as a facultative intracellular pathogen. However, in nature, *Lp* behaves more as an obligate intracellular pathogen and less as a facultative one. That is, in relation to growth inside natural hosts, extracellular replication represents but a minor contribution (Temmerman et al., 2006) to the maintenance of *Lp* populations in freshwater, or to the increase of bulk *Lp* levels (Murga et al., 2001; Kuiper et al., 2004; Declerck et al., 2007, 2009; Fields, 2008). Consequently, intracellular growth is considered a fundamental process in the life cycle of *Lp* in general, and *Lp* differentiation in particular (Garduño, 2008). Amoebae are the preferred *Lp* hosts in the natural environment. Fifteen amoebal species have been reported to support the intracellular growth and differentiation of *Lp* (Hägele et al., 2000, and reviewed by Fields, 1996, 2008).

We have previously discussed the intracellular differentiation of *Lp* (Garduño, 2008), and established that *Lp* has a single developmental program integrated into its life cycle (Garduño et al., 2008), with 14 *Lp* developmental forms reported to date (Rowbotham, 1980; Gress et al., 1980; Faulkner and Garduño, 2002; Greub and Raoult, 2003; Sauer et al., 2005a; Faulkner et al., 2008; Al-Bana et al., 2014) (**Table 1**). Given the complexity of *Lp*'s ecology and the many developmental forms involved, we also proposed the existence of a developmental network (Garduño et al., 2008). This developmental network includes the "accidental" hosts that support the intracellular growth of *Lp* in the context of laboratory investigations, or in the context of human Legionnaires' disease. In this review we will discuss the developmental network of *Lp* and provide as many details as possible, about the many developmental forms that *Lp* produces.

AN OVERVIEW OF THE DEVELOPMENTAL CYCLES AND THE DEVELOPMENTAL NETWORK OF Lp

Key to the establishment of a developmental cycle is the demonstration that the various forms present in it can differentiate into each other, closing a circular process. In the simplest cycle (a biphasic one) one originating form gives rise to another, which in turn differentiates back into the originating one (**Figure 1**). In the case of a multiphasic cycle, more than two forms would

Table 1 | The *Lp* developmental forms that have been identified and reported to date.

Name used (abbreviation)	Main characteristics	Primary references
Exponential phase form (EPF)	Produced extracellularly, non-infectious to host cells, sensitive to stress, replicates actively	Byrne and Swanson, 1998
Stationary phase form (SPF)	Produced extracellularly, infectious to host cells, resistant to stress	Byrne and Swanson, 1998
Filamentous form (FF)	Produced extra- and intra-cellularly, infectious to host cells, forms dense biofilms	Rodgers et al., 1978; Piao et al., 2006
Mature infectious form (MIF)	Produced intracellularly, infectious to host cells, resistant to stress	Garduño et al., 2002
Immature intracellular form (IIF)	Produced in cultured macrophages, morphologically undifferentiated, less infectious and less resistant to stress than MIFs, elongated	Abdelhady and Garduño, 2013
Replicative phase form (RPF)	Produced intracellularly, replicates actively	Faulkner and Garduño, 2002
MIF-EPF intermediate	Produced extracellularly upon germination of mature infectious forms in BYE, shows intraperiplasmic vesicles	Faulkner and Garduño, 2002
MIF-RPF intermediate	Produced intracellularly in response to the presence of amino acids, a precursor to the initiation of replication in the LCV[a]	Sauer et al., 2005a
RPF-MIF intermediates	Produced intracellularly in the late stages of the infection cycle, display unique envelope profiles. Might be similar to IIFs	Faulkner and Garduño, 2002
VBNCC[a] derived from a SPF	Produced extracellularly in response to sustained stress, resuscitates in the presence of amoeba	Steinert et al., 1997; Al-Bana et al., 2014
VBNCC derived from a MIF	Produced extracellularly in response to stress, shows an intact cell ultrastructure, does not resuscitate in amoeba	Al-Bana et al., 2014
VBNCC derived from an EPF	Apparently more fragile than the other VBNCCs mentioned above	Ohno et al., 2003
Pelleted MIFs	Produced by ciliates and amoeba, show unique developmental traits	Berk et al., 1998, 2008
Pelleted VBNCCs	Produced by ciliates, may show unique developmental traits	Al-Bana et al., 2014

[a]*Abbreviations used: LCV, Legionella-containing vacuole; VBNCC, viable but non-culturable cell.*

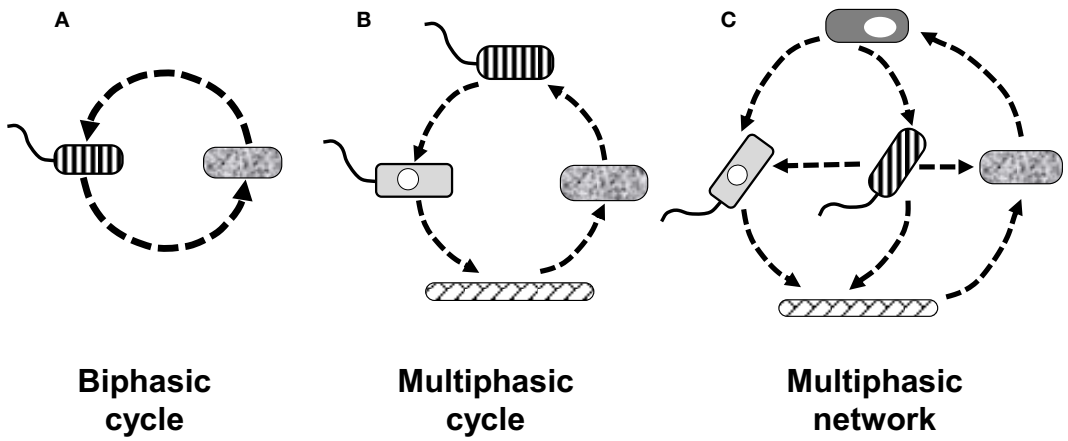

FIGURE 1 | Diagrammatic examples of how the number of forms and their differentiation links define different types of developmental cycles, or a developmental network. (A) Biphasic cycle in which two forms simply alternate into each other. **(B)** Multiphasic cycle showing four forms giving rise to each other in a sequential (linear) manner. **(C)** Multiphasic network of five forms in which the differentiation links are not linear. The differentiation network of *L. pneumophila* includes 14 recognized forms, to date, which are developmentally linked in a non-linear fashion, making the network highly complex.

sequentially differentiate into each other. When the differentiation links are not sequentially circular a developmental network is then established (**Figure 1**).

Extracellular vs. intracellular Lp's developmental cycles

In Section *L. pneumophila* is a Facultative Intracellular Pathogen that Differentiates into Numerous Forms within a Developmental Network, we already defined *Lp* as a facultative intracellular pathogen. Therefore, when discussing the *Lp* differentiation process we need to consider, distinguish and compare the differentiation steps that happen extracellularly, in relation to those linked to intracellular growth or residence. The extracellular development of *Lp* follows a biphasic cycle involving replicative exponential phase forms (EPFs) and transmissive stationary phase forms (SPFs) (reviewed by Molofsky and Swanson, 2004), with differentiation links to viable but non-culturable cells (VBNCCs) and filamentous forms (FFs) (**Table 1**).

Intracellularly, *Lp*'s developmental cycles are multiphasic. Ever since we first described *Lp*'s multiphasic cycle in HeLa cells (Faulkner and Garduño, 2002), we have hypothesized that *Lp* follows many intracellular multiphasic developmental cycles, one per host cell type (Garduño, 2008). This hypothesis stemmed from morphological observations suggesting that *Lp* reaches different developmental endpoints in different host cells (Garduño et al., 2002) and has been now confirmed. That is, we showed that the *Lp* progeny produced in *Acanthamoeba castellanii* is morphologically differentiated and infectious to cells in culture, whereas the progeny produced in human macrophages derived from the U937 or THP-1 cell lines was only partially differentiated (morphologically) and showed infectivity defects (Abdelhady and Garduño, 2013). In addition, as *Lp* interacts with ciliated protozoa of the genus *Tetrahymena* at a temperature of 30°C or lower, it does not replicate (Berk et al., 2008), but still differentiates intracellularly (Faulkner et al., 2008), establishing yet a different developmental cycle with a unique endpoint (Section The Cycle of Packaged *Lp* Forms below).

The developmental network of Lp and why it is necessary to dissect it

In *Lp*'s developmental network, forms within one given developmental cycle, also differentiate into developmental forms that typically belong to another cycle. We refer readers to Figure 4.4 from the Garduño et al. (2008) review, to sample the complexity of the developmental network of *Lp*, as we understood it then. In this review, we will refrain from trying to represent the entire *Lp*'s developmental network, as we currently understand it, in one single figure as it would be too difficult to fit. Instead, in Section Dissecting *Lp*'s Developmental Network we present five cycles that when pieced together should provide a fair representation of the entire developmental network of *Lp*.

THE DIFFERENTIATION PROCESS AND ITS REGULATION

KEY MOLECULAR PLAYERS IN THE DIFFERENTIATION NETWORK OF Lp—AN UPDATE

Differentiation of *Lp* may be implicitly viewed as an adaptation to radically different intracellular and extracellular environments, thus requiring the timely coordination of gene expression to achieve useful phenotypical traits. Not surprisingly, the key molecular players in *Lp* differentiation are regulators that directly or indirectly control the expression of virulence and fitness factors at the transcriptional and (or) post-transcriptional levels. These key molecular players are part of the regulatory pathways shown in **Figure 2**, but it should be acknowledged that these pathways are still not fully elucidated.

ppGpp, RelA, and SpoT

The alarmone guanosine 3′-diphosphate-5′-diphosphate, or ppGpp, is a recognized trigger of the stringent response of bacteria. Although best studied in *E. coli* (Magnusson et al., 2005), ppGpp is key for the differentiation of *Lp* from EPFs to SPFs (Hammer and Swanson, 1999). Produced in response to low nutrient levels, ppGpp is capable of (among a plethora of actions) binding RNA polymerase and altering the polymerase's preference for alternate sigma factors and promoters, and consequently, changing gene expression profiles (Artsimovitch et al., 2004; Magnusson et al., 2005; Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012; Ross et al., 2013). RelA and SpoT are the two enzymes known to synthesize ppGpp in *Lp*, in response to distinct nutritional conditions (Zusman et al., 2002; Dalebroux et al., 2009). In addition, SpoT is a ppGpp hydrolase, responsible for reducing ppGpp levels in *Lp* (Dalebroux et al., 2009). Whereas RelA is a ribosome-associated enzyme that gets activated as a consequence of the ribosomal engagement of uncharged tRNAs (Haseltine et al., 1972; Wendrich et al., 2002), SpoT seems to be activated by a reduction in the rate of fatty acids biosynthesis and(or) increased concentrations of short chain fatty acids (Dalebroux et al., 2009; Edwards et al., 2009). In both *E. coli* and *Lp* the stringent response typically results in upregulation of genes involved in stress resistance and virulence, and a downregulation of genes involved in growth and proliferation. The difference between the stringent responses of these two organisms thus relies on context rather than function. Whereas *E. coli* uses the stringent response primarily to overcome adverse conditions, *Lp* has integrated this response into survival and differentiation. As well, high levels of ppGpp are known to increase the stability and activity of alternative sigma factors including RpoS, one of the major regulators of the stationary phase in *Lp* (reviewed by Dalebroux and Swanson, 2012).

In *Lp*, synthesis of ppGpp seems to be as important as its hydrolysis, as demonstrated by both the fact that *spoT* mutants cannot be obtained in the presence of a functional RelA, and the inability of double *spoT relA* mutants complemented with either a fully functional RelA, or a defective SpoT, to resume intracellular growth, or growth in a nutrient-rich medium (Dalebroux et al., 2009). In both *Lp* and *E. coli* the ability to monitor fatty acid biosynthesis is through an interaction between SpoT and acyl carrier protein, and in *Lp* a functional LetA/S system (see below) is also required (Dalebroux et al., 2009; Edwards et al., 2010). Comparisons between the *relA spoT* double mutant and a *relA* mutant also demonstrate that SpoT plays a role in differentiation, likely as a consequence of the very low levels of ppGpp in the *relA spoT* double mutant (Dalebroux et al., 2009).

DksA

Although ppGpp is a major key inducer of differentiation, other factors act in concert with it to modify, enhance and (or) control the process. DksA is a ribosome-binding protein that acts together with ppGpp to modify the initiation of transcription. Although *dksA* deletion mutants still amass high levels of ppGpp, they are deficient at inducing virulence, cytotoxicity to macrophages, sodium sensitivity and motility (i.e., transmissive traits) in SPFs (Dalebroux et al., 2010). Furthermore, the *dksA* deletion mutant is unable to differentiate in response to propionic acid (a chemical that disturbs fatty acid metabolism). That is, when exposed to propionic acid *dksA* mutants were deficient in motility and escape from phagosomes, suggesting that full differentiation in response to fatty acid perturbations requires DksA. The aforementioned deficiencies are attributed to the absence of DksA-mediated modification of gene expression, as DksA is known to enhance the expression of *fliA*, *flaA*, *rsmZ*, and other regulatory components

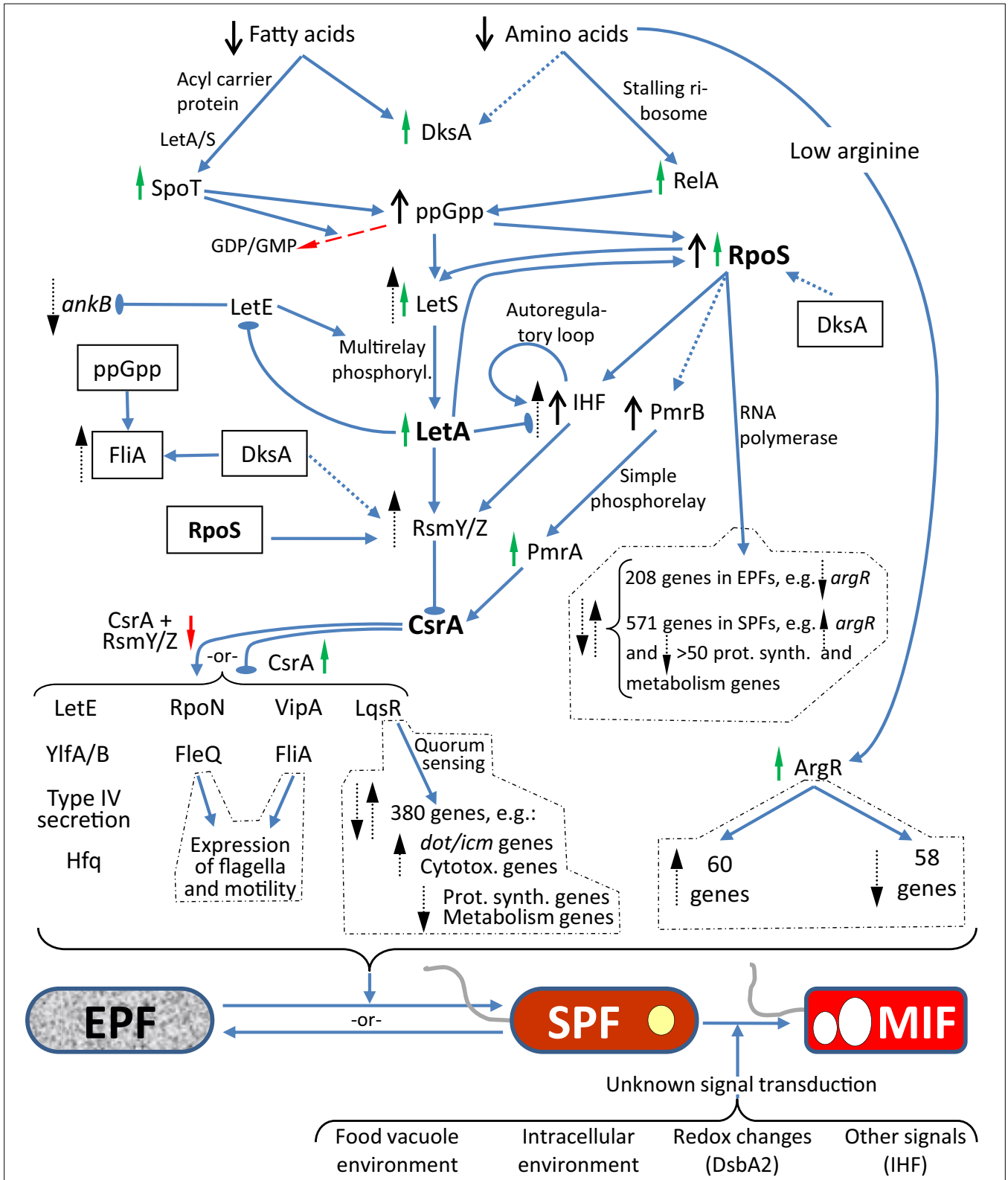

FIGURE 2 | Diagram showing the regulatory pathways of *Lp* differentiation as described in Section The Differentiation Process and Its Regulation of the text. Known confirmed interactions are depicted by blue solid arrows, where pointy arrows indicate positive/inducing effects and oval-headed arrows negative/repressing effects. Dotted blue lines indicate experimentally unproven or indirect links. Master regulators are shown in boldface. The boxed factors were duplicated to be placed in a convenient position to show additional links. RpoS, FleQ, and FliA (regulators of class II, III and class IV flagellar genes, respectively), LqsR

(Continued)

FIGURE 2 | Continued
(through quorum sensing) and ArgR have their own transcriptional regulatory networks (dotted line boxes). The *Lp* forms EPF, SPF, and MIF are as per **Table 1**. The top pathways pertain to the EPF-to-SPF differentiation in a low nutrient environment, but the interactions can be reversed to show the SPF-to-EPF differentiation in a nutrient-rich environment. Black arrows indicate high or low factor levels. Red and green arrows indicate decreased or increased activity of the corresponding factor, respectively. Black dotted line arrows indicate upregulation or downregulation of transcription. Besides being a ppGpp synthase, SpoT is also a ppGpp hydrolase, and this activity is depicted by the red dashed arrow.

(Dalebroux et al., 2010). For instance, inducible expression of *dksA* from a plasmid restored 20–35% motility in a ppGpp-negative, non-motile *Lp* strain, enhancing transcription of *fliA* and *flaA*. DksA also appears to be necessary (at least partially) for survival of *Lp* in stationary phase, as *dksA* mutants grow normally in exponential phase but lose viability in stationary phase relative to the parent *Lp* strain (Dalebroux et al., 2010). In summary, DksA participates in (and complements) ppGpp-mediated processes. Further work on DksA is warranted, as its impact on *Lp* differentiation is still poorly understood.

RpoS

The alternative RNA polymerase sigma factor RpoS is considered a master regulator of *Lp* differentiation from replicative forms (EPFs or RPFs) into transmissive forms (SPFs or MIFs), and has been studied in great extent. In our previous review (Garduño et al., 2008), we presented the major known properties of the *Lp* RpoS, and highlighted the fact that the regulation of *rpoS* expression and RpoS activity, constitutes a complex process different from that described in *E. coli*. Here we will focus on recent (after 2008) data.

The ppGpp-dependant induction of RpoS (see above) is also dependent on DksA in *E. coli* and *Salmonella enterica*, as the deletion of *dksA* results in significantly lower levels of RpoS, even when ppGpp is produced (Brown et al., 2002; Paul et al., 2004). However, it is not known yet whether expression of the *Lp* RpoS is also dependent on DksA. There can be little doubt of the centrality of RpoS to *Lp* differentiation as recent microarray data has confirmed that RpoS significantly alters gene expression, affecting 208 genes in exponential phase, and 571 genes in stationary phase (Hovel-Miner et al., 2009). The expression of genes encoding for secretion substrates of the type IV virulence-related secretion system, Dot/Icm, is modified both positively and negatively in both growth phases, suggesting that RpoS coordinates a shift in the effectors that are released at different stages of the infection cycle (Hovel-Miner et al., 2009). *Lp* RpoS also caused the downregulation in SPFs of over 50 genes related to translation and metabolism. Expression of the small RNAs *rsmY* and *rsmZ* is also enhanced by RpoS, leading to an increase in transmissive traits via the sequestration of CsrA (see below) (Rasis and Segal, 2009). Finally, *rpoS* mutants are unable to differentiate into MIFs in HeLa cells, and either get digested in the intracellular environment of *T. tropicalis* (Faulkner et al., 2008) or do not grow in amoebae (Hales and Shuman, 1999; Abu-Zant et al., 2006).

Although the *Lp* RpoS is clearly important for *Lp* differentiation in stationary phase, we previously discussed that it also plays a functional role in the exponential growth phase. In fact, some of the genes upregulated by RpoS in stationary phase are actually downregulated by RpoS during the exponential growth phase, as confirmed for the metabolic gene *argR* which enhances intracellular growth in amoebae, but not in macrophages (Hovel-Miner et al., 2009). *Lp*'s ArgR is a transcriptional regulator with an unusually large and complex regulon (Hovel-Miner et al., 2010). In the stationary growth phase, ArgR positively affects the expression of 60 genes, and negatively affects the expression of 58 genes, in response to exogenous low arginine concentrations (Hovel-Miner et al., 2010).

Two-component regulatory systems (LetA/S, PmrA/B, and, LqsR/S)

The *Lp* LetA/S system is a two-component regulatory system (2CRS) whose activity is required for full expression of transmissive phenotypes in SPFs. LetA/S is part of the signal transduction pathway that connects nutritional gaging to differentiation responses, and occupies a crossroads position between ppGpp, RpoS, and CsrA (see below and **Figure 2**). Not surprisingly, the LetA/S system was determined to be involved in the regulation of *rpoS*, several *icm* genes, *flaA*, *plaC*, and other genes involved in lipid metabolism, *ralF* and *hfq* (Gal-Mor and Segal, 2003; Lynch et al., 2003; McNealy et al., 2005; Broich et al., 2006). Unlike most 2CRSs, the *Lp* LetA/S system acts as a rheostat (rather than an ON/OFF switch) by virtue of including multiple phosphorylation steps in the phosphorelay pathway, thus making it comparable to the BvgA/BvgS 2CRS of *Bordetella pertussis* (Edwards et al., 2010). High levels of ppGpp activate LetA/S, but the transcription of *letS* is reduced in an *rpoS* mutant, confirming that multiple factors (including LetE) are actually combined to increase the expression and activity of the LetA/S system (Hovel-Miner et al., 2009). The effects of the LetA/S system on induction of transmissive phenotypes are indirect, as LetA/S acts by repressing CsrA (Sahr et al., 2009) by virtue of inducing the expression of the small RNAs *rsmY* and *rsmZ* (Rasis and Segal, 2009), which in turn bind to CsrA to antagonize its activity (see Section CsrA below).

While the precise link between the LetA/S system and LetE remains unsolved, recent information indicates that LetA appears to repress expression of *letE*, as levels of LetE increase threefold in a *letA* mutant (Sahr et al., 2009). LetE is also known to repress *ankB*, giving LetE both positive and negative roles in regulation (Al-Khodor et al., 2008). As indicated above for RpoS, *Lp letA* mutants do not differentiate into MIFs and are digested in *Tetrahymena*, but are able to establish a *Legionella*-containing vacuole and grow well in HeLa cells (Faulkner et al., 2008). In addition, it is known that a *letA* mutant grows well in human macrophages, but not in *A. castellanii* (Gal-Mor and Segal, 2003).

The PmrA/B 2CRS is used in *Lp* to control Dot/Icm secretion and the stress response (Zusman et al., 2007). It has been suggested that the PmrA/B system may have a more global effect on transcription in *Lp*, as microarray data shows that mutations in *pmrA/B* cause the differential expression of 279 genes, including type IV and type II secretion effectors, stress response genes, metabolic genes, and others (Al-Khodor et al., 2009). The

PmrA/B system's link to the differentiation regulatory network comes from the facts that *pmrA/B* mutants exhibited significantly lower levels of *csrA* in both the exponential and stationary growth phases (Al-Khodor et al., 2009), and that *pmrA/B* is regulated by RpoS (Hovel-Miner et al., 2009). Unfortunately, no mechanism for this role has been elucidated at this time.

Finally, the Lqs 2CRS responsible for quorum sensing in *Lp* is also involved in regulating differentiation. Three gene products are key for the function of this system, LqsA (encoding the autoinducer synthase), LqsS (encoding the sensor kinase) and LqsR (encoding the response regulator) (Tiaden et al., 2008). Expression of LqsR is growth phase-dependent and controlled by the combined action of RpoS and LetA (Tiaden et al., 2007), likely through RsmY/Z and CsrA (Section CsrA and **Figure 2**). The Lqs system, in turn, controls the expression of more than 380 genes; of relevance being the upregulation of virulence traits in SPFs (Tiaden et al., 2008). The most recent piece of information regarding the Lqs system is that LqsT, a second sensor kinase besides LqsS, is capable of phosphorylating (and activating) LqsR (Schell et al., 2014). Besides the autoinducer, the signals to which the two sensor kinases respond are not yet elucidated, but their convergence upon a single response regulator strongly suggests an unusual flexibility with enhanced signaling options.

CsrA

The carbon storage regulator CsrA is an RNA-binding protein that recognizes a binding site near the 5′ end of target transcripts (Baker et al., 2002) and blocks their translation. In EPFs, CsrA blocks the translation of transcripts encoding transmissive traits and stationary phase-associated factors, including *rpoN*, *fliA*, *letE*, *ylfA*/*B*, and *vipA* (Forsbach-Birk et al., 2004; Rasis and Segal, 2009). During the stationary growth phase, the LetA/S system is activated by the combined action of ppGpp and other factors (see above), and induces production of the non-coding RNAs RsmY and RsmZ. These small RNAs, predicted early to exist in the *Lp* genome (Kulkarni et al., 2006), are now experimentally confirmed to be transcribed and to bind to CsrA, un-blocking the translation of transcripts encoding transmissive traits (Rasis and Segal, 2009; Sahr et al., 2009). Optimal expression of *rsmY/Z* requires RpoS, confirming the previously suggested link between LetA/S, CsrA, and RpoS within the pathway that regulates differentiation in *Lp* (Hovel-Miner et al., 2009; Rasis and Segal, 2009).

A picture has recently emerged, in which CsrA seems to play an important role in regulating the expression and (or) activity of type IV secretion systems (T4SSs) in *Lp*, including the Dot/Icm virulence system (Rasis and Segal, 2009; Sahr et al., 2009; Nevo et al., 2014). Therefore, the correlation between differentiation and expression of virulence continues to be strengthened. A puzzling fact about the *Lp* pangenome is that it encodes, in a strain-dependent manner, several T4SSs whose genes are either stably integrated into the chromosome, or found within integrative and conjugative elements (ICE). For instance, strain 130b (also known as AA100), carry as many as six T4SSs (Schroeder et al., 2010), five of which reside in horizontally acquired, mobile genetic elements (Gómez-Valero et al., 2011; Wee et al., 2013). All these horizontally acquired T4SSs carry with them homologs of CsrA (Brassinga et al., 2003; Gómez-Valero et al., 2011; Wee et al., 2013; Flynn and Swanson, 2014). Therefore, the number of *csrA* copies would vary between strains, depending on how many genomic island-encoded T4SSs are present. Assuming that there might be functional differences between the various CsrAs present in a given strain, their regulatory flexibility could be astounding. However, it remains to be determined whether this is actually the case or not.

FliA and FleQ

Motility and differentiation are closely linked, simply because the differentiated transmissive *Lp* forms (SPFs and MIFs) are motile, whereas the replicative *Lp* forms (EPFs and RPFs) are not. It has been known that the flagellar sigma factor FliA is required both for the synthesis of flagella and for actual motility, as well as for achieving full virulence (Hammer et al., 2002; Heuner et al., 2002). It is thus not surprising that FliA, and its regulator FleQ (Albert-Weissenberger et al., 2010; Schulz et al., 2012), have found a place within the regulatory pathway of *Lp* differentiation, located downstream of CsrA (Heuner and Albert-Weissenberger, 2008; Sahr et al., 2009). Recent reports indicate that both ppGpp and DksA are required for the activation of the *fliA* promoter (Dalebroux et al., 2010), and have confirmed the role of FliA in virulence, showing that *fliA* mutants cannot compete with wild-type *Lp* during a co-culture assay in *A. castellanii* (Schulz et al., 2012).

DsbA

The bifunctional periplasmic disulfide bond oxidoreductase/isomerase of *Lp*, DsbA, modifies proteins by catalyzing the formation of disulfide bonds between cysteines (Kpadeh et al., 2013). In *Lp* there are two DsbA proteins, the non-essential DsbA1 and the essential and bifunctional DsbA2. When *dsbA2* is modified at the region encoding its active redox site, losses in *Lp* infectivity, intracellular growth and motility are observed (Jameson-Lee et al., 2011). Expression of native *dsbA2* was necessary for virulence and motility, as *Lp* carrying the defective *dsbA2* did not express flagellin and was deficient in Dot/Icm-dependent haemolysis (Jameson-Lee et al., 2011). It thus seems reasonable to propose that DsbA2 plays a role in *Lp* differentiation, as motility and the expression of a functional Dot/Icm system are hallmarks of transmissive *Lp* forms (SPFs and MIFs).

Integration host factor (IHF)

IHF is a heterodimeric DNA-binding protein that by virtue of its DNA-bending ability regulates transcription and recombination in bacteria (reviewed by Dorman, 2009). In *Lp*, IHF participates in the RPF-to-MIF differentiation (Morash et al., 2009) by an unknown mechanism. It is known that RpoS positively regulates the expression of the *ihfA* and *ihfB* genes, and that IHF acts as a positive autoregulator of expression (Pitre et al., 2013). In fact, experimentally confirmed binding sites for RpoS and IHF have been identified in the promoter regions of *ihfA* and *ihfB*. Puzzlingly, the DNA binding sites for the LetA response regulator (see above) and IHF have similar consensus sequences, suggesting that LetA and IHF do compete for these sites. Support for this notion comes from the fact that LetA negatively regulates the transcription of *ihfA* and *ihfB* (Pitre et al., 2013). Finally, IHF was

found to cooperate with LetA in the induction of transcription of RsmY and RsmZ, further implicating IHF as a regulator of *Lp* differentiation. IHF is the third regulator of *Lp* differentiation (the other two being RpoS and LetA) for which mutants do not fully differentiate into MIFs (by morphological criteria), and grow in mammalian cells but not in amoeba (Morash et al., 2009). Thus, we would like to reiterate here our view that *Lp* is under strong selective pressure to differentiate into MIFs inside protozoa, but not in mammalian cells (Faulkner et al., 2008).

DISSECTING Lp'S DEVELOPMENTAL NETWORK

THE VARIOUS Lp DEVELOPMENTAL CYCLES AND FORMS

In this section we have dissected *Lp*'s developmental network into five separate cycles, to highlight the main characteristics and biological relevance of key developmental forms found in the freshwater environment. As explained above (Section The Developmental Network of *Lp* and Why It is Necessary to Dissect It), trying to represent *Lp*'s developmental network in a single diagram would be overchallenging. Therefore, we have grouped closely related forms (**Table 1**) (on the basis of their direct biological relationships and presence in the same environmental niche) that sequentially differentiate into each other, to define each cycle. We believe that the cycles presented cover all the known aspects of *Lp*'s developmental biology. Although in our previous review (Garduño et al., 2008) we discussed FFs as a possible variation of SPFs, FFs have proven to be significantly different from bacillary *Lp* forms, particularly in the way they interact with host cells. In addition, filamentation in *Lp* often occurs in a growth phase-independent manner, thereby warranting the developmental separation of FFs and SPFs, as presented here.

EPFs and SPFs—The Lp extracellular growth cycle

The biphasic extracellular growth cycle that alternates between EPFs and SPFs is schematically represented in **Figure 3**. This cycle happens in artificial, nutrient-rich culture media, and allegedly, within natural microbial communities, e.g., biofilms, where *Lp* could grow at the expense of dead microorganisms (Temmerman et al., 2006) or utilize nutrients released by other bacteria and (or) photosynthetic organisms (Tison et al., 1980; Pope et al., 1982; Bohach and Snyder, 1983a; Wadowsky and Yee, 1983, 1985; Hume and Hann, 1984a; Stout et al., 1985, 1986; Tison, 1987), onto which *Lp* might even physically attach (Bohach and Snyder, 1983b; Hume and Hann, 1984b).

A substantial body of experimental data has been obtained for the differentiation of EPFs and SPFs produced *in vitro* (in broth or agar cultures), but to the best of our knowledge, no experimentation has been reported on the differentiation of naturally produced EPFs and SPFs. *In vitro* EPFs have a typical Gram-negative envelope ultrastructure (Chandler et al., 1979; Faulkner and Garduño, 2002), and appear as slender short rods with a rather homogeneous cell size. The EPF is the *Lp* form that actively replicates in nutrient-rich media at a rate that varies according to growth conditions. EPFs utilize amino acids as their primary carbon and energy source, and are auxotrophic for cysteine (Ewann and Hoffman, 2006; Hoffman, 2008). Therefore, EPFs must rely on gluconeogenesis to synthesize the sugar precursors required for cell wall synthesis (Hoffman, 2008). Although

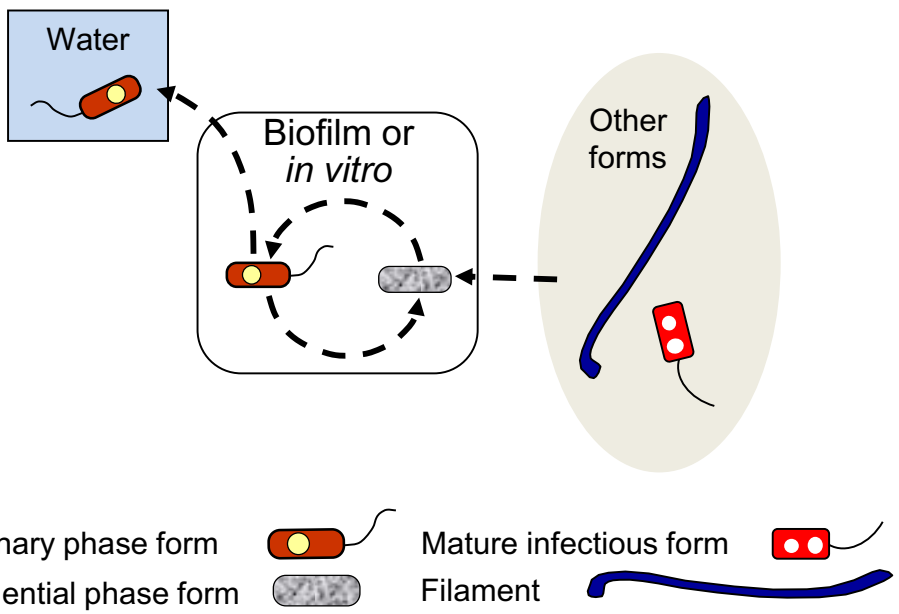

FIGURE 3 | Schematic representation of the exponential phase form (EPF)–stationary phase form (SPF) biphasic developmental cycle (dashed line arrows). EPFs and SPFs simply alternate into each other when *L. pneumophila* grows extracellularly, but SPFs can initiate intracellular cycles (**Figure 5**), or be internalized by ciliates of the genus *Tetrahymena* inside which they differentiate into mature infectious forms (**Figure 7**). SPFs produced in natural biofilms are likely to enter the water environment as planktonic free forms. SPFs and EPFs can produce filaments (**Figure 4**) and viable but non-culturable cells (**Figure 6**). Filaments and mature infectious forms are known to be able to differentiate into EPFs *in vitro*, entering the biphasic developmental cycle.

synthetic media have been formulated for *Lp*, e.g., chemically defined media (Pine et al., 1986, and references within), the best growth of EPFs is always obtained in complex media with added yeast extract. The EPF is reportedly unable to initiate infections in macrophages and does not effectively avoid fusion with lysosomes (Joshi et al., 2001). Furthermore, the EPF is tolerant to salt, a phenotype that has been historically associated with avirulence in *Lp*. Consequently, the EPF is considered to be the replicative, non-infectious *Lp* form produced extracellularly.

In contrast, the SPF is infectious, morphologically heterogeneous (Chandler et al., 1979), and shows partial morphological differentiation features (Garduño et al., 2002) including the presence of cytoplasmic inclusions and invaginations of the inner membrane into the cytoplasm (Faulkner and Garduño, 2002). SPFs express transmissive traits and effectively initiate infections in macrophages, departing from the endocytic pathway shortly after internalization to establish a replicative vacuole (Joshi et al., 2001). The net gain in *Lp* cell numbers in the stationary growth phase is null or negative, however, it is virtually impossible to determine whether a proportion of SPFs in a *Lp* culture actually replicate or not. In spite of these technicalities, the SPF is generally considered non-replicative. As indicated in Section Background, the SPF has constituted the model *Lp* form for studying the molecular mechanisms of *Lp* differentiation into transmissive forms. We have determined that SPFs are metabolically active, consume oxygen in the presence of organic substrates, are infectious to a variety of mammalian cells in culture (Garduño et al., 2002), and remain culturable for long periods in water at room temperature (Al-Bana et al., 2014).

Although SPFs are confirmed transmissive forms of *Lp*, they show many differences with the transmissive *Lp* form produced intracellularly, i.e., MIFs. These differences have been repeatedly emphasized (Garduño et al., 2002, 2008; Faulkner and Garduño, 2002; Garduño, 2008) and will not be reiterated here. However, it is worth mentioning here that SPFs directly differentiate into MIFs inside food vacuoles of the ciliate *Tetrahymena* (Faulkner et al., 2008, also see Section The Cycle of Packaged *Lp* Forms, below), indicating that these two forms are developmentally linked; the SPF being a stable differentiation intermediate between EPFs and MIFs.

In nature, EPFs and SPFs would be likely produced within biofilms, from which they would be released into the freshwater environment. However, these naturally produced forms could have different characteristics in relation to EPFs and SPFs produced *in vitro*. As transmissive *Lp* extracellular forms, SPFs have the potential for causing disease in humans. Also, naturally produced SPFs could initiate infections in freshwater amoeba and be ingested by ciliates, within which they would replicate and (or) differentiate to be released into the water environment as free or pelleted MIFs (Berk et al., 1998; Faulkner et al., 2008).

Filamentous forms (FFs)

We have summarized the developmental links of FFs in **Figure 4**. An anecdotal curiosity is that the very first picture of *Lp* ever published, prominently portrays a FF (McDade et al., 1977). However, the mechanisms that control *Lp* filamentation are poorly understood, as are its potential biological benefits.

Filamentation correlates with enhanced infectivity, persistence and pathogenesis in uropathogenic *E. coli* (UPEC) and *Proteus mirabilis* (Allison et al., 1994; Rosen et al., 2007). The gene *sulA* (encoding a cell cycle check-point protein that is part of the SOS response to stress) mediates filamentation of UPEC *in vivo*, and subvert innate immunity, confirming that a correlation exists between filamentation and pathogenesis (Justice et al., 2006). While *Lp* lacks the *sulA* gene and a *bona fide* SOS response (Charpentier et al., 2011), *Lp*'s differentiation into FFs is still linked to stressful signals such as nutrient limitation (Warren and Miller, 1979), the presence of antibiotics (Smalley et al., 1980; Elliott and Rodgers, 1985), high temperature (Piao et al., 2006), or exposure to UV radiation (Charpentier et al., 2011).

One clue that supports the developmental nature of filamentation comes from a study showing that overexpression of CsrA (the RNA-binding inhibitor of transcript translation, and a master regulator of *Lp* differentiation, **Figure 2**) enhances the production of *Lp* filaments in the post-exponential growth phase *in vitro* (Fettes et al., 2001). However, no mechanistic details on how CsrA regulates cell elongation are available. Other gene products implicated in the production of FFs are the HtpB chaperonin and the putative spermidine transporter PotD. Overexpression of HtpB leads to filamentation in *Lp* and *E. coli* (Garduño and Chong, 2013), and deletion of *potD* completely inhibits filamentation in stationary phase, while the *pot* operon promoter is highly activated in FFs (Nasrallah et al., 2014). Nonetheless, as for CsrA, no mechanism on how HtpB and PotD induce filamentation has been elucidated.

FFs have been observed in the water environment, in lung tissue and in clinical bronchial lavages (Rodgers et al., 1978; Prashar et al., 2012), and we now know that they can initiate intracellular infections in lung epithelial cells (Prashar et al., 2012) and macrophages (Prashar et al., 2013). In fact, the survival of filaments in macrophages correlates with length, so that the longest filaments are the most prone to replicate intracellularly (Prashar et al., 2013). In lung epithelial cells and macrophages, the uptake and early intracellular trafficking mechanisms of FFs are different from those established for bacillary *Lp* forms, and involve β1-integrin and E-cadherin as well as unique membrane, actin and vesicular trafficking rearrangements (Prashar et al., 2012, 2013). These mechanistic differences between bacillary forms and FFs, suggest that FFs express unique bacterial cell surface molecules not present in bacillary *Lp* forms (none of which have been as yet identified), and (or) that the number and presentation of surface proteins is unique due to the dramatically increased surface of FFs.

A larger surface area would also favor the secretion and presentation of extracellular matrix materials required for the formation of biofilms. It is thus not surprising that long FFs actually produce robust *Lp* micelial mat-like biofilms in a temperature- and surface-type-dependent manner (Piao et al., 2006). FFs are not taken up by the ciliate *Tetrahymena tropicalis*, suggesting that by differentiating into FFs, *Lp* could avoid predation by bacteriovorous protozoa that do not support its intracellular growth (Berk et al., 2008). However, the effect of filamentation on the

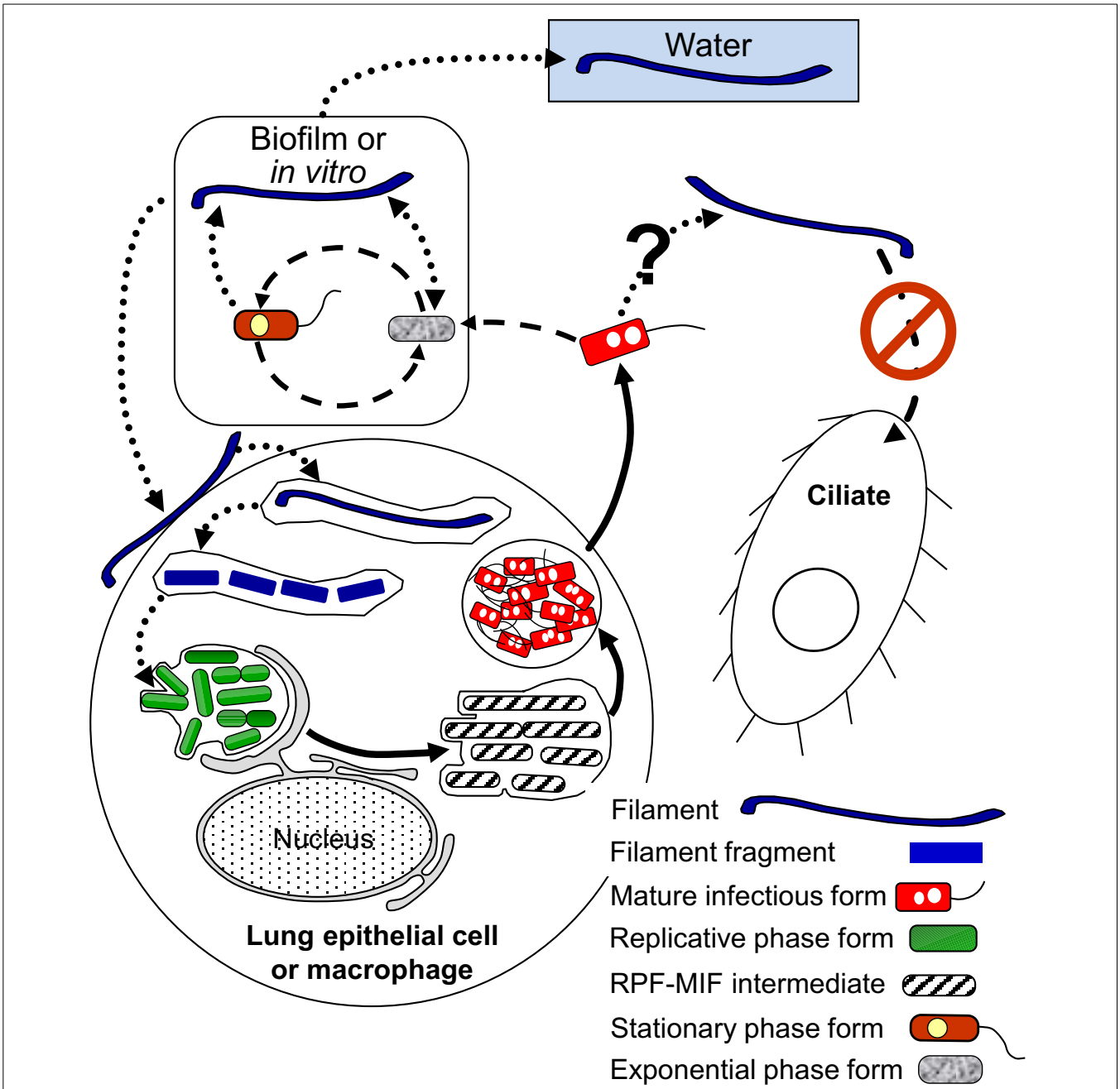

FIGURE 4 | Schematic representation of the multiphasic developmental cycle of filamentous forms (FFs) (round-dotted line arrows). Bacillary exponential phase forms and stationary phase forms produce FFs *in vitro* mainly in response to stress, and filamentation enhances biofilm-formation. FFs are infectious to lung epithelial cells and macrophages. As a consequence of the internalization of FFs by host cells FFs fragment to produce bacillary forms and eventually RPFs. FFs also differentiate into exponential phase forms *in vitro*. It is not known whether mature infectious forms can produce FFs, but free FFs are not internalized by *Tetrahymena* ciliates. Dashed line arrows, solid line arrows, and dash-dot patterned arrows are used to depict steps of the SPF–EPF developmental cycle (**Figure 3**), the MIF–RPF intracellular developmental cycle (**Figure 5**) and the ciliate-pellets developmental cycle (**Figure 7**), respectively.

ability of protists to engulf *Lp* has not been studied in *Lp*'s natural environment. Although preliminary observations in our lab indicate the ability of *A. castellanii* to ingest FFs, the interaction of FFs with freshwater amoebae remains understudied and deserves further attention.

A fascinating event is the recently observed intracellular fragmentation of filaments (i.e., the differentiation of FFs into RPFs), which occurs as a consequence of FF uptake by macrophages (Prashar et al., 2013). This process also occurs extracellularly *in vitro* (Piao et al., 2006) where FFs differentiate into EPFs.

Filaments are reportedly produced from EPFs and SPFs, but production of FFs by other *Lp* forms has not been reported. Finally, we would like to speculate on the significance of FFs in the transmission of Legionnaires' disease. Perhaps in the late stages of the disease, after *Lp* numbers in the lung have been amplified through replication in alveolar macrophages and patients develop a fever (inducing HtpB expression through high temperature), FFs could be commonly present and preferentially be taken by lung epithelial cells, which in turn could serve as reservoirs from which *Lp* could re-enter the environment.

RPFs and MIFs—The Lp intracellular growth cycles

A general representation of the intracellular developmental cycle depicted in **Figure 5** is made possible by the remarkable conservation of intracellular events that characterize *Lp* infections, as described in human monocytes (Horwitz, 1983), mouse macrophages (Yamamoto et al., 1992), several mammalian cell lines (Oldham and Rodgers, 1985) including HeLa cells (Garduño et al., 1998), as well as different species of amoeba (Fields et al., 1989; Abu Kwaik, 1996; Solomon et al., 2000; Greub and Raoult, 2003; Lu and Clarke, 2005). Central in this general intracellular cycle is the MIF, initially named the mature intracellular form (Garduño et al., 1998) and subsequently renamed mature infectious form, to reflect the fact that MIFs persist in the extracellular environment as the predominant transmissive form of *Lp*. Thus, by our definition, the *Lp* progeny produced as a result of an intracellular growth cycle (see Section Extracellular vs. Intracellular *Lp*'s Developmental Cycles above) would be MIFs, which depending on the type of host cell infected, could have reached different developmental endpoints and exit as either free MIFs (which seems to be the most common mechanism, as described by Rowbotham, 1986), MIFs in host-derived membrane-bound

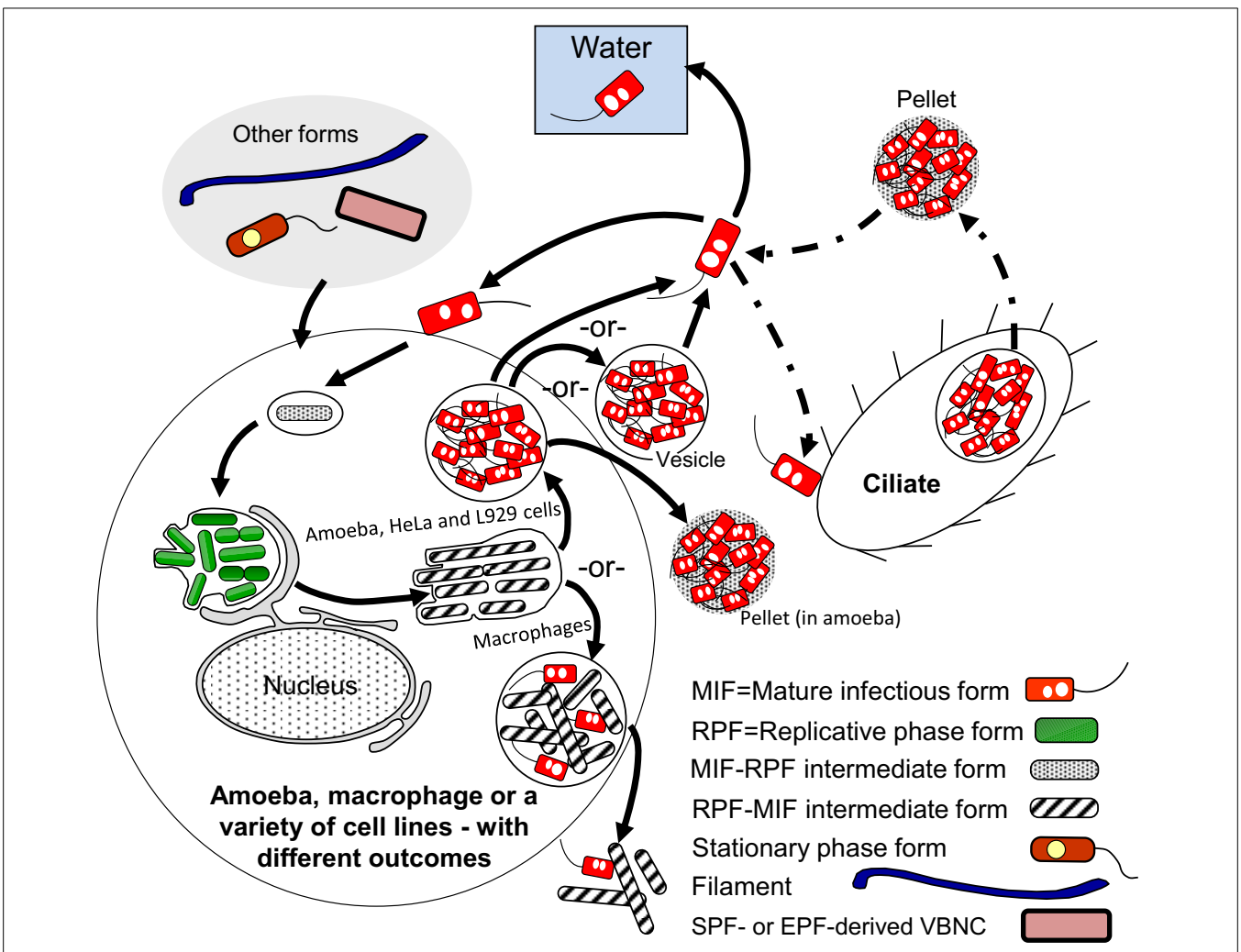

FIGURE 5 | Schematic representation of the replicative phase form (RPF)–mature infectious form (MIF) multiphasic developmental cycle (solid line arrows), which involves distinct morphological intermediate forms between MIFs and RPFs, and between RPFs and MIFs. The cycle branch that happens in the ciliate *Tetrahymena* results in pellets of MIFs, but does not involve bacterial replication. The link to other cycles occurs when extracellular forms ("other forms" oval) initiate an intracellular infection that results in differentiation into RPFs, replication, and later differentiation into MIFs. MIFs, but not RPFs, persist in the water environment. It seems reasonable to surmise that if intracellular growth is the primary means of *L. pneumophila* replication in nature, MIFs would be the most abundant *Lp* transmissive form in the water environment. The dash-dot patterned arrows are used to depict steps of the ciliate-pellets developmental cycle (**Figure 7**).

vesicles [as observed in HeLa cells (Garduño et al., 1998) and amoeba (Rowbotham, 1986; Bouyer et al., 2007)], or pelleted MIFs (wrapped in a combination of multilamellar bodies produced by intra-endosomal budding in protozoa, and undigested bacterial membranous debris, Berk et al., 1998; Marchetti et al., 2004; Paquet et al., 2013).

MIFs exhibit several characteristic morphological and physiological features, which could be regarded as adaptations to both survive in the extracellular environment and remain infectious toward new potential hosts. These adaptations include resistance to environmental stressors (pH, detergents, chlorine, and antibiotics), a thickened cell wall that deviates from the characteristic Gram-negative ultrastructure, large and abundant cytoplasmic inclusions, metabolic dormancy, flagellation, and enhanced infectivity. Although MIFs were originally described in the context of the HeLa cell growth cycle, others (Greub and Raoult, 2003) and we (Abdelhady and Garduño, 2013, in addition to unpublished results) have confirmed that MIFs (with the same morphology and [or] general characteristics observed in HeLa cells) are also produced in amoeba. In addition, a retrospective look at publications regarding the intracellular growth of *Lp* in amoeba, starting with the careful observations of Rowbotham (1986), confirms beyond any doubt the natural production of MIFs in amoebal hosts (also reviewed by Garduño, 2008).

Once internalized by a new host, MIFs quickly adapt to the intracellular host environment and any possible host defense mechanisms triggered by their internalization. This adaptation is a pre-requisite for replication and primarily, albeit not exclusively, is mediated by the Dot/Icm type IV secretion system. The Icm/Dot system secretes a multitude of functionally redundant effectors that act at every stage of the infection process, beginning with the binding of *Lp* to cell surface receptors of the new host, and ending with the exit of progeny from the wasted host cell (reviewed by Hoffmann et al., 2014). In this respect, we have often argued that MIFs must be "infection-ready," carrying a spring-loaded Dot/Icm system (set during the differentiation of RPFs into MIFs) that is released upon contact with a new host cell, allowing the establishment of an intracellular niche within minutes after internalization (Roy et al., 1998).

However, before fully exploiting the newly acquired intracellular niche and beginning replication, MIFs must first induce host mechanisms to transport (or themselves directly transport) nutrients from the host cell cytoplasm into the lumen of the replicative vacuole. Not until these nutrients (primarily amino acids) reach a threshold concentration inside the *Legionella*-containing vacuole (LCV), MIFs can differentiate into RPFs. Among these nutrients, *Lp* must be able to have access to iron (reviewed by Cianciotto, 2007), in addition to nucleosides (Fonseca et al., 2014), to initiate and maintain growth, but amino acids seem to be the primary triggers for differentiation into RPFs.

The collective experimental evidence that supports the role of amino acids in the MIF-to-RPF differentiation is as follows: (i) During *Lp* infection the host amino acid transporter SLC1A5 (putatively responsible for mobilizing cytoplasmic amino acids into the lumen of the LCV) is induced (Wieland et al., 2005). (ii) Human MM6 monocytes with a chemically inactivated, or post-transcriptionally silenced SLC1A5 transporter do not support the growth of *Lp* (Wieland et al., 2005). (iii) A transposon-insertion mutant with a defective amino acid transporter (PhtA, with high affinity for threonine) invades well but does not replicate in murine macrophages. This mutant is also defective at initiating growth *in vitro* (Sauer et al., 2005a). (iv) The growth defects of the *phtA Lp* mutant are reversed by supplying an excess of exogenous amino acids (particularly threonine). (v) The *phtA* mutant remains "locked" as a SPF, predominantly showing transmissive phenotypes (Sauer et al., 2005a). (vi) The concentration of free amino acids is increased in *Lp*-infected cells in an AnkB-dependent manner (Price et al., 2011); AnkB being a LetE-regulated effector of the Icm/Dot system (**Figure 2**) that promotes the degradation of ubiquitinated host cell proteins. (vii) *ankB* mutants cannot initiate intracellular replication in spite of being able to establish an apparently functional LCV, and persist in the LCV as a form that predominantly expresses transmissive phenotypes (Price et al., 2011). (viii) *ankB* mutants (but not a *dotA* mutant) can be rescued and initiate intracellular growth by the addition of exogenous amino acids (Price et al., 2011). (ix) The presence of arginine in the LCV induces major changes in *Lp* gene expression, mediated by the inhibition of ArgR (**Figure 2**), an important transcriptional regulator (Hovel-Miner et al., 2009).

RPFs have a morphology that is indistinguishable, at the ultrastructural level, from that documented for EPFs. That is, both RPFs and EPFs show an envelope ultrastructure that is typical of Gram-negative bacteria, an electron-dense cytoplasm rich in ribosomes, and a lack of cytoplasmic inclusions (Faulkner and Garduño, 2002). RPFs usually show an intimate interaction with the inner face of the LCV membrane. In transmission electron microscopy sections of *Lp*-infected cells, the LCV membrane closely follows the contour of the contained RPFs. This feature is displayed both in protozoan and in mammalian host cells, suggesting that the underlying mechanism involved is conserved among eukaryotes. We propose that such intimate interaction is related to the acquisition of nutrients by RPFs. In this respect, a supply of nutrients must be secured to sustain the active replication of RPFs.

Factors inferred to contribute to the flow of nutrients from the host cell into the LCV's lumen include the AnkB-mediated degradation of host proteins, which results in increased levels of available amino acids (recently reviewed by Price et al., 2014), the transport of amino acids across the LCV membrane (Wieland et al., 2005), and the characteristic association of the LCV with mitochondria and the endoplasmic reticulum (ER), first recognized in electron microscopy studies of *Lp*-infected human monocytes (Horwitz, 1983). We have demonstrated that the *Lp* chaperonin, HtpB, reaches the cytoplasm of host cells and associates with the LCV membrane (Nasrallah et al., 2011; Garduño and Chong, 2013). Furthermore, purified HtpB attached to polystyrene microbeads attracts mitochondria by an unknown mechanism (Chong et al., 2009). The secretion by *Lp* of an eukaryotic-like sphingosine-1-phosphate lyase, LegS2 (Degtyar et al., 2009) and a mitochondrial carrier protein, LncP (Dolezal et al., 2012), both of which localize to mitochondria after secretion, could induce nutrient leaching in the attracted mitochondria. In fact, the secreted LncP localizes to the inner membrane of host cell mitochondria, from where (by means of

LncP's ability to transport nucleotides across proteoliposomes *in vitro*) it is speculated to help in the unidirectional flow of nutrients from mitochondria to the LCV lumen (Dolezal et al., 2012). If this would be the case, mitochondria could be the source of nucleotides to be taken up by RPFs from the LCV by the transporters PhtC/D (Fonseca et al., 2014).

A second experimentally demonstrated ability of HtpB that is related to nutrient acquisition is its specific interaction with host S-adenosyl methionine decarboxylase (SAMDC). We have proposed that this interaction contributes to the intracellular production of elevated levels of polyamines, in turn required for the optimal replication of RPFs (Nasrallah et al., 2011). Finally, the association of the LCV with the ER is thought to be a major contributor of nutrients for RPFs. However, in spite of intensive recent work showing the targeting of Dot/Icm effectors to the ER/Golgi/ER vesicular trafficking, and their specific effects on these processes (which are beyond the scope of this review), the flow of nutrients from the ER to the LCV has not been unequivocally demonstrated, except for the structural incorporation of ER-derived vesicles into the LCV and consequent derivation of LCV membrane from the ER, as reported by Tilney et al. (2001). That is, the cargo of ER-derived vesicles, and the ER membrane itself, could be sources of nutrients and lipids for RPFs.

Replication of RPFs usually result in LCVs that are "packed full" of *Lp* cells, which late in the infection cycle would differentiate into MIFs through a number of morphological intermediates, thereby closing the MIFs-RPFs intracellular growth cycle. One point to emphasize here is that according to Abdelhady and Garduño (2013) (see Section Extracellular vs. Intracellular *Lp*'s Developmental Cycles above), MIFs produced in different hosts could reach different developmental endpoints. This is not surprising because the *Lp* growth cycles followed in different host cells also show unique defining features. For instance, the late stage of *Lp*'s growth in murine macrophages is characterized by the fusion of lysosomes with, and the acidification of, the LCV (Sturgill-Koszycki and Swanson, 2000), whereas in human macrophages this does not happen (Wieland et al., 2004; Sauer et al., 2005b). Moreover, the intracellular environment must not be the same between different amoebal species, because these do not support growth of the same *Lp* serogroups (Rowbotham, 1980). An analysis of the transcriptome of *Lp* in a variety of host cells, in conjunction with a phenotypical characterization of the progeny produced (see Section Potential Molecular Markers for Detection of MIFs below), would significantly enhance our understanding of the impact that particular intracellular host environments have on the differentiation process of *Lp*.

Finally, differentiation of RPFs into MIFs must be a survival and (or) late growth requirement inside protozoa, but not inside mammalian cells. That is, *rpoS*, *letA*, and *ihfAB* mutants with defects in RPF-to-MIF differentiation (refer to Sections RpoS, Two-Component Regulatory Systems (LetA/S, PmrA/B, and LqsR/S), and Integration Host Factor (IHF) above) are able to grow well and release a partially differentiated progeny in mammalian cells, but not in amoeba (Hales and Shuman, 1999; Gal-Mor and Segal, 2003; Abu-Zant et al., 2006; Faulkner et al., 2008; Morash et al., 2009). These mutants are also completely digested in the food vacuoles of *Tetrahymena tropicalis* (Faulkner et al., 2008). Therefore, we propose that, in nature, the *Lp* forms most often found in the freshwater and moist soil environments must be fully differentiated MIFs produced as a result of *Lp*'s growth in protozoa. This would be in sharp contrast to what happens during replication in mammalian cells, where *Lp* is not under a strong selective pressure to differentiate, and would thus produce a mixture of MIFs with a variety of developmental maturities (Abdelhady and Garduño, 2013, **Figure 5**); a factor to consider in explaining (at least in part) the lack of person-to-person transmission of Legionnaires' disease.

Viable but not culturable cells (VBNCCs)

Being unable to sporulate, Gram-negative bacteria survive severe environmental stress by becoming dormant (reviewed by Oliver, 2010). This "standby mode" of survival, known as the viable but non-culturable (VBNC) state, is characterized by a physiological adjustment, perhaps similar to the stringent response, but with more profound consequences, i.e., loss of culturability. Key to the decision of including a discussion on VBNC *Lp* as a distinct developmental form, was to take a side on the controversy of whether the VBNC state results from differentiation or simply from cell injury (Nyström, 2003). That is, on the one hand, it has been argued that VBNC cells (VBNCCs) are no more than injured cells struggling to stay alive for as long as physiologically possible, thereby eliciting stress responses and repair mechanisms in a general manner. On the other hand, entry into the VBNC state is viewed as a purposeful physiological adaptation that requires a coordinated change in gene expression, regulated (at least in part) by the same factors that control stress responses and repair mechanisms. We subscribed to the latter, mainly because our own experimentation with VBNCCs derived from MIFs (that will be described further in this section), suggests that in the VBNC state *Lp* maintains a robust ultrastructure and physiology, which would be difficult to reconcile with the view of injured cells at the brink of death. Due to the important implications of VBNCCs in water quality control and detection of *Lp* in the context of public health, VBNC *Lp* has recently received increased attention. However, many gaps still exist in our understanding of *Lp* VBNCCs, in particular, the molecular mechanisms that orchestrate and control entry into, and exit from, the VBNC state.

The developmental links that we have identified for VBNCCs are shown in **Figure 6**. So far, we know that VBNCCs can be produced from EPFs (Ohno et al., 2003), SPFs (Ohno et al., 2003; Al-Bana et al., 2014) and MIFs (Al-Bana et al., 2014). The characteristics of these VBNCCs are defined by the developmental form that produces them and that is why we depict them as three different entities.

The triggers for VBNCC production (as previously noted for the production of FFs) are stress-related and numerous, but prolonged starvation in water is a natural condition (likely encountered by *Lp* on a regular basis) that consistently induces VBNCC formation in *Lp* (Steinert et al., 1997; Ohno et al., 2003; Al-Bana et al., 2014). Temperature increases and a reduction in the concentration of inorganic ions, results in significant shortening of the time required to enter the starvation-mediated VBNC state in water (Ohno et al., 2003; Al-Bana et al., 2014). Once formed, VBNCCs would persist in the water environment for extended

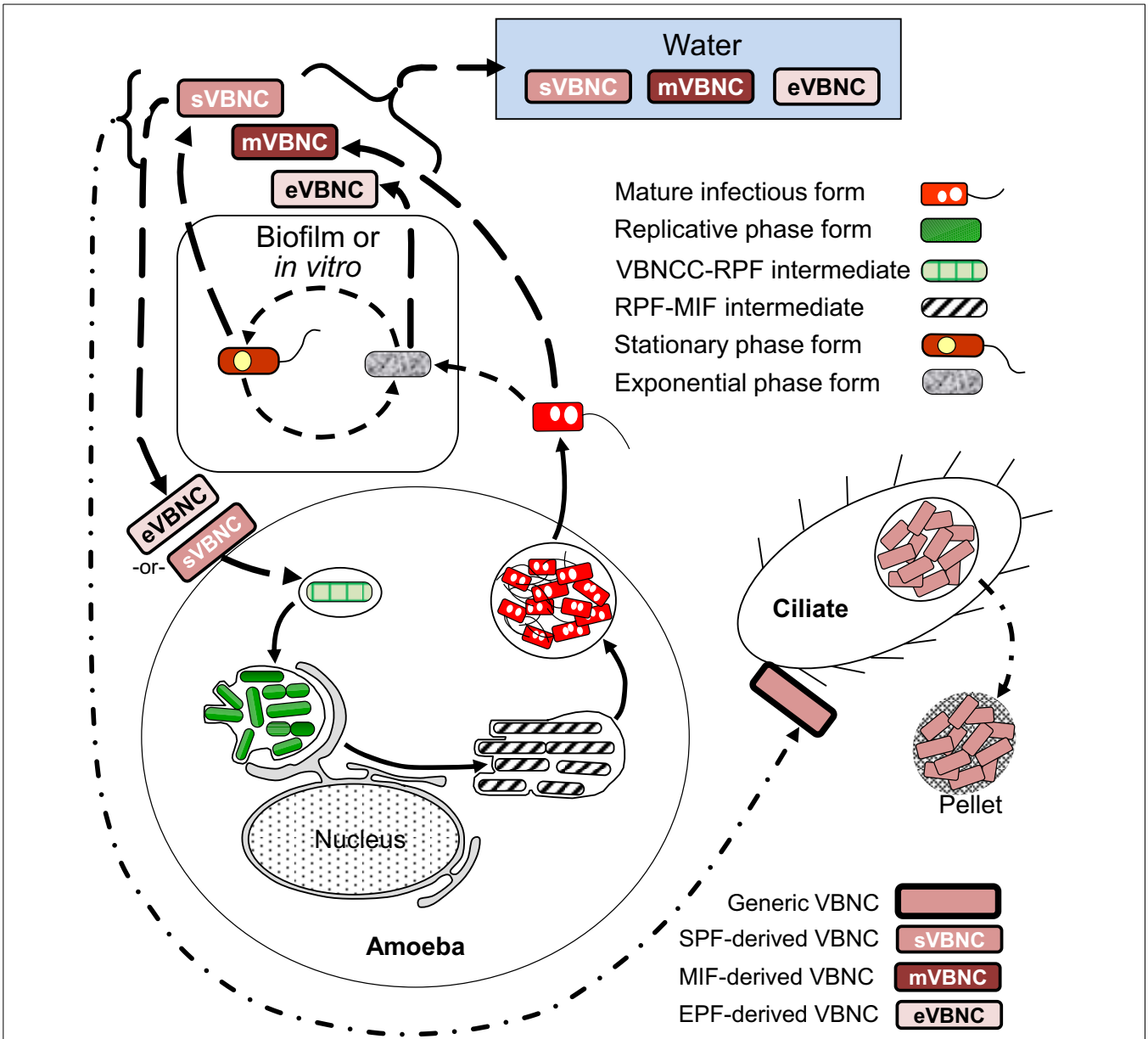

FIGURE 6 | Schematic representation of the developmental cycle of viable but non-culturable cells (VBNCCs) (long-dashed line arrows). Mature infectious forms (MIFs), stationary phase forms and exponential phase forms *in vitro* or in biofilms produce VBNCCs (mVBNC, sVBNC, and eVBNC, respectively) through either a natural process of attrition during cell senescence, or programmed differentiation. Internalization by amoebae resuscitates eVBNC and sVBNC cells, which then differentiate into replicative phase forms and produce a progeny of MIFs. Resuscitation of mVBNC cells has not been reported. mVBNC and sVBNC cells ingested by *Tetrahymena* ciliates survive in food vacuoles and produce pellets, but do not resuscitate in this host. Dashed line arrows, solid line arrows, and dash-dot patterned arrows are used to depict steps of the SPF–EPF developmental cycle (**Figure 3**), the MIF–RPF intracellular developmental cycle (**Figure 5**) and the ciliate-pellets developmental cycle (**Figure 7**), respectively.

periods, until they receive a signal to "wake up" from dormancy, a process known as VBNCC resuscitation.

In our work with VBNCCs derived from SPFs (SPF–VBNCCs) or MIFs (MIF–VBNCCs) in sterile tap water at 45°C (Al-Bana et al., 2014), we observed that cytoplasmic inclusions and a portion of the cytoplasmic material are consumed during the starvation period, so that VBNCCs become thin. Under the transmission electron microscope, the cytoplasm of SPF–VBNCCs shows numerous zones with low electron density and one or two electron-dense spots, and the outer membrane shows a wavy contour with small projections, suggesting that SPF–VBNCCs produce outer membrane vesicles. In contrast, MIF–VBNCCs maintain an electron-dense cytoplasm and an apparently intact envelope that shows the typical traits originally described for MIFs from HeLa cells. The proportion of starved MIFs that enter the VBNC state in water at 45°C is between 70–90%, with no

significant drop in viability after 1 month. It is the combination of this high viability and ultrastructural preservation that made us conclude that formation of MIF–VBNCCs is a purposeful differentiation that *Lp* uses to cope with environmental stress. It only remains to demonstrate that MIF–VBNCCs can resuscitate with high efficiency into RPFs to close the MIF–VBNCC branch of the cycle. Thus, it would be interesting to test on MIF–VBNCCs the newly reported resuscitation method that incorporates the addition of organic scavengers of oxygen radicals (Ducret et al., 2014).

VBNCCs derived from SPFs or EPFs resuscitate in the presence of amoeba (Ohno et al., 2003; Al-Bana et al., 2014). Upon resuscitation in amoeba, it is not clear whether the differentiation of VBNCCs into RPFs includes the formation of intermediate FFs, but starved *Lp*, just before becoming unculturable, profusely produce filaments when placed on nutrient-rich BCYE plates and convert into EPFs (Al-Bana et al., 2014).

One final point in relation to VBNCCs is the controversial discussion of whether VBNC *Lp* is capable of causing Legionnaires' disease in humans. Although the potential for disease transmission exists, infection of mammalian cells by VBNCCs *in vitro* has not been experimentally demonstrated. Regardless, the potential resuscitation of VBNCCs by amoeba is sufficient to implicate VBNCCs as relevant to human health, as this would (i) allow for a repopulation of water systems by *Lp* MIFs following disinfection attempts, and (ii) inhalation of amoeba carrying VBNCCs, could potentially initiate an infection (Brieland et al., 1997).

The cycle of packaged Lp forms

The last cycle to discuss here is the one in which ciliates of the genus *Tetrahymena*, as well as amoeba, participate by ingesting *Lp* cells into food vacuoles, and later expelling the content of such vacuoles in the form of "packaged" fecal pellets containing live *Lp* cells (Denoncourt et al., 2014). The pelleted live *Lp* cells would act as complex infectious particles that can initiate infections after being phagocytosed whole by either amoeba or macrophages, thus closing the cycle to commence a new one as RPFs (**Figure 7**). We decided to include this cycle among the developmental *Lp* network for two main reasons. First, the *Tetrahymena* food vacuoles promote the direct differentiation of SPFs into MIFs in the absence of intracellular replication (Faulkner et al., 2008). Second, packaged MIFs could be an effective way (in addition to biofilms) for *Lp* to persist in the water environment, or even in non-aqueous niches where resistance to desiccation would be afforded by the pellet configuration, and from where new infections could be initiated (in amoeba, or accidentally in humans) (Denoncourt et al., 2014). In this respect, packaged MIFs could be "the" infectious particle hypothesized by Rowbotham (1980, 1986) to transmit Legionnaires' disease, spreading *Lp* from the environment to the human lung in one large installment.

VBNCCs are also pelleted by *Tetrahymena tropicalis* and *Tetrahymena thermophila* (Al-Bana et al., 2014). The pelleted VBNCCs were shown to (i) remain viable by means of a vital fluorescent stain, (ii) preserve their envelope integrity, (iii) become Gimenez-positive (i.e., acquire a bright red color after the Gimenez stain) and (iv) not become structurally degraded (i.e., digested by the ciliates). Resuscitation in *Acanthamoeba* was possible for pelleted SPF–VBNCCs, but not for MIF–VBNCCs, indicating that pelleted SPF–VBNCCs remain infectious.

PRACTICAL IMPLICATIONS

SEARCHING FOR DIFFERENTIATION MARKERS APPLICABLE TO THE DETECTION OF *Lp* FORMS

When we pay attention to what is in all the "Water" boxes of the five developmental cycles presented above (**Figures 3–7**) it becomes apparent that not all the legionellae found in the water environment are the same. Yet, when *Lp* is detected in water samples, it is generally assumed that it exists in a single form; an exception being the awareness that has existed about VBNCCs (Hussong et al., 1987; Hwang et al., 2006; Slimani et al., 2012). But even if one would recognize and accept the existence of the many *Lp* forms, the question is how could these forms be detected and distinguished from each other? Currently available methods are not geared to distinguish between *Lp* forms, except—again—for the practical efforts made to distinguish and quantify VBNCCs using flow (Keserue et al., 2012) or solid-phase (Parthuisot et al., 2011) cytometry. Thus, when *Lp* is detected and quantified in water samples, one cannot tell which *Lp* forms are present, nor their proportions. Because the ecology of each of these forms (as suggested by their developmental cycles) seems to be different (as is their potential to transmit disease), it would be highly desirable to have some useful tools in our analytical toolbox, which could allow us to detect the many *Lp* forms present in water. What follows in the last part of this review, is an account of possible markers (morphological and molecular) that could be used to identify some of the *Lp* forms. It should be noted that *Lp* forms have to be detected in the absence of any culturing step, not to change the developmental stage of the forms present.

Potential molecular markers for detection of MIFs

The MIF-associated protein MagA was first discovered as a 24-kDa protein induced during macrophage infection, which was annotated as "Mip-like protein" (Miyamoto et al., 1993). We re-encountered the protein as one consistently induced to high levels in *Lp* cells placed in water, and later found that its expression was linked to the differentiation of *Lp* into transmissive forms (SPFs and MIFs) (Hiltz et al., 2004), with very high levels produced in MIFs (Garduño et al., 2002; Hiltz et al., 2004). We renamed the protein MagA, to avoid confusion with the macrophage infection potentiator protein Mip (Bachman and Swanson, 2004; Hiltz et al., 2004). However, it turns out that MagA is not a good marker for MIF identification, mainly because its encoding gene is exclusively carried in the Philadelphia *Lp* lineage, as part of a genomic island (Brassinga et al., 2003) recently identified as a mobile integrative conjugative element that confers fitness advantages (Flynn and Swanson, 2014). In addition, MagA is a cytoplasmic protein that would be difficult to target with antibodies for fast detection without culture.

When comparing the 2-D protein profiles of SPFs and MIFs (derived from the Philadelphia-1 strain SVir), we identified 17 MIF-specific protein spots (Garduño et al., 2002). MagA was not picked in this comparison because it is also expressed by SPFs (albeit at much lower levels). When identified by mass

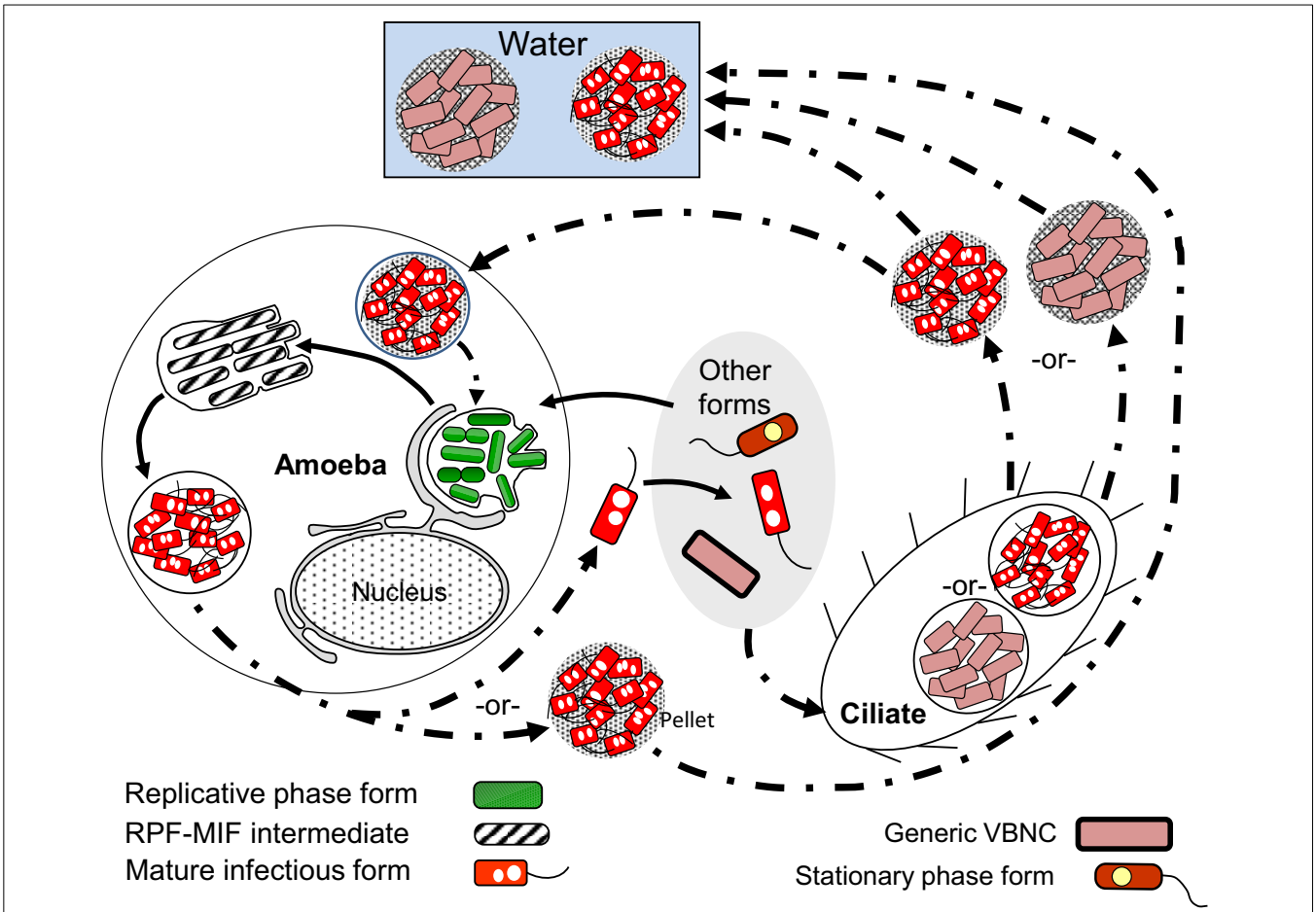

FIGURE 7 | Schematic representation of the ciliate-pellets cycle (dash-dot pattern line arrows) following packaging of *Lp* into pellets by ciliates and amoeba. *Lp* cells inside pellets may be packaged in wraps of degraded *Lp* membranous material and (or) in some protozoa-produced matrix and multilamellar bodies, or in a combination of both. Pellets represent the contents of food vacuoles that have been emptied into the surrounding environment. The reader is reminded that stationary phase forms (SPFs) ingested by *Tetrahymena* ciliates rapidly differentiate into mature infectious forms inside food vacuoles, so that pellets of SPFs are not produced. Pellets should not be confused with free vesicles containing *Lp* progeny still inside a membrane-bound vacuole (e.g., **Figure 5**), released from lysed host cells (protozoan or mammalian). Presence of pellets in water might increase the infectious challenge encountered by vulnerable individuals. Solid line arrows represent steps of the RPF–MIF developmental cycle (**Figure 5**).

spectrometry, some of the spots showed identity to predicted hypothetical proteins (encoded by *lpg0563, 2526, 2755*), and some others included the small heat shock protein HspC2, Mip, 50S ribosomal protein L9, and the 27-kDa outer membrane protein, as well as a lipase, glycyl-tRNA synthetase, and glutaryl-CoA dehydrogenase. However, no further work has been completed to confirm whether these proteins are indeed exclusively expressed in MIFs.

Additional potential markers useful in detecting MIFs could emerge from transcriptomic data obtained 30–60 min after SPFs have been ingested by *Tetrahymena tropicalis*, since this is the time at which SPFs are undergoing a direct differentiation into MIFs, inside the ciliate's food vacuoles (Faulkner et al., 2008). In collaboration with C. Buchrieser (Institut Pasteur, Paris) we have completed this work, but the complete microarray data obtained will be published elsewhere. It was interesting, nonetheless, to find that during the transition into MIFs inside the ciliates, SPFs induced the expression of genes encoding enzymes involved in carbohydrate metabolism. One of these genes (*lpg1669*), encoding a putative amylase, was also found to be grossly transcribed (>150-fold) in amoeba when the microarray data was confirmed using quantitative, reverse-transcriptase PCR. Of great interest is the observation that Lpg1669 was not induced in macrophages, suggesting that in searching for MIF-specific differentiation markers, it might be productive (besides looking at differences between SP and MIF) to look at differences between MIFs produced in different hosts. By comparing the published microarray data for the transcriptome of *Lp* grown in amoeba (Brüggemann et al., 2006) against that of *Lp* grown in macrophages (Faucher et al., 2011) we have identified a list of differentially expressed genes. It should be noted that the transcriptome studies mentioned above use different time points as reference (0 vs. 8 h post-infection as the undifferentiated control). In spite of this limitation, we believe that the analysis is valuable, mainly because the data still highlight major gene expression differences in MIFs obtained in different hosts. Therefore, we focused on a short

Table 2 | Short list of *Lp* genes whose transcription is selectively upregulated in amoebae (MIF transcript level/RPF transcript level >2), but down-regulated or unchanged in human macrophages (MIF transcript level/SPF transcript level <2).

Gene ID	Description	*A. castellanii*[a]	Human macrophages[b]
		T_{14}/T_8	T_{18}/T_0
lpg0910	Enhanced entry protein A	20.25	0.99
lpg0818	ATP-dependent Clp A protease	4.66	0.81
lpg0891	Sensory box protein/GGDEF/EAL domains	10.93	0.83
lpg1356	Enhanced entry protein C	11.63	0.77
lpg1491	Lem9 (Dot/Icm effector)	15.78	1.78
lpg0670	Hypothetical protein	8.94	0.66
lpg1669	Putative α-amylase	17.88	0.87
lpg2228	3-oxoacyl ACP synthase III	7.62	1.55
lpg2316	3-hydroxybutyrate dehydrogenase	8.82	0.74
lpg1540	Universal stress protein A	4.66	1.94
lpg2348	Superoxide dismutase SodC	6.96	0.84
lpg2955	Integration host factor HipB	8.94	0.79
lpg2971	Malate dehydrogenase	12.13	0.60
lpg1639	Hypothetical protein	13.74	1.56
lpg0279	Hypothetical protein	9.45	0.67
lpg2495	Homospermidine synthase	7.26	1.06
lpg1887	Hypothetical protein	11.00	0.91

[a] *Data were obtained from Brüggemann et al. (2006), where T_{14} = 14 h after infection, post-replicative phase (MIFs), and T_8 = 8 h after infection, replicative phase (RPFs).*

[b] *Data were obtained from Faucher et al. (2011), where T_{18} = 18 h after infection, post-replicative phase (MIFs), and T_0 = infection at zero time with SPFs grown in vitro.*

list of genes that are: (i) induced more than 2-fold in amoeba, and (ii) either repressed or unchanged in macrophages (**Table 2**). Reasoning that the MIFs present in the water environment would have emerged from protozoa and not from macrophages, some of the highly induced genes showed in **Table 2** could be useful markers for MIF detection in water samples by reverse transcription PCR, or by immunoaffinity reagents to their gene products.

Detection of VBNCCs

As mentioned in Section Searching for Differentiation Markers Applicable to the Detection of *Lp* Forms above, there are a number of published methods that have potential applications in the detection of *Lp* VBNCCs. These methods exploit the fact that VBNCCs must be positively stained with vital stains while not be able to grow on BCYE agar. However, recent interest has been raised in examining the proteome of VBNCCs (Alleron et al., 2013, Antje Flieger, Robert Koch Institute—personal communication; R. Garduño—unpublished results). Using 2-D gels of ^{35}S-labeled proteins, Alleron et al. (2013) identified nine spots that were present in VBNCCs, but not in SPFs, which included some potential virulence-related proteins. Interestingly, among these nine VBNCC proteins, Mip, the 27-kDa outer membrane protein, and the 50S ribosomal protein L9, were three proteins also found in MIFs as part of our proteomic study (see Section Potential Molecular Markers for Detection of MIFs above), indicating that these particular proteins are not VBNCC-specific. Confirmation of whether the other identified proteins are VBNCC-specific would be useful in potentially improving VBNCC detection, by incorporating the labeling of VBNCC-specific proteins in current cytometry-based methods (Parthuisot et al., 2011; Keserue et al., 2012).

Morphological markers

The most obvious application of morphological markers would be in the detection of FFs and pellets of VBNCCs and MIFs, which would be easily distinguished by microscopy or cytometry. We have used an OmpS-specific antibody (Butler and Hoffman, 1990) and a secondary fluorescent antibody to immuno-label water samples concentrated by filtration through a 0.45 μm-pore membrane. This method easily renders *Lp* visible by fluorescence microscopy, under which FFs and *Lp* pellets are readily spotted.

CONCLUSION

Lp differentiates into 14 developmental forms reported to date (and likely new ones will be described) following a complex developmental network that has been defined and described in this review. Therefore, we urge readers to abandon the common depiction of *Lp*'s differentiation as a biphasic developmental process that alternates between replicative and transmissive forms, mainly because this view is an oversimplification of the actual process.

It is our prediction that in the near future, novel developments will make possible the detection of key *Lp* forms found in water. New knowledge both on the proportion in which these forms appear in different water environments (e.g., cooling towers vs. potable water systems), and on their relative infectivity to cells in culture (or ideally, infectivity to animal models via aerosolization) could help immensely in the proper assessment of risk and the effective control of Legionnaires' disease outbreaks.

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Potential role of bacteria packaging by protozoa in the persistence and transmission of pathogenic bacteria

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Many pathogenic bacteria live in close association with protozoa. These unicellular eukaryotic microorganisms are ubiquitous in various environments. A number of protozoa such as amoebae and ciliates ingest pathogenic bacteria, package them usually in membrane structures, and then release them into the environment. Packaged bacteria are more resistant to various stresses and are more apt to survive than free bacteria. New evidence indicates that protozoa and not bacteria control the packaging process. It is possible that packaging is more common than suspected and may play a major role in the persistence and transmission of pathogenic bacteria. To confirm the role of packaging in the propagation of infections, it is vital that the molecular mechanisms governing the packaging of bacteria by protozoa be identified as well as elements related to the ecology of this process in order to determine whether packaging acts as a Trojan Horse.

Keywords: protozoa, multilamellar body, amoeba, bacteria packaging, *Legionella pneumophila*, mycobacteria, persistence, transmission

INTRODUCTION

The risk of the resurgence of bacterial infections caused by the decreasing effectiveness of antibiotics and the increasing number of people with weakened immune systems (cancer, AIDS, aging, etc.) require our attention (Croft et al., 2007). In this context, it is crucial that we improve our understanding of the behavior of pathogenic bacteria in various environments and their transmission in order to develop effective countermeasures. Since many protozoa interact with pathogenic bacteria in diverse environments, understanding the behavior of pathogens includes elucidating their relationships with protozoa.

Protozoa are unicellular eukaryotes that are ubiquitous in virtually all environments. Many are grazers that feed by ingesting other microorganisms, especially bacteria. Protozoa have been interacting with bacteria for a very long time, and several species have become hosts of pathogenic bacteria in natural environments and in man-made structures (air conditioning units, cooling towers, etc.). Studying these interactions is particularly important given that protozoa, for example amoebae, can serve as natural reservoirs for bacteria such as *Legionella pneumophila* and *Mycobacterium* spp. (Greub and Raoult, 2004). This represents a health risk since these bacteria can be dispersed into the air when aerosols are produced and can cause severe, even lethal, pneumonia when inhaled (Abu Kwaik et al., 1998; Falkinham, 2003; Philippe et al., 2006). *L. pneumophila* can be propagated over long distances (several kilometers) while remaining infectious (Nguyen et al., 2006; Nygard et al., 2008). In addition to *L. pneumophila* and *Mycobacterium* spp., a large number of bacterial species can withstand predation by protozoa and can persist and/or grow in them. A summary of the outcomes reported in the literature for pathogenic bacteria that interact with various protozoa is presented in **Table 1**. The interactions of pathogenic bacteria with protozoa can be advantageous if they can resist predation and digestion by the protozoa. For example, *L. pneumophila* has developed a clever strategy to protect itself from the enzymatic degradation that normally occurs in the endocytic pathway of the host by inducing the formation of replication vacuoles inside protozoa (Richards et al., 2013). By being able to grow and survive inside protozoa, these resistant bacteria are protected from stresses like biocides and antibiotics.

Some investigators have suggested that protozoa may act as a Trojan Horse in the propagation of human pathogenic bacteria (Barker and Brown, 1994; Greub and Raoult, 2004). For example, soon after *L. pneumophila* was discovered, it was suggested that bacteria residing inside amoebae, rather than free bacteria, were the source of legionellosis (Rowbotham, 1980). *Legionella* and mycobacteria associated with *Acanthamoeba* have a greater capability to replicate in macrophages than free bacteria (Cianciotto and Fields, 1992; Cirillo et al., 1994, 1997, 1999; Moffat et al., 1994; Neumeister et al., 2000). *L. pneumophila*, *Mycobacterium* spp., and other amoeba-resisting bacteria may also be able to reside within amoebal cysts (Steinert et al., 1998; Marciano-Cabral and Cabral, 2003; Adekambi et al., 2006; El-Etr et al., 2009; Ben Salah and Drancourt, 2010). Most protozoa can make cysts, often with thick protective walls, which are their resting form, that provide protection from adverse environmental conditions (Greub and Raoult, 2004) and, at the same time, that provide involuntary protection to bacteria inside the cysts. *Mycobacterium*

Table 1 | Fate of bacteria following interactions with protozoa.

Protozoa	Fate of bacteria	References
Acanthamoeba astronyxis	Intracellular survival[a]	Inglis et al., 2000; Marciano-Cabral and Cabral, 2003
	Intracellular multiplication	Marciano-Cabral and Cabral, 2003
	Packaged in expelled vesicles	Marciano-Cabral and Cabral, 2003
	Survival in cysts	Marciano-Cabral and Cabral, 2003
Acanthamoeba castellanii	Intracellular multiplication	Ly and Muller, 1990b; Cirillo et al., 1997; Essig et al., 1997; Winiecka-Krusnell et al., 2002; Abd et al., 2003; Casson et al., 2008; El-Etr et al., 2009; Saeed et al., 2009; Abd et al., 2010; Verhoeven et al., 2010; Lienard et al., 2011
	Intracellular survival[a]	King et al., 1988; Essig et al., 1997; Inglis et al., 2000; La Scola and Raoult, 2001; Winiecka-Krusnell et al., 2002; Abd et al., 2003, 2010; Thomas et al., 2006; Casson et al., 2008; El-Etr et al., 2009; Saeed et al., 2009
	Survival in cysts	Abd et al., 2003, 2010; El-Etr et al., 2009; Saeed et al., 2009; Cateau et al., 2011
	Long-term survival in vegetative forms or dead cells	Abd et al., 2003; Bouyer et al., 2007; El-Etr et al., 2009; Cateau et al., 2011
	Protection from chlorine	King et al., 1988
	Bacteria spore-like formation	La Scola and Raoult, 2001
	Biofilm formation	Verhoeven et al., 2010
	Protection from gentamicin	Bouyer et al., 2007; Saeed et al., 2009; Abd et al., 2010
	Packaged in expelled vesicles	Rowbotham, 1980; Berk et al., 1998
	Protection from biocides	Berk et al., 1998
	Protection from freezing and thawing	Berk et al., 1998
	Enhanced virulence	Cirillo et al., 1997
Acanthamoeba comandoni	Intracellular multiplication	Lienard et al., 2011
Acanthamoeba culbertsoni	Long-term survival in vegetative form	Cateau et al., 2011
	Survival in cysts	Cateau et al., 2011
Acanthamoeba polyphaga	Intracellular multiplication	Kilvington and Price, 1990; Barker et al., 1999; La Scola and Raoult, 1999; Birtles et al., 2000; Ingham et al., 2000; Kahane et al., 2001; La Scola et al., 2004; Evstigneeva et al., 2009; Ben Salah and Drancourt, 2010; Lamrabet et al., 2012
	Intracellular survival[a]	Steinert et al., 1998; Barker et al., 1999; Horn et al., 1999; Ingham et al., 2000; Inglis et al., 2000; La Scola et al., 2000, 2001, 2002, 2003, 2004; Kahane et al., 2001; Adekambi et al., 2006; Medie et al., 2011; Lamrabet et al., 2012; Lamrabet and Drancourt, 2013
	Survival in cysts	Kilvington and Price, 1990; Steinert et al., 1998; Horn et al., 1999; La Scola and Raoult, 1999; Kahane et al., 2001; Adekambi et al., 2006; Ben Salah and Drancourt, 2010; Medie et al., 2011
	Packaged in expelled vesicles	Berk et al., 1998
	Protection from chlorine	Kilvington and Price, 1990; La Scola and Raoult, 1999; Adekambi et al., 2006
	Protection from biocides	Berk et al., 1998
	Protection from freezing and thawing	Berk et al., 1998
	Protection from streptomycin and glutaraldehyde	Medie et al., 2011
Acanthamoeba spp.	Intracellular multiplication	Michel and Hauroder, 1997; Tomov et al., 1999
	Intracellular survival[a]	Drozanski, 1956; Ly and Muller, 1990a; Drozanski, 1991; Amann et al., 1997; Fritsche et al., 1999; Marolda et al., 1999; Tomov et al., 1999; Horn et al., 2001, 2002
	Survival in cysts	Horn et al., 2001, 2002

(Continued)

Table 1 | Continued

Protozoa	Fate of bacteria	References
Colpoda spp.	Packaged in expelled vesicles	Raghu Nadhanan and Thomas, 2014
	Protection from gentamicin and chlorine	Raghu Nadhanan and Thomas, 2014
Dictyostelium discoideum	Intracellular multiplication	Hagele et al., 2000; Solomon and Isberg, 2000; Skriwan et al., 2002; Solomon et al., 2003; Hagedorn and Soldati, 2007; Hagedorn et al., 2009; Lienard et al., 2011
Glaucoma spp.	Intracellular multiplication	Gourabathini et al., 2008
	Packaged in expelled vesicles	Gourabathini et al., 2008
Hartmannella vermiformis	Intracellular multiplication	Lienard et al., 2011
	Intracellular survival[a]	Horn et al., 2000
Naegleria gruberi	Intracellular multiplication	Thom et al., 1992
	Survival in cysts	Thom et al., 1992
Naegleria lovaniensis	Intracellular multiplication	Casson et al., 2008
	Intracellular survival[a]	Casson et al., 2008
Saccamoeba spp.	Intracellular multiplication	Michel et al., 1995
Tetrahymena pyriformis	Intracellular multiplication	King and Shotts, 1988; Ly and Muller, 1990a,b; Gourabathini et al., 2008
	Intracellular survival[a]	King et al., 1988; Ly and Muller, 1990a
	Protection from chlorine	King and Shotts, 1988; King et al., 1988
	Packaged in expelled vesicles	Gourabathini et al., 2008
Tetrahymena tropicalis	Packaged in expelled vesicles	Berk et al., 2008; Koubar et al., 2011
	Long-term survival in vegetative form	Koubar et al., 2011
	Protection from gentamicin	Koubar et al., 2011
	Enhanced virulence	Koubar et al., 2011
Tetrahymena sp.	Intracellular survival	Smith et al., 2012
	Packaged in expelled vesicles	Brandl et al., 2005; Smith et al., 2012
	Long-term survival in vegetative form	Brandl et al., 2005
	Protection from low concentrations of calcium hypochlorite	Brandl et al., 2005

Positive outcomes for bacteria resulting from the interactions with protozoa are listed.

[a] *In vacuoles or the cytoplasm.*

spp. that reside inside cysts can resist 15 mg/L of free chlorine for 24 h (Adekambi et al., 2006). Protozoal cysts may thus be vectors for some bacteria (Ben Salah and Drancourt, 2010).

BACTERIA PACKAGING BY PROTOZOA: A BACTERIAL CAMOUFLAGE

A broad range of phagotrophic protists such as dinoflagellates, ciliates, and amoebae produce and expel vesicles as a part of their normal digestive process (Gezelius, 1959; Hohl, 1965; Allen and Wolf, 1974, 1979; Buck et al., 1990, 2005; Buck and Newton, 1995; Chekabab et al., 2012; Paquet et al., 2013). These expelled vesicles, which are often called fecal pellets or fecal balls, vary in composition, size, and morphology, depending on the protist and the trophic conditions. These pellets, which contain, among things, undigested particulates, and organic nutrients, may play significant roles in the flux of materials in the ecosphere (Buck et al., 1990).

In addition, the long co-evolution of protists and bacterial preys has given rise to survival strategies by the bacteria that enable them to avoid digestion in the normal phagocytic process and to be packaged in the egested pellets. *Acanthamoeba* and *Tetrahymena* protozoa parasitized by *L. pneumophila* expel vesicles or fecal pellets that contain viable bacteria (**Figure 1**) (Rowbotham, 1980; Berk et al., 1998, 2008; Koubar et al., 2011). While bacteria packaging was first observed with *L. pneumophila*, it might be a general phenomenon since a variety of protozoa that expel various species of bacteria packaged in vesicles have been reported (**Table 2**). Amoebae and ciliates appear to be the only two groups of protozoa known to produce extracellular vesicles containing bacteria so far (**Table 2**). The vesicles are produced in the endocytic pathway either on a regular basis or under certain specific conditions (see below). Bacteria that resist lysosomal digestion can be packaged in these structures and can then be expelled outside the cell by exocytosis or, in some cases, following

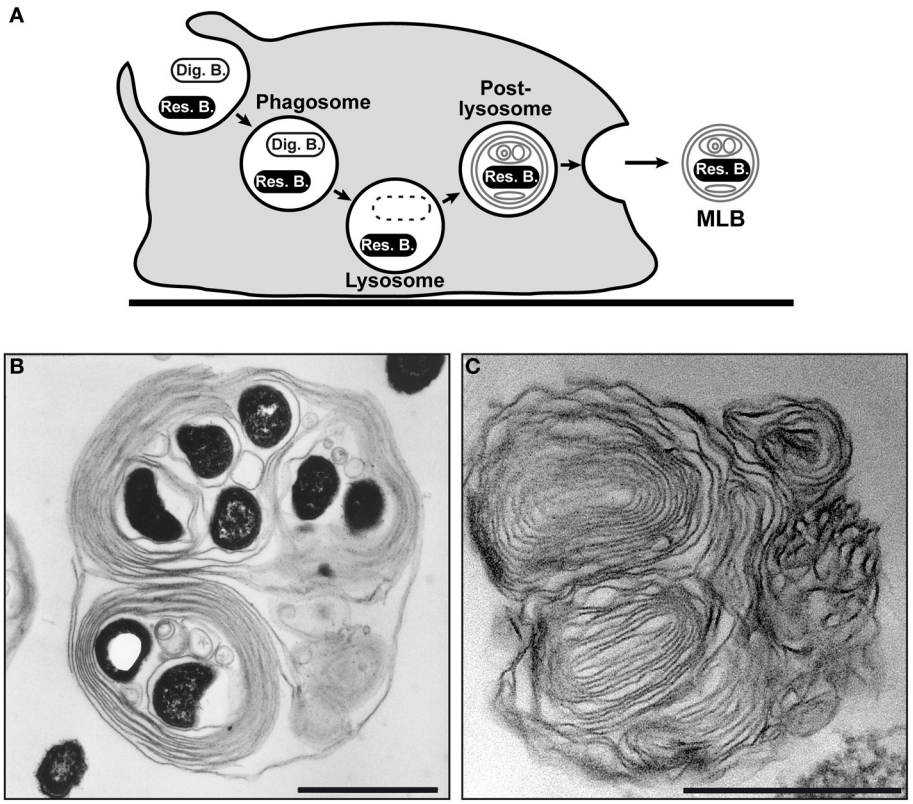

FIGURE 1 | Bacteria packaging by amoebae. (A) Schematic diagram of the packaging process that allows packaged bacteria to resist lysosomal degradation (Res. B), unlike digestible bacteria (Dig. B). The resisting bacteria are packaged in multilamellar bodies (MLB) and are then secreted by the amoebae. **(B)** Transmission electron microscopic image of *L. pneumophila* bacteria (black ovoid forms) packaged in a MLB produced and secreted by *A. castellanii*. Image reproduced from Berk et al. (1998) with the permission of the American Society for Microbiology. **(C)** Transmission electron microscopic image of a MLB devoid of bacteria produced and secreted by *D. discoideum* DH1-10 (Cornillon et al., 2000) grown on digestible bacteria, which were a laboratory strain of *K. aerogenes* (Benghezal et al., 2006). Scale bar = 1 μm in **(B,C)**.

bacteria-dependent cell lysis (**Figure 1**) (Abd et al., 2003; Brandl et al., 2005; Gourabathini et al., 2008).

Packaged bacteria are protected against harsh conditions such as freezing and thawing, chlorine, and the biocides used in cooling towers (Berk et al., 1998; Brandl et al., 2005). *Tetrahymena tropicalis*-packaged *L. pneumophila* display greater gentamicin resistance and long-term survival in nutrient-poor environments, and are more infectious than free bacteria in cultured human pneumocyte cells (Koubar et al., 2011). More recently, it has been reported that viable *Listeria monocytogenes* can be enclosed in fecal pellets produced by *Colpoda* spp. (a ciliate) and that this results in gentamicin and sodium hypochlorite resistance of the bacteria (Raghu Nadhanan and Thomas, 2014). Packaging may thus be a way for the bacteria to persist in the environment. In fact, bacteria packaged by protozoa are more likely to propagate bacterial infections than bacteria-containing cysts. Packaged bacteria are probably the most frequent form of pathogenic bacteria associated with protozoa. Exocytosis is a continuous active process for grazing protozoa and hundreds of bacteria-containing vesicles can be expelled by a single protozoal cell (Berk et al., 1998; Brandl et al., 2005; Gourabathini et al., 2008). However, protozoal cells can form only a single bacteria-containing cyst and while bacteria have been observed in protozoal cysts, they are not always viable in these structures (Gourabathini et al., 2008).

Packages containing bacteria range in size from 2 to 6 μm in diameter (Berk et al., 1998, 2008) and are smaller than vegetative forms of protozoa and even cysts, which can reach diameters of 10–20 μm (Nilsson, 2005). Given that respirable particles (i.e., those able to penetrate into the alveoli) must be under 3.5 μm in diameter (Macher, 1999), packaged bacteria can thus penetrate deeper into the respiratory tract and the alveoli. Respiratory pathogens such as *L. pneumophila* that can be packaged are thus more likely to cause respiratory infections in this form than when included in vegetative forms of protozoa or cysts (Berk et al., 1998). It has been proposed that bacteria that develop inside amoebae may be responsible for some of the 50% of lower respiratory tract infections with unexplained etiologies (Lamoth and Greub, 2009). While the packaging process needs to be studied in greater detail, it cannot be excluded that packaged bacteria may cause some of these lower respiratory tract infections.

BACTERIA PACKAGING: A PROTOZOA-DRIVEN PROCESS

The packages produced by amoebae (**Figure 1B**) and, to a lesser extent by ciliates (see below), are multilamellar bodies (MLBs)

Table 2 | List of bacteria-protozoa combinations where bacteria packaging has been observed.

Bacterium	Protozoa	Output	References
Escherichia coli O157	*Glaucoma* spp. *Tetrahymena pyriformis*	Packaged in expelled vesicles, multiplication, and escape from the vesicles	Gourabathini et al., 2008
	Tetrahymena sp.	Intracellular survival, packaged in expelled vesicles	Smith et al., 2012
Helicobacter pylori	*Acanthamoeba astronyxis*	Packaged in expelled vesicles, intracellular multiplication	Marciano-Cabral and Cabral, 2003
Legionella pneumophila	*Acanthamoeba castellanii* *Acanthamoeba polyphaga*	Packaged in expelled vesicles, resistance to biocides and freezing and thawing	Berk et al., 1998
	Tetrahymena tropicalis	Packaged in expelled vesicles Packaged in expelled vesicles, long-term survival, gentamicin resistance, increased infectivity	Berk et al., 2008 Koubar et al., 2011
Listeria monocytogenes	*Glaucoma* spp.	Packaged in expelled vesicles	Gourabathini et al., 2008
	Colpoda spp.	Packaged in expelled vesicles, resistance to sodium hypochlorite, gentamicin resistance	Raghu Nadhanan and Thomas, 2014
Salmonella enterica	*Glaucoma* sp. *Tetrahymena pyriformis*	Packaged in expelled vesicles	Gourabathini et al., 2008
	Tetrahymena sp.	Packaged in expelled vesicles, enhanced survival, resistance to low concentrations of calcium hypochlorite	Brandl et al., 2005

formed of several concentric layers of lipid membranes containing viable bacteria. MLBs devoid of viable bacteria are also produced (**Figure 1C**), for example by *Acanthamoeba castellanii* (Chekabab et al., 2012), and have been extensively studied using *Dictyostelium discoideum* amoeba (Gezelius, 1959; Mercer and Shaffer, 1960; Gezelius, 1961; Hohl, 1965; Barondes et al., 1985; Cooper et al., 1986; Fukuzawa and Ochiai, 1993; Emslie et al., 1998; Marchetti et al., 2004; Paquet et al., 2013).

To date, the production of MLBs by *D. discoideum* has only been studied in the presence of digestible bacteria (i.e., bacteria that are degraded by the lysosomal enzymes of the endocytic pathway) and not in the presence of undigestible pathogenic bacteria. Interestingly, since *D. discoideum* cells grown in liquid medium in the absence of bacteria produce virtually no MLB (Mercer and Shaffer, 1960; Hohl, 1965; Marchetti et al., 2004; Paquet et al., 2013), it was considered that these MLBs were undigested bacterial remains. However, new evidence has shown that MLB production by *D. discoideum* depends largely on the metabolism of the protozoa even if the presence of digestible bacteria is required to produce MLBs. The analysis of the composition of purified *D. discoideum* MLBs revealed that the lipids in these structures are amoebal in origin, that is, they are mainly produced via amoebal metabolic pathways, even if they are only produced when the amoebae are fed *Klebsiella aerogenes*, a digestible bacterium (Paquet et al., 2013). Even if bacterial remains (glycoconjugates) have also been detected in *D. discoideum* MLBs (Cooper et al., 1986), the lipid composition of these structures suggests that they are not strictly fecal pellets used to dispose of undigested bacterial constituents and that they may play significant roles in amoebal physiology.

The fact that the lipids in the MLBs are amoebal in origin is an argument in favor of the idea that bacteria packaging is under the control of protozoa. However, when *D. discoideum* cells are fed digestible Gram-positive bacteria (**Figure 2**) compared to digestible Gram-negative bacteria (**Figure 1**), the type of food, that is, the type of bacteria ingested, can affect the morphology of the MLBs. Those produced and secreted by *D. discoideum* cells grown on *Bacillus subtilis* and *Micrococcus luteus* (Gram-positive bacteria) are quite different than those from amoebae grown on *K. aerogenes* (Gram-negative bacterium). In the presence of *B. subtilis*, the MLBs have a more heterogeneous structure (**Figures 2A,B**) that can be composed of concentric membrane lamellae, clusters of vesicles in one structure, or a mix of the two. MLBs produced and secreted by cells grown on *M. luteus* contain fewer lamellae and often have a broken appearance (**Figure 2C**). Without being the main actor in bacteria packaging, internalized bacteria may produce factors that influence the process. For example, *L. pneumophila* LepA and LepB proteins appear to be essential for the specific non-lytic release of the bacteria from amoebae (Chen et al., 2004). Do these proteins act as a kind of regulator of the packaging process or only of the exocytosis function? Additional studies are required to evaluate the contribution of bacterial factors to the packaging process.

Less is known about the nature of the packages produced by ciliates than those produced by amoebae. The composition and structure of the packages or pellets produced by ciliates such as *Tetrahymena* spp. differ from those of amoebal MLBs. Packaged bacteria secreted by *Tetrahymena* spp. appear to have various profiles as shown by a transmission electron microscopy study of pellets containing *L. pneumophila* (Berk et al., 2008). The pellets had

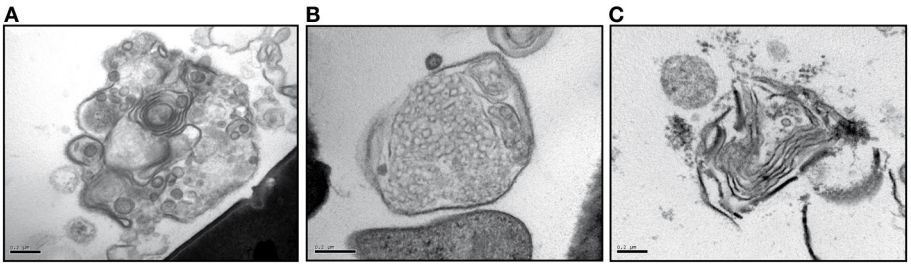

FIGURE 2 | MLBs secreted by *D. discoideum* cells grown on Gram-positive bacteria. Transmission electron microscopic images of MLBs secreted by *D. discoideum* DH1-10 cells (Cornillon et al., 2000) grown on *B. subtilis* (Benghezal et al., 2006) **(A,B)** and *M. luteus* ATCC 4698 **(C)**. One MLB is shown in each panel. Scale bars = 0.2 μm.

three morphologies: (1) bacteria embedded in abundant membranous and vesicular material reminiscent of amoebal MLBs, (2) bacteria surrounded by amorphous material, and (3) bacteria with no apparent electron dense material surrounding them. *Tetrahymena* spp. have also been induced to package *Escherichia coli* O157:H7 (Smith et al., 2012). Scanning electron microscopy has revealed that the pellets produced in this case have a net-like structure surrounding the bacteria and aggregate in flocs due to their stickiness. This suggests that packages produced by ciliates can vary in form and composition as with amoebae. However, it is difficult to determine the extent of the variability without an exhaustive side-by-side comparison of packages produced by various protozoa packaging the same bacterial species.

Like amoeba, ciliates are able to produce MLBs when fed digestible bacteria (Berk et al., 2008). These MLBs are identical to those produced by *D. discoideum* fed digestible bacteria. This suggests that ciliates also control the production of pellets. Immunolabeling has shown that the membranous material in packages produced by *Tetrahymena* spp. is, in part, of bacterial origin (Berk et al., 2008), and indicates that pellets produced by ciliates are likely a mosaic composed of protozoal and bacterial material similar to MLBs produced by amoebae.

The most convincing proof that bacteria packaging is a protozoa-driven process comes from experiments where protozoa are fed synthetic beads. Since the primary characteristic of pathogenic bacterial parasites of protozoa is resistance to enzymatic degradation in phago-lysosomes (Molmeret et al., 2005), the synthetic beads mimic undigestible bacteria but are inert and do not biochemically interact with the protozoa. When fed latex beads, *Tetrahymena* cells produced packaged beads in a net-like matrix similar to that observed with *E. coli* (Smith et al., 2012). Similarly, *D. discoideum* cells grown in presence of polystyrene beads and digestible bacteria produced MLBs containing beads (**Figure 3**). These results are of the utmost important since they indicate that the packaging process is independent of the ingested particle and is mainly under the control of the protozoa as long as the production of packages is metabolically stimulated.

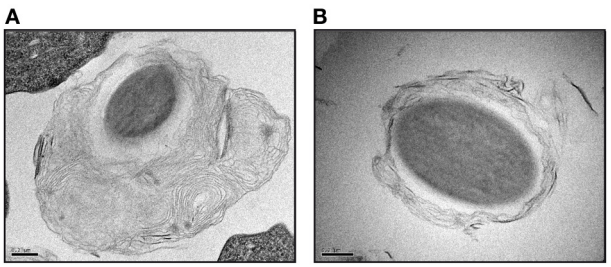

FIGURE 3 | *D. discoideum* cells can package polystyrene beads in secreted MLBs. Transmission electronic micrographic images of polystyrene beads packaged in thick **(A)** and thin **(B)** MLBs after being incubated with *D. discoideum* DH1-10 cells (Cornillon et al., 2000) in the presence of digestible bacteria. Scale bars = 0.2 μm.

HYPOTHESIS AND PERSPECTIVES

Since packages can be produced in abundance by protozoa and provide bacteria enclosed in the structures with much greater resistance to unfavorable conditions, it is tempting to hypothesize that bacteria packaging by protozoa is a general process that contributes to the survival and propagation of pathogenic bacteria in the environment. This process may be an unsuspected source of pathogenic bacteria that could explain many infections, including some of the respiratory tract. The conditions that favor the production of packaged bacteria and their distribution in natural environments and man-made structures are unknown. It will be necessary to identify the environments and conditions in which packaged bacteria are produced in the real world. This cannot be achieved without conducting field studies to first check for the presence of protozoa harboring intracellular bacteria and then to quantitatively assess the presence of packaged bacteria. This information will help in the development of strategies to prevent the spread of pathogenic bacteria. To confirm this hypothesis, many elements need to be addressed.

First, it could be interesting to determine whether other players are involved in bacteria packaging. Even if many protozoa and bacteria are known to participate in this process (**Table 2**), it would be interesting to determine how many other pathogenic and even non-pathogenic bacteria can be packaged as well as which protozoa can perform the packaging. This may be quite difficult since some environments in which bacteria packaging may occur cannot be easily reproduced *in vitro*. In addition, some bacterial species may be packaged by one type of protozoa but not by another. Bacteria packaging may also occur only in presence of a tripartite interaction as with *D. discoideum* where digestible

bacteria are required to produce MLBs containing synthetic beads (**Figure 4**) and with *A. castellanii*, where the production of packaged *L. pneumophila* is enhanced in the presence of digestible *E. coli* (Berk et al., 1998). Environmental conditions likely play a role in the yield of packaged bacteria and even in the process itself. For example, the resistance of *L. pneumophila* to *Acanthamoeba palestinensis* predation can be modulated by environmental conditions such as the incubation temperature (Anand et al., 1983), which may affect the capacity of the bacteria to be included in packages.

Nothing is known about the protozoal mechanisms involved in bacteria packaging since research to date has mainly focused on the impacts of packaging on the bacteria. Apart from the advantages packaging provides to bacteria (**Table 2**), research has also focused on the transcriptional response of *Salmonella enterica* during packaging and the requirement of *L. pneumophila* for a functional Dot/Icm system to resist protozoal degradation and be packaged (Berk et al., 2008; Rehfuss et al., 2011). However, given that the packaging process is under the control of protozoa, a better understanding of the molecular mechanisms of the protozoal endocytic pathway involved in the packaging process is required.

MLB production by *D. discoideum* cells grown in liquid culture can be stimulated by U18666A, a drug that disrupts intracellular cholesterol transport and metabolism in mammalian cells. Under these conditions, MLBs are produced by the invagination of the membrane inside the lysosomal compartments (Marchetti et al., 2004). Inward budding also occurs when *D. discoideum* cells are fed digestible bacteria (**Figure 4**), suggesting that lysosomal membrane proteins may be included in MLBs. In fact, amoebal proteins such cysteine proteinase and discoidin I as well as other unidentified glycosylated proteins have been already detected in secreted MLBs (Barondes et al., 1985; Fukuzawa and Ochiai, 1993; Emslie et al., 1998; Paquet et al., 2013).

The identification of all the protozoal proteins in the MLBs used to package bacteria as well as the biological machinery involved in the process and their characterization will open up new avenues for understanding the packaging process. For example, the identification of the proteins included in MLBs will provide cues to the packaging mechanisms as well as markers for visualizing the packaging process by real-time microscopy. The identification of these proteins and mechanisms will make it possible to develop tools that will help to address important research issues related to bacteria packaging by protozoa such as their presence in the environment and their role in infectious diseases. Understanding the mechanisms should also make it possible to develop chemical inhibitors or modulators of bacteria packaging.

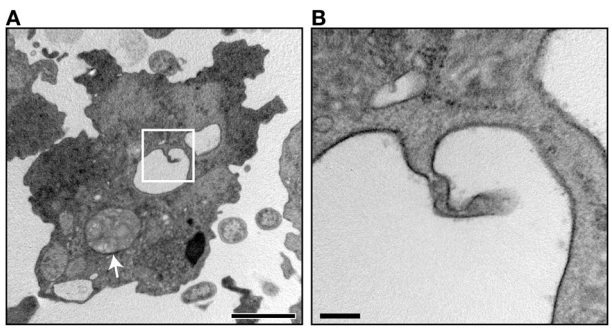

FIGURE 4 | Intra-lysosomal profile of *D. discoideum* cells fed digestible bacteria. (A) Transmission electronic micrographic image of a *D. discoideum* DH1-10 cell (Cornillon et al., 2000) with a lysosomal compartment displaying an intra-lysosomal profile (white square) and a MLB inside a lysosomal compartment (white arrow). B. Magnification of image A showing the inward bud in greater detail. The inward budding is in a lysosomal compartment containing no other electron dense material. The invaginations of the lysosomal membrane are hard to detect in compartments already containing MLBs because the compartments are too crowded. Scale bar = 2 μm in **(A)** and 0.2 μm in **(B)**.

In addition to identifying protozoal proteins in the packages, another approach to understand the mechanisms involved in the process would be to identify the proteins that are essential for the packaging process. Since the production of MLBs is similar to the production of multivesicular bodies (MVBs) by many eukaryotic organisms (Piper and Katzmann, 2007), it is highly likely that the ESCRT complexes involved in MVB biogenesis are also involved in the production of packaged bacteria. Among others, autophagy is probably also associated with MLB production since a link exists between autophagy and MVB biogenesis (Fader and Colombo, 2009).

The *D. discoideum* model appears to be promising for the identification of the genes encoding the proteins involved in the packaging process. Site-directed mutagenesis is a routine procedure with *D. discoideum*, and a mutant of *tom1*, which encodes one of the proteins of an ESCRT-like complex in this amoeba, has already been developed (Blanc et al., 2009). Mutants of the gene encoding Alix, a protein functionally associated with ESCRT complexes, as well as of genes encoding proteins involved in autophagy are also already available (Mattei et al., 2006; Calvo-Garrido et al., 2010). Studying the capacity of these mutants and others that can be generated in the future to produce normal MLBs appears to be a good approach for shedding light on the mechanisms involved in bacteria packaging.

In addition to studying the mechanisms of package formation by protozoa, the impact of packaged bacteria on human health must be investigated. The most probable assumption is that packaged bacteria can be aerosolized, spread over long distances, and play a role in the transmission of respiratory tract infections. Since aerosolization is a major route of transmission of many human pathogens and is also a significant stress factor for microorganisms (Macher, 1999), it is important to determine the relative viability of packaged bacteria compared to free bacteria following aerosolization. It is also important to determine the response of aerosolized packaged bacteria to environmental stresses such as ultraviolet radiation (UV).

Previous studies have provided support for the idea that the intracellular growth of pathogenic bacteria in protozoal hosts increases the invasive potential and virulence of the bacteria in mammals (Cirillo et al., 1994, 1997, 1999; Molmeret et al., 2005). By producing packaged bacteria, protozoa may help bacteria to remain undetected by the immune system following inhalation, which in turn may help them to better adapt to their new environment like the respiratory tract and be more effective in the development of an infection. While the role of bacteria packaging in the infectious process has never been clearly addressed,

T. tropicalis-packaged *L. pneumophila* are much more infectious than free bacteria in cultured human pneumocyte cells (Koubar et al., 2011). Are packaged bacteria more infectious than free bacteria in animal models? How does the immune system response to packaged and free bacteria differ? Answers to these questions are fundamental because protozoa can produce hundreds of packages containing pathogenic bacteria that can persist even after the protozoa have disappeared.

CONCLUSION

Research to date has been limited to descriptions of bacteria packaging by various protozoa. A number of findings suggest that packaging provides the bacteria with an advantage in the environment and can contribute to their pathogenicity. However, many elements need to be clarified to determine whether packaged bacteria are a significant source of infections.

It will be essential to extend the characterization of the role played by bacteria packaging to the persistence of pathogens in the environment and their ability to cause infections, especially of the respiratory tract. More precisely, we require a better understanding of the mechanisms involved in bacteria packaging, the magnitude of this phenomenon in various environments, the enhanced virulence potential related to the increased propagation of bacteria, and their ability to cause infections. The development of packaging markers, protocols for producing packaged bacteria, and methods to detect these bacteria are also needed to better understand this process. Ultimately, if bacteria packaging appears to make an important contribution to the persistence and transmission of pathogenic bacteria, it will be possible to reduce their infectivity and propagation by modulating their interactions with protozoa.

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Life on the outside: role of biofilms in environmental persistence of Shiga-toxin producing *Escherichia coli*

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Escherichia coli is a heterogeneous species that can be part of the normal flora of humans but also include strains of medical importance. Among pathogenic members, Shiga-toxin producing *E. coli* (STEC) are some of the more prominent pathogenic *E. coli* within the public sphere. STEC disease outbreaks are typically associated with contaminated beef, contaminated drinking water, and contaminated fresh produce. These water- and food-borne pathogens usually colonize cattle asymptomatically; cows will shed STEC in their feces and the subsequent fecal contamination of the environment and processing plants is a major concern for food and public safety. This is especially important because STEC can survive for prolonged periods of time outside its host in environments such as water, produce, and farm soil. Biofilms are hypothesized to be important for survival in the environment especially on produce, in rivers, and in processing plants. Several factors involved in biofilm formation such as curli, cellulose, poly-N-acetyl glucosamine, and colanic acid are involved in plant colonization and adherence to different surfaces often found in meat processing plants. In food processing plants, contamination of beef carcasses occurs at different stages of processing and this is often caused by the formation of STEC biofilms on the surface of several pieces of equipment associated with slaughtering and processing. Biofilms protect bacteria against several challenges, including biocides used in industrial processes. STEC biofilms are less sensitive than planktonic cells to several chemical sanitizers such as quaternary ammonium compounds, peroxyacetic acid, and chlorine compounds. Increased resistance to sanitizers by STEC growing in a biofilm is likely to be a source of contamination in the processing plant. This review focuses on the role of biofilm formation by STEC as a means of persistence outside their animal host and factors associated with biofilm formation.

Keywords: STEC, biofilm, sanitizers, processing plant, environment

INTRODUCTION

Escherichia coli is a diverse species of bacterium that includes members of the normal commensal flora of humans and animals but also pathogenic strains of veterinary and medical importance. Pathogenic members are usually classified in two major groups: intestinal *E. coli* (InPEC) and extraintestinal *E. coli* (ExPEC). The latter group is typically responsible for urinary tract infections [uropathogenic *E. coli* (UPEC)], neonatal sepsis, and meningitis in humans and various infectious diseases in animals including mastitis (Kaper et al., 2004; Clements et al., 2012). InPEC are classically divided in 8 sub-groups based on the diseases they cause, their virulence factors, and phylogeny. These 8 pathotypes are: adherent-invasive *E. coli* (AIEC) associated with Crohn's disease, diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga-toxin producing *E. coli* (STEC) that includes enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (including Shigella) (EIEC) (Kaper et al., 2004; Clements et al., 2012). The characteristics of each pathotype have been described in several reviews (Kaper et al., 2004; Croxen and Finlay, 2010; Clements et al., 2012).

STEC are worldwide water- and food-borne pathogens and are some of the more prominent pathogenic *E. coli* within the public sphere (Etcheverria and Padola, 2013). Cattle are an important animal reservoir of STEC and this colonization is typically asymptomatic (Ferens and Hovde, 2011). STEC can also be shed in the feces of sheep, goats, turkeys, and pigs (Heuvelink et al., 1999; Booher et al., 2002; Cornick and Helgerson, 2004; Vu-Khac and Cornick, 2008; Best et al., 2009; La Ragione et al., 2009). STEC disease outbreaks are typically associated with contaminated beef; however unpasteurized milk, contaminated drinking water, contaminated fresh produce, and unpasteurized apple cider have also been implicated (Ferens and Hovde, 2011). In addition to living within animal reservoirs, STEC can persist for prolonged periods of time in the environment, such as in water and farm soil. For example, EHEC can survive for periods greater than 8 months in water contaminated with bovine feces (Ferens and Hovde, 2011).

STEC are also a major concern in food-processing plants and contamination of beef carcasses with STEC may occur during different stages of processing such as slaughtering, dressing, chilling or cutting (Bacon et al., 2003; Koutsoumanis and Sofos, 2004). Therefore, populations of contaminating STEC are likely present on the surface of several pieces of equipment associated with slaughtering and processing. These pieces of equipment may potentially contaminate unadulterated carcasses and fresh meat products (Gill and McGinnis, 2000; Barkocy-Gallagher et al., 2001; Gill et al., 2001; Tutenel et al., 2003). The presence of STEC in beef and food processing plants has been well documented and it has been suggested that the ability to form biofilms on different surfaces is responsible for the distribution and persistence of STEC in meat processing plants (Carpentier and Cerf, 1993; Dewanti and Wong, 1995; Aslam et al., 2004; Rivera-Betancourt et al., 2004). In this review, we will explore the role of biofilm formation by STEC as a means of persistence outside their animal hosts and factors associated with biofilm formation.

GENETIC DIVERSITY OF STEC

The predominant STEC serotype associated with outbreaks is O157:H7. Since it was one of the first serotypes identified as causing hemolytic uremic syndrome (HUS) and the most severe illness, EHEC O157:H7 is the most commonly reported STEC serotype in the media (Etcheverria and Padola, 2013). However, other clinically relevant serotypes have been identified and are commonly called the "the big six," these include serotypes O26, O45, O103, O111, O121, and O145 (Wang et al., 2012). Other serotypes (e.g., O113:H21 and O91:H21) generally do not cause outbreaks but have been associated with sporadic cases of HUS (Karmali et al., 2003). Additionally, a new type of emerging STEC strain was identified after the large HUS outbreak in Germany in 2011 (Frank et al., 2011). This strain belongs to the serotype O104:H4 and combines the chromosomal backbone of a typical EAEC strain with the bacteriophage encoding Stx2 from STEC (Scheutz et al., 2011). The *stx2* gene was presumably acquired via horizontal gene transfer. This atypical Shiga-toxin producing enteroaggregative *E. coli* (STEAEC) strain will not be covered in this review because it does not fit within the classic STEC pathotype.

In addition to serotype diversity within the STEC pathotype, genetic diversity in the O157:H7 serotype is gaining ground as a source of variation in virulence between strains (Bono et al., 2007; Manning et al., 2008; Zhang et al., 2010; Shringi et al., 2012). This phenomenon is observed with different *E. coli* O157 strains, where there is a significant association between clades and the severity and duration of disease (Fukushima et al., 1999; Grant et al., 2008; Manning et al., 2008). Furthermore, geographical distribution also appears to influence the phylogeny of *E. coli* O157 populations and recent findings suggest divergent evolution of EHEC O157 in Australia and the United States (Mellor et al., 2013). Despite this diversity, most studies on STEC biofilm formation are performed with the sequenced reference strain EDL933 that was isolated from meat associated with a USA hemorrhagic colitis outbreak in 1982 (Perna et al., 2001; Manning et al., 2008). Therefore, some of the conclusions may only reflect North American strains rather than strains isolated from other continents.

BIOFILM FORMATION BY STEC

Generally, bacteria do not live freely in suspension (planktonic cells), but in complex communities called biofilms. Biofilms are aggregates of microorganisms (bacteria, fungi, algae, or protozoa) enclosed in a self-produced extracellular polymeric matrix that are attached to a biotic or abiotic surface (Costerton et al., 1999; Hall-Stoodley and Stoodley, 2009; Jacques et al., 2010). Biofilms protect bacteria from several challenges including desiccation, bacteriophages, amoebae, and biocides used in industrial processes (Costerton et al., 1999). With respect to *E. coli* biofilm formation, studies have mostly been performed with K12 strains and have been reviewed in several publications (Beloin et al., 2008; Wood, 2009). EDL933 and MG1655 share a core set of genes, including some genes involved in biofilm formation; as a result, data obtained using K12 strains are often used to infer function for STEC strains. However, such inferences are not always appropriate because there are key differences between the genomes of K12 and EDL933 including the presence of O-islands, lack of type 1 fimbriae production, and the presence of single nucleotide polymorphisms (SNP) (Perna et al., 2001; Roe et al., 2001; Welch et al., 2002; Zhang et al., 2006; Chen et al., 2013). Additionally, the expression and activity of several factors that must act at specific times and at various locations in the biofilm are required for proper biofilm formation (Beloin et al., 2008; Wood, 2009).

DIFFERENCES BETWEEN STEC AND K12 THAT MAY INFLUENCE BIOFILM FORMATION

As stated above, there are key differences between K12 and STEC strains that may have major influences on biofilm formation. For example, the EDL933 genome possesses 177 O-islands (OI), several of which encode fimbrial adhesins (Perna et al., 2001). However, the presence of a gene in a genome does not guarantee its expression. As an example, type 1 fimbriae are associated with biofilm formation in K12 strains, but a deletion in the *fim* regulatory region abolished type 1 fimbriae expression in *E. coli* O157:H7 (Roe et al., 2001; Beloin et al., 2004). Therefore, type 1 fimbriae do not play a role in biofilm formation by *E. coli* O157:H7. These data highlight the fact that findings for K12 do not always represent biological processes for all *E. coli* subtypes. Furthermore, many groups have demonstrated that STEC biofilm formation is more dependent on the strain than the serotype. This could be explained by the presence of SNP that result in premature stop codons in genes encoding adhesins or RpoS, the stationary phase sigma factor that is important for biofilm formation and regulation (Zhang et al., 2006).

In addition to differences at the genomic level, there are key differences in the transcript profiles of K12 and EHEC strains for biological processes involved in the interactions with lettuce leaves (Fink et al., 2012) including genes that may be involved in biofilm formation. Differences in the transcriptomes of K12 and STEC strains could be explained by the presence of additional regulators encoded within genomic islands and changes in promoter regions. For example, the genomic island OI-47 of *E. coli* O157:H7

contains a gene, *vmpA*, coding for a c-di-GMP phosphodiesterase that is specific for EHEC O157:H7 and VmpA was shown to influence the regulation of biofilm formation (Branchu et al., 2013). Furthermore, recent findings have highlighted differences in EHEC and EPEC promoter regions that result in the differential regulation of an outer-membrane protease (Thomassin et al., 2012). Taken together, these differences indicate that biofilm data obtained with K12 strains or other pathotypes are not always directly relevant to STEC strains. Therefore, it is important that biofilm formation be studied in STEC.

STEPS IN BIOFILM FORMATION

Biofilm formation requires specific steps and is typically described as a four-step process: initial contact, attachment, maturation, and dispersion (**Figure 1**).

INITIAL CONTACT

The first step in biofilm formation is reversible attachment to a surface; this is dependent on a balance of attractive and repulsive forces between the bacteria and the surface. Both environmental and bacterial factors are important for this interaction. Attachment is influenced by environmental conditions such as temperature, pH, ionic force of the medium, and the rugosity of the surface in addition to bacterial properties such as hydrophobicity and motility (Fletcher, 1988; Pratt and Kolter, 1998; Danese et al., 2000). Furthermore, flagella-driven motility is considered to be an important factor during the initial step of biofilm formation by *E. coli* because K12 strains lacking flagella do not produce biofilms. Additionally, Chen and collaborators recently suggested that flagella-driven motility is also involved in biofilm formation of non-O157:H7 STEC (Chen et al., 2013).

ATTACHMENT

The second step in biofilm formation is irreversible attachment, which is often influenced by the presence of surface structures such as fimbrial adhesins (Beloin et al., 2004). Many classes of fimbriae have been implicated in the attachment of STEC to surfaces, including type 1 fimbriae, curli, type 4 pili, long polar fimbriae, and F9 fimbriae (Farfan and Torres, 2012).

MATURATION

The third step in biofilm formation is maturation. During biofilm maturation, bacteria continue to multiply and produce extracellular matrix. At this stage, the biofilm adopts a three-dimensional structure. This growth is mostly due to bacterium–bacterium interactions; several surface proteins and extracellular matrix components are involved in bacterial adhesion and biofilm architecture (Beloin et al., 2008). Two important factors for this step have been identified in *E. coli*: autotransporters for cell-cell interactions and exopolysaccharides (EPS) for the matrix architecture (Beloin et al., 2008).

Important factors for biofilm maturation: autotransporters

Autotransporter adhesins, which are members of the type V secretion system, have been associated with autoaggregation and biofilm formation. Screens in STEC strains have identified 9 autotransporter genes: chromosome encoded *agn43, cah, ehaA*, *ehaB*, *ehaD*, *ehaG*, *saa*, and *sab* and plasmid-encoded *espP* (Torres et al., 2002; Wells et al., 2008, 2009; Herold et al., 2009; Puttamreddy et al., 2010). The protein products of each of these genes have been associated with biofilm formation (Torres et al., 2002; Wells et al., 2008, 2009; Herold et al., 2009; Puttamreddy et al., 2010). A comparative study of three autotransporter genes (*agn43*, *cah*, and *ehaA*) among 51 STEC strains found that the presence of autotransporter genes within the genome was variable among STEC serotypes (Biscola et al., 2011). Specifically, *agn43* was present at a higher frequency in non-O157 strains than O157 strains while the frequency of *cah* is higher in O157 strains compared to non-O157 strains (Biscola et al., 2011).

In addition to the autotransporter *espP*, the pO157 plasmid encodes the enterohemolysin translocator *ehxD,* whose protein product was identified as a mediator of biofilm formation, indicating that pO157 is essential for biofilm formation (Puttamreddy et al., 2010). Large plasmids similar to pO157, encoding *espP,*

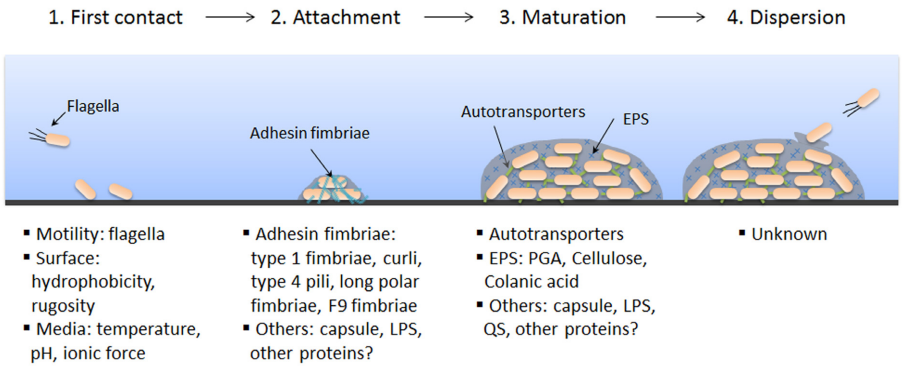

FIGURE 1 | Schematic representation of biofilm formation and STEC factors associated with each step. Biofilm formation is a dynamic and complex process influenced by several bacterial and/or environmental factors. Biofilm formation occurs in four steps: first contact, attachment, maturation, and dispersion. Factors that are known to play a role in STEC biofilm formation are listed below the corresponding step. It should be noted that biofilm formation in STEC is strain dependent and factors presented in this figure are not necessarily representative of all STEC biofilms. PGA, poly-N-acetyl glucosamine; EPS, extracellular polymeric substances; LPS, lipopolysaccharides; QS, quorum sensing.

and *ehxD* can also be found in many non-O157 EHEC strains (Brunder et al., 1999; Caprioli et al., 2005; Verstraete et al., 2013).

Important factors for biofilm maturation: EPS

The *E. coli* biofilm matrix can be composed of three different EPSs: poly-N-acetyl glucosamine (PGA), colanic acid, and/or cellulose. The genes encoding proteins that are involved in the synthesis of these polysaccharides are present in the genomes of STEC strains EDL933 and Sakai (Hayashi et al., 2001; Perna et al., 2001), however, their role in biofilm formation has not been directly established in these strains. However, O157:H7 mutants lacking genes encoding proteins needed to make PGA, cellulose, or colanic acid were unable to adhere to alfalfa sprouts (Matthysse et al., 2008). Furthermore, cellulose production was correlated with biofilm formation in O157 strains (Biscola et al., 2011; Lee et al., 2011). Cellulose production is, however, variable and dependent on both the bacterial strain and environmental conditions (Beloin et al., 2008). Colanic acid is produced by *E. coli* O157:H7, but there is limited data for other STEC serotypes (Beloin et al., 2008). The production of colanic acid protects *E. coli* O157:H7 against osmotic and oxidative stress, suggesting that colanic acid may be implicated in STEC biofilm formation, however, this remains to be tested directly (Yeh and Chen, 2004).

Important factors for biofilm maturation: other factors

Lipopolysaccharides (LPS) and capsules, which are surface structures, have been implicated in biofilm formation by *E. coli*. Mutations affecting LPS synthesis affect the ability of *E. coli* K12 strains to adhere to surfaces and form biofilms (Genevaux et al., 1999; Beloin et al., 2008). Similar observations were noted for *E. coli* O157:H7 strain EDL933, where O-antigen transposon mutants could not form biofilms (Puttamreddy et al., 2010).

Capsules are known to mask bacterial surface adhesins and often have an indirect effect on biofilm formation (Schembri et al., 2004). According to Whitfield, capsule polysaccharides produced by some EHEC strains belong to the *E. coli* group 4 capsule, which is composed of the same sugar repeats as the LPS O-antigen and acetamido sugars in their repeat-unit structures (Whitfield, 2006). The impact of this capsule-type on biofilm formation by STEC has yet to be investigated. If the group 4 capsule has an impact on biofilm formation, its effect might be serotype specific given the diversity of O-antigen structures among STEC. Furthermore, the capsule is only expressed and present under specific laboratory conditions in EDL933 (Shifrin et al., 2008; Thomassin et al., 2013). Therefore, the role of the capsule in *in vivo* biofilm formation might be difficult to evaluate in an *in vitro* setting given that biofilm formation is also highly dependent on growth conditions.

Curli fimbriae are structures that aggregate on the surface of cells, they promote adhesion of *E. coli* to different human cells and biofilm formation on abiotic surfaces (Olsen et al., 1989; Ben Nasr et al., 1996; Vidal et al., 1998; Cookson et al., 2002; Uhlich et al., 2006). Curli expression in some STEC strains has been associated with biofilm formation on polystyrene and stainless steel surfaces (Cookson et al., 2002; Ryu et al., 2004b; Uhlich et al., 2006). However, curli expression, which is strain dependent and serotype independent, is not essential for biofilm formation (Wang et al., 2012). Additionally, curli can interact with cellulose to create networks resulting in the formation of a hydrophobic extracellular matrix (Zogaj et al., 2001; Gualdi et al., 2008). Curli are thought to facilitate initial cell–surface interactions and, subsequent cell–cell interactions (Cookson et al., 2002; Uhlich et al., 2006). Curli are encoded in two divergently transcribed operons: the *csgBA* operon encodes the structural components and the *csgDEFG* operon encodes the regulator (CsgD) and the export machinery (CsgE-G) (Hammar et al., 1995). Both operons are found in the EHEC O157:H7 EDL933 and Sakai reference strains (Hayashi et al., 2001; Perna et al., 2001). Curli production is tightly controlled and complex; several transcriptional regulators (EnvZ/OmpR, CpxR, RcsCDB, RpoS, H-NS, IHF, Crl, and MlrA) and conditions (temperature, osmolarity, pH, and oxygenation) control curli expression, which involves a network of interactions (Dorel et al., 1999; Prigent-Combaret et al., 2001; Brombacher et al., 2003; Gerstel et al., 2003; Jubelin et al., 2005; Vianney et al., 2005). The complex regulatory network of curli expression is thought to be fine-tuned to allow for the colonization of specific niches by *E. coli* (Prigent-Combaret et al., 2001; Kikuchi et al., 2005).

Important factors for biofilm maturation: quorum sensing

During the different steps of biofilm formation the bacterial cell population density fluctuates and gene expression varies. To coordinate gene expression, bacteria communicate using quorum sensing (QS) systems (Walters and Sperandio, 2006). QS systems are based on the secretion and/or recognition of signal molecules called autoinducers (AIs). Three types of AIs have been identified: AI-1, AI-2, and AI-3. Both AI-2 and AI-3 are produced, secreted, and recognized by *E. coli* strains including STEC (Walters and Sperandio, 2006). *E. coli* strains do not produce AI-1; however their genome encodes *sdiA*, the AI-1 sensor, which is a *luxR* homolog. This enables *E. coli*, including STEC strains, to recognize acyl-homoserine lactone (AHL), the signal molecule for AI-1, secreted by others bacterial species. In Sharma et al. (2010) demonstrated that SdiA acts as a repressor of curli and flagellar gene expression. An O157:H7 Δ*sdiA* strain had increased curli fimbriae and biofilm production, suggesting that the AI-1 system has a negative impact on biofilm formation (Sharma et al., 2010). LuxS, a metabolic enzyme also found in STEC strains, is primarily involved in the conversion of ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, which is the precursor for AI-2 (Schauder et al., 2001). Biofilm formation was enhanced when AI-2-like molecules were added to an O157:H7 *luxS* deletion strain (Lu et al., 2005; Bansal et al., 2008; Vikram et al., 2010). Furthermore, AI-3 and host-produced epinephrine/norepinephrine are recognized by the QseBC two component system (Walters and Sperandio, 2006). The addition of epinephrine and norepinephrine increases EHEC motility and biofilm formation, while the addition of indole attenuates these phenotypes (Bansal et al., 2007). Moreover, motility and biofilm formation by a *qseC* deletion strain were reduced by half when compared to the wild type strain (Yang et al., 2014).

DISPERSION

The final step in biofilm formation is the detachment of bacteria from the biofilm and their dispersal, which contributes to the transmission of bacteria. Dispersal is a complex process that involves several environmental signals and effectors and no single dispersal mechanism is used by all bacterial species. As described above, bacteria generally switch from a planktonic to a biofilm lifestyle by sensing environmental changes. Biofilm dispersal has recently been reviewed in detail (Kaplan, 2010). Dispersal is the least understood step in biofilm formation for all bacterial species and has not been investigated for STEC (**Figure 1**). In *E. coli* other than STEC, modulation of crucial surface structures, such as type IV bundle-forming pili (BFP) in EPEC and aggregative adherence fimbriae (AAFs) in EAEC, results in the detachment of bacteria from the biofilm and surface (Knutton et al., 1999; Sheikh et al., 2002; Velarde et al., 2007). For example in EAEC, positively charged AAFs extend away from the surface of the bacterial cell to mediate surface-adherence when dispersin is produced, because dispersin binds to and neutralizes LPS charge (Sheikh et al., 2002; Velarde et al., 2007). When dispersin is down-regulated, the positively charged AAFs collapse on the bacterial surface due to their interaction with negatively charged LPS. As a consequence of this collapse, AAFs no longer adhere to surfaces and the biofilm disperses (Sheikh et al., 2002; Velarde et al., 2007). Mechanisms involved in biofilm detachment are of increased interest, because the understanding of these mechanisms could lead to the development of clinical or industrial tools to remove biofilms.

SURVIVAL IN THE ENVIRONMENT: IS IT BIOFILM MEDIATED?

STEC contamination of the environment and food-processing plant can occur several different ways (**Figure 2**). STEC are typically shed in the feces of cattle and this will contaminate the hide and farm environment (Elder et al., 2000; Aslam et al., 2003). STEC that are present in feces can contaminate manure and, consequently, soils either through manure runoff or manure applied to fields (Gagliardi and Karns, 2000; Solomon et al., 2002; Van Elsas et al., 2011). At this stage, STEC may persist and grow on fresh produce such as lettuce and can be internalized and survive within plant tissue via a mechanism that is not fully understood (Seo and Frank, 1999; Jeter and Matthysse, 2005; Tyler and Triplett, 2008). Furthermore, manure applied to fields often ends up in ground or surface water through runoff; this water is often used to irrigate fields and water crops (Ribeiro et al., 2012). As a consequence, fields and crops that were not treated with manure can become contaminated with STEC. All of these contribute to the contamination and spread of STEC in the environment. STEC can survive in soil, on fresh produce, manure, and river water, which is hypothesized to be associated with the ability of STEC to form biofilms.

SOIL AND MANURE

Survival of *E. coli* O157:H7 in soil and manure is greatly influenced by microbial diversity; EHEC survival is at its highest when diversity is low (Vidovic et al., 2007; Van Overbeek et al., 2010; Ibekwe et al., 2011; Van Elsas et al., 2011). On one hand, survival

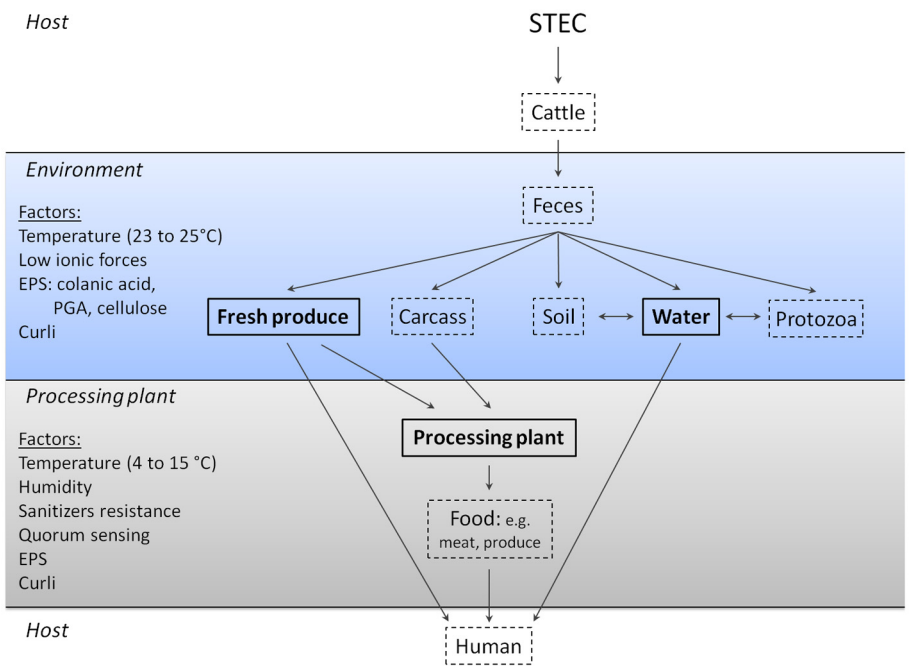

FIGURE 2 | Role of biofilm formation in the transmission and persistence of STEC outside of an animal host. Several studies demonstrated that STEC can persist as a biofilm on fresh produce, in water, and in processing plants. In some cases, factors contributing to biofilm formation have been identified. Texts in bold and framed by solid lines indicate environments where STEC biofilms are identified and participate in the persistence of STEC; dashed lines indicate environments where STEC biofilms are hypothesized to be present. It is currently unknown if STEC biofilm formation plays a role in the colonization of cattle and humans or in STEC survival in feces, soil, protozoans, carcasses, and processed food. PGA, poly-N-acetyl glucosamine; EPS, extracellular polymeric substances; LPS, lipopolysaccharides; QS, quorum sensing.

of STEC within soil and manure is also hypothesized to be associated with the absence of various protozoa that graze on STEC (Ravva et al., 2010). On the other hand certain protozoa are proposed to act as a transmission vehicle for EHEC (Chekabab et al., 2012). Taken together these results strongly suggest that STEC survival is influenced by the environmental microcosm. There is, however, little evidence to indicate that STEC are able to form or integrate into biofilms within manure, soil, or in the farm environment. Therefore, there is a need for studies that investigate the role of biofilms in promoting STEC survival within these environments.

WATER

Biofilms containing STEC have been detected in freshwater streams that drain or are connected to agricultural land (Cooper et al., 2007; Maal-Bared et al., 2013). It is unknown if STEC can act as the pioneer bacteria in environmental biofilms, but this possibility is unlikely because periphytic *E. coli* isolates appear to form biofilms more readily than human and/or bovine isolates (Moreira et al., 2012). The presence of STEC in environmental biofilms may be explained by the finding that biofilm negative *E. coli* O157:H7 strains are able to integrate into pre-established biofilms formed by other *E. coli* strains (Uhlich et al., 2010). Therefore, it is likely that STEC can integrate into pre-existing biofilms.

Mixed-species biofilms formed in rivers and river sediments are also of particular interest because they provide ample opportunity for genetic exchange between bacteria (Maal-Bared et al., 2013). Furthermore, the environment within the biofilm is suitable for the transduction of *stx*-encoding phages carried by STEC (Solheim et al., 2013). The transfer of these phages is responsible for the spread of *stx* genes among *E. coli* species. Such genetic exchanges could contribute to the emergence of new pathogenic *E. coli* and give rise to the next outbreak strain.

FRESH PRODUCE

Several genes and/or structures associated with biofilm formation have been identified as important factors for plant colonization by STEC. For example, PGA, cellulose, and colanic acid play a role in *E. coli* O157 binding to sprouts and tomato root segments (Matthysse et al., 2008). Furthermore, PGA is essential for binding to sprouts and cellulose and colanic acid increase the efficiency of this attachment (Matthysse et al., 2008). These findings provide evidence that the biofilm matrix associated polysaccharides are crucial for attachment to plants. In addition, these EPSs are expressed under environmental conditions [i.e., room temperature (23–25°C)], in low-ionic-strength medium, and during nutrient limitation by several *E. coli* strains (Gottesman and Stout, 1991; Matthysse et al., 2008). The expression of matrix polysaccharides in environments similar to those encountered in the presence of fresh produce further supports the likelihood that biofilm formation plays a role in STEC survival on produce.

Curli fimbriae improve the adherence of *E. coli* O157:H7 to spinach leaves, and interestingly, the improved adherence was found to be independent of cellulose production (Macarisin et al., 2012). Biofilm modulation genes (*ycfR* and *ybiM*) are also significantly up-regulated when *E. coli* O157:H7 interacts with lettuce roots (Hou et al., 2013). Furthermore, a Δ*ycfR* strain was unable to attach to or colonize lettuce roots (Hou et al., 2013). Taken together these studies suggest that certain factors involved in biofilm formation improve the environmental fitness of STEC, especially in the context of plant colonization.

BIOFILMS AND PROTOZOA

Protozoans living in soil, manure, and rivers probably prey on STEC living in the environment (Ravva et al., 2010). Recent studies have shown that EDL933 can survive in *Acanthamoeba castellanii* and replicate within *Acanthamoeba polyphaga*, protozoa commonly found in soil, water, and fecal slurry (Barker et al., 1999; Chekabab et al., 2012). It was suggested that such protozoa could also serve as a transmission vehicle for EHEC (Chekabab et al., 2012). In support, the presence of the Stx-encoding prophage increases the survival of STEC isolates in the presence of *Tetrahymena pyriformis* or *Tetrahymena thermophila* (Steinberg and Levin, 2007; Mauro et al., 2013). Protozoa are known to graze on biofilms and ciliates and flagellates differently influence biofilm communities (Wey et al., 2012). For example, *Glaucoma* and *Tetrahymena* species (ciliates), expel vesicles containing viable *E. coli* O157:H7, whereas *Colpoda steinii* and *Acanthamoeba palestinensis* (flagellates) do not (Gourabathini et al., 2008). Furthermore, protozoans graze less on biofilm communities than on their planktonic cells, suggesting that biofilms may offer some protection from protozoan predation (Wey et al., 2012).

STEC BIOFILMS IN PROCESSING PLANTS, A POTENTIAL SOURCE OF MEAT AND PRODUCE CONTAMINATION

In addition to forming biofilms under environmental conditions and on plants, STEC are able to form biofilms on different surfaces often found in meat processing plants, such as stainless steel, polystyrene, glass, polyurethane, and high-density polyethylene (Dewanti and Wong, 1995; Dourou et al., 2011; Nesse et al., 2013). The introduction of contaminated food into processing plants results in the spread of STEC and contamination. As an example, it was estimated that the prevalence of *E. coli* O157 on cattle entering the slaughter floor may range from 10 to > 70% (Woerner et al., 2006). In the meat industry, contamination of surfaces with STEC can be traced to the entry of contaminated hides. Fecal contamination of hides occurs both directly and indirectly during cattle production and transit. Currently, disinfection protocols are used to try to limit the entry of STEC into slaughterhouses and processing plants. Despite the common use of disinfection protocols, STEC contamination of food still occurs, which according to the Center for Disease Control and Prevention (CDC) and Public Health Agency of Canada (PHAC) shows that disinfections protocols do not always prevent contamination (http://www.cdc.gov/ecoli/ and http://www.phac-aspc.gc.ca/fs-sa/fs-fi/ecoli-eng.php). The persistence of STEC in the presence of disinfectants gives rise to the probability that STEC survive and grow within a biofilm in processing plants (Stopforth et al., 2003; Ryu et al., 2004a; Uhlich et al., 2006; Fouladkhah et al., 2013). In this section we will focus on STEC biofilm formation and associated factors in processing plants.

In the processing plant environment, temperatures are normally controlled and maintained between 4 and 15°C. Many

studies have shown that STEC are able to grow in a biofilm within this temperature range (Dourou et al., 2011; Fouladkhah et al., 2013; Nesse et al., 2013). For example, *E. coli* O157:H7 is able to colonize surfaces in contact with beef at 15°C (non-production hours temperature) and 4°C (production hours temperature) (Dourou et al., 2011; Fouladkhah et al., 2013). Interestingly, *E. coli* O157:H7 attachment increased at 4°C over time in the presence of a fat-lean tissue homogenate (Dourou et al., 2011). Furthermore, *E. coli* O157:H7 EDL933 is able to adhere and produce a dense biofilm on surfaces that are not favorable for its attachment when collagen I is present, which is a muscle fibrous extracellular matrix protein (Chagnot et al., 2013). In addition to form biofilms in meat homogenates, *E. coli* O157:H7 is also able to form biofilms on stainless steel when grown in spinach leaf lysates (Carter et al., 2012). Environmental conditions such as temperature and culture broths containing meat or vegetable residues can affect the expression of genes controlled by QS. For example, it was shown that *E. coli* O157:H7 biofilms produce large amounts of AI-2 when cultured in pork, beef or spinach broth (Silagyi et al., 2009). Based on this evidence, it is possible that QS drives biofilm formation in meat processing plants.

Fouladkhah et al. showed that the use of quaternary ammonium compound-based and peroxyacetic-based chemical sanitizers on biofilms that had matured for 1 week were more effective at 4°C than 25°C. However, these commercial sanitizers used at concentrations recommended to kill planktonic STEC were not able to kill or remove STEC biofilms from stainless steel surfaces (Fouladkhah et al., 2013). Furthermore, curli fimbriae, due to their amyloid properties, can protect bacteria from antibacterial agents like chlorine or quaternary ammonium sanitizers (Uhlich et al., 2006; Wang et al., 2012). It has been shown that tolerance of sanitizers by STEC in biofilms do not depends on serotype but on strain (Wang et al., 2012). It was also shown that at 100% relative humidity (RH), *E. coli* O157:H7 biofilms were more resistant to sanitizers than at lower RH (Bae et al., 2012). Furthermore, large biofilms were more resistant to cleaning and disinfection protocols and repeated treatment could results in the presence of viable but non-culturable *E. coli* O157:H7 that were able to regrow as a biofilm on polyurethane (Marouani-Gadri et al., 2010). Taken together, these data indicate that sanitizer efficacy may be limited against STEC growing within a biofilm community.

Interestingly, non-pathogenic bacteria isolated from processing plants, such as *Comamonas testosterone, Acinetobacter calcoaceticus, Burkholderia caryphylli*, and *Ralstonia insidiosa,* can initiate biofilm formation and may allow *E. coli* O157:H7 to integrate within a pre-formed biofilm, resulting in a mixed biofilm (Marouani-Gadri et al., 2009a; Habimana et al., 2010; Liu et al., 2014). For example, *C. testosteroni* can enhance the ability of *E. coli* O157:H7 to form biofilms (Marouani-Gadri et al., 2009a). The presence of *C. testosteroni* within the biofilm did, however, decrease the number of colony forming units of *E. coli* O157:H7 following chemical treatment when compared to chemical treatment of a single species *E. coli* O157:H7 biofilm (Marouani-Gadri et al., 2010). These data suggest that the presence of non-pathogenic bacterial species has a large influence on the ability of STEC to persist within the processing plant; due to the potential impact of these data, these findings merit further investigation.

The ability to secrete EPS is related to biofilm formation on stainless steel surfaces, but it was shown that overproduction of the EPS inhibits the initial attachment of *E. coli* O157:H7 (Ryu et al., 2004a). EPS production may also protect *E. coli* O157:H7 from sanitizer treatments (Ryu et al., 2004a; Ryu and Beuchat, 2005). As with curli, EPS production may not be essential for biofilm formation on stainless steel by bacterial pathogens, including STEC. In addition, it has been shown that bacteria producing little or no EPS, including *E. coli* O157:H7 could colonize a mature biofilm formed by EPS-producing bacteria (Castonguay et al., 2006; Klayman et al., 2009; Dourou et al., 2011). Although sanitizers are able to reduce or totally kill STEC within biofilms, it is possible that recolonization by STEC or other bacteria will be easier if cleaning protocols do not completely remove the biofilm matrix.

In addition to the protection offered by the biofilm matrix against sanitizers, it is well established that for *E. coli* in general, a slow-growing and dormant subpopulations are highly tolerant to antibacterial treatments (Lewis, 2010). Cells from this subpopulation are called multidrug tolerant persister cells and are dormant variants that emerged from regular cells (Lewis, 2010). The emergence of persister cells occurs at a higher frequency within biofilm populations than planktonic populations (Lewis, 2010). This non-heritable variation could permit STEC to survive the sanitation process and these individual cells could remain encased in the biofilm matrix. These cells could then contribute to the reestablishment of a STEC biofilm or population within the processing plant.

CAN STEC BIOFILMS BE REMOVED?

It is known that STEC biofilms are more resistant to sanitizers than their planktonic counterparts (Wang et al., 2012). In recent years, many studies have focused on cleaning and disinfection procedures using physical and chemical methods. Three primary chemical compounds are used as sanitizers in the food service industry: chlorine-based cleaners, quaternary ammonium, and iodine sanitizers. Because of the toxicity of sanitizer residues and/or increased bacterial resistance to these decontamination reagents (Stopforth et al., 2003; Houari and Di Martino, 2007; Marouani-Gadri et al., 2009b; Hou et al., 2010; Wang et al., 2012), alternative molecules that are preferentially natural with low human and animal toxicity are being tested for their effect on biofilms.

Many essential oils have been shown to have good antibiofilm activity against food-borne pathogens (Giaouris et al., 2013). Perez-Conesa et al. have shown that surfactant micelles loaded with eugenol or carvacrol, two essential oils isolated from clove and thyme, are able to kill *E. coli* O157:H7 inside a biofilm. However, the biofilm matrix remains attached to the surface (Perez-Conesa et al., 2006, 2011), making reformation of a biofilm a dangerous possibility. While essential oils target cell viability, the best way to remove and prevent reformation of a biofilm on a surface is to degrade the EPS surrounding the bacteria by enzymatic treatment (Gibson et al., 1999; Lequette et al., 2010). A combination of an antimicrobial agent to kill cells within the biofilm with a food-grade agent able to remove the entire biofilm matrix could be a solution to reduce and potentially remove *E. coli*

O157:H7 biofilms from processing plants. Others strategies such as bacteriophage treatments of *E. coli* O157:H7 biofilms have also been investigated. The KH1 bacteriophage reduces the population of O157:H7 cells attached to stainless steel, but not those incased within a biofilm matrix (Sharma et al., 2005). The effect of combined techniques such as steam and lactic acid (Ban et al., 2012), aerosolized sanitizers (Park et al., 2012), UV and dry heat (Bae and Lee, 2012) were also studied and have the potential to control STEC O157:H7 biofilms found on surfaces present in the food industry. The best approach for controlling STEC biofilm should kill *E. coli* O157:H7 within the biofilm and remove the biofilm matrix from the contaminated surface. For example, a combination of steam and lactic acid were able to kill *E. coli* O157:H7 and remove the biofilm matrix from stainless steel surfaces (Ban et al., 2012). Further studies should investigate the effect of antibiofilm molecules on the dispersal of biofilms and also focus on mixed biofilms containing both non-pathogenic and STEC bacteria.

CONCLUSION

Contamination of the environment and processing plants with cow feces containing STEC is a major concern for food and public safety, especially since STEC can survive for prolonged periods of time outside its host. Biofilm formation appears to contribute significantly to STEC survival on produce, in rivers, and in processing plants. Several factors involved in biofilm formation such as curli, cellulose, PGA, and colanic acid are involved in plant colonization and attachment to different surfaces often found in meat processing plants. However, the factors involved in STEC survival within biofilms in rivers remain unknown. Furthermore, STEC biofilm formation on farms, in manure, and in soil has not been thoroughly explored despite the presence and persistence of STEC in these environments. The Stx toxin, which is a key factor in human host pathology, also appears to be an important factor for STEC survival against protozoan predation. In the food industry, resistance to sanitizers improves the ability of STEC to persist in the processing plant. Despite the development of new strategies to eradicate biofilms formed by food-borne pathogens, no effective solutions to remove STEC biofilms from surfaces have been identified. Therefore, future research should focus on the identification of factors promoting STEC survival, especially non-O157 STEC, and the persistence of STEC in environmental biofilms on the farm.

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The importance of the viable but non-culturable state in human bacterial pathogens

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Many bacterial species have been found to exist in a viable but non-culturable (VBNC) state since its discovery in 1982. VBNC cells are characterized by a loss of culturability on routine agar, which impairs their detection by conventional plate count techniques. This leads to an underestimation of total viable cells in environmental or clinical samples, and thus poses a risk to public health. In this review, we present recent findings on the VBNC state of human bacterial pathogens. The characteristics of VBNC cells, including the similarities and differences to viable, culturable cells and dead cells, and different detection methods are discussed. Exposure to various stresses can induce the VBNC state, and VBNC cells may be resuscitated back to culturable cells under suitable stimuli. The conditions that trigger the induction of the VBNC state and resuscitation from it are summarized and the mechanisms underlying these two processes are discussed. Last but not least, the significance of VBNC cells and their potential influence on human health are also reviewed.

Keywords: VBNC, stress, resuscitation, virulence, human pathogens, biofilm, antibiotic

INTRODUCTION

CHARACTERISTICS OF VBNC CELLS

Cultivation is one of the most fundamental steps in microbiology, and the plate count technique is one of the standard cultivation methods for the enumeration of viable bacteria (Buck, 1979; Talaro et al., 2002). However, it was first discovered in 1982 that *Escherichia coli* and *Vibrio cholerae* cells could enter a distinct state called the viable but non-culturable (VBNC) state (Xu et al., 1982). Unlike normal cells that are culturable on suitable media and develop into colonies, VBNC cells are living cells that have lost the ability to grow on routine media, on which they normally grow (Oliver, 2000).

Despite their non-culturability on normally permissive media, VBNC cells are not regarded as dead cells because of various dissimilarities. Dead cells have a damaged membrane that is unable to retain chromosomic and plasmidic DNA, while VBNC cells have an intact membrane containing undamaged genetic information (Heidelberg et al., 1997; Cook and Bolster, 2007). The plasmids, if any, are also retained in VBNC cells (Oliver, 2010). While dead cells are metabolically inactive, VBNC cells are metabolically active and carry out respiration (Lleò et al., 2000; Besnard et al., 2002). High ATP level was found in *Listeria monocytogenes* even one year after entering the VBNC state (Lindbäck et al., 2010). Moreover, dead cells do not express genes, while VBNC cells continue transcription and therefore, mRNA production (Lleò et al., 2000). In contrast to dead cells that no longer utilize nutrients, VBNC cells were shown to have continued uptake and incorporation of amino acids into proteins (Lleò et al., 1998).

Although VBNC cells have many general characteristics as a kind of viable cells, they have a lot of physiological and molecular differences from the viable, culturable cells. These differences include cellular morphology, cell wall and membrane composition, metabolism, gene expression, physical and chemical resistances, adhesion properties and virulence potential. In terms of cellular morphology, a reduction in cell size and thus, an increase in the surface area to volume ratio is commonly found in VBNC cells (Rahman et al., 1994; Du et al., 2007), probably as a strategy to minimize energy requirements (Postgate, 1976; Biosca et al., 1996). Apart from cell dwarfing, cell rounding is also found in VBNC cells of many species (e.g., Adams et al., 2003; Cook and Bolster, 2007). *Campylobacter* spp. changes from the characteristic spiral shape in the exponential phase to a coccoid shape in the VBNC state (Thomas et al., 2002). *Burkholderia pseudomallei* and *V. cholerae* cells also changes from rods during exponential growth to cocci in the VBNC state (Inglis and Sagripanti, 2006; Senoh et al., 2010). These morphological changes are commonly found in VBNC cells, however, similar changes are also found in non-VBNC cells that live under stressful conditions, so a change in morphology alone cannot be used as the sole parameter to judge whether the cells are in VBNC state or not (Pinto et al., 2013).

VBNC cells also show marked differences in cell wall and membrane composition, including proteins, fatty acids and peptidoglycan. Rearrangement of the outer membrane subproteome observed in *E. coli* was highly dependent on the conditions used to induce the VBNC state. For example, differential expression of three outer membrane proteins (Omp), antigen 43 β-subunit, TolC, and OmpT, was found in cells exposed to nutrient-limited phosphate buffered saline (PBS), and the most drastic changes showing 106 proteins being modulated were found in those exposed to natural seawater and light (Muela et al., 2008). Significant increases in the percentage of unsaturated fatty acids and fatty acids with less than 16 carbons were found in *V. vulnificus* entering the VBNC state, and significant

changes in the percentage of hexadecanoic, hexadecenoic, and octadecanoic acids were found in the VBNC cells (Day and Oliver, 2004). Moreover, an increase in peptidoglycan cross-linking was observed in VBNC cells of *Enterococcus faecalis* (Signoretto et al., 2000).

VBNC cells have a lower metabolic rate (Shleeva et al., 2004) and a different gene expression profile compared to culturable cells growing in exponential phase. The expression of *ompW* was significantly induced in VBNC cells of *E. coli* (Asakura et al., 2008). In VBNC cells of *V. cholerae*, a study showed that 58 genes related to regulatory functions, cellular processes, energy metabolism as well as transport and binding were induced by more than 5-fold (Asakura et al., 2007a), while another study reported a reduction in 16S rRNA and the mRNA level of *tuf*, *rpoS*, and *relA*, genes responsible for protein synthesis and stress responses (González-Escalona et al., 2006).

In general, VBNC cells have higher physical and chemical resistance than culturable cells, which might be due to their reduced metabolic rate and a cell wall strengthened by increased peptidoglycan cross-linking (Signoretto et al., 2000). In terms of physical stress, VBNC cells of *V. vulnificus* are more resistant to mechanical destruction by sonication (Weichart and Kjelleberg, 1996), and those of *Mycobacterium smegmatis* are more resistant to high temperature (Anuchin et al., 2009). In terms of chemical stress, greater tolerance against low salinity and pH, ethanol, chlorine, and antibiotics was observed in VBNC cells of *V. parahaemolyticus* (Wong and Wang, 2004), *V. vulnificus* (Weichart and Kjelleberg, 1996), *Campylobacter jejuni* (Rowe et al., 1998) and *E. faecalis* (Lleò et al., 2007a), respectively. A recent study has shown that *V. vulnificus* in VBNC state has a higher resistance to a variety of challenges, including heat, low pH, ethanol, antibiotic, heavy metal, oxidative and osmotic stress, than those growing in exponential phase (Nowakowska and Oliver, 2013).

At this point it is necessary to differentiate between VBNC cells and persister cells. Persistence refers to the situation where a subpopulation is resistant to an antibiotic, whereas the bulk of the population is sensitive to this antibiotic. The persister cells are viewed as phenotypic variants in the population. To date, persister cells have only been characterized as being in a non-growing state and are antibiotic tolerant (Helaine and Kugelberg, 2014). In fact, VBNC cells have also been shown to be similarly antibiotic tolerant, as well as exhibiting tolerance to heavy metals, high and low temperature, high and low pH, oxidative and osmotic challenges, and ethanol (Nowakowska and Oliver, 2013). Thus, the distinction between "persisters" and "viable but non culturable" cells appears artificial, and while there has been much published in recent years on the former, the literature on the latter is voluminous, and to date there are no convincing studies indicating they are not the same, or at least variants, of the same phenomenon (Wood et al., 2013). The phenotypes and metabolic programs of persister cells have been discussed in a recent review, and will not be covered here (Amato et al., 2014).

VBNC cells can also show changes in adhesion and virulence properties. For example, VBNC cells of *C. jejuni* retain their ability of attachment to stainless steel (Duffy and Dykes, 2009), while *V. cholerae* has a lower rate of adhesion to human intestinal cells (Pruzzo et al., 2003) and *E. faecalis* is unable to attach to plastic surfaces and initiate biofilm formation (Lleò et al., 2007b). *L. monocytogenes* in a VBNC state cannot cause infection (Cappelier et al., 2005; Lindbäck et al., 2010). In *V. vulnificus*, the progressive reduction of virulence is time dependent (Oliver and Bockian, 1995). Nonetheless, some VBNC cells are still virulent and even cause fatal infections, which may be due to rapid resuscitation into culturable cells in suitable hosts (Oliver and Bockian, 1995; Baffone et al., 2003; Du et al., 2007). The alterations in virulence, adhesion as well as resistance of VBNC cells account for their significance to public health (discussed in section Significances).

THE OCCURRENCE OF VBNC CELLS AND THEIR IMPORTANCE

Many species of bacteria enter the VBNC state when they are exposed to stressful conditions such as starvation and low temperatures (e.g., Biosca et al., 1996; Du et al., 2007), suggesting that this is an adaptive strategy for long-term survival of bacteria under unfavorable environmental conditions (Ducret et al., 2014). This hypothesis is supported by some characteristics of VBNC cells, including higher resistance to exogenous stresses, the ability of long-term survival under stress and the ability of resuscitation. For example, VBNC cells of *V. parahaemolyticus* are more resistant to acidity, allowing them to survive in an adverse environment with low pH (Wong and Wang, 2004). VBNC cells of *V. fluvialis* are viable after six years of starvation, suggesting that bacteria in VBNC state can stay alive over long periods of time, even under continued stress (Amel et al., 2008). More importantly, many species have the ability to resuscitate from the VBNC state back to the culturable state when the stress is removed (e.g., Roszak et al., 1984; Bates and Oliver, 2004). The evidence presented above supports the hypothesis that the VBNC state is a quiescent form of life allowing the organism to wait for suitable conditions to revive (Lleò et al., 2007a). Another hypothesis suggests that the VBNC state is a transitory stage in the degeneration of bacterial population leading to cell death (Thomas et al., 2002; Nyström, 2003). Nonetheless, there is not much evidence supporting the later hypothesis, so the former is generally accepted.

The ability to enter the VBNC state may be advantageous for bacteria, but poses a risk to human health. If VBNC cells are present, the total number of viable bacteria in a sample will be underestimated by the CFU count method due to the inherent non-culturability of VBNC cells. Even worse, if all bacteria in the sample are in VBNC state, the sample may be regarded as germ-free due to non-detection. For bacterial species causing human infections, the underestimation or non-detection of viable cells in quality control samples from the food industry and water distribution systems, or clinical samples may pose serious risks to the public. The risks emerge from the fact that pathogenic bacteria can be avirulent in the VBNC state but regain virulence after resuscitation into culturable cells under suitable conditions (Du et al., 2007). This property of VBNC cells may lead to latency and subsequently, to the recurrence of disease in patients who were thought to be cured (Pai et al., 2000; Rivers and Steck, 2001). Therefore, it is of the utmost importance to understand what species of human pathogens can enter the VBNC state and apply reliable detection methods to quantify the accurate population of viable cells, including both culturable and VBNC cells.

Apart from this, the identification of conditions that can induce bacteria to enter VBNC state and the underlying mechanisms, as well as the understanding of resuscitation conditions and mechanisms are necessary to effectively prevent bacterial infections and cure infected patients. In this review, the detection, induction, resuscitation and significance of VBNC cells will be discussed.

DETECTION OF VBNC CELLS

The two key properties of VBNC cells are viability and non-culturability, so the presence and abundance of VBNC cells can be determined by comparing the number of viable cells to that of culturable cells in the sample. As a general rule, if the number of culturable cells drops to an undetectable level while the number of viable cells remains high, then the population in the sample has become VBNC cells. Therefore, the first major step for the detection of VBNC cells is the estimation of the remaining culturable cells in the sample by a conventional plate count technique. For this step, it is important to use a rich medium without any additional stress, because some cells that may have been injured during exposure to different VBNC-inducing stresses may be unable to grow on selective or differential media with antibiotics or other stresses. These injured cells have a higher sensitivity to growth medium components that are not normally inhibitory, but they are not regarded as VBNC cells as they are culturable on non-selective media (Pinto et al., 2013). Thus, ensuring the most favorable growth condition allows injured cells to be eliminated from the VBNC population. For instance, after exposure to cold temperature, a population of *V. vulnificus* that normally grows on heart infusion (HI) agar supplemented with 20 μl of 3% hydrogen peroxide (H_2O_2) was only able to grow on HI agar with one-tenth concentration of H_2O_2 or less (Bogosian et al., 2000). Similarly, under temperature stress and starvation, *C. jejuni* became non-culturable on Karmali agar, which is a selective medium that suppresses the growth of unwanted bacterial species, but it remained culturable on non-selective Columbia blood agar (Cools et al., 2003).

The second major step for the detection of VBNC cells is the estimation of viable cells. One common method involves the use of a differential staining procedure and direct microscopic enumeration. The LIVE/DEAD® *Bac*Light™ assay consists of two fluorescent dyes with different cell permeability characteristics that can be used to differentiate cells with different membrane integrities (Cunningham et al., 2009). The green fluorescent dye, SYTO®9, penetrates both intact and damaged membrane and thus, labels all cells. The red fluorescent stain, propidium iodide, can only penetrate damaged membranes and label the injured cells and dead cells (Hurst, 1977). As a result, injured cells and dead cells would appear red under an epifluorescence microscope with suitable filter, while the culturable cells and VBNC cells with an intact membrane would appear green. The concentration of each dye and the appropriate parameters must be empirically validated for each bacterial species using appropriate controls to effectively differentiate between live and dead cells. Flow cytometry can be used with LIVE/DEAD® stains to easily obtain quantitative results (Allegra et al., 2008).

A second method is the detection of gene expression by reverse transcription polymerase chain reaction (RT-PCR). Due to the short half-life of mRNA, a positive signal indicates the presence of mRNA and thus, presence of viable cells that carry out transcription (Adams et al., 2003). As reviewed by Trevors (2011), this method is commonly used in many bacterial species to determine the viability of cells. For example, mRNAs from four housekeeping genes and four virulence genes were detected in a sample of *V. parahaemolyticus* after a 15-day incubation in freshwater at 4°C when the culturability was completely lost, suggesting the presence of VBNC cells (Coutard et al., 2007). The gene expression profile also helps understanding the potential phenotypes of VBNC cells. For example, the *cadF* gene, which encodes an outer membrane protein responsible for fibronectin-binding, was found to be continuously expressed in VBNC cells of *C. jejuni* that maintained the ability of adhesion (Patrone et al., 2013). Some studies have recommended specific genes for the detection of viable cells, such as *rfbE* for *E. coli* (Yaron and Matthews, 2002) and *pbp5* for *E. faecalis* (Lleò et al., 2000).

Other approaches can also be used to detect viable cells. The DNase I protection assay, another molecular method, can distinguish viable cells from dead cells, as only the viable cells have intact membranes to protect genomic DNA from digestion by exogenous nucleases. Using this method, Pawlowski et al. (2011) successfully demonstrated the presence of VBNC cells in a sample of *Yersinia pestis* with an undetectable level of culturable cells.

The direct count of viable bacterial cells (DVC) was first described by Kogure et al. (1979). It was found that viable cells cultured in a rich medium with antibiotics do not replicate but elongate, while dead cells remain unchanged. This method is highly dependent on the antibiotic sensitivity of the bacteria, so the use of nalidixic acid as the antibiotic, as was originally reported, may not be suitable for Gram-positive bacteria and some Gram-negative species (Byrd et al., 1991). Instead, aztreonam can be used for *Cytophaga allerginae* and *Serratia marcescens* (Heidelberg et al., 1997), and ciprofloxacin can be used for *L. monocytogenes* (Besnard et al., 2000). After incubation with antibiotics, acridine orange or 4′,6-diamidino-2-phenylindole (DAPI) can be used to stain the cells and illustrate changes in cellular morphology under microscope (Besnard et al., 2000; Du et al., 2007). Since the mechanisms underlying cell elongation in the presence of antibiotics are not fully understood, and the response of different bacteria to antibiotics may vary (Rice et al., 2000), this method is not commonly used.

Since viable cells, but not dead cells, carry out metabolic reactions and respiration, they can also be detected by the *p*-iodonitrotetrazolium violet (INT) assay based on the activity of electron transport system (Rahman et al., 1994). INT is a soluble tetrazolium salt that can compete with oxygen as the final electron acceptor and be reduced to insoluble formazan in metabolically active cells. Therefore, the formation and accumulation of formazan in cells, which appear as dark red precipitates under microscope, indicate the presence of an active electron transport chain, a characteristic of viable cells (Altman, 1970; Zimmermann et al., 1978). Similarly, another tetrazolium salt, 5-cyano-2,3-ditolyltetrazolium chloride (CTC) or the *Bac*Light™ RedoxSensor™ Green can be used (Besnard et al., 2002; Lahtinen et al., 2008). Moreover, the luciferase assay can be used to estimate ATP generation in viable cells (Lindbäck et al., 2010).

Because viable cells incorporate nutrients, they can also be detected by monitoring the uptake and incorporation of radiolabeled amino acids into protein. ^{35}S-labelled methionine,^{3}H-labelled leucine and ^{35}S-labelled cysteine/methionine have been used to detect viable cells in *Shigella dysenteriae*, *E. faecalis* and *Y. pestis*, respectively (Rahman et al., 1994; Lleò et al., 1998; Pawlowski et al., 2011).

BACTERIA WITH A VBNC STATE

In the past 10 years, several reviews have presented findings of bacteria that can exist in a VBNC state. For instance, Rowan (2004) has focused on foodborne and waterborne bacteria, Oliver (2010) has focused on bacteria that are pathogenic to plants, animals or humans and Pinto et al. (2013) have discussed both pathogenic and non-pathogenic bacteria. Currently, up to 85 species of bacteria have been found to exist in VBNC state in different environmental habitats or experimental conditions. Of these 85 species, 18 species are non-pathogenic and 67 species are pathogenic. The non-pathogenic species were generally found in fermented beverages. Sixteen of the pathogenic species cannot infect humans but can infect other organisms, including plants (Alexander et al., 1999; Grey and Steck, 2001; del Campo et al., 2009), fish (Magariños, Romalde, Barja and Toranzo, 1994; Oliver, 2010), and marine invertebrates such as shrimp (Sun et al., 2008), oysters (Williams et al., 2009), corals (Banin et al., 2000; Israely et al., 2001) and sea urchins (Masuda et al., 2004).

In this review, we specifically focus on bacteria that can cause human infections. **Table 1** provides an overview of 51 human pathogens that have been reported to exist in a VBNC state, their inducing conditions, resuscitating conditions and the resuscitation window, if known. The list includes many true pathogens like *E. coli* and *Y. pestis* that cause disease in healthy individuals and may result in fatalities (Gourmelon et al., 1994; Pawlowski et al., 2011). It also includes some opportunistic human pathogens like *Aeromonas hydrophila* and *Agrobacterium tumefaciens* that mainly infect other organisms but also infect immunocompromised patients (Alexander et al., 1999; Rahman et al., 2001). Pathogenic bacteria that can enter a VBNC state have a broad phylogenetic distribution, suggesting that entering VBNC state may be a general strategy adopted by different lineages of bacteria to survive unfavorable conditions.

There is also broad environmental distribution of human pathogens that exist in a VBNC state. They are found in different kinds of water bodies, including seawater (Maalej et al., 2004; Dhiaf et al., 2008), estuarine water (Oliver et al., 1995), stream water (Lemke and Leff, 2006), lake water (Signoretto et al., 2004), ground water (Cook and Bolster, 2007), tap water (Pawlowski et al., 2011) and drinking water (Byrd et al., 1991). Moreover, VBNC cells of *Salmonella typhimurium* were found in soil (Reissbrodt et al., 2002) and those of *E. coli* were found in processed food (Makino et al., 2000). These findings support the idea that entering the VBNC state may be a common adaptive mechanism of bacteria inhabiting different, stressful environments, instead of a specific mechanism limited to bacteria living in a particular niche (Pinto et al., 2013).

INDUCTION OF THE VBNC STATE

CONDITIONS

Although VBNC bacteria may remain viable for long periods of time, these cells lose their ability to grow on classical culture media on which they would normally develop into colonies (Oliver, 2005). It was shown that cells enter the VBNC state as a response to an extensive list of both chemically and environmentally unfavorable conditions (Oliver, 2010), including nutrient starvation (Cook and Bolster, 2007), extreme temperatures (Besnard et al., 2002), incubation outside the pH or salinity ranges that are permissive to cell growth (Cunningham et al., 2009), elevated or lowered osmotic concentrations (Asakura et al., 2008; Wong and Liu, 2008), fluctuating oxygen concentrations (Kana et al., 2008; Mascher et al., 2000), exposure to heavy metals (Ghezzi and Steck, 1999; del Campo et al., 2009), exposure to food preservatives (Quirós et al., 2009) and exposure to white light and UV irradiation (Gourmelon et al., 1994). In addition, treatments normally assumed to be bactericidal may instead result in the induction of the VBNC state in a subpopulation, including pasteurization of milk (Gunasekera et al., 2002) and chlorination of wastewater (Oliver, 2005).

To date, no large-scale studies have been performed to compare the effects of diverse conditions on the induction of VBNC state. Such a study is required to provide new information on the conditions that would lead to faster VBNC state inductions. Pinto et al. (2011) suggested that in *E. coli*, the origin of the strains and the incubation temperature are key factors for the speed at which VBNC cells appear in a bacterial population.

REGULATORS OF THE VBNC STATE

Despite the fact that the formation and the morphological/physiological changes of the VBNC state have been well investigated (Barcina et al., 1997; Colwell and Grimes, 2000), little is known about the genetic control underlying this state. Since, there is a broad range of bacterial species that can enter the VBNC state, it is likely that there is also diversity in the regulatory mechanism. Understanding the molecular control of VBNC state would provide insight into its physiology, and may yield new information to develop novel detection methods or control methods for VBNC bacteria. To our knowledge, no systematic, large-scale screening of mutants for defects in VBNC state induction has ever been performed. Nevertheless, the regulators RpoS and OxyR seem to be important for the induction of VBNC state and are discussed below.

RpoS

A simple approach to identify VBNC genes consists of testing the influence of genes known to be involved in the response to other stressful environmental conditions, upon entry into VBNC state and during the survival of VBNC cells (McDougald et al., 2001). This led Boaretti et al. (2003) and Kusumoto et al. (2012) to analyse the involvement of the major stress regulator RpoS, a sigma factor essential for survival in the stationary phase and the general stress response (Lange and Hengge-Aronis, 1991; Hengge-Aronis, 1993). Indeed, RpoS depletion resulted in faster VBNC state induction in *E. coli* and *Salmonella* spp. (Kusumoto et al., 2012). The parental strain of *E. coli* became non-culturable in 33 days in

Table 1 | The species of human pathogens with a proven VBNC state.

Species	VBNC state inducing factor	Resuscitation condition	Resuscitation window	References
Acinetobacter calcoaceticus	Starvation			Lemke and Leff, 2006
Aeromonas hydrophila	Starvation	Temperature upshift		Rahman et al., 2001; Maalej et al., 2004
Agrobacterium tumefaciens	Starvation, chemicals (copper)			Byrd et al., 1991; Alexander et al., 1999
Arcobacter butzleri	Starvation	Rich medium, NOT temperature upshift	270 days	Fera et al., 2008
Bacillus cereus	Pulsed electric field			Rowan, 2004
Burkholderia cepacia	Starvation			Lemke and Leff, 2006
Burkholderia pseudomallei	Low pH, high temperature, osmotic pressure			Reviewed by Inglis and Sagripanti, 2006
Campylobacter coli	Starvation, low pH, low temperature	Embryonated chicken eggs, NOT rich medium		Thomas et al., 2002; Chaveerach et al., 2003
Campylobacter jejuni	Starvation, low pH, low temperature	Rich medium, rich medium with gas mixture, mouse intestine, embryonated chicken eggs	15 days	Bovill and Mackey, 1997; Thomas et al., 2002; Chaveerach et al., 2003; Cools et al., 2003; Baffone et al., 2006; Cook and Bolster, 2007
Campylobacter lari	Starvation, low temperature			Thomas et al., 2002
Citrobacter freundii	Starvation, high temperature	Rich medium with/ without enterobacterial autoinducer	11 years	Reissbrodt et al., 2002; Dhiaf et al., 2008
Cytophaga allerginae	Aerosolization			Heidelberg et al., 1997
Edwardsiella tarda	Starvation, low temperature	Rich medium with temperature upshift, embryonated chicken eggs		Du et al., 2007
Enterobacter aerogenes	Starvation			Byrd et al., 1991
Enterobacter agglomerans	High temperature	Rich medium with enterobacterial autoinducer		Reissbrodt et al., 2002
Enterobacter cloacae				Oliver, 2010
Enterococcus faecalis (Streptococcus faecalis)	Starvation, low temperature	Rich medium with temperature upshift, embryonated chick eggs, NOT agar with sodium pyruvate/ beef liver catalase/ superoxide dismutase	60 days	Byrd et al., 1991; Lleò et al., 1998, 2001
Enterococcus faecium	Starvation, low temperature	Rich medium, NOT agar with sodium pyruvate/beef liver catalase/ superoxide dismutase	7 days	Lleò et al., 2001
Enterococcus hirae	Starvation, low temperature	Rich medium, NOT agar with sodium pyruvate/ beef liver catalase/ superoxide dismutase	60 days	Lleò et al., 2001
Escherichia coli	Starvation, light, oxidative stress, high temperature, chemicals (chlorination)	Rich medium with enterobacterial autoinducer, minimal medium with amino acids, supernatant of active growing culture, temperature upshift		Gourmelon et al., 1994; Reissbrodt et al., 2002; Oliver et al., 2005; Cook and Bolster, 2007; Asakura et al., 2008
Francisella tularensis				Oliver, 2010
Haemophilus influenzae				Ehrlich et al., 2002
Helicobacter pylori	Starvation	NOT heat shock, NOT agar with catalase		Adams et al., 2003

(Continued)

Table 1 | Continued

Species	VBNC state inducing factor	Resuscitation condition	Resuscitation window	References
Klebsiella aerogenes				Oliver, 2010
Klebsiella planticola	Aerosolization			Heidelberg et al., 1997
Klebsiella pneumoniae	Starvation			Byrd et al., 1991
Legionella pneumophila	Starvation, chemicals (disinfectants NaOCl and NH_2Cl), *Hartmannella vermiformis* supernatant	Amoebae		Steinert et al., 1997; García et al., 2007; Alleron et al., 2008; Buse et al., 2013
Listeria monocytogenes	Starvation, low pH, low temperature, low salinity, chemicals (food preservatives), light, pulsed electric field	NOT rich medium with/ without sodium pyvurate		Besnard et al., 2002; Rowan, 2004; Cunningham et al., 2009; Lindbäck et al., 2010
Micrococcus luteus	Starvation	Rich medium, supernatant of active growing culture	6 months	Mukamolova et al., 1998b
Micrococcus varians (Kocuria varians)				Oliver, 2005
Mycobacterium smegmatis	Starvation, oxygen limitation, altered temperature	Rich medium, supernatant of active growing culture, Rpf		Kuznetsov et al., 2004; Shleeva et al., 2004
Mycobacterium tuberculosis	Starvation, oxygen limitation	Rich medium with catalase	3.5 months	Downing et al., 2005
Pseudomonas aeruginosa	Starvation, low temperature, chemicals (copper)	Temperature upshift, rich medium with copper chelator		Leung et al., 1995; Dwidjosiswojo et al., 2011
Pseudomonas putida	Starvation			Lemke and Leff, 2006
Salmonella enteritidis	Starvation	Rich medium	<21 days	Roszak et al., 1984
Salmonella oranienburg	Starvation, salinity	Rich medium with Rpfs, supernatant from active growing culture		Panutdaporn et al., 2006
Salmonella typhi	Starvation			Cho and Kim, 1999
Salmonella typhimurium	Starvation, low temperature, light, chemical (chlorination)	Rich medium with enterobacterial autoinducer, heat shock, NOT agar with catalase/ temperature upshift		Caro et al., 1999; Reissbrodt et al., 2002; Gupte et al., 2003; Oliver et al., 2005
Serratia marcescens	Aerosolization			Heidelberg et al., 1997
Shigella dysenteriae				Oliver, 1995
Shigella flexneri				Oliver, 1995
Shigella sonnei				Oliver, 1995
Staphylococcus aureus				Zandri et al., 2012
Staphylococcus epidermidis				Zandri et al., 2012
Vibrio alginolyticus				Du et al., 2007
Vibrio cholera	Starvation, low temperature	Human intestine, eukaryotic cell lines, rabbit intestine	110 days	Colwell et al., 1985, 1996; Senoh et al., 2010
Vibrio fluvialis	Starvation	Rich medium	6 years	Amel et al., 2008
Vibrio mimicus				Oliver, 1995
Vibrio parahaemolyticus	Starvation, low temperature, low salinity	Temperature upshift	2 weeks	Bates and Oliver, 2004; Wong and Wang, 2004; Wong et al., 2004
Vibrio vulnificus (type 1 & 2)	Starvation, low temperature	Rich medium, temperature upshift, mice, clams	3 days	Nilsson et al., 1991; Oliver and Bockian, 1995; Oliver et al., 1995; Biosca et al., 1996; Wong and Liu, 2008
Yersinia pestis	Starvation, low temperature	Rich medium		Pawlowski et al., 2011

The inducing factors, resuscitating factors, as well as the longest resuscitation window reported are shown.

an artificial oligotrophic medium incubated at 4°C, whereas the *rpoS* mutant lost its culturability in only 21 days (Boaretti et al., 2003). Moreover, lack of *rpoS* resulted in a decreased ability to stay in VBNC state for long periods of time, resulting in faster cell death (Boaretti et al., 2003). Smith and Oliver (2006) reported that *V. vulnificus* continues to express *rpoS* in the VBNC state, suggesting that the stress-related genes regulated by *rpoS* are also likely to be expressed in VBNC state and lead to cross-protective effects. For example, once the bacteria encounter a stress, they produce a variety of proteins that serve to enhance survival under other stresses (Rangel, 2011).

In *E. coli*, RpoS mediates the expression of 10% of the genome upon exposure to stressful conditions (Gentry et al., 1993). It has been shown that induction of the stress response governed by RpoS involves the production of the alarmone guanosine tetraphosphate or pentaphosphate, ppGpp and pppGpp respectively [termed together as (p)ppGpp], and results in an increase in the amount of RpoS (Gentry et al., 1993). (p)ppGpp is a general indicator of the nutritional status of the cell and it lies on top of the regulatory network (Cashel et al., 1996). In Beta- and Gammaproteobacteria, intracellular levels of (p)ppGpp are modulated by the RelA and SpoT proteins. RelA is a monofunctional alarmone synthase, and SpoT is a bifunctional synthase/hydrolase (Potrykus and Cashel, 2008). Both RelA and SpoT enzymes can synthesize (p)ppGpp, whereas SpoT can also hydrolyze it (Chatterji et al., 1998). It has been shown that, like the *rpoS* mutant, lack of (p)ppGpp production resulted in faster VBNC state induction (Boaretti et al., 2003). Overproducers of (p)ppGpp displayed viability comparable to that of Δ*rpoS* complemented strains or the wild-type strain (Boaretti et al., 2003). These findings suggest that the level of (p)ppGpp may play a crucial role in the accumulation of RpoS, ultimately leading to enhanced stress resistance in VBNC cells. In *V. cholerae*, the entry into VBNC state changes the expression of *relA* which, in turn, modulates the (p)ppGpp level (González-Escalona et al., 2006; Asakura et al., 2007a). This may lead to further modulation of several major systems, including stress-response, replication and virulence systems. It is noteworthy that ppGpp also play a role in the production of persister cells as well (Amato et al., 2014).

OxyR

Cuny et al. (2005) suggested that reactive oxygen species (ROS) play a role in the formation of VBNC cells. When *E. coli* was subjected to H_2O_2 treatment, they entered the VBNC state (Arana et al., 1992). In addition, VBNC *E. coli* shows decreased superoxide dismutase activity, resulting in increased oxidative damage (Desnues et al., 2003). This suggests that regulation of the oxidative stress response is involved in the induction of VBNC state.

OxyR is a LysR-type transcriptional regulator and was first identified in *S. typhimurium* (Christman et al., 1985). It has a characteristic N-terminal DNA-binding domain, and is known to regulate oxidative stress-related genes (Tao et al., 1991; Wei et al., 2012). In *V. vulnificus*, mutation of *oxyR* results in loss of culturability after exposure to cold temperature, which can be relieved by cultivation under anaerobic conditions or with an exogenous supply of catalase (Kong et al., 2004). This study attributed the cold-induced loss of catalase activity to the induction of VBNC state, explaining why *Vibrio* spp. are almost undetectable during the winter months but re-emerge during the summer months, when temperature and nutrient level in seawater increase. In addition, two recent studies have investigated the role of two antioxidative enzymes, the alkyl hydroperoxide reductase subunit C (AhpC) (Wang et al., 2013) and the glutathione S-transferase (GST) (Abe et al., 2007), in the induction and maintenance of the VBNC state in *V. parahaemolyticus* and *V. vulnificus*, respectively (discussed in section Effectors Related to the Oxidative Stress Response). Knowing that OxyR regulates the expression of *ahpC* in several Gram-negative bacteria in response to elevated ROS levels (Charoenlap et al., 2005; Hishinuma et al., 2006), and more recently, the expression of *gst* (*Abgst01*) in *Acinetobacter baumannii* in the presence of organic peroxides (Longkumer et al., 2014), we suggest that OxyR may be involved in the regulatory pathway leading to the induction of VBNC state. Direct evidence, such as quantification of ROS and antioxidative activities during the induction of VBNC state in the wild type and the *oxyR* mutant, is needed to further define the role of ROS in this state.

EFFECTORS OF THE VBNC STATE

Effectors related to the metabolic pathways to obtain energy

One line of research involves detecting proteins present exclusively in cells in the VBNC state (Heim et al., 2002; de Angelis and Gobbetti, 2004). Heim et al. (2002) studied changes in the proteome of *E. faecalis* in VBNC state after a 20-day incubation in lake water at 4°C. Significant differences in protein profiles were observed among the exponentially growing cells, starved cells and VBNC cells. The proteomic analysis showed a significant down regulation of important proteins during the VBNC state: putative proteins enolase (glycolysis), ATP synthase (oxidative phosphorylation) and a homolog of the *Staphylococcus aureus* elongation factor EF-Tu (involved in protein synthesis, cell growth regulation and stress response). The repression of these genes seems in agreement with the decrease in metabolic activity observed in VBNC cells. On the other hand, three proteins were overexpressed in VBNC cells: a putative catabolite regulator protein (CAA09491), a homolog of the *Listeria innocua* elongation factor EF-Ts, and a homolog of the *Streptococcus pneumoniae* fructose bisphosphate aldolase. In this study, the genetic pathways of *E. faecalis* underlying the VBNC response appear to be, in part, the same as those leading to the starvation response, as indicated by the presence of similar expression profiles for certain proteins like enoyl-ACP reductase (phospholipid biosynthesis). Nonetheless, significant differences were also observed in the protein profiles between starved cells and VBNC cells. A protein homologous to the mannose-specific phosphotransferase system (PTS) of *Clostridium acetobutylicum* was only up-regulated in starved cells, and an unidentified protein (30 kDa at pI 6) was only detected in VBNC cells. While the specific function of this unidentified protein is still unclear, a comparison of 2D gels obtained from different cell states suggests that it may be a modified form of enoyl-ACP reductase.

In *E. coli* O157:H7, entry into the VBNC state was observed after 48 h of incubation in PBS containing 0.05% H_2O_2. Under this condition, 11% of the population maintained its membrane integrity. Incubation for 72 h led to a significant reduction in

the number of cells with an intact membrane (Asakura et al., 2007b). The elongation factor EF-Tu, which was under-expressed in *E. faecalis* (Heim et al., 2002), maintained its expression level in *E. coli*, suggesting the maintenance of protein synthesis in the latter (Asakura et al., 2007b). The differences in the expression level of elongation factors between *E. coli* and *E. faecalis* suggest that the species as well as stress conditions are key factors in determining the mechanism involved in the induction of VBNC state.

A second line of research involves comparing the global transcription pattern of VBNC cells with that of cells grown in a rich medium (Asakura et al., 2007a,b). The transcriptomic analysis of *V. vulnificus* VBNC cells following incubation in artificial seawater at 4°C for 70 days has highlighted the induction and repression of hundreds of genes compared to stationary phase cells (Asakura et al., 2007a). The genes whose expression is significantly affected in the VBNC state are classified into the following functional groups: protein synthesis, energy metabolism, cell envelope, cellular processes, regulatory function, amino acid synthesis, and transport proteins. The genes expressed in the VBNC state include those involved in metal ion (iron, magnesium, potassium and cobalamin) transportation, chemotaxis and motility, pilus assembly and chitin utilization. All together, these findings indicate that both the proteomic and transcriptomic profiles of VBNC cells are different from those of cells that are either starved or exponentially growing. This demonstrates that the VBNC state constitutes a physiologically distinct state within the life cycle of a bacterium, which is activated in response to environmental stresses.

Effectors related to the oxidative stress response

The involvement of antioxidative factors in the VBNC state has been investigated (Bogosian et al., 2000; Wai et al., 2000). The addition of catalase or other ROS scavengers, such as sodium pyruvate, to the culture medium improves the culturability of VBNC cultures of *A. hydrophila* (Wai et al., 2000), *E. coli* (Mizunoe et al., 1999), and *V. vulnificus* (Bogosian et al., 2000).

Recently, Wang et al. (2013) investigated the antioxidative activities of alkyl hydroperoxide reductase subunit C (*ahpC1* and *ahpC2*) against H_2O_2 and organic peroxide in *V. parahaemolyticus* in the context of the VBNC state and found that *ahpC2* alone played a significant role in the induction and maintenance of the VBNC state at 4°C. While an earlier study found an enhanced quantity of AhpC2 protein in the VBNC cells (Lai et al., 2009), the latest study showed that the transcripts of both *ahpC1* and *ahpC2* decreased to low levels by approximately three orders of magnitude during the induction of the VBNC state (Wang et al., 2013). The protective function of AhpC2 at 4°C was higher than that of AhpC1. Indeed, the time required to induce and maintain the VBNC state at 4°C in a modified Morita mineral salt solution with 0.5% NaCl in an *ahpC2* mutant and an *ahpC1ahpC2* double mutant was significantly shorter than that for the parental strain and the *ahpC1* mutant (Wang et al., 2013). Complementation with an *ahpC2* gene reversed the effects of the *ahpC2* mutation by increasing the time required for induction and maintenance of the VBNC state (Wang et al., 2013).

GST is another protein related to the oxidative stress response and is also involved in the VBNC state. It is a cytosolic protein that detoxifies endogenous compounds, such as peroxidized lipids, by conjugation with reduced glutathione (Marrs, 1996). In 2007, Abe et al. showed that a mutation causing over-expression of GST was responsible for the suppression of the ability to enter the VBNC state in *V. vulnificus* (Abe et al., 2007). Since GST is linked to the antioxidative response, the authors hypothesized that the induction of VBNC state is directly connected to oxidative stress, and that removing this stress would abolish the entry into VBNC state. This hypothesis was supported by Oliver (2010), who showed an important role of the *V. vulnificus* catalase KatG in its VBNC state. In this bacterium, expression of *katG* is repressed upon the entry into VBNC state, but is induced when cells exit the VBNC state and become culturable again.

Effectors related to the outer membrane proteins

In Gram-negative bacteria, the outer membrane is an important physical and functional barrier separating the inside of cells and their surrounding environment. It consists of phospholipids, lipopolysaccharides and outer membrane proteins (OMPs) (Koebnik et al., 2000). Some OMPs, called porins, allow the passive diffusion of small, charged and uncharged molecules into bacterial cells (Nikaido and Vaara, 1985). In *E. coli*, the major outer membrane proteins are OmpF and OmpC (Misra and Reeves, 1987). Changes in the amount of OMPs in response to environmental stimuli have a major consequence on the survival of *E. coli* in stressful conditions, including starvation, changes in osmolarity and the VBNC state (Nikaido and Vaara, 1985; Özkanca and Flint, 2002; Darcan et al., 2009). In *E.* coli, the highest number of cells entering the VBNC state was found in an *ompCompF* double mutant strain compared to the wild-type and single mutants (Darcan et al., 2009). This is consistent with the phenotype of the *ompCompF* double mutant in *S. typhimurium* that lost culturability and entered the VBNC state when subjected to oxidative stress (Ozkanca et al., 2002).

The EnvZ/OmpR system regulates expression of the OmpF and OmpC proteins (Russo and Silhavy, 1991). OmpR is a cytoplasmic DNA-binding protein (Aiba et al., 1989). EnvZ is an environmental sensor bound to the inner membrane and has both kinase and phosphatase activities (Forst et al., 1987; Igo and Silhavy, 1988). Darcan et al. (2009) showed that the loss of the EnvZ protein has no effect on survival, but prevents the organism from sensing the changes in environment and thus, interfering with the entry into VBNC state. Indeed, *envZ* mutants were found not to enter the VBNC state and stayed culturable for a longer period of time. Nonetheless, it is not clear if this phenotype is specific to the VBNC induction condition which, in this case, was osmotic stress.

RESUSCITATION OF VBNC CELLS

DETERMINATION OF THE RESUSCITATION

It is important to note that bacteria that enter the VBNC state may become culturable again, and thus this state may be reversible. The term "resuscitation" was first used by Roszak et al. (1984) to describe the recovery of non-culturable cells of *S. enteritidis* subsequent to the addition of HI broth. Two decades later, Baffone et al. (2006) defined resuscitation as the reversal of metabolic and physiological changes that characterize VBNC cells. Of the

species of human pathogens that can enter the VBNC state, resuscitation has been reported in only 26 species (17 genera) (**Table 1**). Resuscitation of these species was triggered by a variety of stimuli, such as an increase in temperature, increase in nutrients concentration, and the presence of host cells (discussed in section Factors Stimulating Resuscitation).

The first roadblock that researchers encountered when performing resuscitation studies is the difficulty to differentiate between the resuscitation of VBNC cells and the normal growth of residual culturable cells in a sample. To date, there are no readily available methods to distinguish between culturable cells that arise from resuscitation and those from normal growth after exposure to the stimuli. Therefore, the ideal timing to study resuscitation is when there are no more culturable cells present in the sample. Although the complete absence of culturable cells cannot be guaranteed because of the detection limits, resuscitation experiments are usually conducted after the number of culturable cells drops to an undetectable level, while the number of VBNC cells remains high (Biosca et al., 1996; Cappelier et al., 1999; Chaveerach et al., 2003; Downing et al., 2005). Some studies have carried out extra steps to minimize the number of culturable cells in the sample. For instance, Whitesides and Oliver (1997) performed serial dilutions to further reduce the proportion of culturable cells, and Lleò et al. (1998) used minimal inhibitory concentrations of antibiotics to kill the remaining culturable cells, but not the more resistant VBNC cells before starting resuscitation. Nonetheless, the use of antibiotics is not recommended, as it was found that antibiotics can prohibit the resuscitation of *E. faecalis* from the VBNC state by influencing cell wall biosynthesis (Lleò et al., 2007a).

FACTORS AFFECTING RESUSCITATION

Resuscitation has only been proven in half of the human pathogens that exist in the VBNC state, but this does not mean the others cannot be resuscitated at all. It is likely that the resuscitation requirements for the latter were never met. For example, Chaveerach et al. (2003) were able to resuscitate *C. jejuni* by incubating the VBNC cells in embryonated chicken eggs but not in rich medium, showing that the condition or stimulus required for resuscitation could be very specific. In fact, many factors can influence the successfulness of resuscitation, such as the strain used, the age of VBNC cells, the conditions that induced the VBNC state and, of course, the conditions provided for resuscitation (Pinto et al., 2011). A comprehensive study done by Pinto et al. (2011) clearly demonstrated the effects of these four factors on the resuscitation of *E. coli*. Their study showed that different strains of *E. coli* in a VBNC state can be resuscitated in different media, indicating that the resuscitation process is highly dependent on the strains and resuscitating conditions. It was also found that resuscitation only occurs within a limited period of time after entry into the VBNC state, so it is dependent on the age of VBNC cells. Furthermore, this study demonstrated that cells that were induced into a VBNC state at 4°C could be resuscitated by adding rich media, but those induced at room temperature could not be resuscitated by any of the 41 media tested, indicating that resuscitation also depends on the inducing conditions. Apart from the direct effects on resuscitation, the strain of bacteria may also interact with other factors regulating resuscitation. For example, the strain of *V. parahaemolyticus* can affect the period of time that the VBNC cells remain resuscitable (Wong et al., 2004), and the strain of *S. typhimurium* can affect the conditions required for resuscitation (Reissbrodt et al., 2002).

Pinto et al. (2013) first introduced the term "resuscitation window," which is defined as the period of time in which VBNC cells maintain their ability to resuscitate under suitable stimuli. When exposed to the inducing condition, different cells in a sample enter a VBNC state at different times. It was hypothesized that VBNC cells of the same species have a fixed resuscitation window, so the older VBNC cells will lose their resuscitation ability earlier than the younger VBNC cells, resulting in a reduction of total resuscitable cells over time. This is supported by Senoh et al. (2010), who reported a gradual reduction in the number of resuscitable cells from 74 to 91 days after *V. cholerae* entered a VBNC state. A similar reduction in the number of resuscitable cells over time in a VBNC population was also observed in *E. faecalis* and *E. hirae* (Lleò et al., 2001).

In most published studies, the cells' ability of resuscitation is tested right after the whole population has become VBNC. Therefore, not much information is available on the length of resuscitation window of different bacteria. Some studies report the successful resuscitation of VBNC cells at a particular age but do not repeat the resuscitation experiment at later time points, so the reported resuscitation window may be underestimated (Oliver and Bockian, 1995; Downing et al., 2005; Amel et al., 2008). There are only a few studies that have tried to determine the exact resuscitation window by studying the resuscitation of VBNC cells at different ages. Fera et al. (2008) investigated the VBNC cells of *Arcobacter butzleri* at four different ages and deduced that cells younger than 270 days can be resuscitated by addition of rich medium. Mukamolova et al. (1998b) found that 3-month-old and 6-month-old VBNC cells of *Micrococcus luteus* can be resuscitated within two months and 10 days, respectively, suggesting that the resuscitation window of this species is around six months. According to the available information, there is great variation in the resuscitation windows between different species (**Table 1**), ranging from 4 days in *S. enteritidis* (Roszak et al., 1984) to 11 years in *Citrobacter freundii* (Dhiaf et al., 2008).

FACTORS STIMULATING RESUSCITATION

Rich medium was the first stimulus found to resuscitate VBNC cells in *S. enteritidis* (Roszak et al., 1984). Since then, additional studies have examined a variety of stimuli that can trigger resuscitation. It was found that resuscitation can be mediated by a physical stimulus like temperature upshift (Maalej et al., 2004), and different kinds of chemical stimuli including gas mixtures (Bovill and Mackey, 1997), amino acids (Pinto et al., 2011), rich media (Amel et al., 2008), supernatant from actively growing cultures (Mukamolova et al., 1998b) or compounds secreted by actively growing cells (Reissbrodt et al., 2002; Panutdaporn et al., 2006), as well as the presence of host cells (Chaveerach et al., 2003). As mentioned previously, these stimuli may not be applicable to resuscitate all species or even the same species in different trials because of other interacting factors. For instance, temperature upshift can resuscitate VBNC cells of *Pseudomonas*

aeruginosa (Leung et al., 1995) but not *A. butzleri* and *Helicobacter pylori* (Adams et al., 2003; Fera et al., 2008). Rich medium resuscitated *C. jejuni* in the study of Cools et al. (2003) but not in other studies (Chaveerach et al., 2003; Cook and Bolster, 2007). Among the human pathogens, *C. jejuni*, *E. coli*, and *V. vulnificus* are the three species that have been resuscitated by the greatest variety of stimuli (**Table 1**).

Interestingly, several studies have reported the unsuccessful resuscitation of VBNC cells after adding sodium pyruvate, catalase or superoxide dismutase onto agar plates (Lleò et al., 2001; Adams et al., 2003; Gupte et al., 2003; Lindbäck et al., 2010). These antioxidants can neutralize or prevent the formation of ROS such as H_2O_2 in the medium. These findings further prove that VBNC cells, in contrast to injured cells, do not lose their culturability solely because of sensitivity to ROS (Lleò et al., 2001). Some studies seem to contradict this view, showing that non-culturable cells can be recovered when agar plates are supplemented with ROS scavengers. For example, *S. typhimurium* was recovered by ferrioxamine E and oxyrase (Reissbrodt et al., 2002), *V. vulnificus* was recovered by catalase and pyruvate (Oliver, 2010), and *Legionella pneumophila* was recovered by pyruvate and glutamate (Ducret et al., 2014). However, further analysis of these studies show that the antioxidants only recovered a subpopulation (P1) of the non-culturable cells. In *S. typhimurium*, another subpopulation (P2) of non-culturable cells, which could not be recovered by supplementing with antioxidants, was resuscitated by another well-known resuscitation stimulus, an autoinducer (discussed in section Mechanisms of Resuscitation). These results suggest that P1 is composed of injured cells and P2 is composed of VBNC cells, so the increase in the number of culturable cells on antioxidant-supplemented agar was probably due to the recovery of injured cells instead of the resuscitation of VBNC cells.

MECHANISMS OF RESUSCITATION

Host cells

The mechanisms underlying the resuscitation of VBNC cells remain largely unknown, especially for those triggered by host cells due to complicated bacteria-host interactions that are likely to play a major role. Bacteria in the VBNC state have been found to be resuscitated by amoebae (García et al., 2007), eukaryotic cell lines (Senoh et al., 2010), clams (Birbari et al., 2000), embryonated chicken eggs (Cappelier et al., 1999), mice (Baffone et al., 2006), rabbits (Colwell et al., 1985) and human volunteers (Colwell et al., 1996). These cells or animals are typically the natural host of the resuscitated bacteria. For example, the two species of amoeba, *Acanthamoeba castellanii* and *A. polyphaga*, that were shown to resuscitate VBNC cells of *L. pneumophila*, are the natural hosts of this bacterium (Steinert et al., 1997; García et al., 2007). Within the list of potential hosts, embryonated chicken egg can resuscitate the most species of VBNC cells, including *Campylobacter coli* (Chaveerach et al., 2003), *C. jejuni* (Cappelier et al., 1999), *Edwardsiella tarda* (Du et al., 2007) and *E. faecalis* (Lleò et al., 1998). This may be because of the high nutrient content of the yolk sac and/or the warm temperature during incubation.

Removal of stress and presence of specific compounds

Apart from resuscitation triggered by host cells, two requirements were proposed as being crucial for the occurrence of resuscitation. The first is the removal of external stress. It is known that bacteria enter a VBNC state under stressful conditions such as starvation and cold temperature, the two most common inducers (**Table 1**). Therefore, the addition of rich medium and/or the upshift of incubating temperature may allow the resuscitation of VBNC cells through elimination of the existing stress. Optimal medium concentration and incubation temperature are required for successful resuscitation. For starvation-induced VBNC cells of *M. luteus*, the proportion of cells being resuscitated depends strongly on the concentration of yeast extract in the medium. The optimal concentration depends on the age of VBNC cells, and a high concentration of yeast extract may damage the membranes of resuscitated cells, and consequently, affect their culturability (Mukamolova et al., 1998b). Similar to medium concentration, a temperature that is too high may prohibit resuscitation, as VBNC cells of *V. parahaemolyticus* that were induced by low temperature could be resuscitated at 22°C but not at 37°C (Wong et al., 2004). In contrary, more resuscitation of VBNC *E. coli* was observed at 37°C than at 25°C (Pinto et al., 2011), suggesting that the optimal temperature is species-dependent. Another evidence supporting the importance of stress removal in resuscitation was presented by Dwidjosiswojo et al. (2011). In their study, the VBNC cells of *P. aeruginosa* that were induced by copper ions were fully resuscitated in a solution containing the copper chelator diethyldithiocarbamate within 14 days.

The second hypothesis explaining resuscitation triggers is the presence of specific compounds as a signal. The compounds that were found to resuscitate VBNC cells include amino acids, resuscitation-promoting factors (Rpfs) and autoinducers (Mukamolova et al., 1998a; Reissbrodt et al., 2002; Pinto et al., 2011). Pinto et al. (2011) proposed that the resuscitation of VBNC cells is somehow similar to the germination of dormant spores, which can be triggered by specific amino acids (Atluri et al., 2006). Therefore, they tested the ability of VBNC *E. coli* to resuscitate under a minimal medium supplemented with different amino acids, and found that a combination of leucine, glutamine, methionine and threonine would be sufficient to trigger resuscitation of strain Eco3 (Pinto et al., 2011). It was suggested that these amino acids may bind to receptors on the cell surface or be transported into the cells to initiate resuscitation (Pinto et al., 2013).

Rpfs. Rpf was first described by Mukamolova et al. (1998a) as a protein produced by *Micrococcus luteus* that can stimulate bacterial growth by reducing the lag phase and resuscitating VBNC cells in picomolar concentrations. This extracellular protein has been purified from the supernatant of *M. luteus* cultures growing in lactate minimal medium. It was found to have cross-species activity as it can affect the growth of five different *Micrococcus* species. Shleeva et al. (2004) have demonstrated the importance of Rpf by various methods. They successfully resuscitated the VBNC cells of *Mycobacterium smegmatis* by adding recombinant Rpf protein produced by *M. luteus*, co-culturing with *M. luteus* that secretes Rpf, as well as inserting a plasmid containing the *M.*

luteus rpf gene for endogenous synthesis of Rpf. This study further suggested that *M. smegmatis* can produce its own Rpf-like protein, as the supernatant from a growing culture was able to resuscitate its VBNC cells.

In contrast to these two species, *Mycobacterium tuberculosis* produces five Rpf-like proteins, which are encoded by *rpfA* to *rpfE*. These genes were thought to be functionally redundant and dispensable for growth, as the proteins seemed to have similar characteristics and properties (Mukamolova et al., 2002) and the deletion of all five genes did not affect the growth in rich medium (Kana et al., 2008). However, these five *rpf* genes were expressed differently during exponential growth, exposure to stress and early resuscitation, suggesting they play different roles in bacterial growth and survival (Gupta et al., 2010). Moreover, the deletion of three *rpf* genes prohibited the growth in mice as well as the resuscitation, demonstrating their functions cannot be compensated by the other two *rpf* genes, which were still present in this mutant strain (Downing et al., 2005). Kana et al. (2008) also reported that different combinations of *rpf* gene deletions result in distinct changes to colony formation on agar plates, sensitivity to the detergent sodium dodecylsulphate and virulence in mice. All these findings suggest that the five Rpf proteins produced by *M. tuberculosis* are only partially redundant.

Three models have been proposed to explain the mechanism underlying Rpf-mediated resuscitation (Pinto et al., 2013). The first model suggests that Rpfs are cell-signaling molecules that are secreted by actively growing cells which can bind to the cell surface receptors on VBNC cells to initiate resuscitation. This model was first proposed by Mukamolova et al. (1998a) as Rpf, similar to other cell-signaling molecules, can stimulate cell growth and is likely to be involved in the control of cell replication.

The second model suggests that, instead of binding to receptors, Rpfs degrade or remodel the cell wall peptidoglycan of VBNC cells, thereby triggering resuscitation. Although no studies have demonstrated the molecular mechanisms underlying resuscitation triggered by peptidoglycan alteration, this model is supported by the fact that all Rpfs contain a conserved domain that is highly homologous to lysozyme and transglycosylase, which are both known to degrade the peptidoglycan of bacterial cell walls (Cohen-Gonsaud et al., 2005). Moreover, RpfB and RpfE were found to interact with Rpf-interacting protein A (RipA), which is a peptidoglycan hydrolase (Hett et al., 2007; Kana et al., 2008). The ability of Rpfs to degrade peptidoglycan via hydrolysis was recently demonstrated (Mukamolova et al., 2006), and this ability seems to be responsible for the resuscitation of *M. smegmatis* (Telkov et al., 2006). This suggests that the alteration of cell wall peptidoglycan may be crucial for the resuscitation of VBNC cells. It is noteworthy that the modification of the peptidoglycan layer was previously found to be involved in the induction of the VBNC state (Signoretto et al., 2000).

The third model is also based on the cleavage of peptidoglycan by Rpfs. However, instead of direct remodeling of the cell wall of VBNC cells, it suggests that the Rpfs cleave the peptidoglycan layer of the Rpf-producing cells and release small peptidoglycan fragments that bind to cell surface receptors of VBNC cells, thereby triggering resuscitation. This model was proposed because previous studies showed that some Rpfs are bound to the cell wall of Rpf-producing cells instead of being released into the growth medium (Mukamolova et al., 1998a; Koltunov et al., 2010). It was subsequently found that peptidoglycan fragments, either generated from Rpf digestion or ultrasonication, can stimulate the resuscitation of *Micrococcus* spp. (Nikitushkin et al., 2013). PknB is a Ser/Thr membrane kinase with an extracytoplasmic domain that binds peptidoglycan fragments. This protein may be responsible for the resuscitation mechanism, as it was demonstrated that a synthetic muropeptide with high affinity to PknB had a moderate effect on resuscitation (Mir et al., 2011). This model may also provide clues about the mechanism underlying resuscitation in animal models, since lysozymes produced by the immune system damage the peptidoglycan layer and could lead to the release of peptidoglycan subunits.

Autoinducers. Unlike the Rpfs that were only described in *Micrococcus* and *Mycobacterium* spp., heat-stable autoinducers are produced by a range of Gram-negative bacteria and some Gram-positive bacteria. They were first found in cultures of *E. coli* growing in serum-SAPI medium supplemented with the human catecholamine hormone, norepinephrine (Freestone et al., 1999). Two years later, they were also found in cultures growing in four other kinds of media without the addition of norepinephrine (Freestone et al., 2001). Apart from thermal stability, these autoinducers are dialyzable, acid- and alkali-stable as well as protease-resistant (Weichart and Kell, 2001). *E. coli* were found to produce at least two kinds of autoinducer, AI-2 and AI-3 (Sperandio et al., 2003), which enter the target cells via a TonB-dependent receptor (Freestone et al., 2001). In *V. vulnificus*, the addition of a LuxR inhibitor has been shown to delay the resuscitation mediated by AI-2, suggesting that this autoinducer is sensed by the master quorum sensing regulator SmcR (a LuxR homolog) (Ayrapetyan et al., 2014). In the same study, the authors found that RpoS is also important for AI-2 mediated resuscitation, and proposed that increased level of AI-2 stimulates *rpoS* expression through the action of LuxR, leading to resuscitation.

Similar to Rpfs, autoinducers can stimulate bacterial growth by reducing the lag phase (Freestone et al., 1999). In addition, they can be extracted from the supernatants of bacterial cultures and initiate resuscitation of VBNC cells of other bacterial species, demonstrating cross-species activity. It was found that the autoinducers from *E. coli* can resuscitate its own VBNC cells (Pinto et al., 2011), while the enterobacterial autoinducers from *Y. ruckeri* can resuscitate four species including *C. freundii*, *E. coli*, *Enterobacter agglomerans*, and *S. typhimurium* (Reissbrodt et al., 2002).

Uncertainties waiting to be resolved

The production of Rpfs and autoinducers by bacteria and the importance of these factors in resuscitation have raised another question: Does the resuscitation of VBNC cells require external stimuli or simply rely on factors produced by themselves or other bacterial species? Epstein (2009) proposed that bacteria can awake randomly without relying on environmental stimuli. According to this hypothesis, VBNC cells may resuscitate randomly, dying if the conditions are still unfavorable, or surviving if the conditions are permissive for growth. If the cells survive, they may divide to start a new population and/or secrete a signal

to resuscitate other VBNC cells. Interestingly, a recent study have found that the level of autoinducer AI-2 in *V. vulnificus* reaches a peak 5 h after temperature upshift (a resuscitation-stimulating environmental stimulus), but the resuscitation of VBNC cells only became detectable an additional 2 h later (Ayrapetyan et al., 2014). The authors agreed to Epstein's hypothesis and suggested that resuscitation of the major population occurs after sensing the signal produced by a small, undetectable population of cells that resuscitated stochastically. Another possibility is that the signal is produced by cells remaining in VBNC state in response to a permissive environment, but no supporting or refuting evidences of this theory have ever been shown. Even if the signals have been proven to be produced by resuscitated cells, further investigation is still required to validate Epstein's hypothesis, as the current findings cannot confirm whether an undetectable number of cells resuscitated randomly before, or particularly after, the environment become suitable. The only conclusion that can be drawn is that particular environmental condition (e.g., temperature upshift or nutrient availability) is the prerequisite for the production of Rpfs or autoinducers and the resuscitation of the major population of VBNC cells.

Since the discovery of VBNC cells in the 1980s, few studies have investigated the molecular mechanisms underlying the process of resuscitation. It is known that the expression of *ompW* can regulate resuscitation in *E. coli* (Asakura et al., 2008) and the expression of *luxS* can affect AI-2 production and by consequence, resuscitation in *V. vulnificus* (Ayrapetyan et al., 2014). However, much more work is needed in order to understand the detailed mechanisms by which this gene and other genes induce the resuscitation of VBNC cells.

SIGNIFICANCES

VBNC: CHANGING THE FACE OF PATHOGEN DETECTION

More than 80 bacterial phyla have been identified to date; however, only about half of these have members that can be cultured in the laboratory (Pace, 2009; Stewart, 2012). Evidently, the history of microbiology has had a vested interest in microorganisms that have a direct influence on human health and food production, so most of the culturable species are either probiotics, pathogens, or those related to food spoilage. Until the discovery of the VBNC state (Xu et al., 1982), the presence and viability of these microorganisms were equated with their culturability on defined media. It is now increasingly clear that the potential threat presented by a given bacterial pathogen is not completely represented by its culturability on artificial media. Alternatives to culturing bacterial agents of disease use nucleic acid and antigen-based tests. While these are faster than culture methods, they are not without limitations making it worthwhile to invest energy into ameliorating current culture techniques (Cronquist et al., 2012; Jones and Gerner-Smidt, 2012). As discussed above, various VBNC-inducing conditions have been identified to date and these conditions are most likely just the tip of the iceberg. Research on the VBNC state is still in its infancy and in all likelihood, there are many more stresses that can induce a non-culturable state that are yet to be identified.

Entry into a VBNC state is considered as a bacterial response to exogenous stress. While these stresses may originate from the natural environment, what raises even more concern are the stresses induced by antimicrobial therapies and disinfectants used by humans to cure infections, obtain safe food and water sources, and sanitize environments. These may trigger bacteria to enter a VBNC state, especially when used inaccurately or in sub-lethal amounts. Noncompliance to drug regimens have been identified as a cause for the emergence of multidrug resistant strains of bacteria and this may well be a trigger for the VBNC state in some pathogens like *M. tuberculosis* (Kardas, 2002; Anderson et al., 2013).

More importantly, many documented VBNC inducing conditions are those in which humans encounter pathogens. For example, monochloramine treatment, which is used to sterilize drinking water, renders *L. pneumophila* VBNC, as does prolonged incubation in tap water (Steinert et al., 1997; Ducret et al., 2014). *M. tuberculosis* becomes VBNC under hypoxic conditions, theorized to be a stress encountered by the bacterium inside the tubercules of *M. tuberculosis* carriers (Fattorini et al., 2013; Manina and McKinney, 2013). *V. vulnificus*, a food-borne pathogen, enters the VBNC state in refrigerating temperatures (Nowakowska and Oliver, 2013).

Diagnosis of disease and identification of etiological agents have been and still are highly dependent upon culture techniques. The inability to culture microorganisms is then a major impasse for proper diagnosis of diseases and subsequent treatments, thus posing serious problems to pathogen detection, not only in the environment but also in food and water sources. As such, potentially dangerous contaminations can elude detection. In fact, VBNC bacteria have been found in human urine samples that were previously considered sterile (Anderson et al., 2001). Moreover, uncovering the secrets of the VBNC state may also provide clues to improve culture methods for the environmental bacteria that remain uncultivable as only 1% of the environmental bacterial population is thought to be cultured to date (Bloomfield et al., 1998). Giving the lack of novel antibiotics, enhanced culture techniques may lead to the discovery of new antimicrobial compounds.

VBNC AND BIOFILM

Biofilms are surface-attached, sessile bacterial communities enclosed in an extracellular matrix (ECM) (O'Toole et al., 2000). It has been estimated that 95% of bacteria present in water systems are found in biofilms (Flemming et al., 2002). Since water is a major source of contamination in food and an integral part of food production, and its contamination is the root of several nosocomial infections, focus on VBNC cells inside biofilms is of particular interest. While Kell and Young (2000) argue that the basis for the difference between dormancy and the VBNC state has been attributed to differences in metabolic activity, the literature suggests that both terms are describing the same physiological state of the cell. In the case of dormancy, the halt of cell division (leading to non-culturability) is viewed as a product of the dormant state while the VBNC state is defined by the inability of a cell to grow on laboratory media. Until our understanding of VBNC is deepened, this differentiation seems to be based on semantics and not necessarily on the actual presence of two different states.

Several reports show that the heterotrophic plate count of biofilms from water samples do not reflect the total cell counts and this difficulty has been attributed to a VBNC status achieved within the biofilm (Wingender and Flemming, 2004; Lee et al., 2007). To date, little focus has been placed on studying VBNC cells in biofilms, but the prevalence and the threats associated with biofilms in medical settings will likely result in more rigorous research into this area of biofilm study. For example, *C. jejuni*, a food-borne bacterium, forms VBNC cells in biofilms where biocide resistance is increased (Newell and Fearnley, 2003). *S. epidermidis* in biofilms was found to enter a VBNC state when grown in media with an excess of glucose (Cerca et al., 2011). Interestingly, these VBNC cells induced a lower production of the cytokines TNF-α and IL-6, and replicated in macrophages to a lesser extent than their culturable counterparts leading to lower macrophage death. While the authors conclude that VBNC cells lead to a lower activation of macrophages, the CFU counts were only taken up to 9 h post-infection, which may not have been sufficiently long enough for resuscitation inside the host cells. VBNC *Staphylococcus aureus* and *S. epidermidis* cells have also been isolated from biofilms inside catheters (Zandri et al., 2012). More specifically, Pasquaroli et al. (2013) found that *S. aureus* entered a VBNC state upon exposure to antibiotics. However, subsequent resuscitation in response to sodium pyruvate makes it unclear whether the VBNC cells reported were, in fact, injured cells. More recently, *L. monocytogenes*, another important food-borne pathogen, was also shown to produce VBNC cells in biofilms (Gião and Keevil, 2014).

The microenvironment inside the biofilm structure is subjected to oxygen, pH and nutritional stresses; there is a lower nutrient availability and higher waste concentration deeper inside the biofilm than in the periphery (Stewart and Franklin, 2008). It is well established that bacteria in biofilms are starved and this starvation response confers antibiotic resistance (Nguyen et al., 2011). Such bacteria are thought to be thousand times more resistant to antimicrobials than their planktonic counterparts (Costerton et al., 1999). In multispecies biofilms, the culturability of *L. pneumophila* is influenced by the presence of other species (Gião et al., 2011). It is clear that much more rigorous research needs to be conducted to study the formation and impact of VBNC cells in biofilms.

MYCOBACTERIUM TUBERCULOSIS: AN EXAMPLE OF NON-CULTURABLE CELLS *IN VIVO*

The entry into the VBNC state has been identified in response to simulated environmental stresses and there is evidence of resuscitation in response to co-culture with host cells or their supernatants (Senoh et al., 2012). *M. tuberculosis* is known to enter VBNC state and resuscitate during infection of the host (i.e., human lungs). It is also well established that once the selective pressures for latency is absent, mainly in the form of immunosuppression, these dormant bacteria can cause active tuberculosis (TB). The VBNC state of *M. tuberculosis* (Mtb) is also known as latency or dormancy, and contributes to over two million Mtb carriers around the world (Gengenbacher and Kaufmann, 2012).

Once it invades alveolar macrophages, *M. tuberculosis* can hijack host cell machinery to halt phagosome maturation (Crowle et al., 1991; Sturgill-Koszycki et al., 1994; Vergne et al., 2004), but *Mycobacterium* spp. have also been shown to endure the stresses of a mature phagolysosome (Via et al., 1998). Latent TB is the result of solid granulomas containing *M. tuberculosis*, which is considered as VBNC cells because of decreased culturability (Gengenbacher and Kaufmann, 2012; Reece and Kaufmann, 2012). When the bacterium is non-culturable inside the human lung and alveolar macrophages, it does not cause any diagnosable symptoms until reactivation. Both *in vitro* (Wayne model) and *in vivo* (Cornell model) models are used to generate VBNC cells of *M. tuberculosis* in order to study bacterial and host factors contributing to the VBNC status (Wayne and Hayes, 1996; Scanga et al., 1999).

In the past 20 years, a growing body of research has been investigating Rpfs in *M. tuberculosis* and related mycobacteria that are at the root of latent TB reactivation (see section Mechanisms of Resuscitation). Mtb-derived Rpfs have been shown to increase the culturability of VBNC bacteria in clinical sputum samples (Mukamolova et al., 2010). More recently, highly specific free fatty acids have been identified as playing a role in a regulatory cascade involving Rpf proteins and adenylate cyclase, leading to the resuscitation of *M. tuberculosis* cells (Shleeva et al., 2013). The use of resuscitation knowledge in treating latent TB infections may be the next step in eliminating or reducing the disease burden.

ANTIBACTERIAL RESISTANCE OF VBNC CELLS

Because VBNC bacteria have a low metabolic rate, it is reasonable that antibacterials that target activities or components of active cells would be less effective against them. VBNC cells inside biofilms have an additional protection by the ECM. Moreover, the hypothesis that the VBNC state is an adaptation to stressful environments means that, by definition, these cells are likely to be less sensitive to exogenous stress. In *M. tuberculosis*, VBNC cells are relatively insensitive to isoniazid, which is an antibacterial drug targeting cell wall synthesis (involved in replication of active cell), but it is still used as a long-term therapy to treat the latent phase of disease (Manina and McKinney, 2013). The effectiveness of isoniazid in the long-term supports the idea that VBNC cells have in fact, adopted a slow metabolism. Therefore, the VBNC state may favor the development of drug resistance when strict drug regimens are not followed by allowing the bacterium to adapt before resuscitation. Oliver (2010) reviewed the antibiotic resistance in the VBNC state of some bacteria including *E. faecalis*, *E. coli*, *Haemophilus influenza*, *H. pylori* and *M. smegmatis*, all of which demonstrated an increased resistance to antibiotics. In addition, VBNC cells of *E. coli* are more resistant to sonication and that of *C. jejuni* are more heat resistant than non-VBNC cells (Klančnik et al., 2009; Zhao et al., 2013). Recently, an exponential phase cell culture and a VBNC cell culture of an environmental genotype strain of *V. vulnificus* were exposed to different challenges. It was found that the VBNC cells are more tolerant to a list of exogenous stresses, including high temperature (50°C for 1 h), ethanol (final concentration of 13% for 1 h), high salinity (*c.* 290 ppt for 2 h), oxidative stress (0.2 mM H_2O_2 for 1 h), acidity (pH 3 for 25 min), antibiotics (ampicillin or chloremphenical for 4 h) and zinc (3.4 mM $ZnSO_47H_2O$ for 1 h) (Nowakowska and Oliver, 2013). Interestingly, the same study demonstrated a

lower resistance in VBNC cells of the clinical strain than those of the environmental strain, and proposed that these differences are based on the expression of certain genes (e.g., *relA* and *katG*).

VIRULENCE AND VBNC IN OTHER PATHOGENS

The recurrence of *M. tuberculosis* infections clearly proves that VBNC cells in the latent phase of the disease are able to retain or, in the least, regain their virulence potential upon resuscitation. In fact, since the VBNC state is a slow metabolic state, it would seem more likely that the observed virulence is a result of reactivation of the normal function of the cell (Oliver, 2010). In support of virulence maintenance in the VBNC state, VBNC *L. pneumophila* has been demonstrated to retain the capacity to infect the amoeba used for resuscitation, demonstrating virulence toward its natural host (Steinert et al., 1997; Al-Bana et al., 2013). Interestingly, another *L. pneumophila* strain showed continued production of virulence-related proteins in VBNC cells but could not be resuscitated in the same amoeba (Alleron et al., 2013). VBNC cells of *Vibrio* spp. have the ability to cause disease after resuscitation in their respective hosts (Baffone et al., 2003; Sun et al., 2008) and a microarray analysis of four *Vibrio* spp. revealed that a number of virulence and toxin genes were expressed in the VBNC state (Vora et al., 2005). VBNC *S. typhi* was also able to infect and cause disease in mouse models after resuscitation (Zeng et al., 2013). Much like the vibrios, *E. coli* O157:H7 was shown to produce Shiga-like toxins, but the toxin levels produced were dependent upon the age and the inducing condition of the VBNC cells (Liu et al., 2010).

These data on VBNC virulence suggests that, much like VBNC inducing stimuli and resuscitation factors, the expression and maintenance of virulence in different species of VBNC cells can vary greatly. *P. aeruginosa* is an important pathogen in terms of the disease burden and mortality associated with infection. It colonizes lung tissues of cystic fibrosis patients and is difficult to completely eradicate as it resides in biofilms in the lungs (Mulcahy et al., 2013). Given that VBNC cells can occur inside biofilms, relatively little progress has been made to study resuscitation factors of *P. aeruginosa* in this environment.

This capacity of resuscitation with no apparent loss of virulence potential evidently brings about concerns regarding the presence of VBNC bacteria not only in a medical context with respiratory pathogens like *M. tuberculosis*, *L. pneumophila*, and *P. aeruginosa* but also in relation to food safety (Dinu and Bach, 2011). In the case of *P. aeruginosa*, great strides toward reducing the bacterial load in CF patients could be made by inducing resuscitation of the VBNC bacteria inside the human host which would then be more susceptible to antibiotics. It is an avenue of research that is worth exploring.

CONCLUSION

After decades of study, it is clear that the VBNC state is both an important tool for the survival of bacteria and a dangerous aspect of bacterial pathogens for the host. The knowledge about the VBNC state comes from research on a variety of bacteria and highlights the complexity of this mechanism of adaptation. What seems clear is that induction and resuscitation of the VBNC state are highly variable across bacterial species and in some cases, strains. However, the basic genetic mechanisms may share a common theme and further research into this field will help tie up the loose ends that exist in this area. The ability to avoid conditions that lead to resuscitation, or the development of drugs that induce resuscitation during antibiotherapy could have a major impact on the consequence of the VBNC state in chronic infectious diseases. Development of new, inexpensive methods to easily detect cells in the VBNC state is needed to increase food safety. In conclusion, the potential applications of VBNC research are significant to prevent food- and water-borne infections, and find new treatments to cure chronic bacterial infections.

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The role of metabolism in bacterial persistence

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Bacterial persisters are phenotypic variants with extraordinary tolerances toward antibiotics. Persister survival has been attributed to inhibition of essential cell functions during antibiotic stress, followed by reversal of the process and resumption of growth upon removal of the antibiotic. Metabolism plays a critical role in this process, since it participates in the entry, maintenance, and exit from the persister phenotype. Here, we review the experimental evidence that demonstrates the importance of metabolism to persistence, highlight the successes and potential of targeting metabolism in the search for anti-persister therapies, and discuss the current methods and challenges to understand persister physiology.

Keywords: bacterial persistence, metabolism, antibiotic tolerance, ppGpp, nutrient environment

INTRODUCTION

Bacterial cultures contain a small subpopulation of cells that cannot readily be killed by antibiotics (Bigger, 1944). These cells have been named persisters, and their existence can be detected from antibiotic kill curves, where the first, rapid killing regime represents the death of normal cells and the second, slower killing regime indicates the presence of persisters (Balaban et al., 2004; Kint et al., 2012). Further, when these survivors are cultured, they produce populations with antibiotic sensitivities identical to those of the original culture. This establishes persistence as a phenotypic trait, unique from antibiotic resistance where genetic determinants allow growth at higher antibiotic concentrations. Persisters are an important health concern because they are enriched in biofilms and thought to underlie the proclivity of biofilm infections to relapse following the conclusion of antibiotic therapy (Lewis, 2008, 2010). Persisters have proven to be difficult to analyze due to their transient nature, low abundance, and similarity to the viable but non-culturable (VBNC) cell-type (Roostalu et al., 2008; Orman and Brynildsen, 2013b). However, strong evidence, in the form of genetic- and microscopy-based data (Balaban et al., 2004; Lewis, 2010; Maisonneuve et al., 2013), exists to support that, while under antibiotic stress, persister tolerances are derived from inactivity of essential cell functions. While this is not always the case, as demonstrated in a study of isoniazid (a prodrug requiring activation) (Wakamoto et al., 2013), and dormancy is not essential for persistence prior to antibiotic stress (Orman and Brynildsen, 2013a), prolonged survival to the majority of antibiotics, in the absence of genetic mutations, requires inactivity of the antibiotic's primary target. To achieve and maintain this state, as well as reverse the process to repopulate environments, coordinated metabolic action is needed. Namely, metabolism would participate in cessation of essential functions, be needed to maintain culturability (e.g., sustain a minimal adenylate charge: [ATP + 0.5ADP]/[ATP + ADP + AMP] (Chapman et al., 1971), repair/resynthesize damaged proteins (Nystrom and Gustavsson, 1998)), and reactivate the cell during reawakening (**Figure 1**). We refer to this process as the persister metabolic program and summarize the accumulated evidence substantiating the importance of metabolism to the persister phenotype as well as current methods and challenges to studying the metabolism of these rare and transient phenotypic variants.

GENOMIC STUDIES IDENTIFY METABOLIC GENES AS IMPORTANT TO THE PERSISTER PHENOTYPE

Perturbations to genes that encode enzymes or regulators of metabolism have frequently been found to alter persister levels (**Table 1**). In one of the initial genomic screens for persistence, a library was generated through digestion of the *Escherichia coli* chromosome, ligation of the fragments into plasmids, and transformation of the library into *E. coli* (Spoering et al., 2006). Upon successive rounds of ampicillin (AMP) treatment and culturing of survivors, a plasmid carrying *glpD*, encoding G3P-dehydrogenase that converts glycerol-3-phosphate (G3P) to dihydroxyacetone-phosphate (DHAP), was found to increase the abundance of persisters. Further analysis identified additional enzymes in G3P metabolism important for persistence to AMP, ofloxacin (OFL), and ciprofloxacin (CIP) (**Table 1**). The importance of G3P to *E. coli* persistence was further supported by a transposon mutant screen where *glpD* mutants were found to increase persistence

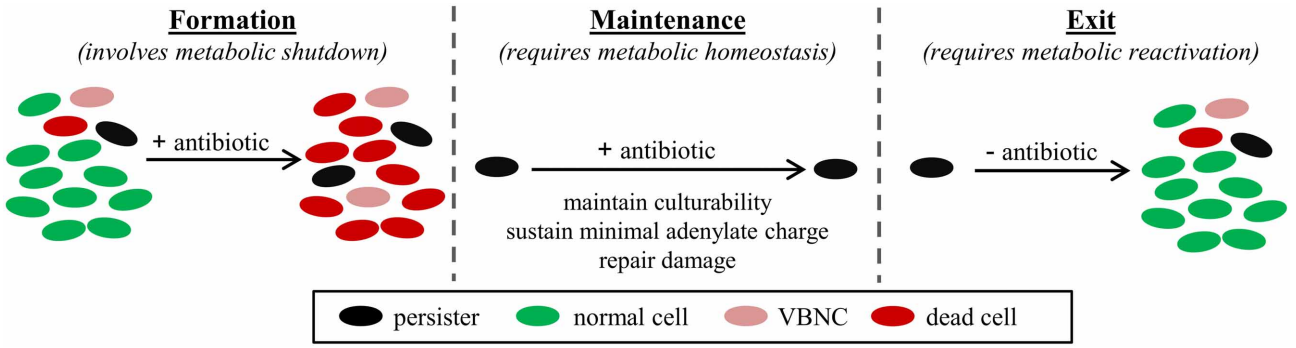

FIGURE 1 | Persister metabolic program. Persisters can be pre-existing in a bacterial population (Balaban et al., 2004), formed in response to stress, such as stresses that activate the stringent response (Amato et al., 2013), and induced during antibiotic treatment (Dörr et al., 2009; Orman and Brynildsen, 2013a). Maintenance of the persister state for the duration of the antibiotic treatment requires temporary inhibition of essential cell functions; however, persisters must remain culturable, which requires a minimal adenylate charge to be sustained (Chapman et al., 1971) and damage to be repaired (Nystrom and Gustavsson, 1998). Upon removal of the antibiotic, persisters exit their tolerant state and give rise to a bacterial population of identical antibiotic susceptibility as the original population (Balaban et al., 2004).

after successive rounds of selection on LB-AMP agar (Girgis et al., 2012). This effect was attributed to elevated levels of methylglyoxal, a toxic compound derived from DHAP. Interestingly, these observations, where GlpD inactivation increased persistence, were opposite to those of Spoering and colleagues. However, we note that G3P is a highly connected metabolite, given its proximity to central metabolism, interaction with the quinone pool, and use as a precursor for phospholipid biosynthesis. Therefore, different assay conditions may explain the variable impacts on persistence.

Beyond G3P, genomic studies have found that mutations perturbing amino acid (AA) metabolism significantly influence persistence (**Table 1**). Screening of an *E. coli* transposon library for persistence to ticarcillin (TIC) or OFL identified 18 mutants with increased persister levels, and of those, 16 mapped to genes involved in AA biosynthesis (Bernier et al., 2013). *Pseudomonas aeruginosa* screens have also uncovered disruptions in AA metabolism as important to persistence. Mutation of PA4115, a putative lysine decarboxylase, was found to increase persistence to carbenicillin (CB) (Manuel et al., 2010), whereas mutation of *pheA*, which is also involved in AA metabolism, was found to increase persistence to OFL (De Groote et al., 2009). These studies suggest that AA metabolism is a critical mediator of persistence, and as one would expect, the stringent response, a major metabolic regulatory system controlled by ppGpp and its transcriptional partner DksA, also mediates persistence (Korch et al., 2003; Viducic et al., 2006; Fung et al., 2010; Nguyen et al., 2011; Amato et al., 2013; Maisonneuve et al., 2013). This influence was also detected in a screen where Δ*dksA* was found to produce far fewer persisters toward OFL (Hansen et al., 2008).

The third major metabolic system that has been shown to impact persistence is energy metabolism. A screen of an *E. coli* transposon library found that deactivation of *phoU* reduced persistence (Li and Zhang, 2007). PhoU is a negative regulator of the phosphate operon, and its inactivation led to a hyperactive metabolic state. In a screen of the Keio collection for AMP persistence, Δ*sucB* and Δ*ubiF* were found to produce lower persister levels (Ma et al., 2010). SucB participates in the TCA cycle, whereas UbiF is an enzyme in ubiquinone biosynthesis, and deactivation of either of these genes leads to deficient energy production. Interestingly, these studies point to both metabolic hyperactivity and inhibition as methods to reduce persistence. One interpretation of these results could be that metabolic hyperactivity reduces entry into the persister state, whereas inhibition of energy production prevents exit from the phenotype. Regardless, energy generation appears to be a critical process to the persister metabolic program.

Collectively, these studies have provided a wealth of evidence on the importance of metabolism to bacterial persistence, even though they have sampled only a fraction of the mutational landscape. The details of how each genetic perturbation affects entry into, maintenance of, or exit from the persister state largely remains to be elucidated; however, it is clear that G3P, AA metabolism, and energy production are all important to defining persistence in a bacterial population.

PERSISTER LEVELS DEPEND ON THE NUTRITIONAL ENVIRONMENT

In addition to genetic evidence, the importance of metabolism to persistence has been supported by the impact of nutrient availability on persister levels. The most comprehensive investigation in this regard explored how the absence of AAs, glucose, ammonium, phosphate, and nucleobases altered persistence to AMP, OFL, and gentamicin (GEN) in *E. coli* (Fung et al., 2010). This study concluded that AA deprivation often increases persistence, mirroring the results from genomic screens that found mutations in AA metabolism to enhance persistence (**Table 1**). In a study of persister awakening, the number of *E. coli* persisters to AMP and norfloxacin (NOR) were found to be higher when the same stationary-phase culture was inoculated into media unable to support rapid growth resumption (minimal glycerol) in comparison to media with rapid regrowth (LB and minimal glucose) (Joers et al., 2010). Similarly, *E. coli* biofilms have been reported to exhibit higher tolerance to OFL or TIC in fresh media lacking

Table 1 | Metabolism-associated genes identified through genomic studies to influence persistence.

Organism	Gene[α]	Gene function	Metabolic role	Mutation	Antibiotic[β]	Culture conditions[γ]	Persistence outcome	References
E. coli	*argE*	Acetylornithine deacetylase	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*argH*	Argininosuccinate lyase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*aroE*	Shikimate 5-dehydrogenase	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*atpA*	F_o/F_1 ATP synthase subunit α	Energy production	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*atpF*	F_o/F_1 ATP synthase subunit B	Energy production	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*cysD*	Sulfate adenylyltransferase subunit 2	AA metabolism	Deletion	TIC	STAT	Increase	Bernier et al., 2013
				Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*dksA*	Transcription regulation	Stringent response regulation	Deletion	OFL, CIP, STM, AMP	STAT, EXP	Decrease	Hansen et al., 2008
E. coli	*galU*	Glucose-1-phosphate uridylyltransferase	Glycogen metabolism	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*glpABC*	Anaerobic G3P dehydrogenase	G3P metabolism	Deletion	CIP	STAT	Decrease	Spoering et al., 2006
E. coli	*glpD*	G3P dehydrogenase	G3P metabolism	Mutation	AMP	Plates	Increase	Girgis et al., 2012
				Deletion	CIP	STAT	Decrease	Spoering et al., 2006
				Over-expression	AMP, OFL	EXP	Increase	Spoering et al., 2006
E. coli	*hipA*	Serine/threonine kinase	Stringent response regulation	Mutation	AMP, CYC, PHM	EXP, Plates	Increase	Moyed and Bertrand, 1983
E. coli	*hipB*	Transcriptional repressor, antitoxin	Stringent response regulation	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*hisG*	ATP phosphoribosyl-transferase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*ilvA*	Threonine dehydratase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*ilvC*	Ketol-acid reductoisomerase	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*livJ*	Leucine/isoleucine/valine transporter	AA transport	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*leuB*	3-Isopropylmalate dehydrogenase	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*leuC*	Isopropylmalate isomerase large subunit	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
				Deletion	TIC	STAT	Increase	Bernier et al., 2013
				Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013

(Continued)

Table 1 | Continued

Organism	Gene[α]	Gene function	Metabolic role	Mutation	Antibiotic[β]	Culture conditions[γ]	Persistence outcome	References
E. coli	*lysA*	Diaminopimelate decarboxylase, PLP-binding	AA metabolism	Deletion	TIC	STAT	Increase	Bernier et al., 2013
				Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*metA*	Homoserine *O*-succinyltransferase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*pheA*	Fused chorismate mutase P/prephenate dehydratase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*phoU*	Pho operon repressor	Phosphate metabolism	Mutation	AMP, NOR, PZA, AMP/GEN	STAT, EXP	Decrease	Li and Zhang, 2007
				Deletion	AMP, NOR	STAT, EXP	Decrease	Li and Zhang, 2007
E. coli	*proA*	γ-Glutamyl phosphate reductase	AA metabolism	Mutation	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*proC*	Pyrroline-5-carboxylate reductase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*sucB*	Dihydrolipoamide acetyltransferase	Energy production	Deletion	AMP, GEN	STAT, EXP	Decrease	Ma et al., 2010
E. coli	*tktA*	Transketolase	Energy production	Mutation	AMP	Plates	Increase	Girgis et al., 2012
E. coli	*trpA*	Tryptophan synthase subunit α	AA metabolism	Deletion	TIC	Biofilm	Increase	Bernier et al., 2013
E. coli	*tyrA*	Fused chorismate mutase T/prephenate dehydratase	AA metabolism	Deletion	TIC, OFL	Biofilm	Increase	Bernier et al., 2013
E. coli	*ubiF*	2-Octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone hydroxylase	Energy production	Deletion	AMP, GEN	STAT, EXP	Decrease	Ma et al., 2010
E. coli	*ygfA*	5-Formyltetrahydrofolate cyclo-ligase	Coenzyme biosynthesis	Deletion	OFL, CIP, STM, AMP	STAT, EXP	Decrease	Hansen et al., 2008
P. aeruginosa	PA14_13680	Putative short-chain dehydrogenase	Unknown	Mutation	OFL	STAT	Increase	De Groote et al., 2009
P. aeruginosa	PA14_17880	Acetyl-CoA acetyltransferase	Fatty acid and phospholipid metabolism	Mutation	OFL	STAT	Decrease	De Groote et al., 2009
P. aeruginosa	PA4115	Lysine decarboxylase	AA metabolism	Mutation	CB	EXP, Plates	Increase	Manuel et al., 2010
				Deletion	CB, TIC	EXP	Increase	Manuel et al., 2010
P. aeruginosa	*pheA*	Prephenate dehydratase	AA metabolism	Mutation	OFL	STAT	Increase	De Groote et al., 2009

(Continued)

Table 1 | Continued

Organism	Gene[α]	Gene function	Metabolic role	Mutation	Antibiotic[β]	Culture conditions[γ]	Persistence outcome	References
P. aeruginosa	*spuC*	Putrescine aminotransferase	Polyamine metabolism	Mutation	OFL	STAT	Decrease	De Groote et al., 2009
P. aeruginosa	*ycgM*	Putative fumarylaceto-acetate hydrolase family protein	Secondary metabolite biosynthesis, transport, and catabolism	Mutation	OFL	STAT	Increase	De Groote et al., 2009
S. mutans	*fruA*	Fructan hydrolase	Carbohydrate metabolism	Fragment over-expression	OFL	STAT	Decrease	Leung and Lévesque, 2012
S. mutans	*pfl*	Pyruvate formate-lyase	Energy production	Fragment over-expression	OFL	STAT	Increase	Leung and Lévesque, 2012
S. mutans	*scrA/scrB*	Sucrose-specific IIABC PTS component/Sucrose-6-phosphate hydrolase	Carbohydrate metabolism	Promoter over-expression	OFL	STAT	Increase	Leung and Lévesque, 2012
S. mutans	*scrR*	Sucrose-PTS operon repressor	Carbohydrate metabolism	Deletion	OFL	STAT	Increase	Leung and Lévesque, 2012
S. mutans	SMU.1278	Putative phosphoglycolate phosphatase	Unknown	Fragment over-expression	OFL	STAT	Increase	Leung and Lévesque, 2012

[α]Genes listed in this table are those associated with metabolism that were found to influence persistence in studies that included a genomic screen. Genes not directly connected to metabolism are not listed here, and metabolic genes identified in non-genomic studies are also not presented. [β]Antibiotic abbreviations: ampicillin (AMP), carbenicillin (CB), ciprofloxacin (CIP), cycloserine (CYC), gentamicin (GEN), norfloxacin (NOR), ofloxacin (OFL), phosphomycin (PHM), pyrazinamide (PZA), streptomycin (STM), ticarcillin (TIC). [γ]Culture conditions describe the bacterial growth state at the time of antibiotic exposure: exponential (EXP), stationary (STAT), agar plates (Plates), biofilm.

glucose, in comparison to controls with glucose (Bernier et al., 2013). Further support derives from the numerous studies that have shown that nutrient-limited stationary phase and biofilm cultures produce higher persister levels than their exponentially growing counterparts (Spoering and Lewis, 2001; Keren et al., 2004a; Lechner et al., 2012; Bernier et al., 2013). However, it is important to note that high density phenotypes such as quorum signaling may also contribute to persistence in such populations (Möker et al., 2010; Vega et al., 2012).

Taken together, these studies demonstrate that the nutritional environment directly influences persistence, suggesting a central role for metabolism in the persistence phenotype. Further, the mechanisms by which these nutritional stresses enhance persistence have been investigated, and ppGpp has been found to be a key mediator of this process.

ppGpp, THE METABOLITE CONTROLLER OF PERSISTENCE

ppGpp and the transcriptional regulator DksA are global regulators of metabolism (Traxler et al., 2006; Dalebroux and Swanson, 2012) that are critical mediators of persistence (Korch et al., 2003; Hansen et al., 2008; Amato et al., 2013; Bokinsky et al., 2013; Germain et al., 2013; Maisonneuve et al., 2013). In *E. coli*, AA limitation stimulates the ribosome-associated RelA to synthesize ppGpp, whereas various stress conditions, such as carbon (Xiao et al., 1991) and fatty acid starvation (Seyfzadeh et al., 1993), stimulate ppGpp synthesis from the cytoplasmic SpoT, which also encodes the sole ppGpp hydrolase. In conjunction with DksA, ppGpp interacts with RNA polymerase to inhibit transcription from stable ribosomal RNA promoters, while simultaneously upregulating transcription of AA biosynthesis and stress response genes (Potrykus and Cashel, 2008; Dalebroux and Swanson, 2012). ppGpp was initially associated with persistence through *hipA7*, a toxin mutant that required ppGpp for its elevated persister phenotype (Korch et al., 2003). Recent work on the native HipA has also shown that its impact on persistence requires ppGpp (Bokinsky et al., 2013; Germain et al., 2013). ppGpp can also increase persistence through its inhibition of exopolyphosphatase (*ppx*), a modulator of the antitoxin degrading Lon protease (Maisonneuve et al., 2013). Additionally, we have demonstrated that RelA, SpoT, and DksA mediate persister formation in response to carbon source transitions (Amato et al., 2013). In particular, we found that the ppGpp biochemical network can act as a metabolic toxin–antitoxin module, where ppGpp is the metabolite toxin and SpoT is its enzymatic antitoxin. We demonstrated that increased ppGpp levels resulted in growth arrest and increased persistence, which could be reverted by SpoT coexpression, and using a mathematical model, we showed that the ppGpp biochemical network can exhibit bistability, where one subpopulation corresponds to normal cells (low ppGpp) and the other to persisters (high ppGpp). Interestingly, RelA–SpoT also demonstrate the prototypical conditional essentiality of a classical toxin–antitoxin system, where the toxin (*relA*) can be deleted, but the antitoxin (*spoT*) can only be removed in a strain without the toxin. In addition to *E. coli*, the stringent response has been shown to impact persistence in other organisms as well. In *P. aeruginosa*, RelA, SpoT, and DksA have all been found to impact persistence (Viducic et al., 2006; Nguyen et al., 2011), whereas in *Mycobacterium tuberculosis*, ppGpp was required for long term survival in an *in vitro* starvation and murine model (Primm et al., 2000; Dahl et al., 2003). Further, the mycobacterial stringent response was shown to exhibit bistability (Ghosh et al., 2011), supporting the assertion that ppGpp is a possible source of phenotypic heterogeneity. In addition, in *Staphylococcus aureus*, ppGpp has been shown to mediate antibiotic tolerance in response to cell envelope stress (Geiger et al., 2014).

These studies demonstrate the importance of the stringent response to persistence and highlight a prevalent mechanism by which metabolic stress can induce persistence. Considering this evidence supporting a central role for ppGpp in persistence, it is attractive to propose that an inhibitor of ppGpp synthesis, such as Relacin (Wexselblatt et al., 2012), or an activator of ppGpp hydrolysis could be effective therapeutics against persisters (Amato et al., 2013; Maisonneuve et al., 2013).

PERSISTER METABOLISM AS A SOURCE OF ELIMINATION STRATEGIES

To date, only a limited number of methods to kill persisters have been discovered, and interestingly, persister metabolism plays a vital role in each approach. For example, the first method, which we co-developed, used metabolites to stimulate proton motive force (pmf) generation in persisters, enabling aminoglycoside (AG) transport and their subsequent killing of *E. coli* and *S. aureus* persisters (Allison et al., 2011b). The participation of persister metabolism was confirmed with genetic mutants and chemical inhibitors, and subsequent studies have found the method to also be effective against *P. aeruginosa* persisters (Barraud et al., 2013). Another method was identified by Kim and colleagues, who screened a chemical library and found that a chemical named C10 promoted fluoroquinolone killing of *E. coli* persisters by stimulating their reversion to a replicating state (Kim et al., 2011). In another study, the quorum-sensing (QS) inhibitor BF8 facilitated elimination of *P. aeruginosa* persisters when combined with CIP or tobramycin (TOB) (Pan et al., 2012). However, upon further analysis, it was discovered that the effect of BF8 was likely due to reactivation of metabolism rather than inhibition of QS. Interestingly, BF8 has also been found to reduce *E. coli* persister levels when combined with OFL, tetracycline (TET), TOB, or GEN (Pan et al., 2013). Recently, another method to eliminate *S. aureus* persisters was discovered by leveraging knowledge that energy levels are low in persisters (Conlon et al., 2013). Specifically, ADEP4, which renders the ClpP protease ATP-independent, led to non-specific protein degradation and death in energy-depleted persisters. Taken together, these studies show that targeting persister metabolism holds great potential for the elimination of these dangerous bacteria and that greater knowledge of persister metabolism will facilitate the discovery of novel therapeutic strategies.

METHODS TO MEASURE PERSISTER METABOLISM

Given the potential of persister metabolism to yield anti-persister therapeutics, enhanced metabolic knowledge of these phenotypic variants is desirable. However, direct measurement of metabolites in persisters or assessment of their metabolic activities using conventional approaches, such as mass spectrometry and

formazan-based colorimetric assays, are not currently possible due to isolation difficulties (Roostalu et al., 2008; Kint et al., 2012; Orman and Brynildsen, 2013a,b). Although several methods can provide persister-enriched samples (Keren et al., 2004b; Shah et al., 2006), such samples still contain many more other cell-types, such as normal cells and VBNCs, than persisters, and thus are of limited utility for metabolic measurements (Orman and Brynildsen, 2013b). Indeed, the major limitation to segregating persisters from a heterogeneous population is their similarity to VBNCs, which are often more highly abundant. Both VBNCs and persisters stain as live cells, harbor metabolic activity, and are non-growing under antibiotic stress. The only present difference between these subpopulations is that persisters resume growth on standard media, though we note that some VBNCs can regain culturability on non-standard media (Oliver, 2005) suggesting a role for the post-antibiotic environment in defining those cells that survive. Given these technical limitations, we have developed two methods to quantify persister metabolism. The first uses fluorescence activated cell sorting (FACS), a fluorescent measure of metabolic activity, and persistence assays to evaluate the metabolic status of persisters (Orman and Brynildsen, 2013a). This study, which provided the first direct measurement of persister metabolism, demonstrated that *E. coli* persisters largely contained low cellular reductase activity prior to antibiotic stress, confirming previous assumptions about the metabolic activity of the persister state. The second method leveraged the phenomenon by which specific metabolites enabled AG killing of persisters (Allison et al., 2011b). AG potentiation offered a rapid way to measure the breadth of persister metabolic activities (Orman and Brynildsen, 2013b), since the phenomenon relies on persister catabolism of nutrients for pmf generation. Persister metabolic activities are inferred from culturability on standard media, the distinguishing feature between VBNCs and persisters, thereby allowing investigation of persister metabolism even in the presence of VBNCs. This method enabled identification of nutrients metabolized by persisters to different antibiotics (AMP, OFL) during distinct growth stages (exponential, stationary), and thus allowed quantification of heterogeneity in persister metabolism. From these investigations, we demonstrated that glycerol and glucose are the most ubiquitously used carbon sources by various types of persisters, suggesting that the enzymes required for their catabolism are broadly available in persisters.

CHALLENGES IN THE STUDY OF PERSISTER METABOLISM

The technical hurdles associated with isolation of persisters have hindered understanding of the persister metabolic program and other aspects of persister physiology, including their transcriptome and proteome content. However, FACS offers a technical opportunity to discriminate between VBNC and persister phenotypes. For instance, mixed populations of VBNCs and persisters can be segregated from antibiotic-treated cultures using FACS (Roostalu et al., 2008; Orman and Brynildsen, 2013b), and since VBNCs are much more abundant than persisters in these samples, VBNC physiology can be quantified and potential biomarkers to discriminate between these two cell-types can be found. Nevertheless, any distinguishing features may be condition-specific, since numerous mechanisms can contribute to persister formation (Dhar and McKinney, 2007; Allison et al., 2011a; Balaban, 2011). Indeed, activation of particular pathways will depend on the environment and antibiotic used (Li and Zhang, 2007; Luidalepp et al., 2011), and different formation mechanisms may be active in different growth stages, giving rise to persister heterogeneity, where multiple, distinct persister subpopulations, each with its own unique antibiotic tolerances, coexist in a bacterial culture (Allison et al., 2011a). As a result of heterogeneity, any isolation technique may only capture a fraction of the persisters present, yielding a limited sample of the persister population. Single-cell analysis techniques offer means to interrogate individual cells (Iino et al., 2012, 2013); however, the identification of persisters before they exit their non-replicative state is not presently possible. Perhaps a viable path forward is to study model persisters generated following the over-expression of genes that have been shown to increase persister levels (Korch and Hill, 2006; Vázquez-Laslop et al., 2006). Quantifying metabolic changes in these model systems may provide insight into the physiology and metabolic capabilities of different types of persisters (Bokinsky et al., 2013).

CONCLUSION

Persisters embody a medically important bacterial phenotype that relies on metabolism to establish and maintain a dormant, tolerant state during antibiotic stress, and exit that state upon removal of antibiotics (**Figure 1**). Considerable experimental evidence has accumulated substantiating the importance of metabolism to persistence, and the participation of metabolism in current persister eradication methods provides a convincing argument that enhanced knowledge of the persister metabolic program will accelerate the discovery of additional elimination strategies. However, isolation difficulties impede progress in the understanding of persister physiology, including metabolism. Two potential paths forward are to improve isolation techniques by studying the differences between persisters and VBNCs and to use model persisters to define the breadth and landscape of the persister metabolic program.

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