STRESS RESPONSE MECHANISMS OF BACTERIAL PATHOGENS

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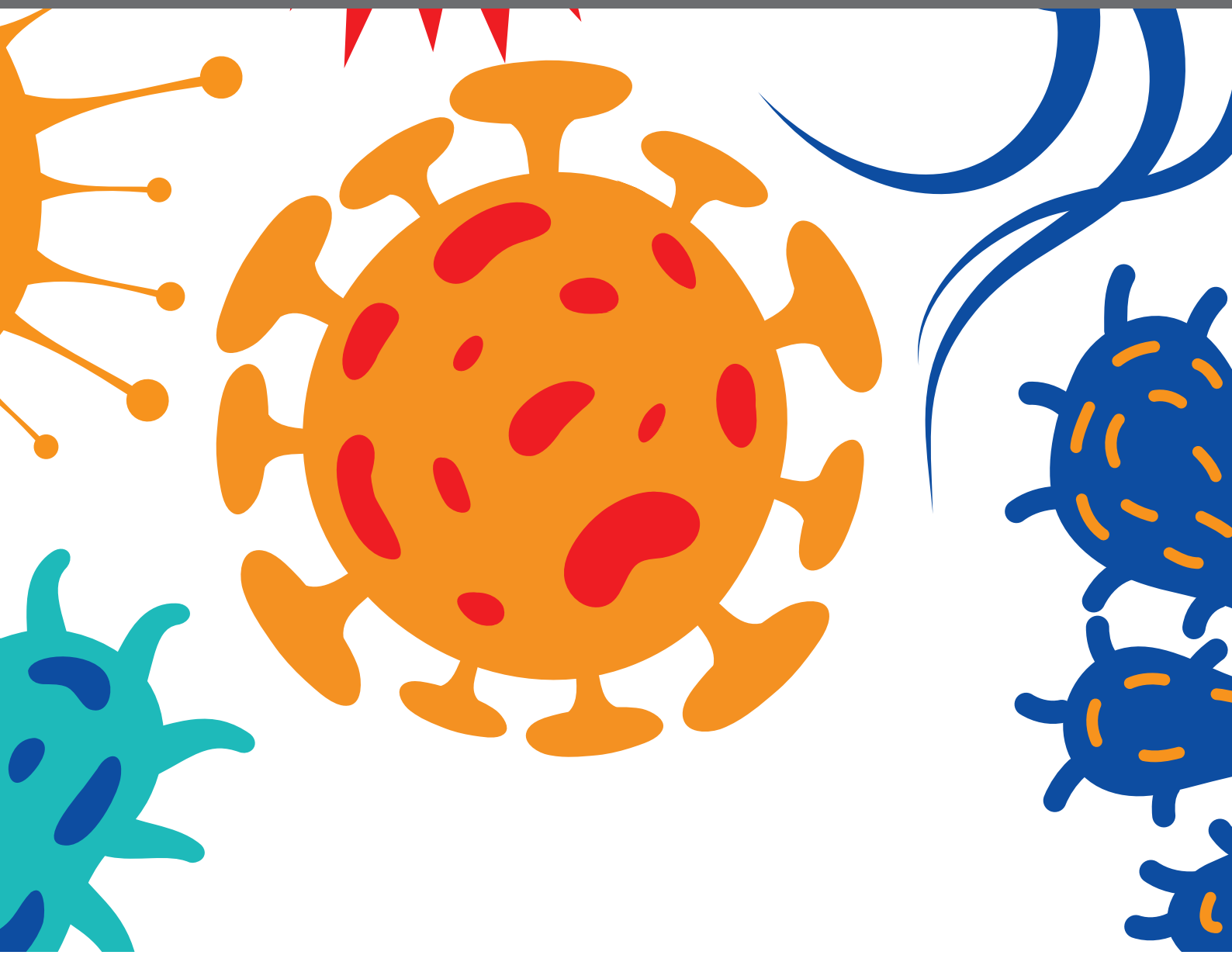

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STRESS RESPONSE MECHANISMS OF BACTERIAL PATHOGENS

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A critical factor for bacterial survival in any environment is the ability to sense and respond appropriately to insults that cause stress to the cell, threatening its survival. Most of these stressors first affect the outer surface of the bacterial cell, are sensed in some way, and defense measures are enacted in response. If the bacteria successfully respond to an encountered stress, they survive and multiply. If they are unsuccessful or inefficient in their response, it can result in death. Efficiently responding to factors that induce stress is especially important for bacteria that inhabit environments that are constantly changing, or for those that inhabit more than one biological niche. In addition, bacterial species that associate with humans and other organisms must be able to overcome stresses that are produced by the host immune response in order to colonize and cause disease. The wide variety of stressors encountered by bacteria has resulted in countless strategies that are used by pathogens to overcome these insults, which we continue to identify. Clearly, a better understanding of these stress response mechanisms may be useful for developing new strategies to combat bacteria that cause certain infectious diseases.

This Research Topic aims to highlight our increasing understanding of mechanisms by which bacteria sense and respond to stresses encountered in the host or other environments. Examples of stress response mechanisms of interest include, but are not limited to those that respond to antimicrobials, host immune responses, or environmental changes.

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MINI REVIEW
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Regulation of Transcription Termination of Small RNAs and by Small RNAs: Molecular Mechanisms and Biological Functions

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Accurate and efficient transcription termination is an important step for cells to generate functional RNA transcripts. In bacteria, two mechanisms are responsible for terminating transcription: intrinsic (Rho-independent) termination and Rho-dependent termination. Growing examples suggest that neither type of transcription termination is static, but instead are highly dynamic and regulated. Regulatory small RNAs (sRNAs) are key players in bacterial stress responses, are frequently expressed under specific growth conditions, and are predominantly terminated through the intrinsic termination mechanism. Once made, sRNAs can base-pair with mRNA targets and regulate mRNA translation and stability. Recent findings suggest that alterations in the efficiency of intrinsic termination for sRNAs under various growth conditions may affect the availability of a given sRNA and the ability of the sRNA to function properly. Moreover, alterations of mRNA structure, translation, and accessibility by sRNAs have the potential to impact the access of Rho factor to mRNAs and thus termination of the mRNA. Indeed, recent studies have revealed that some sRNAs can modulate target gene expression by stimulating or inhibiting Rho-dependent termination, thus expanding the regulatory power of bacterial sRNAs. Here we review the current knowledge on intrinsic termination of sRNAs and sRNA-mediated Rho-dependent termination of protein coding genes in bacteria.

Keywords: SgrS, ChiX, SraL, Hfq, CsrA, Rho

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BACTERIAL SRNA FUNCTION AND THE ROLE OF HFQ

Critical processes in bacteria, including those necessary for pathogenesis, are frequently regulated at multiple levels. Small regulatory RNAs play important roles in both bacteria and hosts. The bacterial small RNAs (sRNAs) function by base-pairing to target mRNAs, resulting in stimulation or inhibition of mRNA stability and translation. These sRNAs are usually around 100 nt long, and use a short seed region (8–15 nt) to form an RNA duplex with mRNA for regulation (Storz et al., 2011; Wagner and Romby, 2015). In *E. coli* and many related bacteria, sRNAs often act in concert with the RNA chaperone Hfq. Mutations in *hfq* are associated with attenuated virulence in many

pathogens, suggesting critical roles for sRNAs in pathogenesis (Chao and Vogel, 2010), although, in a few instances, Hfq can act independently of sRNAs [see, for instance (Chen and Gottesman, 2017)].

Hfq, an Lsm/Sm family RNA binding protein, forms a ring-shaped homohexamer. Hfq binds to and stabilizes sRNAs and promotes their pairing with mRNA targets (Vogel and Luisi, 2011; Updegrove et al., 2016). Three different surfaces on the hexamer have been shown to be important for RNA binding. The proximal face binds polyU sequences, and mutations on this surface disrupt sRNA binding *in vitro* and sRNA stability *in vivo* (Otaka et al., 2011; Sauer and Weichenrieder, 2011). The distal face binds AAN repeats (Robinson et al., 2014), frequently found on mRNA targets of sRNAs. Some RNAs bind to the rim of Hfq through AU rich regions; the rim has also been implicated in helping bring sRNA and mRNA together (Panja et al., 2013). Most sRNAs are quite stable in the cell when bound to Hfq, but are presumably displaced from Hfq and degraded after pairing to mRNA targets (Massé et al., 2003; Schu et al., 2015). Thus, sRNA function and stability depend on the ability to bind Hfq properly.

Here we focus on sRNAs in *Escherichia coli*, one of the species in which these regulators have been well studied. sRNAs are usually expressed from dedicated and well-regulated promoters, but in some cases, sRNAs are processed from the 3′ end of mRNAs, and are thus dependent on the upstream mRNA promoters for expression (Miyakoshi et al., 2015b; Kavita et al., 2018). Regulation of sRNA promoters is often in response to specific cellular stresses, contributing to the cells' ability to adapt to changing environments. For instance, the iron-responsive RyhB sRNA is induced in response to iron deficiency and negatively regulates multiple iron binding proteins, thus helping the cell save iron for critical proteins (Massé and Gottesman, 2002). SgrS, on the other hand, is an sRNA made in response to accumulation of toxic sugar phosphates; it negatively regulates genes involved in the import of the sugar phosphates into the cell, while upregulating a phosphatase gene critical for detoxification of phosphosugar stress (Papenfort et al., 2013; Bobrovskyy and Vanderpool, 2014).

Regardless of whether an sRNA is processed from a longer transcript or not, a critical feature that enables sRNAs to bind Hfq is the presence of a Rho-independent terminator at its 3′ end (Otaka et al., 2011; Morita et al., 2017). This requirement suggests that transcription termination is crucial for both sRNA biogenesis and function. Recent work highlighting this process of sRNA termination, ways in which intrinsic terminators of sRNAs may be distinct from other intrinsic terminators, and the ways in which termination may be regulated are discussed in the first part of this review.

sRNAs, by binding to mRNAs, can affect many aspects of mRNA folding, translation, and decay, as well as access of RNA binding proteins. While sRNAs frequently act to alter ribosome binding and translation initiation, recent findings highlight how sRNAs can affect gene expression by blocking or facilitating premature Rho-dependent transcription termination within the genes of target mRNAs. We review the characteristics of these interactions in the second part of this review.

RHO-INDEPENDENT TERMINATION

Transcription termination that is independent of termination factors is known as Rho-independent termination, also called intrinsic termination [reviewed in Roberts (in press)]. Essential elements of a Rho-independent terminator consist of a GC-rich dyad repeat that forms a stem-loop (hairpin) structure followed by a T-rich stretch, generating a U-rich tail in the RNA after termination (Adhya and Gottesman, 1978). Rho-independent termination is achieved by formation of the stem-loop structure, which is facilitated by RNA polymerase pausing during transcription of the T-rich tract (Ray-Soni et al., 2016). The T-rich stretch is highly conserved among Rho-independent terminators in bacteria, while sequences of the stem-loop seem not to be conserved except for their GC-rich characteristic. Interestingly, T-rich stretches can be found in terminators for both eukaryotic RNA polymerase III and archaeal RNA polymerase (Arimbasseri et al., 2013; Maier and Marchfelder, 2019), suggesting that the intrinsic termination within a T-stretch is a fundamental characteristic of termination pathways. Although many of the T-rich stretches contain four to eight Ts, they are frequently disrupted with other nucleotides (d'Aubenton Carafa et al., 1990; Chen et al., 2013). Intrinsic termination can be directly measured *in vitro* by appearance of properly terminated transcripts, and *in vivo* by reporters to measure termination read-through [see, for instance (Morita et al., 2017)].

Characteristics of sRNA Rho-Independent Terminators

Transcription of genes encoding sRNAs are generally terminated by Rho-independent termination (Livny and Waldor, 2007). A notable feature of Rho-independent terminators of sRNAs is a consecutive T stretch longer than seven nucleotides, which is not necessarily found in all Rho-independent terminators (Otaka et al., 2011; Morita et al., 2017). The fact that discontinuous and relatively short T-rich stretches are found at many Rho-independent terminators implies that seven or more Ts found in sRNA terminators is not required for transcription termination. The transcribed long T stretch, i.e., a polyU tail of seven or more Us, is the primary element responsible for the binding of sRNAs to Hfq (**Figure 1A**). Studies of sRNA SgrS and others demonstrated that shortening the polyT stretch to four Ts in terminators of sRNA genes no longer produced functional sRNAs *in vivo* (Otaka et al., 2011). Additionally, the individual consecutive six uridines, including the last U, bind to the proximal face of the Hfq hexamer *in vitro* (Sauer and Weichenrieder, 2011). Consistent with these findings, deep sequencing analyses of Hfq-binding sRNAs verified that polyU tails of sRNAs longer than six Us are primary binding sites for Hfq *in vivo* (Holmqvist et al., 2016; Melamed et al., 2016) (**Figure 1Bi**). It should be noted that even shortening an *sgrS* variant from eight Ts to six Ts generated less functional SgrS, implying that six Us is not long enough to produce a fully functional sRNA (Otaka et al., 2011). Because, from a structural perspective, the six U tail seems to be sufficient for binding to Hfq, a question remains what the role of extra U(s) plays in sRNA function *in vivo*. In addition, some sRNAs possess

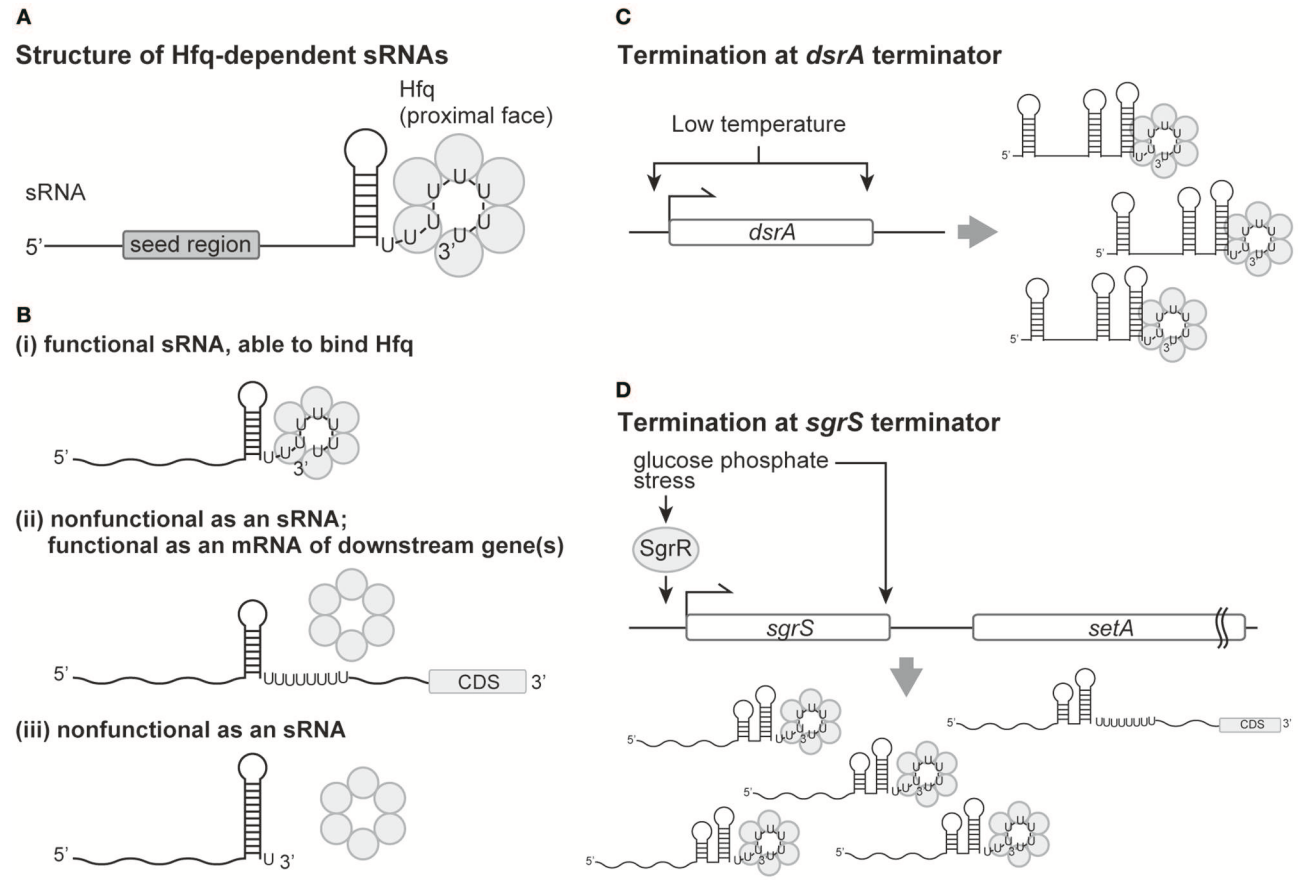

FIGURE 1 | Production of the polyU tail of sRNAs by Rho-independent termination and the role of a polyU tail in Hfq binding and sRNA function. **(A)** Functional structure of an Hfq-dependent sRNA. The polyU tail of seven or longer is responsible for binding to the Hfq proximal face. The seed region is defined as the region which pairs with target mRNAs and is generally located 5′ to the terminator. **(B) (i)**: Termination at the seventh or longer position within the T stretch is necessary for functional sRNAs. **(ii)**: Transcripts with 3′ extensions resulting from termination site read-through bind poorly to Hfq, resulting in non-functional sRNAs. **(iii)**: The transcripts with the U tail shortened by premature termination are no longer able to bind Hfq, resulting in non-functional sRNAs. **(C)** Low temperature increases both the level of transcription initiation and the termination efficiency for the gene encoding the DsrA sRNA. **(D)** Termination efficiency at the *sgrS* terminator is improved by glucose-phosphate stress, which also induces transcription initiation of *sgrS* by SgrR, a transcriptional regulator. Increased transcription termination will result in more production of SgrS and less expression of the downstream *setA* mRNA.

a discontinuous U-rich tail disrupted with other nucleotides (Otaka et al., 2011; Morita et al., 2017). Effects of these discontinuities on the function and/or production of sRNAs also remain to be studied.

In contrast to a conserved polyT stretch, nucleotide sequences for the stem-loop structure seem not to be conserved between intrinsic terminators (Ishikawa et al., 2012; Morita et al., 2017). In fact, the SgrS sRNA was found to be fully functional with a heterologous terminator with a long polyU tail and hairpin stability approximately equal to that of the native terminator (Otaka et al., 2011). The stem-loop structure itself is critical for sRNA function, likely because without this structure termination efficiency is disrupted, resulting in 3′-extended transcripts. Nucleotide substitutions in the DsrA and RybB terminators that reduce thermodynamic stability of the stem were isolated as mutations affecting their function (Sledjeski and Gottesman, 1995; Balbontín et al., 2010). Consistent with the structure of Hfq specifically binding to the hydroxyl group at the 3′-end of the RNA *in vitro* (Sauer and Weichenrieder, 2011), a recent study found that read-through products of SgrS and RyhB with a polyU tract internal to the transcript did not bind Hfq *in vivo* (Morita et al., 2015). These findings suggest that 3′-extended transcripts resulting from read-through no longer function as sRNAs (**Figure 1Bii**). One sRNA, DicF, was reported to be produced both via termination and via 3′-processing by RNase III from a longer read-through transcript (Faubladier et al., 1990). However, a *dicF* coding region expressed from a plasmid and lacking the downstream processing site still generated functional DicF *in vivo* (Balasubramanian et al., 2016), suggesting that the intrinsic termination pathway is sufficient for the production of DicF, as with other sRNAs.

Another important feature of the terminator stem-loop structure is the strength of the stem, which affects the position of termination. A moderate but not too strong stem is needed to

generate a polyU tail of seven nucleotides or longer. Extension of SgrS and RyhB terminator stems with additional G-C pairs resulted in premature termination and the generation of non-functional transcripts with polyU tails shorter than six (Morita et al., 2017; **Figure 1Biii**).

Based on the sRNAs studied thus far, the intrinsic terminators of sRNAs are expected to have unique features, forming a subset of the Rho-independent terminators in *E. coli* and possibly other bacteria. These features—a polyT stretch of seven or more and a moderate-strength stem-loop that enables termination after a stretch of seven or more Ts—can help in identifying potential Hfq-dependent sRNAs from genomic sequence analysis, and possibly help in distinguishing RNA transcripts that encode short proteins from those that have the potential to act as sRNAs. This would also be useful for the design and engineering of synthetic sRNAs for the control of specific gene expression. The absence of the critical characteristics for Hfq binding in the intrinsic terminators for mRNAs might prevent these mRNAs from binding to and blocking the proximal face of Hfq, where sRNAs must bind.

Regulation of Rho-Independent Termination of sRNAs

A growing number of studies have found that sRNAs can be encoded upstream of ORFs, where the promoter upstream of the sRNA is responsible for expression of the downstream ORF. In these cases, expression of the ORF requires that transcription continue through the sRNA termination sequences, leading to a mRNA in which the embedded sRNA is non-functional. SgrS is co-transcribed with the downstream gene *setA* encoding a SET (sugar efflux transporter) family protein, although SgrS requires transcription termination at its own terminator for function (Sun and Vanderpool, 2011; Morita et al., 2015). SroC, one of the 3′ derived Hfq-binding sRNAs, is encoded within the *gltIJKL* operon encoding a Glu/Asp ABC transporter and likely needs to terminate at its own Rho-independent terminator for function (Vogel et al., 2003; Miyakoshi et al., 2015a). Given that Rho-independent termination impacts functional sRNA production, the downstream genes are assumed to be expressed discordantly from these sRNAs. Although the biological significance of the discordant expression of sRNAs and the downstream genes remains to be determined, a recent study revealed that such discordant expression by internal Rho-independent termination occurs frequently within operons and contributes to preferred expression levels for individual proteins (Lalanne et al., 2018).

Intriguingly, growing evidence suggests that the efficiency of transcription termination at Rho-independent terminators of sRNAs can be regulated by the same physiological and/or stress signals that induce initiation of sRNA transcription. Termination efficiency at the terminator for the DsrA sRNA, a positive regulator of RpoS translation, is increased at low temperature, where the *dsrA* promoter is most active (Sledjeski et al., 1996; **Figure 1C**). Similarly, an increase in termination efficiency at the SgrS and RyhB terminators was observed under conditions of both the cognate stress for transcriptional induction of these sRNAs and non-cognate stresses (Morita et al., 2015; **Figure 1D**). One can envision that this increase in termination efficiency would result in more effective production of sRNAs under specific conditions.

Previous studies on Rho-independent termination provide clues to the molecular mechanism by which Rho-independent termination is regulated. Lower levels of UTP nucleotide were found to improve transcription termination at several Rho-independent terminators *in vitro* (Farnham et al., 1982; McDowell et al., 1994). The density of RNA polymerase on an mRNA was found to influence the rate of transcription elongation/termination at Rho-independent terminators *in vivo* and *in vitro*, with higher transcription (more RNA polymerases) decreasing pausing and termination (Jacquet and Reiss, 1992; Epshtein and Nudler, 2003). Therefore, this linkage between promoter strength (transcription initiation) and termination provides a way in which stresses that decrease overall transcription activity on a given gene may result in increases in the termination efficiency. In addition, specific protein factors can be involved in regulation of Rho-independent termination. NusA is an essential protein which affects termination efficiency at Rho-independent terminators in multiple ways, by contributing to RNA polymerase pausing and by helping form and stabilizing RNA structures upon termination (Nudler and Gottesman, 2002; Guo et al., 2018; Holmqvist and Vogel, 2018), and thus could regulate termination in response to stresses. Hfq can be excluded as a candidate factor because it seems not to be involved in the termination at sRNA terminators (Morita et al., 2015).

Most *in vivo* experiments on Rho-independent termination have been carried out with protein-coding genes as templates. The situation at the sRNA terminators might be different from that at the mRNA terminators because the protein-coding genes are typically longer than sRNA genes. For instance, for short sRNA-encoding transcripts, dissociation of the sigma factor from the RNA polymerase, usually assumed to occur soon after transcription initiation, might not occur before the polymerase reaches the intrinsic terminator, possibly changing the efficiency of termination. A recent long-read RNA sequencing strategy enabled analysis of intact transcripts and revealed that the degree of readthrough of several mRNA terminators also varied between growth conditions (Yan et al., 2018). A critical question for future investigation will be how Rho-independent terminators are modulated and whether these mechanisms of modulation are specific to sRNA terminators, or also affect those for mRNAs.

RHO-DEPENDENT TRANSCRIPTION TERMINATION

In *E. coli*, 20–30% of transcription events are terminated in a Rho-dependent manner [(Peters et al., 2009, 2012); reviewed in Roberts (in press)]. While Rho is generally not used to form the 3′ ends of regulatory sRNAs, its ability to terminate mRNAs can be regulated by sRNAs.

Transcription termination factor Rho is an ATP-dependent RNA helicase/translocase, which can bind to a sequence motif

called the Rho utilization (*rut*) site on the nascent transcript, translocate along the naked RNA and dissociate the elongation complex to terminate transcription (Grylak-Mielnicka et al., 2016; Ray-Soni et al., 2016). A typical *rut* site is a single-stranded, ribosome-free, cytosine-rich/low-guanine RNA sequence with a length of ∼60–80 nucleotides. Thus, molecular or cellular processes that change ribosome occupancy on mRNA or the single-stranded nature of an RNA stretch at or adjacent to the *rut* site may regulate Rho's function. Rho forms a homohexamer ring, and uses its N-terminal OB-like protein fold to bind cytosine-rich sequences, while a region near the C-terminal part of the protein binds to the RNA threaded in the channel; the latter binding event activates Rho's ATPase activity, driving its translocation (Skordalakes and Berger, 2006). Several models of Rho-dependent transcription termination have been proposed (Peters et al., 2011; Ray-Soni et al., 2016), but the molecular details in terms of the mechanism of Rho translocation and RNA polymerase dissociation on Rho-dependent terminators remain to be fully determined. *In vitro* approaches to study Rho-dependent termination and its regulation by sRNAs have been recently described (Nadiras et al., 2018). *In vivo* approaches primarily depend on defining Rho-dependent termination with Rho-specific inhibitors such as bicyclomycin, and evaluating effects of sRNAs on mRNA expression by measuring read-through products with reporter assays, quantitative PCR or deep sequencing (Hussein et al., 2015; Elgamal et al., 2016; Sedlyarova et al., 2016).

Rho-Dependent Transcription Termination Affected by sRNAs and RNA-Binding Proteins

Rho-dependent termination at the 3′ end of protein-coding genes is well documented, and this process is not believed to be significantly regulated. Rho can also terminate transcription inside genes and within operons, and this is likely a highly regulated process given the fact that the translation status of mRNAs can directly affect Rho access to *rut* sites inside coding sequences (Adhya and Gottesman, 1978). Genome-wide analysis of RNA Polymerase redistribution in the presence of the Rho-specific inhibitor bicyclomycin (BCM) found that Rho terminates transcription intragenically within ∼100 genes (Peters et al., 2009, 2012). The observation of such intragenic termination sites raised the possibility that factors that can affect translation, such as sRNAs or RNA-binding proteins, may regulate Rho-dependent termination at these sites.

A seminal study documented sRNA-mediated regulation of Rho-dependent termination in *Salmonella* (Bossi et al., 2012). ChiX is an Hfq-dependent sRNA that can bind to the *chiP* mRNA, encoding the outer membrane channel (porin) for chitosugars, at its ribosome binding site, inhibit translation and trigger *chiP* mRNA decay (Figueroa-Bossi et al., 2009). Interestingly, ChiX-mediated translation inhibition has a secondary effect. By blocking entry of ribosomes on *chiP* mRNA, ChiX binding exposes an intragenic *rut* site for Rho loading, leading to premature termination of transcription inside the *chiP* coding sequence (**Figure 2A**). This regulation not only reinforces the inhibition of *chiP* translation but also leads to a polarity effect, inhibiting expression of the downstream *chiQ* gene in the same operon (Bossi et al., 2012). When chitosugars are available and thus the transporter is needed, ChiX is destroyed by interaction with an RNA decoy, induced dependent on chitosugar sensing (Figueroa-Bossi et al., 2009; Overgaard et al., 2009). Therefore, regulation coordinates with other transcriptional circuits to ensure genes responsible for utilization of chitin-derived oligosaccharides are switched on only when chitosugars are present. In a second example, the *E. coli* Spot 42 sRNA base pairs with the *galK* leader, the third gene in the *galETKM* operon, resulting in translational inhibition of *galK* as well as an increased Rho-dependent transcription termination, possibly by interfering with translational coupling between ribosomes translating through *galT* with those initiating translation of *galK*, and thus allowing Rho access (Wang et al., 2015; **Figure 2A**). This type of regulation allows discoordinate expression of operon genes in response to metabolic needs of the cells.

In the cases described above, sRNAs facilitate Rho access to *rut* sites by interfering with mRNA translation, consistent with the consensus that mRNA sequences devoid of translating ribosomes are preferable targets for Rho binding and regulation. However, sRNAs can also positively regulate genes, raising the possibility that in some cases, sRNAs might antagonize Rho function by blocking Rho binding and termination. In addition to the 3′ end of genes and the intragenic regions containing *rut* sites, long 5′ UTRs (>80 nt) of mRNAs are another reservoir of potential sites for Rho-dependent termination. Transcriptomic analyses of RNA samples from *E. coli* treated with sublethal concentrations of Rho inhibitor BCM (Sedlyarova et al., 2016) suggested that, out of the 1,200 5′ UTRs longer than 80 nts, at least 250 were targets for Rho-dependent termination. One such 5′ UTR is the 567 nt leader of *rpoS*, an mRNA known to be positively regulated by sRNAs (Battesti et al., 2011). In-depth analysis of regulation in the *rpoS* 5′ UTR revealed that the three base-pairing sRNAs (RprA, DsrA, and ArcZ) that activate *rpoS* mRNA translation can antagonize Rho-dependent premature termination in the *rpoS* leader (Sedlyarova et al., 2016; **Figure 2B**). Such a regulatory mechanism is likely not limited to *rpoS*, but may apply to many other putative targets of these three sRNAs, as well as other positively regulated mRNAs. As optimal growth of *E. coli* in the stationary stress condition relies on expression of the stationary sigma factor RpoS, the sRNA-mediated upregulation of RpoS production both via increasing ribosome entry and via anti-Rho-dependent premature termination provides an important way for sRNAs to contribute to the response to stress.

In another example of an sRNA preventing Rho-dependent termination, the *Salmonella* SraL sRNA was reported to base-pair with the leader for *rho* mRNA, protecting it from transcription attenuation and thus increasing levels of full-length *rho* mRNA (Silva et al., 2019; **Figure 2B**). While the physiological role of this regulatory interaction is not known, it raises the possibility that under certain conditions the cell might modulate Rho levels and thus Rho-dependent termination via the SraL sRNA. For both the *rpoS* and *rho* leader, it is still not clear how

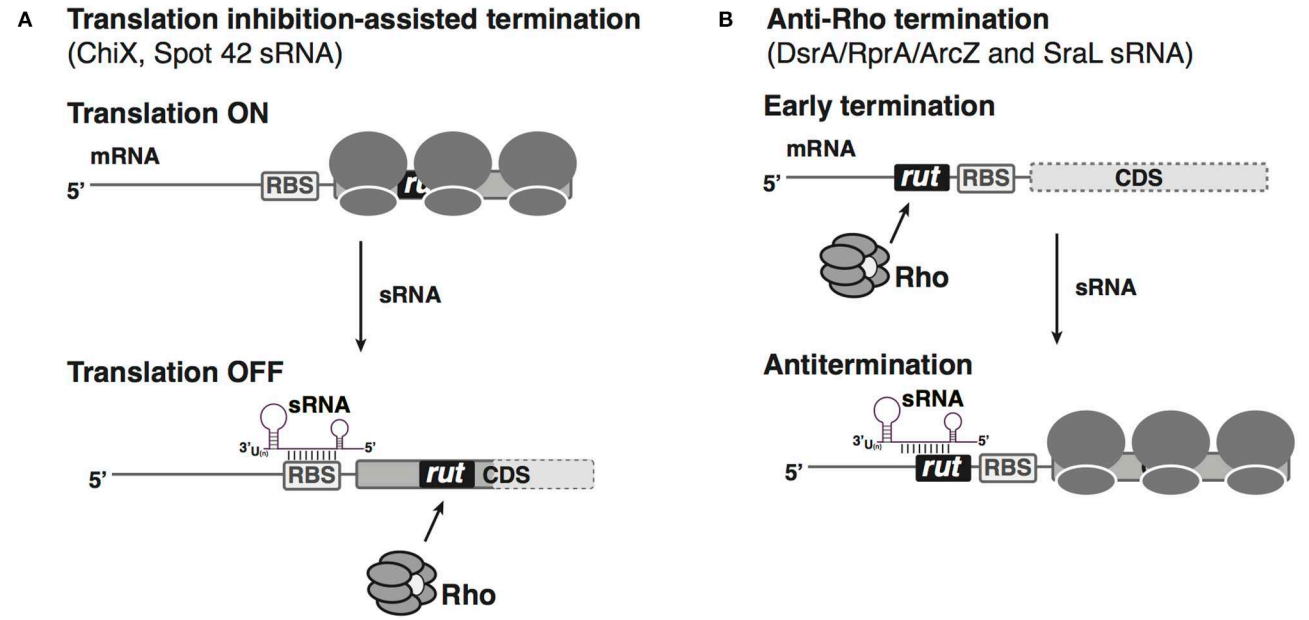

FIGURE 2 | sRNA-based negative or positive regulation of Rho-dependent transcription attenuation in bacteria. **(A)** Translation inhibition by sRNA assists Rho-dependent termination. When the accumulation of *Salmonella* ChiX or *E. coli* Spot 42 sRNA is low, the mRNAs *chiPQ* and *galETKM* are translated well. When ChiX or Spot42 sRNA is produced at high levels, under specific environmental conditions, sRNA annealing blocks the ribosome binding site of the corresponding mRNA target (*chiP* or *galK*, respectively); reduced translation allows Rho loading onto a cryptic *rut* site to terminate transcription inside the genes (dotted pale portion of box represents untranscribed portion of the mRNA). **(B)** Antagonization of Rho-dependent attenuation by sRNAs. In genes with long 5′ UTRs, *rut* sites can allow Rho termination, blocking expression of the downstream gene (dotted box represents untranscribed coding region). sRNAs binding within the 5′ UTR can block Rho access to the *rut* sites, thus acting as anti-terminators for the downstream CDS.

sRNA binding prevents Rho-dependent premature termination. In both cases, the sRNA binds upstream of the RBS site, and in the absence of corresponding sRNAs, Rho can prematurely terminate transcription in the leader. Therefore, it is very likely that sRNA blocks Rho loading by occluding a *rut* site, either directly by overlapping the site, or indirectly via RNA structure rearrangements. The latter regulatory mechanism is exploited by the *E. coli* CsrA RNA-binding protein in regulating *pgaABCD* operon expression (Figueroa-Bossi et al., 2014). *pgaA* mRNA is prematurely terminated in its leader in a Rho-dependent manner, and this regulation relies on CsrA binding to a region in the long *pgaA* leader; CsrA binding refolds the *pgaA* leader, exposing an intragenic *rut* site for Rho binding to prematurely terminate transcription.

In addition to the role of Hfq in chaperoning sRNA stability and function, sRNA-independent roles of Hfq also have been reported (Wagner and Romby, 2015; Chen and Gottesman, 2017). Rabhi et al. (2011) elegantly demonstrated that Hfq, as a general RNA-binding protein, can antagonize Rho-dependent transcription termination at a prototypical terminator (λtR1) both *in vitro* and *in vivo*. The antitermination activity of Hfq depends on (1) its distal face binding to an A-rich sequence motif upstream of *rut* sites in λtR1, and (2) physical interaction of Hfq with Rho protein; this interaction can block Rho binding to other proteins, such as NusG, which has been shown to stimulate Rho termination at some terminators (Mitra et al., 2017). In this case, Hfq directly modulates Rho's RNA-DNA unwinding activity, rather than simply blocking access of Rho to RNA. Such a regulation is distinct from the roles that sRNAs and CsrA play in regulating Rho access to RNAs. Nonetheless, these reports of both negative and positive regulation of Rho-dependent termination by Hfq-dependent sRNAs or RNA-binding proteins reinforce the idea that RNA-based regulation of termination is widely exploited by bacteria.

Autoregulation of Rho

Rho is an abundant protein in many bacteria, including *E. coli* (Grylak-Mielnicka et al., 2016). It is autoregulated through a transcription attenuation mechanism at its own leader in *E. coli* and in other bacteria (Barik et al., 1985; Matsumoto et al., 1986; Ingham et al., 1999), emphasizing the importance of cells maintaining an appropriate level of Rho and presumably of Rho-dependent termination. In *Rhodobacter capsulatus*, Rho is significantly induced under anaerobic growth (Jäger et al., 2004), suggesting a role of Rho in response to changing environments. The recent finding of an sRNA, SraL, that regulates Rho expression, described above (Silva et al., 2019), further highlights the likelihood that cells adjust termination under some conditions by adjusting Rho availability.

SUMMARY AND ISSUES FOR THE FUTURE

sRNA research in the last 20 years have led to a growing appreciation of the ways in which small regulatory RNAs

participate in stress circuits in bacteria. In the simplest terms, a sRNA will be induced in response to a stress, and it will pair and regulate the expression of mRNAs encoding proteins that help the cell deal with stress, and will thus contribute in significant ways to the recovery from the stress (Holmqvist and Wagner, 2017; Fröhlich and Gottesman, 2018). Here we have reviewed recent studies that highlight the complexity of this process and its intersection with transcription termination. In particular:

1) While transcription initiation remains the most important contributor to sRNA synthesis and accumulation, proper termination of the sRNA transcript is equally important for its function. The requirements for efficient termination and binding of the nascent sRNA to Hfq have likely selected for particular characteristics of intrinsic terminators for sRNA-encoding genes.
2) Recent work shows that the efficiency of proper intrinsic termination at sRNA terminators can be changed by cellular stress, by mechanisms still to be explored. It remains to be determined if these changes in termination affect intrinsic termination in general or are specific to sRNA terminators. For sRNAs, however, stress that changes the efficiency of termination will affect the fraction of transcripts that become functional sRNAs. This provides new insight into the mechanism of Rho-independent termination, which has been considered a simple, unregulated process.
3) Rho-dependent termination requires access of Rho to single-stranded RNA, and thus sRNAs that block translation may open up Rho entry sites within genes, reinforcing negative regulation of the mRNAs. sRNA binding to otherwise untranslated RNAs (for instance, in 5′ UTRs) can positively regulate downstream ORFs by blocking Rho entry.

The extent and importance of these levels of regulation are just beginning to be realized. Much still remains to be addressed to fully understand the effect of sRNAs on transcription termination. Unlike translational repression and mRNA degradation, effects on termination must occur rapidly, during the transcription process. This suggests that sRNA pairing with mRNAs must be happening co-transcriptionally; how often does that happen? Are there properties of the particular mRNAs, sRNAs, or transcriptional machinery that will favor this? In addition to the trans-acting sRNAs discussed here, the cis-regulatory riboswitches can also control gene expression through modulation of Rho-dependent transcription termination (Hollands et al., 2012; Proshkin et al., 2014; Bastet et al., 2018). It seems likely that, in addition to uncovering new levels of stress regulation, these studies may lead to a deeper understanding of transcription termination itself.

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frontiers in Cellular and Infection Microbiology

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Regulated Proteolysis in *Vibrio cholerae* Allowing Rapid Adaptation to Stress Conditions

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The lifecycle of the causative agent of the severe secretory diarrheal disease cholera, *Vibrio cholerae*, is characterized by the transition between two dissimilar habitats, i.e., as a natural inhabitant of aquatic ecosystems and as a pathogen in the human gastrointestinal tract. *Vibrio cholerae* faces diverse stressors along its lifecycle, which require effective adaptation mechanisms to facilitate the survival fitness. Not surprisingly, the pathogen's transcriptome undergoes global changes during the different stages of the lifecycle. Moreover, recent evidence indicates that several of the transcription factors (i.e., ToxR, TcpP, and ToxT) and alternative sigma factors (i.e., FliA, RpoS, and RpoE) involved in transcriptional regulations along the lifecycle are controlled by regulated proteolysis. This post-translational control ensures a fast strategy by the pathogen to control cellular checkpoints and thereby rapidly respond to changing conditions. In this review, we discuss selected targets for regulated proteolysis activated by various stressors, which represent a key feature for fast adaptation of *V. cholerae*.

Keywords: post-translational regulation, stressor, Lon, Clp, DegS, DegP YaeL, tail-specific protease

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A BRIEF SURVEY OF REGULATORY EVENTS ALONG *Vibrio cholerae*'s LIFECYCLE

Vibrio cholerae spends much of its lifecycle outside of the host in estuarine and costal aquatic reservoirs with a geographical range from tropics to temperate waters world-wide. Along its interepidemic persistence in the aquatic reservoirs, *V. cholerae* faces temperature shifts, osmotic stress, bacterivorous predators and nutrient limitation (Lutz et al., 2013; List et al., 2018). *Vibrio cholerae* employs several strategies to cope with these numerous stressors. In particular, biofilm formation has been highlighted as a key factor for environmental survival and transmission of *V. cholerae* (comprehensively reviewed in Yildiz and Visick, 2009; Teschler et al., 2015). A central player of biofilm regulation is the transcriptional repressor HapR, which acts negatively on biofilm formation via repression of exopolysaccharide synthesis. Additionally, HapR is a quorum sensing key regulator affecting virulence factor expression and natural competence (Ng and Bassler, 2009). Since transcription of *hapR* is also activated by the alternative sigma factor RpoS, the pathways mentioned above are regulated by central physiological signals, like cell density, or carbon concentration. Biofilm-associated bacteria are generally better protected against host-derived stressors ranging from digestive enzymes, acidic pH to antimicrobial substances and exhibit hyperinfectivity in the murine model (Tamayo et al., 2010; Seper et al., 2011). Thus, biofilm clumps

are a likely form by which clinically relevant *V. cholerae* initiate outbreaks (Colwell et al., 2003; Hall-Stoodley and Stoodley, 2005).

Upon oral ingestion, *V. cholerae* passages through the stomach to finally reach the small intestine, representing the primary site of colonization. Intestinal stimuli induce expression of virulence factors such as the toxin coregulated pilus (TCP) and the cholera toxin (CTX) (Childers and Klose, 2007; Matson et al., 2007). TCP represents the main colonization factor responsible for adherence to epithelial cells, while CTX constitutively activates adenylate cyclase of the host, causing a massive water efflux into the intestinal lumen known as rice-water stool (Sharp et al., 1971; Burns et al., 1983; Miller et al., 1987; Taylor et al., 1987; Herrington et al., 1988; Fishmann, 1990).

The expression of virulence factors is controlled by a complex regulatory cascade. It includes the membrane-bound transcription complexes ToxR/S and TcpP/H as well as the cytosolic transcription factor ToxT (Childers and Klose, 2007). Most of the virulence factors, e.g., CTX and TCP, are regulated by the ToxT-dependent pathway in response to intestinal stimuli, such as temperature and sodium bicarbonate (Thomson and Withey, 2014; Weber et al., 2014). In addition, ToxR can directly regulate several genes independently of ToxT. For example, ToxR inversely regulates the expression of the porins OmpU and OmpT, which plays an essential role to achieve bile resistance and full colonization fitness *in vivo* (Provenzano and Klose, 2000).

Once adapted to the intestinal conditions, *V. cholerae* starts to massively proliferate and the patient develops a severe secretory diarrhea, releasing the bacteria back into the aquatic environment. Transcriptional control of a defined set of genes at the late stage of infection facilitates the transition of *V. cholerae* into the aquatic reservoir (Schild et al., 2007). Under these conditions HapR and RpoS coordinate a drastic shift in the gene expression profile also known as "mucosal escape response" (Nielsen et al., 2006).

As highlighted above, adaptation to diverse conditions along the lifecycle is achieved by spatio-temporal induction of gene expression. However, termination of a regulatory pathway could be equally important to facilitate transition to the next stage of the lifecycle. An effective way to remove factors involved in gene transcription is regulated proteolysis, comprising directed degradation of defined effectors by specific proteases (Mahmoud and Chien, 2018). Indeed, regulated proteolysis has been reported as a control mechanism for several transcriptional effectors (i.e., FliA, ToxR, TcpP, ToxT, RpoS, and RpoE) along the lifecycle of *V. cholerae* (**Figure 1** and **Table 1**), which will be discussed with an emphasis on the physiological impact and players involved.

THE ALTERNATIVE SIGMA FACTOR FliA (σ^{28}) IS DEGRADED BY Lon

In addition to virulence factor expression, flagella-dependent motility contributes to virulence of *V. cholerae*. The single polar flagellum is required to approach and penetrate the mucosal layer of the intestinal epithelium (Freter and Jones, 1976; Freter and O'Brien, 1981; Lee et al., 2001; Butler and Camilli, 2005). Entrance into the viscous mucosal layer puts substantial shear force on the rotating flagellum, which eventually breaks. As a result, the anti-sigma factor FlgM, usually bound to the alternative sigma factor FliA and preventing its association with the RNA polymerase, is released through the broken flagellar apparatus (Correa et al., 2004; Liu et al., 2008). Decreasing levels of FlgM result in derepression of FliA important for activation of flagella biosynthesis. Although this is an efficient feedback mechanism to sense damage of the flagellum and initiate its repair, it is dispensable in the *in vivo* setting once *V. cholerae* has penetrated through the mucosal layer. FliA inhibits virulence factor expression in *V. cholerae* by a so far unknown mechanism (Syed et al., 2009). Therefore, efficient removal of FliA from the cytosol is essential to allow full virulence expression in early stages of infection. A recent study demonstrated that FliA of *V. cholerae* is target for rapid proteolysis via the AAA+ (ATPase associated with a variety of cellular activities) protease Lon in the absence of its anti-sigma factor FlgM (Pressler et al., 2016). Under virulence-inducing conditions [i.e., *in vitro* cultivation using AKI conditions (Iwanaga and Yamamoto, 1985; Iwanaga et al., 1986)], the Lon-dependent proteolysis facilitates cholera toxin production in the presence of a damaged flagellum. Thus, the rapid removal of FliA via Lon provides a first molecular explanation for high virulence expression upon mucosal penetration at early stages of infection. Concordantly, FliA of *Escherichia coli* is also a target for Lon-mediated proteolysis and can be protected by the anti-sigma factor FlgM (Barembruch and Hengge, 2007). Thus, the FliA-FlgM-Lon feedback circuit could represent a conserved mechanism for correct flagella assembly and repair upon flagellar damage.

REGULATED PROTEOLYSIS OF ToxR IS MEDIATED BY DegS, DegP AND RseP

Vibrio cholerae persistence and virulence are coordinated by a complex network that has been historically referred as the "ToxR-regulon" (Matson et al., 2007). ToxR is a single component signal transduction regulator comprising the N-terminal winged helix-turn-helix domain promoting DNA-binding, a transmembrane domain and the C-terminal periplasmic sensor domain (Miller et al., 1987). ToxR binds multiple AT-rich promoter proximal regions termed ToxR-boxes and is involved in transcriptional control of more than 100 genes (Miller et al., 1987; Bina et al., 2003; Goss et al., 2013). Molecular activation mechanisms for the membrane bound transcription factor ToxR are limited. Although, it has been recently shown that the transcriptional activation by the ToxR-like protein CadC in *E. coli* and binding to its operator sites, follows a model termed diffusion and capture mechanism (Brameyer et al., 2019). ToxR and TcpP (discussed below), together with their respective co-transcribed interaction partners ToxS and TcpH, are both required for maximal *toxT* expression, whereas ToxRS mediates outer membrane porin (OMP) expression directly (Higgins and DiRita, 1994; Häse and Mekalanos, 1998; Bina et al., 2003; Childers and Klose, 2007). The two major porins of *V. cholerae*, OmpT and OmpU are inversely regulated by

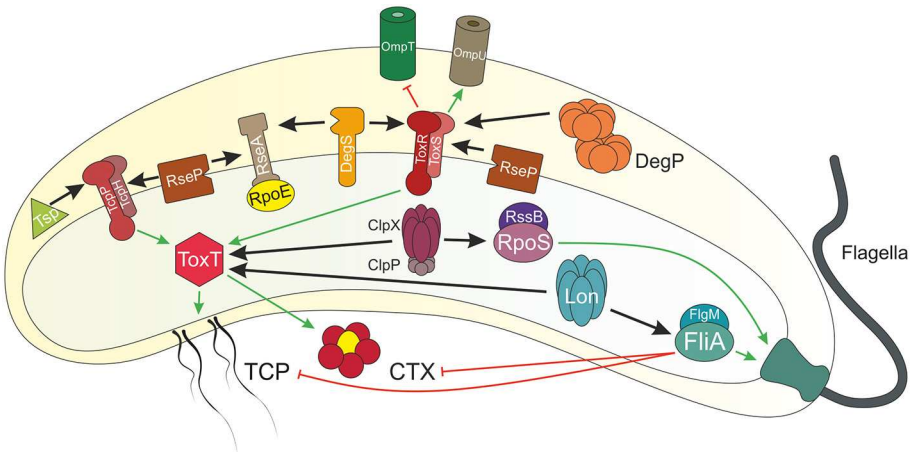

FIGURE 1 | Overview of regulated proteolysis in *V. cholerae*. Shown is a *V. cholerae* cell with proteins illustrated by icons. Regulated proteolysis is indicated by black arrows. Transcriptional activation is highlighted in green and repression in red. At early stages of infection, the single polar flagellum of *V. cholerae* breaks by entering the viscous mucosal layer of the small intestine. Thereby, levels of the anti-sigma factor FlgM decreases within the cell and the alternative sigma factor FliA activates transcription of flagella biosynthesis genes. Besides of this repair mechanism, FliA also inhibits virulence genes expression, e.g., *tcp* and *ctx*. The AAA+ protease Lon degrades FliA in absence of FlgM to achieve full virulence at early stage infection. The membrane embedded transcriptional regulators and their respective partner proteins ToxRS and TcpPH activate *toxT* expression, which in turn encodes for the master regulator for transcription of the downstream genes *tcp* and *ctx*. Furthermore, ToxRS also coordinate the inverse regulation of the outer membrane porins OmpU and OmpT, in order to build up a resistance to bile salts. Both regulators are targets of regulated intramembrane proteolysis (RIP). ToxR periplasmic domain is cut by the site-1 proteases DegS and DegP, followed by site-2 protease RseP. Commensurately, Tsp mediates TcpP degradation as a site-1 protease, which in turn triggers RseP. The half-life of the virulence master regulator ToxT is controlled by the AAA+ proteases Lon and ClpXP. Additionally, the proteases DegS and RseP also act on the transmembrane anti-sigma factor RseA under envelope stress conditions, e.g., cellular or environmental changes, in order to release the alternative sigma factor RpoE to the cytosol, eventually to activate transcription of *degP*, *rpoE* itself or genes encoding for the T2SS. The alternative sigma factor RpoS is responsible to cope with starvation conditions, e.g., high (p)pGpp levels. At low levels of (p)pGpp, the anti-sigma factor RssB is bound to RpoS, leading to proteolysis mediated by ClpXP. At late stages of infection, RpoS is required to activate mucosal escape response by inducing the expression of chemotaxis and motility genes.

TABLE 1 | Examples for regulated proteolysis in *V. cholerae* (for details see text).

Target for proteolysis	Binding partners/(anti-)sigma factors	Protease(s)	Physiological role/regulated pathways	Trigger for proteolysis
FliA (σ^{28})	FlgM	Lon	Motility, virulence	Broken flagellum
ToxR	ToxS	DegS, DegP, RseP	Persistence, virulence	Alkaline pH in combination with starvation
TcpP	TcpH	Tsp, RseP	Virulence	Non-virulence-inducing conditions
ToxT	–	ClpXP, Lon	Virulence	High temperature, alkaline pH
RseA	RpoE (σ^{E} or σ^{24})	DegS, RseP	Envelope stress response	Misfolded periplasmic protein
RpoS (σ^{S} or σ^{38})	RssB	ClpXP	Motility, chemotaxis, biofilm	Non-starvation condition

ToxR. The *ompT* expression is repressed, whereby *ompU* is strongly induced by ToxR under nutrient rich conditions or bile salts (i.e., sodium deoxycholate), facilitating resistance toward antimicrobial compounds (Miller and Mekalanos, 1988; Provenzano et al., 2000; Mathur et al., 2007; Lembke et al., 2018). Unlike the remainder of the regulon, *ompT* is the only verified gene negatively regulated by ToxR and is derepressed under nutrient limiting conditions (Li et al., 2000). In general, *toxR* is constitutively expressed, environmental and stress stimuli may modulate the expression of ToxR regulated genes (Miller and Mekalanos, 1988). Molecular activation mechanisms for ToxR transcriptional activity are largely unknown. The two periplasmic cysteine residues (Cys236 and Cys293) of ToxR influence ToxR regulated gene expression. More specifically, DsbAB-mediated intramolecular disulfide bond and homodimer formation increase ToxR transcription factor activity (Ottemann and Mekalanos, 1996; Fengler et al., 2012; Lembke et al., 2018). Interventions in these cysteine residues (e.g., cysteine to serine substitution, reducing conditions) decrease ToxR transcription factor activity and consequently abolish the ability of proper porin gene regulation, but does not affect *toxT* transcription (Fengler et al., 2012; Lembke et al., 2018). The cysteine-reduced ToxR is a trigger to stimulate site-1 mediated proteolysis by DegS and DegP, hence resulting in ToxR degradation, most effective in strains lacking *toxS* (Lembke et al., 2018). Regulated intramembrane proteolysis (RIP) control of ToxR seems to play a physiologically important role for *V. cholerae* to properly adapt to changing environmental conditions (Almagro-Moreno et al.,

2015a,b). Upon transition into a dormant stage in presence of unfavorable stress conditions, e.g., alkaline pH and nutrient limitation, ToxR becomes a substrate for RIP by the site-2 protease RseP (YaeL), which belongs to the RpoE response system (see below). ToxS also plays a major role in protecting ToxR from proteolysis under these conditions at late stationary phase (Almagro-Moreno et al., 2015b). A point mutation in ToxS ($ToxS^{L33S}$) even triggers ToxR proteolysis comprising several site-1 proteases, including DegS, DegP, VesC, and TapA (Almagro-Moreno et al., 2015b). The two major routes of ToxR proteolysis, one responding toward the redox state and the other being sensitive to an alkaline pH and starvation, can be inhibited by bile salts, which are present in the human gut and are also known to strengthen ToxRS interaction (Midgett et al., 2017; Lembke et al., 2018). The RIP of ToxR is a highly versatile 2-step process, leading to a clearance of ToxR molecules and eventually to a termination of ToxR dependent gene regulation.

TcpP IS A SUBSTRATE OF Tsp AND RseP PROTEASES

A second membrane-bound transcription factor that coordinates expression of *toxT* is TcpP. Like ToxR, TcpP is a bitopic protein containing a carboxy-terminal periplasmic domain and an amino-terminal cytoplasmic DNA-binding domain similar to transcription activators of the OmpR/PhoB-family (Martínez-Hackert and Stock, 1997). TcpP functions together with TcpH, a membrane protein that interacts with the periplasmic domain of TcpP. In order to activate transcription of *toxT*, ToxR recruits TcpP to the *toxT* promoter region through protein-protein interaction, where TcpP binds two pentameric repeats located between positions-53 and -38 relative to the *toxT* transcription start site (Krukonis and DiRita, 2003; Goss et al., 2010). TcpP levels in the bacterial cell are regulated both transcriptionally and post-transcriptionally. Upon entering the human intestine, environmental signals activate expression of *tcpPH* through AphA and AphB (Kovacikova and Skorupski, 1999; Skorupski and Taylor, 1999). Alternatively, under conditions that do not activate virulence gene expression, TcpP is degraded by RIP. The site-1 protease that first acts to cleave TcpP within its periplasmic domain is Tsp (tail-specific protease) (Teoh et al., 2015). Tsp is a serine protease that generally controls protein quality and gene regulation, and is rarely associated with RIP mechanisms. After the initial cleavage, TcpP becomes a substrate for the site-2 protease RseP, a membrane-localized metalloprotease that cuts within the transmembrane domain (Matson and DiRita, 2005). This cleavage and removal from the inner membrane inactivates TcpP, halting expression of *toxT* and downstream virulence genes. TcpP is normally protected from degradation through its interaction with TcpH under virulence-gene inducing conditions (Beck et al., 2004). Disruption of a periplasmic disulfide bond in TcpP results in instability of the protein, even in the presence of TcpH (Morgan et al., 2016). In addition, disruption of these periplasmic cysteines causes TcpH to also become unstable, suggesting a role for these intramolecular disulfide bonds in the TcpP-TcpH interaction (Morgan et al., 2016). Furthermore, transcriptionally active TcpP-homodimers are formed by an intermolecular disulfide bond via Cys207 in presence of the bile salt taurocholate (Yang et al., 2013). Heterodimers between TcpP and ToxR depend on the periplasmic thiol-disulfide-oxidoreductase DsbA and are enhanced by anaerobic growth conditions resulting in virulence gene induction (Fan et al., 2014). It should be noted that the outcome of ToxR and TcpP RIP is unusual in that it functions to inactivate a membrane-bound regulator and halt transcription. In the case of the RpoE-pathway (see below) and others, RIP results in transcriptional activation of downstream genes.

ClpXP AND Lon MEDIATE ToxT PROTEOLYSIS

ToxT was identified as a central transcription factor, activating expression of important virulence genes, e.g., encoding for TCP and CTX (DiRita and Mekalanos, 1991). ToxT is a crucial checkpoint, thereby its own synthesis is under complex control, i.e., by endogenous and exogenous factors as reviewed elsewhere (Weber and Klose, 2011). Exogenous signals negatively control ToxT activity, such as bile-derived unsaturated fatty acids (Plecha and Withey, 2015), or positively, like sodium carbonate (Thomson and Withey, 2014). Additionally, a 5′ mRNA thermometer control element of *toxT* allows access of ribosomes to the Shine-Dalgarno sequence at 37°C, but not at 20°C (Weber et al., 2014). ToxT becomes a substrate for proteolysis during virulence gene expression, reducing ToxT protein half-life (Abuaita and Withey, 2011). Precise timing of ToxT activity is crucial for the colonization success, e.g., by determining the duration and intensity of virulence gene expression. This was best monitored under *in vivo* conditions (Lee et al., 1999) and by a microarray series performed on cells grown under virulence activating conditions *in vitro* analyzing 13 time points within a 6 h period (Kanjilal et al., 2010). ToxT is part of a positive forward feedback loop and therefore positively autoregulated (Yu and DiRita, 1999). ToxT proteolysis is one mechanism to terminate its activity, which is mediated by AAA+ proteases including Lon, ClpXP, and others (Abuaita and Withey, 2011). Proteolytic instability of ToxT is regulated via stressors like high temperature and alkaline pH, and depends on an unstructured region located at amino acid positions 100-109 (Abuaita and Withey, 2011; Thomson et al., 2015). Thereby, ToxT itself harbors a protease sensitive response domain, which may trigger proteolysis depending on the listed exogenous conditions.

THE RpoE (σ^E OR σ^{24})-DEPENDENT STRESS RESPONSE REQUIRES PROTEOLYSIS BY DegS AND RseP

The periplasmic protease/chaperone DegP and the membrane embedded proteases DegS and RseP are essential to react to environmental and cellular changes in Gram-negative bacteria (reviewed by Alba and Gross, 2004; Rowley et al., 2006).

They are part of the envelope stress response mediated by the alternative sigma factor RpoE, first described in *E. coli* upon high temperature conditions, exposure to ethanol, or the overproduction of OMPs (Erickson and Gross, 1989; Wang and Kaguni, 1989; Mecsas et al., 1993). In absence of such stimuli, the N-terminal cytoplasmic portion of the integral membrane bound anti-sigma factor RseA captures RpoE, retaining it to the inner membrane (De Las Peñas et al., 1997; Missiakas et al., 1997; Campbell et al., 2003). The stepwise cleavage of RseA is characteristic of RIP (Ehrmann and Clausen, 2004). The site-1 protease DegS senses misfolded C-terminal portions of OMPs with its PDZ-domain (Walsh et al., 2003; Wilken et al., 2004). In *V. cholerae,* OmpU is the essential stress sensor for membrane damaging and misfolded periplasmic proteins, e.g., in presence of antimicrobial peptides to activate a RpoE-dependent resistance. The signal transduction is based on the exposure of OmpU C-terminal YDF motifs, which interact with the PDZ-domain of DegS and hence activate RpoE activity (Mathur et al., 2007). DegS is a member of the DegS/HtrA2-subfamily of oligomeric serine HtrA proteases that possesses an N-terminal transmembrane portion, a catalytic serine protease domain and a C-terminal PDZ-domain (Clausen et al., 2002). DegS is the only known protease targeting RseA in *E. coli*. Additionally, in *V. cholerae* DegS also finds the sulfide-thiol reduced ToxR as its substrate (Lembke et al., 2018). DegS is activated by the interaction between C-terminal OMP peptides and its PDZ-domain. Refolding of DegS is induced resulting in proteolytic activity and the cleavage of RseA at its periplasmic portion, which triggers a second cut by the site-2 protease RseP at the cytoplasmic portion of RseA. RseP is a zinc metalloprotease of the inner membrane, harboring highly conserved HEXXH and LDG motifs and a PDZ-domain (Rudner et al., 1999; Kanehara et al., 2001; Drew et al., 2002). Eventually, RpoE is liberated into the cytoplasm where it assembles into the RNA polymerase holoenzyme. The first identified RpoE-dependent promoter in *V. cholerae* is located upstream of the *rpoErseABC* operon and harbors high similarity with consensus sequences of RpoE-regulated promoters in *E. coli* (Kovacikova and Skorupski, 2002). Comparative microarray analyses of a *rseA* deletion strain and wild type (WT) indicate that *degP* is also under RpoE-control in *V. cholerae* (Ding et al., 2004). Additionally, in a *degS* deletion strain less RpoE is released from RseA and consequently the RpoE-response is decreased, resulting in significantly reduced transcription of *degP* compared to WT (Lembke et al., 2018).

DegP is a periplasmic heat-shock protein, which is highly conserved across species and can act as both chaperone and protease (Spiess et al., 1999). DegP belongs to the HtrA-family of PDZ-domain containing proteases (Kolmar et al., 1996; Krojer et al., 2008a). Its structure is formed by trimer subunits that assemble to proteolytically inactive hexamers (Krojer et al., 2002). In presence of unfolded protein substrates, active DegP builds up dodecamers or icosatetramers (Jiang et al., 2008; Krojer et al., 2008b).

The type II secretion system (T2SS) of *V. cholerae*, encoded by two different *eps* operons (Sandkvist, 2001), is required for the secretion of enzymes and cholera toxin into the environment (Korotkov et al., 2012). Interestingly, a deletion of *eps* genes causes outer membrane damage which in turn activates RpoE-dependent response (Sikora et al., 2007). Furthermore, RpoE is also responsible for expression of the T2SS in *V. cholerae,* essential for release of important effectors along the lifecycle like the CTX or biofilm adhesion factors (Zielke et al., 2014). Concordantly, *rpoE* deletion strains are significantly attenuated in the murine model compared to WT (Kovacikova and Skorupski, 2002).

THE ALTERNATIVE SIGMA FACTOR RpoS (σ^S OR σ^{38}) IS TARGETED BY THE ClpXP PROTEASE

RpoS is a hallmark of a proteolysis-controlled regulator and was mainly characterized in *E. coli* to be responsible to counteract starvation conditions (Hengge-Aronis, 2002). Degradation of RpoS is under control of its specific proteolysis targeting factor, termed RssB (Muffler et al., 1996), which is activated by the kinase ArcB (Mika and Hengge, 2005) leading to a proteolytic complex comprising ClpXP, phosphorylated RssB, and RpoS (Becker et al., 1999; Zhou et al., 2001; Stüdemann et al., 2003). Anti-adaptor proteins, termed Ira (inhibitor of RssB activity) are identified to block RssB activity, thus stabilizing RpoS. Ira proteins (Battesti et al., 2013) respond to specific physiological stress conditions (Hryckowian et al., 2014), as well as to the accumulation of intracellular metabolites (Battesti et al., 2015). In *V. cholerae* the role of RpoS is less clear as a *rpoS*-mutant only exhibits minor defects in intestinal colonization (Yildiz and Schoolnik, 1998; Merrell et al., 2000). However, the "mucosal escape" of *V. cholerae* at late stages of the infection depends on RpoS regulated gene expression (Nielsen et al., 2006). This phenotype is marked by activation of chemotaxis and motility genes resulting in detachment from the mucosal surface and entrance into the gastrointestinal lumen (Nielsen et al., 2006). While flagellar motility is crucial to direct *V. cholerae* to the mucosal layer, the bacteria enter a non-motile state upon mucosal penetration (Liu et al., 2008). After infection progression, nutrients in the gut decline, thereby starvation and high cell density may trigger *rpoS* expression. RpoS activates chemotaxis and motility gene expression, subsequently resulting in the mucosal escape phenotype. Moreover, RpoS is responsible for biofilm dispersal in a hydrodynamic model (Müller et al., 2007) or for biofilm escape (Wurm et al., 2017).

Termination of the RpoS-program is less clear, but likely involves proteolytic control (Wurm et al., 2017). Under laboratory conditions, RpoS-levels can increase if bacteria are stressed by a shift from rich into poor nutrient conditions. The enhanced ppGpp concentrations activate *rpoS* transcription leading to high RpoS-levels, which in turn activate chemotaxis and motility gene expression (Wurm et al., 2017). Interestingly, as soon as ppGpp-levels decline, *rpoS* transcription stops and RpoS-proteolysis is subsequently activated by a RssB homolog and the ClpXP-protease complex (Wurm et al., 2017).

To date, *rpoS* expression control seems to be conserved in *Enterobacteriacae* and other Gram-negative bacteria (e.g., *Vibrio* and *Pseudomonas*). Stringent control, mRNA

stability and ClpXP- or Lon-mediated proteolysis control *rpoS* expression levels. In contrast, RpoS-mediated responses are completely different in such species, leading to physiological changes like persistence, motility, and virulence (Schellhorn, 2014).

CONCLUDING REMARKS

While transcriptional regulation along the lifecycle of *V. cholerae* has been intensively studied, we are just beginning to identify and understand post-translational control elements. Recent reports highlight that regulated proteolysis of alternative sigma factors or transcriptional regulators is involved in blockage or termination of gene expression comprising virulence, transmission, and environmental persistence conditions.

Regulated proteolysis events during *V. cholerae*'s lifecycle are summarized in **Table 1**. To conclude, FliA proteolysis ensures full virulence induction during initial colonization upon penetration of *V. cholerae* through the intestinal mucus. Meanwhile, the virulence cascade starts to operate, i.e., the ToxT positive forward loop regulation is activated. At some point during the end of the colonization phase and shortly before onset of mucosal escape, this virulence regulatory loop needs to be terminated via proteolysis of TcpP, ToxR, and ToxT (**Figure 1** and **Table 1**). During colonization stage, RpoE is released via RIP of RseA and mediates host protective strategies. Regulated proteolysis maintains low RpoS levels during colonization, while starvation conditions inhibit RpoS proteolysis via RssB. Elevated RpoS levels at late infection stages subsequently initiate the mucosal escape mechanism.

Several important questions remain to be addressed to understand such complex post-translational regulation events. For example, what are the specific triggers initiating or preventing regulated proteolysis? What are the associated anti-proteolytic or targeting proteolysis factors, which protect proteins from degradation or attract key-proteases such as Lon, ClpXP, and DegS? With FlgM, RssB, RseA, ToxS, or TcpH probably only a fraction of such factors have been identified. Due to established intestinal infection and environmental persistence assays as well as its rapid proliferation requiring fast adaptation, *V. cholerae* is a valuable model to study and identify the proteolytic regulatory networks to gain deeper insights into the pathogen's bacterial physiology.

AUTHOR CONTRIBUTIONS

NP wrote parts of the RpoE chapter and RpoS chapter as well as designed **Figure 1**. ML wrote parts of the ToxR chapter. KP wrote parts of the introduction and FliA chapter. JM wrote the Tcp chapter. JR wrote parts of the RpoE, RpoS, ToxR chapter, and the ToxT chapter. SS was the coordinating author involved in writing and editing of all chapters as well as finalizing the manuscript for submission.

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Peptidylarginine Deiminase Inhibitors Reduce Bacterial Membrane Vesicle Release and Sensitize Bacteria to Antibiotic Treatment

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Outer membrane and membrane vesicles (OMV/MV) are released from bacteria and participate in cell communication, biofilm formation and host-pathogen interactions. Peptidylarginine deiminases (PADs) are phylogenetically conserved enzymes that catalyze post-translational deimination/citrullination of proteins, causing structural and functional changes in target proteins. PADs also play major roles in the regulation of eukaryotic extracellular vesicle release. Here we show phylogenetically conserved pathways of PAD-mediated OMV/MV release in bacteria and describe deiminated/citrullinated proteins in *E. coli* and their derived OMV/MVs. Furthermore, we show that PAD inhibitors can be used to effectively reduce OMV/MV release, both in Gram-negative and Gram-positive bacteria. Importantly, this resulted in enhanced antibiotic sensitivity of both *E. coli* and *S. aureus* to a range of antibiotics tested. Our findings reveal novel strategies for applying pharmacological OMV/MV-inhibition to reduce antibiotic resistance.

Keywords: outer-membrane vesicles (OMVs), peptidylarginine deiminase (PAD), deimination/citrullination, antibiotic sensitivity, *E. coli* VCS257, *S. aureus* subsp. *aureus* Rosenbach

INTRODUCTION

Outer membrane vesicles (OMVs), and membrane vesicles (MVs), are released from Gram-negative and Gram-positive bacteria and participate in bacterial communication, facilitating the transfer of cargo molecules (Dorward and Garon, 1990; Li et al., 1998; Fulsundar et al., 2014; Jan, 2017; Toyofuku et al., 2019). OMVs are released in greater abundance from Gram-negative than Gram-positive bacteria, are crucial for bacterial survival and form part of the stress response (McBroom and Kuehn, 2007; Macdonald and Kuehn, 2013; Jan, 2017). Research on bacterial OMVs has grown rapidly in recent years, including their use as bioengineered drug delivery vehicles (Gujrati et al., 2014; Bitto and Kaparakis-Liaskos, 2017) and in vaccine development (Gaillard et al., 2014; Choi et al., 2015; Alves et al., 2016; Raeven et al., 2016; Wang et al., 2017).

Peptidylarginine deiminases (PADs) are a group of calcium-activated enzymes that are preserved throughout phylogeny from bacteria to mammals and catalyze the post-translational deimination/citrullination of arginine residues to citrulline, causing structural, and functional changes in target proteins (Vossenaar et al., 2003; Wang and Wang, 2013; Witalison et al., 2015; Magnadóttir et al., 2018). Five mammalian PAD isozymes have been identified which participate in physiological and pathophysiological processes, including autoimmune and neurodegenerative diseases, cancer and sepsis (Wang and Wang, 2013; Witalison et al., 2015; Kosgodage et al., 2017, 2018; Lange et al., 2017; Biron et al., 2018; Costa et al., 2018). Recent studies have highlighted novel PAD-mediated mechanisms of extracellular vesicle (EV) release in eukaryotic cells (Kholia et al., 2015; Kosgodage et al., 2017, 2018; Gavinho et al., 2019) but a link to conserved mechanisms in bacterial OMV/MV release has hitherto not been made. While several PAD isozymes, with different preferences for target proteins, are present higher in phylogeny, in bacteria only one PAD form has been described. For example in *Porphyromonas gingivalis*, a Gram-negative bacterium, the association of PAD and its citrullinome has been linked to neo-epitope generation in oral cavity disease and rheumatoid arthritis (Maresz et al., 2013; Gully et al., 2014; Stobernack et al., 2016; Bereta et al., 2019). However, although an arginine deiminase (AD) has been identified in the *Eschericia coli* genome (GenBank: EDV68547.1), also a Gram-negative bacterium, no significant data is available to confirm the presence of an associated citrullinome. Furthermore, an AD has also been identified in *Staphylococcus aureus* (GenBank: BBA25170.1), a Gram-positive bacterium.

Our previous studies established that EV release from cancer cells is largely PAD-driven, can be effectively inhibited using pharmacological PAD inhibitors and that such inhibition sensitizes cancer cells to chemotherapy (Kholia et al., 2015; Kosgodage et al., 2017, 2018). Therefore, we set out to investigate if this could be a phylogenetically conserved mechanism and in the same vein, be exploited to sensitize bacteria to antibiotics.

The role of OMVs in biofilm formation and in protecting biofilms via adsorption of antimicrobial agents has indeed been previously recognized (Schooling and Beveridge, 2006; Manning and Kuehn, 2011; Toyofuku et al., 2019). Thus, application of OMV inhibition could potentially lower resistance to antibiotics and be useful in minimizing multi-drug resistance associated with antibiotic treatment.

Using a range of antibiotics, we determined the effect of several PAD-specific inhibitors on changes in OMV/MV release and on antibiotic sensitivity of Gram-negative (*E. coli* VCS257) and Gram-positive bacteria (*S. aureus* subsp. *aureus* Rosenbach).

The PAD inhibitors tested were first generation pan-PAD inhibitor Cl-amidine (Luo et al., 2006), second generation pan-PAD inhibitor BB-Cl-amidine (Knight et al., 2014), PAD2 inhibitor AMF30a (Muth et al., 2017), and PAD4 inhibitor GSK199 (Lewis et al., 2015). The following range of antibiotics was tested in combination with the OMV/MV inhibitors: **(i)** Colistin (Polymyxin E), which acts on the lipoglycans and endotoxins of the Gram-negative bacterial cell membrane (Falagas et al., 2005; Livermore et al., 2011; Yahav et al., 2012; Yu et al., 2019); **(ii)** Vancomycin, which alters the permeability of the cell membrane and selectively inhibits ribonucleic acid synthesis (Watanakunakorn, 1984). It is effective against Gram-positive bacteria, including *Staphylococcus, Streptococcus, and Listeria* and prescribed for serious skin, blood-borne and joint infections as well as meningitis caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Ng et al., 2014); **(iii)** Rifampicin, which inhibits DNA-dependent RNA polymerase activity, suppressing the initiation of RNA synthesis (Campbell et al., 2001). It is effective against a broad spectrum of bacteria, mainly Gram-positive cocci (van Ingen et al., 2011); **(iv)** Kanamycin, which binds to the bacterial 30S ribosomal subunit, causing misreading of t-RNA and inhibition of bacterial protein synthesis (Hoerr et al., 2016). It is active against most Gram-negative bacteria and some Gram-positive bacteria (Salian et al., 2012); **(v)** Erythromycin, which inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits (Ianaro et al., 2000). It is effective against Gram-positive bacteria including *Staphylococci*, *Streptococci* and *Pneumococci* and Gram-negative sporing and non-sporing gut anaerobes, such as *E. coli* (Jelić and Antolović, 2016).

Here we show that OMV/MV release can be regulated via PAD-mediated pathways both in *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach and that this can be exploited to enhance antibiotic effectivity of selected antibiotics in both Gram-negative and Gram-positive bacteria. Furthermore, in *E. coli* we identified deiminated/citrullinated proteins both in the bacterial cells and in derived OMVs, indicative of bacterial communication via lateral transfer of deiminated proteins.

MATERIALS AND METHODS

Preparation of Outer Membrane Vesicles (OMVs) and Membrane Vesicles (MVs)

E. coli (VCS257, Agilent, La Jolla, CA) and *S. aureus* subsp. *aureus* Rosenbach (ATCC 29247; CDC73-57501) cultures were grown for 24 h at 37°C (static culture). The growth phase before vesicle isolation was exponential, as assessed by optical density (OD600) before overnight incubation and for 4 h the following day, to ensure that bacteria were in log phase; the volume of the cultures was 20 ml. For OMV/MV-associated experiments, the bacterial growth medium (Luria-Bertani (LB) broth) and Dulbecco's phosphate buffered saline (DPBS)S were pre-treated before use by ultracentrifugation at 100,000 g (SW60Ti rotor, Beckmann L60 ultracentrifuge) for 24 h, to ensure that the medium used was minimally contaminated with extracellular vesicles (EVs).

The OMV/MVs were isolated from the supernatant of the bacterial culture medium as follows: The supernatant was initially centrifuged once at 400 *g* (F-34-6-38 rotor, Eppendorf 5804, U.S.A.) for 10 min to remove the cells. Thereafter, the supernatant was centrifuged at 4,000 *g* (F-34-6-38 rotor, Eppendorf 5804) for 1 h at 4°C to remove cell debris. The resultant supernatant was then centrifuged at 100,000 *g* (SW60Ti rotor, Beckmann L60 ultracentrifuge, Beckman Coulter, U.S.A.) for 1 h at 4°C for isolation of OMVs. The isolated OMV/MV

pellet was then resuspended in Dulbecco's phosphate-buffered saline (DPBS; ultracentrifuged and sterile filtered using a 0.22 μm filter) and filtered through an 0.45 μm filter before the second ultracentrifigation step at 100,000 *g* for 1 h at 4°C. The resulting OMV/MV pellet was thereafter resuspended in 100 μl sterile filtered (0.22 μm) DPBS and the isolated OMV/MV pellets were either used immediately, or stored at −80°C for further experiments.

Nanoparticle Tracking Analysis (NTA) of OMV/MV

For NTA analysis, isolated OMV and MV pellets, respectively, prepared as described above, were resupended in 100 μl sterile filtered DPBS and then diluted 1/200 before quantification to assess vesicle size, based on Brownian motion, using the Nanosight LM10, with a 405 nm diode laser (Malvern, U.K.). Numbers of particles per frame were kept at approximately 30, and five individual 60 s videos were recorded using a sCMOS camera for each sample to create the respective size distribution histograms. OMVs/MVs were further characterized by transmission electron microscopy (TEM) and Western blotting as described below.

Transmission Electron Microscopy Imaging

A suspension of isolated OMVs/MVs (1.4×10^8 vesicles/ml) was used for TEM imaging. Mesh copper grids were prepared with glow discharged carbon support films and 10 μl of OMV/MV samples applied to the grid and incubated for 2 min. The grids were then washed five times with 50 μl of 1% aqueous uranyl acetate. The last drop was left to incubate on the grid for 1.5 min before being wicked off by torn filter paper. Grids were left to dry for 5 min before being viewed. Micrographs were taken with a JEOL JEM 1230 transmission electron microscope (JEOL, Japan) operated at 80 kV at a range of magnification mainly around a magnification of 80,000–100,000. Digital images were recorded on a Morada CCD camera (EMSIS, Germany) and processed via iTEM (EMSIS, Germany).

Western Blotting for Membrane Vesicle Characterization

Protein was isolated from the OMV/MV pellets using Bacterial Protein Extraction Reagent (B-PER, ThermoFisher Scientific, U.K.), pipetting gently and shaking the pellets on ice for 2 h, where after samples were centrifuged at 16,000 *g* at 4°C for 20 min and the resulting supernatant collected for protein analysis. Samples were electrophoresed under reducing conditions by SDS-PAGE on 4–20% TGX gels (BioRad, U.K.), followed by semi-dry Western blotting. Membranes were blocked in 5% BSA in TBS-T at RT for 1 h. The membranes were incubated overnight at 4°C with the anti-OmpC (Outer-membrane protein C antibody; orb6940, Biorbyt, U.K.; diluted 1/1,000 in TBS-T). The membranes were washed 3 × 10 min in TBS-T, incubated for 1 h in anti-rabbit-HRP conjugated secondary antibody (BioRad, U.K.) at RT, followed by visualization using ECL (Amersham, U.K.) and the UVP BioDoc-ITTM System (U.K.).

Immunoprecipitation (IP) and LC-MS/MS Analysis of Deiminated and PAD4 Bound Proteins From *E. coli* VCS257 Cells and Derived OMVs

To determine the presence of deiminated/citrullinated proteins and PAD4 bound proteins from *E. coli* VCS257 and derived OMVs, protein extracts were prepared using Bacterial Protein Extraction Reagent (B-PER, ThermoFisher Scientific, U.K.), according to the manufacturer's instructions. In brief, bacterial cells and OMVs were centrifuged at 5,000 g for 10 min to obtain a pellet. Two microliter of lysozyme and 2 μL of DNase I per 1 mL of B-PER Reagent was added along with EDTA-free protease inhibitors (ThermoFisher Scientific). Four mL of B-PER Reagent per gram of cell pellet was added. The suspension was pipetted up and down until it was homogeneous. The suspension was incubated for 10–15 min at room temperature. The lysate was centrifuged at 16,000 *g* for 5 min to separate soluble proteins from the insoluble proteins. Proteins were thereafter immunoprecipitated, using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.), according to the manufacturer's instructions, in conjunction with the pan-deimination F95 (MABN328, Merck) antibody (both for *E. coli* and derived OMVs) or the PAD4 (ab96758, Abcam) antibody (for *E. coli* cells only). The F95 pan-deimination specific antibody has been developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002). F95 or PAD4 bound proteins, respectively, were eluted from the columns according to the manufacturer's instructions (MERCK), using the supplied elution buffer (non-reducing or a reducing elution buffer, which was supplemented with 5% beta-mercapthoethanol) and thereafter further analyzed by Western blotting, under reducing conditions, and by liquid chromatography-mass spectrometry (LC-MS/MS).

Western Blotting Analysis of Citrullinated/Deiminated Proteins From *E. coli* VCS257 Cells and Derived OMVs

In order to compare protein profiles of deiminated proteins between *E. coli* and *E. coli* OMVs, total protein lysates and IP protein eluates were subjected to Western blot analysis. Briefly, samples were boiled for 5 min at 100°C in 2x Laemmli sample buffer (BioRad, U.K.). Protein (20 μg per sample) was separated by SDS-PAGE using 4–20% Mini-Protean TGX protein gels (BioRad, U.K) and transferred to nitrocellulose membranes. Even loading was assessed using Ponceau S staining (Sigma, U.K.) and membranes were then blocked in 5% bovine serum albumin (BSA) in Tris buffered saline with 0.1% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the primary antibodies: pan-deimination antibody F95 (1:2,000; MABN328), PAD2 (1:1,000; ab50257), PAD3 (1:1,000; ab169479) and PAD4 (1:1,000; ab96758), respectively. Membranes were washed three times in TBS-T, incubated at room temperature for 1 h with HRP-conjugated secondary antibodies: anti-mouse IgM and anti-rabbit IgG, respectively (both 1:4,000; BioRad, U.K.), followed by six 10 min washes in TBS-T, before visualization

using ECL (Amersham, U.K.) and the UVP BioDoc-IT™ System (U.K.).

Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Analysis

For identification of deiminated/citrullinated proteins and PAD4 bound proteins, respectively, in *E. coli* cell lysate and OMV lysates, the F95 and PAD4 bound eluates were subjected to LC-MS/MS analysis, which was carried out at the Proteomics Service at the Barts Cancer Institute (Queen Mary University, U.K.). For identification of protein hits the peak list files were submitted to in-house Mascot (Matrix Science).

Effects of PAD Inhibitors on Bacterial OMV/MV Release and Cell Viability

E. coli and *S. aureus* were cultivated using EV-free Müeller-Hinton broth for 24 h. Culture condition were as follows: An inoculate of 0.1 ml of bacteria were grown at exponential phase overnight, as assessed by OD600; the volume of the cultures was 20 ml. The cells were washed using DPBS at 4,000 g for 10 min and seeded in triplicate in micro centrifuge tubes. The PAD inhibitors were added in triplicates and incubated for 1 h at 37°C as follows: PAD2 inhibitor AMF30a (5 μM), PAD4 inhibitor, GSK199 (10 μM), pan-PAD inhibitor Cl-amidine (50 μM), and BB-Cl-amidine (5 μM). Cell viability of bacteria in the presence of the different PAD inhibitors was assessed by counting the number (no) of surviving bacterial colonies on the plates. The no. of colonies (viable cells), inoculating volume and dilution factor of the bacterial culture used was used to calculate the viable cells in colony forming unit (cfu/ml) (cfu/ml = (no. of colonies x dilution factor)/volume of culture plate). OMV/MV isolation following treatment was carried out as described above and changes in OMV/MV release were assessed by quantifying numbers of OMVs/MVs by NTA analysis using the Nanosight LM10 as described above. The experiment was repeated three times and replicate histograms were averaged.

Disc Diffusion Test

E. coli VCS257 and *S. aureus* subsp. *aureus* Rosenbach nutrient agar plates were prepared and sterile paper disks were soaked in the PAD inhibitors at the same concentrations as before. Culture medium was 10 ml, log growth phase of bacteria was assessed by OD600, and the inoculum concentration was 0.1 ml. Discs were impregnated with the antibiotics (all from Sigma-Aldrich) at the following concentrations: colistin (10 μg/ml), rifampicin (15 μg/ml), erythromycin (15 μg/ml), kanamycin (1,000 μg/ml), and vancomycin (5 μg/ml). The inhibitor discs were placed in the middle of the agar plates, while the antibiotic discs were placed equi-distant to the respective inhibitor discs to be tested. The Kirby-Bauer test was used to assess the zone of inhibition after 24 h. **Supplementary Figure 4** shows the agar plates containing the disks, following completion of the test.

MIC Measurement of Colistin and Vancomycin in *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

MIC values for colistin and vancomycin were tested for *E. coli* and *S. aureus*, respectively. *E. coli* and *S. aureus* suspensions were prepared in Müller Hinton Broth according to Iqbal et al. (2013). For *E. coli*, the concentration of colistin was based on previously published MIC values for colistin, ranging between 2 and 16 μg/ml (Moskowitz et al., 2010; Rojas et al., 2017), and here this range was further expanded between 0.015 μg/ml to 64 μg/ml. MIC values for vancomycin have previously been recorded in the range of 1.5–2 μg/ml (Maclayton et al., 2006; Kshetry et al., 2016; Goldstein et al., 2018) and in some studies a MIC value of 8 μg/ml and higher has been reported (Alam et al., 2014; Lepe et al., 2014). Therefore, for *S. aureus* a MIC confirmation test starting from a concentration of 8 μg/ml was carried out here and thereafter the MIC was considered accordingly. The doubling dilution method was used to obtain triplicates of concentrations used for the antibiotics and a triplicate of control omitting antibiotic was also prepared. The plates were incubated overnight at 37°C and the MIC values of antibiotic treated control wells were compared to wells where PAD inhibitors were applied in combination with the antibiotics.

Statistical Analysis

The histograms and graphs were prepared and statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, U.S.A.). One-way ANOVA and *t*-test analysis were performed, followed by Tukey's *post-hoc* analysis. Significant differences were considered as $p \leq 0.05$ and histograms represent mean of data, with error bars representing standard error of mean (SEM).

RESULTS

Characterization of Bacterial OMVs/MVs

A poly-dispersed population of OMVs was verified by nanoparticle tracking (NTA) analysis, with the majority of vesicles released in the 20–300 nm range for *E. coli* VCS257, and similarly in the 37–300 nm range for *S. aureus* subsp. *aureus* Rosenbach (**Figures 1A1**, **2**). This was further confirmed by transmission electron microscopy, assessing overall morphology and providing average size estimations (**Figures 1B1**, **2**). Morphological analysis by TEM revealed OMVs, including with inner and outer membranes visible, for *E. coli* (**Figure 1B1**) and typical MVs for *S. aureus* (**Figure 1B2**). Western blotting verified the presence of the OMV-specific marker OmpC (**Figure 1C**). Changes in OMV size distribution after PAD-inhibitor treatment in *E. coli* was reflected in a change in NTA profile as shown in **Figures 1D–G** and further discussed below.

PAD Inhibitors Inhibit OMV Release in the Gram-Negative Bacterium *E. coli* VCS257

Effects of the various PAD inhibitors on OMV release in *E. coli* are shown in **Figure 2**. The PAD4-specific inhibitor GSK199

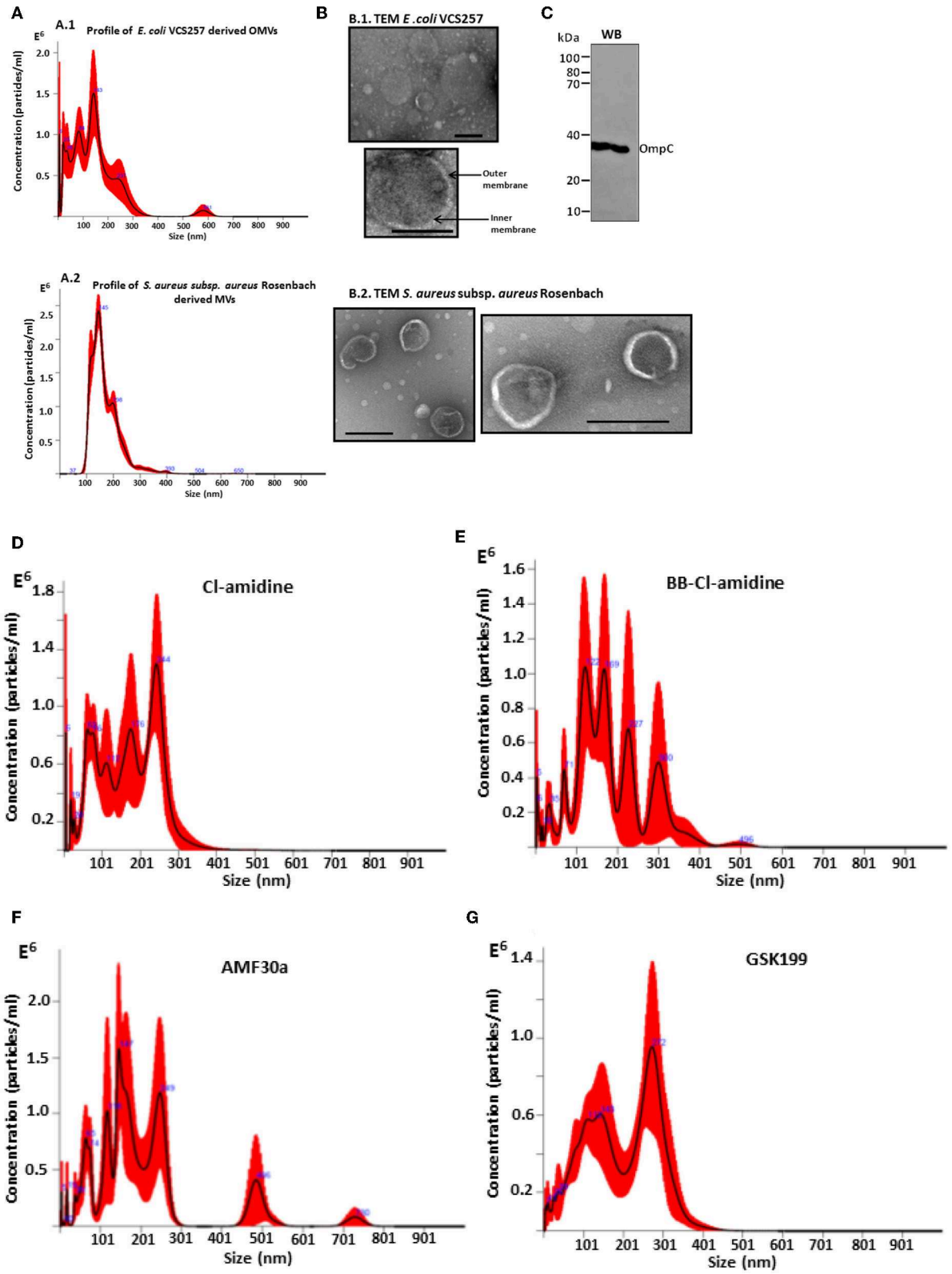

FIGURE 1 | Characterization of *E. coli* VCS257 OMVs and *S. aureus* subsp. *aureus* Rosenbach MVs using NTA, TEM and Western blotting analysis. **(A)** NTA curves, obtained by Nanosight analysis, showing OMVs released from *E. coli* **(A1)** and *S. aureus* **(A2)** under standard conditions; **(B)** Negative stain TEM micrographs of *E. coli* OMVs **(B1)** show the presence of a poly-dispersed sample ranging in size from mainly 20 nm to 320 nm (scale bars represent 200 nm), including vesicles showing

(Continued)

FIGURE 1 | inner and outer membranes for *E.coli* VCS257 OMVs **(B1)** and MVs from *S. aureus* subsp. *aureus* Rosenbach **(B2)**. **(C)** OMV-specific marker OmpC verified by Western blotting. **(D–G)** NTA profiles of OMVs released from *E. coli* VCS257 following PAD inhibitor treatment. OMV release profile from *E. coli* treated for 1 h with PAD inhibitors as follows: **(D)** Cl-amidine (first generation pan-PAD inhibitor; 50 μM); **(E)** BB-Cl-amidine (second generation pan-PAD inhibitor; 5 μM); **(F)** AMF30a (PAD2 inhibitor; 5 μM); **(G)** GSK199 (PAD4 inhibitor; 10 μM).

showed most potent OMV inhibition with a 66.4% reduction in OMV release ($p = 0.0001$), but also most significantly affected cell viability (23.9% reduction, $p = 0.0008$). BB-Cl-amidine was the second most potent inhibitor with a 53.8% reduction in OMV release ($p < 0.0001$) and a reduction, albeit non-significant ($p = 0.1351$), of 19.3% in cell viability, as measured by CFU. Cl-amidine resulted in a 42.4% inhibition of OMV release ($p = 0.0001$) and an 18.2% decrease in cell viability ($p = 0.0111$). The PAD2-specific inhibitor AMF30a was less effective with a 28.2% reduction in OMV release ($p = 0.0116$) and caused a 14.7% reduction in cell viability ($p = 0.0283$) (**Figure 2B**). Furthermore, in addition to reduced total OMV numbers released after PAD inhibitor treatment, the following changes in OMV profiles were observed by NTA analysis: Cl-amidine treated *E. coli* released OMVs in the size range 20–400 nm, BB-Cl-amidine treated cells showed OMV release in the 20–500 nm size range, while AMF30a (PAD2 inhibitor) treatment resulted in an additional notable peak of OMVs at 501 nm and a second smaller peak at 701 nm. GSK199 (PAD4 inhibitor) treatment resulted in an OMV release profile of 20–400 nm, similar to that for Cl-amidine (**Figures 1D–G**).

PAD Inhibitors Inhibit Membrane Vesicle (MV) Release in *S. aureus* subsp. *aureus* Rosenbach

The same range of PAD inhibitors as used with *E. coli* was used to examine the effect on membrane vesicle (MV) release from a Gram-positive bacterium, *S. aureus* subsp. *aureus* Rosenbach (**Figure 2C**). PAD4-specific inhibitor GSK199 resulted in the highest inhibition of MV release, with 22.5% reduction ($p = 0.0018$), while PAD2 inhibitor AMF30a showed only 3.4% reduction in OMV release ($p = 0.0606$). First generation pan-PAD inhibitor Cl-amidine resulted in a 12.5% inhibition of MV release ($p = 0.0016$), and also had the lowest negative impact on *S. aureus* cell viability (3.3%, $p = 0.0835$; **Figure 2D**), while second generation pan-PAD inhibitor BB-Cl-amidine showed 7.6% MV inhibition ($p = 0.0061$) (**Figures 2C,D**).

Phylogenetic Reconstruction of *E. coli* and *S. aureus* PAD/AD

Two well supported clades were formed within the Neighbor-joining phylogeny (**Figure 3**) based on multiple sequence alignment of the whole amino acid sequences (using Clustal Omega), suggesting that E. coli PAD/AD (arginine deiminase RRM86073.1) is most closely related to human PAD2 (**Figure 3A**). The E. coli PAD/AD has a shorter, 406 amino acid (aa) sequence (arginine deiminase EDV68547.1), compared to human PAD2 (NP031391), PAD3 (AIC56498), and PAD4 (AIC56076), which are 665, 663, and 664 aa, respectively. Various single, fully conserved residues are found, while some conservation of similarity between E. coli PAD/AD with human PAD2 and PAD3 is visible that scores >0.5 in the Gonner PAM matrix (**Supplementary Figure 1**). For S. aureus, two well-supported clades were also formed within the Neighbor-joining phylogeny (**Figure 3B**), suggesting that S. aureus PAD/AD (arginine deiminase BBA25170) is also most closely related to human PAD2. The S. aureus bacterial PAD/AD has a shorter, 411 aa sequence (BBA25170), similar to that seen for E. coli PAD/AD, compared to the human PAD2, PAD3, and PAD4 isozymes, which are approximately 664 aa. Various single, fully conserved residues are found, while some conservation of similarity between S. aureus PAD/AD to human PAD2 and PAD3 is visible that scores >0.5 in the Gonner PAM matrix (**Supplementary Figure 2**).

Detection of PAD/AD Protein and Citrullinated/Deiminated Protein Products in *E. coli* VCS257

Using Western blotting analysis, *E. coli* cell and *E. coli* OMV protein extracts were assessed for total deiminated proteins, using the anti-peptidyl-citrulline F95 antibody (MABN328, Merck, U.K.), as well as for cross-reactivity with human PAD2 (ab50257), PAD3 (ab169479), and PAD4 (ab96758) antibodies (**Figure 4**). For total deiminated proteins (F95), bands in the size range of 15–120 kDa were revealed in both *E. coli* cells and derived OMVs (**Figure 4A**). The presence of a PAD/AD-like protein was verified in *E. coli* and derived OMVs, by the detection of an expected band at 40–50 kDa, representative of a bacterial PAD/AD (**Figure 4B**).

Immunoprecipitation of Deiminated Proteins From *E. coli* VCS257 and Derived OMVs

Deiminated proteins from *E. coli* and derived OMVs were immunoprecipitated using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions and the anti-peptidyl-citrulline F95 antibody (MABN328, Merck) (**Figures 4C,D** and **Supplementary Figure 3**). As the PAD4 inhibitor GSK199 was here found to be the most effective OMV inhibitor (**Figure 2A**), immunoprecipitation using the PAD4 antibody was also carried out on *E. coli* cell protein extracts, for identification of putative PAD4 bound proteins (**Figure 4** and **Supplementary Figure 3**). A range of F95 enriched bands was seen in both *E. coli* and OMV samples (**Figure 4C**). In addition, the PAD4 enriched eluate showed an F95 positive band in the expected size range of an *E. coli* PAD/AD at 45 kDa (**Figure 4C**, lane 6, arrow), thus indicating that *E. coli* PAD/AD itself may also be deiminated. This band was more prominent when the PAD4 antibody was used for immunoprecipitation from the *E. coli* protein lysate, showing a very strong band just below 50 kDa

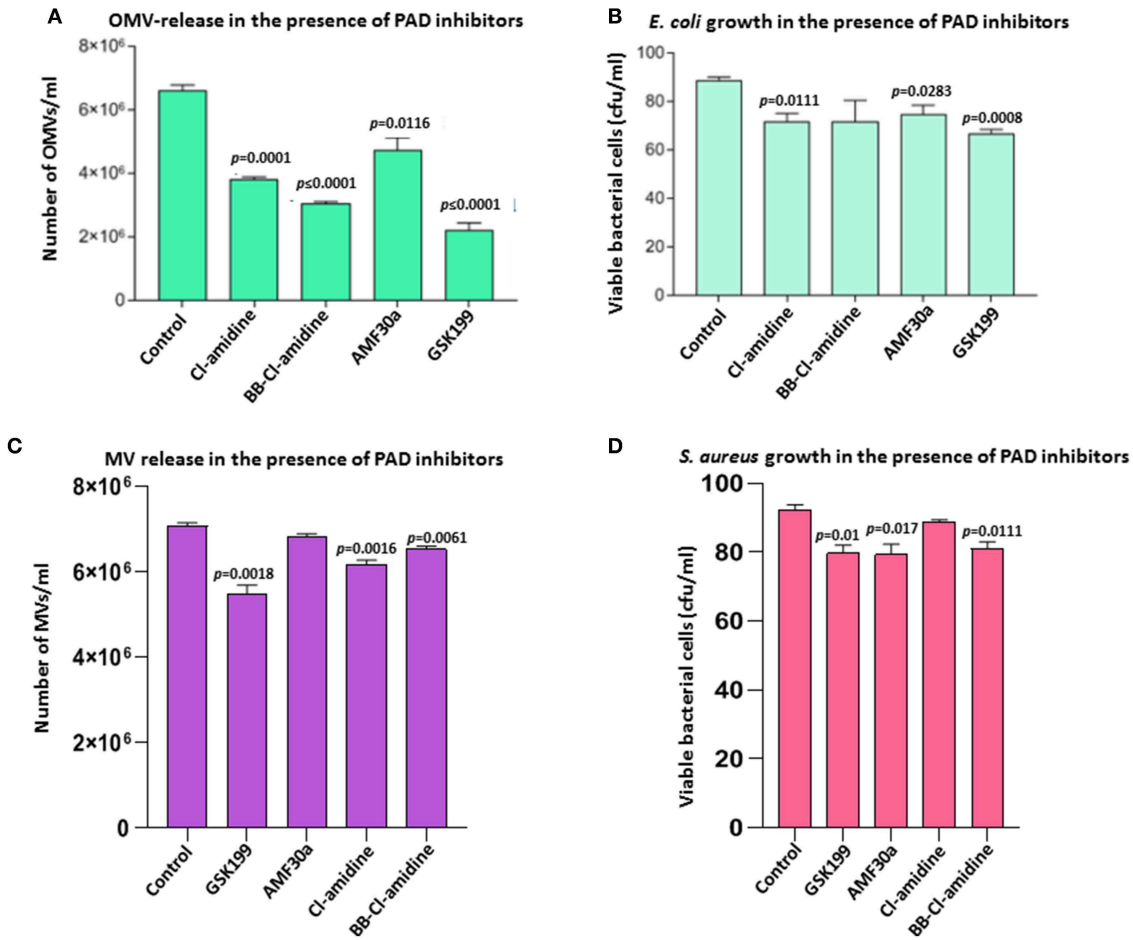

FIGURE 2 | Effects of PAD inhibitors on OMV release from *E. coli* VCS257 and MV release from *S. aureus* subsp. *aureus* Rosenbach. **(A)** All PAD inhibitors showed significant OMV inhibition in *E. coli* compared to untreated controls. PAD4 inhibitor GSK199 and second generation pan-PAD inhibitor BB-Cl-amidine were the strongest inhibitors of OMV release. The PAD2-specific inhibitor AMF30a was less effective. **(B)** *E. coli* viability after 24 h PAD inhibitor treatment, represented as CFU. **(C)** PAD4 inhibitor GSK199 was the most effective MV inhibitor in *S. aureus*, reducing MV release by 22.5%. **(D)** *S. aureus* viability, represented as CFU, after 24 h treatment with PAD inhibitors is shown. The experiment was repeated thrice and the data presented are mean ± SEM of the results; exact *p*-values are indicated. Concentration of PAD-inhibitors used was as follows: PAD2 inhibitor AMF30a (5 μM), PAD4 inhibitor, GSK199 (10 μM), pan-PAD inhibitors Cl-amidine (50 μM) and BB-Cl-amidine (5 μM).

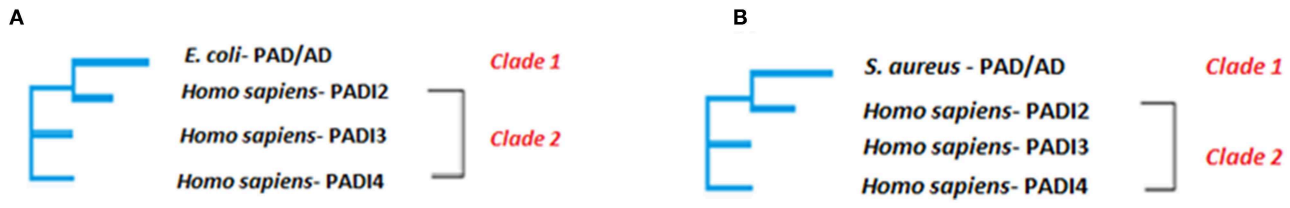

FIGURE 3 | Neighbor joining tree. The phylogenetic clustering of *E. coli* **(A)** and *S. aureus* **(B)** PAD/AD, respectively is shown. The evolutionary analysis was inferred using the Neighbor-Joining method under the conditions of the Poisson distance correction model in MEGA6. Bootstrap values >50 based on 1,000 replicates are shown as nodal support. Clade 1 contains *E. coli* (GenBank: EDV68547.1) PAD/AD and Clade 2 contains human PAD2 (GenBank:NP031391), PAD3 (GenBank: AIC56498) and PAD4 (GenBank: AIC56076).

(**Figure 4D**, lanes 5–6). When testing the PAD4 bound eluate with the F95 deimination antibody, several additional positive bands were detected and may possibly represent deiminated proteins co-immunoprecipitating with the PAD4 antibody (**Figure 4C**; lanes 5 and 6). The F95 eluate from OMVs though also showed a reaction with the PAD4 antibody (**Figure 4D**;

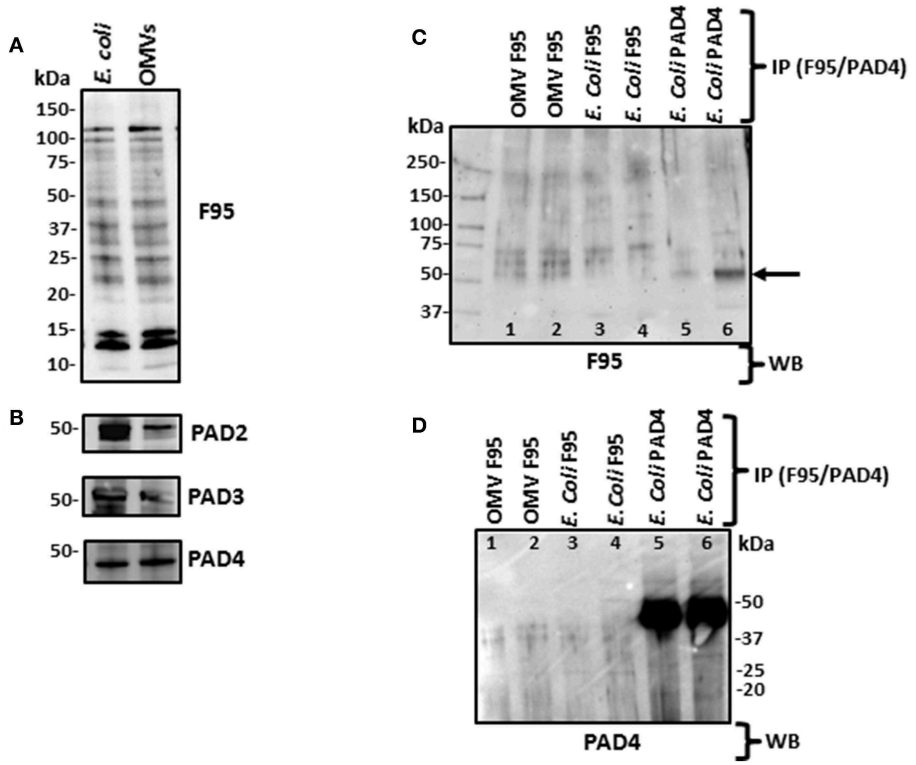

FIGURE 4 | Western blotting of total deiminated proteins and PAD/AD in *E. coli* VCS257 cells and derived OMVs. **(A)** *E. coli* and derived OMVs were analyzed for total deiminated proteins (F95) as well as for cross-reactivity with human PAD2, PAD3 and PAD4. Bands in the size range of 15–120 kDa were revealed for the pan-deimination antibody (F95). **(B)** The human PAD2, 3 and 4 antibodies reacted with a band of 40–50 kDa as expected for a bacterial PAD/AD homolog. Lane 1, *E. coli* total protein extract; Lane 2, *E. coli* OMV protein extract. **(C,D)** Immunoprecipitated deiminated proteins (F95) and PAD4 bound proteins from *E. coli* VCS257 and derived OMVs. IP was performed on *E. coli* total protein and *E. coli* derived OMVs, using F95 and PAD4 antibodies, respectively. **(C)** Immunoprecipitated fractions (F95 enriched and PAD4 bound, respectively) were tested with the pan-deimination F95 antibody. A range of deiminated/citrullinated proteins was observed in *E. coli* cells and derived OMVs. Lanes 5 and 6 indicate a deiminated band at 50 kDa corresponding to *E.* coli PAD/AD (arrow) For lanes 5 and 6 the F95 enriched eluate was eluted using non-reducing and reducing elution buffer, respectively, and both F95-bound eluates obtained were thereafter run under reducing conditions in the gel. **(D)** The same fractions were tested with the PAD4 antibody. Prominent bands around the 50 kDa region (lanes 5–6) correspond to a predicted size of *E. coli* PAD/AD. Faint bands of similar sizes are also observed in the OMV F95-enriched samples (lanes 1–2) which may suggest the presence of a PAD/AD which is deiminated in both *E. coli* and derived OMVs. The protein standard is indicated in kDa on all blots. For identification of protein hits as assessed by LC-MS/MS for the F95 and PAD4 bound eluates, see **Tables 1**, **2**.

lanes 3 and 4), and therefore may indicate a deiminated PAD/AD and further deiminated proteins bound to the PAD/AD in *E. coli* derived OMVs. Such auto-deimination of the bacterial PAD/AD will though require further investigation to be fully confirmed.

Liquid Chromatography–Mass Spectrometry (LC-MS/MS) Analysis of Deiminated Proteins From *E. coli* VCS257 and Derived OMVs

Immunoprecipitated proteins (F95 enriched and PAD4 bound) from *E. coli* cells and derived OMVs were analyzed by LC-MS/MS (**Figures 5**, **6** and **Tables 1**, **2**). Nine deiminated proteins were identified in the F95 enriched *E. coli* OMV protein sample (**Figures 5**, **6A**). The 30s ribosomal protein S15 was unique to OMVs. As threonine-tRNA ligase (*thrS*), has not been reported in *E. coli* OMVs before, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (https://string-db.org/) was carried out to assess if *thrS* is interconnected with the other proteins in the OMV associated proteins (**Figure 6B**). This showed five of the nine proteins to be associated with each other, at least through text mining. Furthermore, the *E. coli* OMV-associated *thrS* is co-expressed with 30s ribosomal protein S15 (*rpsO)*, 30s ribosomal protein S4 (*rpsD*) and 50S ribosomal protein L22 (*rplY*), as well as being experimentally determined to interact with *rplY* (**Figure 6B**).

Table 1 lists proteins identified in *E. coli* cell samples that were unique in F95 enriched eluates. **Table 2A** lists proteins that were identified in the PAD4 enriched eluates from *E. coli* cells, indicative of PAD4 bound proteins. **Table 2B** summarizes common proteins identified in all three eluates (F95 enriched eluate from *E. coli* cells, F95 enriched eluate from OMVs, PAD4 enriched eluate from *E. coli* cells); proteins are shown that had two or more peptides identified and a protein score of >50.

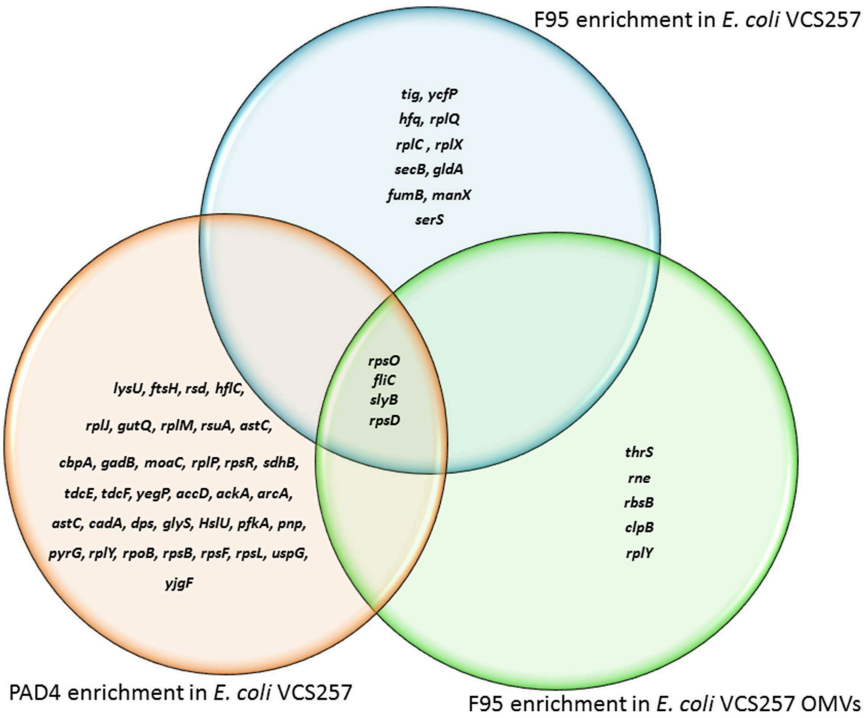

FIGURE 5 | Deiminated proteins and PAD4 bound proteins identified in *E. coli* VCS257 cells and derived OMVs. The Venn diagram shows F95 enriched proteins identified from *E. coli* cells and *E. coli* OMVs, as well as PAD4 bound proteins identified from *E. coli* cells. Four proteins were common to all three eluates (for further details on protein identification see **Tables 1**, **2**).

PAD Inhibitor Treatment Enhances Antibiotic Sensitivity of *E. coli* VCS257

Kanamycin was the most effective antibiotic against *E. coli* in the absence of PAD inhibitors (**Figure 7A**). In the presence of PAD inhibitors, GSK199 most significantly enhanced the effects of erythromycin, measured as a percentage increase in the zone of inhibition on the lawn of *E. coli*, by 88.9% ($p = 0.0025$); that of rifampicin by 56.7% ($p = 0.0561$) and that of colistin by 14.6% ($p = 0.2495$). BB-Cl-amidine was most effective when used in combination with rifampicin, increasing the zone of inhibition by 106.45% ($p = 0.0025$) and that of kanamycin by 65.6% ($p = 0.0186$). Cl-amidine caused enhancement of antibiotic sensitivity in combination with rifampicin (43.8%; $p = 0.045$), erythromycin (35.6%; $p = 0.0572$), and kanamycin (38.8%; $p = 0.0390$), compared to antibiotic alone (**Figure 8**). AMF30a was not effective in sensitizing *E. coli* to any of the antibiotics tested (see **Supplementary Figure 4A**, showing the agar plates). Importantly, there were no zones of inhibition seen in the agar plates which were only treated with the inhibitor discs, thus ruling out any inhibition on bacterial growth in the absence of antibiotics (**Supplementary Figure 4B**).

PAD Inhibitors Enhance Antibiotic Sensitivity of *S. aureus* subsp. *aureus* Rosenbach

PAD inhibitors significantly enhanced antibiotic effectivity against *S. aureus* (**Figure 7B**). For erythromycin, BB-Cl-amidine significantly enhanced antibacterial effects, by 18.2% ($p = 0.0234$). For vancomycin, GSK199 was most effective, with a 69.3% increase in antibiotic effectivity ($p = 0.0250$), while Cl-amidine was also significant (42.7%, $p = 0.0354$). (**Figure 7B**). For rifampicin, GSK199 and Cl-amidine significantly increased the zone of inhibition (10.1%; $p = 0.0202$) and 6.4%; $p = 0.0239$, respectively). The antibacterial effects of kanamycin were also significantly increased by both Cl-amidine and BB-Cl-amidine by 20.8% ($p = 0.0055$) and 28.9%; ($p = 0.0101$), respectively. For colistin, Cl-amidine increased the zone of inhibition by 21.5% ($p = 0.0444$). There were no zones of inhibition seen in the agar plates treated with the PAD inhibitor discs alone, indicating no effect on bacterial growth (**Supplementary Figures 4C,D**).

PAD Inhibitors Decrease MIC Value of Colistin Against *E. coli* VCS257

As the minimum inhibitory concentration (MIC) value of colistin against Gram-negative bacteria has been inconsistently reported in the literature it was further investigated here (**Figure 8A**). BB-Cl-amidine and Cl-amidine resulted in 91.6% ($p = 0.0001$) and 87.4% ($p = 0.0002$) reduction in MIC, respectively, while GSK199 lowered the MIC by 76% ($p = 0.0004$) (**Figure 8A**). There were no zones of inhibition seen in the plates which were only treated with the inhibitor discs, indicating no bactericidal effect due to inhibitors alone.

A

UniProt ID	Protein name	Gene name	MS2 Intensity	Score (p<0.05)
Q1R6H3	30S ribosomal protein S15	*rpsO*	67181.14	585.62
P04949	Flagellin	*fliC*	9488.724	653.24
P0A906	Outer membrane lipoprotein	*slyB*	10565.21	632.79
Q1R636	30S ribosomal protein S4	*rpsD*	3554.363	289.35
B7L6J2	Threonine--tRNA ligase	*thrS*	8718.552	371.78
P21513	Ribonuclease E	*rne*	8574.855	369.58
P02925	D-ribose-binding periplasmic protein	*rbsB*	6973.666	270.86
P63285	Chaperone protein ClpB	*clpB*	30537.26	227.63
B5YWX8	50S ribosomal protein L25	*rplY*	4326.5	51.12

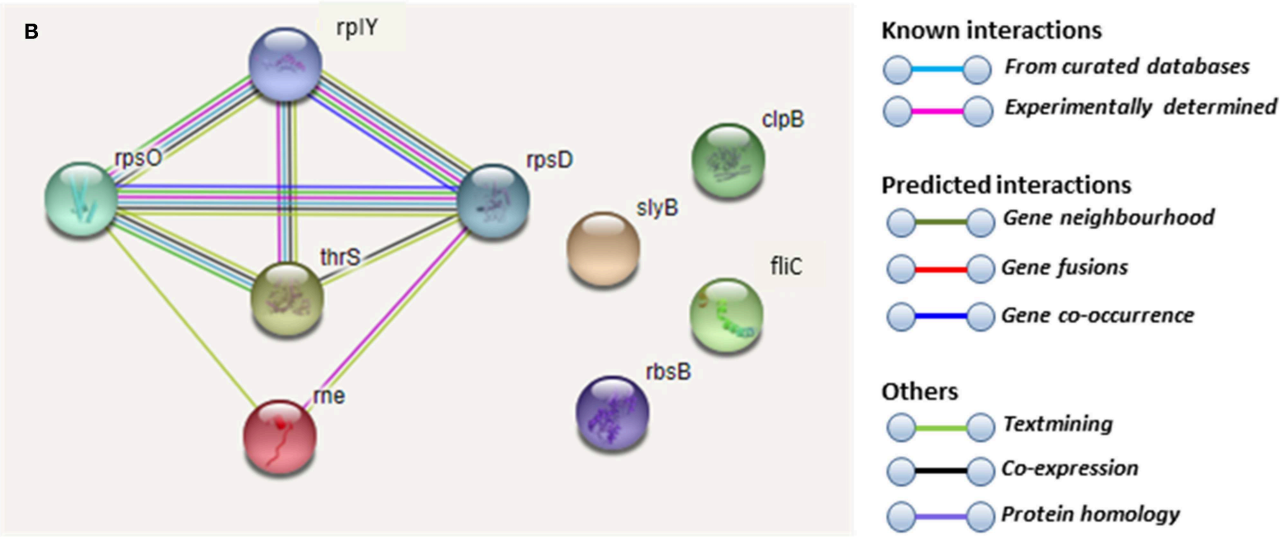

FIGURE 6 | Deiminated proteins in E. coli VCS257 OMVs. Deiminated proteins were isolated by immunoprecipitation using the F95 antibody and analyzed by LC-MS/MS. **(A)**. All deiminated proteins identified in the OMV sample, with a score >50 are listed. Ions score is −10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores >16 indicated identity or extensive homology (p<0.05). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20. Values with 2 or more peptides per protein and a score of >50 were considered. **(B)**. String analysis of the deiminated proteins identified in E. coli OMVs. Out of 9 proteins, 5 were associated with each other, at least through text mining.

PAD Inhibitors Decrease MIC Value of Vancomycin Against *S. aureus* subsp. *aureus* Rosenbach

The MIC value of vancomycin against *S. aureus* has been inconsistently reported in the literature and was thus investigated further. **Figure 8B** shows the effect of the most effective MV-inhibiting PAD inhibitors on the MIC of vancomycin; Cl-amidine showed a 62.5% reduction ($p = 0.0022$) while GSK199 lowered MIC by 25% ($p = 0.0161$) (**Figure 8B**). There was no effect on MIC of colistin in the presence of inhibitors alone.

DISCUSSION

For analysis of bacterial OMV/MVs in this study, isolation and quantification approaches using ultracentrifugation and NTA analysis were used, similar as performed by other groups (McCaig et al., 2013; Klimentova and Stulik, 2015; Roier et al., 2016). OMVs have previously been reported to fall mainly in the size range 10–300 nm (Kulkarni et al., 2015; Huang et al., 2016), and similar profiles were observed here. OMVs were also further characterized morphologically using TEM, and by Western blotting analysis using the outer membrane specific marker, OmpC (**Figure 1**). Furthermore, some change in OMV profile was observed in response to PAD inhibitor treatment, and this varied between inhibitors used, showing a change in shift of vesicle size populations after treatment with the different PAD inhibitors.

Here, an *E. coli* VCS257 citrullinome was identified for the first time, using F95 enrichment and LC-MS/MS analysis, confirming the presence of deiminated/citrullinated proteins in *E. coli* cells and *E. coli* derived OMVs. In bacteria, studies on PAD/AD

TABLE 1 | Deiminated proteins identified by F95 enrichment in *E. coli* VCS257cells.

UniProt ID	Protein name	Abbreviation	MS2 Intensity	Score ($p < 0.05$)[a]
B7MQF2	Trigger factor	*tig*	81193	192.72
B7NKH1	UPF0227 protein YcfP	*ycfP*	11941	166.86
B7MSJ0	RNA-binding protein Hfq	*hfq*	256917	132.47
Q1R638	50S ribosomal protein L17	*rplQ*	126430	128.85
Q1R602	50S ribosomal protein L3	*rplC*	69461	254.77
Q1R619	50S ribosomal protein L24	*rplX*	6484.4	75.48
B7N255	Protein-export protein SecB	*secB*	136512	77.044
P14407	Fumarate hydratase class I, anaerobic	*fumB*	15233	78.27
P0A9S6	Glycerol dehydrogenase	*gldA*	220449	441.19
P69799	PTS system mannose-specific EIIAB component	*manX*	94232	56.71
B7MRV6	Serine-tRNA ligase	*serS*	16845	55.11

[a] *Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores >16 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20. Values with 2 or more peptides per protein and a score of >50 were considered.*

Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody and analyzed by LC-MS/MS. Peak files were submitted to Mascot.

homologs have been limited and hitherto mainly reported in *P. gingivalis* (Mangat et al., 2010; Bielecka et al., 2014); a *Gingivalis* citrullinome has also been described (Stobernack et al., 2016). An *E. coli* PAD/AD-like protein was detected here at approximatly 40–50 kDa, similar to that found in *P. gingivalis* (Bielecka et al., 2014; Gabarrini et al., 2018), while in comparison human PADs are 72–75 kDa (Vossenaar et al., 2003; Kosgodage et al., 2018). Multiple sequence analysis of *E. coli* and *S. aureus* suggested that PAD/AD of *E. coli* and *S. aureus* are most closely related to human PAD2.

Interestingly, for inhibiting OMV/MV release from Gram-negative and Gram-positive bacteria the PAD4-specific inhibitor GSK199 was most effective while the PAD2-specific inhibitor AMF30a was least effective. Therefore, it may be postulated that the tertiary conformation of both *E. coli* and *S. aureus* PAD/ADs may be more similar to human PAD4, although the amino acid sequence alignment analysis indicates more similarity to PAD2, and this will require further investigation. In *P. gingivalis,* also a Gram-negative bacterium, PAD is believed to be evolutionarily only remotely related to human PAD2 despite the fact that both catalyze the same chemical reaction (Rodríguez et al., 2009; Bereta et al., 2019) and furthermore, point-mutation variants with differing deimination activity have also been reported in *Gingivalis* (Gabarrini et al., 2018; Bereta et al., 2019).

Here, F95 enrichment analysis revealed a range of deiminated proteins, both in *E. coli* cells and their derived OMVs, indicating lateral transfer of deiminated proteins via OMVs. Besides roles in bacterial communication, this may possibly also affect host-pathogen interactions, including immune evasion via modification of the host's proteins. For example, citrullination/deimination of complement component C5a, upon treatment with *P. gingivalis* OMVs, as opposed to *in vitro* treatment of C5a with PAD, has been shown to contribute to bacterial immune evasion by decreasing the chemotactic ability of neutrophils (Bielecka et al., 2014).

The citrullinome of *E. coli* VCS257 observed here, revealed indeed a range of metabolic, stress-response related and membrane proteins, therefore indicating diverse roles for protein deimination in bacterial cell function. Deiminated proteins identified in *E. coli* derived OMVs included threonine-tRNA ligase, which has not been described as being deiminated in bacterial OMVs before, but has been shown to be in OMVs from *Streptococcus suis* (Haas and Grenier, 2015), albeit not in deiminated form. Threonine-tRNA ligase, also known as threonine-tRNA synthetase, belongs to the family of aminoacyl-tRNA synthetases, which are involved in RNA splicing and transcriptional and translational regulation. They link amino acids to their cognate transfer RNAs (tRNA) in aminoacylation reactions, that establish the connection between a specific amino acid and a nucleotide triplet anticodon embedded in the tRNA (Schimmel, 2008). Also, 30S ribosomal protein was identified as being deiminated in OMVs. It is one of the primary rRNA binding proteins that bind directly to 16S rRNA, where it helps nucleate assembly of the platform of the 30S subunit by binding and bridging several RNA helices of the 16S rRNA (Smith et al., 2018). In addition, 30S ribosomal protein S4 (*rpsD*) and S15 (*rpsO*) were identified as deiminated both in the *E. coli* cells as well as their derived OMVs. Previously, 40S ribosomal protein has been reported as a substrate of PAD4 mediated deimination/citrullination in HEK 293Tcells (Guo et al., 2011).

Furthermore, evidence of a PAD/AD-like protein exported in *E. coli* VCS257 derived OMVs was found. When probing the PAD4 enriched eluate with the F95 antibody, a faint positive band in the expected range for a putative bacterial PAD/AD, in the region of 50 kDa region was observed, indicating that possibly the *E. coli* PAD/AD may be deiminated itself, although the LC MS-MS analysis did not reveal a PAD/AD-like protein hit. Such a possibility of PAD/AD auto-deimination would though align with previous studies on auto-deimination of mammalian PAD4 (Slack et al., 2011) but will require further in-depth investigation. In a recent study on OMVs derived from *P. gingivalis*, it was found that *Gingivalis* PAD was abundant in OMVs, although no assessment was made of a deiminated PAD form or of deiminated proteins exported in OMVs (Gabarrini et al., 2018).

The effect of PAD inhibitors on OMV/MV release, as previously established for EV release in eukaryotic cells (Kholia et al., 2015; Kosgodage et al., 2017, 2018; Gavinho et al., 2019),

TABLE 2A | PAD4 bound proteins identified in *E. coli* VCS257 cells.

UniProt ID	Protein name	Abbreviation	MS2 Intensity	Score[a] ($p<0.05$)
P0A895	Lysine-tRNA ligase, heat inducible	*lysU*	321997	300.12
Q8X9L0	ATP-dependent zinc metalloprotease FtsH	*ftsH*	1E+06	151.09
B7MRC1	Regulator of sigma D	*rsd*	23402	247.53
P0ABC5	Modulator of FtsH protease HflC	*hflC*	66850	189.23
Q1R5V0	50S ribosomal protein L10	*rplJ*	478659	218.99
Q8X4S5	Arabinose 5-phosphate isomerase GutQ	*gutQ*	486219	120.37
Q1R6A9	50S ribosomal protein L13	*rplM*	99365	135.62
P0AA45	Ribosomal small subunit pseudouridine synthase A	*rsuA*	50727	162.27
A7ZML6	Succinylornithine transaminase	*astC*	232079	73.58
B7MPT2	Curved DNA binding protein	*cbpA*	6123.2	90.68
P69911	Glutamate decarboxylase beta	*gadB*	387378	72.46
A7ZY39	Cyclic pyranopterin monophosphate synthase accessory protein	*moaC*	17232	67.03
Q1R613	50S ribosomal protein L16	*rplP*	475140	95.53
Q1R358	30S ribosomal protein S18	*rpsR*	312269	103.87
P07014	Succinate dehydrogenase iron-sulfur subunit	*sdhB*	48651	157.85
P42632	PFL-like enzyme TdcE	*tdcE*	29499	66.01
P0AGL3	Putative reactive intermediate deaminase TdcF	*tdcF*	161746	80.57
Q8X7I0	UPF0339 protein YegP	*yegP*	6121.7	87.63
Q0TFD0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit-β	*accD*	156916	99.12
P0A6A5	Acetate kinase	*ackA*	79146	70.42
P0A9Q3	Aerobic respiration control protein ArcA	*arcA*	19035	60.23
A8A0U0	Succinylornithine transaminase	*astC*	28022	73.51
P0A9H4	Inducible lysine decarboxylase	*cadA*	716567	78.26
Q8FJM0	DNA protection during starvation protein	*dps*	51562	65.43
B7N1L1	Glycine-tRNA ligase beta subunit	*glyS*	23006	87.76
B7N2S2	ATP-dependent protease ATPase subunit HslU	*HslU*	398703	52.01
B7N2Q7	ATP-dependent 6-phosphofructokinase isozyme 1	*pfkA*	57552	62.75
B7UJ59	Polyribonucleotide nucleotidyltransferase	*Pnp*	5914.7	70.94
B7MZ76	CTP synthase	*pyrG*	17163	59.39
B6I184	50S ribosomal protein L25	*rplY*	56618	57.85
Q0TA78	DNA-directed RNA polymerase subunit beta	*rpoB*	102175	72.91
B6HZE3	30S ribosomal protein S2	*rpsB*	226035	68.17
B7NGD4	30S ribosomal protein S6	*rpsF*	151007	61.19
C4ZUJ7	30S ribosomal protein S12	*rpsL*	396281	110.55
Q8XBT3	Universal stress protein G	*uspG*	36115	159.67
P0AF94	2-iminobutanoate/2-iminopropanoate deaminase	*yjgF*	22468	105.18

[a]*Ions score is −10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 16 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20. Values with 2 or more peptides per protein and a score of >50 were considered.*

Proteins were isolated by immunoprecipitation using the PAD4 antibody (ab96758) and analyzed by LC-MS/MS. Peak files were submitted to Mascot.

TABLE 2B | Proteins identified both in F95 and PAD4 enriched samples of *E. coli* VCS257 cells and derived OMVs.

			MS2 intensity			
UniProt ID	Protein name	Abbreviation	*E. coli* F95	*E. coli* PAD4	OMV F95	Score[a] ($p<0.05$)
Q1R6H3	30S ribosomal protein S15	*rpsO*	128707	61762	67181	585.62
P04949	Flagellin	*fliC*	704599	1E+06	9488.7	653.24
P0A906	Outer membrane lipoprotein SlyB	*slyB*	289574	555800	10565	632.79
Q1R636	30S ribosomal protein S4	*rpsD*	22719	44378	3554.4	289.35

[a]*Ions score is −10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 16 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20. Values with 2 or more peptides per protein and a score of >50 were considered.*

Proteins commonly identified by LC-MS/MS in both F95 and PAD4 enriched protein eluates are listed.

reveals a phylogenetically conserved pathway from bacteria to mammals. This is also in line with findings that many commonly expressed proteins including chaperone proteins, ribonuclease, outer membrane lipoprotein, 50S ribosomal protein L22 and flagellin are believed to be targets of protein deimination (Huang et al., 2016; Claushuis et al., 2018). Importantly, the present study shows that PAD inhibitors can be used to enhance antibiotic sensitivity of selected antibiotics. PAD4 inhibitor GSK199, alongside the pan-PAD inhibitors were effective for OMV/MV inhibition and sensitization to antibiotic treatment. Differences in mechanisms of action for the selected PAD inhibitors used in this study may need to be considered, both with regards to inhibiting vesicle release and the synergism observed with each antibiotic. Interestingly, while PAD4-inhibitor GSK199 was overall the strongest OMV/MV inhibitor, albeit the pan-PAD inhibitors showed a similar trend, in some cases the pan-PAD inhibitors (Cl-am and BB-Cl-am) were more effective in sensitization to antibiotic treatment. It must also be taken into consideration that BB-Cl-amidine and Cl-amidine are hydrophilic, while AMF30a and GSK199 are hydrophobic compounds. Furthermore, GSK199 is highly lipophilic which may facilitate its uptake in the cell. The difference in hydrophobicity of the PAD inhibitors could have played a role in cell penetration, in addition to differing in specificity for inhibition of the bacterial PAD/AD, and this may therefore also have affected OMV/MV release. A recent review has demonstrated the presence of different types of vesicles released from both Gram-negative and Gram-positive bacteria, indicating also that the composition of the cell membrane plays a role in vesiculation (Toyofuku et al., 2019). The presence of a thickened peptidoglycan cell wall in Gram-positive bacteria restricts the penetration of most drugs into the cells, which suggests the need of an alternative receptor-mediated transport system (Liu et al., 2018). However, the high lipid content of the Gram-negative cell membrane with a thin layer of peptidoglycan increases membrane fluidity thus facilitating OMV release (Roier et al., 2016). This may also facilitate the bacterial cell wall penetration of lipid soluble drugs and elicit a more effective response, as indeed observed here for GSK199.

While PAD4-mediated neutrophil extracellular trap (NET) formation is a well-known bactericidal and anti-pathogenic mechanism of the immune system (Li et al., 2010; Claushuis et al., 2018; Magnadóttir et al., 2018), we have now revealed here another antibacterial mechanism, namely via PAD/AD-mediated inhibition of bacterial OMV/MV release. Bacteria may indeed utilize their PAD/AD in several ways for modulation of the host immune system and immune invasion, including via the generation of neo-epitopes, modification of host's immune proteins, such as C5a, and also through the release of OMV/MVs. While many studies on OMVs have been based on Gram-negative bacterial species (Pérez-Cruz et al., 2013; Bonnington and Kuehn, 2016; Roier et al., 2016), MV release from *S. aureus* has also been shown by different groups (Lee et al., 2009; Gurung et al., 2011). Interestingly, MV secretion and improper vancomycin treatment have been correlated with biofilm formation by methicillin-resistant *Staphylococcus aureus* (MRSA) (He et al., 2017).

When assessing the effectivity of PAD inhibition to enhance susceptibility of Gram-positive and Gram-negative bacterial species to antibiotic treatment, *E. coli* VCS257 were rendered more sensitive to erythromycin, rifampicin, kanamycin and colistin, while *S. aureus* subsp. *aureus* Rosenbach became more sensitive to all five antibiotics tested. This increased sensitivity in both bacterial species varied though somewhat, depending on the PAD-specific inhibitors used. It must also be noted that while the zone of inhibition was statistically significant in *S. aureus*, the proportional differences observed in antibiotic sensitization were not as high as those observed in some cases for *E. coli* and therefore it will require further investigation how physiologically significant such lower, albeit statistically significant, effects are. It can also be postulated that bacteria may use PAD/AD mediated deimination as a mode of a hitherto non-described microbial strategy to evade antibiotic action, and this might also explain some differences observed with the different PAD inhibitors and will require further in-depth investigation. There was no antibacterial effect of vancomycin on *E. coli* VCS257, confirming its limited effectiveness on Gram-negative species and the previously established resistance of *E. coli* to vancomycin, due to its inability to significantly penetrate the outer membrane (Zhou et al., 2015). Here, colistin and vancomycin effectivity was found to be enhanced in the presence of PAD inhibitors in *E. coli* and *S. aureus* respectively, decreasing MIC value at varying degrees. This indicates that lower concentrations of the antibiotic may be used to treat infections with minimal damage to healthy cells. It has previously been shown that the presence of calcium decreases the bactericidal effect of colistin on *Paenibacillus polymyxa*, which suggests that Ca^{2+} modulates a protective barrier against colistin (Yu et al., 2015). As the PADs are calcium-activated enzymes, it can be postulated that downstream PAD/AD activation and resulting OMV/MV release via a PAD/AD pathway may be a measure of bacterial defense against colistin treatment.

Our current study is the first to describe effects of PAD inhibitors on OMV/MV release in both a Gram-positive and Gram-negative bacterial species. PAD/AD inhibition approaches in bacteria have previously been discussed in relation to oral *Gingivalis* and the association to initiation of autoimmunity via the generation of neo-epitopes (Mangat et al., 2010; Montgomery et al., 2016). Pharmacological inhibition of PADs, using Cl-amidine or a PAD2/PAD4 inhibitor has also been shown to improve survival in several murine models of sepsis and LPS-induced endotoxemia (Zhao et al., 2016; Biron et al., 2017; Liang et al., 2018). Here we have shown that PAD inhibition had a significant effect on antibiotic sensitization in both species, albeit to a lower extent in *S. aureus*. Therefore, further identification and assessment of candidate OMV/MV inhibitors may allow for tailored application according to bacteria type and specific antibiotics.

Previous studies have discussed the use of OMVs for example as drug delivery vehicles (Ellis and Kuehn, 2010;

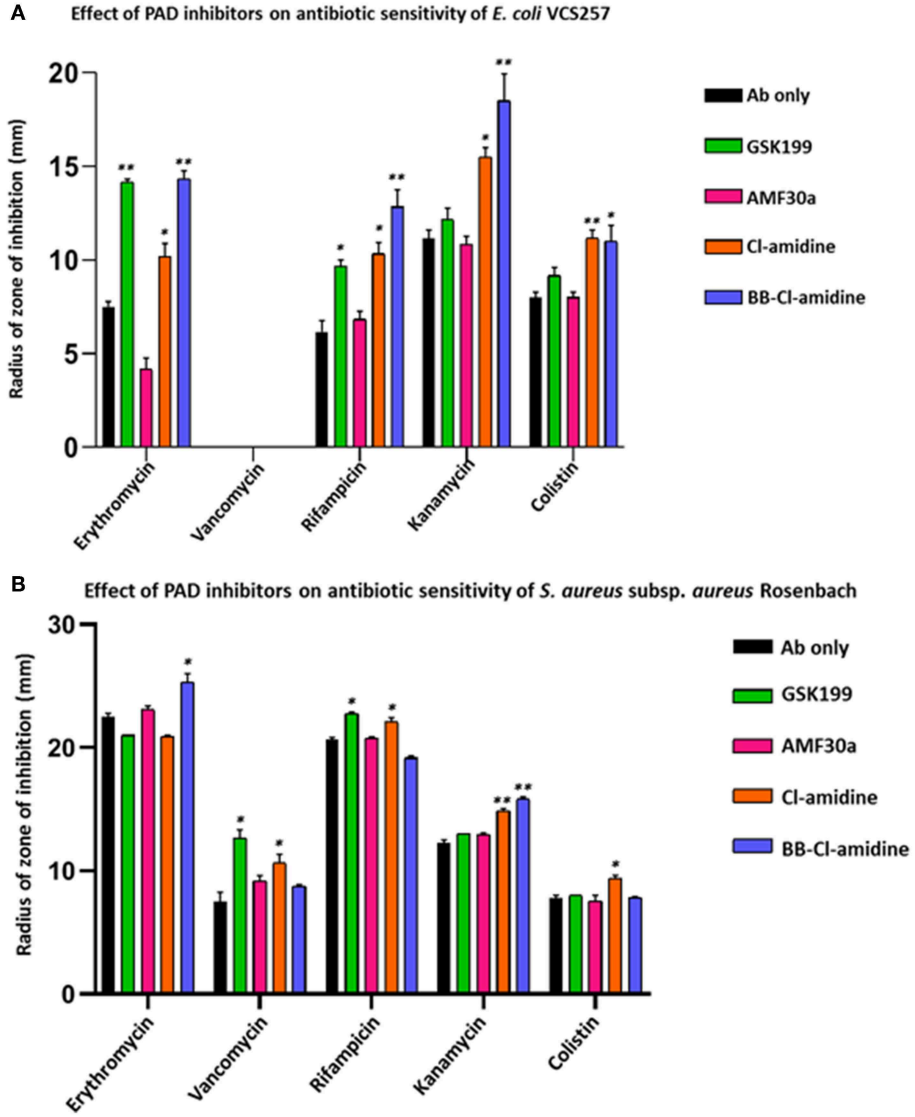

FIGURE 7 | PAD inhibitors increase antibiotic sensitivity of *E. coli* VCS257 *and S. aureus* subsp. *aureus* Rosenbach. **(A)** In *E. coli*, all antibiotics (Ab), except for vancomycin, had significantly increased zones of inhibition in the presence of PAD inhibitors. BB-Cl-amidine, Cl-amidine and GSK199 were effective, increasing antibiotic sensitivity of *E. coli* to erythromycin, rifampicin, kanamycin and colistin. Vancomycin was not effective against Gram-negative bacterial colonies and did not result in a zone of inhibition in the presence of any of the PAD inhibitors. **(B)** In *S. aureus*, vancomycin, rifampicin, kanamycin and colistin significantly increased their zones of inhibition in the presence of PAD inhibitors. BB-Cl-amidine enhanced bactericidal effects of erythromycin, while vancomycin was enhanced by GSK199 and Cl-amidine. Anti-bacterial effects of rifampicin were enhanced by GSK199 and Cl-amidine. Kanamycin's zone of inhibition was increased by Cl-amidine and BB-Cl-amidine. For colistin, the zone of inhibition was increased by Cl-amidine. Concentration of PAD-inhibitors used was as follows: PAD2 inhibitor AMF30a (5 μM), PAD4 inhibitor, GSK199 (10 μM), pan-PAD inhibitors Cl-amidine (50 μM) and BB-Cl-amidine (5 μM). The experiments were carried out three times and the data presented are mean ± SEM of the results (*$p \leq 0.05$; **$p \leq 0.01$).

Gujrati et al., 2014; Gerritzen et al., 2017; Jain and Pillai, 2017; Jan, 2017; Wang et al., 2018). OMVs have also been tested as delivery vehicles for targeted gene silencing using siRNA-packaged OMVs (Alves et al., 2016), although the exact mechanism for packaging proteins and other reagents in OMVs still remains to be fully understood. There is also an increasing interest in identification of OMV sub-populations (Pérez-Cruz et al., 2016; Bonnington and Kuehn, 2017; Turner et al., 2018; Cooke et al., 2019; Toyofuku et al., 2019; Zavan et al., 2019), as well as in assessing the importance of OMV size for cellular uptake and entry (Turner et al., 2018). Therefore, changes observed here in the NTA spectra of OMVs in response to PAD inhibitor treatment (**Figures 1D–G**) may be of some interest in addition to the observed reduction in total amounts of OMV/MVs released.

For the first time, the potential of using PAD inhibitors to enhance antibiotic sensitivity has been assessed, in both a

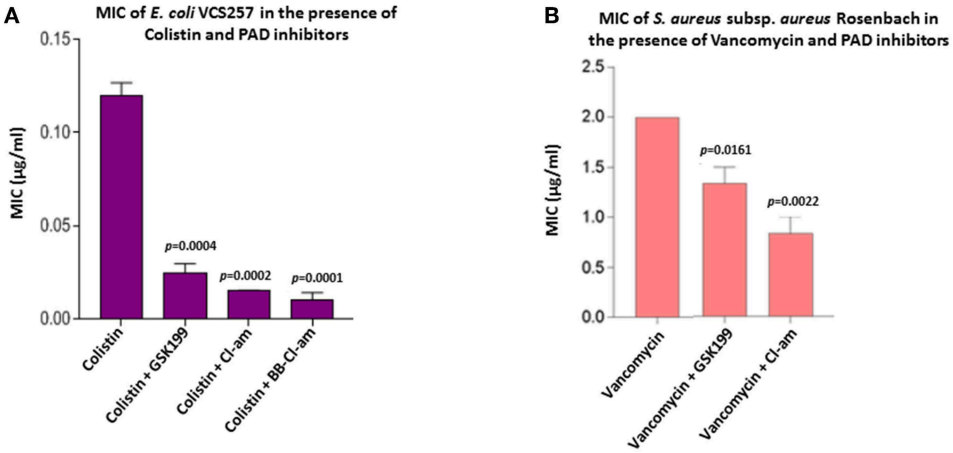

FIGURE 8 | PAD inhibitors reduce the MIC value of colistin for *E. coli* VCS257 and MIC value of vancomycin for *S. aureus* subsp. *aureus* Rosenbach. **(A)** In *E. coli*, BB-Cl-amidine was most effective at lowering the MIC value of colistin, reducing MIC by 91.6%. Cl-amidine decreased MIC by 87.4%, being the second most effective OMV-inhibitor lowering the MIC value of colistin. GSK199 lowered MIC by 76%. The experiment was repeated thrice and the data presented are mean ± SEM of the results; exact *p*-values are indicated. **(B)** In *S. aureus*, Cl-amidine increased the effectiveness of vancomycin by at least 62.5%, while GSK199 resulted in a 25% decrease in MIC. The experiments were repeated thrice and the data presented are mean ± SEM of the results; exact *p*-values are shown.

Gram-positive and Gram-negative bacterial species, revealing a phylogenetically conserved PAD/AD pathway of membrane vesicle release. This may open avenues for tailored OMV/MV inhibition in combination with selected antibiotics, according to bacterial type, to regulate biofilm formation and tackle antibiotic resistance.

CONCLUSION

This study reveals a phylogenetically conserved PAD/AD pathway of OMV/MV release in bacteria that can be pharmacologically modulated to sensitize bacteria to antibiotic treatment. For the first time, a citrullinome of *E. coli* VCS257 and associated OMVs is described, indicating lateral transfer of deiminated proteins via OMVs. Our findings highlight new applications for PAD inhibitors to regulate OMV/MV release and to enhance antibiotic sensitivity in both Gram-positive and Gram-negative bacteria.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

UK, PM, GM, IK, DB, BA, and SL performed the experiments. UK, SL, and JI analyzed the data. PM, GM, IK, AN, SL, and JI provided resources. UK, SL, and JI designed the study and wrote the manuscript. All authors critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00227/full#supplementary-material

Supplementary Figure 1 | Multiple sequence alignment of *E. coli* AD and human PADs. The evolutionary relationship between *E. coli* AD and human PAD2, 3 and 4 is shown; (*) indicates positions which have a single, fully conserved residue; (:) indicates conservation between groups of strongly similar properties - scoring >0.5 in the Gonnet PAM 250 matrix; (.) indicates conservation between groups of weakly similar properties, scoring <0.5 in the Gonnet PAM 250 matrix.

Supplementary Figure 2 | Multiple sequence alignment of *S. aureus* AD and human PADs. The evolutionary relationship between *S. aureus* AD and human PAD2, 3 and 4 is shown; (*) indicates positions which have a single, fully conserved residue; (:) indicates conservation between groups of strongly similar properties—scoring >0.5 in the Gonnet PAM 250 matrix; (.) indicates conservation between groups of weakly similar properties, - scoring <0.5 in the Gonnet PAM 250 matrix.

Supplementary Figure 3 | Western blotting of immunoprecipitated deiminated proteins (F95) and PAD4 bound proteins from *E. coli* VCS257 and derived OMVs.

(A) Same figure as 4C, but also showing unbound fractions (flow-through) from OMVs and *E. coli* after IP, using the F95 antibody. **(B)** Same figure as 4D, but also showing unbound fractions (flow-through) from OMVs and *E. coli* after IP, using the PAD4 antibody.

Supplementary Figure 4 | Agar plates showing the Kirby-Bauer disk diffusion test for *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach. **(A)** Disk diffusion test for *E. coli* VCS257. **(B)** Effects of PAD inhibitor disks alone on *E. coli* are shown. **(C)** Disk diffusion test for *S. aureus* subsp. *aureus* Rosenbach. **(D)** Effects of PAD inhibitors alone on *S. aureus* are shown. Disks containing the following antibiotics are indicated: erythromycin **(E)**, vancomycin (V), Rifampicin (R), kanamycin (K), colistin (C). PAD inhibitors used were GSK199 (10 μM; PAD4 inhibitor), Cl-amidine (50 μM; pan-PAD inhibitor), BB-Cl-amidine (5 μM; pan-PAD inhibitor), AMF30a (5 μM; PAD2 inhibitor).

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oqxAB-Positive IncHI2 Plasmid pHXY0908 Increase *Salmonella enterica* Serotype Typhimurium Strains Tolerance to Ciprofloxacin

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Salmonella enterica serotype Typhimurium is a major global food-borne pathogen and causes life-threatening infections. Although the resistance mechanisms to fluoroquinolones in *S.* Typhimurium had been well-defined, tolerance to fluoroquinolones and the associated mechanism for this are obscure. In the current work, we investigated an *oqxAB*-positive plasmid pHXY0908 and analyzed its role in *S.* Typhimurium tolerance to ciprofloxacin using time-kill, transcriptome sequencing and real-time PCR. *S.* Typhimurium ATCC14028 could survive under lethal concentrations of ciprofloxacin after acquiring plasmid pHXY0908. Transcriptome sequence analysis showed the chromosomal genes were systematically regulated after acquiring this plasmid suggesting an interaction between chromosome and plasmid. Additionally, the chromosomal efflux pump genes *acrB*, *acrA*, *tolC*, and *yceE* were up-regulated after acquiring plasmid pHXY0908 suggesting that these efflux pumps may contribute to the survival of ATCC14028 exposed to the lethal concentrations of ciprofloxacin. In conclusion, this is the first known report demonstrating that an IncHI2 type plasmid harboring *oqxAB* could assist *S.* Typhimurium survival under lethal concentrations of ciprofloxacin.

Keywords: *oqxAB*, IncHI2, plasmids, *Salmonella*, tolerance

INTRODUCTION

Salmonella enterica Typhimurium is a major global food-borne pathogen, causing a wide spectrum of human and animal diseases including acute gastroenteritis, bacteremia, and extra intestinally localized infections involving many organs (Coburn et al., 2007). Poultry, pigs, cattle, and reptiles are *S.* Typhimurium reservoirs and humans generally become infected by eating undercooked or contaminated food (Gomez et al., 1997).

Although intestinal infections caused by non-typhoid *Salmonella* are usually self-limiting, effective antimicrobial therapy is essential if invasive infection occurs (Hohmann, 2011). The extensive use of antimicrobials in humans and animals has led to an increase in multi-drug resistance among numerous bacterial strains. In particular, multidrug resistant (MDR) *Salmonella* isolates such as *S.* Typhimurium monophasic variant (*S.* 4,[5],12:i:-), *S.* Typhimurium DT104 and *S.* Rissen are major global public health problems (Threlfall, 2000; Hopkins et al., 2010; Mather et al., 2013; Gomes-Neves et al., 2014). Due to increasing resistance to the conventional antimicrobial agents such as ampicillin, chloramphenicol, and trimethoprim/sulfonamides, fluoroquinolones such as ciprofloxacin for the treatment of severe invasive salmonellosis has become more common (Hohmann, 2011). The use of fluoroquinolones has also led to a rapid increase in reduced susceptibility of *S.* Typhimurium to these therapeutics. MDR *S.* Typhimurium with reduced ciprofloxacin susceptibility has become common in China (Li et al., 2013a; Wong and Chen, 2013).

Resistance to fluoroquinolones is mainly due to point mutations in the quinolone resistance-determining region (QRDR) of the gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes. The efflux pump AcrAB-TolC can decrease susceptibility to drugs from seven antibiotic classes such as fluoroquinolones, tetracyclines, rifamycins, oxazolidinones, macrolides, and so on (Schuster et al., 2017). The efflux function of AcrAB-TolC is not only used for antibiotics but also small molecules, such as metabolins, dyes, detergents, organic solutes and even bile salts (Nikaido, 1996; Pos, 2009; Oswald et al., 2016). In addition, plasmid-mediated quinolone resistance (PMQR), including derivatives of quinolone resistance proteins (Qnr), aminoglycoside acetyltransferase Aac(6′)-Ib-cr, and quinolone efflux pumps QepA and OqxAB, have also been described in fluoroquinolone-resistant *S.* Typhimurium isolates (Strahilevitz et al., 2009; Poirel et al., 2012). Additionally, tolerance to ciprofloxacin has been observed that was primarily linked to mutations in *gyrA* and *parC* (Dahiya et al., 2014). Even though tolerance of *Salmonella* is most often attributed to the action of efflux pumps the evidence linking PMQR genes and fluoroquinolone tolerance is scant (Webber et al., 2006; Thorrold et al., 2007).

Plasmids play an important role in the dissemination of antimicrobial resistance genes (Carattoli, 2009) and different incompatibility group plasmids have been examined for their roles in MDR *S.* Typhimurium (Fernandez et al., 2007; Zaidi et al., 2011). In our previous study, we identified that spread of *oqxAB* was predominately due to transferable MDR IncHI2 pHXY0908-like plasmids in *S.* Typhimurium (Li et al., 2013a). Interestingly, we found that possession of plasmid pHXY0908 was correlated with the treatment failure to avian salmonellosis using enrofloxacin at a routine dosage. This was in spite of the fact that this plasmid could directly confer only low-level fluoroquinolone resistance (Chen et al., 2016). In the current study, we examined potential mechanisms involved in *S.* Typhimurium survival to the ciprofloxacin selective pressure after acquisition of plasmid pHXY0908.

MATERIALS AND METHODS

Bacterial Strains

S. Typhimurium ATCC14028 and *S.* Typhimurium ATCC14028-bearing plasmid pHXY0908 (ATCC14028-pHXY0908) were used as the test strains in the present study. IncHI2 plasmid pHXY0908 harboring *oqxAB* confers a multi-drug resistance phenotype. The ATCC14028-bearing plasmid pHXY0908 were obtained by electroporation of the transferable pHXY0908 into *S.* Typhimurium ATCC14028 as previously described (Chen et al., 2016, 2017).

Minimum Inhibitory Concentration (MIC) and Mutant Prevention Concentration (MPC) Determinations

The MIC of ciprofloxacin was determined for *S.* Typhimurium ATCC14028 and *S.* Typhimurium ATCC14028-bearing plasmid pHXY0908 by the standard broth microdilution methods according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (M100-S25). The breakpoint criteria used to determine ciprofloxacin phenotype in *Salmonella spp.* was based on the CLSI breakpoint criteria [$\leq$0.06 μg/mL (susceptible), 0.12–0.5 μg/mL (intermediate), and $\geq$1 μg/mL (resistant)]. *E. coli* ATCC25922 was used as a quality control strain.

The MPC values were determined as previously described (Allou et al., 2009). In summary, each strain was grown at 37°C in antibiotic-free Mueller Hinton (MH) broth for ∼6 h, until an OD_{600} of ∼1.0 was reached (corresponding to ∼10^9 CFU/mL). Cultures (20 mL) were centrifuged at 4,000 × g for 15 min. The supernatant was discarded and the pellet containing ∼10^{10} CFU/mL was suspended in 2 mL of sterile MH broth. MH agar plates containing ciprofloxacin at levels ranging from 0.002 to 32 μg/mL (diluted in $\log_2$ series) against each strain were inoculated with 100 μL of cell suspension and incubated at 37°C for 96 h. The MPC was recorded as the lowest ciprofloxacin concentration at which no colonies grew on an agar plate after 96 h.

Time-Kill Experiments

Time-kill curve kinetics assays were conducted using MH broth containing ciprofloxacin levels equaling 1 × MIC, 2 × MIC, 4 × MIC, and 8 × MIC of the strains tested. Antibiotic-free broth was evaluated in parallel as a control. Cultures were incubated at 37°C with shaking. Viable counts were determined by serial dilution after 0, 3, 6, 9, and 24 h of incubation and by plating 100 μL of the control, test cultures, or with dilutions at the indicated times onto MH agar plates. Colony counts were determined after 24 h of incubation.

Plasmid Sequencing

DNA of plasmid pHXY0908 was sequenced using the Single Molecule Real Time (SMRT) DNA Sequencing approach. After filtering *S.* Typhimurium ATCC14028 chromosomal DNA data, the remaining reads were assembled by HGAP2.2.0 method (Chin et al., 2013). Open reading frames (ORF) were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html)

and annotation was performed using RAST tools (Aziz et al., 2008). The sequence comparison and map generation was performed using BLAST (http://blast.ncbi.nlm.nih.gov) and Easyfig version 2.1 (Sullivan et al., 2011). The annotated sequence of pHXY0908 has been submitted to the GenBank nucleotide sequence database under the accession number KM877269.

Transcriptome Sequencing and Sequence Analysis

ATCC14028 and ATCC14028-pHXY0908 were cultured in LB broth with and without 1/2 × MIC levels of ciprofloxacin. Total RNA was extracted as previously described (Li et al., 2013b). A pooled sample from 3 independent experiments was used for RNA-seq. Ribo-Zero rRNA Removal Kit (Gram-Negative Bacteria) (Epicentre, Madison, WI, USA) was used to remove rRNA from total bacterial RNA. The library was constructed using an Illumina TruSeq RNA sample Prep Kit v2 as previously described (Wang et al., 2013). In brief, mRNA was fragmented into lengths of 200∼300 bp and first and second strand cDNA was synthesized. The short cDNA fragments were purified and end repaired and tailed with single A (adenine) addition. Adapters were ligated to the A-tailed cDNA fragments and ligated. These cDNA fragments were enriched by 12 PCR cycles. Purified libraries were quantified using a by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and validated using an Agilent 2100 Bioanalyzer (Agilent, Beijing, China). Libraries were sequence by the Illumina Hiseq-2000 for 90 cycles. The reads that passed the Illumina quality filter were kept for sequence analysis.

High quality reads were mapped to *S.* Typhimurium strain 14028 genome (downloaded from https://www.ncbi.nlm.nih.gov/nuccore/267991652/) and plasmid pHXY09080 sequence by using SOAP aligner/SOAP2 (Li et al., 2009) with 5 max alignment error. The mRNA abundance was normalized using rpkM (reads per kilobase per million reads) (Mortazavi et al., 2008). Gene differential expression analysis was performed as previously described (Audic and Claverie, 1997). The genes with <0.001 FDR and >2-fold change or <0.5-fold were detected as differentially expressed genes (DEG). The RNA-seq data had been submitted to SRA database (Accession number: PRJNA544622, https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA544622).

Gene Ontology Enrichment Analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis, and Functional Protein Association Networks Analysis

The DEGs were performed Gene Ontology enrichment analysis by MATLAB bioinformatics toolbox (MathWorks, Natick, MA, USA). The gene ontology annotations were downloaded from Gene Ontology Consortium (http://www.geneontology.org/page/download-go-annotations). KEGG pathway enrichment analysis was performed by ClueGO and CluePedia which are Cytoscape apps (Bindea et al., 2009, 2013). The functional protein association networks were constructed by STRING with high confidence and hided disconnected nodes in the network (Szklarczyk et al., 2017).

TABLE 1 | Ciprofloxacin MICs and MPCs of the *Salmonella* Typhimurium strains used in this study.

Strain	MIC (mg/L)	MPC (mg/L)	MPC/MIC ratio
ATCC14028	0.03	0.5	16
ATCC14028-pHXY0908	0.125	2	16

Each value is the mean ± SD derived from three independent experiments.

Validation the DEGs by qRT-PCR

Total bacterial RNA was obtained and reverse transcribed into cDNA as described (Li et al., 2013b). The qRT-PCR was performed with SYBR Premix Ex Taq (Takara, Dalian, China) in an iQ5 thermal cycler (Biorad, Hercules, CA) according to the manufacturer's instructions. The cycling conditions were as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, at 55°C for 1 min and 72°C for 1 min with a final step of 72°C for 5 min. Melting curves were read from 60 to 95°C in steps of 1°C. Normalized expression levels of the target gene transcripts were calculated relative to 16S rRNA using the $-\Delta\Delta$CT method. The primers used for gene amplification are listed in **Table S1**.

Statistics

All the *in vitro* experiments described above were repeated at least three times. Geometric means were used to express the results for MICs and MPCs and the means ± standard deviations (SD) were calculated for CFU counts.

RESULTS

MIC, MPC, and Time-Kill Curve Assays

Ciprofloxacin MIC and MPC values were increased by 4-fold when *S.* Typhimurium ATCC14028 acquired plasmid pHXY0908. However, the MPC/MIC ratios between these strains did not differ (**Table 1**).

However, time-kill curve assays indicated a survival advantages at 1×, 2×, and 4× ciprofloxacin MIC levels for the plasmid bearing strain compared to the parental strain. In addition, the antibacterial activity of ciprofloxacin significantly decreased over the 3 h antibiotic exposure period against ATCC14028 strain harboring plasmid pHXY0908. Interestingly, the bacterial concentration of ATCC14028-pHXY0908 increased after 3 h and after 6 h under the 1 × MIC. The CFU of ATCC14028-pHXY0908 was almost identical with ATCC14028-pHXY0908 cultured without ciprofloxacin for 24 h (**Figure 1**).

General Features of Plasmid pHXY0908

Plasmid pHXY0908 is a circular molecule of 249,144 bp with an average G + C content of 46.22% and harboring 265 predicted ORFs (**Figure 2A**). The backbone region of pHXY0908 is closely related to other IncHI2 plasmids including pHK0653 (KT334335) from *Salmonella* enterica, plasmid R478 (BX664015) from *Serratia marcescens* and pAPEC-O1-R (DQ517526) from

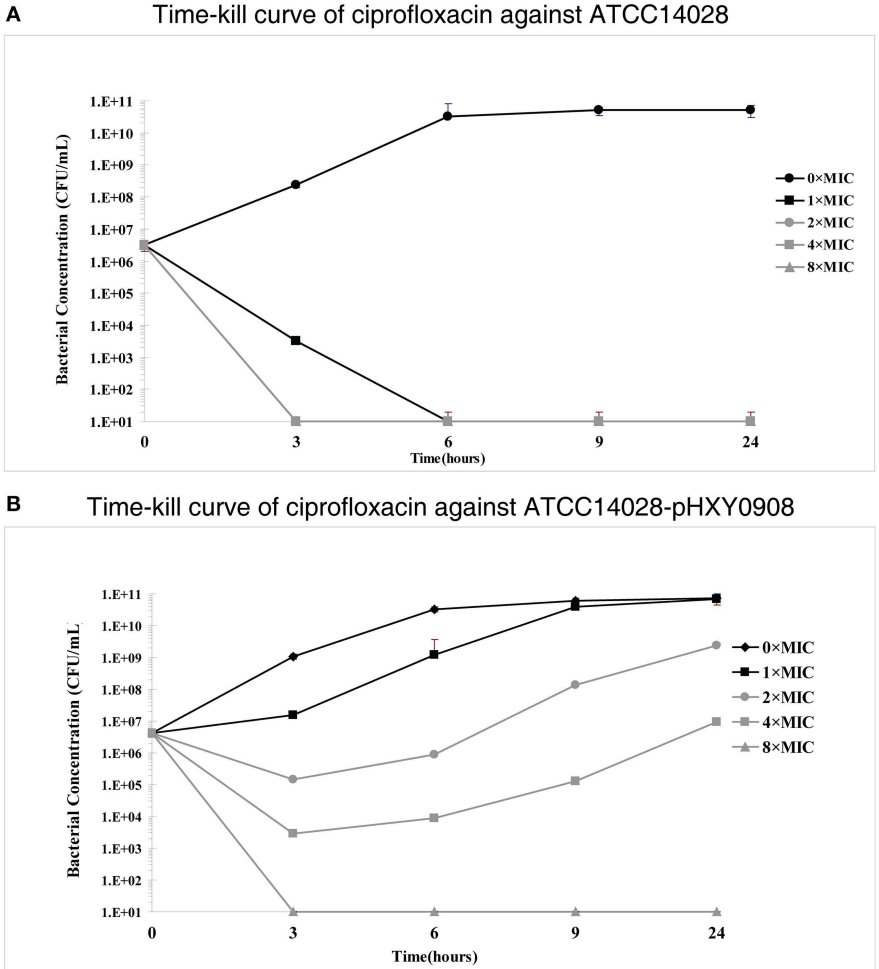

FIGURE 1 | *In vitro* time-kill studies of ciprofloxacin **(A)** *Salmonella* Typhimurium ATCC14028 and **(B)** and ATCC14028-pHXY0908 1 × MIC is equivalent to 0.03125 mg/L for ATCC14028 and 0.125 mg/L for ATCC14028-pHXY0908. The lines and markers of 2 × MIC and 8 × MIC coincided with 4 × MIC in **(A)**.

an extraintestinal pathogenic *Escherichia coli* (**Figure 2B**). These backbone regions contained the replication gene *repHI2*, the plasmid maintenance and partitioning modules *parA-parB* and *hipAB* and two *tra* transfer regions. In addition to the typical backbone, the 52,876 bp MDR region of pHXY0908 contained 9 IS*26* elements flanking four main segments (**Figure S1**). The first segment containing an *oqxAB* cassette flanked by IS*26* elements was first identified in the IncX1 type plasmid pOLA52 from *E. coli*. The second segment between IS*26*-6 and IS*26*-7 contained the antibiotic resistance genes *sul3*, *aadA1*, *cmlA*, and *aadA2* and was identical to plasmid pND11_107 (HQ114281). The third segment containing insertion sequences IS*26*-7 and IS*26*-8 and the structure *tnp21-floR-sul2*-IS4-*aacC4*-IS*26*, identical to that of pK1HV (HF545434) from *Klebsiella pneumoniae*. The fourth segment was similar to plasmids pSTA155 (NG041621) and pAPEC-O1-R (DQ517526) and included *aac(6′)-Ib-cr* adjacent to an unusual class 1 integron containing $bla_{\mathrm{OXA-1}}$, *catB3*, *arr3*, *qacEΔ1*, and *sul1*. Importantly, *oqxAB,* and *aac(6′)-Ib-cr* could confer low-level resistance to fluoroquinolones.

Transcriptional Regulation of ATCC14028 After Acquiring pHXY0908

The transcriptional profiles of ATCC14028 with and without pHXY0908 were obtained using transcriptome sequencing. Both strains were cultured in the presence and absence of 1/2 × MIC concentrations of ciprofloxacin. We quantified the levels of 4,687 and 4,671 genes in ATCC14028 and ATCC14028-pHXY0908, respectively. The 283 genes were only expressed in ATCC14028 and the 267 genes were only expressed in ATCC14028-pHXY0908 (**Figure 3A**). We found 411 chromosomal DEGs between ATCC14028-pHXY0908 and ATCC14028 (**Figure 3C**). And 223 plasmid genes were expressed in ATCC14028-pHXY0908. The plasmid situated efflux pump *oqxA* and *oqxB* genes were highly expressed in ATCC14028-pHXY0908. Interestingly, the chromosomal efflux pump gene *acrB* was significantly up-regulated (2.5-fold, $P < 10^{-32}$) in the plasmid-bearing strain. The *acrA* gene which product composed efflux pump with AcrB protein was also slightly up-regulated (1.6 fold, $P < 10^{-32}$). The *tolC* gene was also significantly up-regulated as same as *acrB* (2.16 fold, $P = 1.5 \times 10^{-11}$).

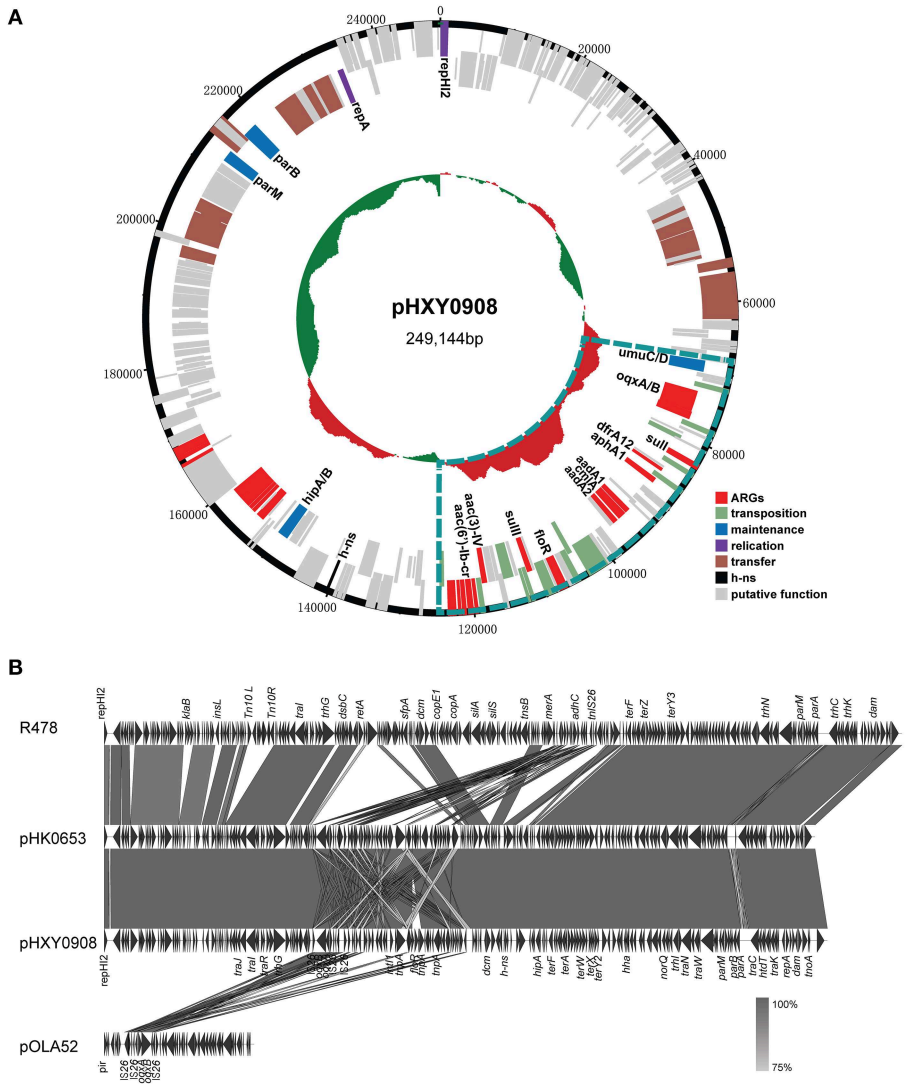

FIGURE 2 | Genetic map and linear comparisons for pHXY0908. **(A)** Genetic map of pHXY0908. The central region of the map is G+C plot of the nucleotide sequence. Boxes represent ORFs predicted with RAST tools and are color coded after their predicted functions. The blue lines represent the multidrug-resistant regions. **(B)** Linear comparison of pHXY0908, R478, pHK0653, and *oqxAB*-positive plasmid pOLA52. The arrows represent ORF position and transcriptional direction. Regions of homology are shaded in gray and functional regions are indicated above and below the linear maps.

The gene expression profiles of these strains also differed with the strains cultured in the presence of ciprofloxacin. We quantified the expression of 4,473 and 4,686 genes in ATCC14028-CIP and ATCC14028-pHXY0908-CIP, respectively. The 167 genes were only expressed in ATCC14028-CIP and the 416 genes were only expressed in ATCC14028-pHXY0908-CIP (**Figure 3B**). There were 1029 chromosomal DEGs between ATCC14028-pHXY0908-CIP and ATCC14028-CIP. And 224 plasmid genes were expressed in ATCC14028-pHXY0908-CIP. The *acrB* and *tolC* genes were up-regulated in ATCC14028-pHXY0908-CIP, but we found no difference for *acrA*. Furthermore, we found that many chromosomal efflux pump genes were up-regulated significantly in ATCC14028-pHXY0908-CIP compared with ATCC14028-CIP, for example, *ydgF*, *ydgE*, *ybjY*, *yceE*, *sugE*, and *yohM* (**Figure 3D**).

The GO and KEGG Enrichment Analysis of Chromosomal DEGs of ATCC14028 After Acquiring pHXY0908

The transcriptional profile of ATCC14028 was changed after acquiring pHXY0908. GO enrichment analysis indicated that chromosomal DEGs of ATCC14028-pHXY0908 vs. ATCC14028 were enriched for some biological processes. These included sulfur incorporation into metallo-sulfur clusters, sulfate assimilation, 6-sulfoquinovose (1-) metabolism, sulfur compounds in catabolic process, type III protein secretion (**Figure 4A**). Interestingly, six biological processes in the top ten enriched biological processes were related to sulfide. pHXY0908 contained the thiol:disulfide interchange protein DsbC that is responsible for the formation of disulfide bonds and this

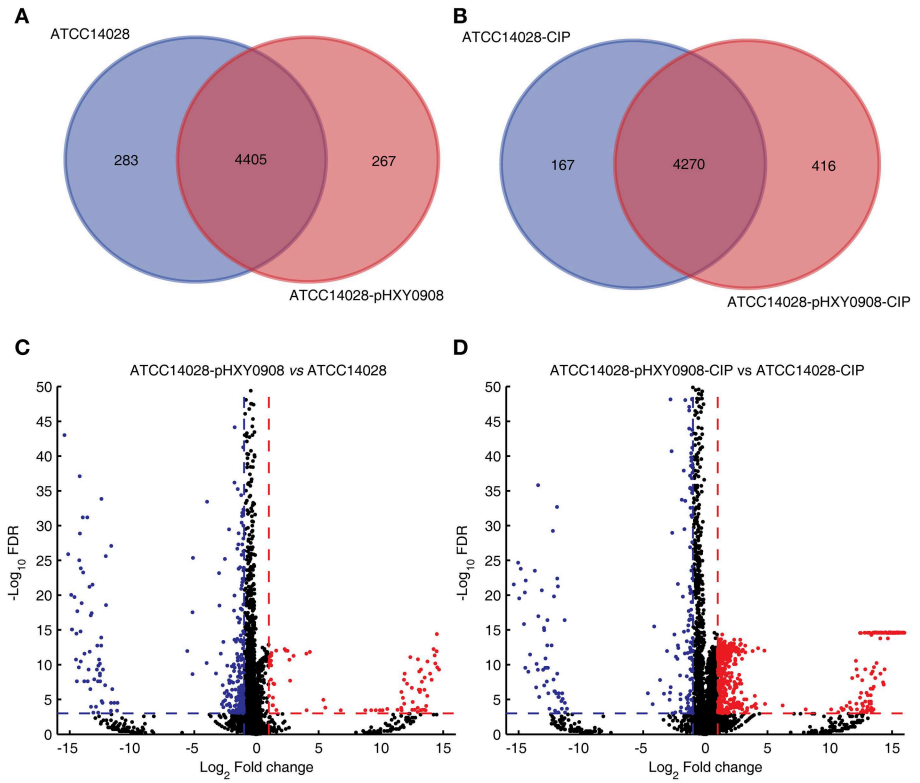

FIGURE 3 | Venn diagram of expressed genes and volcano plot. **(A)** Venn diagram of expressed genes in ATCC14028 and ATCC14028-pHXY0908. **(B)** Venn diagram of expressed genes in ATCC14028-CIP and ATCC14028-pHXY0908-CIP. **(C)** Volcano plot of ATCC14028-pHXY0908 vs. ATCC14028. **(D)** Volcano plot of ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP. The blue plot in volcano plot represented down-regulated genes (Log_2 Fold change ≤ -1 and *FDR* < 0.001). The red plot in volcano plot represented up-regulated genes (Log_2 Fold change ≥ 1 and *FDR* < 0.001).

process is related to sulfur metabolism (Mangold et al., 2011). This suggests that ATCC14028 may regulate genes expression to support the plasmid function. In addition, sulfur-mediated biological process could provide a new way for ciprofloxacin biodegradation (Jia et al., 2018).

The chromosomal DEGs of ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP were enriched in four transcriptional biological processes and two electron transport chain processes (**Figure 4B**). After acquiring pHXY0908, ATCC14028 could activate transcription of resistance genes including efflux pump genes under ciprofloxacin selective pressure. This indicates that pHXY0908 can influence the gene expression of ATCC14028 to resist ciprofloxacin selective pressure.

The chromosomal DEGs of ATCC14028-pHXY0908 vs. ATCC14028 were enriched in four KEGG pathways, including phosphotransferase system (PTS), fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism and citrate cycle (TCA cycle) (**Figure 5A**). Additionally, the KEGG pathways enrichment analysis for DEGs of ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP is C5-branched dibasic acid metabolism and phosphotransferase system (PTS) (**Figure 5B**). The phosphotransferase system (PTS) serves as a complex protein kinase system that regulates a wide variety of transport, metabolic and mutagenic processes as well as the expression of numerous genes (Saier, 2015). That indicated the pHXY0908 influenced the gene expression, the substance metabolism and energy metabolism in the ATCC14028.

The Functional Protein Association Networks Analysis for DEGs of ATCC14028 After Acquiring pHXY0908

The functional protein association networks were constructed by chromosomal DEGs of ATCC14028 after acquiring pHXY0908. A majority of DEGs were constructed into two main networks. This suggests pHXY0908 may systematically regulate ATCC14028 functional protein association networks rather than at an independent gene expression level. The largest sub-network that included the genes *spaI*, *invA*, *sicA*, and *sipB* is related to invasion and type III secretion systems. Another sub-network that included *cysA*, *cysH,* and *cysI* was related to sulfur metabolism. These two main functional networks were consistent with the GO enrichment analysis and pHXY0908 function (**Figure 6**). Additionally, the networks of ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP DEGs also contained the sub-networks related to invasion and type III secretion systems, sulfur metabolism, energy metabolism, and other substance metabolism (**Figure S2**).

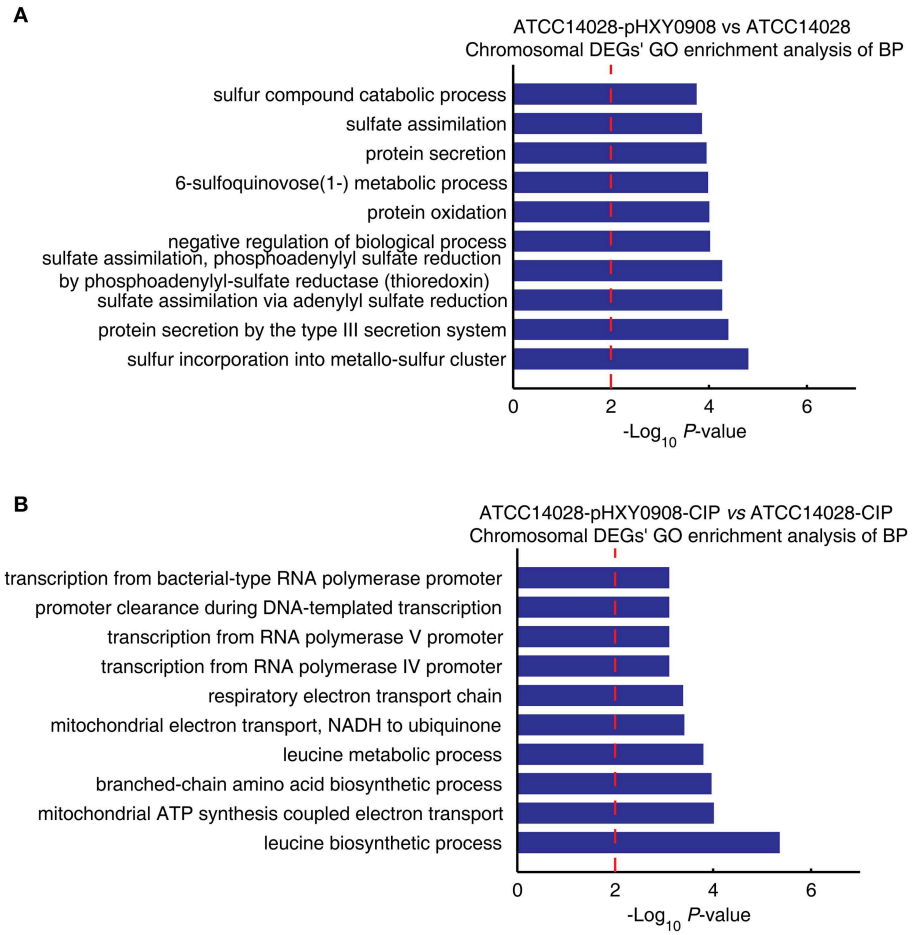

FIGURE 4 | The chromosomal DEGs GO enrichment analysis of biological processes. **(A)** ATCC14028-pHXY0908 vs. ATCC14028 and **(B)** ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP **(B)**. The top 10 biological processes are shown. The dotted red line indicates the *P*-value threshold of 0.01.

RNA-seq Data Validated by qRT-PCR

Five randomly selected DEGs that including *dam*, *csgD*, *csgE*, *STM14-1453*, and *acrB*, and five genes which we interested, including *acrA*, *tolC*, *yceE*, *sicA*, and *cysA*, were validated by using qRT-PCR. The qRT-PCR results had a good correlation with different expression analysis (**Figure S3**, $R = 0.9793$, $P = 7.7606 \times 10^{-7}$ in ATCC14028-pHXY0908 vs. ATCC14028; $R = 0.9761$, $P = 1.3851 \times 10^{-6}$ in ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP). This indicated that the RNA-seq and the associated data analysis represented actual gene expression changes.

DISCUSSION

This study demonstrated that an MDR IncHI2 type plasmid encoding OqxAB could contribute to the survival under ciprofloxacin selective pressure of *S.* Typhimurium strains that had acquired plasmid pHXY0908. Although the MIC and MPC increased after acquiring pHXY0908, the same MPC/MIC ratios for both ATCC14028 and ATCC14028-pHXY0908 indicated that pHXY0908 did not increase ability of ciprofloxacin for selecting resistant mutation in ATCC14028. The time-kill curve assays demonstrated that ciprofloxacin concentrations >MIC values for *S.* Typhimurium ATCC14028 were lethal while the plasmid-bearing strain could survive up to 4 × MIC. Thus, we further explored the implied survival mechanism of *S.* Typhimurium under drug pressure after acquiring an *oqxAB*-positive IncHI2 type plasmid. The same MPC/MIC suggested that the genes expression regulation may be the potential mechanism instead of genes mutation.

Firstly, we determined the complete nucleotide sequence of plasmid pHXY0908.

DNA sequencing further confirmed that this plasmid belonged to the IncHI2 group and showed high similarity to the backbone region of R478, one of the prototypes of IncHI2 plasmid groups (Garcia-Fernandez and Carattoli, 2010). In addition to the conserved backbone, pHXY0908 harbored a multidrug resistance region composed of *oqxAB*, *aac(6′)-Ib-cr* as well as nine other ARGs and included a wide range of mobile genetic elements and notably, IS*26*. Except for

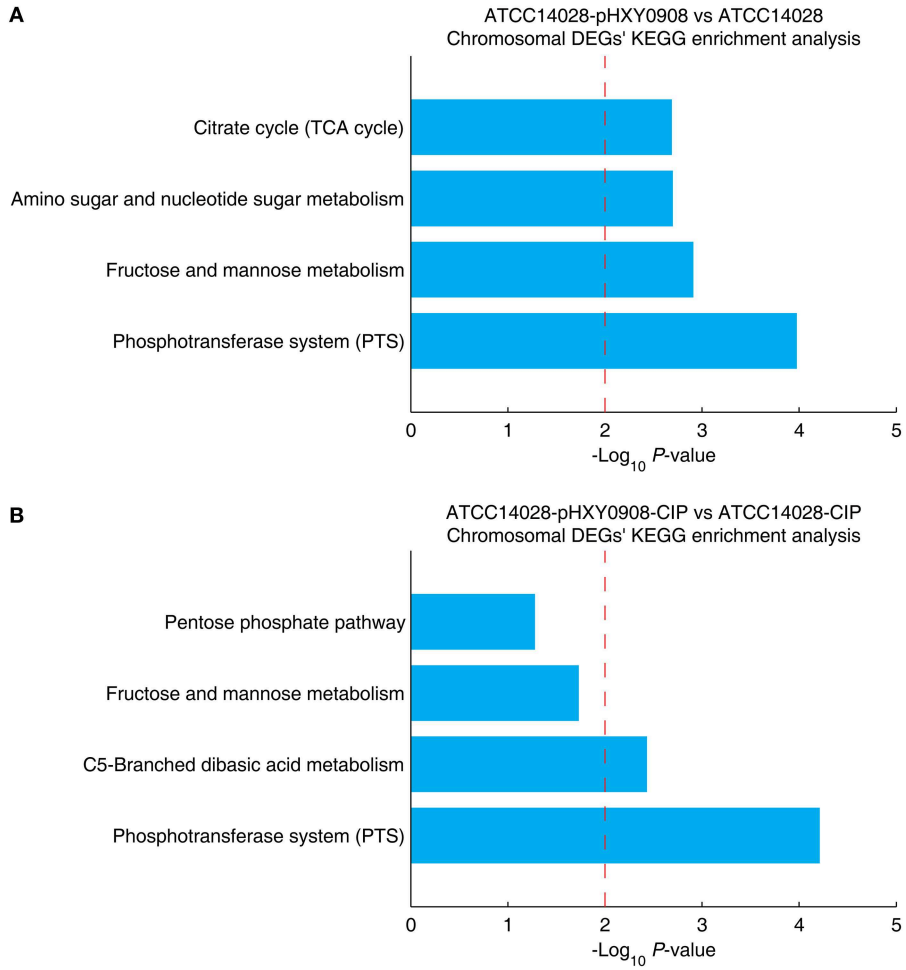

FIGURE 5 | The chromosomal DEGs KEGG pathway enrichment analysis. **(A)** ATCC14028-pHXY0908 vs. ATCC14028 and **(B)** ATCC14028-pHXY0908-CIP vs. ATCC14028-CIP **(B)**. The dotted red line indicates the *P*-value threshold of 0.01.

oqxAB and *aac(6′)-Ib-cr*, none of the other known genes are involved in fluoroquinolone resistance although many hypothetical proteins were found on pHXY0908. In addition, pHXY0908 showed a surprisingly high degree of homology (100% coverage, 99% identity) with the IncHI2 plasmid pHK0653 from an *S.* Typhimurium strain of human origin and pHK0653-like plasmids that were identified as the key vectors responsible for *oqxAB* transmission among *Salmonella* species (Wong et al., 2016).

Plasmid pHXY0908 harbored diverse ARGs including *oqxAB* and *aac(6′)-Ib-cr* that could confer only low-level fluoroquinolone resistance. However, the gene features of pHXY0908 cannot explain the reason for ATCC14028 survival under the lethal concentrations of ciprofloxacin up to 4 × MIC. To study the influence of pHXY0908, we measured the transcriptional profiles of ATCC14028 with and without pHXY0908 cultured in the presence and absence of 1/2 MIC concentrations of ciprofloxacin. ATCC14028 could not grow at >1 × MIC, so we selected a 1/2 MIC level of ciprofloxacin to study the transcriptional regulation of ATCC14028 by pHXY0908. The 1/2 MIC concentrations allowed bacterial growth but also induced stress responses that are sub-MIC and often used in the study of antibiotic resistance (Patkari and Mehra, 2013; Heo et al., 2014; Zhong et al., 2015; Aedo and Tomasz, 2016). The transcriptome data show that 283 chromosomal genes were not expressed after ATCC14028 acquiring pHXY0908. Similarly, there are 167 chromosomal genes were not expressed in ATCC14028-pHXY0908-CIP compared with ATCC14028-CIP. The bacteria will synthesize proteins encoded by plasmid leading the cost of resources. The bacteria may turn off some chromosomal genes expression to fit this situation. These genes were included in DEGs to analyze the function. Due to the ciprofloxacin inhibits the activity of gyrase and topoisomerase IV, we analyzed the expression of *gyrA*, *gyrB*, *parC*, and *parE*. These four genes were not differently expressed in ATCC14028-pHXY0908 compared with ATCC14028. The *gyrA* was down-regulated in ATCC14028-pHXY0908-CIP compared with ATCC14028-CIP. However,

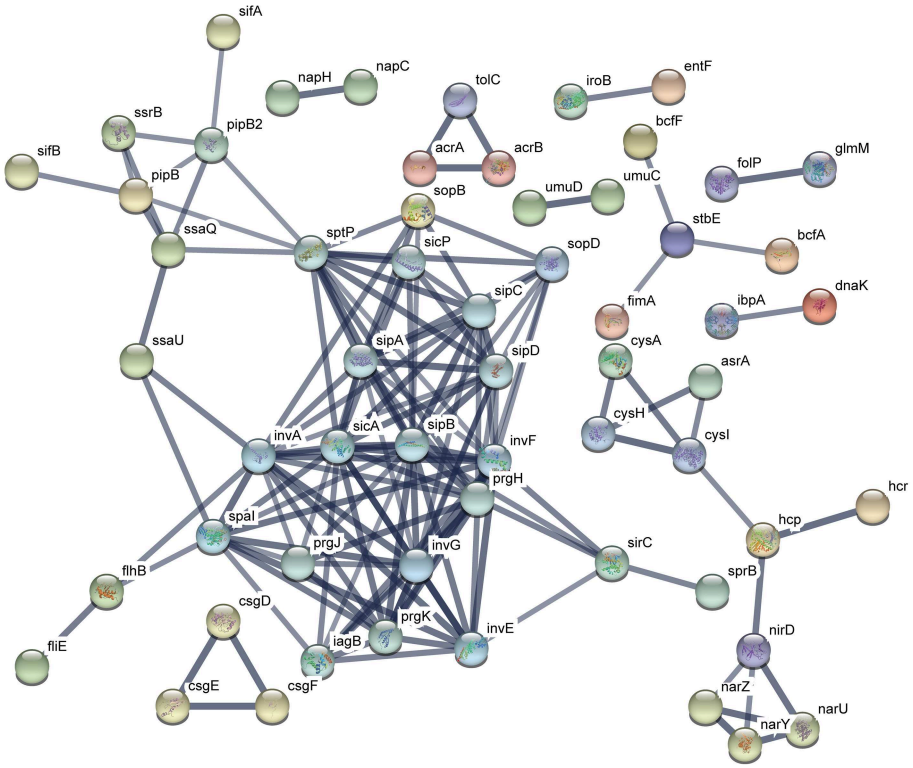

FIGURE 6 | Functional protein association networks of chromosomal DEGs after acquiring pHXY0908. Each node is a protein. Each edge represents protein-protein associations. The thicker edges indicate higher levels of confidence.

other three genes were not differently expressed in ATCC14028-pHXY0908-CIP compared with ATCC14028-CIP. The gyrA down-regulated in ATCC14028-pHXY0908-CIP compared with ATCC14028-CIP may due to the resistant proteins encoded by pHXY0908 could decreased the ciprofloxacin pressure in the cell. The GO enrichment analysis of DEGs indicated that pHXY0908 influenced chromosomal gene expression to support plasmid function and resist ciprofloxacin selective pressure. This indicated an interaction between the chromosome and plasmid. And the KEGG pathway enrichment analysis of DEGs indicated that pHXY0908 influence ATCC14028 genes expression and metabolism. Furthermore, the pHXY0908 systematically regulated ATCC14028 functional protein association networks. Intriguingly, the chromosomal efflux pump genes were up-regulated after acquiring the plasmid and these included *acrB*, *acrA*, and *tolC*. Except for AcrAB-TolC efflux pump, the *ydgF*, *ydgE*, *ybjY*, *yceE*, *sugE*, and *yohM* were especially up-regulated when cultured under the 1/2 MIC levels of ciprofloxacin. The multidrug efflux pump AcrAB-tolC system decreases susceptibility to fluoroquinolones (Piddock, 1999) and *yceE* encoding the multidrug transporter subunit MdtG contributes to fluoroquinolone-resistance (Fàbrega et al., 2010). The *ydgF* and *ydgE* encoded MdtI and MdtJ, respectively. These two proteins compose multidrug efflux pump MdtIJ which excretes polyamine (Higashi et al., 2008; Leuzzi et al., 2015). The *ybjY* codes macrolide transporter subunit MacA. The *sugE* was reported the contribution for tributyltin (TBT) and antimicrobial resistance (He et al., 2011; Cruz et al., 2013). And the *yohM* encodes a membrane-bound polypeptide conferring increased nickel and cobalt resistance (Rodrigue et al., 2005). The up-regulation of chromosomal efflux pump genes may be caused by the accumulation of intermediate metabolites for plasmid DNA replication and proteins synthesis. In addition, another potential mechanism is some plasmid proteins regulated chromosomal genes. It is worthy of further investigations. The chromosomal efflux pump up-regulated by pHXY0908 may be the one of the reasons for survival under the lethal concentrations of ciprofloxacin. Excepted the efflux function, the synthesis of chromosomal efflux pump proteins, the activity of efflux pumps and the encoding of plasmid genes may cost much energy, so that the growth of ATCC14028 may be influenced. The slow grown speed lead to the tolerance to fluoroquinolone (Evans et al., 1991; Brauner et al., 2016). The change of ATP level and grown rate of the ATCC14028 after acquiring pHXY0908 may be measured in the further researches. However, the specific molecular mechanism of the tolerance phenotype is not clear. In the further investigations, we will use functional genome technologies combined our plasmid gene expression profiles to detected the plasmid gene caused the tolerance to ciprofloxacin.

Taken together, we found that chromosomal genes were systematically regulated after ATCC14028 acquired the IncHI2

type MDR plasmid pHXY0908. ATCC14028 could survive under the ciprofloxacin lethal concentrations may be attributed to an up-regulation of chromosomal efflux pump genes after acquiring pHXY0908.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

XLL carried out the study design, data analysis, and manuscript writing. XW carried out the sequence experiments and was involved in the preparation of the manuscript. XL and JX carried out the RT-qPCR experiments. LF, JS, XPL, and YL contributed to the study design. All authors approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00242/full#supplementary-material

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The *Salmonella* Specific, σ^E-Regulated, STM1250 and AgsA, Function With the sHsps IbpA and IbpB, to Counter Oxidative Stress and Survive Macrophage Killing

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The host presents an array of environments which induce bacterial stress including changes in pH, antimicrobial compounds and reactive oxygen species. The bacterial envelope sits at the interface between the intracellular and extracellular environment and its maintenance is essential for *Salmonella* cell viability under a range of conditions, including during infection. In this study, we aimed to understand the contribution of the σ^H- and σ^E-regulated small heat shock proteins IbpA, IbpB, and AgsA and the putative σ^E-regulated stress response protein STM1250 to the *Salmonella* envelope stress response. Due to shared sequence identity, regulatory overlap, and the specificity of STM1250 and AgsA to *Salmonella* sp., we hypothesized that functional overlap exists between these four stress response proteins, which might afford a selective advantage during *Salmonella* exposure to stress. We present here new roles for three small heat shock proteins and a putative stress response protein in *Salmonella* that are not limited to heat shock. We have shown that, compared to WT, a quadruple mutant is significantly more sensitive to hydrogen peroxide, has a lower minimum bactericidal concentration to the cationic antimicrobial peptide polymyxin B, and is attenuated in macrophages.

Keywords: *Salmonella*, envelope stress, oxidative stress, *rpoE*, sHsp

INTRODUCTION

Salmonella sp. are major causes of morbidity and mortality worldwide. In 2010, it was estimated that non-typhoidal *Salmonella* (NTS) was responsible for 93.9 million cases of disease and 155,000 deaths per year (Majowicz et al., 2010). Emphasizing the global significance of *Salmonella* infection, the World Health Organization priority pathogens list (Tacconelli et al., 2018) details fluoroquinolone resistant *Salmonella* to be of high priority for research. As a result, a better understanding of the mechanisms of *Salmonella* infection and survival in the host will direct research into new therapeutics for this prevalent pathogen.

During the *Salmonella* life cycle a range of precarious environments are encountered, both within the host and the environment, that have the potential to be bacteriostatic and/or bactericidal in nature. Such conditions need to be detected and transduced to allow appropriate transcriptional responses to be elicited that counter the stress. During mammalian infection, *Salmonella* encounters highly stressful environments; macrophages are sites of reactive oxygen

species (ROS) and nitric oxide (NO), and intestinal antimicrobial peptides can disrupt the outer membrane (OM) causing serious cellular damage. The cell envelope, formed of the OM, periplasmic space, peptidoglycan layer, and inner membrane, is a crucial barrier between the bacteria and its extracellular environment. Thus, maintenance of this barrier is essential to survival and significantly contributes to the success of *Salmonella* as an intracellular pathogen.

The extracytoplasmic sigma factor σ^E (RpoE) (reviewed in Rowley et al., 2006), is an important gatekeeper for maintenance of the cell envelope, detecting stresses and subsequent perturbations to OM and periplasmic proteostasis. In the intracellular pathogen *Salmonella*, *rpoE* is upregulated within macrophages (Eriksson et al., 2003) and regulates genes required for resistance to oxidative stress and heat (Testerman et al., 2002). Moreover, it has been shown previously that σ^E is extremely important for *Salmonella* during intracellular survival in macrophages and in murine infection (Humphreys et al., 1999). Well-characterized σ^E-regulated genes make significant contributions to envelope maintenance and the infection process (Humphreys et al., 2003; Lewis et al., 2009; Rowley et al., 2011). Additionally, in the serovar responsible for typhoid fever, *S.* Typhi, *rpoE* is important for intracellular invasion and survival (Zhang et al., 2016). Clearly, the σ^E regulon is of great importance to multiple stages of *Salmonella* host colonization across different serovars. That given, there is much we do not understand about the contribution of a number of σ^E-regulated genes to the envelope stress response (ESR) and infection.

Identification of the σ^E regulon in *Salmonella* highlighted a number of genes of unknown function (Skovierova et al., 2006) including *ibpA*, *ibpB*, *agsA*, and the putative cytoplasmic protein *STM1250*. In addition to σ^E regulation, *ibpA, ibpB,* and *agsA* are also regulated by the sigma factor σ^H. Although primarily induced by heat shock, members of the σ^H regulon have been linked to bacterial pathogenesis (Roncarati and Scarlato, 2017).

IbpA and IbpB (from herein referred to as IbpAB) are well-conserved across species of Gram-negative bacteria. These proteins share 50% amino acid sequence identity and were first observed in *E. coli* to be highly expressed and associated with inclusion bodies (inclusion body protein) during expression of heterologous proteins (Allen et al., 1992). As members of the small heat shock protein (sHsp) family, IbpA and IbpB are 15 and 16 kDa respectively and contain a C-terminal α-crystallin domain, a characteristic feature of sHsps (Nakamoto and Vígh, 2007). Furthermore, the *ibpA* 5′-untranslated region (UTR) encodes a ROSE (repression of heat shock gene expression)-like RNA thermometer enabling its temperature controlled expression (Waldminghaus et al., 2009). IbpAB have been shown to associate with endogenous proteins in *E. coli* following heat shock (Laskowska et al., 1996); however, limited phenotypic or functional studies exist for these proteins in *Salmonella* and to date no contribution to infection has been identified.

Conversely, STM1250 and AgsA are unique to *Salmonella* spp. (Skovierova et al., 2006). AgsA (aggregation-supressing protein A) is a 17 kDa sHsp with 32% amino acid sequence identity to IbpA and 31% to IbpB (Tomoyasu et al., 2003). Like *ibpA*, the *agsA* 5′-UTR contains an RNA thermometer for temperature control of expression (Waldminghaus et al., 2007). The 10 kDa protein STM1250 is not a member of the sHsp family, lacking the α-crystallin domain. However, *STM1250* has been shown to form an operon with *agsA* and the two genes are separated by only 151 bp (Skovierova et al., 2006).

In this study we aimed to understand the contribution of the stress-induced small heat shock proteins IbpA, IbpB, AgsA, and the *Salmonella* specific putative chaperone STM1250, to the ESR. Despite their description as sHsps we hypothesize that IbpAB and AgsA, in cooperation with STM1250, are involved in tolerance to multiple stresses. Interestingly, *ibpAB* and *STM1250* are highly expressed during intracellular infection and all genes are expressed under osmotic stress (Kröger et al., 2013; Canals et al., 2019). Additional published gene expression and TraDIS libraries provide interesting insights into the potential roles of these genes. Chaudhuri et al. (2013) identified that a *STM1251* TraDIS mutant is attenuated in cattle, but not chickens, swine or mice. Together, these data support that these genes may function beyond tolerance to heat shock.

Based on sequence identity, shared regulation by σ^E and σ^H, and genomic location, we predict functional overlap exists between these proteins, and the putative stress responsive protein STM1250, that in *Salmonella*, is not limited to surviving heat shock. To investigate this, we have subjected deletion mutants to conditions known to perturb envelope homeostasis and present new roles for these stress responsive proteins. We have demonstrated that an Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant is attenuated in macrophages and is more sensitive to hydrogen peroxide (H_2O_2) -induced oxidative stress and the cationic antimicrobial peptide polymyxin B, compared to WT.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are detailed in **Table 1**. Bacteria were maintained on LB agar and overnight cultures were grown in LB broth at 37°C with aeration, supplemented with 50 μg/mL kanamycin, 30 μg/mL chloramphenicol, or 100 μg/mL ampicillin where required. Bacterial growth curves were performed in 24 well plates in a SpectraMax M5 microplate reader with culture volumes of 1 mL. An overnight culture was diluted to OD_{600} 0.1 in LB. Where stated, 6 mM hydrogen peroxide (H_2O_2, Sigma), 1,000–4,000 U/mL bovine catalase (Sigma), 3 mM $CuCl_2$, 0.5 μM potassium tellurite (K_2TeO_3), 30 mM paraquat (methyl viologen, Sigma), or 1 mM indole was added to each well. The plate was incubated at 37°C and OD_{600} reading taken every hour, with 3 s of agitation before each reading.

Construction of Deletion Mutants

Mutants were generated by lambda Red recombination (Datsenko and Wanner, 2000). Chloramphenicol or kanamycin antibiotic resistance cassettes were amplified by PCR from the plasmids pKD3 or pKD4, respectively, using Biomix Red (Bioline) and mutant primers in **Supplementary Table S1**. The PCR product was transformed into SL1344 harboring the pKD46 plasmid. Transformants were screened by colony PCR

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain	Description	Source
STRAINS		
SL1344	*Salmonella enterica* serovar Typhimurium 4/74, *hisG, rpsL*	Hoiseth and Stocker, 1981
GVB2551	SL1344 Δ*fkpA*Δ*surA*Δ*ppiAD*	Gift from Professor Mark Roberts, University of Glasgow
Δ*ibpAB*	SL1344 Δ*ibpA-B* :: Kan	This study
Δ*STM1250*Δ*agsA*	SL1344 Δ*STM1250*Δ*agsA* :: Cm	This study
Δ*ibpAB*Δ*agsA*	SL1344 Δ*ibpA-B* :: Kan Δ*agsA* :: Cm	This study
Δ*ibpAB* Δ*STM1250*Δ*agsA*	SL1344 Δ*ibpA-B* :: Kan Δ*STM1250*Δ*agsA* :: Cm	This study
Δ*rpoE*	SL1344 Δ*rpoE* :: Kan	Humphreys et al., 1999
PLASMIDS		
pKD46	λ-Red helper plasmid carrying γ, β, and *exo* genes under control of P_{araB} promoter. Temperature sensitive replication. AmpR	Datsenko and Wanner, 2000
pKD3	pANT-Sγ derivative, FRT-flanked CmR	Datsenko and Wanner, 2000
pKD4	pANT-Sγ derivative, FRT-flanked KanR	Datsenko and Wanner, 2000

using verification primers (**Supplementary Table S1**). Successful mutants were transduced into a clean WT background by P22 transduction. Triple and quadruple mutants were generated by transducing a Δ*agsA* or Δ*STM1250*Δ*agsA* mutation into the SL1344 Δ*ibpAB* background.

Sequence Alignments

Amino acid sequences were retrieved from NCBI. Sequences were aligned using M-Coffee online alignment tool (Notredame et al., 2000) and shaded figure of multiple-alignments generated using the ExPASy BoxShade online tool (https://embnet.vital-it.ch/software/BOX_form.html).

Temperature Shock Assays

Bacteria were cultured in LB at 37°C for 3 h to mid-log phase. Cultures were then incubated at 10 or 50°C. At each time point, a 1 mL sample was taken, serially diluted in PBS and spotted onto LB agar plates in 10 μL spots. Plates were incubated O/N at 37°C and percentage survival for each time point calculated by comparing to the 0-h non-shocked control.

Determination of Minimum Bactericidal Concentration

Minimum bactericidal concentration (MBC) assays were performed in 96-well plates according to the Clinical and Laboratory Standards Institute guidelines. Briefly, an O/N culture was diluted to OD_{600} 0.08 in LB and 100 μL added to each well. Polymyxin B was added to a final concentration of either 16 or 12 μg/mL and 1:2 serial dilutions performed across the plate. Bacteria were incubated at 37°C O/N, for at least 18 h before reading OD_{600}.

Disc Diffusion and Vancomycin Sensitivity Assays

For disc diffusion assays, an O/N culture was diluted 1:100 in LB and incubated at 37°C with aeration for 1 h. A 0.75% (w/v) agarose top agar was prepared in LB, and 4 mL top agar inoculated with 100 μL culture. Sterile whatman discs were impregnated with 10 μL 10% (w/v) SDS, 1% (v/v) Triton X-100 or 3% (v/v) H_2O_2.

For vancomycin sensitivity assays, O/N cultures were standardized to OD_{600} 1.0, diluted to 1×10^{-7} CFU/mL and 10 μL spots of each dilution plated onto LB agar containing 65 μg/mL vancomycin hydrochloride (Alfa Aesar, Thermo Fisher Scientific).

Gentamicin Protection Assay

RAW264.7 murine macrophages were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 2 mM L-glutamine (Sigma). Cells were grown at 37°C in a 5% CO_2 (v/v) atmosphere.

RAW264.7 were seeded into 24-well plates at a density of 10^6 cells/mL and incubated for 3 h before addition of IFN-γ (1,000 U) for activation. Cells were grown for a further 21 h before infection. Bacteria were cultured on LB agar plates and macrophages infected with a bacterial dose at a multiplicity of infection of 10 to 1 cells. After 1 h of infection, cells were treated with 100 μg/mL gentamicin (Invitrogen) for 1 h. Samples for 2 h time point were washed twice in PBS, lysed with 1 mL of 1% (v/v) Triton X-100 and 0.1% (v/v) SDS in PBS and plated on LB agar for CFU/mL counts. Samples for 24 h time point were incubated in 10 μg/mL gentamicin for a further 22 h then lysed as previous. For inhibition of NADPH oxidase, 250 μM apocynin (acetovanillone, 4-hydroxy-3-methoxyacetophenon, Sigma) was added with the bacterial dose, with each gentamicin treatment and after 8 h for the 24 h infection samples.

Statistical Analysis

Data were analyzed using GraphPad Prism version 8 software and statistical analysis performed by one-way ANOVA or student *t*-test, as stated in the figure legends.

RESULTS

STM1250 and AgsA Have Divergently Evolved Across *Salmonella* Serovars

The sHsps *ibpA* and *ibpB* are highly conserved across all enteric Gram-negative bacteria; however, *STM1250* and *agsA* are unique to *Salmonella* (Skovierova et al., 2006). The *Salmonella* genus can be divided into two distinct species, *S. bongori* and *S. enterica*. While *S. bongori* rarely causes infection in humans and is a commensal of cold-blooded animals (Fookes et al., 2011), serovars of *S. enterica* subspecies *enterica* are responsible for infections in a broad range of mammalian hosts. These hosts include humans, chickens, swine and cattle. We sought to determine whether these *Salmonella* specific genes are conserved across different *Salmonella* serovars. Amino acid sequences obtained from the NCBI database were aligned using M-Coffee (Notredame et al., 2000) and Expasy Boxshade (https://embnet.vital-it.ch/software/BOX_form.html) online tools. Alignments are presented in **Supplementary Figure S1**.

AgsA is highly conserved and shown to be part of the core genome across all serovars tested in this study. Conversely, alignment of the amino acid sequence of *S.* Typhimurium STM1250 showed different levels of homology across the serovars tested. Between 99 and 100% identity was observed between *S.* Typhimurium, the invasive NTS serovar D23580 and the non-invasive serovar *S.* Enteritidis and 92% identity with *S.* Typhi. However, the *S.* Typhimurium STM1250 sequence was not found to be conserved in *S.* Paratyphi, *S.* Choleraesuis, *S.* Newport, or *S.* Dublin. As such, we cannot observe any patterns of identity that enable *STM1250* to be associated with a specific invasive/non-invasive disease type, although it appears that *STM1250* is more highly conserved in serovars which cause disease in humans rather than other mammals. Interestingly, *S. bongori STM1250* is annotated as a pseudogene suggesting that functional *STM1250* arose after divergence of the *S. enterica* species. These data point toward a role for *STM1250* in *S. enterica* mammalian infection.

Salmonella Survival at 50 and 10°C Requires IbpAB and AgsA but Not STM1250

The IbpAB sHsps have been shown to bind to aggregated proteins in *E. coli* following heat shock (Laskowska et al., 1996); however, they are not essential for survival at 50°C (Thomas and Baneyx, 1998). We aimed to determine whether this is also true for *Salmonella ibpAB,* and furthermore, determine whether survival of an *STM1250agsA* double mutant is affected at high temperatures. STM1250 is not currently described as a sHsp; however, Hsu-Ming et al. (2012) identified that *STM1250* is upregulated during recovery from heat shock at 55°C.

Bacteria were grown to mid-log phase at 37°C and then transferred to a water bath at 50°C. In agreement with previous studies in *E. coli* (Thomas and Baneyx, 1998), the Δ*ibpAB* double mutant was unaffected at 50°C. Furthermore, survival of the Δ*STM1250*Δ*agsA* double mutant was also equivalent to WT (**Figure 1A**). As a result, we constructed triple (Δ*ibpAB*Δ*agsA*) and quadruple (Δ*ibpAB*Δ*STM1250*Δ*agsA*) deletion mutants to test our proposed hypothesis of functional redundancy. Survival of the triple deletion mutant in *Salmonella* was compromised after heat shock (Tomoyasu et al., 2003). Our data agreed with this finding; survival of the triple mutant was significantly reduced compared to WT at 50°C (**Figure 1B**). Survival of the quadruple mutant was not significantly different to the triple mutant (**Figure 1B**), suggesting there is no additive contribution of *STM1250* to surviving heat shock at this temperature.

The ability of the quadruple deletion mutant to grow at high temperature, rather than survive shock, was also tested. We observed that at 37 and 47°C, the mutant grew equally to WT (**Figures 1C,D**) suggesting that the reduced survival phenotype following heat shock, resulting from loss of *ibpAB* and *agsA*, is limited to perturbations induced by sudden heat shock or at least sudden changes in temperature. Considering this, we also investigated the ability of the triple and quadruple deletion mutants to survive cold shock at 10°C. Cold shock is one of the major inducers of *rpoE* (Miticka et al., 2003) and incubation at low temperatures is known to disrupt bacterial membrane fluidity (Barria et al., 2013). Over the course of 2 h, growth of all strains was observed (**Figure 1E**). However, there was a significant increase, particularly after 30 min, in WT growth compared to the triple and quadruple mutant (**Figure 1F**), but again no significant difference between the triple and quadruple mutant, indicating no role for STM1250 in responding to temperature-based damage.

Deletion of the σ^E-Regulated *ibpAB*, *STM1250* and *agsA* Does Not Impact Overall OM Integrity in *Salmonella*

The proteins in this study are predicted to be cytoplasmic, although *ibpA* has been detected in the OM and S-fraction of *E. coli* following heat shock (Kuczynska-Wisnik et al., 2002). Moreover, due to their regulation by σ^E, we hypothesized that *ibpA, ibpB, STM1250,* and *agsA* may be involved in maintaining OM integrity. We tested this by measuring the survival of WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* after exposure to 65 μg/mL vancomycin, 1% Triton X-100 and 10 % SDS, compounds known to be excluded by an intact OM. Both WT and the quadruple mutant were resistant to Triton X-100 and SDS in disc diffusion assays (data not shown). Vancomycin is unable to traverse the Gram-negative bacterial membrane; however, the deletion of σ^E-regulated chaperones (Δ*fkpA*Δ*ppiAD*Δ*surA*) has been shown to increase sensitivity in *E. coli* via loss of maintenance of the cell envelope (Justice et al., 2005). No significant difference was observed in the ability of Δ*ibpAB*Δ*STM1250*Δ*agsA* to grow on LB agar containing vancomycin; however, survival of Δ*fkpA*Δ*ppiAD*Δ*surA* was significantly reduced (**Figure 2**). These data suggest that no overall alteration to the permeability or integrity of the OM is caused by the loss of the IbpAB, STM1250, and AgsA stress responsive proteins.

Lag Phase of the Δ*ibpAB*Δ*STM1250*Δ*agsA* Mutant Is Extended by H_2O_2 Oxidative Stress

Heat shock proteins are known to overlap in function with hydrogen peroxide (H_2O_2) induced proteins (Morgan et al., 1986) and the σ^E regulon is important for *Salmonella* resistance

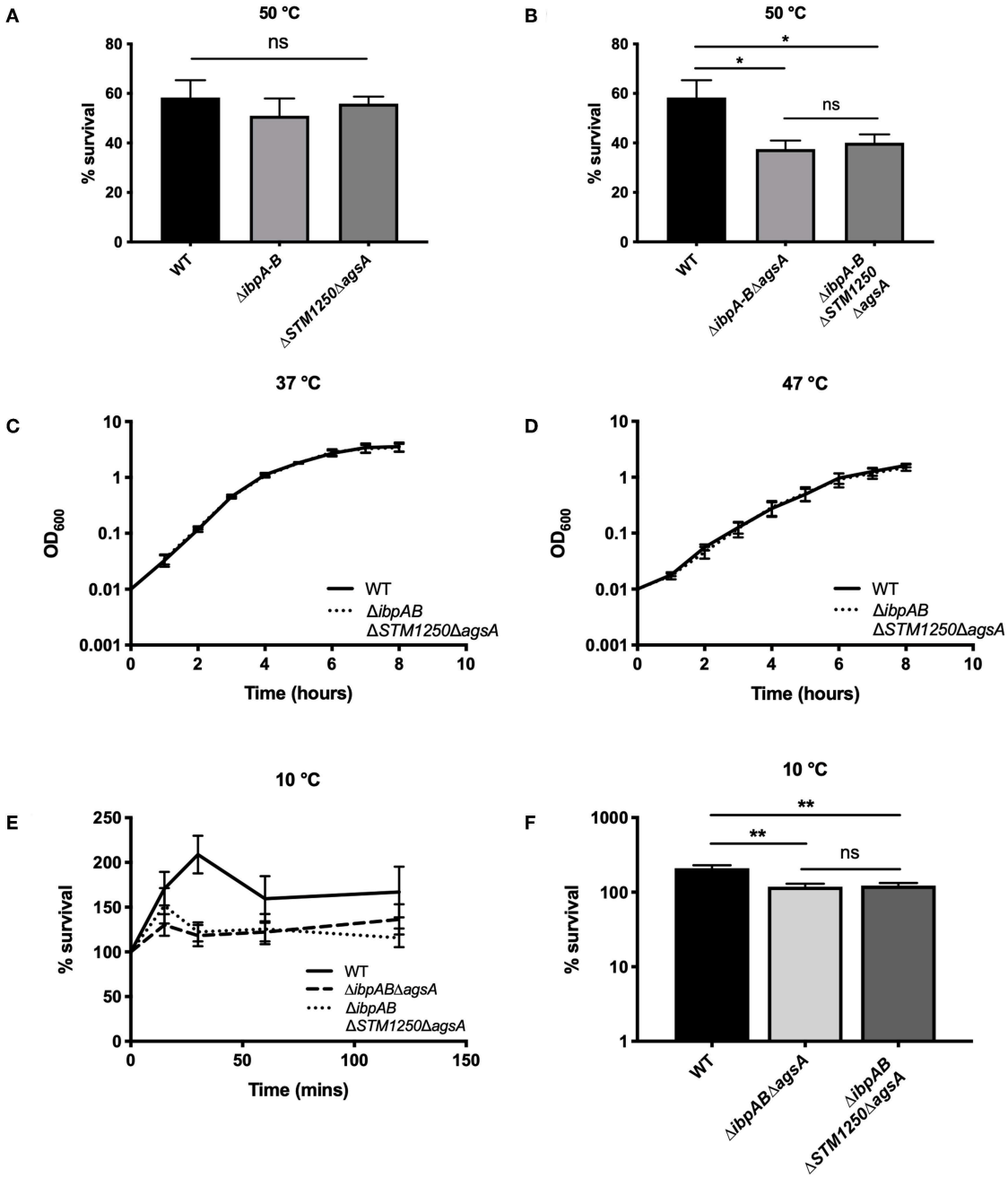

FIGURE 1 | *ibpAB* and *agsA* contribute to *S.* Typhimurium survival following sudden changes in temperature. **(A)** Percentage survival following a 4 h incubation at 50°C. Bacteria were grown for 3 h at 37°C before incubation at 50°C. Survival of the double mutants are equal to WT. **(B)** Percentage survival following a 4 h incubation at 50°C. Bacteria were grown for 3 h at 37°C before incubation at 50°C. Survival of the triple and quadruple mutant were significantly reduced compared to WT. **(C)** Growth of WT and the quadruple mutant are equivalent at 37°C and **(D)** 47°C. **(E)** Percentage survival over a 2 h incubation at 10°C. Bacteria were grown for 2 h at 37°C before incubation at 10°C. **(F)** Survival of the triple and quadruple mutant is significantly reduced after 30 min at 10°C. Data are the means of three separate experiments performed in duplicate. Error bars represent SEM. Data analyzed by one-way ANOVA with Tukey's multiple comparisons test, $^{*}p < 0.05$ and $^{**}p < 0.005$.

to oxidative stress (Testerman et al., 2002). Both *ibpA* and *ibpB* are strongly induced by H_2O_2 in *E. coli* (Zheng et al., 2001). However, to date, there is no specific link between sHsps and tolerance to oxidative stress in *Salmonella.* We aimed to determine whether the proteins of interest in this study are involved in resistance to oxidative stress using H_2O_2.

Bacteria were grown in a plate reader in the presence of 6 mM H_2O_2 and the OD_{600} read hourly. The quadruple mutant

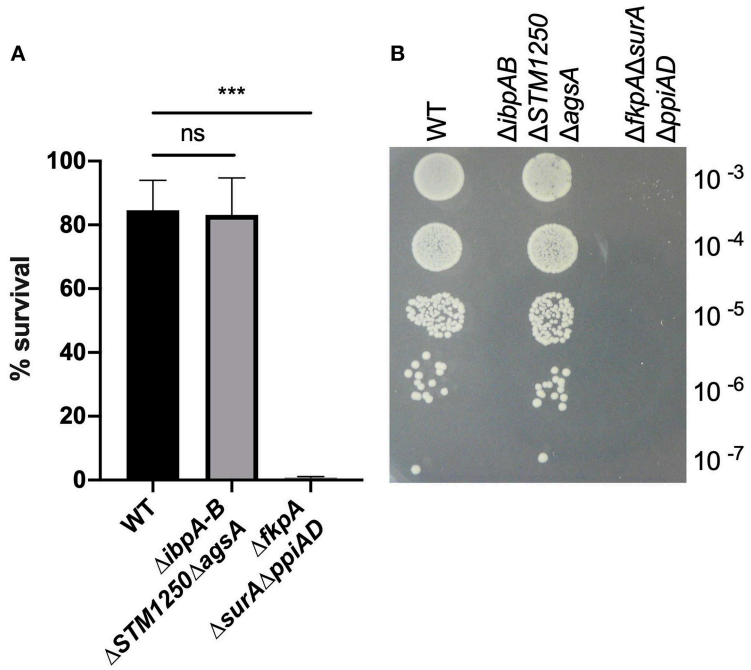

FIGURE 2 | The quadruple deletion mutant is not sensitive to 65 μg/mL vancomycin. O/N cultures were serially diluted in PBS and 10 μL spotted onto LB agar either with or without 65 μg/mL vancomycin. **(A)** Colonies grown on vancomycin expressed as a percentage of colonies on LB agar. **(B)** Representative image of growth on vancomycin. Data are the means of three separate experiments performed in duplicate. Error bars show SEM. Data analyzed by one-way ANOVA with Tukey's multiple comparisons test, $^{***}p < 0.0005$.

showed the greatest sensitivity to H_2O_2 compared to WT (**Figure 3A** and **Supplementary Figure S2**) with an extended lag phase, indicative of H_2O_2-induced stress during bacterial growth (Watson and Schubert, 1969). This phenotype was also observed for the Δ*rpoE* control strain and the growth rate constant of the quadruple mutant and Δ*rpoE* control was significantly reduced in the first 2 h compared to WT (**Figure 3C**). Growth of all strains was restored to non-treated levels by the addition of the hydrogen peroxide scavenger, bovine catalase (**Figures 3B,C**).

Sensitivity to H_2O_2 was also tested by disc diffusion assay. In agreement with growth curve findings, the quadruple mutant was more sensitive to 3% H_2O_2 compared to WT with a significant difference in the area of inhibition (**Figure 4**).

Previously, *ibpAB* have been linked to oxidative stress resistance in *E. coli* (Kitagawa et al., 2000, 2002) and an *E. coli ibpAB* mutant is more sensitive to $CuCl_2$-induced oxidative stress (Matuszewska et al., 2008). Moreover, the *ibpA* promoter is more active in the presence of potassium tellurite (K_2TeO_3), a further inducer of bacterial oxidative stress (Pérez et al., 2007). Since these studies were performed on *E. coli,* we sought to determine whether the *Salmonella* Δ*ibpAB* mutant and quadruple deletion strain in this study were sensitive to $CuCl_2$ or K_2TeO_3. In addition, in order to further determine whether the increased sensitivity to oxidative stress of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is limited to H_2O_2, bacteria were also challenged with the oxidizing agent methyl viologen (paraquat). *Salmonella* was grown in the presence of 30 mM paraquat (**Figure 5A**), 3 mM $CuCl_2$ (**Figure 5B**), or 0.5 μM K_2TeO_3 (**Figure 5C**) for 24 h. We observed no differences in sensitivity or initial growth rate constant (μ h^{-1}) between WT or the quadruple mutant for these alternative bio-oxidants.

Additionally, in a previous study, *agsA* was found to be upregulated in the presence of 1 mM indole (Nikaido et al., 2012); however, no specific contribution of *agsA* to tolerance of increased levels of indole has been shown. Indole is produced by bacteria from tryptophan by tryptophanase (TnaA). *Salmonella* lacks the TnaA enzyme and is, therefore, unable to produce indole. Interestingly, Garbe et al. (2000) showed that exposure to indole induced expression of antioxidant proteins in *E. coli*. The proposed mechanism described that the lipophile indole dissolves within membrane lipids, affecting membrane integrity and enabling quinones to interact with oxygen, subsequently leading to the generation of superoxide (Garbe et al., 2000). In the current study, exposure of WT and mutant *Salmonella* to 1 mM indole did not significantly affect the growth rate constant or overall growth over 24 h (**Figure 5D**).

The Quadruple Mutant Is More Sensitive to Polymyxin B With a Lower MBC Range Than WT *S.* Typhimurium

Polymyxin B is a cationic antimicrobial peptide and binds to the negative charge of lipopolysaccharide (LPS) on the

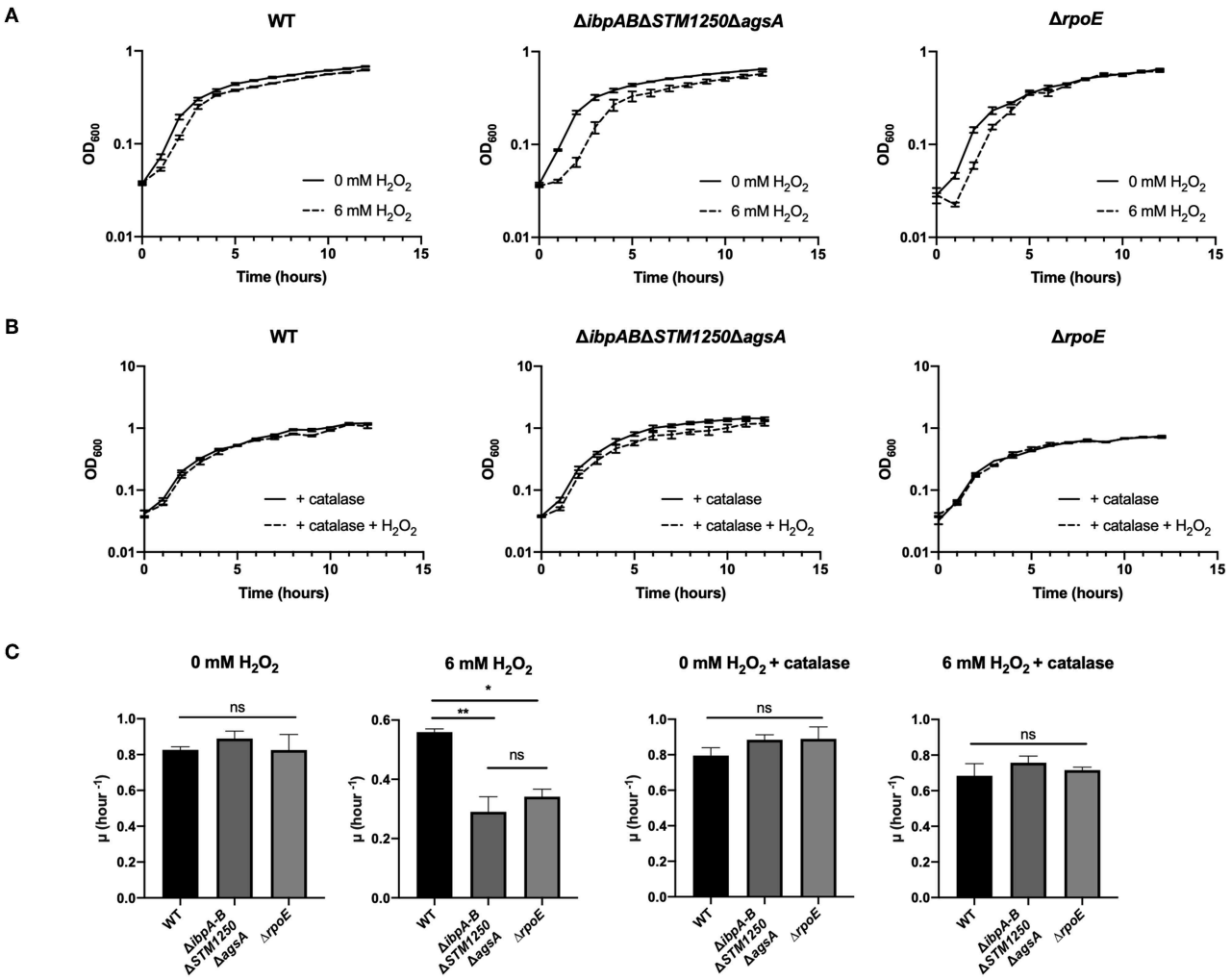

FIGURE 3 | The quadruple deletion mutant is more sensitive to 6 mM H_2O_2 compared to WT. Bacteria were grown in a plate reader for 12 h at 37°C. **(A)** Bacteria grown either with or without 6 mM H_2O_2. OD_{600} readings taken hourly. **(B)** Bacteria grown in the presence of 1,000–4,000 U/mL bovine catalase and either with or without 6 mM H_2O_2. OD_{600} readings taken hourly. **(C)** Initial growth rate constants for bacterial growth in the presence/absence of 6 mM H_2O_2 and/or 1,000–4,000 U/mL bovine catalase. Data are the means of three separate experiments performed in duplicate. Error bars show SEM. Data analyzed by one-way ANOVA with Tukey's multiple comparisons test, ns $p > 0.05$, $^*p < 0.05$ and $^{**}p < 0.005$ vs. WT.

bacterial cell surface, causing disruption of the outer membrane. During host infection, cationic antimicrobial peptides (cAMPs) are released by intestinal epithelial cells (IECs) as part of the host immune defense (reviewed in Muniz et al., 2012). As a result, resistance to cAMPs is highly important to enteric bacteria.

Polymyxin B MBC assays were performed with WT and mutant strains. After an O/N incubation in a range of polymyxin B concentrations, the quadruple mutant and Δ*rpoE* control had a lower MBC range than WT (**Figure 6A** and **Supplementary Table S2**). In addition, overall growth of the quadruple mutant and Δ*rpoE* was limited significantly at 2 μg/mL compared to WT (**Figure 6B**) while no significant differences were observed between WT and the remaining mutants tested.

IbpAB, STM1250, and AgsA Are Critically Important for *Salmonella* Intracellular Survival

Resistance to oxidative stress is an important contributor to the ability of *Salmonella* to cause infection. σ^E-regulated genes are important for *Salmonella* intracellular survival (Humphreys et al., 1999). As indicated in **Figure 1**, increased expression of *ibpAB*, *STM1250,* and *agsA* has been previously observed during intracellular macrophage infection and further studies have indicated their upregulation during intracellular survival (Eriksson et al., 2003).

In this study, IFN-γ activated RAW264.7 macrophages were infected with bacteria at an MOI of 10:1 and the number of intracellular CFU/mL determined at 2 and 24 h post-infection.

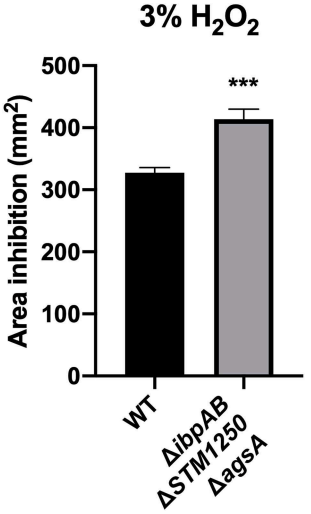

FIGURE 4 | The quadruple mutant is more sensitive to 3% H_2O_2. Bacteria were cultured in top agar on LB and sterile discs impregnated with 3% H_2O_2. Data are the means of three separate experiments performed in triplicate. Error bars show SEM. Data analyzed by Student's *t*-test, $^{***}p < 0.0005$.

Indeed, survival of the quadruple mutant was reduced compared to WT after 2 and 24 h of infection with significantly lower intracellular CFUs/mL (**Figure 7A**). Interestingly, after 2 h of infection, intracellular CFU/mL of Δ*ibpAB*Δ*STM1250*Δ*agsA* and Δ*rpoE* were also significantly reduced compared to Δ*ibpAB,* indicating a functional role for STM1250/AgsA in compensating for loss of IbpAB.

Macrophages are known to release ROS as a bactericidal mechanism and, therefore, our H_2O_2-sensitive quadruple mutant strain may be more susceptible to ROS killing within the macrophage. To investigate this, the macrophage NADPH oxidase was inhibited with 250 μM apocynin. Following a 2 and 24 h infection, the survival of the quadruple mutant was restored to WT levels (**Figure 7B**).

DISCUSSION

Detection of stresses and subsequent responses, to nullify and repair stress-induced damage, are critical to the ability of *S.* Typhimurium to cause infection. The envelope stress response regulator, σ^E, required for *Salmonella* survival in macrophages and a mouse typhoid model (Humphreys et al., 1999), controls expression of a wide array of genes (Skovierova et al., 2006), which must, therefore, include systems which contribute to infection. However, many of the σ^E-regulated genes of *Salmonella* are of unknown function. These genes may be of yet unrecognized importance to bacterial stress survival and pathogenesis, particularly where functional overlap exists, a common emerging theme in stress response biology. In this study, we aimed to investigate the roles of the σ^E- and σ^H-regulated IbpA, IbpB, and AgsA and σ^E-regulated STM1250 in *S.* Typhimurium. Due to shared regulation and sequence identity, we hypothesized that functional overlap occurs between these proteins. We predicted that limited understanding of the roles of these proteins in *Salmonella* exists, because when single deletions are made no phenotypes are observed. To counter this, in this study, we have generated mutants incorporating deletions in up to four of the genes of interest to enable a better understanding of their roles during *Salmonella* stress survival and infection.

IbpA has been implicated with heat shock survival in other species, with a *Pseudomonas putida ibpA* mutant presenting a growth defect at 40°C (Krajewski et al., 2013). However, our *S.* Typhimurium Δ*ibpAB* mutant did not show reduced survival at 50°C, in agreement with previous studies in *E. coli* (Thomas and Baneyx, 1998). Additionally, it has been shown previously that a *Salmonella* Δ*agsA* mutant is unaffected by heat shock at 70°C (Tomoyasu et al., 2003) and in this study we found that an Δ*STM1250*Δ*agsA* double mutant survival is equal to WT and Δ*ibpAB* at 50°C. However, when a triple mutant, Δ*ibpAB*Δ*agsA* was subjected to heat shock, survival rate compared to WT was significantly reduced. In order to further investigate proposed functional redundancy, we also subjected a Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant to heat shock and observed a significant decrease in survival compared to WT but no further reduction compared to the triple mutant. These data suggested that STM1250 does not function as a sHsp in *Salmonella*. This finding can largely be explained by the fact that STM1250 does not possess the characteristic α-crystallin domain seen in sHsps. We observed that the quadruple mutant grew equally as well as WT at elevated temperature and, therefore, proposed that reduced survival was only a result of sudden changes in temperature. Indeed, following cold shock at 10°C, the triple and quadruple mutant showed significantly reduced growth compared to WT. Cold shock has been shown to induce phospholipid phase separation and subsequently decrease membrane fluidity (Barria et al., 2013). Therefore, the proteins in this study may function to maintain integrity and fluidity during extreme temperature changes, although, the precise mechanism behind this in *Salmonella* remains unclear.

Due to previous studies linking sHsps to oxidative stress resistance and the σ^E regulon of importance for survival against oxidative stress, we proposed that functional overlap may occur in the presence of oxidizing agents. We observed a reduced rate of growth of our Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant during the first 2 h in the presence of hydrogen peroxide. *ibpAB* have been linked to oxidative stress survival in a number of species including *E. coli* (Kitagawa et al., 2002) and *Yersinia pestis* (Pradel et al., 2014). Interestingly, van der Heijden et al. (2016) showed reduced survival of *Salmonella* Δ*ibpA* following a 2 h challenge with H_2O_2. We did not observe any differences between WT and *ibpAB* in this study; these conflicting findings may be due to the parent strain used. The previous study utilized an $HpxF^-$ catalase and peroxidase negative background strain and this would certainly affect the response to H_2O_2 in WT. IbpA is proposed to bind to OmpC in *E. coli* (Butland et al., 2005). Moreover, an increase in H_2O_2 influx in an *S.* Typhimurium *ibpA* mutant has been shown and this was dependent on the OmpC porin (van der Heijden et al., 2016). In our study, deletion of *ibpA*, in conjunction with *ibpB*, *STM1250,* and *agsA*, may result in a higher influx of H_2O_2, which coupled to deletion of *ibpB,*

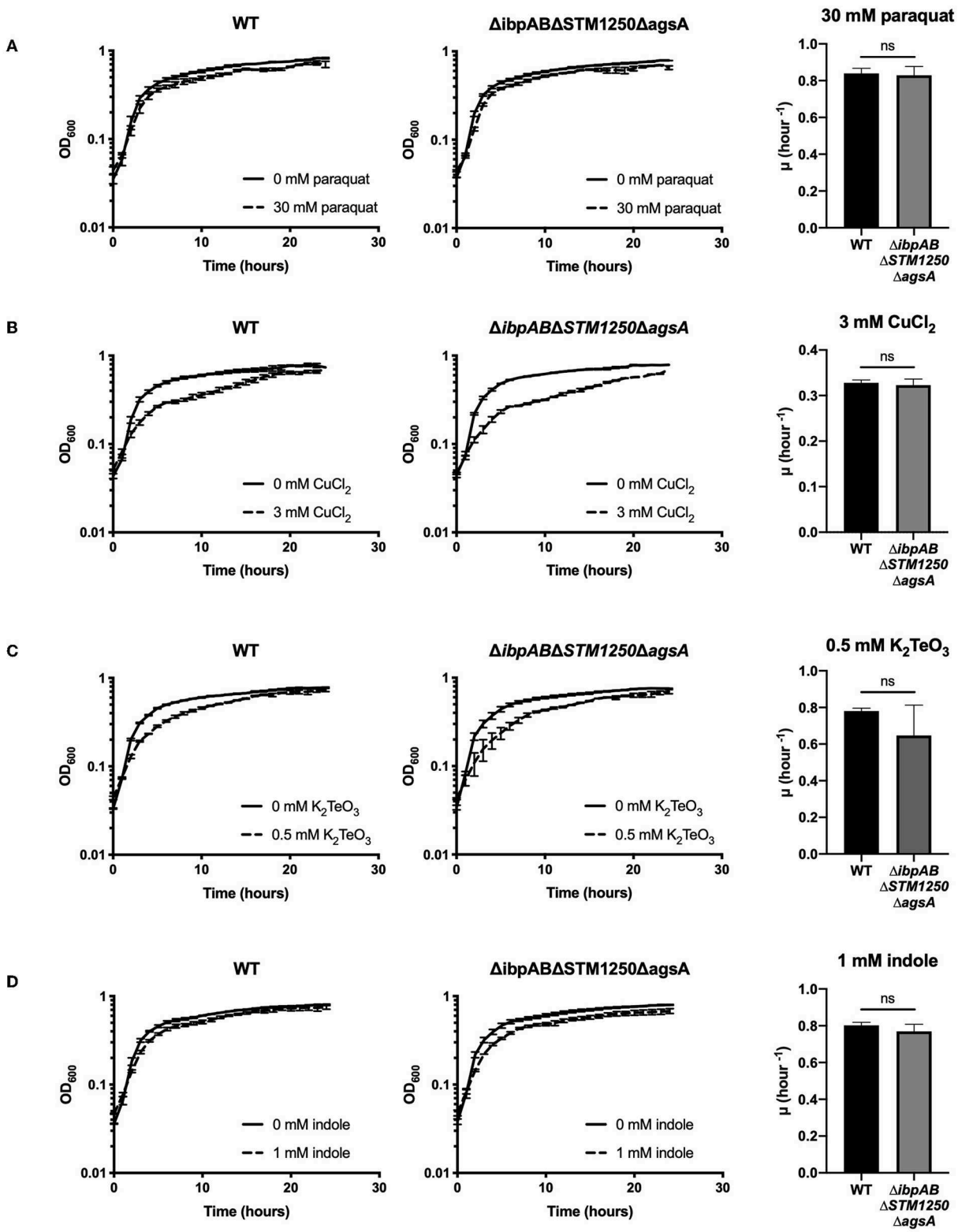

FIGURE 5 | Deletion of ibpAB, STM1250, and agsA has no effect on overall growth or initial growth rate (μ hour^{-1}) in the presence of 30 mM paraquat, 3 mM CuCl2, 0.5 μM K2TeO3, or 1 mM indole. Bacteria were grown in a plate reader at 37°C. Graphs show growth over 24 h or initial growth rate for WT and the quadruple mutant in the presence of **(A)** 30 mM paraquat (methyl viologen) **(B)** 3 mM CuCl2 **(C)** 0.5 μM K2TeO3 or **(D)** 1 mM indole. Data are the means of three separate experiments performed in duplicate. Error bars represent SEM. Data analyzed by one- way ANOVA with Tukey's multiple comparisons test, ns $p > 0.05$.

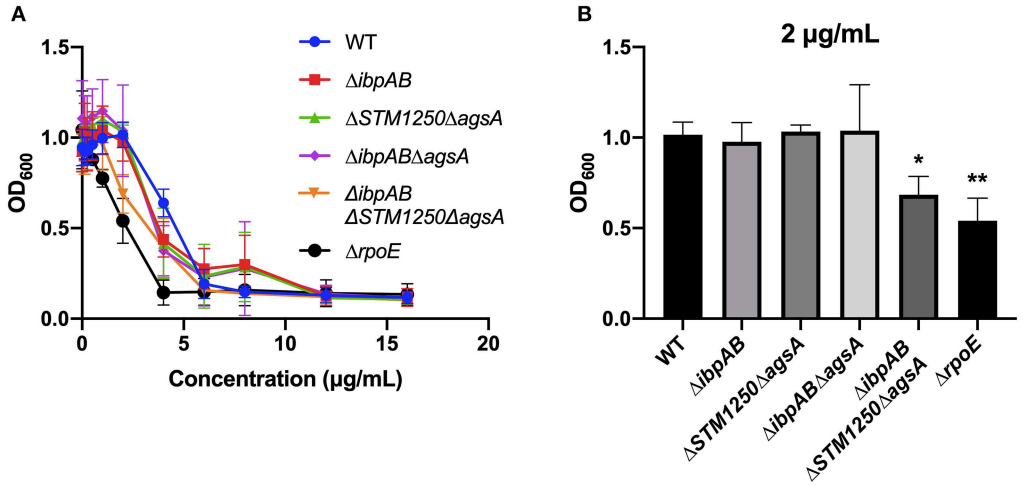

FIGURE 6 | Deletion of *ibpAB, STM1250,* and *agsA* increases sensitivity of *Salmonella* to polymyxin B. **(A)** MBC assays were performed in 96-well plates containing different concentrations of polymyxin B. **(B)** A 2 μg/mL concentration of polymyxin B significantly limited the overall growth of Δ*ibpAB*Δ*STM1250*Δ*agsA* and an Δ*rpoE* control. Data are the means of three separate experiments performed in quadruplicate. Data analyzed by one-way ANOVA with Dunnett's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.005$ vs. WT.

STM1250, and *agsA* could explain the reduced growth rate of the quadruple mutant. Future work is required to elucidate the mechanism behind the contribution of STM1250 and AgsA to tolerance of H_2O_2 stress and it would be interesting to investigate *STM1250* expression in the triple mutant. Intracellular ROS and protein oxidation levels could also be measured for the different mutants to investigate H_2O_2 uptake and the downstream effects in the absence of the stress response proteins. Interestingly, exposure to alternative inducers of oxidative stress, $CuCl_2$, K_2TeO_3, paraquat, and indole, did not affect the growth of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant. These findings suggest that the sensitivity of the quadruple mutant is specifically limited to oxidative stress induced by H_2O_2 in the growth conditions tested in this study.

The mutants in this study were not sensitive to the OM-targeting compounds vancomycin, SDS or Triton X-100 detergents. The cAMP polymyxin B can also be used to investigate OM integrity as it binds to the cell surface LPS and subsequently disrupts the OM. σ^E and the two-component system PhoPQ regulate genes needed for resistance to cAMPs (Matamouros and Miller, 2015). IbpAB have been shown to associate with the OM (Laskowska et al., 1996) and a PhoP motif has been identified upstream of *STM1250* in *S.* Typhimurium (Monsieurs et al., 2005). We investigated whether the mutants generated in this study were susceptible to polymyxin B. MBC analyses identified that the quadruple mutant has a polymyxin B MBC in the range of 2–4 μg/mL while the MBC for the WT is between 1 and 2 μg/mL. Subsequently, overall growth of the quadruple mutant was significantly lower than WT in the presence of 2 μg/mL polymyxin B. Previous studies have shown that the LPS profile of an Δ*rpoE* mutant does not differ in comparison to the WT (Humphreys et al., 1999) and further work will be required to determine whether this is also the case for the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant in the current study. Interestingly, previous studies in *Acinetobacter baumannii* have identified that cAMPs such as polymyxin B can induce the production of intracellular hydroxyl radicals ($OH^{\bullet}$) (Sampson et al., 2012). Further work will be required to determine the levels of intracellular $OH^{\bullet}$ in the WT and mutant strains in this study, following cAMP treatment, to investigate whether the limited growth in the presence of polymyxin B is linked to the oxidative stress phenotypes we have reported.

Multiple studies have indicated a role for *ibpAB, STM1250,* and *agsA* during intracellular survival. Previously published expression data highlighted high expression of *ibpAB* and *STM1250* within macrophages (Canals et al., 2019) and *agsA* has also been observed to be upregulated over the course of a 21 h macrophage infection (Eriksson et al., 2003). Furthermore, overexpression of *ibpAB* in a non-pathogenic strain of *E. coli* was shown to increase survival of this strain within macrophages (Goeser et al., 2015). Macrophages are sites of oxidative and nitrosative stress. The NADPH-dependent oxidase Phox produces superoxide while the inducible nitric oxide synthase (iNOS) is responsible for the production of nitric oxide. In this study, survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly reduced after 2 and 24 h of infection. Additionally, the intracellular CFU/mL of the quadruple mutant was also significantly lower than that of the Δ*ibpAB* mutant after 2 h. This is indicative or functional redundancy between these two stress response operons and is a possible reason for the lack of data on these genes via global mutagenesis studies. Together, with the slow growth in the presence of H_2O_2, we hypothesized that the reduction in survival could be, in part, due to macrophage induced oxidative stress. Apocynin was used to inhibit NADPH oxidase activity, and led to loss of the intracellular survival phenotype observed for the quadruple mutant in untreated cells. This chemical complementation indicates that IbpAB, STM1250, and AgsA function to protect *Salmonella* against macrophage

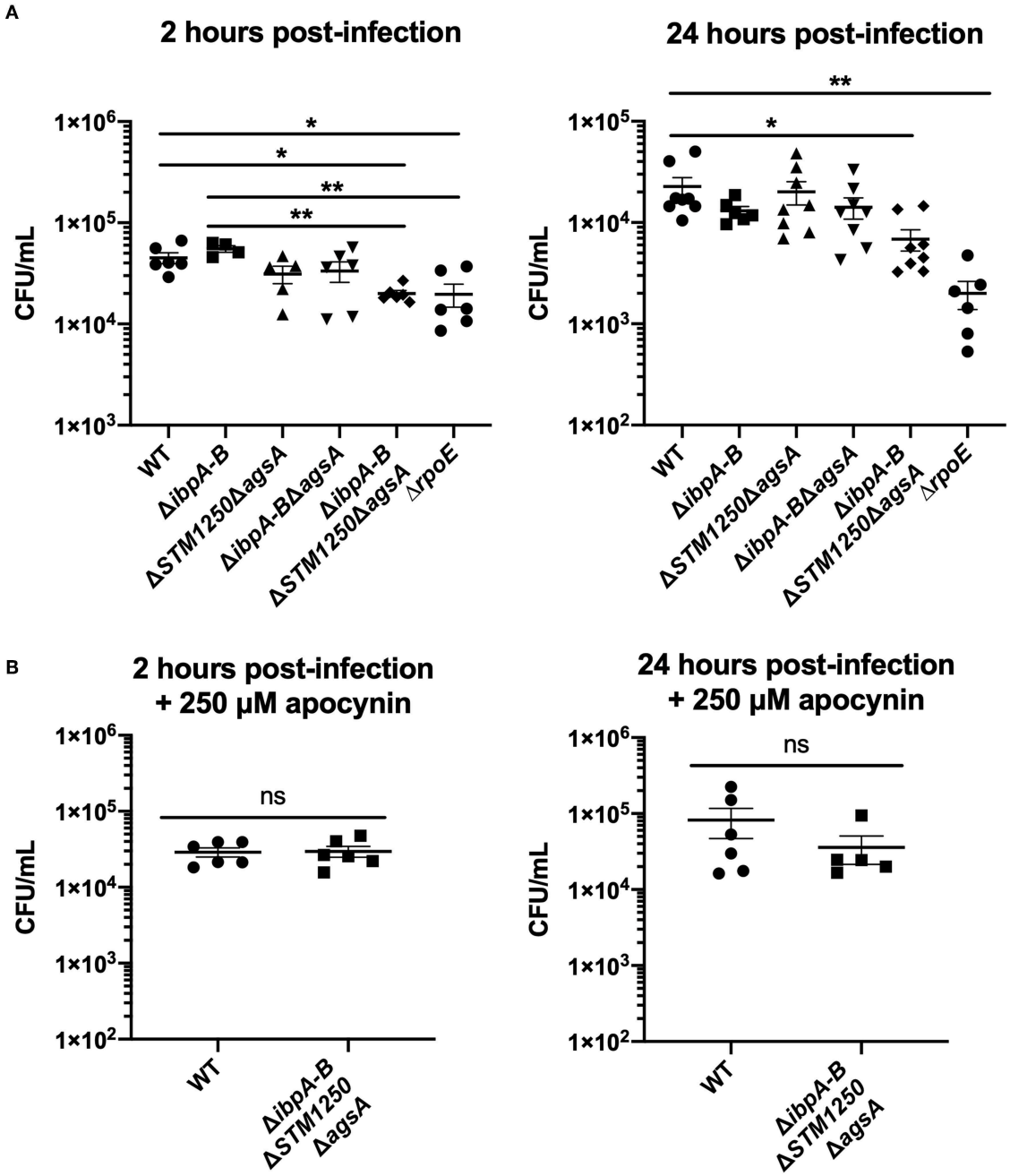

FIGURE 7 | The quadruple deletion mutant is attenuated in IFN-γ activated RAW264.7 macrophages after 2 and 24 h of infection but can be rescued by inhibition of the macrophage NADPH oxidase. **(A)** Macrophages infected for 2 or 24 h, data represents intracellular CFU/mL at each time point. **(B)** IFN-γ activated macrophages in the presence of apocynin, infected for 2 and 24 h. Data are the means of three separate experiments performed in duplicate. Error bars show SEM. Data analyzed by **(A)** one-way ANOVA with Tukey's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.005$, **(B)** Student's *t*-test, ns $p > 0.05$.

induced oxidative stress. In addition to an involvement in protection against ROS, all genes of interest in this study have been previously shown to be upregulated by nitric oxide exposure (Richardson et al., 2011). Further work is required to determine whether our quadruple mutant is affected by exposure to nitrosative stress as well as oxidative stress.

The genomic location of *STM1250* and *agsA* may provide insight into their roles in *S.* Typhimurium. Upstream of *STM1250* is *Salmonella* Pathogenicity Island 11 (SPI-11) (*STM1239, envF, msgA, envE, cspH, pagD, pagC, and pliC*). These proteins are highly important to virulence, with PagC required for serum resistance in *S.* Choleraesuis (Nishio et al., 2005) and PagD

and MsgA important for intracellular survival (Gunn et al., 1995). Furthermore, genes within SPI-11 are regulated by σ^E and PhoPQ ESRs. Interestingly, *STM1250* is also regulated by Fis, a global regulator of virulence genes (Wang et al., 2013). The regulation of *STM1250* and *agsA,* as well as their proximity to SPI-11, points toward their role in infection. Interestingly, Canals et al. (2019) identified upregulation of *STM1250* in macrophages and, furthermore, observed a 4-fold increase in expression of *STM1250* in the invasive NTS (iNTS) serovar ST313. We suggested that STM1250 could not be associated with disease type based on our sequence homology analysis yet these data do suggest a role for STM1250 for iNTS serovars. This will require further investigation using a ST313 *STM1250* deletion strain.

In summary, this paper indicates new roles for sHsps, aside from resistance to heat shock stress. Overall, we demonstrate that these sHsps should instead be described as stress responsive proteins. We have demonstrated functional overlap between IbpA, IbpB, STM1250, and AgsA and for the first time have identified a role for these proteins during *Salmonella* intracellular survival. Ongoing work will include further study of STM1250, including biochemical characterization to better elucidate the mechanism by which these proteins function to tolerate oxidative stress and intracellular survival. Ultimately, an improved understanding of the role of stress response proteins during infection and survival of environmental stress, with particular emphasis on those with functional overlap, will identify whether these proteins are novel therapeutic targets.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

CH and GR designed the study. CH and EP performed the experimental work and CH analyzed the data. CH and GR wrote the manuscript. All authors read and reviewed the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00263/full#supplementary-material

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Resuscitation of Dormant "Non-culturable" *Mycobacterium tuberculosis* Is Characterized by Immediate Transcriptional Burst

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Under unfavorable conditions such as host immune responses and environmental stresses, human pathogen *Mycobacterium tuberculosis* may acquire the dormancy phenotype characterized by "non-culturability" and a substantial decrease of metabolic activity and global transcription rates. Here, we found that the transition of *M. tuberculosis* from the dormant "non-culturable" (NC) cells to fully replicating population *in vitro* occurred not earlier than 7 days after the start of the resuscitation process, with predominant resuscitation over this time interval evidenced by shortening apparent generation time up to 2.8 h at the beginning of resuscitation. The early resuscitation phase was characterized by constant, albeit low, incorporation of radioactive uracil, indicating *de novo* transcription immediately after the removal of the stress factor, which resulted in significant changes of the *M. tuberculosis* transcriptional profile already after the first 24 h of resuscitation. This early response included transcriptional upregulation of genes encoding enzymes of fatty acid synthase system type I (FASI) and type II (FASII) responsible for fatty acid/mycolic acid biosynthesis, and regulatory genes, including *whiB6* encoding a redox-sensing transcription factor. The second resuscitation phase took place 4 days after the resuscitation onset, i.e., still before the start of active cell division, and included activation of central metabolism genes encoding NADH dehydrogenases, ATP-synthases, and ribosomal proteins. Our results demonstrate, for the first time, that the resuscitation of dormant NC *M. tuberculosis* is characterized by immediate activation of *de novo* transcription followed by the upregulation of genes controlling key metabolic pathways and then, cell multiplication.

Keywords: resuscitation, *M. tuberculosis*, non-culturability, dormancy, transcriptional burst, small non-coding RNAs

INTRODUCTION

One-third of the human population is estimated to be latently infected with *Mycobacterium tuberculosis*, a causative agent of tuberculosis (TB), without overt disease symptoms (World Health Organization, 2018). Being under immune control of the host, latent TB infection presents a constant risk of disease reactivation, which constitutes 10% over a lifetime in the general

population and 10% per year in immunocompromised patients (Pawlowski et al., 2012; O'Garra et al., 2013; Scriba et al., 2016; Veatch and Kaushal, 2018). The clinical status of latent TB is traditionally associated with the transition of *M. tuberculosis* to a dormant state in response to non-optimal growth conditions *in vivo* due to activation of the host immune response (Zhang, 2004). Dormancy is a specific physiological state characterized by significant cessation of metabolic activity and growth, whereas resuscitation from dormancy is a process of restoring cell activity followed by bacterial multiplication, which in case of *M. tuberculosis* can lead to disease progression. Therefore, understanding the mechanisms underlying *M. tuberculosis* resuscitation may provide clues to the development of control measures in order to inhibit reactivation of latent infection, reduce disease severity in infected patients, and prevent pathogen transmission in the population.

Although there are several *in vivo* models of *M. tuberculosis* latency and reactivation, including mice (McCune and Tompsett, 1956; Scanga et al., 1999; Radaeva et al., 2005), guinea pigs (Ordway et al., 2010), and rabbits (Manabe et al., 2008; Subbian et al., 2012), they do not reproduce disease pathology and immune control observed in humans. The cellular composition of murine TB granulomas is similar to that of human granulomas, with the exception of the absence of multinucleated giant cells, however, mouse TB lesions lack tissue necrosis, which is the pathological hallmark of human TB granulomas (Dutta and Karakousis, 2014). Granulomas in guinea pigs and rabbits more closely approximate the human granuloma; while TB granulomas in standard mouse models are not hypoxic, tissue hypoxia is observed in those of guinea pigs and rabbits (Dutta and Karakousis, 2014). TB models in non-human primates most closely resemble human disease (Kaushal et al., 2012; Peña and Ho, 2015, 2016), however, they are expensive and time-consuming. Furthermore, the existing *in vivo* TB reactivation models are aimed to study host immune response after the reactivation of infection rather than the mechanisms of bacterial resuscitation (Mehra et al., 2011; Foreman et al., 2016).

In vitro modeling is a cost-effective strategy, which enables investigation of *M. tuberculosis* resuscitation process in detail and identify principal molecular players, providing necessary clues for further *in vivo* experiments (Veatch and Kaushal, 2018). However, despite extensive attempts for modeling of *M. tuberculosis* dormancy *in vitro*, including the well-known Wayne model of non-replicating state under hypoxic conditions (Wayne, 1976; Wayne and Hayes, 1996), dormant bacilli obtained in the majority of *in vitro* models were fully culturable, whereas bacteria isolated from *in vivo* models of latent TB are "non-culturable" (NC) (Dhillon et al., 2004; Biketov et al., 2007). "Non-culturability" is a specific term for cells that are temporary unable to grow on standard solid media and become culturable only after resuscitation (Oliver, 2005). Therefore, *in vitro* models of *M. tuberculosis* dormancy should reproduce the phenomenon of "non-culturability" and more adequately imitate latent TB in humans and animals. Recently, we have developed an *in vitro* model of *M. tuberculosis* dormancy in K^+-limiting conditions, in which the bacteria acquired the NC phenotype, high tolerance to rifampicin and isoniazid, and significant (up to 1×10^7 cells/ml) recovery potential (Salina E. et al., 2014; Ignatov et al., 2015). Using the model of dormancy under K^+ deficiency, we found that the adaptation of *M. tuberculosis* to the NC state was characterized by global transcriptional repression and maintenance of a pool of stable/stored transcripts in dormant cells (Ignatov et al., 2015).

Still, little is known about the resuscitation of dormant *M. tuberculosis* and the mechanisms underlying the transition from the NC to multiplication state. Most studies on *M. tuberculosis* resuscitation modeled reactivation by re-aeration of dormant cells obtained under hypoxia, thus studying only one stress parameter—oxygen deficiency (Du et al., 2016; Iona et al., 2016). Transcription analysis revealed that re-aeration affected multiple metabolic pathways, including downregulation of persistence-associated regulons and upregulation of pathways involved in DNA repair and recombination and synthesis of major cell wall components (Du et al., 2016; Iona et al., 2016), indicating physiological transformation of *M. tuberculosis* in preparation for cell division.

In our previous study, we examined transcriptomic changes in NC *M. tuberculosis* during resuscitation after potassium re-introduction using microarray technology, which revealed a lag phase in the transcriptional initiation of resuscitating cells that lasted for at least 4 days (Salina E. G. et al., 2014). However, according to the level of radioactive uracil incorporation, transcriptional activity was triggered in resuscitating *M. tuberculosis* immediately after switching to growth-favoring conditions. It can be speculated that the failure to detect activation of gene expression at the early resuscitation phase of dormant NC bacilli characterized by global transcriptional repression (Ignatov et al., 2015) can be attributed to the limited sensitivity of the microarray assay (Shendure, 2008). Therefore, in this study, we examined early transcriptional response of *M. tuberculosis* to resuscitation using a more sensitive RNA-seq approach.

Here, we report, for the first time, that the resuscitation of NC *M. tuberculosis* from dormancy involves two-stage transcriptional activation, including immediate *de novo* mRNA synthesis, that precedes cell multiplication. This scenario involves activation of lipid metabolism and cell defense mechanisms followed by the induction of central metabolic reactions.

MATERIALS AND METHODS

Bacteria and Media

Dormant NC *M. tuberculosis* was obtained as described previously (Ignatov et al., 2015). Briefly, *M. tuberculosis* strain H37Rv was initially grown from frozen stocks for 10 days in Sauton medium containing (per liter): 0.5 g KH_2PO_4, 1.4 g $MgSO_4 \cdot 7H_2O$, 4 g L-asparagine, 60 ml glycerol, 0.05 g ferric ammonium citrate, 2 g sodium citrate, 0.1 ml 1% $ZnSO_4$, pH 7.0 (adjusted with 1 M NaOH) and supplemented with ADC and 0.05% Tween 80, at 37°C with agitation (200 rpm). The starter culture was inoculated into fresh medium (same composition) and incubated for another 10 days with agitation (200 rpm) until its optical density at 600 nm (OD600) reached 4.0. These bacteria were then inoculated (5×10^5 cells/ml) into K+-deficient Sauton

medium (containing 8.9 g $Na_2HPO_4 \cdot 12\ H_2O$ instead of 0.5 g KH_2PO_4) and grown at 37°C, with agitation (200 rpm). After 14–15 days of culture, when CFU started to decrease, rifampicin (5 μg/ml) was added to eliminate culturable bacteria and to obtain the NC population with the "zero-CFU" phenotype.

Resuscitation of "Non-culturable" Cells

Dormant NC cells were harvested by centrifugation (20 min at 5,000 rpm), washed twice with fresh Sauton media and diluted 5 times from initial culture volume with "resuscitation media" which is standard Sauton medium containing 0.6% glycerol (Shleeva et al., 2011) with ADC and Tween-80 (0.05% v/v), and supplemented with an equal volume of used culture supernatant, prepared as previously described. Bacterial cultures were incubated with agitation (150 rpm) at 37°C and harvested at appropriate time points for colony forming units (CFU) and most probable numbers (MPN) counting, radioactive uracil incorporation, DPI-reductase activity measurement and isolation of RNA. In some cases, resuscitation procedure was performed in the presence of 5 μg/ml of rifampicin.

Cell Viability Estimation

To assess cell viability, 10-fold serial dilutions of *M. tuberculosis* cultures were plated in triplicate onto solidified Sauton agar supplemented with ADC and incubated at 37°C for 25 days, after which CFUs were counted. To assess the proportion of bacteria with the ability to resuscitate in liquid medium by MPN assay, 10-fold bacterial dilutions were resuspended in ADC-supplemented Sauton medium diluted 1:1 (v/v; final glycerol concentration, 0.6%) and seeded into 48-well Corning microplates, which were incubated statically at 37°C for 30 days. The wells with visible bacterial growth were counted as positive, and MPN values were calculated using standard statistical methods (de Man, 1975).

Incorporation of Radioactively Labeled Uracil

One μl of 5,6-3H uracil (1 mCi) was added to 1 ml culture samples and incubated at 37°C with agitation for 20 h. Two hundred microliters of this culture was placed in 3 ml 7% ice-cold CCl_3COOH and incubated at 0°C for 20 min, followed by filtration through a glass microfiber filter (Whatman). Precipitated cells were washed with 3 ml 7% CCl_3COOH and 6 ml 96% ethanol. Filters were placed in 10 ml scintillation mixture; impulse counts were determined by LS analyzer (Beckman Instruments) and expressed as counts per minute (cpm).

DPI-Reductase Activity Measurement

The activity of the initial part of respiratory chain (Complex I) of cells was evaluated by estimation of their activity to reduce the artificial electron acceptor−2,6-dichlorophenol-indophenol (DPI) in the presence of menadione by measurement of color changes in the optical density at 600 nm. The reaction mixture (1 ml) contained: 0.5 mM 2,6-DPI; 0.145 mM menadione and *M. tuberculosis* cell suspension (1×10^7 cells) in 0.1 M phosphate buffer pH 7.0 at 37°C. The activity was calculated as pmols of DPI on 1 ml of cell suspension per minute, defining it as the average of the results of 3 measurements, while the relative error did not exceed 5%.

Isolation of RNA

Bacterial cultures were rapidly cooled on ice, centrifuged, and total RNA was isolated by phenol-chloroform extraction after cell disruption with BeadBeater (BioSpec Products) as previously described (Rustad et al., 2009). After isolation, RNA was treated with Turbo DNase (Life Technologies) to remove traces of genomic DNA, and purified with the RNeasy mini kit (Qiagen). Amounts and purity of RNA were determined spectrophotometrically; integrity of RNA was assessed in 1% agarose gel. Semi-quantitative evaluation of rRNA in total RNA samples was performed by Gel-Pro Analyzer software (Meyers Instruments).

Illumina Sequencing

RNA samples were depleted of 16S and 23S rRNA using Ribo-Zero rRNA Removal Kit for Gram-Positive Bacteria (Epicenter). Sequencing libraries were generated using the resulting ribosomal transcript-depleted RNA and the TruSeq Stranded mRNA Library Prep Kit (Illumina) (Run 1) or NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (Run 2) according to the manufacturers' protocol. Sequencing was performed using the Illumina HiSeq 4000 (Run 1) or HiSeq 2500 (Run 2). Experiments were performed in triplicates.

Processing of RNA-seq Data

After quality control evaluation and trimming of bad qualitative reads the reads were mapped on the reference *M. tuberculosis* genome (AL123456.3, http://www.ncbi.nlm.nih.gov/) by Bowtie2 (Langmead and Salzberg, 2012). The alignment was performed with the "–local" option, which allows leaving 5′ and 3′ ends uncharted. Calculation of the mapped reads for all genes was performed using functions of the Feature Counts package (Liao et al., 2014) built into the author's script. Gene expression was represented in form of reads per kilobase per million (RPKM), for the calculation of which only unambiguously mapping reads were used. Differentially expressed genes were identified by the software package DESeq2 (Love et al., 2014). The genes were considered to be differentially expressed, if the *p*-adjusted value was <0.1, and the expression change module (FC, Fold change) was not <3. Further distribution of genes according functional categories was performed using the Mycobrowser database (https://mycobrowser.epfl.ch/).

Quantitative Real-Time PCR

One microgram of total RNA was used for cDNA synthesis with random hexanucleotides and SuperScript III reverse transcriptase (Life Technologies). Quantitative PCR was performed using qPCRmix-HS SYBR (Evrogen) and the Light Cycler 480 real-time PCR system (Roche); cycling conditions were as follows: 95°C for 20 s, 60°C for 20 s, 72°C for 30 s, repeat 40 times; primers are listed in **Table S1**. In the end of amplification, a dissociation curve was plotted to confirm

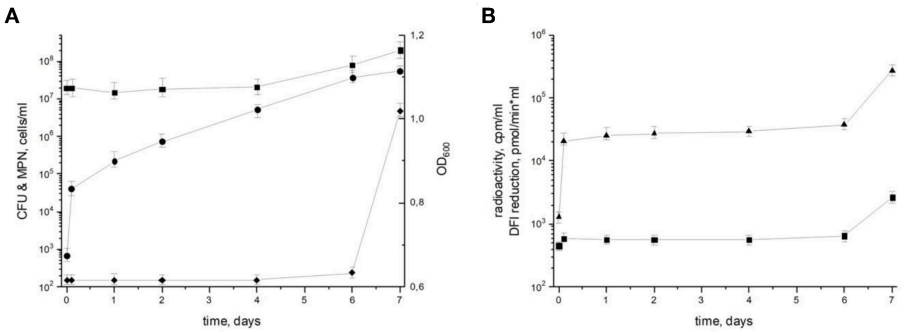

FIGURE 1 | Resuscitation of dormant non-culturable *M. tuberculosis* bacilli. Changes in cell characteristics during the transition from NC to actively growing state. **(A)** Most probable number (squares) MPN/ml; colony forming units (circles) CFU/ml; optical density (diamonds) OD_{600}. **(B)** Radioactive incorporation of uracil (triangles) cpm/ml; DPI reduction (squares) pmol/(min × ml). This experiment was repeated five times with similar results.

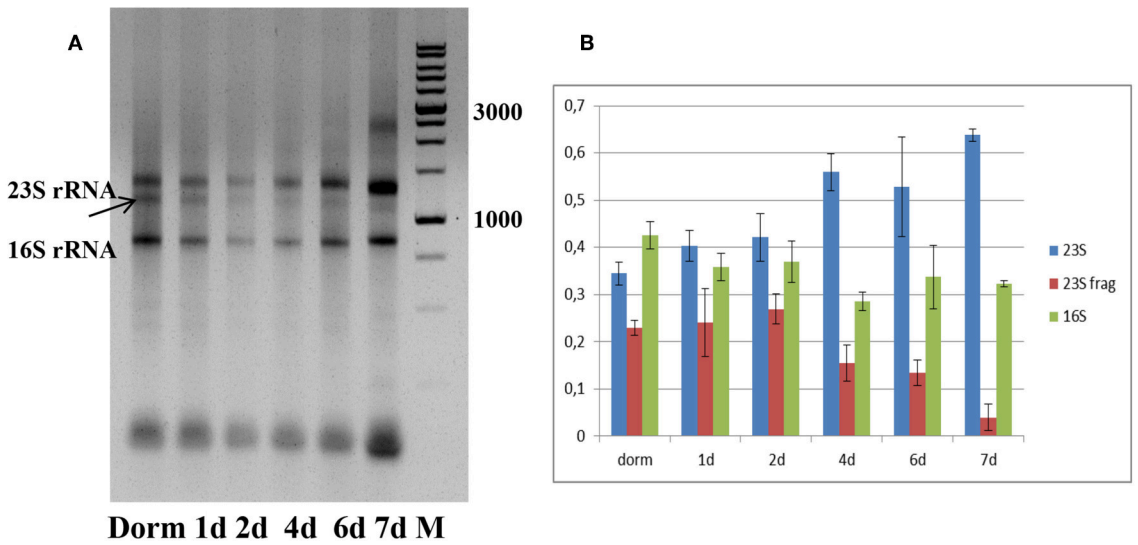

FIGURE 2 | Ribosomal RNA status during resuscitation. **(A)** 1.0% agarose gel profiling of total RNA samples. Arrow indicates the 23S rRNA fragment characteristic for *M. tuberculosis* NC state. M−1 kb DNA ladder (SibEnzyme, Russia). **(B)** Changes in the proportion of 23S/23S fragment/16S rRNAs in the process of resuscitation. The proportions obtained by image 2A processing by Gel-Pro Analyzer software. The results of three independent RNA isolation experiments were presented.

specificity of the product. All real-time experiments were repeated in triplicate. The results were normalized against the 16S rRNA gene.

RESULTS

Dynamics of NC *M. tuberculosis* Resuscitation

To characterize the resuscitation phenomenon and the transition of dormant *M. tuberculosis* from the NC state ("zero-CFU" phenotype) to the multiplication phase, dormant NC bacilli were washed and inoculated into fresh resuscitation medium. Although there were no changes in the optical density (OD) 600 or most probable number (MPN) corresponding to the maximum count of potentially viably cells in the population until day 7 (the time point when cell multiplication begins), the number of culturable bacteria estimated by colony forming units (CFU) increased by about 10 times immediately after washing and continued to rise, reaching 5.7×10^7 CFU/ml at day 7 (**Figure 1**). Estimation of the apparent generation time revealed its gradual increase from 2.8 h (from NC state to day 1 of resuscitation) to 16 h (from day 6 to 7 of resuscitation). As the generation time for replicating *M. tuberculosis* in this medium is 18–20 h, its shortening indicates the prevalence of resuscitation over multiplication during transition of NC *M. tuberculosis* to replicating state. In addition, the level of metabolic activity assessed by incorporation of radioactively labeled uracil and DPI-reductase activity, which reflects functioning of the initial part of the respiratory chain, was constantly low until a steep increase at day 7, which also supports the predominance of resuscitation over cell division up to day 6 (**Figure 1**). The constant number of potentially viable cells both in NC state and

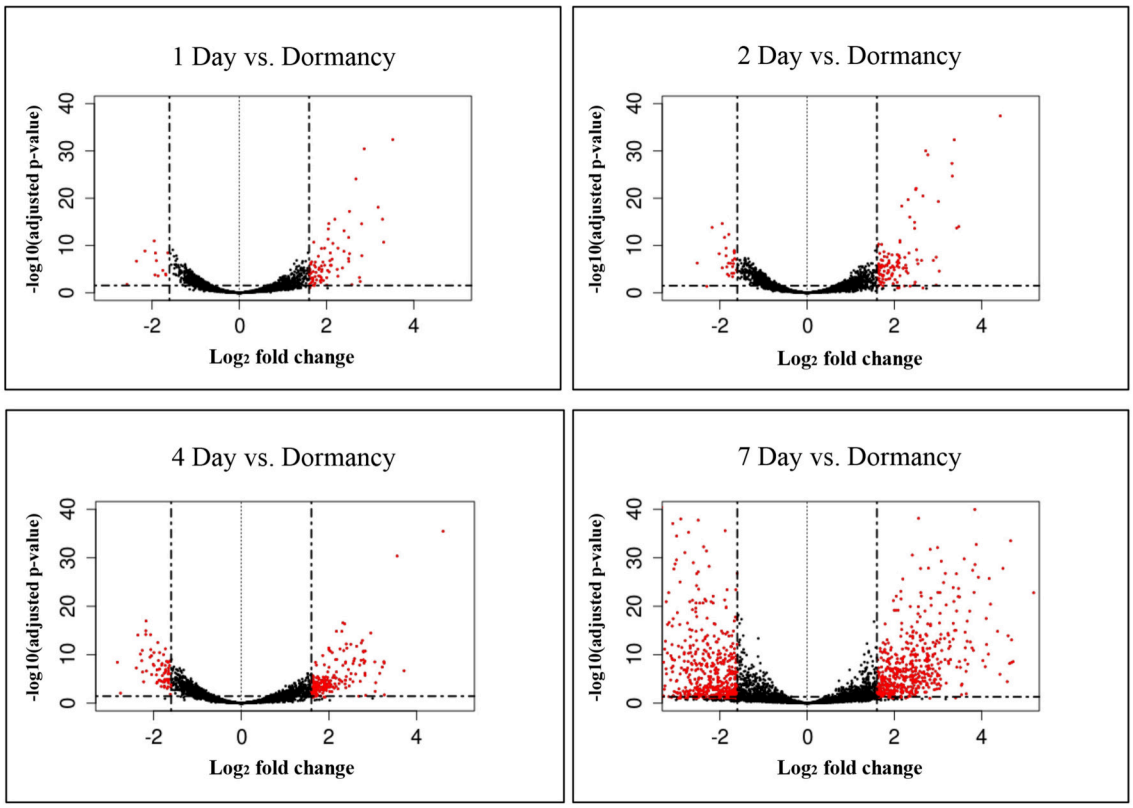

FIGURE 3 | Volcano plots of differentially expressed genes. Fold changes between genes were plotted. Criteria of >3 x change (log2FC > 1.6) in expression and <0.1 p-adj-value were used to define significantly changed genes. Differentially expressed genes which met the criteria are shown in red, and those which were expressed at lower levels are shown in black.

resuscitating mycobacteria estimated by MPN assay also supports the notion of resuscitation preceding multiplication.

Ribosomal RNA Status

Total RNA profiling in dormant NC *M. tuberculosis* (Dorm) revealed the presence of an additional fragment below the 23S rRNA band (**Figure 2A**), which was likely a product of specific 23S rRNA cleavage characteristic for NC *M. tuberculosis* (Ignatov et al., 2015). This 23S rRNA fragment was detected over the whole resuscitation period—from dormancy to day 7 (**Figure 2A**).

We estimated the relative ratio of 23S, 23S fragment and 16S rRNAs at each time point of resuscitation of bacterial culture (**Figure 2B**), and found that it was very similar in dormancy and at days 1 and 2. From day 4 we noticed the increase of intact 23S rRNA amount and corresponding decrease of 23S fragment amount. This tendency is most pronounced at day7, where integrity of 23S rRNA is the highest.

RNA-seq Analysis

The constant, albeit low incorporation of radioactive uracil indicative of *de novo* transcription in resuscitating cells immediately after the resuscitation start (**Figure 1**) prompted us to perform dynamic transcriptome profiling over the resuscitation period by RNA-seq at 5 time points: dormant NC state, days 1, 2, 4, and 7. Using the software package DESeq2, we identified genes differentially expressed in resuscitating compared to dormant *M. tuberculosis* (**Table S2**). The results indicated that the total number of differentially expressed genes gradually increased over time, reaching maximum at day 7. Volcano plots clearly demonstrate that according to the number of upregulated transcribed genes and transcription intensity ($\log_2$FC $\geq$ 1.6), the resuscitation process could be divided into two phases: before and after day 4 (**Figure 3**). The Venn diagram shows the number of differentially expressed protein-coding genes ($\log_2$FC $\geq$ 1.6) on different time points over 7-day resuscitation and their intersections revealed that 21 genes were expressed throughout the whole period, 21were common for days 1 and 2, 45 started expression at day 2, 93—at day 4, and 399—at day 7 (**Figure 4**).

The functional analysis of differentially expressed genes was performed using the Mycobrowser database and the results for genes with expression fold change exceeding 3 ($\log_2$FC $\geq$ 1.6) are shown in **Table S2**.

Transcriptional Changes at the Early Resuscitation Phase

Comparison of RPKM values for individual genes at days 1, 2, 4, and 7 with those at the NC state and expression analysis

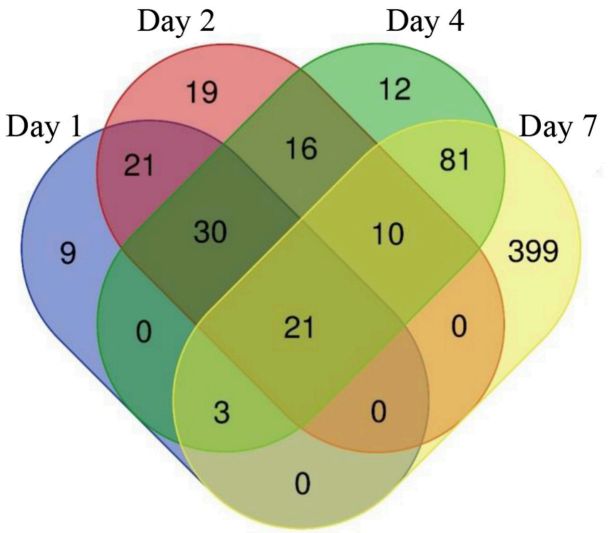

FIGURE 4 | Venn diagram showing the number of differentially expressed genes in days 1, 2, 4, and 7 comparing to dormant NC state.

revealed two groups of genes differing in transcription dynamics (**Table S2**): the first contained genes activated at days 1 and 2 and possibly downregulated later, and the second—those activated after day 4.

In the first group, 84 protein-coding genes showed more then 3-fold upregulation at day 1. The prominent increase was observed for genes encoding enzymes of fatty acid synthase system type I (FASI) and type II (FASII) (**Figure 5**) responsible for biosynthesis of fatty/mycolic acids (Singh et al., 2011), including diacylglycerol kinase (Rv2252), meromycolate extension acyl carrier protein (Rv2244, *acpM*), holo acyl-carrier protein synthase (Rv2523c, *acpS*), malonyl CoA-acyl carrier protein transacylase (Rv0649, *fabD*), keto-acyl-carrier protein synthases (Rv2245, *kasA* and Rv2246, *kasB*), atty acid synthase (Rv2524c, *fas*), acetyl/propionyl-CoA carboxylase (Rv2247, *accD6*), thioesterase (Rv2928, *tesA*), polyketide synthase associated proteins (Rv1528c, *papA4*), and enoyl-CoA hydratase (Rv1472, *echA12*).

The genes encoding redox-sensing transcription factor WhiB6 and transcriptional regulator Rv3830c belonging to the TetR-family were also considerably induced after 24 h of resuscitation. Similar expression shifts were observed for the *pe13* (Rv1195), *ppe18* (Rv1196), *esxK* (Rv1197), and *esxL* (Rv1198) genes, coding the components of the ESX-5b secretion system (Shah and Briken, 2016).

Several genes of mycobacterial defense systems, such as *hsp* (Rv0251c) encoding a molecular chaperone heat shock protein, *clpB* (Rv0384c) encoding endopeptidase which removes oxidized proteins, and antitoxin *vapB12* (Rv1721c) encoding antitoxin, were upregulated at the resuscitation start. A similar activation pattern was observed for *trxB1*(Rv1471) coding for thioredoxin, which participates in various redox reactions through reversible oxidation of dithiol, and *msrB* (Rv2674) coding for peptide

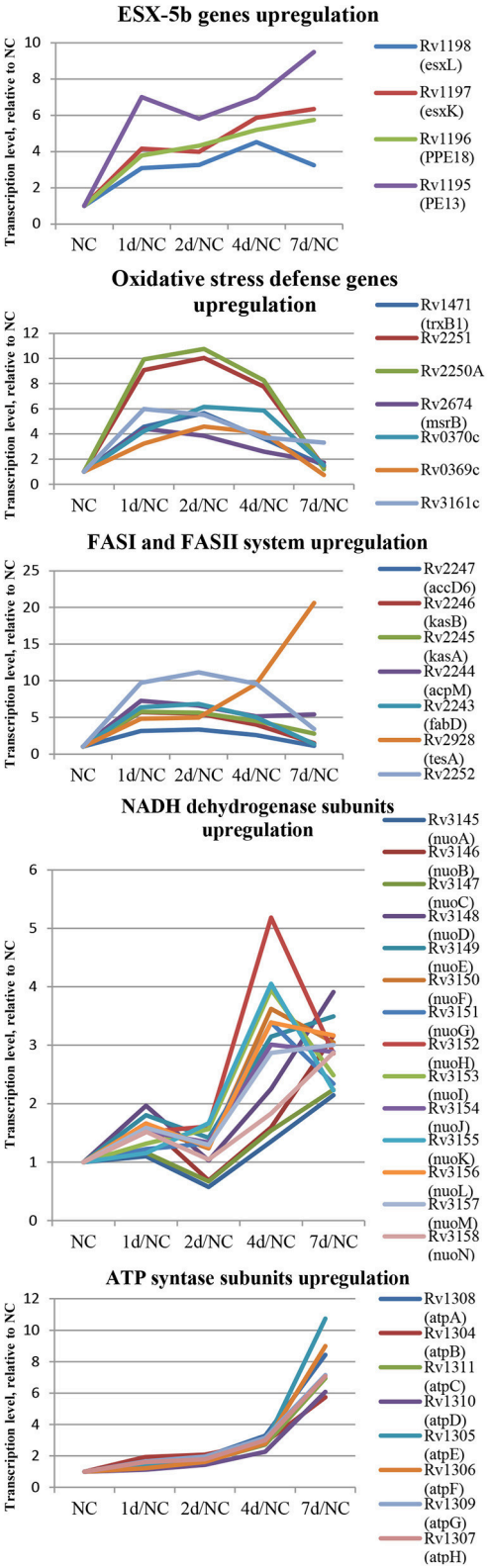

FIGURE 5 | Dynamics of several functional groups expression during resuscitation. Differential expression is given relative to dormant NC state.

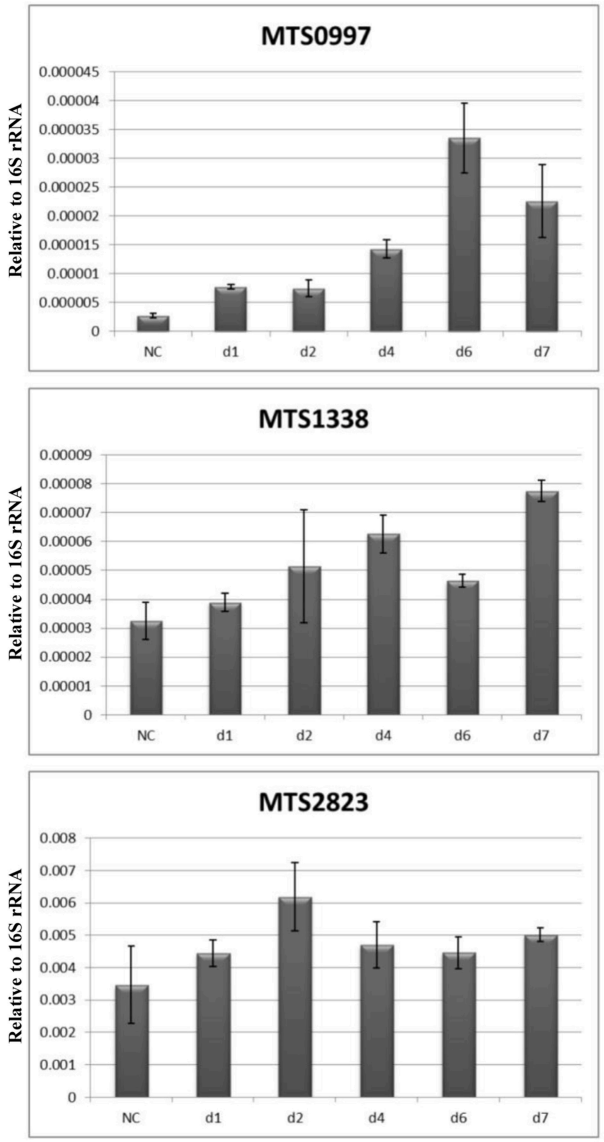

FIGURE 6 | Dynamics of ncRNAs expression during resuscitation. qPCR data are normalized to 16S rRNA transcription level.

methionine sulphoxide reductase, a repair enzyme for proteins inactivated by oxidation. In addition, the expression of genes encoding some enzymes involved in redox reactions, including dioxygenase Rv3161c, oxidoreductases Rv3352c, Rv0369c, and Rv0370c as well as electron acceptors flavoproteins Rv2250A and Rv2251 was also upregulated, indicating intensification of redox processes in the early resuscitation phase. The *pyrE* (Rv0382c) gene for orotate phosphoribosyl transferase involved in pyrimidine biosynthesis was also significantly activated.

The transcriptional profile of *M. tuberculosis* at resuscitation day 2 closely resembled that at day 1, however, more genes (107) were upregulated by >3-fold. In particular, the transcription activity of the majority of genes listed above, namely, those belonging to FAS I and FAS II, fluctuated at the same level or tended to be slightly higher than at 24 h. Transcription of *whiB6* and *hsp* tended to increase further at day 2.

Transcriptional Changes at the Late Resuscitation Phase

In the second group, the spectrum of genes activated at the late phase of resuscitation (days 4–7) was significantly different from that in the first group. Among the late-response genes, there were those related to central metabolism pathways, such as NADH dehydrogenase subunits *nuoEFGHIJKLM* (Rv3149-Rv3157). Activation of *qcrA* (Rv2195, iron-sulfur protein), *qcrB* (Rv2196), and *qcrC* (Rv2197, ubiquinol-cytochrome C reductase), and *ctaE* (Rv2193, cytochrome C oxidase) indicated initiation of aerobic respiration at day 4. The *atpA-G* (Rv1304-Rv1311) genes coding for the ATPase complex were also upregulated. Activation of enzymes belonging to the tricarboxylic acid cycle: *sdhAB* (Rv3318, Rv3319, succinate dehydrogenase subunit), *acn* (Rv1475c, aconitase), *sucCD* (Rv0951, Rv0952, succinyl-CoA synthetase subunits), *gltA2* (Rv0896, citrate synthase I), *ndkA* (Rv2245c, nucleoside phosphate kinase participating in nucleoside biosynthesis), and *pntAb* and *pntB* (Rv0156, Rv0157, transhydrogenase subunits providing transhydrogenation between NADH and NADP) clearly indicated initiation of energy metabolism after resuscitation day 4.

The expression of genes coding for the enzymes of the tryptophan biosynthesis pathway (*trpABCS,* Rv1611-Rv1613, and Rv3336c, respectively) and proteins of the antigen 85 complex [antigen 85 and cord-factor, *fbpA (*Rv3804c) and *fbpD* (Rv3803c), respectively] was also increased. The upregulation of *rmlBC* (Rv3464-Rv3465, rhamnose biosynthesis) and *ppiA* (Rv0009, peptidyl-prolyl cis-trans isomerase accelerating protein folding) indicated the start of central metabolic processes, whereas that of *ahpCD* (Rv2428-Rv2429, involved in oxidative stress response) suggested unbalanced metabolism at this stage of resuscitation.

To confirm the RNA-seq data, we assessed the expression dynamics of selected genes by qPCR using the 16S rRNA gene as a reference. During resuscitation, the correlation between the RNA-seq (RPKM) and qPCR data was moderate to high as evidenced by Spearman correlation coefficient from 0.60 to 0.95 (**Table S3**).

Overall, these results reveal that transcriptional changes in resuscitating *M. tuberculosis* may occur in two steps: at first, activation of fatty acid biosynthesis, defense systems, and transcriptional regulators is provided, and then, the induction of central metabolism and respiration takes places prior to cell multiplication (**Figure 5**).

Non-coding Transcriptome

Recent studies have reported upregulation of non-coding (nc) RNAs in *M. tuberculosis* under different stress conditions (Arnvig and Young, 2012; Ignatov et al., 2015; Del Portillo et al., 2018). Thus, we have previously found that MTS0997, MTS1338, and MTS2823 were the most abundant ncRNAs in dormant NC mycobacteria subjected to potassium deficiency (Ignatov et al., 2015). During resuscitation, only one ncRNA, MrsI (MTB000142, ncRv11846), was upregulated: 4-fold at day 1 and 6- to 7-fold at days 2 and 4 (**Table S2**). MTS2823 (MTB000078)

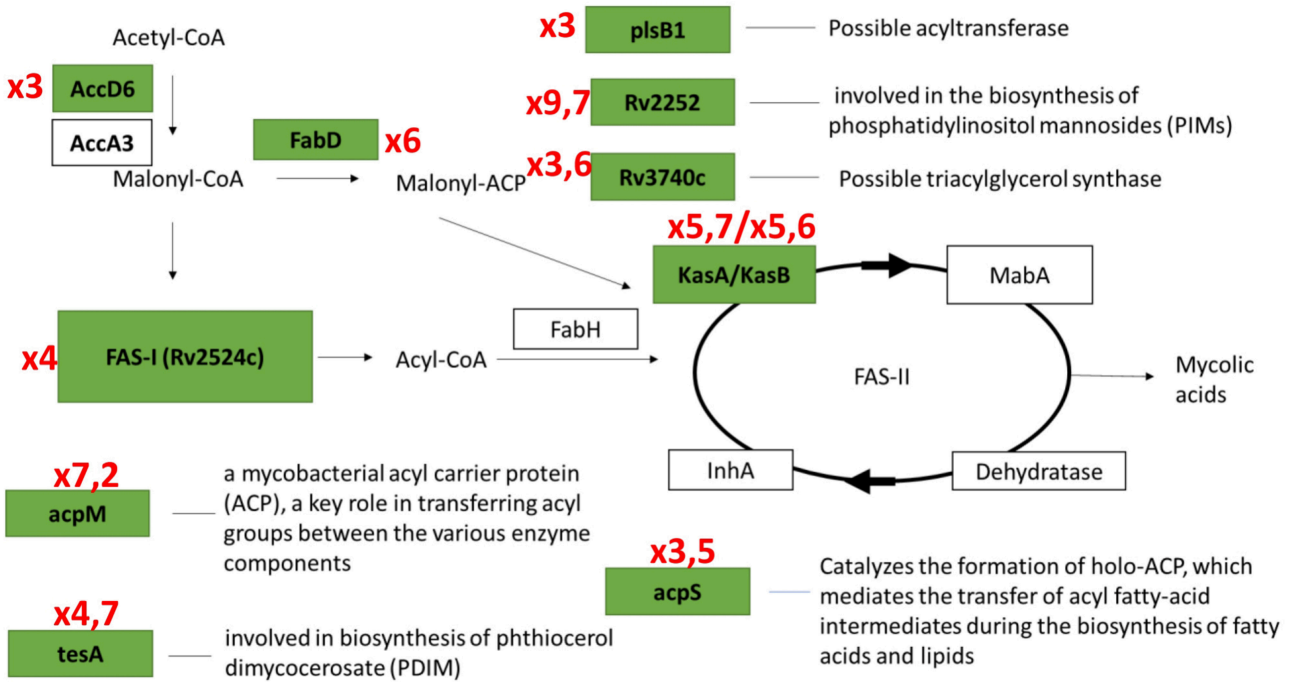

FIGURE 7 | Transcriptional activation of genes involved in fatty acids biosynthesis systems (FASI/FASII) in the early resuscitation phase. Components upregulated at Day1 are given in green boxes. Red numbers designate transcriptional fold changes comparing to dormant NC state.

and MTS0997 (mcr11, MTB000063) were downregulated 4-fold at day 1 and this level remained constant, whereas MTS1338 expression changes were not statistically significant.

These data were further validated by qPCR at the NC stage and at days 1, 2, 4, and 7. The results revealed that the expression of MST2823 was unchanged, that of MTS1338 was slightly upregulated at day 4 and increased by 2- to 3-fold at day 7, and that of MTS0997 also increased by about 10-fold at day 7 (**Figure 6**).

Confirmation of the *de novo* Transcription at the Early Resuscitation Phase

To confirm the early transcriptional response and *de novo* mRNA synthesis at resuscitation, we treated resuscitating *M. tuberculosis* by rifampicin, a blocker of RNA-polymerase activity. The results revealed no changes in the OD600 and metabolic activity until day 7, and even a log reduction in CFU by day 2, indicating that transcriptional blockade inhibited resuscitation (**Figure S1**).

The expression of seven most upregulated genes representing the early transcriptional response was examined by qPCR at days 1 and 2 in resuscitating *M. tuberculosis* treated or not with rifampicin and compared to that in NC bacteria. The following genes were analyzed: Rv2243 (*fabD*), Rv2246 (*kasB*), Rv3160, Rv0251c (*hsp*), Rv0384 (*clpB*), Rv2674 (*msrB*), and Rv1471 (*trxB1*). Changes observed in the transcription of these genes at resuscitation days 1 and 2 were abolished by rifampicin treatment (**Figure S2A**), confirming the *de novo* mRNA synthesis at the early stages of *M. tuberculosis* resuscitation.

Finally, we performed qPCR analysis of ncRNAs and found that rifampicin caused no difference in the transcription rate of ncRNAs MTS2823 and MTS1338 (**Figure S2B**), indicating that these ncRNAs are stable and not being newly synthesized during resuscitation. However, the upregulation of MTS0997 and MrsI observed at days 1 and 2 was completely blocked by rifampicin, indicating that their *de novo* synthesis is required at the early resuscitation stage of *M. tuberculosis*.

DISCUSSION

In the present study, we examined the resuscitation of dormant *M. tuberculosis* driven to the NC phenotype by K^+ deficiency. K^+ is crucial for the maintenance of an electrochemical gradient and proton motive force in the membrane and for the regulation of intracellular pH and osmotic pressure in both eukaryotic and bacterial cells (Epstein, 2003). In *M. tuberculosis*, low K^+ concentrations result in the inability to maintain acceptable intracellular pH levels in mildly acidic conditions (Sturgill-Koszycki et al., 1994), thus decreasing cell viability (Rao et al., 2008).

Transcriptional response in several *in vivo* studies (Karakousis et al., 2004; Rachman and Kaufmann, 2007; Garton et al., 2008) may point out potassium deficiency for *M. tuberculosis*. Therefore, we suggested that low K^+ concentration may be considered as a trigger for development dormant phenotype *in vivo*. We proved experimentally, that K^+ deficiency *in vitro* induced a dormant phenotype which is characterized by

(i) significant decrease in metabolic activity, (ii) tolerance to antibiotics, (iii) changed morphology, (iv) "non-culturability" (Salina E. G. et al., 2014). NC mycobacteria obtained in the K^+ deficiency model are characterized by a high recovery potential and could be resuscitated in Sauton medium diluted 1:1 with culture supernatant (**Figure 1**). According to the MPN assay, nearly 50% of NC *M. tuberculosis* population could be driven to the culturable state (Ignatov et al., 2015). According to **Figure 1**, resuscitation of dormant cells in liquid medium takes at least 6 days. Evidently, that during this period both resuscitation and further cell replication can take place. However, significant shortening of apparent generation time, especially in the very beginning of the reactivation process, steep increase in metabolic activity (radioactive uracil incorporation and DPI reduction) at the same period followed by a plateau, and constant numbers of potentially viable cells (MPN assay) rather indicate the significant prevalence of resuscitation over replication at the first 48 h after the removal of the stress factor. However, the impact of cell multiplication on the overall process cannot be excluded, with increased proportion of replication to the end of resuscitation period.

Earlier, we found that the transition of *M. tuberculosis* to the NC state was accompanied by significant global downregulation of transcriptional activity: by at least 30-fold compared to replicating cells (Ignatov et al., 2015). In this study, we identified, for the first time, the phenomenon of "transcriptional burst" characterized by sharp transcriptional activation of certain genes and significant increase (by 10- to 20-fold) of *de novo* mRNA synthesis at the first 24 h, i.e., time corresponding to one generation (**Figure 5** and **Table S3**), which is consistent with global transcriptional repression during transition to dormancy (Ignatov et al., 2015). The sensitivity of transcriptional activation to rifampicin indicates participation of RNA polymerase, which is fairly stable and present in cells dormant for long periods of time (Trutneva, personal communication). However, the mechanisms underlying the early transcriptional activation in resuscitating *M. tuberculosis* are unclear and require further investigation.

Results of transcriptome analysis by RNA-seq revealed two groups of genes activated during resuscitation of NC *M. tuberculosis*. In the first group, transcriptional upregulation occurred immediately after stress removal (day 1), suggesting that these genes participate in the early processes necessary for resuscitation rather than multiplication. Interestingly, the majority of genes in fatty acid synthase (FASI and FASII) systems belong to this early activated group (**Figure 7**). Mycolic acids (2-alkyl, 3-hydroxy long-chain fatty acids) are the major component of the mycobacterial cell wall, constituting about 50% of the dry weight (Barry, 2001), and are crucial for survival and pathogenesis of *M. tuberculosis*, providing resistance to antibiotics and dehydration (Takayama et al., 2005). Our previous results indicate that unlike dormant *M. tuberculosis* isolated from sputum (Garton et al., 2008), NC mycobacteria obtained under K^+ deficiency have lower lipid content than replicating cells (Salina et al., 2010), suggesting that the activation of fatty acids biosynthesis during resuscitation may compensate the lack of lipids in dormancy providing augmentation of cell wall components for further bacilli multiplication.

We also observed transcriptional induction of heat-shock protein Hsp, a molecular chaperone, and endopeptidase ClpB involved in degradation of misfolded and oxidized proteins and protein turnover. Clp proteases have been identified as factors specific to *M. tuberculosis* reactivation (Sherrid et al., 2010; McGillivray et al., 2015; Du et al., 2016) and mycobacteria lacking ClpB were found to be less fit to recover from the stationary phase or antibiotic exposure *in vitro* and to have lower virulence in mice than the wild type (Vaubourgeix et al., 2015).

WhiB6 belonging to the WhiB superfamily of small global transcriptional regulators is known to control aerobic and anaerobic metabolism, cell division, and virulence of *M. tuberculosis* (Chen et al., 2016). Transcriptional regulators Rv3830c and Rv3160c of the TetR protein family are involved in the regulation of multidrug efflux pumps, response to osmotic stress and toxic chemicals, control of catabolic pathways and differentiation processes, and antibiotic resistance of pathogenic bacteria, including *M. tuberculosis* (Ramos et al., 2005). Therefore, the upregulation of these genes during reactivation from dormancy may reflect metabolic adjustment in resuscitating cells, in particular, neutralization of toxic component produced in catabolic reactions.

We paid special attention to small ncRNAs known to control transcription and translation in bacteria. Typically, ncRNAs are expressed in response to external factors, enabling bacterial adaptation to changing environmental conditions and regulating the key stages of pathogenesis. In our study, significant increase was observed only for MrsI, whose *de novo* (rifampicin-sensitive) synthesis was steady up to the cell multiplication phase at day 7. This ncRNA was recently characterized as a mediator of iron-sparing response but is also activated by other macrophage-related stress factors, and it is suggested that MrsI has an anticipatory function, preparing mycobacteria to potentially unfavorable conditions (Gerrick et al., 2018).

Another ncRNA, MTS0997, was upregulated to a much less extent; however, its *de novo* synthesis was also confirmed. MTS0997 was shown to be involved in the control of *M. tuberculosis* lipid metabolism and was overexpressed under hypoxia in the presence of fatty acids (Aguilar-Ayala et al., 2017; Del Portillo et al., 2018). Given sharp upregulation of FASI/FASII fatty acids synthesis systems at the very start of resuscitation, the increase in MTS0997 expression suggests its involvement in the induction of lipid metabolism in mycobacteria after dormancy.

Two other ncRNAs, MTS1338 and MTS2823, were shown to be significantly represented in the NC *M. tuberculosis* transcriptome. MTS1338 is highly expressed during the stationary growth phase (Arnvig et al., 2011) and at dormancy (Ignatov et al., 2015) and was shown to be a part of the DosR regulon activated in hypoxia (Moores et al., 2017), suggesting its role in the maintenance of *M. tuberculosis* survivability in unfavorable conditions.

MTS2823 belonging to 6S ncRNAs is the most abundant ncRNA in the stationary phase of *M. tuberculosis* (Arnvig et al., 2011). Its *M. smegmatis* homolog Ms1 influences the RNApolymerase level and is suggested to sequester

the RNA polymerase core in a cache of inactive enzymes, which can be reactivated when needed (Sikova et al., 2019). Such a mechanism could be critical when the demand for RNA polymerase activity increases after environmental changes such as nutrient availability and outgrowth from the stationary phase. Our quantitative analysis demonstrated constant levels of MTS2823 and its very slight increase of MTS1338 only after resuscitation day 4, which should be mainly due to high ncRNA stability (Ignatov et al., 2015; Moores et al., 2017), rather than *de novo* synthesis. Therefore, we can conclude that MTS1338 and MTS2823 are not involved in the resuscitation process after K^+-limiting conditions but are preserved through the NC state as stable transcripts accumulated at the time of active *M. tuberculosis* growth.

After 4 days of resuscitation, a group of "late" genes, including those regulating central metabolism (respiration, ATP-synthesis, TCA cycle activity, and translation), are activated, suggesting their role in the initiation of cell division. It is interesting that the five genes coding for resuscitation-promoting factors (Rpfs) did not show significant activation before the start of cell division; only *rpfE* was significantly upregulated at day 7, which coincides with the multiplication onset. In our previous study, we found that only *rpfB* was induced after 8 days of resuscitation from the NC state (Salina E. G. et al., 2014). These findings are consistent with late activation of Rpfs during resuscitation of *M. smegmatis* (Shleeva et al., 2013).

In conclusion, the application of RNA-seq in combination with qPCR enabled us to detect transcriptional changes and *de novo* mRNA synthesis at the early stage of *M. tuberculosis* resuscitation from the dormant NC state, which preceded the activation of genes controlling central metabolism. Our findings revealed, for the first time, the occurrence of an immediate transcriptional burst at the very start of pathogen resuscitation, which is followed by two-phase changes in the expression profile and then, cell multiplication. The described phenomenon of transcriptional activation at the early stage of *M. tuberculosis* recovery from dormancy warrants further investigation to provide comprehensive understanding of the mechanisms underlying pathogen transition from dormancy to replication, which is crucial for combating latent tuberculosis.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI Sequence Read Archive (SRA) database (Accession No. SRR8816592, SRR8816593, SRR8816588, SRR8816589, SRR8816590, SRR8816591, SRR8816605, SRR8816602, SRR8816594, SRR8816595, SRR8816596, SRR8816600, SRR8816603, SRR8816597, SRR8816601, SRR8816604, SRR8816598, SRR8816599).

AUTHOR CONTRIBUTIONS

TA, AK, and ES conceived and designed the experiments and wrote the manuscript. ES, AG, OB, YS, and IM performed the experiments. AK, TA, ES, and AG analyzed the data. ES, TA, and AG prepared figures and graphs. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00272/full#supplementary-material

Table S1 | Oligonucleotides used in the study.

Table S2 | NGS data.

Table S3 | Confirmation of NGS data by qPCR. Two different sets of genes were checked for changes in transcripts level in days 2, 4, and 7 vs. dormancy (1), and days 1, 2 vs. dormancy (2). Spearman correlation coefficients between NGS and PCR data were calculated.

Figure S1 | Resuscitation of dormant non-culturable *M. tuberculosis* bacilli in the presence of rifampicin. Changes in cell characteristics in the absence (in black) and in the presence (in red) of 5 μg/ml of rifampicin: colony forming units (circles), CFU/ml; radioactive incorporation of uracil (triangles) cpm/ml; optical density (diamonds) OD_{600}. This experiment was repeated five times with similar results.

Figure S2 | Expression of several protein-coding genes **(A)** and ncRNAs **(B)** at early stages of resuscitation in bacterial cultures with and without rifampicin (rif) treatment. qPCR data are normalized to 16S rRNA transcription level.

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Assessing the Contribution of an HtrA Family Serine Protease During *Borrelia turicatae* Mammalian Infection

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Tick-borne relapsing fever (TBRF), characterized by recurring febrile episodes, is globally distributed and among the most common bacterial infections in some African countries. Despite the public health concern that this disease represents, little is known regarding the virulence determinants required by TBRF *Borrelia* during infection. Because the chromosomes of TBRF *Borrelia* show extensive colinearity with those of Lyme disease (LD) *Borrelia*, the exceptions represent unique genes encoding proteins that are potentially essential to the disparate enzootic cycles of these two groups of spirochetes. One such exception is a gene encoding an HtrA family protease, BtpA, that is present in TBRF *Borrelia*, but not in LD spirochetes. Previous work suggested that *btpA* orthologs may be important for resistance to stresses faced during mammalian infection. Herein, proteomic analyses of the TBRF spirochete, *Borrelia turicatae*, demonstrated that BtpA, as well as proteins encoded by adjacent genes in the *B. turicatae* genome, were produced in response to culture at mammalian body temperature, suggesting a role in mammalian infection. Further, transcriptional analyses revealed that *btpA* was expressed with the genes immediately upstream and downstream as part of an operon. To directly assess if *btpA* is involved in resistance to environmental stresses, *btpA* deletion mutants were generated. *btpA* mutants demonstrated no growth defect in response to heat shock, but were more sensitive to oxidative stress produced by *t*-butyl peroxide compared to wild-type *B. turicatae.* Finally, *btpA* mutants were fully infectious in a murine relapsing fever (RF) infection model. These results indicate that BtpA is either not required for mammalian infection, or that compensatory mechanisms exist in TBRF spirochetes to combat environmental stresses encountered during mammalian infection in the absence of BtpA.

Keywords: *Borrelia*, relapsing fever, relapsing fever borrelia, BtpA, HtrA, BhpA, oxidative stress

INTRODUCTION

Relapsing fever (RF), caused by spirochetes belonging to the genus *Borrelia*, is characterized by recurrent febrile episodes accompanied by non-specific symptoms including headache, nausea, vomiting, and diarrhea (Ross and Milne, 1904; Dutton et al., 1905; Dworkin et al., 1998, 2008). In more severe cases, infection with RF spirochetes can be associated with other manifestations including jaundice, meningitis, acute respiratory distress syndrome, and perinatal mortality (Jongen et al., 1997; Dworkin et al., 1998; Centers for Disease and Prevention, 2007). Tickborne RF (TBRF) is globally distributed with high prevalence in several endemic areas (Cutler, 2010). Accordingly, TBRF is the most common bacterial infection in Senegal and the most prevalent cause of fever in rural Zaire (Dupont et al., 1997; Vial et al., 2006). Moreover, the actual incidence of TBRF may be even higher than reported in many studies, as TBRF cases often go unreported or are misdiagnosed as another disease, such as malaria, in endemic regions of Africa (Dworkin et al., 1998; Nordstrand et al., 2007; Cutler, 2010; Schwan et al., 2012; Talagrand-Reboul et al., 2018). Despite the public health concern that TBRF represents, and the fact that the etiologic agent of TBRF was first described over 100 years ago (Ross and Milne, 1904; Dutton et al., 1905), our knowledge regarding virulence determinants utilized by the causative *Borrelia* spirochetes is limited.

RF and Lyme disease (LD) *Borrelia* are evolutionarily related spirochetes with chromosomes that are largely colinear (Hyde and Johnson, 1986; Fraser et al., 1997; Guyard et al., 2006; Lescot et al., 2008; Miller et al., 2013). In fact, comparison of the chromosomes of these two groups revealed only 17 genes unique to RF spirochetes and 13 genes unique to LD spirochetes (Lescot et al., 2008). Despite this similarity, TBRF and LD *Borrelia* have very disparate enzootic cycles and cause unique diseases. With respect to the enzootic cycle, most TBRF spirochetes are transmitted by *Ornithodoros* soft ticks, whereas LD spirochetes are spread by *Ixodes* hard ticks (Davis, 1936, 1941; Burgdorfer et al., 1982; Barbour, 2018; Barbour and Schwan, 2019). TBRF spirochetes colonize both the midgut and salivary glands of the *Ornithodoros* tick and are transmitted to the mammalian host within seconds of tick attachment (Schwan and Piesman, 2002; Boyle et al., 2014). Alternatively, LD spirochetes primarily colonize the tick midgut and migrate to the salivary glands only after the tick begins feeding (Ribeiro et al., 1987). Therefore, colonized *Ixodes* ticks must feed more than 24 h to transmit spirochetes (Piesman et al., 1987). During mammalian infection with TBRF spirochetes, characteristic recurring bacteremic episodes occur with spirochetes reaching numbers as high as 10^8 bacteria/mL in the blood (Stoenner et al., 1982; Cadavid et al., 1994; Pennington et al., 1997; Dworkin et al., 1998, 2008; Cutler, 2010). Conversely, during mammalian infection by LD spirochetes, bacteremia primarily occurs early during infection and at relatively lower levels (10^3-10^4 bacteria/mL of blood), during which bacteria are disseminating to distal tissues (Wang et al., 2002). Therefore, whereas symptoms of TBRF are predominantly due to high amounts of bacteria present in the bloodstream, LD symptoms are often the result of specific tissue colonization. These differences suggest that TBRF and LD spirochetes have evolved to encode unique tick colonization factors and virulence factors essential for their distinct enzootic cycles and associated disease courses.

While the chromosomes of TBRF and LD *Borrelia* species are mostly colinear, their chromosomes also contain a subset of conserved genes that are only found in either TBRF or LD spirochetes (Hyde and Johnson, 1986; Fraser et al., 1997; Guyard et al., 2006; Lescot et al., 2008; Miller et al., 2013). It has been hypothesized that the chromosomal genes unique to TBRF or LD *Borrelia* may encode important bacterial factors that contribute to the distinct aspects of the enzootic cycles and disease courses of the two groups of *Borrelia* (Guyard et al., 2006). *bt0790A*, designated *btpA* in *Borrelia turicatae* by Guyard et al., encodes an HtrA (high temperature requirement) family serine protease found in the chromosomes of TBRF spirochetes, but not LD spirochetes (Guyard et al., 2006). The HtrA family of serine proteases are important for the pathogenesis of several species of bacteria, including *Salmonella typhimurium, Yersinia enterocolitica*, and *Listeria monocytogenes* (Johnson et al., 1991; Li et al., 1996; Pallen and Wren, 1997; Schafer et al., 1999; Clausen et al., 2002; Raivio, 2005; Wilson et al., 2006; Ingmer and Brondsted, 2009). Periplasmic HtrA proteases serve as stress-response chaperones that assist in polypeptide folding or as proteases involved in turnover of improperly folded proteins (Strauch and Beckwith, 1988; Spiess et al., 1999; Rizzitello et al., 2001). In light of this key physiological role, it is not surprising that *htrA* mutants of many bacteria are more sensitive to environmental stresses encountered during human infection that result in misfolded proteins, including increased temperature and oxidative stress (Lipinska et al., 1989; Johnson et al., 1991; Elzer et al., 1994; Li et al., 1996; Cortes et al., 2002; Brondsted et al., 2005; Wilson et al., 2006). Guyard et al. demonstrated that BhpA, the *Borrelia hermsii* ortholog of BtpA that shares 89% identity, has caseinlolytic activity, and is located intracellularly, presumably in the periplasm (Guyard et al., 2006). Guyard et al. also showed elevated transcription of *bhpA* when bacteria were cultured at mammalian body temperature relative to a temperature representative of an unfed tick, suggesting a possible role during mammalian infection. Finally, they demonstrated that heterologous expression of *bhpA* in *Borrelia burgdorferi,* a LD spirochete, rendered the bacteria more resistant to oxidative stress, and neutrophil-mediated killing *in vitro* (Guyard et al., 2006). It was therefore hypothesized that these unique proteases in TBRF spirochetes play a role in turnover of proteins damaged by the immune response [e.g., reactive oxygen species (ROS) and reactive nitrogen species (RNS)] and facilitate the capacity for these bacteria to achieve high-level bacteremia relative to LD spirochetes (Guyard et al., 2006).

Herein, we aimed to further the work of Guyard et al. (2006). Proteomic analyses revealed that, in *B. turicatae*, BtpA and proteins encoded by adjacent chromosomal genes are produced in response to growth at mammalian body temperature. Subsequent transcriptional analyses demonstrated that *btpA* is expressed in an operon with immediately upstream and downstream genes, which encode a hypothetical protein and thymidine kinase, respectively. Further, the analogous region

of the *B. burgdorferi* chromosome was also co-transcribed, suggesting that the operonic nature of this region of the chromosome is conserved among diverse *Borrelia* species. Importantly, in the previous study, attempts to delete *bhpA* in *B. hermsii* were unsuccessful, leading to the suggestion that this protease may be required for bacterial viability (Guyard et al., 2006). However, we were able to generate mutants lacking *btpA* in the TBRF spirochete, *B. turicatae*, and directly assess the importance of this protease *in vitro* under environmental stresses, and *in vivo* during mammalian infection. *btpA* mutants did not exhibit increased susceptibility to high culture temperature like *htrA* mutants of several other bacteria. Furthermore, *btpA* mutants were equally resistant to oxidative stress produced by hydrogen peroxide (H_2O_2) and nitrosative stress produced by the nitric oxide donor diethylamine NONOate (DEA/NO) relative to wild-type *B. turicatae*, however, exhibited slightly increased susceptibility to *t*-butyl peroxide. Lastly, *in vivo* murine infection experiments demonstrated that mutants are able to establish initial bloodstream infection and cause subsequent bacteremic relapses. These data imply, contradictory to previous work, that BtpA is not required by TBRF spirochetes to facilitate mammalian infection.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are detailed in **Table 1**. *Escherichia coli* strain TOP10F' (Life Technologies, Carlsbad, CA) was utilized for cloning. *E. coli* cultures were grown at 37°C in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin or 5 μg/mL gentamicin when necessary. Low passage strains of *B. turicatae* [strain 91E135 (Oz1)] and *B. burgdorferi* (strain 297) were used for this study (Taylor et al., 1991; Hughes et al., 1992; Schwan et al., 2005). Specifically, wild-type *B. turicatae* and *B. burgdorferi* were passaged no more than twice from the original frozen stock, and *B. turicatae btpA* mutants were passaged no more than twice after clonal isolation (see below). *B. turicatae* was cultured at 35°C with 3% CO_2 in modified Barbour-Stoenner-Kelly (mBSK) medium with 12% rabbit serum at a pH of 7.6 (Barbour, 1984; Battisti et al., 2008), except during oxidative and nitrosative stress susceptibility assays (below). For *B. turicatae* growth temperature experiments, all culture conditions were identical to those above with the exception of temperature, which was increased to 37, 39, or 41°C. For the temperature shift experiment, culture conditions were identical to the parameters above, except cultures were incubated at 23°C for seven days, followed by a shift to 37°C. Selection of *B. turicatae* transformants was achieved by supplementing mBSK medium with 40 μg/mL gentamicin. *B. burgdorferi* was cultured at 35°C in 3% CO_2 in BSK-II medium with 6% rabbit serum at a pH of 7.6 (Barbour, 1984; Pollack et al., 1993).

Reverse Transcription-PCR (RT-PCR) Analyses

RT-PCR was performed to evaluate possible transcriptional linkage of chromosomal segments spanning *bt0790-bt0791* in *B. turicatae,* and *bb0790-bb0791* in *B. burgdorferi*, as well as to assess transcription of *btpA* and adjacent genes. RNA was extracted as previously described (Blevins et al., 2007; Groshong et al., 2014). Briefly, cultures of *B. turicatae* or *B. burgdorferi* were grown to late exponential phase followed by treatment with 10% (vol/vol) RNA stop solution (Bernstein et al., 2002; Blevins et al., 2007). Cells were collected by centrifugation and stored at −80°C until RNA extraction was performed. Total RNA was isolated with TRIzol reagent (ThermoFisher Scientific, Waltham, MA), and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instructions. RNA was then treated with RNase-free DNase I (Qiagen) to eliminate possible DNA contamination. Absence of contaminating genomic DNA (gDNA) was confirmed by PCR utilizing primers specific for an internal region of the *flaB* gene (*B. turicatae* primers: 5′ BtFlaB and 3′ BtFlaB; *B. burgdorferi* primers: 5′ Chrom and 3′ Chrom), and EmeraldAmp GT PCR Master Mix (TaKaRa Bio, Mountain View, CA). Primers used in this study are described in **Table 2**.

Purified RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) or SuperScript IV VILO Master Mix (ThermoFisher Scientific) according to the manufacturers' protocols. As a negative control, a mock reaction was conducted in the absence of reverse transcriptase. cDNA was then used as template for PCRs with EmeraldAmp GT PCR Master Mix and primers either annealing to adjacent genes to assess transcriptional linkage or primers annealing to internal regions of genes to evaluate transcription (**Table 2**). For the *bt0790-bt0791* RT-PCR linkage reaction however, PrimeSTAR Max DNA polymerase (TaKaRa Bio) was used in place of EmeraldAmp GT PCR Master Mix due to the need to generate a larger PCR product. Wild-type *B. turicatae* or *B. burgdorferi* gDNA was used as an amplification control where appropriate. PCR products were separated by gel electrophoresis in 0.8% agarose and stained with ethidium bromide for DNA visualization.

Quantitative Real-Time PCR (qRT-PCR) Analyses

qRT-PCR was used to measure transcription of genes in the *btpA*-containing operon in *btpA* mutant spirochetes relative to wild-type *B. turicatae*. RNA was isolated and converted to cDNA as described above. Primers were designed to detect transcription of *bt0790* (primers: *bt0790* IDT SYBR FWD and *bt0790* IDT SYBR REV), *btpA* (primers: *bt0790A* IDT SYBR FWD and *bt0790A* IDT SYBR REV), and *bt0791* (primers: *bt0791* IDT SYBR FWD and *bt0791* IDT SYBR REV), as well as *flaB* (primers: 5′ Bt*flaB* SYBR/ABI and 3′ Bt*flaB* SYBR/ABI) as a control gene. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) was used per the manufacturer's instructions. Briefly, a master mix was made so that 19 μL contained 10 μL of the above 2X supermix, 1 μL of each primer at a concentration of 10 μM, and 7 μL of nuclease free water. 19 μL aliquots of the master mix were then distributed into wells in a 96-well real-time PCR reaction plate. 1 μL of cDNA template at a concentration of 100 ng/μL was then added to the 19 μL of master mix in each well, resulting in a final primer concentration of 500 nM. To check for DNA contamination, no template control (NTC)

TABLE 1 | Plasmids and strains used in this study.

Plasmid or strain	Description[a,b]	Source
PLASMID		
pGEM-T Easy	TA cloning vector; Amp^r	Promega
pUAMS4	pGEM-T Easy::P*flgB-aacC1* (*Asc*I-flanked); Gent^r, Amp^r	This study
pUAMS169	pGEM-T Easy::*aacC1* (Flanked with 5′ *NdeI* and 3′ *AscI*); Gent^r, Amp^r	This study
pUAMS177A	*btpA*::P*flgB-aacC1* mutagenesis construct; Gent^r, Amp^r	This study
pUAMS238	*btpA*::*aacC1* mutagenesis construct; Gent^r, Amp^r	This study
STRAIN		
B. turicatae 91E135 (Oz1)	*B. turicatae* tick isolate	Taylor et al., 1991; Schwan et al., 2005
B. burgdorferi 297	*B. burgdorferi* human isolate	Hughes et al., 1992
E. coli TOP10F'	F′ [*lacI*^{q}Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 *nupG* Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1*	Life Technologies
B. turicatae btpA::P*flgB-aacC1*	*btpA* mutant with gentamicin resistance marker under transcriptional control of the *flgB* promoter	This study
B. turicatae btpA::*aacC1*	*btpA* mutant with promoterless gentamicin resistance marker	This study

[a]*Amp, ampicillin.*
[b]*Gent, gentamicin.*

reactions were conducted in which 1 μL of nuclease free water was added to the 19 μL of master mix instead of cDNA template. Reactions were performed using the QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific), and reaction conditions included an initial polymerase activation step at 95°C for 30 s, followed by 40 cycles of DNA denaturation at 95°C for 10 s and primer annealing/DNA extension at 60°C for 30 s. Three technical replicates were conducted for each experiment, and two biological replicates were performed. Data was analyzed using the $\Delta\Delta C_t$ method as previously described (Livak and Schmittgen, 2001).

Proteomic Analysis

To evaluate temperature-dependent differences in *B. turicatae* protein production, wild-type *B. turicatae* cultures were inoculated at an initial density of 10^4 bacteria/mL and grown at either 37°C or 23°C to the late exponential growth phase. Spirochetes were collected by centrifugation, washed two times in cold saline, and then prepared for SDS-PAGE. For each sample, a volume of whole cell lysates equivalent to approximately 3×10^7 spirochetes was loaded in a 4–20% Mini-PROTEAN TGX gel (Bio-Rad Laboratories), and proteins were separated by electrophoresis. After SDS-PAGE, whole lanes were excised in slices, and subjected to in-gel trypsin digestion of proteins. Proteins were identified and quantified by LC-MS/MS based on spectral counts of tryptic peptides for the proteins of interest. Sample preparation, protein identification, and analysis was carried out by the UAMS Proteomics Core as previously described (Zielinska et al., 2012; Byrum et al., 2018). Proteins were then identified using Mascot version 2.5.1 (Matrix Science, Boston, MA) and the *B. turicatae* strain 91E315 database (GenBank assembly accession: GCA_000012085.2). Comparisons were performed on two biological replicates for 37°C cultures and three biological replicates for 23°C cultures.

Generation of *btpA* Mutants

Allelic exchange mutagenesis was used to inactivate *btpA* in *B. turicatae* (Lopez et al., 2013). Primers used are described in **Table 2**. All PCRs for cloning were performed with high-fidelity PrimeSTAR Max DNA polymerase. The gentamicin resistance cassette, P*flgB-aacC1*, was generated by fusing the promoter for the *B. turicatae* flagellar basal body rod protein (*flgB*; *bt0294*) to the *aacC1* resistance marker and introducing AscI sites flanking the cassette (primers: 5′ Bt*flgB-Asc*I and 3′ Gent-*Asc*I) (Battisti et al., 2008). The resistance cassette was then TA-cloned into pGEM-T Easy (Promega Corp., Fitchburg, WI). The *btpA*::P*flgB-aacC1* mutational construct was generated by amplifying a 5′ flanking region (primers: 5′ F1 *bt0790A* KO and 3′ F1 *bt0790A* KO_*Asc*I), and a 3′ flanking region (primers: 5′ F2 *bt0790A* KO_*Asc*I and 3′ F2 *bt0790A* KO_*BssH*II). The respective amplicons were subsequently TA-cloned into pGEM-T Easy and sequence confirmed. The 5′- and 3′-flanking region fragments were ligated together with the P*flgB-aacC1* cassette between them to yield the final *btpA*::P*flgB-aacC1* mutational construct, pUAMS177A.

Derivation of the construct to inactivate *btpA* with a promoterless *aacC1* marker was similar to that described above. A 5′ flanking region (primers: 5′ F1 *bt0790A* KO and 3′ F1 *bt0790A* KO_*Nde*I) and a 3′ flanking region (primers: 5′ F2 *bt0790A* KO_*Asc*I_v2 and 3′ F2 *bt0790A* KO_*BssH*II) were PCR amplified (**Table 2**). The promoterless marker was generated by amplifying the *aacC1* open reading frame (ORF) from P*flgB-aacC1* with primers that introduced 5′ NdeI and 3′ AscI restriction sites (primers: 5′ PromLess Bt Gent and 3′ Gent-*Asc*I). The respective amplicons were subsequently TA-cloned into pGEM-T Easy and sequence confirmed. The 5′- and 3′-flanking region fragments were ligated together with the *aacC1* ORF between them to generate the final *btpA*::*aacC1* allelic exchange construct,

TABLE 2 | Primers and probe used in this study.

Primer designation	Sequence[a,b]	Purpose
5′ BtFlaB	CTGGAATGGGTGTTGCAGGA	RT-PCR; PCR Screening
3′ BtFlaB	CTCCCTCTTGTTGTGCACCT	RT-PCR; PCR Screening
5′ Chrom	GATTATCAATCATAATACATCAGC	RT-PCR
3′ Chrom	TCTAAGCAATGACAAACATATTGG	RT-PCR
5′ *bt0790-790A* link RT-PCR (P1—**Figure 1**)	CCCTGTTCGTTATGAAAATGCTTTGCTTGG	RT-PCR
3′ *bt0790-790A* link RT-PCR (P2—**Figure 1**)	GCAAAGAAACTCGCAAGCATTGGGTCC	RT-PCR
5′ *bt0790A-791* link RT-PCR (P3—**Figure 1**)	GCTCCTAATTCTCCTGCAGATATTGGG	RT-PCR
3′ *bt0790A-791* link RT-PCR (P4—**Figure 1**)	GGCCTACATCAAAAGAATCACCTGC	RT-PCR
5′ *bt0790A* ORF RT-PCR (P3—**Figure 2**)	TGTAAGACTTCCAAGAGGCAAGGG	RT-PCR; PCR Screening
3′ *bt0790A* ORF RT-PCR (P4—**Figure 2**)	TTTCATTTACCACAGGTCCACCGG	RT-PCR; PCR Screening
5′ *bt0790* ORF RT-PCR	GGGAAGTTTTAGTAAAGTGTTAGCCG	RT-PCR
3′ *bt0790* ORF RT-PCR	ACTGCGGCATTATTTTTGTCATCTACA	RT-PCR
5′ *bt0791* ORF RT-PCR_v2	CCATTACCAAGGGACGTAGAAA	RT-PCR
3′ *bt0791* ORF RT-PCR_v2	ACAATCTCATCACCACCAACA	RT-PCR
5′ *bb0790-bb0791* link (P5—**Figure 1**)	GGCTGCAATTTTAATAGTTTTGGGATC	RT-PCR
3′ *bb0790-bb0791* link (P6—**Figure 1**)	CGTTCATCATAAAAACATGCCTCATC	RT-PCR
bt0790 IDT SYBR FWD	ACTTGATTTACATGAGACTTGAAGC	qRT-PCR
bt0790 IDT SYBR REV	AAAGAGTCGGCTAACACTTTACT	qRT-PCR
bt0790A IDT SYBR FWD	CCAATGCTTGCGAGTTTCTTT	qRT-PCR
bt0790A IDT SYBR REV	CCCATATCCACCCAAACTGTTA	qRT-PCR
bt0791 IDT SYBR FWD	CCATTACCAAGGGACGTAGAAAC	qRT-PCR
bt0791 IDT SYBR REV	TCACTTCCACCGCCTCTATAA	qRT-PCR
5′ Bt*flaB* SYBR/ABI	AAAAACAGCTGAAGAGCTTGGAAT	qRT-PCR
3′ Bt*flaB* SYBR/ABI	CACCCACATGTACTCTTAATGTCCAT	qRT-PCR
5′ F1 *bt0790A* KO	CACCTGATGAAGCTTATATGTTTTTTA	Mutagenesis Cloning
3′ F1 *bt0790A* KO_*Asc*I	**GGCGCGCC**CTCTACTCTCAGCAAGCATCATACC	Mutagenesis Cloning
5′ F2 *bt0790A* KO_*Asc*I	**GGCGCGCC**AATATTATTGAGAGTATTATAGAG	Mutagenesis Cloning
3′ F2 *bt0790A* KO_*BssH*II	**GCGCGC**TCCCTACCAACAAGATAATGATGCC	Mutagenesis Cloning
3′ F1 *bt0790A* KO_*Nde*I	**CATATG**CTCTCAAAAGCTAATTAATGTTATGATAG	Mutagenesis Cloning
5′ F2 *bt0790A* KO_*Asc*I_v2	**GGCGCGCC**GCTAAGGTCTTTGCAAATGGTCTTGGTG	Mutagenesis Cloning
5′ Bt*flgB-Asc*I	**GGCGCGCC**AGCACCCGGTAGCAAGTTAAAAAAATTTG	Mutagenesis Cloning
3′ Gent-*Asc*I	**GGCGCGCC**TTAGGTGGCGGTACTTGGGTCG	Mutagenesis Cloning
5′ PromLess Bt Gent	GGCGCGCCATAGAGGGT**CATATG**TTACGCAGCAGC	Mutagenesis Cloning
5′ *aacC1* diag	GCAACGATGTTACGCAGCAG	PCR Screening
3′ *aacC1* diag	GCATCACTTCTTCCCGTATGC	PCR Screening
5′ Bt0790A ext diag_V2 (P1—**Figure 2**)	GATAAAGGAGTTTTGAAAGTTAAGAAAG	PCR Screening
3′ Bt0790A ext diag_V2 (P2—**Figure 2**)	CATAAAGCAATAAGACAAACACTCTCT	PCR Screening
Bt*flaB* F	CCAGCATCATTAGCTGGATCAC	qPCR
Bt*flaB* R	GTTGTGCACCTTCCTGAGC	qPCR
Bt*flaB*-Probe	/5YakYel/TGCAGGTGA/ZEN/AGGTGCGCAGGTT/3IABkFQ/	qPCR

[a] *Relevant restriction sites are indicated by bold lettering.*
[b] *YakYel, 5′ Yakima Yellow dye; ZEN, ZEN internal quencher; IABkFQ, Iowa Black FQ 3′ quencher.*

pUAMS238. The *aacC1* ORF was introduced at the start codon of *bt0790A*.

Constructs were electroporated into wild-type *B. turicatae*, transformants were selected for with gentamicin, and clones were obtained via serial dilution plating as previously described (Lopez et al., 2013). Disruption of the gene in both mutants was genotypically confirmed using PCR with primers (**Table 2**) that amplify an internal segment of *btpA*

(primers: 5′ Bt0790A ORF RT-PCR and 3′ Bt0790A ORF RT-PCR), a segment flanking the deleted region (primers: 5′ Bt0790A ext diag_V2 and 3′ Bt0790A ext diag_V2), an internal segment of the *aacC1* ORF (primers: 5′ *aacC1* diag and 3′ *aacC1* diag), and an internal segment of the *flaB* gene (primers: 5′ BtFlaB and 3′ BtFlaB) as a positive amplification control.

Oxidative and Nitrosative Stress Susceptibility Assays

Low passage *B. turicatae* strains were grown to stationary phase ($\sim 2 \times 10^8$ cells/mL) under aerobic conditions (5% CO_2, 18% O_2) at 34°C in modified pyruvate-free mBSK media (Troxell et al., 2014). Strains were pelleted by centrifugation and resuspended to a density of 1–2 $\times$ 10^7 cells/mL in modified pyruvate-free mBSK media. One mL aliquots were transferred to 5-mL polypropylene culture tubes (Midwest Scientific, Valley Park, MO) and cultured in the presence or absence of H_2O_2, *t*-butyl peroxide, or diethylamine NONOate (Cayman Chemical, Ann Arbor, MI) for 2 h under aerobic conditions at 34°C. Following incubation, serial dilutions of the cultures were prepared in mBSK and were plated on semi-solid mBSK media with 12% rabbit serum in 6-well culture plates as described previously (Raffel et al., 2018). Colony forming units (CFUs) were enumerated after 8–10 days of incubation. Percent survival was determined by dividing CFUs from the 2 h timepoint samples by the CFUs from the 0 h timepoint. Data are presented as mean ± standard deviation (SD).

Murine Infections

Murine infections were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, the Public Health Science Policy on Humane Care and Use of Laboratory Animals, and the Animal Welfare Act. The protocol was approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee (IACUC). Wild-type *B. turicatae* and *B. turicatae btpA* mutants were passaged no more than two times from the original frozen stocks. Groups of 4- to 6-week-old, female Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were used in this study. Wild-type *B. turicatae*, *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1* strains were grown to mid- to late-log phase, enumerated by dark-field microscopy, and diluted in fresh mBSK media to a concentration of 10^3 spirochetes/mL. 100 μL of this dilution (containing 10^2 total spirochetes) was then intradermally/subcutaneously injected into mice in the thoracic region. Daily blood samples, taken via tail venipuncture, were collected on days 3 to 14 post-infection for bacterial burden quantitative PCR (qPCR) assays as previously described (Mccoy et al., 2010; Boyle et al., 2014). 2.5 μL of blood was immediately combined with 47.5 μL of SideStep Lysis & Stabilization Buffer (Agilent Technologies, Santa Clara, CA) and stored at −80°C (Mccoy et al., 2010; Boyle et al., 2014). For preparation of qPCR standard curve samples, described below, blood was collected from uninfected female Swiss Webster mice by brachial artery bleed and combined with SideStep Lysis & Stabilization Buffer at a blood-to-buffer ratio of 1:18.

qPCR for Bacterial Burdens

TaqMan-based qPCR analysis was conducted as initially described by McCoy et al. for *B. hermsii* (Mccoy et al., 2010) and Boyle et al. for *B. turicatae* (Boyle et al., 2014), but with some modifications. 17 μL of master mix containing the following components was added to a 96-well real time PCR plate; 10 μL 2x SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories), 0.8 μL of each forward and reverse primer at a concentration of 10 μM (primers: Bt*flaB* F and Bt*flaB* R), 1.2 μL of probe at a concentration of 5 μM [probe: Bt*flaB* probe labeled with 5′ Yamika Yellow and double-quenched with an internal ZEN quencher and 3′ Iowa Black FQ quencher (Integrated DNA Technologies, Coralville, IA)], and 4.2 μL of nuclease free water. 3 μL of the blood sample in lysis/stabilization buffer (see above) was then added to each well; resulting in a final concentration of 400 nM for each primer and 300 nM for the probe. Samples for standard curves were generated by centrifuging 1 mL of late-exponential phase *B. turicatae* culture at 6,000 × g for 15 min at room temperature. Supernatant was discarded and cells were resuspended in 1 mL of phosphate-buffered saline with 5 mM $MgCl_2$ (PBS-$MgCl_2$). This wash step was then repeated two more times. Following the final centrifugation, spirochetes were resuspended in 500 μL PBS-$MgCl_2$ and enumerated by dark-field microscopy. This suspension was then diluted with PBS-$MgCl_2$ to a density of 10^8 spirochetes/mL, and a range of 10-fold serial dilutions were prepared from 10^4 to 10^8 spirochetes/mL. For purposes of a NTC, nuclease free water was diluted 10-fold in PBS-$MgCl_2$. Preparations from 10^4 to 10^8 spirochetes/mL and the NTC were spiked into naïve blood in lysis/stabilization buffer (see above) at a ratio of 1:19. 3 μL of this final preparation were added to 17 μL of the above master mix and used to generate a standard curve for qPCR. All sample and standard curve reactions were conducted in triplicate. Real-time qPCR was performed using the QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific). The run method consisted of an initial 50°C hold for 2 min followed by a polymerase activation step at 95°C for 10 min. DNA amplification was performed by running 40 cycles consisting of DNA denaturation at 95°C for 15 s and primer annealing/DNA extension at 60°C for 60 s. Data obtained was subsequently imported into Prism version 6 (GraphPad Software, San Diego, CA), graphed, and analyzed.

Statistical Methods

To compare maximum bacterial burdens in the blood of infected mice (qPCR results) and survival following exposure to ROS/RNS, analysis of variance (ANOVA) models were used with Tukey's procedure for pairwise comparisons. $p < 0.05$ were considered statistically significant. For qRT-PCR analyses, mean and standard error of the mean (SEM) for fold-change was calculated based on the $\Delta\Delta C_t$ values before log-transformation (i.e., evaluating the $2^{-\Delta\Delta Ct}$ term) as previously described (Livak and Schmittgen, 2001). Statistical tests were performed using Prism version 6 (GraphPad Software).

RESULTS

BtpA Is Produced in Response to Mammalian Body Temperature

In the LD spirochete, *B. burgdorferi*, numerous proteins important for mammalian infection are produced when the bacteria are cultured at mammalian body temperature (37°C), whereas, proteins involved in tick colonization are produced in response to culture at a temperature representative of an unfed tick (23°C) (Schwan et al., 1995; Stevenson et al., 1995; Schwan and Piesman, 2000; Yang et al., 2000; Ojaimi et al., 2003). Guyard et al. demonstrated that, in *B. hermsii*, *bhpA* transcription is significantly higher at 37°C relative to 23°C, suggesting a potential role for BhpA during mammalian infection. Additionally, Guyard et al. showed production of BhpA at 23, 34, and 37°C by immunoblot, but did not quantify possible temperature-dependent differences (Guyard et al., 2006). We therefore sought to assess whether production of BtpA in *B. turicatae* was elevated at 37°C relative to 23°C. To this end, wild-type *B. turicatae* was cultured at these two temperatures and bacterial proteins were separated by SDS-PAGE. Gel slices were prepared from the lanes and proteins in each slice were identified and quantified via LC-MS/MS. Results for the proteins encoded by *btpA* and adjacent genes are presented in **Table 3**. BtpA-derived peptides were only detected at 37°C and not 23°C, supporting the hypothesis that BtpA plays a potential role during mammalian infection. Similarly, BT0790 and BT0791, a hypothetical protein and thymidine kinase, respectively, were produced at ∼3-fold more at mammalian body temperature relative to 23°C. In contrast, BT0789 and BT0792 were produced at similar levels between these two temperatures. These results agree with the transcriptional results of Guyard et al. (2006), and suggest that BtpA, as well as BT0790 and BT0791, may be important for mammalian infection.

btpA Is Transcribed in an Operon

Analysis of the *B. turicatae* genome annotation revealed that the *btpA* ORF is encoded on the same DNA strand as *bt0790* and *bt0791*. Additionally, *btpA* is located 14 bp downstream of *bt0790* and overlaps 34 bp with *bt0791* (Hyde and Johnson, 1986; Fraser et al., 1997; Penningon et al., 1999; Guyard et al., 2006; Miller et al., 2013). Due to the proximity of *btpA* to the immediately adjacent genes and similar protein production patterns of BT0790, BtpA, and BT0791 (higher production at 37°C relative to 23°C), it was hypothesized that *bt0790*, *btpA*, and *bt0791* may be in an operon. To test this, RT-PCR was performed with primers designed to amplify across intergenic regions of adjacent ORFs within the potential operon (**Figure 1A**), as well as to amplify an internal region of *flaB* as a positive RT control. RT-PCR for *flaB* generated an amplicon of appropriate size, confirming successful RNA isolation and cDNA synthesis from *in vitro*-grown *B. turicatae* (**Figure 1B**). Amplification products were also obtained from reactions to assess linkage of *bt0790* to *btpA* and *btpA* to *bt0791*. Furthermore, an amplification product was observed linking *bt0790* to *bt0791*, indicating that *btpA* is transcribed with the immediately upstream (*bt0790*) and downstream (*bt0791*) genes.

Because of the colinear nature of *Borrelia* chromosomes, we hypothesized that the analogous region of LD spirochete genomes would also be co-transcribed. To test this, RT-PCR analyses were performed using *B. burgdorferi*. Reactions were conducted to amplify across the intergenic region between *bb0790* and *bb0791*, as well as an internal region of *flaB* as a positive control (**Figure 1C**). RT-PCR results indicated transcriptional linkage of *bb0790* and *bb0791*, suggesting that the operonic nature of this region of the chromosome is conserved among diverse *Borrelia* species.

Inactivation of *btpA* in *B. turicatae*

Because Guyard et al. were unable to inactivate *bhpA* in *B. hermsii*, they could not directly test their hypothesis that BhpA is important for mammalian infection (Guyard et al., 2006). Their inability to mutate *bhpA* also led them to hypothesize that this protease might be required for bacterial viability. Contrary to the findings of Guyard et al., we were able to mutate *btpA* using two different allelic exchange-based mutational approaches (Lopez et al., 2013). The *btpA*::P*flgB-aacC1* mutant was made by replacing a 1012-bp internal region of the *btpA* ORF with an *aacC1* gentamicin resistance gene under transcriptional control of the promoter for the flagellar basal body rod protein (*flgB*) (**Figure 2A**). Considering that *btpA* is encoded in an operon, this mutational approach has the potential to be problematic as the *flgB* promoter could cause overexpression of the thymidine kinase gene, *bt0791*, that is downstream of *btpA*. To our knowledge, detrimental effects due to overexpression of a native thymidine kinase in prokaryotes has not been reported. However, to circumvent potential complications, an alternative mutational construct was also employed to generate a second independent *btpA* mutant. In this construct, designated *btpA*::*aacC1*, a promoterless *aacC1* marker was fused at the start codon of the *btpA* ORF to replace 1,446 bp of the 1,641-bp coding region (**Figure 2B**). In the *btpA*::*aacC1* mutant, the *aacC1* resistance gene is under transcriptional control of the native promoter of the *bt0790-bt0791* operon, thus reducing the possibility of detrimental polar mutation effects. Genotypic confirmation of the *btpA* mutants was performed by PCR amplifying a chromosomal region flanking the inserted antibiotic resistance marker, as well as internal regions of *btpA*, *aacC1*, and *flaB* (a positive control gene) (**Figure 2C**). In both *btpA* mutants, amplicons of the expected sizes were observed, and primers specific for an internal region of *btpA* only generated an amplicon with gDNA for wild-type *B. turicatae*. In addition, the *aacC1* marker was only detected in the *btpA* mutants. RT-PCR analyses were also conducted to confirm both the absence of *btpA* transcription in mutants and that the mutational strategies utilized did not significantly affect expression of other genes in the *bt0790-bt0791* operon (**Figure 2D**). In wild-type *B. turicatae*, *bt0790*, *btpA*, and *bt0791* transcription was readily detectable. As expected in both mutants however, *btpA* transcript was absent, whereas *bt0790* and *bt0791* were expressed. Successful generation of *btpA* mutants indicates that BtpA is not required for TBRF spirochete viability, as was previously hypothesized (Guyard

TABLE 3 | Impact of cultivation temperature on production of BtpA and proteins encoded by adjacent chromosomal genes in *B. turicatae*.

Protein	23°C average spectral counts (± SEM)	37°C average spectral counts (± SEM)	Fold change in production (37°C/23°C spectral counts)	Identity
BT0789	104.30 ± 4.92	83.80 ± 1.58	0.80	FtsH family protease
BT0790	2.16 ± 0.09	6.62 ± 0.13	3.06	Hypothetical protein
BT0790A (BtpA)	0.00 ± 0.00	3.85 ± 0.48	>3.85*	HtrA family serine protease
BT0791	0.67 ± 0.67	3.33 ± 1.17	4.97	Thymidine kinase
BT0792	1.49 ± 0.75	1.10 ± 0.02	0.74	Hypothetical protein

**Only detected at 37°C.*
SEM; Standard error of the mean.

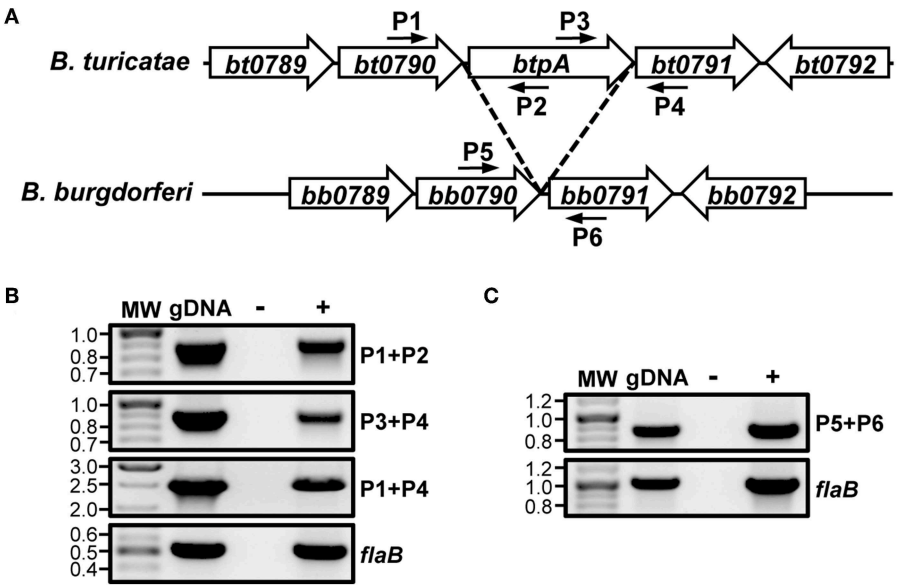

FIGURE 1 | *btpA* is transcriptionally linked to *bt0790* and *bt0791*. **(A)** Selected regions of *B. turicatae* and *B. burgdorferi* chromosomes; adapted from Guyard et al. (2006). Numbered smaller arrows indicate primers used in **(B,C)**. **(B)** Transcriptional linkage of *btpA* to *bt0790* and *bt0791* in *B. turicatae.* RNA was isolated from wild-type *B. turicatae* culture and converted to cDNA (+). Mock reactions without reverse transcriptase were performed as a negative control (–). Wild-type *B. turicatae* genomic DNA (gDNA) was included as a positive control for each reaction. Approximate locations of primers in the genome are indicated by smaller arrows and numbers in **(A)**. PCRs were performed to amplify intergenic regions between genes within the suspected operon: *bt0790-btpA* (P1+P2, 886 bp), *btpA-bt0791* (P3+P4, 887 bp), and *bt0790-bt0791* (P1+P4, 2,527 bp). Another PCR was performed to amplify an internal region of *flaB* (519 bp). MW denotes the DNA standard, and numbers to the left indicate molecular weights in kb. **(C)** Transcriptional linkage of *bb0790* and *bb0791* in *B. burgdorferi.* RNA was isolated from *B. burgdorferi* culture and converted to cDNA (+). Mock reactions without reverse transcriptase were performed as a negative control (–). *B. burgdorferi* genomic DNA (gDNA) was included as a positive control for each reaction. Approximate locations of primers in the genome are indicated by smaller arrows and numbers in **(A)**. A PCR was performed to amplify an intergenic region between *bb0790-bb0791* (P5+P6, 861 bp). Another PCR was performed to amplify an internal region of *flaB* (1,006 bp). MW denotes the DNA standard, and numbers to the left indicate molecular weights in kb.

et al., 2006), and allowed us to directly assess the importance of this protease in *B. turicatae* resistance to environmental stresses and during mammalian infection.

Because *btpA* is transcribed as part of an operon, and RT-PCR is only semi-quantitative, qRT-PCR analyses were conducted in order to detect possible polar effects associated with mutation of *btpA*. To this end, RNA was isolated from wild-type, *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1* strains, converted to cDNA, and expression of genes in the *btpA*-containing operon (*bt0790*, *btpA*, and *bt0791*) was quantified (**Figure 3**). As expected, *btpA* expression was not detected in *btpA* mutants. In addition, expression of *bt0790* was similar between wild-type, *btpA*::P*flgB-aacC1* (mean = 0.96; SEM = 0.87–1.06), and *btpA*::*aacC1* (mean = 1.02; SEM = 0.79–1.32) strains. However, expression of *bt0791* was increased approximately 1.53-fold (SEM = 1.40–1.68) in the *btpA*::P*flgB-aacC1* mutant, consistent with our hypothesis that the P*flgB* promoter may drive overexpression of this downstream gene. In contrast, in the *btpA*::*aacC1* mutant, a 0.44-fold (SEM = 0.41–0.47) decrease in expression relative to wild-type *B turicatae* was observed. These observations suggest that subtle polar mutations were introduced with regard to *bt0791* expression via both mutational strategies. Therefore, it was important that the

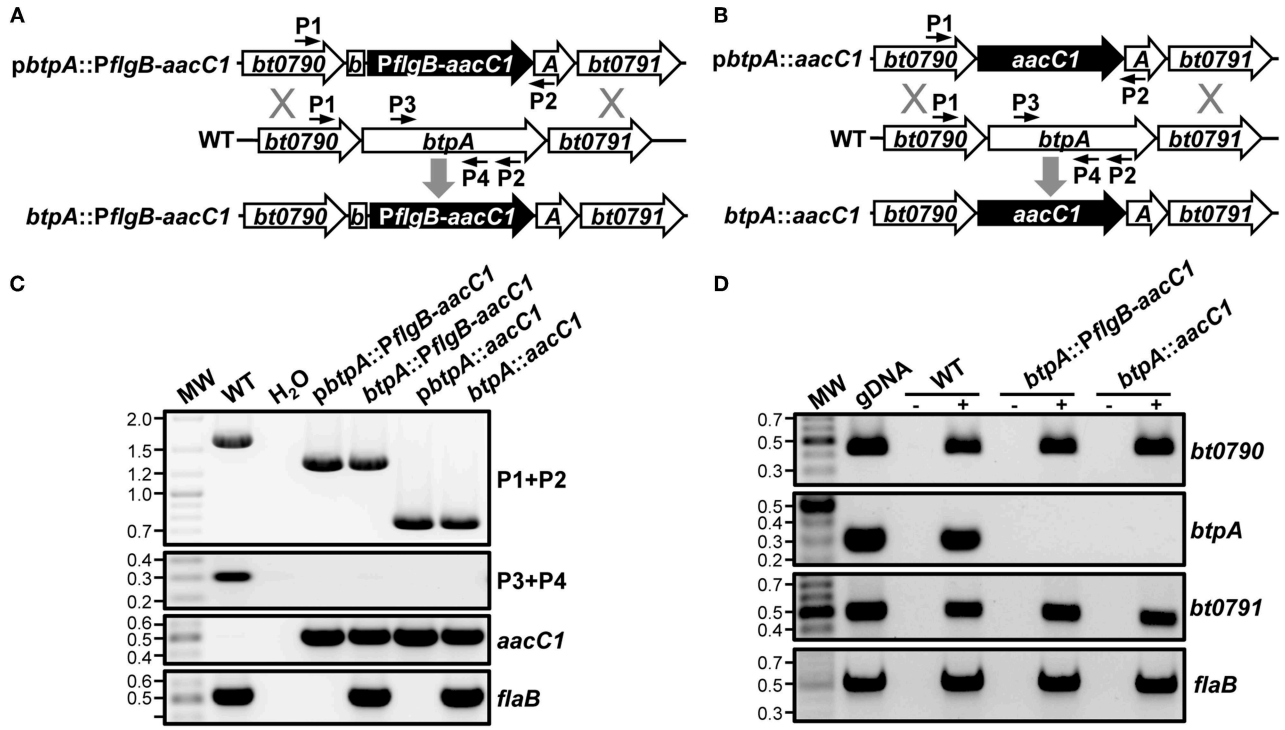

FIGURE 2 | Generation of *B. turicatae btpA* mutants. **(A)** Organization of *btpA*::P*flgB-aacC1* mutant. A segment of *btpA* in wild-type *B. turicatae* (WT) was replaced with the *aacC1* gentamicin resistance marker expressed from the *B. turicatae flgB* promoter (*btpA*::P*flgB-aacC1*) via allelic exchange. **(B)** Organization of *btpA*::*aacC1* mutant. A segment of *btpA* in wild-type *B. turicatae* (WT) was replaced with a promoterless *aacC1* gentamicin resistance marker via allelic exchange. **(C)** Genotypic confirmation of *btpA* mutants. PCRs were conducted to amplify a region flanking the insertion site of the *aacC1* marker in *btpA* (P1+P2; WT, 1,649 bp; *btpA*::P*flgB-aacC1,* 1,319 bp; or *btpA*::*aacC1*, 745 bp) or internal segments of *btpA* (P3+P4; 305 bp), *aacC1* (689 bp), and *flaB* (519 bp). PCRs were conducted using genomic DNA (gDNA) from wild-type *B. turicatae* (WT), *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1*, as well as the plasmids used to generate the mutants. PCRs were also conducted with no DNA as a purity control (H_2O). MW denotes the DNA standard, and numbers to the left indicate molecular weights in kb. **(D)** Transcriptional confirmation of *btpA* mutants. RNA was isolated from wild-type *B. turicatae* (WT), *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1* culture and converted to cDNA (+). Mock reactions without reverse transcriptase were performed as a control (–). Wild-type *B. turicatae* genomic DNA (gDNA) was included as a positive control for each reaction. PCRs were conducted with cDNA to amplify internal regions of *bt0790* (450 bp), *btpA* (305 bp), *bt0791* (505 bp), and *flaB* (519 bp). MW denotes the DNA standard, and numbers to the left indicate molecular weights in kb.

following experiments assessing *in vitro* and *in vivo* phenotypes associated with loss of *btpA* were conducted using both mutants in case slightly increased or decreased expression of *bt0791* in *btpA*::P*flgB-aacC1* and *btpA*::*aacC1* strains, respectively, could account for any phenotypes observed.

Sensitivity to High Temperature Is Not Altered in *btpA* Mutants

High temperature requirement (HtrA) family proteases are designated as such because *htrA* mutants in *E. coli* exhibit a decreased growth rate when cultured at elevated temperatures (Lipinska et al., 1989). Subsequently, *htrA* mutants of several other bacteria, including *Y. enterocolitica*, *Campylobacter jejuni*, and *Brucella abortus*, were found to possess similar growth defects (Elzer et al., 1994; Li et al., 1996; Brondsted et al., 2005). To first evaluate whether *btpA* is required for adaptation of *B. turicatae* to elevated culture temperatures, wild-type, *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1 B. turicatae* strains were grown at standard culture temperature (35°C), as well as two higher temperatures (**Figures 4A–C**), and counted daily. At the normal growth temperature, *btpA* mutants had similar growth to wild-type *B. turicatae*. Interestingly, *btpA* mutants also grew comparable to wild-type bacteria at 37 and 39°C. However, when we attempted to grow all strains at temperatures exceeding 39°C, no growth was observed (data not shown). To evaluate whether the *btpA* mutants exhibit a growth defect in response to the environmental temperature changes experienced by *B. turicatae* upon transition from tick to mammal, an experiment was conducted in which wild-type and *btpA* mutant strains were cultured at 23°C before being shifted to 37°C. Specifically, cultures were inoculated at an initial density of 5×10^5 bacteria/mL, incubated at 23°C for seven days, and then shifted to a temperature of 37°C. *B. turicatae* cultured at 23°C do not demonstrate significant growth. Therefore, a higher inoculation density was required to monitor bacterial concentration and viability by dark-field microscopy at 23°C prior to culture at 37°C. In these growth comparisons, both wild-type *B. turicatae* and the *btpA* mutants demonstrated minimal growth at 23°C. Upon shifting the cultures to 37°C, the two *btpA* mutants and wild-type parent grew similarly

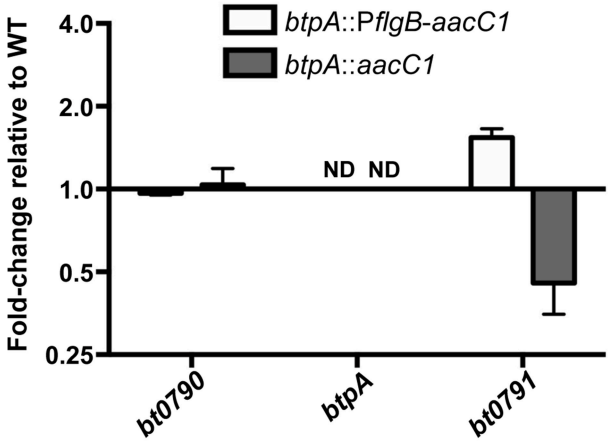

FIGURE 3 | *btpA* mutants exhibit modest changes in *bt0791* expression. cDNA was generated from *in vitro* cultured wild-type, *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1 B. turicatae* RNA and used for qRT-PCR analyses to determine possible expression changes in *bt0790*, *btpA*, and *bt0791*. Displayed are results from two independent biological replicates, and error bars represent standard error of the mean (SEM) with respect to fold-change values. Not detected, ND.

(**Figure 4D**), indicating that *btpA* is not required by *B. turicatae* to survive environmental temperature shifts encountered during tick-to-mammal transmission. Collectively, these results suggest that, unlike HtrA family homologs in other bacterial species, BtpA is not required for resistance to heat shock *in vitro*.

btpA Confers Resistance to Oxidative Stress Produced by *t*-butyl Peroxide

Guyard et al. demonstrated that heterologously expressing *bhpA* in the LD spirochete, *B. burgdorferi*, increased its resistance to oxidative stresses in the form of *t*-butyl peroxide and diamide (Guyard et al., 2006). This finding led them to conclude that BhpA and orthologous proteins of TBRF spirochetes could play a role in resistance to oxidative stresses faced during bloodstream infection. Therefore, we hypothesized that *btpA* mutants would be more susceptible to ROS/RNS. To assess this hypothesis, the susceptibility of wild-type, *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1 B. turicatae* strains to killing by H_2O_2, *t*-butyl peroxide, and the NO donor diethylamine NONOate (DEA/NO) were compared. All three strains exhibited similar levels of survival following challenge with 0.25 mM H_2O_2 and 1.25 mM DEA/NO (**Figure 5**). In contrast, exposure of cultures to 2.5 mM *t*-butyl peroxide resulted in an ∼6-fold decrease in survival of the *btpA*-deficient *B. turicatae* strains (**Figure 5**). This latter finding is consistent with the previously described role of *bhpA* in defense against oxidative stress (Guyard et al., 2006).

btpA Is Not Required for Mammalian Infection

We hypothesized that BtpA would be important for resistance to environmental stresses based on previous work (Guyard et al., 2006). However, we were unable to detect a growth defect in *btpA* mutants after incubation at increased culture temperature

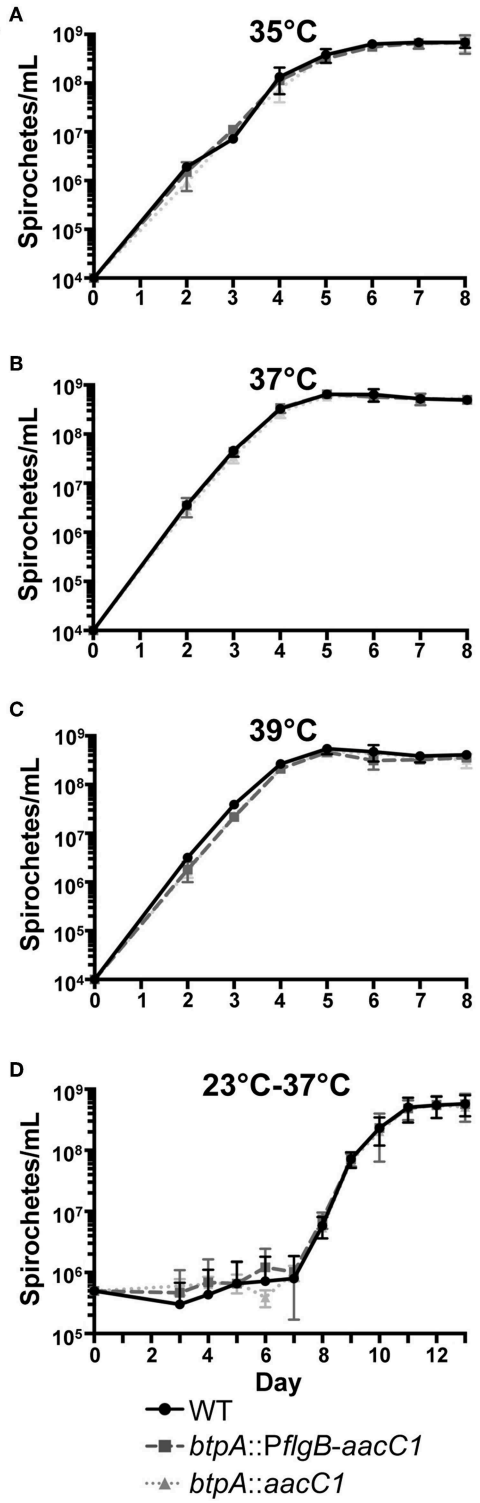

FIGURE 4 | *btpA* mutants do not exhibit attenuated growth at elevated temperature. **(A–C)** *B. turicatae* wild-type (WT), *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1* cultures were inoculated at an initial density of 10^4 spirochetes/mL, grown at **(A)** 35°C, **(B)** 37°C, or **(C)** 39°C, and then counted daily by dark-field microscopy beginning on day 2 post-inoculation.

(Continued)

FIGURE 4 | (D) *B. turicatae* wild-type (WT), *btpA*::P*flgB-aacC1*, and *btpA*::*aacC1* cultures were inoculated at an initial density of 5×10^5 spirochetes/mL at 23°C and then counted daily by dark-field microscopy beginning on day 3 post-inoculation. On day 7, cultures were shifted to a temperature of 37°C. Displayed are the results of two independent biological replicates, and error bars represent SEM.

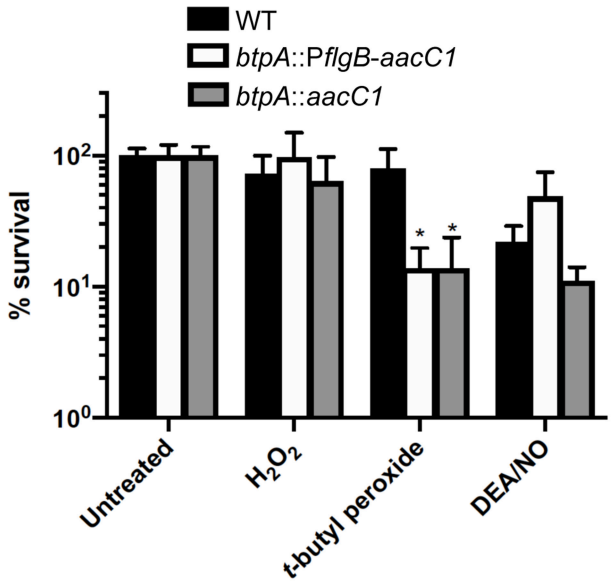

FIGURE 5 | *btpA* contributes to resistance to *t*-butyl peroxide. *B. turicatae* strains were cultured in pyruvate-free mBSK in the presence or absence of 0.25 mM H_2O_2, 2.5 mM *t*-butyl peroxide, or 1.25 mM diethylamine NONOate (DEA/NO) for 2 h under aerobic conditions (5% CO_2, 18% O_2). Following treatment, serial dilutions were prepared in mBSK with 7 mM sodium pyruvate and plated on solid mBSK media. Data represent the mean percent survival ± standard deviation (SD) of 4 to 6 biological replicates. $^*p < 0.05$ compared to wild-type *B. turicatae*.

and only observed a slight increase in the sensitivity of the *btpA* mutants to oxidative stress produced by *t*-butyl peroxide. Although the *in vitro* defects observed in the *btpA* mutants were relatively modest, BtpA may still be important for survival of the multiple, simultaneous stresses the bacteria face during mammalian infection. To assess if BtpA is required for both initial bacteremia and subsequent relapses, a murine model of RF was utilized. Mice were intradermally inoculated with 100 spirochetes and infection was allowed to proceed until the mice were sacrificed at day 14. This dose was selected because 100 spirochetes is the lowest challenge dose of wild-type *B. turicatae* with which we are able to consistently establish infection in mice (data not shown). This timing allows for detection of the initial peak in spirochetemia (approximately days 4–6), as well as at least one subsequent relapse. Blood samples were collected daily, and bloodstream bacterial burden was quantified by qPCR (**Figure 6**). In both WT and *btpA* mutant strains, the maximum bacterial burden was 10^5-10^7 bacteria/mL. Additionally, relapse kinetics in WT and mutant strains were similar, with initial bacteremic peaks occurring between days 4–6 and first relapse occurring before day 12. Although the overall maximum bacterial burdens in the mice infected with the *btpA* mutants do appear to be slightly lower by comparison to the mice infected with WT parent, these differences were not statistically significant. Based on these infection results, *btpA* is not required for initial infection or subsequent bacterial relapse, suggesting that *btpA* is dispensable for the mammalian phase of the *B. turicatae* enzootic cycle.

DISCUSSION

Although RF represents a significant public health issue worldwide (Dupont et al., 1997; Vial et al., 2006; Talagrand-Reboul et al., 2018), very little information exists regarding specific virulence determinants that RF *Borrelia* spirochetes require to navigate their enzootic cycle. Guyard et al. proposed that an HtrA family protease (BtpA and orthologs) unique to TBRF spirochetes may serve an essential function during mammalian infection (Guyard et al., 2006). In support of this hypothesis, Guyard et al. demonstrated that *bhpA* was more highly expressed when *B. hermsii* was cultured at mammalian body temperature. To confirm these results in *B. turicatae*, we conducted a proteomic analysis with wild-type *B. turicatae* cultured at 37°C and 23°C to identify differences in protein production. BtpA was only detected at 37°C and not 23°C, consistent with the *bhpA* expression results. In addition, proteins encoded by genes immediately adjacent to *btpA* were also produced more than 3-fold more at mammalian body temperature relative to 23°C, leading us to hypothesize that *btpA* was co-transcribed with *bt0790* and *bt0791*. RT-PCR analyses confirmed this hypothesis. It should be noted that there are two caveats associated with this conclusion. First, transcriptional patterns do not always directly correlate with protein levels. Yet, the failure to detect BtpA at 23°C, while detecting BT0790 and BT0791, could suggest that these three genes don't comprise an operon. However, there were low average spectral counts obtained for BT0790, BtpA, and BT0791 at 23°C (2.16, 0.00, and 0.67, respectively). Thus, the absence of BtpA is likely due to protein levels at 23°C approaching the limit of detection for the analysis. Importantly though, BT0790, BtpA, and BT0791 were consistently detected at 37°C, which agreed with the prior transcriptional studies (Guyard et al., 2006). Second, replacing a portion of the *btpA* ORF with a promoterless antibiotic resistance marker appeared to decrease transcription of *bt0791*. This observation could indicate that an independent promoter, which is partly responsible for transcription of *bt0791*, exists within the region of the *btpA* ORF that was also disrupted in the *btpA*::*aacC1* mutant. Nonetheless, we still observed transcriptional linkage between *btpA* and *bt0791* in addition to the regulatory trends seen in temperature-dependent protein production; again, the latter agreed with the prior transcriptional studies (Guyard et al., 2006). Further studies are required to determine if a second alternative promoter also controls transcription of *bt0791*. Interestingly, we found that the operonic content of the region of the chromosome encoding *bt0790-bt0791* is conserved in the colinear *B. burgdorferi* chromosome. Though these are divergent

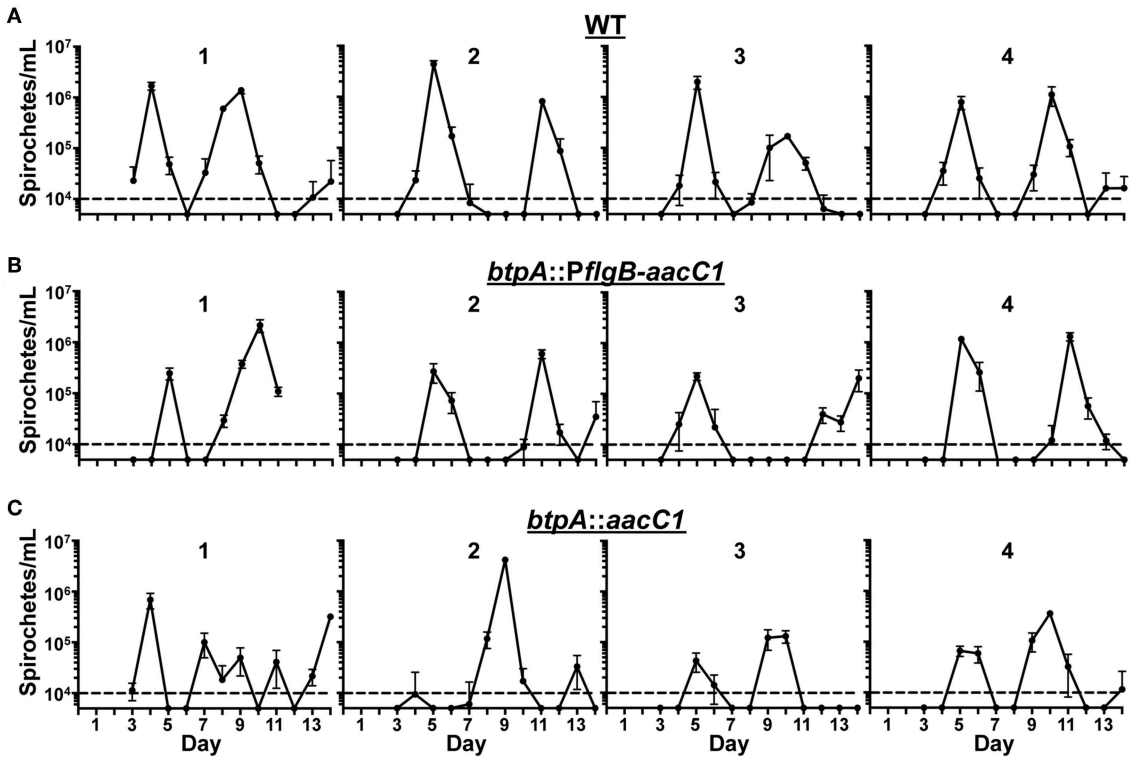

FIGURE 6 | *btpA* is dispensable for mammalian infection. Groups of four mice were inoculated with a dose of 10^2 spirochetes of the following strains; **(A)** wild-type (WT), **(B)** *btpA*::P*flgB-aacC1,* and **(C)** *btpA*::*aacC1*. Blood was collected daily between days 3 and 14, and spirochetemic burdens were then quantified by qPCR. Numbers above the graphs identify the mouse in each of the experimental groups. The dashed line represents the limit of detection of this assay (10^4 spirochetes/mL), and error bars represent SEM.

Borrelia species, transcriptional similarities may imply conserved regulatory mechanisms. Accordingly, differential regulation in response to culture temperature has been noted in both LD and TBRF spirochetes (Schwan et al., 1995; Stevenson et al., 1995; Schwan and Hinnebusch, 1998; Schwan and Piesman, 2000; Yang et al., 2000; Revel et al., 2002; Ojaimi et al., 2003; Guyard et al., 2006; Marcsisin et al., 2012; Wilder et al., 2016; Neelakanta et al., 2017). However, the regulatory pathways mediating temperature-dependent changes in gene regulation in TBRF spirochetes remain unknown.

Guyard et al. was unable to directly test the hypothesis that the BtpA homolog of *B. hermsii*, BhpA, was important for resistance to environmental stresses *in vitro* or for mammalian infection due to an inability to generate a *bhpA* mutant (Guyard et al., 2006). In the LD spirochete, *B. burgdorferi*, molecular genetics are commonly used as a tool to assess the roles of proteins during the enzootic cycle (Rosa et al., 2005; Groshong and Blevins, 2014; Drecktrah and Samuels, 2018). However, as TBRF is relatively understudied, genetic manipulation of TBRF spirochetes is less frequently published. In fact, at the time of publication of the findings by Guyard et al., successful genetic manipulation of TBRF spirochetes had yet to be reported (Guyard et al., 2006; Battisti et al., 2008; Fine et al., 2011; Lopez et al., 2013). Moreover, genetic manipulation in TBRF spirochetes is still in its infancy, as the *btpA* mutants reported herein represent just the third publication of targeted mutagenesis in *B. turicatae* and the eighth publication utilizing targeted mutagenesis in all TBRF spirochetes (Battisti et al., 2008; Fine et al., 2011, 2014; Lopez et al., 2013; Raffel et al., 2014; James et al., 2016; Krishnavajhala et al., 2017). Additionally, the *btpA*::*aacC1* mutant is the first published use of a promoterless resistance marker for a mutagenesis approach in TBRF spirochetes. Using newly established means of genetically manipulating *B. turicatae*, we were able to extend the work of Guyard et al. and directly test whether BtpA is an important virulence factor in TBRF spirochetes mediating resistance to stresses faced during mammalian infection.

Based on phenotypes associated with *htrA* mutation in other bacteria and heterologous expression experiments performed by Guyard et al., we hypothesized that *btpA* mutants would exhibit increased sensitivity to heat shock and oxidative stress (Lipinska et al., 1989; Johnson et al., 1991; Elzer et al., 1994; Li et al., 1996; Cortes et al., 2002; Brondsted et al., 2005; Guyard et al., 2006; Wilson et al., 2006). However, no differences in growth were identified when the *btpA* mutants were cultured at higher temperatures. Interestingly though, we found that *B. turicatae* is unable to grow *in vitro* at temperatures exceeding 39°C. This differs from *B. burgdorferi sensu lato* strains, which can grow at temperatures up to 41°C (Hubalek et al., 1998), and is especially surprising given the high body temperatures associated

with TBRF [up to 41.7°C (107°F) in humans] (Dworkin et al., 1998; Talagrand-Reboul et al., 2018). Further studies are required, however, to assess if *in vitro* sensitivity to elevated culture temperature is unique to *B. turicatae* or if this is common among TBRF spirochetes. Next, to evaluate susceptibility of *btpA* mutants to oxidizing agents, a semi-solid media plating-based approach was utilized (Raffel et al., 2018; Bourret et al., 2019). Bourret et al. recently showed that TBRF spirochetes are significantly more resistant to H_2O_2 relative to *B. burgdorferi*, hypothesizing that BtpA could be involved in this differential susceptibility (Bourret et al., 2019). Interestingly though, we failed to identify a role for BtpA in resistance to oxidative stress produced by H_2O_2. The ability of *B. turicatae* to resist higher levels of H_2O_2 relative to *B. burgdorferi* appears to therefore be independent of BtpA, indicating the existence of other unidentified mechanisms found in TBRF *Borrelia*, but not in LD *Borrelia*, that are involved in resistance to ROS. In contrast, we observed a modest contribution of BtpA in the defense of *B. turicatae* against *t*-butyl peroxide. This difference is likely due to the fact that organic peroxides primarily target polyunsaturated fatty acids in *Borrelia* cell membranes (Boylan et al., 2008), while oxidative stress produced by H_2O_2 occurs after it diffuses across the cell envelope and presumably produces hydroxyl radicals ($^{\bullet}OH$) as the result of Fenton chemistry (Imlay, 2003). Future studies will be required to determine whether BtpA protects the *B. turicatae* cell membrane against lipid peroxidation caused by organic peroxides, or whether it confers resistance to oxidative stress by a different mechanism.

A potential limitation of this study with respect to interpretation of the phenotype of *btpA* mutants upon treatment with *t*-butyl peroxide is the absence of genetic complementation. Complementation would ensure that this increased sensitivity can be directly attributed to the loss of *btpA* specifically, and not due to an off-target effect associated with either randomly acquired mutations or polar effects introduced via mutational strategies. However, as noted above, genetic manipulation of TBRF spirochetes is still in its infancy. As such, successful complementation of a targeted mutagenesis approach in TBRF spirochetes has only been reported in one study using *B. hermsii* (Raffel et al., 2014). Importantly, this study utilized a method of plasmid incompatibility to perform complementation of a gene on a small (28-kb) linear plasmid. This method requires integration of an antibiotic resistance marker into the plasmid of interest in the wild-type *Borrelia* strain. Genomic DNA, isolated from a bacterial clone containing the plasmid with the resistance marker, is transformed into the mutant strain in order to replace the mutated plasmid with a plasmid containing the wild-type copy of the gene. This method is not possible in the case of our study however, as *btpA* is encoded on the ∼1 Mb chromosome. Other possible complementation methods, such as site-specific integration and use of a shuttle vector, are also problematic. First, a stable shuttle vector has not yet been described for use in *B. turicatae*. Second, *cis*-based complementation by integration and restoration of *btpA* at the original location in the genome is complicated by the location of the gene in the middle of an operon, as well as organization of the genes flanking the operon. Additionally, integration of the gene into another site in the *btpA* mutant genome is complicated by the lack of a commonly used integration site that has been empirically proven not to lead to polar effects. The region analogous to the *bb0445-bb0446* integration approach is not conserved in *B. turicatae* and contains a unique gene in this intergenic region (Li et al., 2007; Promnares et al., 2009; Yang et al., 2009; Zhang et al., 2009; Pitzer et al., 2011; Moon et al., 2016). While a green fluorescent protein allele has been integrated into the genome of *B. turicatae*, integration occurred at an unknown location, and thus could lead to other potential off-target effects (Krishnavajhala et al., 2017). The lack of complementation in this study highlights the need for further development of genetic tools for use in *B. turicatae*. Because restoration of *btpA* expression in the mutants will be essential for studies intended to delineate the role of *btpA* during tick colonization and transmission, we are currently developing an alternative site-specific integration approach that will be suitable for complementation in *B. turicatae*. To overcome the lack of complementation, our study used two independent clones generated with different mutational constructs, and similar phenotypes were seen with each mutant. The probability that these independently generated mutants have similar randomly acquired mutations is not likely. We did observe subtle changes in expression of *bt0791* in each of the *btpA* mutants, indicating polar effects may have been introduced by the mutational strategies used. However, *bt0791* expression was slightly increased in the *btpA*::P*flgB-aacC1* mutant, whereas expression was slightly decreased in the *btpA*::*aacC1* mutant. The observation that these mutants exhibited similar oxidative stress phenotypes, but contained subtle, but opposite, changes in *bt0791* expression indicates that polar mutations are likely not the reason for decreased resistance to *t*-butyl peroxide. Additionally, bacterial thymidine kinases are not known to be involved in resistance to oxidative stress, further arguing against the *t*-butyl peroxide phenotype being associated with polar effects. Finally, our results demonstrating decreased resistance to *t*-butyl peroxide agree with previous heterologous expression experiments (Guyard et al., 2006). Therefore, these findings support the supposition that the mutants' increased sensitivity to *t*-butyl peroxide is due to loss of *btpA* and not an off-target effect, despite lack of complementation.

We observed only modest defects in the ability of *btpA* mutants to withstand environmental stresses *in vitro*, but it was still possible that BtpA could be required for mammalian infection. However, using a murine model of RF, we found that BtpA was not required for the mammalian phase of the enzootic cycle. The modest defects in the *btpA* mutants *in vitro* and their lack of attenuation *in vivo* suggest that compensatory mechanisms exist that render BtpA dispensable in these assays. Interestingly, there is another HtrA family serine protease encoded in the genomes of TBRF spirochetes. BT0104, referred to hereafter as BtHtrA, is a chromosome-encoded protein that is conserved among TBRF and LD *Borrelia* (Hyde and Johnson, 1986; Fraser et al., 1997; Penningon et al., 1999; Guyard et al., 2006; Miller et al., 2013). BtHtrA and TBRF spirochete orthologs have not been investigated, but the *B. burgdorferi* homolog, BB0104/BbHtrA, has been extensively studied (Coleman et al., 2013, 2016, 2018; Gherardini, 2013; Kariu et al., 2013; Russell

and Johnson, 2013; Russell et al., 2013; Ye et al., 2016). Several of the substrates recognized by BbHtrA have been identified as virulence factors of *B. burgdorferi*, including BB0323, P66, BmpD, and CheX, and BbHtrA appears to also play a key physiological role, as mutants demonstrate morphological and structural defects (Coleman et al., 2013; Kariu et al., 2013; Ye et al., 2016). In line with a role in physiology and processing of virulence factors, BbHtrA is required for mammalian infection of *B. burgdorferi* (Ye et al., 2016). Intriguingly, while the function of BbHtrA has not been investigated with respect to oxidative and nitrosative stresses, *bb0104* mutants do exhibit a growth defect at 37°C (Ye et al., 2016). These observations may indicate that BtHtrA could compensate for loss of *btpA* with respect to heat shock, mammalian infection, and possibly ROS/RNS resistance. However, two observations contradict this possibility. First, alignment of BtHtrA and BtpA revealed only 19.8% identity (Hyde and Johnson, 1986; Fraser et al., 1997; Penningon et al., 1999; Guyard et al., 2006; Miller et al., 2013). Similarly, BhHtrA and BhpA of *B. hermsii* share only 19.3% identity (Guyard et al., 2006). Furthermore, as mentioned by Guyard et al., BtpA homologs have a >100 bp C-terminal extension of unknown function not present in other HtrA family proteases (Guyard et al., 2006). Second, cellular localization experiments of BbHtrA of *B. burgdorferi* indicate surface localization (Russell and Johnson, 2013), as well as presence in the periplasm (Kariu et al., 2013). BhpA of *B. hermsii*, however, is only located intracellularly (Guyard et al., 2006). The lack of identity of BtHtrA and BtpA homologs and the difference in cellular localization indicates likely functional distinction between these two proteins.

While BtpA is dispensable for mammalian infection, it remains possible that BtpA has a function that is critical during another aspect of the TBRF spirochete enzootic cycle. Specifically, BtpA might be required for vector acquisition, vector colonization, or transmission from the vector to the mammal. Interestingly, Bourret et al. recently demonstrated that the salivary glands of *Ornithodoros turicata*, the tick vector for *B. turicatae*, were a highly oxidative environment (Bourret et al., 2019). Because TBRF spirochetes persistently colonize both the salivary glands and midgut of ticks, whereas LD spirochetes only persistently colonize the midgut, this may lead to an increased need for proteins involved in resistance to oxidative stress for TBRF spirochetes. We observed only a modest increase in the susceptibility of *btpA* mutant strains to *t*-butyl peroxide *in vitro*, but this assay does not fully recapitulate what TBRF spirochetes encounter in the tick environment. First, we treated the spirochetes with oxidative agents for 2 hours and then tested survival by plating. However, TBRF spirochetes have been noted to remain viable and infectious in unfed *Ornithodoros* ticks for several years (Barbour and Schwan, 2019). Therefore, if BtpA is required by *B. turicatae* to resist prolonged exposure to oxidative stress, the assay utilized may not detect this. Second, there are likely multiple assaults faced by TBRF spirochetes in the tick salivary glands that cannot be simulated by an *in vitro* oxidative stress assay. In addition to genes related directly to oxidative stress being transcribed in the *Ornithodoros* salivary glands (Bourret et al., 2019), Araujo et al. recently reported that several other anti-microbial genes are expressed in Argasid ticks, including microplusins, which can affect protein folding (Araujo et al., 2019). Perhaps the combined pressures of oxidative stress and other assaults renders BtpA essential for survival in the salivary glands. Finally, it is also possible that BtpA could be important for *B. turicatae* transmission from tick to mammal. It should be noted that the dose used for inoculation of mice in our murine infection experiments (10^2 bacteria) is at least 10-fold higher than the number of spirochetes that can possibly be deposited into the skin during *Ornithodoros* tick feeding (Boyle et al., 2014). Therefore, it is conceivable that this higher dose could overcome a potential necessity for BtpA upon entry into the mammal, as more spirochetes would increase the chances of at least one bacterium surviving the initial infection event and associated mammalian immune defenses. Future studies will evaluate the capacity of *btpA* mutants to successfully complete the tick phase of the *O. turicata-B. turicatae* enzootic cycle.

In summary, we found that BtpA, as well as proteins encoded by adjacent chromosomal genes (BT0790 and BT0791), are produced in response to culture at mammalian body temperature, consistent with a role in mammalian infection and prior transcriptional studies. The genes encoding BT0790, BtpA, and Bt0791 were subsequently shown to be transcribed in an operon. To determine if BtpA was required for resistance to environmental stresses and during mammalian infection, we inactivated *btpA* in *B. turicatae* using two mutational strategies. *btpA* mutants showed no defect in response to heat shock. However, the mutants did exhibit a modest increase in sensitivity to the oxidative agent, *t*-butyl peroxide, suggesting a possible role for BtpA in resistance to oxidative stress. Finally, *btpA* mutants were fully infectious in a murine model of RF. Future studies will determine if BtpA is required for acquisition/colonization in the tick and subsequent transmission from tick to mammal.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JB, CJ-L, TB, and JEL contributed to project conception, design of the study, or oversight of experiments. CJ-L, AZ, CR, and JIL performed experiments in the study. JB, CJ-L, and TB performed the data analysis and statistical tests and wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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yqhG Contributes to Oxidative Stress Resistance and Virulence of Uropathogenic *Escherichia coli* and Identification of Other Genes Altering Expression of Type 1 Fimbriae

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Urinary tract infections (UTIs) are common bacterial infections and the vast majority of UTIs are caused by extraintestinal pathogenic *Escherichia coli* (ExPEC) strains referred to as uropathogenic *E. coli* (UPEC). Successful colonization of the human urinary tract by UPEC is mediated by secreted or surface exposed virulence factors—toxins, iron transport systems, and adhesins, such as type 1 fimbriae (pili). To identify factors involved in the expression of type 1 fimbriae, we constructed a chromosomal transcriptional reporter consisting of *lux* under the control of the fimbrial promoter region, *fimS* and this construct was inserted into the reference UPEC strain CFT073 genome at the *att*Tn7 site. This *fimS* reporter strain was used to generate a Tn*10* transposon mutant library, coupled with high-throughput sequencing to identify genes that affect the expression of type 1 fimbriae. Transposon insertion sites were linked to genes involved in protein fate and synthesis, energy metabolism, adherence, transcriptional regulation, and transport. We showed that YqhG, a predicted periplasmic protein, is one of the important mediators that contribute to the decreased expression of type 1 fimbriae in UPEC strain CFT073. The Δ*yqhG* mutant had reduced expression of type 1 fimbriae and a decreased capacity to colonize the murine urinary tract. Reduced expression of type 1 fimbriae correlated with an increased bias for orientation of the *fim* switch in the OFF position. Interestingly, the Δ*yqhG* mutant was more motile than the WT strain and was also significantly more sensitive to hydrogen peroxide. Taken together, loss of *yqhG* may decrease virulence in the urinary tract due to a decrease in production of type 1 fimbriae and a greater sensitivity to oxidative stress.

Keywords: *Escherichia coli*, urinary tract, type 1 fimbriae, luciferase, stress

INTRODUCTION

Urinary tract infections (UTIs) can occur throughout the urinary tract within the urethra, bladder, ureters, or kidneys. UTIs are a common infectious disease with over 150 million cases documented worldwide each year (Stamm and Norrby, 2001; Foxman, 2014). Furthermore, the overwhelming majority of uncomplicated UTI cases (≥80%) are caused by extraintestinal pathogenic

Escherichia coli (ExPEC), referred to as uropathogenic *E. coli* (UPEC) (Ronald, 2002), which can belong to a diversity of phylogenetic groups or sequence types (Russo and Johnson, 2000; Chen et al., 2013). An estimated 50% of women experience at least one UTI during their life, and up to a quarter of those women are prone to recurrent UTIs (Foxman, 2002). Adherence of UPEC to host cells is a key event in initiating UTI pathogenesis and is important for overcoming strong urine flow, and to promote urinary tract colonization (Ulett et al., 2013; Flores-Mireles et al., 2015).

Fimbriae (pili) are filamentous structures that can mediate adherence of bacteria to host cell receptors (Mulvey et al., 1998; Nielubowicz and Mobley, 2010). For instance, type 1 fimbriae are critical for colonization of the bladder by UPEC (Gunther et al., 2002), to directly stimulate UPEC invasion into epithelial cells and aid in formation of intracellular reservoirs that may contribute to recurrent infection (Mysorekar and Hultgren, 2006). Type 1 fimbriae encoded by the *fimAICDFGH* (*fim*) genes, are one of the best-characterized UPEC chaperone-usher fimbriae. These fimbriae are produced by most *E. coli* strains including UPEC (Buchanan et al., 1985; Sivick and Mobley, 2010). The *fimA* gene encodes the type 1 major fimbrial subunit and the tip-located adhesin, encoded by *fimH*, mediates binding to α-d-mannosylated receptors, such as uroplakins, which are abundant in the bladder and other host surfaces containing mannosides (Connell et al., 1996; Wu et al., 1996). The *fim* promoter is located on a 314-bp invertible DNA promoter element (*fimS*), the orientation of which determines the transcriptional status (ON or OFF) (Abraham et al., 1985; Bjarke Olsen and Klemm, 1994). The switching orientation of *fimS* is controlled by two recombinases. FimB, which promotes inversion in both orientations, and FimE, which mediates the switching from phase-on to phase-off (Klemm, 1986; Gally et al., 1996). Two additional recombinases, IpuA and IpbA, present in UPEC strain CFT073 can also mediate switching of the invertible element independent of FimB and FimE (Bryan et al., 2006). Regulation of *fim* genes is affected by multiple environmental factors (including pH, osmolarity, temperature and oxygen levels) and at least three regulatory proteins are directly implicated (Lrp, IHF, and H-NS) (Unden and Kleefeld, 2004).

To establish a UTI, UPEC strains must resist environmental stresses in the bladder and kidneys including pH stress and wide fluctuations in osmolarity (Culham et al., 2001; Cahill et al., 2003). The high osmolality, high urea concentration, acidic pH and organic acids in urine can limit the growth and survival of *E. coli* within the urinary tract (Mulvey et al., 2000). Thus, an important aspect of UPEC virulence is the capacity to resist high osmolality and the denaturing effects of urea, and rapidly adapt to changes and stress encountered in these niches during establishment of the infection.

Previously, we showed that interference with phosphate homeostasis decreased the expression of type 1 fimbriae in strain CFT073 and attenuated UPEC virulence (Crépin et al., 2012b). The phosphate-specific transport (Pst) system negatively regulates the activity of the two-component signal transduction system PhoBR and also transports inorganic phosphate. Inactivation of the Pst system results in constitutive activation of PhoBR regardless of environmental phosphate availability (Wanner, 1996). As such, a *pst* mutant responds as if it is always under phosphate-limiting conditions. In UPEC, we showed that decreased virulence of a *pst* mutant is largely due to reduced expression of type 1 fimbriae. In avian pathogenic *E. coli* (APEC), an altered membrane homeostasis was also observed in a *pst* mutant (Lamarche and Harel, 2010) and caused increased sensitivity to acid, cationic antimicrobial peptides, and serum (Lamarche et al., 2005; Crépin et al., 2008; Bertrand et al., 2010).

In order to identify genes affecting the expression of type 1 fimbriae, we constructed a transcriptional luciferase (*lux*) reporter consisting of *lux* under the control of the *fimS* invertible promoter and this construct was inserted into the CFT073 genome at the *att*Tn7 site. This *fimS* reporter containing strain was used to generate a Tn*10* transposon mutant library, coupled with high-throughput sequencing to identify the location of transposon insertions that altered the expression of type 1 fimbriae. In this report, we show that YqhG is one of the important mediators that contribute to decreased expression of type 1 fimbriae in UPEC strain CFT073. Our results demonstrated that the deletion of *yqhG* in CFT073 reduced the expression of type 1 fimbriae and reduced urinary tract colonization of the *yqhG* mutant in the murine model. We also examined whether deletion of *yqhG* as well as the *pst* system also reduced resistance to environmental stresses, suggesting that altered expression of type 1 fimbriae can be linked to changes in bacterial adaptation to environmental stresses, such as oxidative or osmotic stress.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Plasmids

E. coli strains and plasmids used in this study are listed in **Table 1**. *E. coli* CFT073 was isolated from the blood and urine of a patient with acute pyelonephritis (Mobley et al., 1990). Bacteria were routinely grown in lysogeny broth (LB) (Alpha Bioscience, Baltimore, MD) at 37°C and in human urine. Urine was obtained from healthy female volunteers, 20–40 years old, with no occurrence of a UTI or antibiotic use within the last 2 months prior to collection. A protocol for obtaining biological samples from human donors was reviewed and approved by the ethics committee—Comité d'éthique en recherche (CER 19-507) of INRS. Urine was immediately filter sterilized (0.2-μm pore size), pooled, and frozen at −80°C and used within 2 weeks of sampling. Antibiotics and reagents when required were added at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol 30 μg/ml and diaminopimelic acid (DAP), 50 μg/ml.

Construction of the *fim-lux* Reporter Fusions

E. coli CFT073 harboring the *fimS* reporter was obtained by site-specific transposition of the *fimS-lux* genes at the chromosomal *att*Tn*7* site as described by Crépin et al. (2012a). Briefly, the promoterless *lux* operon of *Photorhabdus luminescens*

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
STRAINS		
CFT073	UPEC wild-type pyelonephritis strain (O6:K2:H1)	Mobley et al., 1990; Welch et al., 2002
QT1324	CFT073 Δ*oxyR*::Km; Km^r	Crépin et al., 2012b
QT1911	CFT073 *ΔpstSCA::FRT*	Crépin et al., 2012b
QT2087	MGN-617 + pLOF/Km; Ap^r, Km^r	Crépin et al., 2017
QT2117	QT1911::Tn*7*T-Gm::*pstSCA*; Gm^r	Crépin et al., 2012b
QT2138	CFT073 *ΔfimAICDFGH*::km; Km^r	Crépin et al., 2012b
QT2496	CFT073 + pSTNSK, Km^r	Crépin et al., 2012a
QT4791	χ7213 + pGP-Tn7-Cm-*PfimA* L-ON *luxCDABE*, Ap^r, Cm^r, Km^r	This study
QT4792	χ7213 + pGP-Tn7-Cm-*PfimA* L-ON *luxCDABE*, Ap^r, Cm^r, Km^r	This study
QT4793	χ7213 + pGP-Tn7-Cm-*PfimA* phase variable *luxCDABE*, Ap^r, Cm^r, Km^r	This study
QT4794	QT2496::Tn7T-Cm::*PfimA L-ON luxCDABE*, Cm^r	This study
QT4795	QT2496::Tn7T-Cm::*PfimA-L-OFF luxCDABE*, Cm^r	This study
QT4796	QT2496::Tn7T-Cm::*PfimA* phase variable *luxCDABE,* Cm^r	This study
QT4976	CFT073 *ΔpstSCA*::FRT +pSTNSK, Km^r	This study
QT5018	QT4976::Tn7T-Cm- *PfimA* phase variable *luxCDABE*, Cm^r	This study
QT5134 (BW25123)	*E. coli* BW25123, *yqhG::Km*	Baba et al., 2006
QT5178	CFT073 *ΔyqhG*::FRT	This study
QT5235	QT5178::Tn7T-Cm::*yqhGH,* Cm^r	This study
χ7213 (MGN-617)	*thi thr leu tonA lacY glnV supE ΔasdA4 recA*::RP4 2-Tc::Mu [λpir], Km^r	Kaniga et al., 1998
PLASMIDS		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	Datsenko and Wanner, 2000
pGP-Tn7-Cm	pGP-Tn7-FRT::Cm, Ap^r, Cm^r	Crépin et al., 2012a
pSTNSK-	pST76-K::*tnsABCD*, Km^r	Crépin et al., 2012a
pIJ461	pGP-Tn7-Cm::*luxCDABE;* Ap^r, Cm^r	This study
pIJ514	pGP-Tn7-Cm::*luxCDABE*; *rbs* Ap^r, Cm^r	This study
pIJ516	pGP-Tn7-Cm::*PfimA* L-ON *luxCDABE*, Ap^r, Cm^r	This study
pIJ517	pGP-Tn7-Cm::*PfimA*-L-OFF *luxCDABE*, Ap^r, Cm^r	This study
pIJ518	pGP-Tn7-Cm::*PfimA* phase variable *luxCDABE*, Ap^r, Cm^r	This study
pIJ543	pGP-Tn7-Cm::*yqhGH*, Ap^r, Cm^r	This study
pKD3	Template plasmid for the amplification of the *cat* gene bordered by FRT sites	Datsenko and Wanner, 2000
pKD4	Template plasmid for the amplification of the *km* cassette bordered by FRT sites	Datsenko and Wanner, 2000
pKD46	λ-Red recombinase plasmid Ts replicon; Ap^r	Datsenko and Wanner, 2000
pLOF/km	Tn*10*-based transposon vector delivery plasmid; Ap^r Km^r	Herrero et al., 1990

(*luxCDABE*) (Allen, 2000) was amplified with the primers CMD1733 and CMD1734 (**Supplemental Table 1**). This DNA fragment was digested with KpnI and ApaI (New England Biolabs), purified with the Biobasic kit and ligated into the multiple-cloning sites (MCS) of the mini-Tn*7*-containing vector pGP-Tn7-Cm, generating the vector pGP-Tn7-*lux* (pIJ461). An optimized ribosome-binding site, RBS, was then added to plasmid pIJ461 to generate the pIJ514 vector. Then, the plasmid pIJ514 was used to generate the vectors, pIJ516 *pfimA* phase L-ON, pIJ517 pfimA phase L-OFF and pIJ518 *pfimA* variable phase. The *fim* promoter from strain CFT073 was amplified by PCR with primers CMD1645 and CMD1646 (see **Supplemental Table 1**). Genomic DNA, digested with the restriction enzymes *Eco*RI and SalI, and ligated to pIJ514 previously digested with EcoRI and *Xho*I. Transformation into *E. coli* DH5α cells was followed by selection on LB plates containing chloramphenicol. Using the approach described by Gunther et al. (2002), point mutations in primer CMD1133 were introduced to block the promoter switch in the ON position, digested with the restriction enzymes SmaI and SalI, and ligated to pIJ514 plasmid digested with SmaI and XhoI. The resulting vectors pIJ516, pIJ517, pIJ518 were transformed in *E. coli* SM10 λ*pir*-derivative strain MGN-617.

Strain MGN-617 (pGP-Tn7-*fimS-lux*) was conjugated overnight with strain CFT073, containing plasmid pSTNSK, which encodes the Tn*7 tnsABCD* transposase genes, at 30°C on LB agar plates supplemented with DAP. Following overnight culture, the bacteria from agar plates were suspended in 1 ml of phosphate-buffered saline (PBS), washed twice in PBS, serially diluted, and cultured on LB agar supplemented with gentamicin, and incubated at 37°C. Colonies that grew were then tested

for sensitivity to kanamycin and ampicillin, indicating the likelihood of integration at *att*Tn7 and loss of the transposase-encoding plasmid pSTNSK. Insertion of Tn*7* into the *att*Tn7 site was verified by PCR (primers CMD26 and CMD1416 (see **Supplemental Table 1**).

Testing the *fim-lux* Fusions Under Different pH Conditions

To measure the changes following growth in media at different pH, LB was buffered using 0.1 M Na_2HPO_4-NaH_2PO_4 buffer. The media were prepared with a pH ranging between 4.4 and 7.0. *E. coli* containing *pfimA phase-variable, pfimA L-ON* and *pfimA-L-OFF lux* fusions were incubated overnight at 37°C, 250 rpm in 5 ml LB medium. The next day 3 μl of each overnight culture was transferred to 180 μL of buffered LB at a specific pH and incubated with agitation until mid-logarithmic phase had been reached in 96-well plates at 37°C. The luminescence and $O.D._{600nm}$ was measured each 15 min for 4 h using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek Instruments Inc.). The luminescence results were reported as relative luminescence units (RLU). The luminescence readings were normalized to the $O.D._{600nm}$ values. The luminescence of *lux* fusion-containing strains streaked on LB agar plates was recorded with a ChemiDoc XRS system equipped with Quantity One 1-D analysis software (Syngene Chemi Genius), with an integration time of 30 s.

Transposon Mutagenesis

Transposon mutagenesis was performed as described by Simms and Mobley (2008). Briefly, the MGN-617/pLOF-Km donor strain and recipient strain CFT073 carrying *fimS* phase variable reporter were cultured overnight (O/N) at 37°C in LB with appropriate antibiotics and supplements. Mixed cultures were prepared as a 1:4 donor-to-recipient ratio, placed onto LB agar plates supplemented with IPTG and DAP, and incubated O/N at 37°C. After incubation, cells were suspended in 1 ml of PBS, washed twice in PBS, serially diluted, and plated onto LB agar supplemented with kanamycin and incubated O/N at 37°C to select for the recovery of kanamycin-resistant transposon mutants of the CFT073 *fimS-lux* recipient strains. Colonies were screened for susceptibility to ampicillin to confirm loss of pLOF-Km.

Measurement of Luminescence of Insertion Mutants in CFT073 Carrying *PfimA-lux*

Transposon mutants of CFT073 carrying *fimS-lux* were cultured at 250 rpm in 150 μL of LB in a 96-well plate (Corning White With Clear Flat Bottom), and luminescence was measured at $O.D._{600nm}$. In total, 5,904 transformants were analyzed. The luminescence readings were normalized to the $O.D._{600}$ values to account for any differences in growth. Mutants with disrupted genes that resulted in higher or lower levels of luminescence than the WT *pfim-lux*-fusion containing strain, were confirmed phenotypically by quantification of type 1 fimbriae by yeast agglutination assays as described below. Mutants of interest were then streaked on LB agar over three successive rounds of subculture and then stored individually in 25% glycerol at −80°C.

Evaluation of Type 1 Fimbriae Production

The production of type 1 fimbriae was determined by yeast agglutination assay (Crépin et al., 2008). Briefly, the transposon mutants were cultured at 37°C in LB broth or human urine to mid-log phase. In our experiment, log-phase (period of steady-state growth in LB) is estimated to occur at OD_{600} (optical density at 600 nm) between 0.6 and 0.8 However, for growth in urine, cells reach a stationary phase at an OD_{600} of 0.5–0.9. As such, we used a growth of cells to an OD_{600} of 0.6 for cells for mid-log growth in LB and an OD_{600} of 0.4 for mid-log growth in human urine. Following centrifugation, 40 μl of an initial suspension of $\sim 2 \times 10^{11}$ cells ml^{-1} in PBS was transferred and serially diluted 2-fold in microtiter wells containing equal volumes of a 3% commercial yeast suspension in PBS. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive aggregation reaction.

Site-Specific Integration of Tn*10*

Genomic DNA of 32 clones was extracted from cultures using phenol-chloroform. DNA was sequenced at the Génome Québec Innovation Centre, McGill University. DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). DNA samples were generated using the NEB Next Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) as per the manufacture protocol. TruSeq adapters and PCR primers were purchased from IDT. Size selection of libraries containing the desired insert size was obtained using SPRI select beads (Beckman Coulter). Briefly, genomic DNA was fragmented and tagged with adapter sequence via one enzymatic reaction (tagmentation). We initially amplified by PCR the region between the end of the insertion (primer Tn_pLOF-Km-CS1:(5′ACACT GACGACATGGTTCTACAcgttgcgctgcccggattac 3′ [transposon-specific sequence is in lowercase])), and the Illumina adapter with primer 2 (5′ TACGGTAGCAGAGACTTGGTCTCTAG-CATAGAGTGCGTAGCTCTGCT 3′) to enrich for transposon insertion sites and allow multiplex sequencing. The thermocycler program was 94°C for 2 min, 94°C for 30 s, 55°C for 30 s 72°C for 30 s for 33 cycles and 72°C for 7 min. Each library was prepared with a unique Illumina barcode. We amplified this region to add the Illumina adapters for MiSeq sequencing: PE1-CS1 (AATGATACGGCGACCACCGA-GATCTACACTGACGACATGGTTCTACA) and primer 2. The libraries were then pooled in equimolar concentration and sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 Kit (500-cycles).

After determining the location of the transposon, the clones in the pool carrying the specific mutations were determined using a primer complementary to the transposon end and another primer complementary to the identified transposon-interrupted gene. Following DNA amplification of each clone by PCR, we

were able to determine which specific clones contained some of the identified site-specific insertions.

Construction of Site-Directed Mutants and Complementation of Strains

Mutations were introduced by lambda-red recombination as described using plasmids pKD3 and pKD4 as templates for chloramphenicol and kanamycin resistance cassettes, respectively (Datsenko and Wanner, 2000). Primers are listed in **Supplemental Table 1**. Antibiotic cassettes flanked by FLP recombination target (FRT) sites were excised by introduction of vector pCP20 expressing the FLP recombinase (Cherepanov and Wackernagel, 1995).

Preparation of Fimbrial Extracts and Western Blotting

Preparation of fimbrial extracts and western blotting were performed as described previously (Crépin et al., 2008), with anti-type 1 fimbriae serum from *E. coli* strain B_{AM}. Briefly, after the growth to log phase, the bacteria were harvested and resuspended in 5 ml of 150 mM NaCl-0.5 mM Tris-HCl (pH 7.8). Following incubation at 56°c for 1 h and centrifugation (3,000 × *g* for 10 min), the aliquot was precipitated with 10% trichloroacetic acid. Followed by centrifugation 20,000 × *g* for 15 min at 4°C, the pellet was washed twice with 0.5 M Tris-HCl-0.5 M EDTA (pH 12.0) and resuspended in 0.5 M Tris-EDTA.

Further, fimbrial extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were stained with Coomassie brilliant blue and transferred to a nitrocellulose membranes (Bio-Rad) for 60 min at 100 V. The membrane was blocked with supplemented with 0.05% Tween 20 (Pierce). Incubations with primary rabbit anti-*fim* (1:5,000) and secondary goat anti-rabbit (1:25,000) antibodies were carried out for 1 h at room temperature. SuperSignal West Pico chemiluminescent substrate (Pierce) was used for detection.

Detection and Quantification of the On/Off State of the *fimS* Region

The orientation of the *fimS* region was determined as described previously (Müller et al., 2009). Briefly, the *fimS* region was PCR amplified with the primers CMD1258 and CMD1259 (see **Supplemental Table 1**), to produce a 650 bp fragment. The DNA was then digested with HinfI and analyzed on a 2% agarose gel. Following digestion, the ON orientation produces fragments of 128 and 522 bp, whereas the OFF orientation generates fragments of 411 and 239 bp. Quantification of the ratio of cells in ON or OFF position was performed as described (Wu and Outten, 2009). The WT strain was cultured statically for 48 h at 37°C for use as a control for increased orientation of the ON position. The WT strain was also cultured for 24 h on LB agar plates at room temperature as a control to favor orientation in the OFF position.

Experimental UTI in CBA/J Mice

The animal study was reviewed and approved by the animal ethics evaluation committee—Comité Institutionel de Protection des Animaux (CIPA No 1608-02) of INRS. The murine experimental UTIs were carried out as described previously (Hagberg et al., 1983), using a single-strain infection model. Prior to inoculation, strains were grown for 16 h at 37°C with shaking (250 rpm) in 55 ml of LB medium. Six-weeks-old CBA/J female mice were inoculated through a catheter inserted in the urethra with 20 µl of the pellet containing 2×10^9 CFU of either UPEC strain CFT073, CFT073 Δ*yqhG* (QT5178) or the complemented strain (QT5235). After 48 h, mice were euthanized; kidneys and bladders were sampled, homogenized, diluted, and plated on MacConkey agar for enumeration of colonies.

Adhesion Assays

5,637 human bladder cells (ATCC HTB-9) were grown in RPMI 1640 medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/liter glucose, and 1.5 g/liter sodium bicarbonate. For the assays 5,637 cells were grown to confluency in RPMI 1640 and 2×10^5 cells/well were distributed in 24-well plates. Strain CFT073 and isogenic mutants were grown in LB medium at 37°C to the mid-log phase of growth (O.D. 0.6). Immediately before infection, cultures were washed with PBS, suspended in medium and inoculated at a multiplicity of infection (MOI) of 10 CFU per epithelial cell. Bacteria-epithelial cell contact was enhanced by a centrifugation at 600 × g for 5 min. After 2 h, cells were washed three times and lysed with PBS−0.1% sodium deoxycholate (DOC), serially diluted, and plated on LB agar plates. Quantification of cell-associated bacteria was performed as previously described (Martinez et al., 2000). To block adherence mediated by type 1 fimbriae, 2.5% α-d-mannopyranose was added.

Motility Assay

Motility assays were as previously described (Lane et al., 2005) with modification. Following overnight growth at 37°C, strains were cultured at 37°C in LB broth to mid-log phase. Strains were stabbed into the surface of soft agar (1% tryptone, 0.5% NaCl, 0.25% agar) using an inoculating needle. Care was taken not to touch the bottom of the plate during inoculation to ensure only swimming motility was assessed. After 16 h of incubation, the diameters of motility zones were measured. Three independent motility experiments for each strain were performed. Results were analyzed using a paired *t*-test.

Growth Under Conditions of Osmotic Stress

Strains were tested for growth under conditions of osmotic stress using NaCl or urea. Cultures were diluted 1:100 from overnight cultures grown in LB, grown until mid-log phase with shaking. They were serially diluted and plated on LB agar alone and LB agar supplemented with 0.3 M NaCl, 0.6 M NaCl, 0.3 M urea, or 0.6 M urea. Colonies were enumerated, and growth under each condition was compared to growth on LB agar.

Hydrogen Peroxide Sensitivity Assay

Sensitivity to H_2O_2 was determined by using an agar overlay diffusion method (Boyer et al., 2002). Briefly, overnight cultures were used to inoculate (1/100) fresh LB medium, and incubated until the $O.D._{600}$ reached 0.6. Then, 100 µl of each culture

were mixed with 3 ml molten top agar and poured onto an LB agar plate. A 6-mm-diameter Whatman filter disk containing 10 µl 30% H_2O_2 was placed on the agar surface and plates were incubated overnight at 37°C. Inhibition zone diameters were then measured.

Statistical Analyses

Statistical tests were obtained using the Prism 7.04 software package (GraphPad Software). Statistically significance between two groups was determined by unpaired *t*-test and comparison among three or more groups was obtained by one-way analysis of variance (ANOVA). For the independent infections, comparisons of the CFU mL^{-1} or CFU g^{-1} distributions were analyzed using the Mann–Whitney test.

RESULTS

The Single-Copy Integrated CFT073 *fimS-lux* Reporter System

To identify systems that alter the expression of type 1 fimbriae, we used a promoterless *lux* reporter system fused to the *fim* type 1 fimbriae promoter region, *fimS*. A *luxCDABE* reporter system originally from *Photorhabdus luminescens* (Allen, 2000) was used. The system encodes all the enzymes needed to produce a luminescent signal. The *lux* genes were introduced into the pGP-Tn7-*Cm* vector. An optimized ribosome binding site, *RBS*, was added to plasmid pIJ461 to generate pIJ514 vector. Further, we generated phase variable, *pfimA phase variable-lux* (pIJ518) to measure the expression of type 1 fimbriae in various conditions (**Figure 1A**). Using the same approach as described by Gunther et al. (2002), point mutations were introduced in order to lock the promoter in the ON- and OFF-position, respectively to generate *pfimA L-ON-lux* (pIJ516) and *pfimA L-OFF-lux (*pIJ517) (**Figure 1**).

Analysis of the *fim-lux* Fusions in UPEC CFT073 Grown Under Different pH Conditions

To better characterize the reporter system upon dynamic transcriptional changes, we set out to assay the response kinetics of the *pfimA* promoter, driving the expression of the type 1 fimbriae using the *lux* reporter system. This promoter has been extensively studied in *E. coli* and used to validate a *lux* reporter system and *lacZYA* fusion implemented on a single copy number plasmid (Schwan et al., 2002; Schwan and Ding, 2017). Thus, it has been reported that growth conditions play a substantial role in the ability of *E. coli* cells to undergo phase variation and alter expression of type 1 fimbriae. Of note, transcription of all of the *fim* genes was shown to be repressed in a low pH environment (Schwan et al., 2002). Following a similar protocol, the *fim-lux* reporter containing variant of CFT073 or its isogenic *pst* mutant was grown in LB adjusted at different pH conditions ranging from 4.4 to 7. To verify any differences in expression, the *E. coli* cells were grown to mid-logarithmic phase (OD_{600} of 0.6). The *fimS*-Locked ON fusion had the highest expression level and did not vary regardless of the pH during growth (**Figure 2A**). However, the neutral pH influenced the expression of the *fim* switch. A shift from pH 4.4 to a neutral pH of 7 in LB media resulted in a 2-fold increase in expression of the *fimS* promoter. Decrease in pH also diminished the production of type 1 fimbriae (**Supplemental Figure 1**). In addition, no significant change in growth rates was observed for growth of the CFT073 parent strain and the P*fimA*-Locked ON and P*fimA* variable *lux* derivatives, suggesting that the expression of the *lux* operon and its gene products had no adverse effects on bacterial growth (**Supplemental Figure 2**).

CFT073 derivatives carrying the *PfimA* promoter in either direct (*PfimA*-Locked ON) or opposite (*PfimA*-Locked OFF) orientation with respect to the *luxCDABE* operon were streaked on LB agar plates, together with the CFT073 strain and a *pst* mutant carrying the *PfimA* phase-variable *lux* fusion. Stronger emission was detected from the *fimS*-Locked ON fusion (**Figure 2B**), with single colonies producing a strong signal. By contrast, there was no luminescence signal detected in the negative control carrying the *PfimA-Locked-OFF* fusion or the CFT073 strain or its *pst* mutant carrying the *PfimA*-phase-variable-*lux fusion*, although a dim light emission could be detected for CFT073 containing the *PfimA*-phase-variable fusion after longer exposure. In addition, the stability of the *fimS*-Locked-ON-*lux* strain was evaluated after 10 passages without antibiotic selection and the maintenance and luminescence expression of this fusion was found to be stable.

The expression of *lux* derivatives was also compared in LB liquid media overnight. As expected, there was a high signal for the *fimS*-Locked ON strain (QT4794), no signal for the *fimS*-Locked *OFF* strain (QT4795) and an intermediate signal for the *fimS-lux* variable strain (QT4796) (**Figure 2C**). In contrast, the signal for Δ*pst, fimS-phase variable-lux* (QT5018) increased significantly (**Figure 2C**) when compared with the same mutant at OD_{600} 0.6 (**Figure 2A**). This result was similar to what was previously observed (**Supplemental Figure 1**) (Crépin et al., 2012b).

Screening for Transposon Mutants With Increased or Decreased *lux* Expression and Altered Production of Type 1 Fimbriae

To identify genes affecting the expression of type 1 fimbriae, *E. coli* CFT073 carrying the *fimS* phase-variable reporter (**Figure 1B**) was subjected to transposon mutagenesis. A mini-Tn*10* transposon carrying a kanamycin resistance marker pLOF/Km (Herrero et al., 1990) was randomly inserted into the bacterial chromosome. A transposon bank was screened for luminescence. Clones demonstrating increased or decreased light emission were identified. Transposon insertions that produced high or low levels of luminescence may identify genes encoding transcriptional repressors or activators of *fim*. Transposon insertions that result in low levels of luminescence suggest that the disrupted genes may promote *fim* transcription and production of type 1 fimbriae. A total of 5,904 transposon mutants were generated and stored individually in 96-well plates stored at −80°C. The mutants were assessed for luminescence at $O.D._{600nm}$ 0.6 with shaking at 37°C (**Figure 3A**).

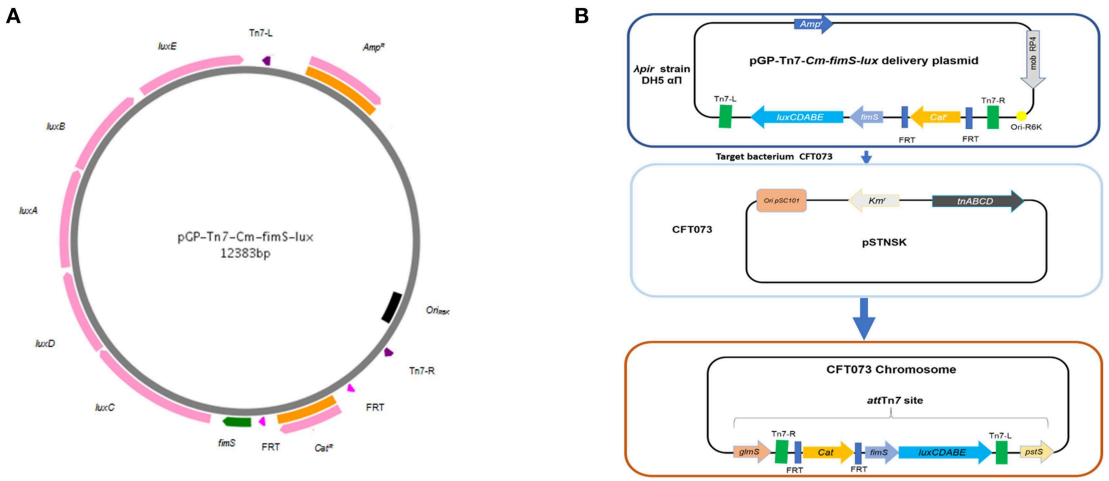

FIGURE 1 | Methods for site-specific insertion of *fimS-lux* fusions using mini-Tn 7-*lux* vector. **(A)** The mobilizable suicide vector pGP-Tn7-*Cm-fimS-lux* contains the conjugative transfer Mob RP4 and the *ori* R6K. A multiple cloning site is integrated between the two Tn*7* ends, where the promoterless *lux* reporter system is fused to the fimbrial promoter region, *fimS*. **(B)** Tn7-based transposition at the chromosomal *att*Tn7 site was achieved by conjugation, where the donor strain (*E. coli* SM10λ*pir*) harbored the mini-Tn7 vector and the recipient strain CFT073 contained the thermosensitive suicide vector pSTNSK pSC101-temperature sensitive origin and transposases *tnsABCD*. The Tn*7* transposon integrates at the site-specific *att*Tn*7*, located downstream of the highly conserved *glmS* gene.

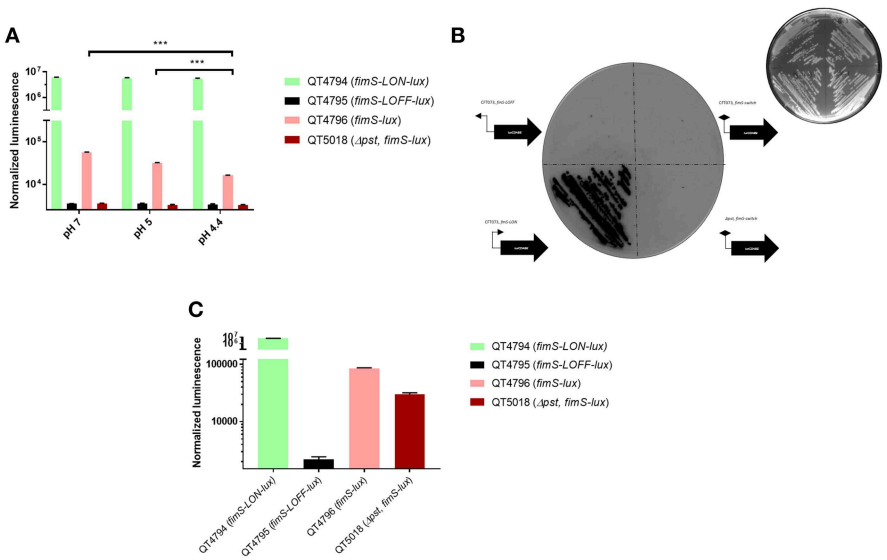

FIGURE 2 | Response of the *lux* reporter system in exponential-phase, overnight cultures and on agar. **(A)** Effects of pH on *fimS*-phase variable, *fimS*-Locked ON and *fimS*-Locked OFF expression was determined with *luxABCDE* transcriptional fusions in strain CFT073. Luminescence was normalized to the $O.D._{600nm}$ of the culture compared to CFT073; means ± standard deviations are indicated from at least three separate runs. **(B)** Inverted darkfield image of luminescence emitted on plates by CFT073 and *pst* reporter strains carrying *PfimA* from the constitutively expressed promoter (*fimS-LON*) or inverted promoter (*fimS-LOFF*) or switch variable (*fimS*-phase variable) direction with respect to *luxCDABE*. Colonies were grown overnight at 37°C and imaged using white light 400 ms (top right corner) or luminescence imaging for 30 s exposure time. Exposure images were acquired through ChemiDoc XRS system using high sensitivity chemiluminescence settings. **(C)** Response of the *lux* reporter system in overnight cultures in LB broth. Statistical significance was calculated by the Student *t*-test: $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

A subset of 65 transposon mutants demonstrating the highest and lowest levels of luminescence compared to the control CFT073 (attTn7 *PfimA-lux*) were further evaluated. Mutants that produced less luminescence than the control strain were further tested for levels of production of type 1 fimbriae by yeast agglutination assay (Crépin et al., 2012b). From this secondary screen, 48 transposon mutants were confirmed and there was at least a 4-fold increase or decrease in yeast agglutination ($p<0.0001$) (**Figure 3B**). Some of these mutants are currently being investigated to determine the transposon

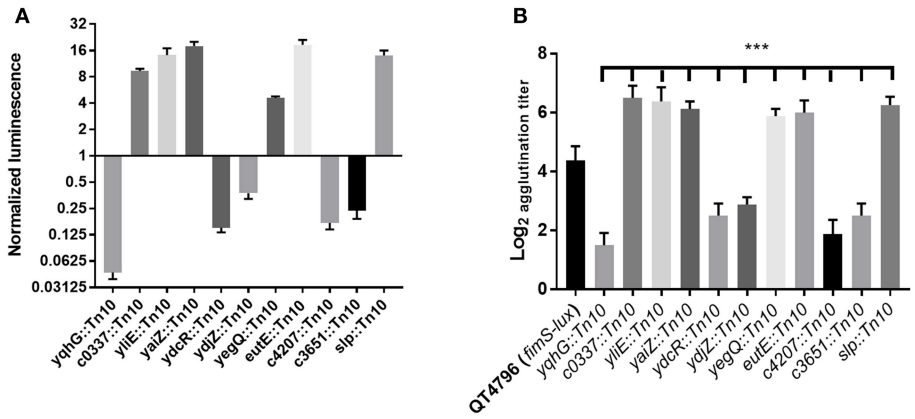

FIGURE 3 | Screening of transposon mutants based on *lux* expression and the production of type 1 fimbriae. **(A)** A decrease or increase of luminescence corresponding to the expression of type 1 fimbriae was observed in the transposon mutants, **(B)** Production of type 1 fimbriae in transposon mutants cultured to the mid-log phase in LB broth determined by yeast agglutination. The QT4796 (*fimS-lux*) strain was used as a control and showed baseline luminescence. Results are the mean values and standard deviations for three biological experiments. Statistical significance was calculated by the Student *t*-test: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

insertion site locations. In addition, 32 of the individual mutants were pooled and their transposon insertions were determined (**Supplemental Figure 3**) as described in the Materials and Methods section.

Identification of Mutations Affecting Type 1 Fimbriae Expression

Analysis of the 32 mutants generated 6,733 sequence reads that mapped to the CFT073 genome. The insertions were linked to genes involved in protein fate and synthesis, energy metabolism, adherence, transcriptional regulation, and transport, regulatory functions, etc. (**Table 2**). A number of transposon mutations were also inserted in genes predicted to encode proteins of unknown function (**Supplemental Figure 3**).

Insertions Within Genes Contributing to Amino Acid Biosynthesis and Metabolism

Several clones carried an insertion within genes with metabolic functions. For instance, one of these clones was inserted in *tdcB* (**Table 2**) that encodes a catabolic threonine dehydratase involved in the first step in threonine degradation. It is one of several enzymes carrying out the first step in the anaerobic breakdown of L-threonine to propionate (Umbarger and Brown, 1957). However, TdcB is activated by cAMP. Following a transition from aerobic to anaerobic growth, cAMP levels rise dramatically, which leads to increased expression of *tdcB* and consequently high levels of catabolic threonine deaminase (Hobert and Datta, 1983).

Similarly, another insertion was in *nadB* (Tritz et al., 1970) which is involved in NAD biosynthesis under both aerobic and anaerobic conditions (Messner and Imlay, 2002). Another mutant had an insertion in a pathogen-specific iron acquisition gene, *iucC*, which encodes a protein required for synthesis of aerobactin (de Lorenzo and Neilands, 1986). Likewise other insertion sites were *yecK,* encoding a membrane anchored pentaheme *c*-type cytochrome involved in an anaerobic respiratory system (Gon et al., 2000); *eutE* which codes for a protein that increases the level of acetylating acetaldehyde dehydrogenase activity (Rodriguez and Atsumi, 2012) and *sseB* involved in increased rhodanese activity (Hama et al., 1994).

Insertions Within Genes Encoding Other Fimbrial Adhesins, Transporters, or Outer Membrane Proteins in *E. coli* CFT073

A number of mutants with altered *lux* expression were found to contain insertions in genes encoding fimbriae, outer membrane proteins and transport proteins. Among these mutants, three carried insertions in fimbrial systems. One insertion was identified in *fimD* which is the usher of the chaperone-usher pathway of type 1 fimbriae (**Supplemental Figure 3**) (Klemm and Christiansen, 1990), the other mutant had an insertion in *aufG* which codes for a putative fimbrial-like adhesin protein, and another insertion was identified in c1933 (*ydeS*) encoding the minor fimbrial subunit of F9 fimbriae (**Table 2**). These loci have been previously characterized (Buckles et al., 2004; Ulett et al., 2007). We also identified insertions in genes encoding a putative ABC transporter periplasmic binding protein, *ydcS*, a predicted outer membrane protein, *yaiO,* and an outer membrane porin, *nmpC* (**Table 2**). Chaperone genes involved in protein fate, such as *dnaJ* coding a chaperone protein were also identified. DnaJ acts as a sensor for non-native proteins (Siegenthaler and Christen, 2006) and is involved in many cytoplasmic events, such as promotion of protein folding and translocation of nascent polypeptides (Hartl, 1996).

Insertions Within Regulatory Genes

Further, sequencing also identified insertions in well-known regulatory genes, such as *lrp* (Calvo and Matthews, 1994). Insertion within *fimB*, which mediates switching in both directions was also identified. An insertion was identified within

TABLE 2 | Transposon mutants with altered p*fimA::lux* expression in CFT073*.

Insertion site[a]	CDS	Gene symbol	Gene product description
ENERGY METABOLISM			
yecK	*c2287*	*yecK*	Cytochrome c-type protein torY
eutE	*c2980*	*eutE*	Ethanol amine utilization protein eutE
sseB	*c3047*	*sseB*	Serine sensitivity enhancing B (SseB)
nadB	*c3098*	*nadB*	L-aspartate oxidase
iucC	*c3625*	*iucC*	IucC protein
tdcB	*c3875*	*tdcB*	Threonine dehydratase
FIMBRIAL ADHESINS, TRANSPORTERS, OR OUTER MEMBRANE PROTEINS			
ydeS	*c1933*		ydeS—fimbrial-like protein ydeS precursor (minor subunit proteins F9 fimbriae)
aufG	*c4207*	*aufG*	Putative fimbrial adhesin precursor
fimD	*c5396*	*fimD*	Outer membrane usher protein fimD precursor
dnaJ	*c0020*	*dnaJ*	Chaperone protein DnaJ
yaiO	*c0467*		Outer membrane protein YaiO
ydcS	*c1864*	*ydcS*	ABC transporter periplasmic-binding protein (polyhydroxybutyrate synthase)
nmpC	*c2348*	*nmpC*	Outer membrane porin protein nmpC precursor
emrK	*c2904*	*emrK*	Multidrug resistance protein K
Adjacent to *kpsM*	*-c3698*	*kpsM*	Capsule synthesis
slp	*c4304*	*slp*	Outer membrane protein slp precursor
ytfR	*c5326*	*ytfR*	Putative ATP-binding component of a transport system
REGULATORS			
yliE	*c0918*	*yliE*	Putative c-di-GMP phosphodiesterase PdeI
lrp	*c1026*	*lrp*	Leucine-responsive transcriptional regulator
rstA	*c2000*	*rstA*	DNA-binding transcriptional regulator RstA
	c3750		Putative regulator
kguS	*c5041*	*KguS*	α-ketoglutarate utilization sensor
fimB	*c5391*	*fimB*	FimB recombinase regulator for *fimA*
UNKNOWN FUNCTION			
	c0337		Putative conserved protein
yaiZ	*c0486*	*yaiZ*	Hypothetical protein
	c1269		Hypothetical protein
	c1555		Putative DNA N-6-adenine-methyltransferase of bacteriophage
ynjA	*c2154*	*ynjA*	Hypothetical protein
yegQ	*c2611*	*yegQ*	Putative protease yegQ
yqhG	*c3747*	*yqhG*	Hypothetical protein YqhG precursor
Adjacent to *yqhG*	*c3746-c3747*	*yqhG*	Hypothetical protein YqhG precursor

List includes insertions identified that had at least 4-fold greater or 4-fold less relative light units compared to the CFT073 control level of lux expression. Genetic locus with the closest match to the sequence interrupted by the transposon in each mutant. Locations of specific Tn insertions are presented in* ***Supplemental Figure 3**.*
[a]*Genes in bold are present in the genome of E. coli K-12 strain MG1655 genome.*

the *c0918* (*yliE*) gene (**Table 2**). *yliE* encodes a hypothetical conserved inner membrane protein which contains a phosphodiesterase EAL domain. This protein is involved in hydrolysis of the bacterial second messenger cyclic di-GMP (c-di-GMP), a key factor in processes, such as flagellar motility, biofilm formation, the cell cycle, and virulence of pathogenic bacteria (Jenal and Malone, 2006; Römling et al., 2013). Similarly, another clone contained an insertion in *c5041* (*kguS*) which was identified as a sensor protein of a two-component signaling system involved in α-ketoglutarate utilization. This system is involved in utilization of α-ketoglutarate, an abundant metabolite during UPEC infection, which regulates target genes that encode α-ketoglutarate dehydrogenase and a succinyl-CoA synthetase. This two-component signaling system has been shown to be important for UPEC fitness during UTI (Cai et al., 2013). Also, *rstA*, a member of the two-component regulatory system RstB/RstA (Calvo and Matthews, 1994) was disrupted in one of the mutants.

Insertions Within Hypothetical Genes

Several of the mutations disrupted genes predicted to encode hypothetical proteins of unknown function, and the roles of such genes for *E. coli* physiology as well as their influence on expression of type 1 fimbriae and UPEC pathogenesis are unknown (**Table 2**). Sequence analysis identified an independent insertion within the *yqhG* gene and an insertion immediately

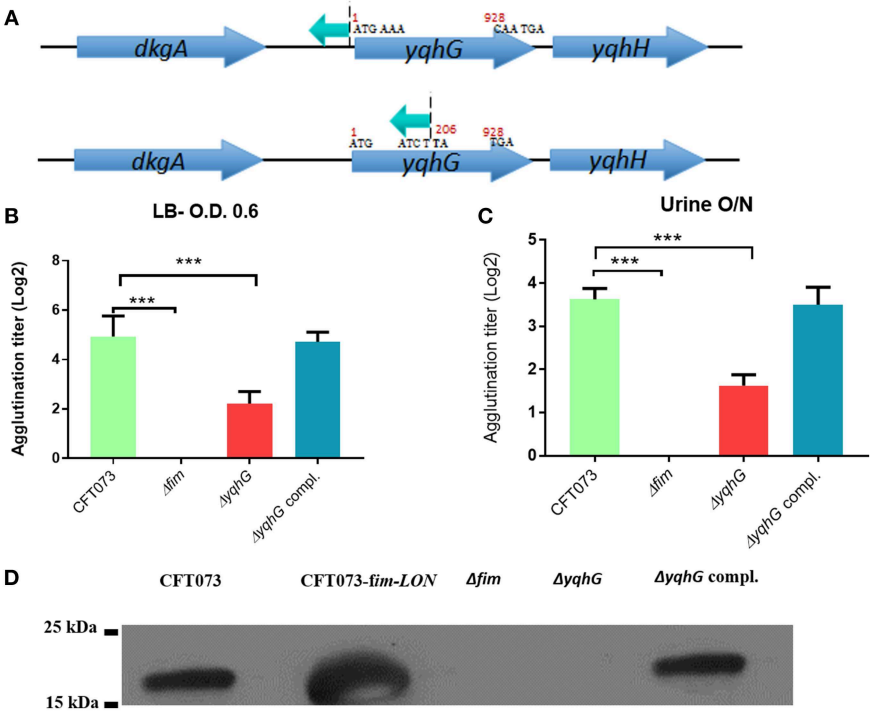

FIGURE 4 | Inactivation of the *yqhG* gene reduced expression of type 1 fimbriae. **(A)** Schematic representation of a transposon insertion which caused a decrease in expression of type 1 fimbriae. **(B)** Production of type 1 fimbriae in strains cultured to the mid-log phase of growth in LB broth and O/N in urine. The Δ*fim* strain was used as a negative control and showed no agglutination. **(C)** Production of type 1 fimbriae in strains cultured O/N in human urine. **(D)** Western blot of fimbrial extracts of strains cultured to the mid-log phase of growth in LB broth. Results are the mean values and standard deviations for six biological experiments. Statistical significance was calculated by the Student *t*-test: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

upstream of the *yqhG* coding region (**Figure 4A**), strongly suggesting the involvement of YqhG in type 1 fimbriae production. Thus, we focused the rest of our investigation on this gene of unknown function and its role for production of type 1 fimbriae and UPEC pathogenesis.

Disruption of *yqhG* Reduces Expression of Type 1 Fimbriae

A transposon was inserted in the opposite orientation of *yqhG* in one mutant and in the middle of the gene in another mutant (**Figure 4A**), suggesting that the *yqhG* gene was involved in regulation of expression of type 1 fimbriae. Since type 1 fimbriae contribute to UPEC pathogenicity (Nielubowicz and Mobley, 2010), the production of type 1 fimbriae was then evaluated by yeast agglutination at the mid-log phase of growth in LB (O.D 0.6) and urine (O.D 0.4). As expected, mutations within *yqhG* had an effect on the production of type 1 fimbriae (**Figure 4B**). After mid-log growth with shaking in LB, and overnight growth in human urine with shaking, the agglutination titer of CFT073Δ*yqhG* was reduced by 4-fold as compared to strain CFT073. Agglutination titers of the complemented mutant regained titers similar to that of the WT strain (**Figure 4B**). To confirm that yeast agglutination was mediated by type 1 fimbriae, the assay was also performed in the presence of 2.5% mannopyranose, which blocks type 1 fimbriae-mediated agglutination. As expected, no yeast agglutination was observed when 2.5% mannopyranose was added to the bacteria. These results indicate that the mutation of *yqhG* caused a substantial decrease of type 1 fimbriae expression. Since the *yqhG* mutant demonstrated a defect in type 1 fimbriae production (**Figure 4B**), we further investigated the ability of the mutant to grow in human urine. Loss of the *yqhG* in strain CFT073 did not affect growth in human urine.

To confirm that production of type 1 fimbriae was reduced in the *yqhG* mutant, Western blotting against the type 1 major subunit FimA was determined. Western blotting confirmed an important decrease of the FimA protein in the *yqhG* mutant compared to parent strain CFT073 and the complemented strain (**Figure 4D**).

The *yqhG* Mutant Demonstrates Reduced Adherence to Human Bladder Epithelial Cells

The adherence of the *yqhG* mutant to 5,637 human bladder epithelial cells was compared to that of the parental strain CFT073. **Figure 5A** shows that the adherence of the Δ*yqhG* mutant was reduced ∼2-fold compared to that of WT strain. Further, the decrease in epithelial cell adherence was rescued by complementation of the *yqhG* gene. Addition of 2.5% α-d-mannopyranose to block the effect of type 1 fimbriae greatly

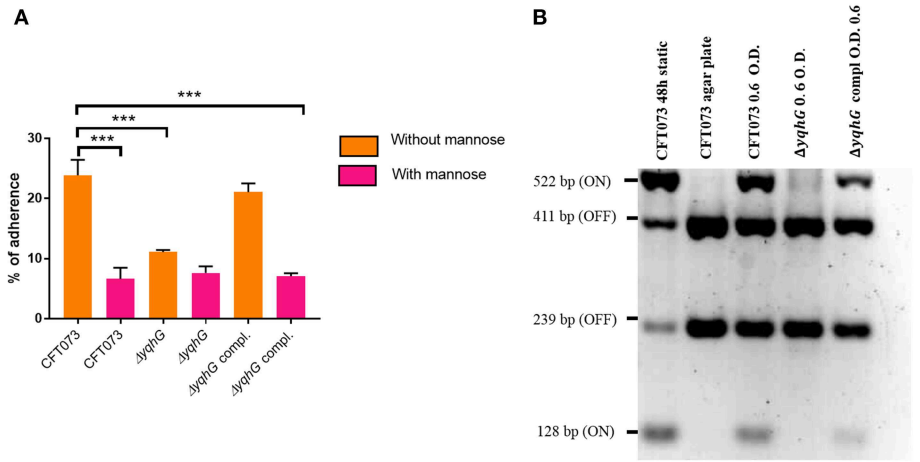

FIGURE 5 | (A) Effect of inactivation of *yqhG* and production of type 1 fimbriae on adherence of uropathogenic *E. coli* CFT073 to human bladder epithelial cells *in vitro*. Adherence of strain CFT073 and its derivatives to human 5,637 bladder epithelial cells in the presence or absence of 2.5% α-d-mannopyranose was determined. **(B)** Effect of inactivation of *yqhG* on orientation of the *fim* promoter switch (*fimS*) *in vitro*. The *fimS* region was PCR amplified, and the product was digested with HinfI. Fragments of different sizes indicate the ON or OFF orientation. All results shown are the mean values and standard deviations for four biological experiments. Statistical significance was calculated by the Student *t*-test **(A)**. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

reduced cell association of all strains tested in the cell association assay (**Figure 5A**).

To determine if the reduction of type 1 fimbriae was due to orientation bias of the phase-variable promoter, we determined orientation of the *fimS* promoter region. The orientation of *fimS* was evaluated in strains grown under agitation to mid-log phase in LB broth. Using the procedure described by Stentebjerg-Olesen et al. (2000), we observed that the *fim* promoter clearly had an increased bias for the OFF position in the *yqhG* mutant (**Figure 5B**).

The *yqhG* Mutant Demonstrates Reduced Bladder and Kidney Colonization in Mice

Type 1 fimbriae are important for ExPEC colonization of the bladder during UTIs. Since the *yqhG* mutant demonstrated decreased type 1 fimbriae production, we tested its capacity to cause urinary tract infection in the CBA/J mouse model. Forty-eight hours after urethral inoculation, the *yqhG* mutant was attenuated 100-fold in bladder and 10,000-fold in kidneys ($P < 0.0001$) compared to the WT parent strain (**Figure 6**). Further, the complemented mutant regained the capacity of colonization. This reduction in colonization from inactivation of *yghG* could be due to reduced production of type 1 fimbriae, and potentially other changes in the *ΔyqhG* mutant that could decrease colonization of the murine urinary tract (**Figure 6**).

The *yqhG* Mutant Demonstrates Increased Motility

Since flagella and swimming motility play a pivotal role in colonization and persistence in the urinary tract (Lane et al., 2005), we investigated whether the loss of *yqhG* affected swimming motility. The *yqhG* mutant was considerably more motile than the UPEC CFT073 parental strain. Complementation of the *yqhG* mutant (strain QT5235) effectively restored motility to wild-type levels. Further, a *fimS* Locked-ON reference strain was shown to be non-motile in the swimming assays (**Figure 7**).

yqhG Contributes to Oxidative Stress Resistance

During the course of a UTI, UPEC come across a variety of environmental stresses that can potentially limit survival and growth (Mulvey et al., 2000). Envelope stress response pathways are likely to be critical for UPEC, in order to detect and respond to potentially harmful environmental insults during the course of infection. In an effort to determine the mechanism by which *yqhG* influences type 1 fimbriae expression, we first sought to determine if this gene is involved in hydrogen peroxide resistance. Our observations revealed that the Δ*yqhG* strain was more sensitive to oxidative killing compared to wild-type strain CFT073. This result indicates that *yqhG* contributes to resistance to oxidative stress mediated by hydrogen peroxide (**Table 3**). Further, screening of specific transposon mutants that were also shown to have decreased expression of type 1 fimbriae including clones with insertions in the *slp or yegQ* genes also showed increased sensitivity to hydrogen peroxide-mediated oxidative stress (**Table 3**).

The *pst* Mutant of UPEC CFT073 Is Also Sensitive to Osmotic and Oxidative Stress

In a previous study, in avian pathogenic *E. coli* (APEC) strain χ7122, inactivation of the *pst* system mimicked phosphate-limiting conditions and caused pleiotropic effects (Lamarche et al., 2008). In the APEC *pst* mutant, membrane homeostasis was altered and included modification of phospholipids (Lamarche et al., 2008). Accordingly, the same mutant appeared to modulate the expression of some genes regulating antioxidant activities

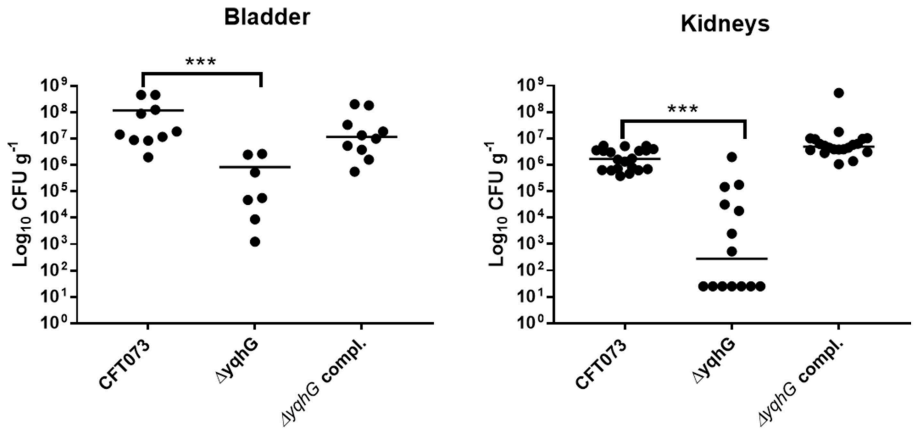

FIGURE 6 | Deletion of *yqhG* reduces colonization of the murine urinary tract. CBA/J mice were infected transurethrally and the animals were euthanized, and organs were collected 48 h post-infection. Each data point represents a sample from an individual mouse, and horizontal bars indicate the medians. Two independent infections were performed: with CFT073 WT and CFT073Δ*yqhG* and with CFT073 WT and CFT073Δ*yqhG*-Tn7T-Cm::*yqhGH*. Each kidney was sampled separately (Mann-Whitney test). *$P < 0.05$; **$P < 0.005$; ***$P < 0.0001$.

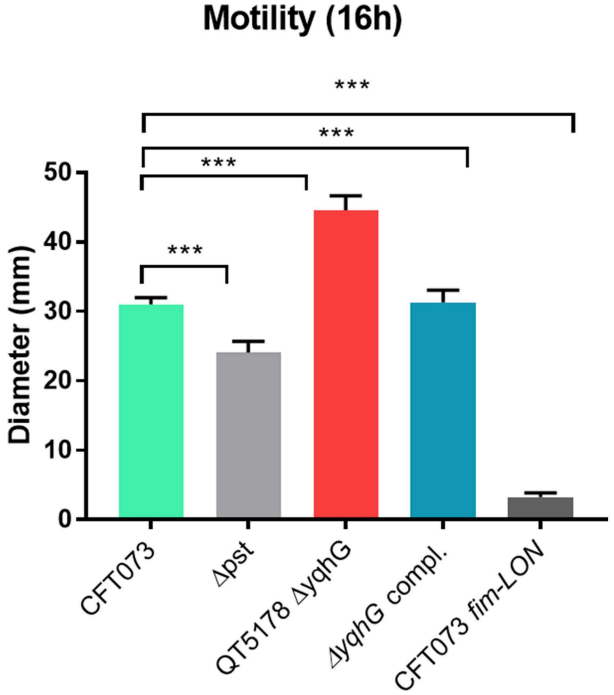

FIGURE 7 | Effect of deletion of *yqhG* on motility. Diameters of swimming motility of CFT073, mutants and complemented mutant. Data represent the averages of five separate experiments. Error bars represent the SEM. Significant differences in motility between mutants and complemented mutants were determined using a paired Student *t*-test. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0001$.

TABLE 3 | Growth inhibition zones of UPEC CFT073, isogenic mutants, and complemented strains exposed to 10 μl of hydrogen peroxide.

Strain	Mean diameter inhibition zone (mm) in LB ± SD[a]
CFT073	25.56 ± 0.49
Δ*pst*	**31.25 ± 0.70**
Δ*pst* compl.	26.0625 ± 0.41
Δ*yqhG*	**28.62 ± 0.51**
Δ*yqhG* compl.	25.12 ± 0.58
QT1324 (*oxyR::Km*)	**39.35 ± 1.04**
QT4937 (*slp::Tn10*)	**28.93 ± 0.67**
QT4940 (*yegQ::Tn10*)	**28.87 ± 0.23**

Data presented are the means ± standard deviations for eight independent experiments. The compound used was 10 μl of H_2O_2 (30%) on LB or agar plates.
[a] *Mean of eight determinations per strain. All strains were tested in parallel each day. Values indicated in bold text are significantly different, $P < 0.05$, from the mean for the wild-type strain as calculated by Student's t-test.*

(Crépin et al., 2008). In the UPEC strain CFT073 *pst* mutant, attenuation of urinary tract virulence was shown to mainly be attributed to reduced production of type 1 fimbriae (Crépin et al., 2012b). As stress resistance is crucial for the survival of UPEC strains in the host, we also evaluated the capacity of the UPEC CFT073-derived *pst* mutant to resist oxidative and osmotic stresses. Sensitivity to hydrogen peroxide was analyzed from exponential growth cultures of CFT073 and the Δ*pst* derivative by using the H_2O_2 agar overlay diffusion method. Under phosphate-sufficient conditions (LB), the *pst* mutant was more sensitive to oxidative stress, as diameters of inhibition zones were significantly larger with the *pst* mutant than with the CFT073 parental strain ($P < 0.001$) (**Table 2**). Complementation of the Δ*pstCAB* mutant restored the wild-type phenotype (**Table 3**). Given the significant decrease in the colonization of the mouse bladder by the *pst* mutant, we also tested whether this strain displayed increased susceptibility to osmotic stress by using an established protocol (Pavanelo et al., 2018). We, therefore, tested the growth of the wild-type, *pst* mutant, and a complemented mutant on modified LB agar containing different concentrations

of NaCl or urea. Both strains were able to grow on LB agar with 0.3 M NaCl (**Figure 8A**) and with 0.3 M urea (**Figure 8B**), and no strain could grow on LB agar with 1 M NaCl and 1 M urea. In contrast, the *pst* mutant was significantly more sensitive to 0.6 M NaCl (**Figure 8C**) and 0.6 M urea (**Figure 8D**) compared to parent strain CFT073. The complemented mutant grew similarly to the WT strain (**Figure 8**). Taken together, the *pst* mutant was found to show increased sensitivity to both oxidative and osmotic stresses.

DISCUSSION

The ability to adhere to host epithelial cells is an important factor for initial colonization and persistence during a UTI. Therefore, type 1 fimbriae are crucial for the establishment of UPEC infections (Schaeffer et al., 1987). Bacterial adherence to the uroepithelium limits the effect of shear forces produced by urine flow and thereby improves colonization (Thomas et al., 2002). Urine is considered to be a nutrient limiting environment with relatively low levels of available sugars and metabolites. Consequently, UPEC metabolism is tightly regulated and highly responsive to nutrient availability, and UPEC adapted to utilization of a wide range of nutrients from this nutrient-limited environment (Mann et al., 2017). Further, most bacteria are unable to thrive within the urinary tract environment due to its high osmolarity, elevated urea concentrations, low pH, and limited iron availability (Mann et al., 2017). Due to this transition, bacteria that enter the urinary tract encounter a harsh environment and are subject to numerous stresses, and stringent competition due to a drastic reduction in the abundance of nutrients. Further, environmental cues, such as pH and osmolality, have been shown to regulate *fim* genes and to affect the orientation of the *fim* switch (Schwan et al., 2002).

The expression of type 1 fimbriae is controlled by an invertible DNA element that upon inversion changes the promoter orientation through two site-specific recombinases, FimB and FimE. Thus, affecting the transcription of the *fimAICDFGH* genes (Abraham et al., 1985). This inversion phenomenon known as phase variation, reversibly switches between the expression of type 1 fimbriae (Phase-ON) and loss of expression (Phase-OFF) (Klemm, 1986). Besides the *fim* gene cluster, other genes and their gene products contribute to the expression of type 1 fimbriae. The global regulator histone-like nucleoid-structuring protein (H-NS), integration host factor (IHF), leucine-responsive protein (Lrp), and cyclic adenosine monophosphate (cAMP) receptor protein (CRP)/cAMP, control the expression of type 1 fimbriae directly and indirectly (Blomfield et al., 1997; Olsen et al., 1998; Kelly et al., 2006). Other proteins affect type 1 fimbriae expression in *E. coli* such us OmpX (Otto and Hermansson, 2004), IbeA, and IbeT (Cortes et al., 2008), although mechanisms of control are unknown. The regulatory alarmone, ppGpp, has been connected to the regulation of the *fim* operon (Åberg et al., 2006). Alteration of phosphate metabolism through inactivation of the phosphate specific transporter (*pst)* was shown to contribute to expression of type 1 fimbriae and attenuated UPEC virulence (Crépin et al., 2012b). Further, inactivation of *pst* was linked to increased production of the signaling molecule c-di-GMP, which in turn decreased the expression of type 1 fimbriae in UPEC CFT073 (Crépin et al., 2017). It has been shown that under a slightly acidic pH and low salt growth conditions found on the vaginal surface, that proteins, such as SlyA or RcsB may activate *fimB* and prevent H-NS from binding, allowing type 1 fimbriae to be expressed on the surface of the UPEC cells (Schwan et al., 2007; McVicker et al., 2011). Recently, the *treA* gene coding for a periplasmic trehalase that contributes to osmotic stress resistance was also shown to affect type 1 fimbriae production in an APEC strain and this mutation significantly reduced adherence to and invasion of epithelial cell and bladder colonization in a murine model of UTI (Pavanelo et al., 2018). Taken together, there is a body of evidence indicating that type 1 fimbriae expression is important for UPEC colonization of the urinary tract and that multiple factors, including adaptation to osmotic and oxidative stress, that influence expression of these fimbriae play a role in *E. coli* UTI pathogenesis.

Given the importance of type 1 fimbriae and control of its expression, in the present report we devised a means to randomly identify different genes involved in the regulation of type 1 fimbriae by using a *luxCDABE* reporter fusion and genetic screening of a transposon bank. We constructed a *fimS-lux* reporter fusion integrated on a single-copy at the chromosomal *att*Tn*7* of pyelonephritis strain CFT073 (**Figure 1B**). Integrating the *lux* reporter into the chromosome results in a reduced level of promoter expression compared to fusions on multi-copy vectors, but has the added advantage of stability of the signal during long term localization studies and more relevant comparison to regulatory effects on the native *fim* switch, as the single-copy fusion will not be as biased by titration of regulator proteins or recombinases that could occur with multi-copy fusion vectors. Moreover, the luciferase bioluminescence system may overcome some limitations of fluorescent reporters used in *in vivo* imaging, such us background signals associated with cellular autofluorescence, poor penetration of the excitation wavelength, slow turnover of the fluorescent protein, etc. (Riedel et al., 2007).

The *fimS-lux* fusion was shown to undergo a change in signal in accordance to changes in regulation of the *fimA* promoter observed under different pH conditions (**Figure 2A**), demonstrating its potential value at investigating environmental cues that could affect transcription of the *fim* gene cluster. A shift from pH 4.4 to a neutral pH 7.0 resulted in a significant increase of *fimS* expression. Furthermore, and in accordance with previous reports (Schwan et al., 2002; Schwan and Ding, 2017), the expression of *fimS* was increased when bacteria were cultured in LB (neutral pH) and decreased at lower pH (**Figure 2A**). The *in vitro* results with the *fim-lux* fusions were similar to previous studies (Schwan et al., 2002; Schwan and Ding, 2017), and helped validate the reporter system that we developed. Our *lux* fusion was highly sensitive with low background noise, which makes this reporter rapid enough to enable a delicate monitoring of quick response kinetics. Indeed, such vectors have also been developed for transposon-based systems with random integration of *lux* into the chromosomes of both Gram-negative and Gram-positive bacteria (de Lorenzo et al., 1990; Francis et al., 2001). However, these systems are based on random integration of the transposon

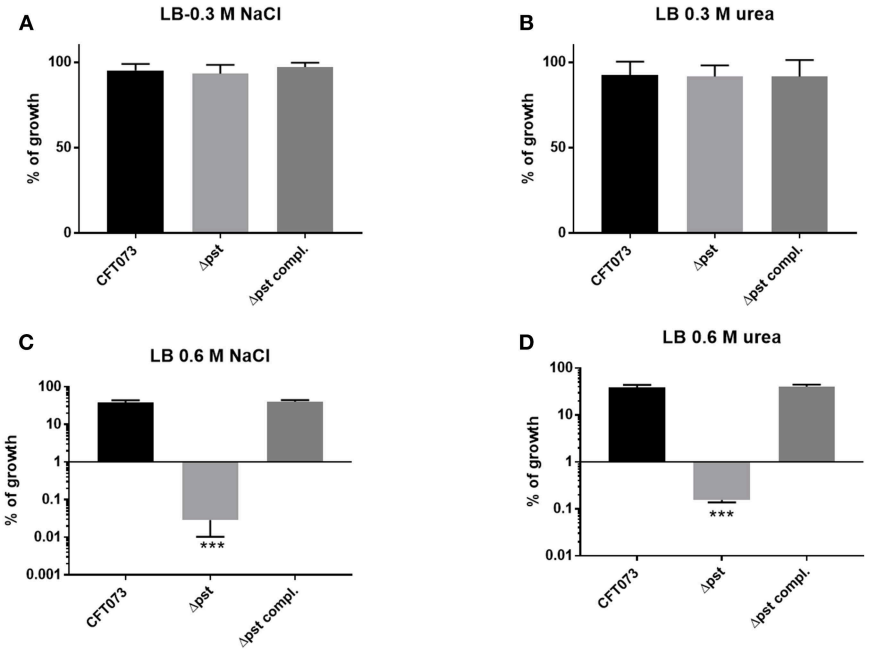

FIGURE 8 | Growth in conditions of osmotic stress. Strains were grown under shaking in LB medium until mid-log phase ($O.D._{600}$ 0.6) and plated on LB agar (taken as 100% growth) and **(A)** LB agar with 0.3 M of NaCl, **(B)** 0.3 M of urea, **(C)** 0.6 M NaCl, and **(D)** 0.6 M of urea. Graphs show the mean of growth relative to regular LB with standard error bars. Assays were performed three times in duplicates (Kruskal-Wallis test). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

into the chromosome, followed by selection of clones which retain viability but demonstrate high levels of *lux* expression. Hence, our *lux* system seems more efficient with single-copy integration of recombinant genes at the *att*Tn*7* site that does not require selective pressure and can be used in a variety of *Enterobacteriaceae*. This system provides a useful tool for studying promoter regulation without introducing unforeseen genetic changes that influence the behavior of the strain *in vitro* and *in vivo*. The application of the *lux* reporter in combination with transposon mutagenesis and high-throughput sequencing herein provided a novel and valid approach to identify specific genes and begin to dissect genetic pathways linked to expression of type 1 fimbriae.

Our transposon library screening resulted in the identification of numerous insertions that deregulated expression of type 1 fimbriae. In this study, we initially screened 5,904 transposon mutants by measuring the level of luminescence following growth on LB broth and phenotypic screening for production of type 1 fimbriae by using a yeast agglutination assay (**Figure 3**). Here, we searched for genes with a significant increase or decrease in luminescence levels from specific transposon mutants compared to the control strain (CFT073, *fimS-lux*). The insertion of transposons in specific clones were shown to repress or activate *lux* expression *fimS* and hence deregulate type 1 fimbriae production (**Figure 3A**). Using high throughput sequencing, we then mapped the Tn*10* insertion sites of these mutants (**Supplemental Figure 3**), leading to the identification of numerous genes that significantly altered type 1 fimbriae production (**Table 2**). In addition to known structural and regulatory genes, genes identified included those involved in biogenesis of type 1 fimbriae and other fimbriae, amino acid biosynthesis, membrane transport, chaperones involved in protein fate and genes that are currently of unknown function. Confirmation of the role of these genes in type 1 fimbriae expression via the construction and characterization of specific mutants will be required to more clearly determine their mechanisms of action in the regulation of expression type 1 fimbriae and potential roles in UTI pathogenesis. Several clones had insertions within genes with metabolic functions. One of these clones disrupted *tdcB* (catabolic threonine dehydratase). Many nutrient transport systems and genes related to carbohydrate metabolism have been reported to be involved in the virulence of ExPEC strains. For example, the periplasmic trehalase *treA* affects type 1 fimbriae production and virulence of APEC strain MT78 (Pavanelo et al., 2018). The metabolic *frz* operon has also been shown to link the metabolic capacity of ExPEC with expression of genes required for adherence to the bladder epithelium; the presence of the *frz* operon favors the ON orientation of the invertible type 1 fimbriae promoter (Rouquet et al., 2009). The two-component-system (TCS), KguSR, involved in the control of utilization of α-ketoglutarate, has also been shown to be important for UPEC fitness during UTI (Cai et al., 2013). Interestingly, this system was disrupted in our mutant bank. As such, the roles of this TCS in type 1 fimbriae expression in UPEC merit further investigation. Moreover, proteins involved in the transport and catabolism of sialic acid, xylose, arabinose, and the biosynthesis of arginine and serine are highly expressed in UPEC cultured in human urine (Alteri et al., 2009). It has

also been reported that sialic acid regulates type 1 fimbriae by inhibiting FimB switching in UPEC (Sohanpal et al., 2004). Tn insertions also disrupted well-studied regulators, Lrp, a global regulator of genes involved in metabolic functions within *E. coli* including fimbriae production (Brinkman et al., 2003; Kelly et al., 2006) and *fimB* which regulates phase variable expression of the *fim* operon (Blomfield et al., 1997). It is intriguing to see a potential connection of iron acquisition mechanisms and type 1 fimbriae production as disruption of the aerobactin precursor *iucC* (de Lorenzo and Neilands, 1986) affected the fimbriae production. However, the specific mechanisms underlying how certain genes involved in metabolism influence the expression of type 1 fimbriae and to what extent this alone may influence UPEC pathogenesis during UTI remain to be elucidated.

Further, some of the genes in our bank have been reported earlier for the correlation with strong virulence and pathogenesis of UPEC. Loss of *nadB* rendered NAD auxotrophy and contributes to the virulence in *Shigella* (Prunier et al., 2007) however the colonization of CFT073 and UTI89 in murine UTI model was not influenced by NAD auxotrophy (Li et al., 2012). We identified an insertion in *rstA* which is a part of low pH sensing RstBA two- component system which is directly involved in the regulation of *csgD*, which encodes a regulator responsible for curli and cellulose production (Ogasawara et al., 2010). Interestingly, mutation in *emrK* in *E. coli* caused a significant drop in biofilm formation (Matsumura et al., 2011). So, these observations show the possible correlation between inhibition of fimbriae and a reduction in biofilm formation.

This relation has been documented before in terms of aerobic respiration in the bladder. Biofilm production in UPEC has been shown to be affected by different terminal electron acceptors (Eberly et al., 2017). In addition, type 1 fimbriae production was reduced in the absence of oxygen, and UPEC strain UTI89 had increased type 1 fimbriae production on the air exposed region of biofilm due to increased oxygen level (Floyd et al., 2015). In conjugation with biofilm forming capacity, cytochrome *bd* provided a fitness advantage for UTI89 under hypoxic growth conditions as well as increased nitric oxide tolerance (Beebout et al., 2019). So, our insertion in cytochrome c-type gene, *yecK/torY*, may also affect the adherence due to type 1 fimbriae as well as respiration in various oxygen gradient in the complex biofilm community.

Interestingly, many of the genes identified in our bank that influenced type 1 fimbriae expression are uncharacterized genes of hypothetical or unknown function. We were particularly interested in further investigating the role of the *yqhG* gene encoding a hypothetical protein containing DUF3828 domains that is conserved in *E. coli*. Although, the function of this gene is still unknown, transcription of *yqhG* was shown to be positively regulated by the BglJ-RcsB complex (Salscheider et al., 2013) in *E. coli* K-12. A putative binding site for BglJ-RcsB with a significant score was also identified upstream of the *yqhG* promoter (Salscheider et al., 2013). Interestingly, RcsB is the response regulator of the Rcs phosphorelay which is conserved in *Enterobacteriaceae*. RcsB plays a pleiotropic role in the control of biofilm formation, motility behavior and responds to membrane stress, specifically outer membrane stress, and is best known for its positive regulatory effect on capsule synthesis (Majdalani and Gottesman, 2005; Majdalani et al., 2005). Further, BglJ is a positive DNA-binding transcriptional regulator of transport and utilization of the aromatic β-glucosides arbutin and salicin (Madhusudan et al., 2005). It has been shown that it is completely dependent on RcsB (Venkatesh et al., 2010). It will therefore be of interest to further investigate potential regulatory links between these regulators and *yqhG* expression in UPEC.

A more pronounced reduction of yeast agglutination occurred when the *yqhG* mutant was grown to mid-log phase in LB or urine compared to O/N growth. Similarly, other mutations in genes, such as the *pst* system also resulted in a marked change in expression of type 1 fimbriae during growth to mid-log phase compared to after overnight static growth (Crépin et al., 2012b). Because the CFT073 Δ*yqhG* mutant displayed a decreased capacity to agglutinate yeast in LB and urine (**Figures 4B,C**), we performed Western blotting against type 1 fimbriae to investigate any effect on type 1 fimbriae production. As shown in **Figure 4D** in comparison to the WT strain, the mutant had a reduced production of type 1 fimbriae. This reduction in type 1 fimbriae was also in line an increased bias for the OFF position with *fim* promoter in the *yqhG* mutant in LB broth at $O.D._{600nm}$ 0.6 (**Figure 5B**). Interestingly, we also found that the *yqhG* mutant was more motile than the WT strain (**Figure 7**). UPEC strains coordinately regulate motility and adherence to mediate colonization and dissemination during the pathogenesis of UTIs. It is widely believed that when adhesin genes are expressed, motility genes are repressed and *vice versa* (Simms and Mobley, 2008). They represent opposing forces. Thus, by mediating adherence, fimbriae would promote a sessile state and flagellar-based motility would be expected to be decreased. By contrast, increased motility by flagella would reduce the ability of bacteria to adhere at one site. Accordingly Bryan et al. (2006) and Lane et al. (2007) have shown that constitutive expression of type 1 fimbriae (CFT073 *fim* L-ON) leads to repression of swimming motility in strain CFT073. The *yqhG* mutant also exhibited increased binding to Congo Red on agar plates, suggesting a possible increase in cellulose and curli production compared to the wild-type strain. The decreased expression of type 1 fimbriae may therefore also lead to increased production of other adhesins or biofilm associated factors under certain growth conditions. Further experiments, will be required to confirm whether loss of *yqhG* may promote expression of other fimbriae while reducing expression of type 1 fimbriae.

In the murine UTI model, the *yqhG* mutant was attenuated (**Figure 5**). Loss of *yghG* in CFT073 caused an important decrease in colonization of both the bladder and kidneys, whereas loss of type 1 fimbriae by deletion of the *fim* operon, mainly results in a decrease in colonization of the bladder in the mouse model (Gunther et al., 2002). As such, we investigated whether loss of *yqhG* also affected resistance to certain stress conditions including oxidative stress and osmolarity. A Δ*yqhG* derivative of CFT073 was significantly more sensitive than its wild-type parent to oxidative stress from H_2O_2 challenge (**Table 3**), and complementation of this mutant with a single-copy of *yqhG* restored wild-type resistance to H_2O_2 killing. Although, it is clear that YqhG plays a role in regulating expression of type 1 fimbriae

and promoting adherence of UPEC CFT073 to host cells, YqhG likely plays a greater role in UPEC, including adaptation to environmental stresses, as it is also required for resistance to oxidative stress. A plausible mechanism for this drop could be due to reduced catalase activity in the mutant to counteract the oxidative burst from immune cells during infection. The *yghG* mutant colonies also demonstrated less bubbling upon addition of H_2O_2 compared to wild type. So, mutation in *yqhG* may also lead to increased sensitivity to reactive oxygen radicals as well as reduced production of type 1 fimbriae. Nevertheless, our results shed new light on the importance of *yqhG* for UPEC virulence, and it will be of interest to further elucidate what other factors that are, directly or indirectly, regulated by *yqhG*, including type 1 fimbriae, and determine its importance for resistance of *E. coli* to host innate immune response during infection.

Finally, we investigated whether inactivation of *pst,* in addition to repression of expression of type 1 fimbriae also reduced resistance to stresses including oxidative and osmotic stress. Decreased virulence of the *pst* mutant of UPEC CFT073 was mainly attributed to the decreased expression of type 1 fimbriae (Crépin et al., 2012b). The ability sense, adapt to, and resist different types of stress can also play an important role in regulation of gene expression, including regulation of type 1 fimbriae. As such, we investigated the capacity of the *pst* mutant to resist oxidative and osmotic stresses. The conditions were chosen to simulate the effects of NaCl and urea that UPEC cells may encounter during colonization of the urinary tract. Urea can permeate through the cell membrane and destabilize the native structure of proteins inside cells (Withman et al., 2013). According to our results, CFT073 showed a higher resistance to 0.6 M urea and 0.6 NaCl than the *pst* mutant grown to mid-log phase (**Figures 7C,D**). Similarly, the *pst* mutant was much more sensitive to hydrogen peroxide than the CFT073 parent strain (**Table 3**). Therefore, inactivation of *pst* in CFT073 resulted in increased sensitivity to both osmotic and oxidative stress, and this may importantly also in part be linked to changes in levels of expression of type 1 fimbriae. In APEC strain χ7122, inactivation of the Pst system induced deregulation of phosphate sensing and important changes in cell surface composition that led to reduced virulence in a chicken infection model, decreased production of type 1 fimbriae and lower resistance to oxidative stress (Lamarche et al., 2005; Crépin et al., 2008). Several studies concerning osmotic stress and expression of type 1 fimbriae were also reported for UPEC. In UPEC strain NU149, type 1 fimbriae expression was downregulated under osmotic stress caused by NaCl (Schwan et al., 2002). Further, loss of *treA* in ExPEC strain MT78 also resulted in a change in osmotic resistance to urea, concomitant with a decreased expression of type 1 fimbriae and reduced urinary tract colonization in the mouse model (Pavanelo et al., 2018). Transcriptome analyses also showed upregulation of type 1 fimbriae expression and the genes that are regulated by the osmotic stress response in CFT073 during UTI (Snyder et al., 2004).

Overall, generation of a transposon bank and a single-copy *lux*-based reporter fusion system integrated at the *att*Tn7 site in UPEC strain CFT073, has led to successful identification of insertions in a number of genes including heretofore unknown sites that altered expression of type 1 fimbriae. Interestingly, these insertions include a variety of genes involved in a diversity of functions including protein fate and synthesis, energy metabolism, adherence, transcriptional regulation, and transport, and genes of hypothetical or unknown function including *yqhG*, that we have shown to play an important role in UPEC colonization in the mouse model as well as resistance to oxidative stress. It will be of interest to more fully elucidate how some of these different systems influence expression of type 1 fimbriae as well as their potential roles in metabolism and bacterial regulatory networks as well as sensing, adaptation and resistance to environmental stresses, such as osmotic and oxidative stresses that may be encountered during the course of colonization and infection of the host.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

HB was the primary author and performed most of the experiments and writing of the manuscript. PP and HH contributed to some of the experiments and writing of the text. SH contributed the technical assistance including mouse infections with other co-authors. CD conceived the planning of the study, design of experiments, mentored the researchers, and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00312/full#supplementary-material

Supplemental Figure 1 | Production of type 1 fimbriae by uropathogenic *E. coli* CFT073 and its derivative strains. **(A)** Production of type 1 fimbriae in strains cultured to the mid-log phase of growth in LB broth in different pH **(B)** O/N culture **(C)** in LB plate. The CFT073 *fim*-LON strain was used as a positive control for agglutination. Results are the mean values and standard deviations for three biological experiments. Statistical significance was calculated by the one-way ANOVA. $*P < 0.05$; $**P < 0.005$; $***P < 0.0001$.

Supplemental Figure 2 | (A) Growth curve optical density and **(B)** viable counts (CFU/ml) of wild type CFT073 and its P*fimA*-Locked ON and P*fimA* variable *lux* derivatives. The cells were grown in LB broth. There was no significant differences in the growth phases of all strains.

Supplemental Figure 3 | Transposon mutants with affected expression of type 1 fimbriae in CFT073. Genetic locus with the closest match to the sequence interrupted by the transposon in each mutant are represented with an arrow (teal) in their respective insertion site.

Supplemental Table 1 | Primers used in this study.

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REVIEW
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Maintaining Integrity Under Stress: Envelope Stress Response Regulation of Pathogenesis in Gram-Negative Bacteria

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The Gram-negative bacterial envelope is an essential interface between the intracellular and harsh extracellular environment. Envelope stress responses (ESRs) are crucial to the maintenance of this barrier and function to detect and respond to perturbations in the envelope, caused by environmental stresses. Pathogenic bacteria are exposed to an array of challenging and stressful conditions during their lifecycle and, in particular, during infection of a host. As such, maintenance of envelope homeostasis is essential to their ability to successfully cause infection. This review will discuss our current understanding of the σ^E- and Cpx-regulated ESRs, with a specific focus on their role in the virulence of a number of model pathogens.

Keywords: Gram-negative bacteria, envelope stress, pathogenesis, sigmaE response, Cpx response

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INTRODUCTION

Bacteria encounter numerous harsh and fluctuating environments during their lifecycle. This is of particular concern to pathogenic bacteria which must be able to tolerate the challenging conditions and immune response within a host. Detection of potentially harmful changes in the environment, or damage caused by such conditions, are inducers of bacterial stress responses. The ability of a bacterium to sense and withstand these environmental stresses is crucial to its survival, particularly within a host, and to establish and maintain infection. Within the host, a bacterium is threatened by a milieu of noxious stresses including temperature and pH changes, antimicrobial compounds, bile salts, oxidative and nitrosative stress, and nutrient starvation. In addition, successful colonization and infection of hosts requires careful regulation of a multitude of virulence factors, many of which are envelope-localized.

Consisting of an inner membrane (IM), periplasmic space (PP) containing a thin peptidoglycan (PG) layer and an outer membrane (OM), the Gram-negative bacterial envelope functions as the interface between the cell and the extracellular environment. It serves as the gate for import and export, barrier to harmful substances, protects against shifting environmental conditions and is the location of many essential metabolic processes. The envelope is first subjected to harsh environmental conditions and must be able to withstand the associated stresses. The ability to maintain envelope homeostasis and quickly repair any damage to ensure integrity is dependent on envelope stress responses (ESRs).

ESRs are able to perceive the presence of extracellular stress and the disruption of periplasmic homeostasis that can arise as a result (e.g., damage to proteins in the outer membrane or periplasm). The ESRs are comprised of a series of regulatory cascades, which have independent

and overlapping stimuli, as well as regulon members. They are grouped into two component signal transduction (2CST) systems and RNA polymerase-associating alternative sigma factors. Much of our understanding of the mechanisms and regulons of the ESRs have arisen from extensive work in *Escherichia coli*. The best characterized ESRs are regulated through the σ^E pathway and the 2CST system CpxRA, central to the responses to outer membrane/periplasmic and inner membrane stress, respectively. In addition, other key ESRs include the regulator of capsule synthesis (Rcs) response, responding, in broad terms, to outer membrane and peptidoglycan stresses (Guo and Sun, 2017; Wall et al., 2018) and the phage shock protein (Psp) response, responding to disruptions at the IM (reviewed in Darwin, 2013; Flores-Kim and Darwin, 2016). Further 2CST systems exist, in addition to CpxAR, including ZraSR and BaeSR. Studies describe ZraSR as a zinc responsive system (Appia-Ayme et al., 2012; Petit-Härtlein et al., 2015), but this 2CST system has not been studied in depth and its precise physiological role remains to be determined. BaeSR on the other hand, responds to antibiotics/toxic compounds (Raffa and Raivio, 2002; Macritchie and Raivio, 2009; Appia-Ayme et al., 2011). Though not thought of as strictly responding to envelope stress, other regulatory systems such as the PhoPQ and EnvZ/OmpR two-component systems regulate processes that impact the ability of bacteria to adapt to envelope stress.

This review will summarize our current understanding of the σ^E and Cpx ESRs. While by no means the only ESRs relevant to bacterial pathogenesis, a large body of work has been conducted in characterizing the mechanism and function of these two ESRs in various Gram-negative bacteria. The aim of this review is to explain the basic mechanisms of these systems, as well as discuss their relevance to physiology and pathogenesis in several Gram-negative bacteria. In particular, we wish to highlight recent findings since this topic was last reviewed (Rowley et al., 2006; Raivio, 2014; Fang et al., 2016).

ENVELOPE BIOGENESIS

Being the barrier between the intracellular and extracellular environment, proper formation and maintenance of the envelope is essential to bacterial viability during all stages of a pathogen's lifecycle. Envelope biogenesis is a complex process which involves several machineries (Silhavy et al., 2010). Here we briefly describe those that transport, fold and assemble envelope proteins, since misfolding and/or mis-localization of such proteins has been directly linked to induction of both the σ^E and Cpx ESRs.

Proteins destined for all compartments of the envelope begin their life in the cytoplasm. As such, these proteins must be transported into or across the IM. Two different pathways exist for protein transport across the IM; the Sec translocase pathway and the twin arginine translocase (Tat) pathway. The vast majority of secreted proteins utilize the Sec pathway (reviewed in Kuhn et al., 2017; Tsirigotaki et al., 2017), while the Tat system (reviewed in Lee et al., 2006; Palmer and Berks, 2012) transports proteins which have already been folded in the cytoplasm and those which also contain metal cofactors. Briefly, an unfolded protein is targeted to the Sec translocase machinery through the recognition of an N-terminal signal peptide. SecYEG forms the IM channel through which unfolded proteins are transported. Transport across the IM is energized by the ATPase SecA. Proteins that are inserted into the IM can be inserted by SecYEG itself or utilize the IM protein YidC to assist in insertion. Once secreted across the IM, proteins can be modified and translocated in several different ways. A number of factors including the IM protein insertase YidC, together with proteases and associated regulators, play roles in the biogenesis and quality control of IM proteins (Akiyama, 2009; Luirink et al., 2012).

Lipoproteins are a special class of envelope proteins that are acylated at their N-terminus and can be localized to either the IM or the OM (reviewed in Okuda and Tokuda, 2011; Szewczyk and Collet, 2016; Narita and Tokuda, 2017). In brief, after being secreted across the IM, lipoproteins undergo multiple modification steps that result in the addition of several acyl chains to an N-terminal cysteine residue. OM-destined lipoproteins are trafficked to the OM by the Lol pathway. An IM complex consisting of the ATP-binding cassette (ABC) transporter LolCDE recognizes OM-destined lipoproteins based on the presence of an aspartate residue at the +2 residue and energizes the transfer of these proteins to a periplasmic chaperone LolA. LolA transports OM lipoproteins to the OM, where LolB, itself an OM lipoprotein, receives and inserts OM lipoproteins into the OM. As OM lipoproteins have been implicated in promoting the virulence of some Gram-negative organisms, the Lol pathway presents a potential target for the development of new therapies. For example, the surface-exposed lipoproteins of *Neisseria* spp. play roles in pathogenesis, such as immune evasion (reviewed in Hooda et al., 2017). More broadly, lipoproteins make up key members of envelope biogenesis machineries, such as the BAM complex (see below), and can indirectly impact many envelope-localized virulence determinants.

Outer membrane biogenesis has been reviewed extensively elsewhere (Ruiz et al., 2006; Bos et al., 2007; Plummer and Fleming, 2016; Konovalova et al., 2017) therefore it will only be briefly described in this review for relevance to understand the contribution of the ESR to this process. All unfolded outer membrane proteins (uOMPs) are translocated via the Sec system. Once the uOMP reaches the periplasm, the signal sequence is cleaved and the nascent OMP is delivered by periplasmic chaperones (SurA and Skp) to the β-barrel assembly machinery (BAM) complex for folding and insertion into the outer membrane.

The BAM complex consists of five major components: BamA (YaeT), BamB (YfgL), BamC (NlpB), BamD (YfiO), and BamE (SmpA) (Plummer and Fleming, 2016). The genes encoding the BAM complex components are all regulated by σ^E (Skovierova et al., 2006; Lewis et al., 2008) and are discussed below. BamA, a member of the Omp85 protein family and originally identified in *Neisseria meningitidis,* is highly conserved across Gram-negative bacteria and is essential to the complex and bacterial viability (Voulhoux et al., 2003). Interestingly, while BamA is essential across all Gram-negative bacteria, the essentiality of the other four lipoprotein components varies; for example, BamD is essential for complex function in *E. coli* yet is not

essential in *Salmonella* (Fardini et al., 2009). BamA formation is dependent on BamD with depletions in this lipoprotein resulting in BamA misfolding (Misra et al., 2015). In addition, BamB and BamD have been shown to bind unfolded BamA and assist in its localization and insertion into the outer membrane (Hagan et al., 2013).

Aside from its essentiality, there are numerous links between the BAM complex and pathogenicity. *Salmonella bamE* mutants are attenuated in mice (Lewis et al., 2008) and *Salmonella* Enteritidis *bamB* and *bamD* deletions render the pathogen less virulent with reduced expression of flagella and the type III secretion system (T3SS) (Amy et al., 2004; Fardini et al., 2007, 2009). In *Yersinia enterocolitica,* a *bamB* mutant is attenuated in mice with a significant reduction in the spleen bacterial burden after 3 days (Weirich et al., 2017). Additionally, a *bamE* deletion leads to OMP defects and rifampicin sensitivity in *E. coli* (Sklar et al., 2007a).

Recently, a number of studies have identified small molecule inhibitors of components of the BAM complex. Hagan et al. (2015) identified a 15-mer peptide fragment of BamA that binds to BamD. Since BamA formation is dependent on BamD and BamA is required for cell viability, substrate competition between the small molecule and the BamA substrate resulted in growth defects and increased sensitivity to vancomycin and rifampicin. BamA has been identified as a viable target for therapeutics with the development of a monoclonal antibody (MAB1) which binds to the extracellular loop of BamA and affects OM integrity (Storek et al., 2018). Furthermore, inhibition of *Pseudomonas* BamA by bacteriocins has also been observed, specifically lectin like bacteriocins (LlpAs). Bacteria often secrete these toxic compounds in order to selectively kill related bacteria and Ghequire et al. (2018) identified that LlpA resistant *Pseudomonas* carried mutations in the extracellular loop of BamA.

Put together, studies such as these support the consensus that understanding the process of OM biogenesis and its maintenance may provide new therapeutic routes against these pathogens.

THE EXTRACYTOPLASMIC SIGMA FACTOR σ^E

Maintenance of the outer membrane depends largely on the extracytoplasmic sigma factor σ^E (*rpoE*) (Rouvière et al., 1995). This ESR senses misfolded OMPs within the outer membrane and periplasmic space. Activation of the σ^E pathway involves a series of proteolytic cleavage events (**Figure 1**). Inducers of the σ^E pathway are summarized in Rowley et al. (2006) and include: oxidative stress, heat shock, carbon starvation and biofilm formation. More recently, acid stress (Muller et al., 2009) ultraviolet A (UVA) radiation, P22 phage and hypo-osmotic

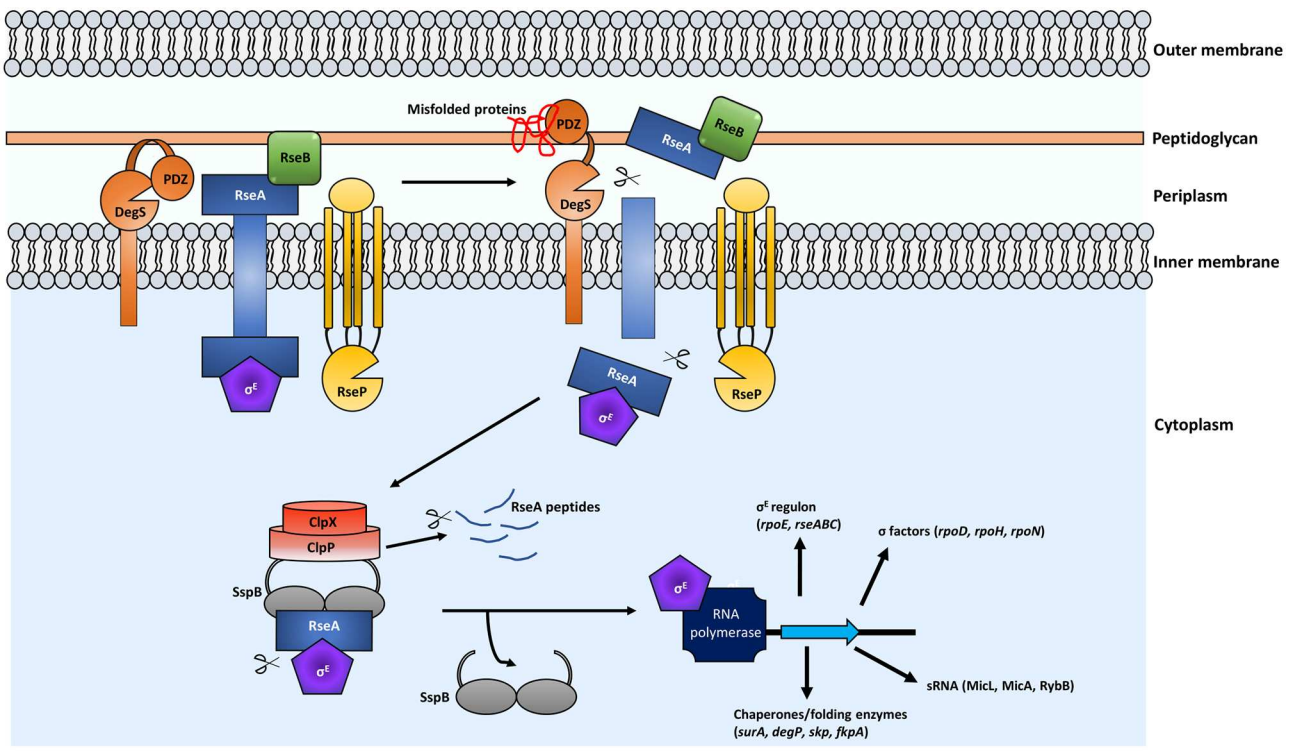

FIGURE 1 | Regulated intramembrane proteolysis (RIP) leads to activation of the σ^E ESR. In the presence of misfolded proteins, a conformational change as a result of misfolded proteins binding to the PDZ domain of DegS occurs. Subsequently the protease domain of DegS is exposed and the periplasmic domain of RseA is cleaved. RseP then cleaves RseA at the cytoplasmic domain, releasing the σ^E-bound RseA portion into the cytoplasm. Binding of the adaptor protein SspB to RseA-σ^E recruits the ClpXP protease for degradation of RseA and release of free σ^E. σ^E binds to RNA polymerase and transcription of the σ^E regulon is induced.

shock have also been shown to induce the σ^E ESR (Amar et al., 2018). Generally, activation of the σ^E pathway is due to accumulation of misfolded and/or mis-translocated OMPs or LPS within the periplasm (Rowley et al., 2006). In laboratory *E. coli* strains and *Yersinia enterocolitica, rpoE* is an essential gene (De Las Peñas et al., 1997a; Heusipp et al., 2003), although suppressor mutations do enable *rpoE* mutants to be viable (De Las Peñas et al., 1997a). Insertion mutations in *ydcQ*, a putative DNA binding protein (Button et al., 2007), and two genes of unknown function *yhbW* and *ptsN* (Hayden and Ades, 2008) have been identified to enable the *rpoE* deletion in *E. coli* to be tolerated. Numerous studies have shown that *rpoE* is not essential in other bacteria including *Salmonella* (Humphreys et al., 1999; Skovierova et al., 2006), although it does make a major contribution to their virulence. Interestingly, it has recently been reported that loss of the LPS O-antigen renders an *rpoE* deletion lethal in *Salmonella* (Amar et al., 2018). The authors propose that when present, the LPS O-antigen provides protection to the *Salmonella* OM allowing an *rpoE* deletion, and presumably the OM defects that result, to be tolerated.

Encoded within the *rpoE* operon are the σ^E inhibitors *rseA* and *rseB* (Dartigalongue et al., 2001). Activation of the σ^E ESR occurs via regulated intramembrane proteolysis (RIP) and begins at the transmembrane protein RseA (**Figure 1**). Under normal physiological conditions, RseA is bound to σ^E and as such, sequesters it from interacting with RNA polymerase to influence gene expression. RseB is also a negative regulator of the σ^E pathway and when bound to RseA it increases affinity of this complex for σ^E, resulting in inhibition of the pathway (De Las Peñas et al., 1997b; Collinet et al., 2000; Ahuja et al., 2009; Chaba et al., 2011) (**Figure 1**).

In the presence of misfolded proteins, the σ^E cascade begins with the cytoplasmic cleavage of RseA by the protease DegS (Ades et al., 1999; Alba et al., 2002; Kanehara et al., 2002; Li et al., 2009). The DegS site-1 protease (S1P) is a transmembrane protein embedded within the IM (**Figure 1**). The presence of uOMPs in the periplasm induces conformational changes in DegS, via interaction between the C-terminus of the uOMPs with the PDZ protease domain of DegS. This conformational change exposes the protease region for RseA cleavage (Walsh et al., 2003; Sohn et al., 2009). DegS is essential in *E. coli* laboratory strains however construction of a *degS* mutant has been reported in certain extraintestinal *E. coli* strains, *Salmonella* Typhimurium (Rowley et al., 2005), and *Vibrio cholerae* (Mathur et al., 2007). Although viable, *S.* Typhimurium *degS* mutants are defective in their ability to colonize the host and cause infection (Redford et al., 2003) while *V. cholerae degS* mutants are unable to activate the σ^E cascade following antimicrobial peptide (AMP) treatment (Mathur et al., 2007). Interestingly, *degS* mutants can still mount a σ^E response to certain stresses (Rowley et al., 2005). In *S.* Typhimurium, σ^E can be activated by acid stress independently of DegS and misfolded proteins (Muller et al., 2009).

Following cleavage by DegS, the RseP (YaeL) protease is recruited and the cytoplasmic portion of RseA, bound to σ^E, is released into the cytoplasm (Alba et al., 2002; Kanehara et al., 2002) (**Figure 1**). RseP is a site-2 protease (S2P), therefore, its cleavage of RseA can only occur after cleavage by the S1P. Li et al. (2009) identified that S1P cleavage leads to S2P cleavage due to the exposure of a hydrophobic amino acid (Val-148) at the carboxyl-terminus of RseA. Inhibition of RseP prevents cleavage of RseA and results in a lethal sequestration of σ^E. Interestingly, a sRNA, RseX, was shown to reduce the levels of OMPs OmpA and OmpC when overexpressed and this led to survival of *rseP* mutant cells in *E. coli* (Douchin et al., 2006). Recently, Konovalova et al. (2018) identified a eukaryotic matrix metalloprotease (MMP) inhibitor which targets RseP and leads to toxic uOMP accumulation. This was found to be due to loss of σ^E-regulated sRNAs, MicA and RybB, which play an important role in downregulating expression of uOMPs (see **Figure 2** and described in detail below).

Following release of RseA bound σ^E, the adaptor SspB binds to the complex and recruits the ATP-dependent ClpXP protease (**Figure 1**) (Flynn et al., 2004; Baker and Sauer, 2012). ClpX is an AAA+ ATPase and ClpP is a peptidase (Baker and Sauer, 2012) and in the final stage of the σ^E cascade the protein complex releases σ^E from RseA (Chaba et al., 2007). Analysis of the roles of the ClpXP protease provides evidence that individual components of the σ^E pathway play important roles in bacterial pathogenesis with *Salmonella clpX* and *clpP* mutants being attenuated in macrophages and BALB/c mice (Yamamoto et al., 2001).

Once released from RseA, free σ^E binds RNA polymerase as a cofactor, subsequently inducing expression of the σ^E regulon (**Figure 1**). The σ^E regulon has been linked with virulence in a number of pathogens (discussed below). The function of proteins that form part of the σ^E regulon across many bacterial species include those for DNA repair, metabolism, OM biogenesis and chaperones and proteases required for maintaining periplasmic homeostasis (Dartigalongue et al., 2001; Rhodius et al., 2006; Skovierova et al., 2006).

σ^E-regulated chaperones and proteases are of importance to OM biogenesis and the tolerance of extracytoplasmic stress particularly during host infection (see below). The major σ^E-regulated chaperones are Skp, SurA, FkpA, and HtrA (DegP). The function of these proteins has been studied extensively in *E. coli,* however they are well-conserved across multiple Gram-negative species including *E. coli*, *Salmonella,* and *Yersinia* sp.

SurA possesses peptidyl-prolyl cis/trans isomerase (PPIase) activity which folds proteins via catalysis of the rate-limiting cis/trans isomerization of peptidyl bonds around proline residues (bacterial PPIases are reviewed in Ünal and Steinert, 2014). In addition to, and independently of, its PPIase activity SurA also functions as a periplasmic chaperone (Behrens et al., 2001). A major role of the periplasmic chaperones is to prevent aggregation of uOMPs; however, SurA and a further chaperone Skp, encoded within the *bamA* genomic region, have been shown to play a role in uOMP folding in cooperation with the BAM complex. Studies in *E. coli* have shown that SurA interacts with BamA in order to deliver uOMPs (Sklar et al., 2007b; Vuong et al., 2008), while Skp assists in the folding of OmpA and Skp-OmpA complexes interact with BamA (Patel and Kleinschmidt, 2013). Interestingly, double deletion mutants of *skp* and *surA* result in a lethal phenotype and as a result, it is understood that they function in two different but overlapping pathways of

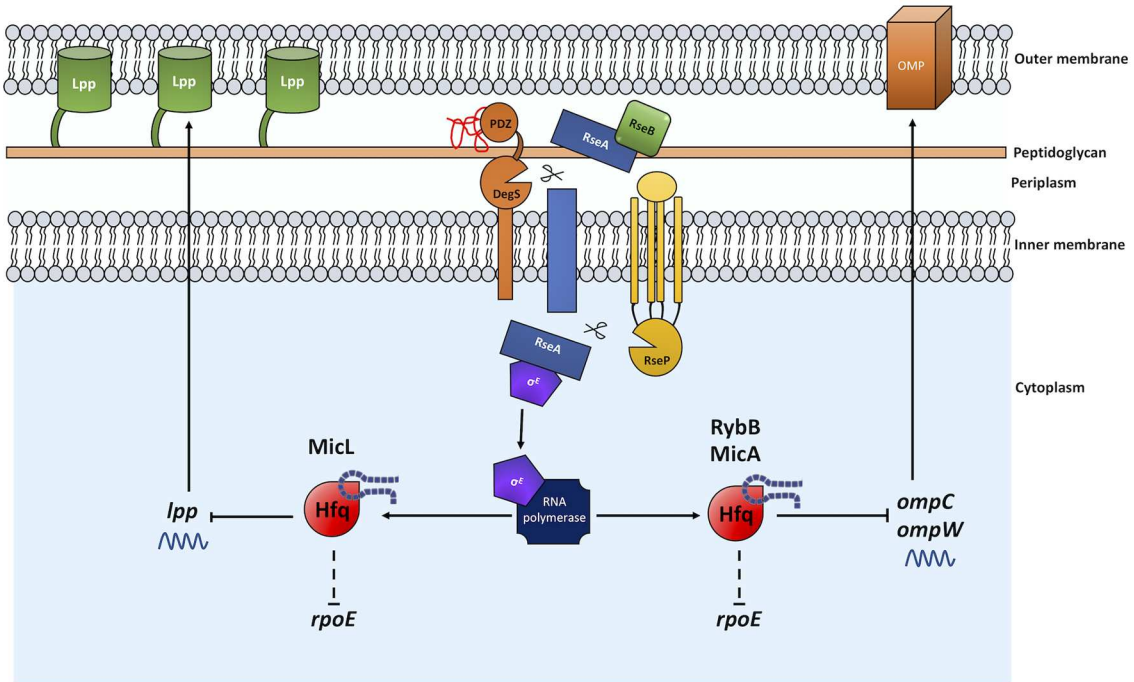

FIGURE 2 | The sRNAs MicL, RybB, and MicA exert control over OM homeostasis and the σ^E envelope stress response. The presence of misfolded OMPs activates the σ^E ESR through a proteolytic cleavage cascade as described in **Figure 1**. The σ^E regulon includes the Hfq-associating sRNAs *micL, rybB, and micA.* MicL inhibits *lpp* mRNA translation leading to a reduction in Lpp production. RybB and MicA reduce *ompC* and *ompW* mRNA levels, reducing the flux of uOMPs into the periplasm during stress and σ^E inducing conditions. Normal arrows represent positive regulation and blunt arrows indicate negative regulation. Dashed lines indicate where regulation is indirect and/or where intermediate factors are unknown.

OM biogenesis (Sklar et al., 2007b). Despite this, overexpression of *fkpA* can compensate for the lethal phenotype and enables *Salmonella* growth at 37°C (Ge et al., 2014). Like SurA, FkpA is a PPIase and chaperone and its chaperone activity is independent of PPIase activity. In addition to a role in OM biogenesis, overexpression of FkpA was shown to rescue *degP* (*htrA*) mutants during heat shock (Arie et al., 2001). DegP is unique in its ability to function as both a chaperone and a protease and this switch is dependent on temperature. At low temperatures, DegP is a chaperone transporting misfolded and unfolded OMPs; however, at higher temperatures it possesses protease function degrading proteins that are beyond repair (Spiess et al., 1999).

In addition to chaperones and proteases, encoded within the *rpoE* regulon are a number of sRNAs with regulatory functions. sRNAs bind to specific mRNA targets and subsequently activate or repress the mRNA through affecting its stability or by inhibiting its translation (Holmqvist and Wagner, 2017). sRNAs have been shown to regulate the σ^E, CpxRA, and Rcs ESRs (reviewed in Frohlich and Gottesman, 2018). The sRNAs regulated by σ^E are MicA (SraD), RybB and MicL (RyeF/SlrA) and they function to regulate the σ^E response and expression of a group of OMPs and lipoproteins (**Figure 2**). Ultimately, the sRNAs regulated by σ^E downregulate other processes, such as uOMP production, that may otherwise lead to continued activation of the ESR. All σ^E-regulated sRNAs associate with the RNA chaperone Hfq and inactivation of Hfq is an activator of the σ^E ESR (Figueroa-Bossi et al., 2006; Sittka et al., 2007; Klein and Raina, 2015). This Hfq-dependent regulation does indeed warrant further study in order to better understand the underlying mechanisms. In *Salmonella,* loss of Hfq has been shown to result in an increase in DegS-dependent cleavage of RseA, and this is likely due to increased accumulation of uOMPs (Figueroa-Bossi et al., 2006). In addition, Guisbert et al. (2007) identified that downregulation of the mRNA of eight OMPs (*tsx*, *fiu*, *ompX*, *ompA*, *ompF*, *lpp*, *ompC*, *yhcN*) is dependent on Hfq. Therefore, through association with sRNAs that function to downregulate uOMP production (described in more detail below) and control the σ^E response, Hfq can indirectly inactivate σ^E.

MicL is an 80 nt transcript, processed from a primary transcript of 308 nt, and is located within the *cutC* gene. Also referred to as SlrA (suppressing lap RNA), MicL can function as a multicopy suppressor of *lapA lapB* mutations (the Lap proteins are essential in the process of LPS synthesis and for cell viability) (Klein et al., 2014). To date, the only known mRNA target of MicL is *lpp* (Guo et al., 2014). The *lpp* gene encodes an OM lipoprotein, commonly referred to as Lpp or Braun's lipoprotein (Braun, 1975), which is covalently attached to the peptidoglycan layer. Lpp is the most abundant protein in *E. coli* and functions to stabilize the cell envelope through its OM-peptidoglycan interaction (for recent review, see Asmar and Collet, 2018). MicL targets *lpp* by preventing translation of its mRNA, thereby inhibiting Lpp protein production (Guo et al., 2014). This could

be seen as counterproductive; why would σ^E, a mechanism in place to maintain OM integrity, prevent production of a cell envelope stabilizing protein? Studies indicate that in doing so, demand on the Lol lipoprotein assembly machinery is lessened. This, in turn, enables increased production of the BamD lipoprotein and LPS assembly components (primarily LptE), thus increasing the folding of uOMPs and LPS production in the periplasm, which would otherwise further induce σ^E (Guo et al., 2014).

The remaining sRNAs MicA and RybB were first identified in *E. coli* as ∼70 nt and ∼80 nt, respectively. Since, these sRNAs have also been identified and shown to be conserved in *Salmonella* (Papenfort et al., 2006). These sRNAs overlap in function and downregulate expression of outer membrane porins in an Hfq-dependent manner. MicA is responsible for the decrease in *ompA* mRNA, particularly during stationary phase when σ^E is highly active (Vogel and Papenfort, 2006). RybB has been shown to specifically decrease *ompC* and *ompW* mRNA levels via Hfq (Johansen et al., 2006). As a result, these sRNAs contribute to the maintenance of envelope homeostasis via downregulation of OMP production, in a similar fashion to MicL, reducing transport of uOMPs into the periplasm during conditions of stress when the periplasmic uOMP content may already be elevated.

Interestingly, MicA negatively regulates the response regulator PhoP of the PhoPQ 2CST via base-pairing within the *phoP* translation initiation site (Coornaert et al., 2010). The contribution of the PhoPQ 2CST system to OM remodeling in *Salmonella* has been well-documented (Ernst et al., 2001; Dalebroux and Miller, 2014; Dalebroux et al., 2014) and cross-talk between this system and the σ^E ESR provides further evidence that different ESR pathways overlap. This connection emphasizes the necessity of a coordination of responses that modify and maintain the envelope during infection and bacterial stress. The negative regulation of the PhoPQ 2CST system by σ^E demonstrates that under some ESR-inducing conditions, it may be detrimental to the bacterium for certain ESRs to be simultaneously active.

σ^E and Bacterial Pathogenesis

Salmonella

Salmonella is an intracellular pathogen capable of causing infection in both humans and animals. Serovars of *Salmonella* sp. can reside in different niches and vary in the type of infection they cause. *S.* Typhimurium and *S.* Enteritidis are both examples of enteric bacteria typically causing food poisoning, while *S.* Typhi is responsible for the more serious and systemic Typhoid fever.

A large number of σ^E-regulated proteins, chaperones and PPIases have been implicated in *Salmonella* virulence (**Figure 3A**). During infection, *Salmonella* resides within macrophages in the *Salmonella* containing vacuole (SCV), a site of ROS production and associated stress. It has been shown previously that σ^E is important for survival within macrophages and in a murine infection model (Humphreys et al., 1999). *rpoE* is upregulated in macrophages (Eriksson et al., 2003) and the σ^E regulon is required for *Salmonella* resistance to oxidative stress (Testerman et al., 2002; Li et al., 2015a). Furthermore, in the invasive serovar *S.* Typhi, responsible for the systemic infection Typhoid fever, *rpoE* mutants are attenuated for invasion and intracellular survival. In addition, expression of the pathogenicity islands SPI-1 and SPI-2, encoding the T3SSs required for invasion and intracellular survival, are reduced (Xie et al., 2016; Zhang et al., 2016). σ^E also plays an important role in the downregulation of cellular processes. The anti-$FlhC_2D_4$ complex factor RflP (YdiV) is activated by σ^E and RflP functions to target the $FlhC_2D_4$ master regulator of flagellar synthesis to the ClpXP protease for degradation in *S.* Typhimurium (Spöring et al., 2018). As such, in this non-typhoidal serovar, σ^E is involved in the downregulation of *Salmonella* motility. Ultimately, research suggests that downregulation of flagellar synthesis can aid in host immune evasion, thus increasing bacterial fitness during infection. Conversely, in *S.* Typhi, σ^E has been found to promote flagellar gene expression, during osmotic stress, via upregulation of *fliA* (Du et al., 2011). The authors of this study propose that RpoE may respond to the hyperosmotic environment in the intestinal lumen and increase motility to enable *S.* Typhi invasion of epithelial M cells. These interesting discrepancies indicate that σ^E may be involved in differences between localized and systemic *Salmonella* infections. Much of the groundwork in understanding the role of σ^E in *Salmonella* pathogenesis has been performed in *S.* Typhimurium; however, these data indicate the continuing need to expand these studies into other serovars, especially those which cause invasive and systemic disease.

Further links between σ^E and coordination of virulence gene expression have also been shown: the SPI-2 pathogenicity island is, in part, regulated by the SsrAB 2CST system and an *S.* Typhimurium *rpoE* mutant has reduced expression of the SsrB-dependent secreted effector protein SseB (Osborne and Coombes, 2009). As described above, encoded within the *bamA* region is the σ^E-regulated periplasmic chaperone, *skp*. Rowley et al. (2011) identified significant attenuation of a *skp* mutant in a murine infection model. Additionally, *fkpA* mutants of *Salmonella* Copenhagen show reduced levels of intracellular survival (Horne et al., 1997), although studies in *S.* Typhimurium demonstrate that *fkpA* mutants are unaffected for intracellular survival, unless also combined with a deletion in *surA* or *degP* (Humphreys et al., 2003). Deletion of *surA* leads to loss of adhesion and invasion of epithelial cells by *S.* Typhimurium (Sydenham et al., 2000). In addition, the *surA* mutant was identified as an attenuated live vaccine. Sydenham et al. (2000) showed that when mice were challenged with WT *S.* Typhimurium, following a challenge with the *surA* mutant, the WT was unable to colonize the host. These findings certainly point toward an important role for σ^E-regulated periplasmic chaperones in infection and also for the identification of new therapeutic targets.

In addition to the proteins regulated by σ^E, the sRNAs have also been linked to virulence. MicA and RybB are both upregulated inside macrophages (Srikumar et al., 2015). Furthermore, MicA has a role in biofilm formation in *Salmonella* and this was understood to be due to MicA targeting of *ompA* and *phoP* mRNAs (Kint et al., 2010).

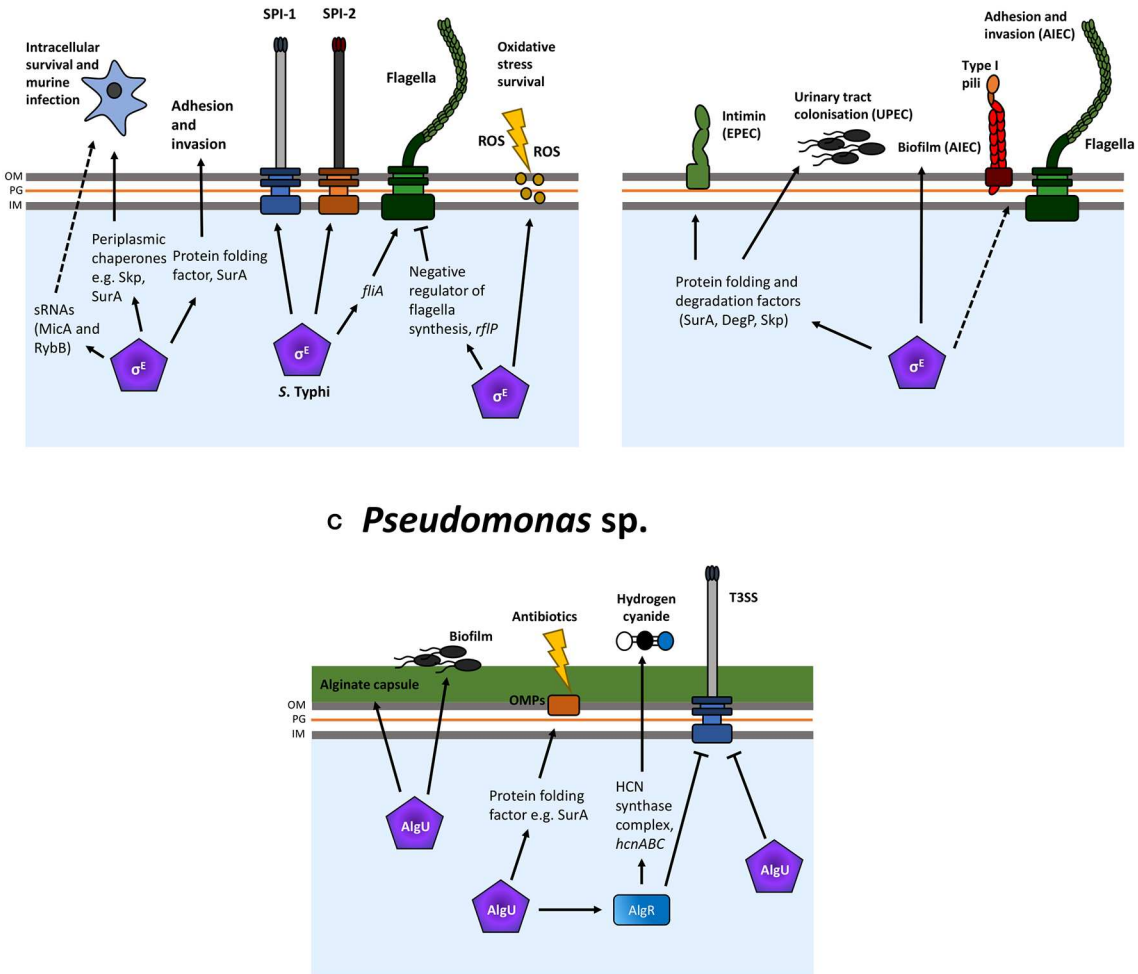

FIGURE 3 | σ^E-regulation of virulence factors in *Escherichia coli*, *Salmonella enterica,* and *Pseudomonas* spp. The σ^E ESR regulates many virulence factors and surface structures, such as adhesins, in multiple bacteria such as *Salmonella enterica* **(A)**, *E. coli* **(B)**, and *Pseudomonas* spp. **(C)**, primarily via the upregulation of periplasmic folding factors. Additionally, a number of bacteria-specific factors are regulated by this ESR. These include hydrogen cyanide in *Pseudomonas* sp. and the *Salmonella* pathogenicity islands (SPIs), SPI-1, and SPI-2. Normal arrows represent positive regulation and blunt arrows indicate negative regulation. Dashed lines indicate where regulation is indirect and/or where intermediate factors are unknown.

Aside from a role in pathogenicity and intracellular survival, *rpoE* is also required for *Salmonella* resistance to antimicrobial peptides (AMPs) during infection. Specifically, *rpoE* mutants were shown to be reduced in survival when challenged with the bactericidal/permeability-increasing protein (BPI)-derived peptide P2 and the murine α-defensin cryptdin-4 (Crp4), both of which are known to disrupt the cell envelope (Crouch et al., 2005).

Studies identifying the role of previously uncharacterized σ^E-regulated genes are continually emerging. Recently, Morris et al. (2018) demonstrated that the σ^E-regulated lipoprotein YraP contributes to OM integrity and the ability to cause murine infection. Results showed that infection of mice with a *yraP* mutant resulted in significant attenuation, particularly in the liver, compared to WT. However, the *yraP* mutant strain was not attenuated for adhesion, invasion or intracellular survival in J774, Caco-2 or primary bone marrow-derived macrophages. As such, the precise mechanism behind the attenuation of Δ*yraP* in mice is yet to be elucidated.

Escherichia coli

The role of σ^E in the pathogenesis of *E. coli* has not been studied to the same extent as in *Salmonella,* potentially because *rpoE* is an essential gene in laboratory *E. coli*. However, a small number of studies have demonstrated a role for σ^E-regulated chaperones in the virulence of different pathogenic *E. coli* strains (**Figure 3B**).

Extraintestinal *E. coli* strains are capable of colonizing and causing infection outside of their usual intestinal niche. Uropathogenic *E. coli* (UPEC) is one such example and is a major

cause of urinary tract infections (UTIs). It has been described that the σ^E-regulated chaperones *degP* and *skp* are important for *E. coli* urinary tract colonization (Redford and Welch, 2006). Enteropathogenic *E. coli* (EPEC) is a major cause of infant diarrhea and its ability to adhere to intestinal enterocytes is central to its pathogenesis (Walsham et al., 2016). The adhesin intimin is required for attachment to epithelial cells and it has been shown that the σ^E-regulated chaperones SurA, DegP and Skp are needed for the insertion of intimin into the OM (Bodelon et al., 2009).

Another *E. coli* pathovar, adherent-invasive *Escherichia coli* (AIEC), is capable of invading intestinal epithelial cells (Yang et al., 2017). σ^E is required for biofilm formation of Crohn's disease associated AIEC; inhibition of σ^E by overexpression of RseAB resulted in a significant reduction of AIEC biofilm formation (Chassaing and Darfeuille-Michaud, 2013). In addition, σ^E is important for AIEC adhesion and invasion of epithelial cells (Rolhion et al., 2007; Chassaing and Darfeuille-Michaud, 2013). Studies demonstrate that inhibition of σ^E leads to a reduction in adhesion and invasion and that this is in part, due to reduced expression of flagella and type 1 pili. However, the exact mechanisms behind the observed phenotypes are yet to be fully elucidated (Chassaing and Darfeuille-Michaud, 2013). In contrast to σ^E, other stress responses including the 2CST system CpxAR have been implicated in *E. coli* virulence to a greater extent and this will be described in detail below.

Pseudomonas

Pseudomonas aeruginosa is an opportunistic pathogen associated with high morbidity and mortality in patients with underlying respiratory disease such as cystic fibrosis (Gellatly and Hancock, 2013). *P. aeruginosa* strains exist in mucoid or non-mucoid forms but generally environmental strains and those which lead to initial colonization of the host are non-mucoid in nature (Rao et al., 2011). It has been demonstrated that the conversion to the mucoid form occurs during host colonization and it appears that the conditions, particularly in the CF lung, positively select for mucoid *P. aeruginosa*. As expected, mucoid strains are most commonly associated with CF patients; however, they have also been isolated from non-CF patients (Govan and Deretic, 1996).

The conversion from non-mucoid to mucoid is mediated by mutations in *mucA*. The *mucA* gene forms part of the *algU, mucA, mucB, mucC* operon which is homologous to the *rpoE rseA rseB rseC* operon in *E. coli* (Rowley et al., 2006). Normally, MucA sequesters AlgU activity (as does RseA for RpoE); however, mutations in *mucA*, typically *mucA22*, result in constitutive *algU* activation (Mathee et al., 1999). The *algU* regulator, sharing 79% amino acid sequence homology with *E. coli* σ^E (Potvin et al., 2008), controls expression of the alginate biosynthesis operon, formed of 12 genes for biosynthesis and export which starts at *algD*. Induction of the AlgU pathway results in production of the exopolysaccharide alginate and is of particular importance for bacterial protection and persistence in the lung (**Figure 3C**).

Activation of the AlgU pathway occurs via RIP (reviewed in Damron and Goldberg, 2012), as does activation of the σ^E pathway. In addition, *Pseudomonas* has functional equivalents of the *E. coli* proteases DegP, DegS and RseP; MucD, AlgW, and MucP, respectively (Pandey et al., 2016). Further to this, the ClpXP proteases are conserved in *Pseudomonas* and are required to release AlgU from MucA in the cytoplasm (Qiu et al., 2008).

Yu et al. (1995) showed that *Pseudomonas algU* mutants can be complemented with *E. coli rpoE*, and this complementation provided the *algU* mutant with resistance to paraquat-induced ROS stress. It has not yet been shown whether the lethal *rpoE* deletion in *E. coli* can be rescued by *algU*.

Environmental stress has been shown to induce mutations in *mucA*. Mathee et al. (1999) described that when a non-mucoid strain, PA01, was grown in a biofilm treated with hydrogen peroxide, mucoid variants with mutations in *mucA* formed. The authors propose that these findings suggest anti-oxidant therapy may be a therapeutic option for patients, as this may aid in preventing the switch from non-mucoid to mucoid.

In addition to regulating alginate production, AlgU regulates genes required for virulence (**Figure 3C**). Interestingly, an *algU* deletion increases systemic virulence (Yu et al., 1996), hence it appears that *algU* is required for infection in the respiratory but not systemic environment. The ability to form a biofilm is a major virulence factor of many bacterial pathogens and AlgU is important for biofilm formation, specifically in non-mucoid strains (Bazire et al., 2010). AlgR is a component of the 2CST system AlgZR and is regulated by AlgU (Okkotsu et al., 2014). AlgR, in turn, regulates hydrogen cyanide (HCN) production which studies indicate is an important *Pseudomonas* virulence factor. Produced under low oxygen conditions, HCN has been shown to be responsible for killing in the *Caenorhabditis elegans* infection model (Gallagher and Manoil, 2001). Additionally, both AlgR and AlgU have been shown to suppresses the *Pseudomonas* T3SS in mucoid strains (Okkotsu et al., 2014).

Several of the chaperones and proteases involved in maintenance of OM and periplasmic homeostasis in *E. coli* and *Salmonella* are conserved in *Pseudomonas* sp. A recent paper described a *surA* deletion re-sensitizes a MDR strain to antibiotics, suggesting that SurA could be a promising therapeutic target (Klein et al., 2019). Moreover, in support of this conclusion, deletion of *surA* also increased sensitivity to normal human serum.

Conclusions and Further Evidence of Roles of σ^E in Bacterial Virulence

The σ^E ESR has been implicated in other bacterial species in addition to those described above. Unfortunately, due to limitations in space, a detailed discussion is beyond the scope of this particular review. In brief, additional examples of a role for σ^E in pathogenesis include work in *Vibrio cholerae* and *Yersinia* sp. In *Vibrio cholerae*, an *rpoE* deletion resulted in a highly attenuated strain that was unable to colonize the intestine (Kovacikova and Skorupski, 2002). Moreover, a σ^E-regulated sRNA, VrrA, inhibits production of OmpA and subsequently inhibits outer membrane vesicle (OMV) formation (Song and Wai, 2009). VrrA was also shown to control biofilm formation through translational repression of *rbmC,* a *V. cholerae* specific biofilm matrix protein. When VrrA was overexpressed, biofilm levels were reduced and, as such, VrrA may assist in the

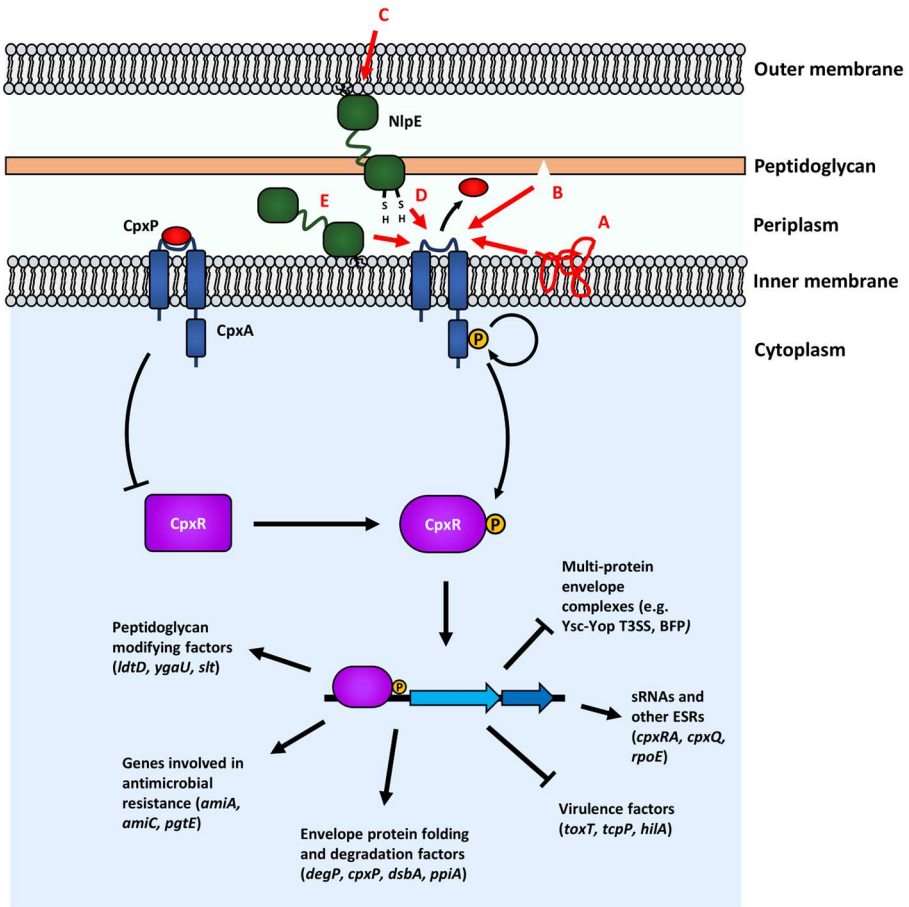

FIGURE 4 | The Cpx response senses and responds to a variety of envelope stresses. Proposed inducing cues of the Cpx response are shown with red arrows and corresponding red lettering: (A) stresses that lead to protein misfolding; (B) damage to peptidoglycan; (C) surface signals; (D) monitoring periplasmic redox states; (E) OM lipoprotein trafficking defects. In the absence of induction, the phosphatase activity of CpxA keeps CpxR unphphosphorylated. Upon induction, phosphotransfer from phosphorylated CpxA to CpxR leads to CpxR-P modulation of transcription of genes involved in several processes. Genes shown are representative, but not exhaustive of the Cpx regulon.

transition of *V. cholerae* from attachment in the intestine to shedding and uptake by new hosts (Song et al., 2014). In *Yersinia* sp. the σ^E-regulated chaperone SurA is required for *Y. pseudotuberculosis* adhesion to HeLa cells (Obi and Francis, 2013) and a *Y. pestis surA* mutant is attenuated in mice (Southern et al., 2016). The vast array of studies highlighted in this review demonstrate that σ^E is of extreme importance to not only survival of Gram-negative bacteria, through the maintenance of the extracytoplasmic OM, but also to the ability to cause such successful and varied infections.

THE CpxRA TWO-COMPONENT SYSTEM

The Cpx (conjugative pilus expression) response is a widely-conserved ESR in Gram-negative bacteria. As a canonical 2CST system, signaling in the Cpx response occurs through the sensor histidine kinase (SHK) CpxA and the response regulator CpxR (**Figure 4**). Under non-inducing conditions, the phosphatase activity of CpxA keeps CpxR in an unphosphorylated state (Raivio and Silhavy, 1997). Under inducing conditions, CpxA autophosphorylates and transfers the phosphate group to CpxR. One of the earliest findings implicating the Cpx response in a concrete role in responding to envelope stress was the finding that activated alleles of *cpxA* suppress the toxicity of the IM-localized fusion protein LamB-LacZ-PhoA (Cosma et al., 1995). Since then, much work has been done to characterize the function of the Cpx response in mitigating envelope stress (which is more comprehensively reviewed in Vogt and Raivio, 2012; Raivio, 2014). A variety of inducing cues activate the Cpx response, many, although not all, of which are thought to affect the integrity of the envelope and/or protein-folding outside of the cytoplasm. These include alkaline pH (Danese and Silhavy, 1998), aberrant expression of the Pap pilus (Jones et al., 1997), adhesion to hydrophobic surfaces (Otto and Silhavy, 2002), antimicrobial peptides (Audrain et al., 2013), and copper (Yamamoto and Ishihama, 2006). Some of the best characterized members of the Cpx regulon are genes that are involved in envelope protein folding and degradation, further suggesting a role for the

Cpx response in maintaining the integrity of the envelope by monitoring and responding to stress due to misfolded envelope proteins. These Cpx-regulated genes include those encoding the periplasmic chaperones CpxP and Spy (Danese and Silhavy, 1998; Raivio et al., 2000), the chaperone/protease DegP/HtrA (Danese et al., 1995), the disulfide bond-forming oxidoreductase DsbA (Pogliano et al., 1997), peptidyl-prolyl isomerase PpiA (Pogliano et al., 1997), IM protease HtpX and YccA, a factor that modulates IM proteolytic activity (Shimohata et al., 2002; Yamamoto and Ishihama, 2006; Raivio et al., 2013). The Cpx response also appears to regulate other ESRs, such as the operon encoding the regulatory components of the σ^E response (*rpoE-rseABC*), which is negatively regulated (Price and Raivio, 2009). It is not clear why the σ^E response is negatively regulated by the Cpx response, given that both the Cpx and σ^E responses respond to stress related to protein misfolding in the envelope. Future studies investigating this cross-regulation may shed more light onto the specific role of these ESRs and the purpose of this regulatory antagonism.

Although ostensibly a "two-component" system, other regulatory factors, such as the periplasmic protein CpxP and the OM lipoprotein NlpE are known to regulate signaling in some capacity in the Cpx response. Overexpression of NlpE is a well-known inducer of the Cpx response in *E. coli* (Danese et al., 1995; Snyder et al., 1995; Danese and Silhavy, 1998). However, NlpE is not essential in signaling all, indeed most, Cpx-inducing cues, such as PapG overexpression (DiGiuseppe and Silhavy, 2003). The crystal structure of NlpE was solved by Hirano et al. (2007) and was shown to possess two distinct β-barrel domains at its N- and C-terminus connected by an unstructured flexible linker. Comparisons of NlpE homologs in Gram-negative organisms reveal that there are two broad types of NlpE: type I (possessing the full-length protein) and type II (lacking the C-terminal domain) (Hirano et al., 2007).

One of the roles that NlpE is thought to play is as a signaler of adhesion to hydrophobic abiotic surfaces, as well as host cell surfaces (Otto and Silhavy, 2002; Shimizu et al., 2016). The structure of NlpE and its implication in sensing surface signals suggest that during signaling, conformational changes in NlpE could lead to the C-terminus "reaching down" and interacting with the sensing domain of CpxA (Hirano et al., 2007). However, the precise mechanism of NlpE signaling to the Cpx response remains largely unknown, although recent papers, that will be discussed shortly, present new insights into the function of NlpE. Interestingly, multi-drug resistant (MDR) strains of *Acinetobacter baumannii* were found to express increased levels of NlpE and this was implicated in higher biofilm formation on abiotic surfaces, despite the fact that the *A. baumannii* NlpE is type II (i.e., lacks a C-terminal domain) (Siroy et al., 2006).

Studies in both the non-pathogenic K-12 strain MC4100 and enterohemorrhagic *E. coli* (EHEC) have suggested NlpE functions as a sensor of surface adhesion both to hydrophobic glass surfaces (K-12, EHEC) and undifferentiated Caco-2 cells (EHEC) (Otto and Silhavy, 2002; Shimizu et al., 2016). Sensing surface adhesion is thought to be an important initial step in the ability of a bacteria to colonize both biotic and abiotic surfaces, particularly as a biofilm (reviewed in Belas, 2014). In line with this, surface sensing by NlpE was implicated by Shimizu et al. (2016) as an important regulatory event in promoting virulence factor expression in EHEC. However, the role of the Cpx response in surface sensing was recently disputed by Kimkes and Heinemann (2018) who did not find induction of Cpx-regulated *yebE*- or *cpxP*-GFP fluorescent reporters in response to adhesion to hydrophobic glass in a microfluidics setup nor when they attempted to recreate the original experimental conditions of Otto and Silhavy (2002). However, a close examination of these studies reveals a number of methodological differences that make it difficult to make a conclusive verdict as to whether or not NlpE is involved in surface sensing. Clearly, these conflicting conclusions point to a need for further study clarifying the role of NlpE in surface adhesion.

Recent studies have pointed to other potential roles for NlpE, specifically, as a sensor for monitoring stress related to lipoprotein trafficking and periplasmic redox state (Grabowicz and Silhavy, 2017; Delhaye et al., 2019). Deleting *lolB*, which encodes for the OM receptor lipoprotein responsible for inserting lipoproteins trafficked to the OM, has a severe deleterious effect on cell growth presumably because essential OM lipoproteins are not trafficked properly (Tanaka et al., 2001; Grabowicz and Silhavy, 2017). Deleting *cpxR* or *nlpE* in genetic backgrounds that suppress this toxicity, restores toxicity (Grabowicz and Silhavy, 2017). Conversely, activating CpxA is able to restore growth in the deleterious *lolB* null background suggesting that the Cpx response, through NlpE, is able to sense lipoprotein trafficking defects and mitigate this stress (Grabowicz and Silhavy, 2017). This makes sense, as NlpE is itself an OM lipoprotein and its own trafficking would be affected by lipoprotein trafficking defects. This model is further supported by work showing that the N-terminal domain of NlpE, which would be physically close to CpxA at the IM in the absence of trafficking to the OM, physically interacts with and is able to activate CpxA (Delhaye et al., 2019). Furthermore, it is known that forcing NlpE to localize to the IM induces the Cpx response (Miyadai et al., 2004; Delhaye et al., 2016). Future studies should work to characterize what Cpx regulon members are responsible for mitigating stress related to lipoprotein mistrafficking. Grabowicz and Silhavy (2017) raised the intriguing possibility that in the absence of the canonical Lol trafficking pathway, an alternative, Cpx-regulated pathway might exist to traffick lipoproteins, as essential OM lipoproteins such as BamD are still trafficked to the OM in the absence of LolB during Cpx-activation.

In addition to this novel role as a sensor for lipoprotein trafficking, NlpE may also act as a sensor for stress related to protein folding. Disulfide bonds in periplasmic proteins are introduced by DsbA, the absence of which leads to Cpx activation (Bardwell et al., 1991; Delhaye et al., 2019). This activation is dependent on the presence of NlpE and NlpE lacking C-terminal cysteine residues (and therefore without its normal disulfide bond) activates the response, suggesting that the function the of the C-terminal domain of NlpE is at least partly as an indicator of periplasmic redox state (Delhaye et al., 2019). It should be noted that the C-terminal cysteine residues of NlpE are conserved across Gram-negative bacteria that possess type I NlpE, suggesting that this function of NlpE may be important in several different organisms (Hirano et al., 2007).

These studies present a model in which NlpE acts as a "Swiss army knife," with distinct domains involved in sensing distinct stresses and signaling to CpxA to maintain the integrity of the envelope. Although not directly regulating virulence in most cases, these functions of NlpE have potential ramifications on pathogenesis of bacteria at various stages of infection. The transition from the environment to the host may increase oxidative stress or alter periplasmic redox status, necessitating a means to monitor and respond to these changes. A bacterial cell must "know" that it is in contact with an appropriate surface to express virulence factors or form biofilms, making surface sensing a key initial step during infection. Lipoproteins play a key role in the biogenesis of many envelope components, including virulence factors such as secretion machineries, and as such, monitoring lipoprotein trafficking is important for their proper expression. Overall, further studies of the signaling functions of NlpE may reveal important insights into the role that the Cpx response plays as a whole during infection.

CpxP is a periplasmic protein that bears structural homology to the Cpx-regulated periplasmic chaperone Spy (Kwon et al., 2010). The role of CpxP in signaling is thought to be primarily negative. *cpxP* is one of the most highly expressed members of the Cpx regulon upon activation but overexpression of CpxP inhibits activation of CpxA (Raivio et al., 1999, 2013; DiGiuseppe and Silhavy, 2003). This inhibition likely occurs through direct interaction with the periplasmic sensing domain of CpxA (Raivio et al., 1999, 2000; Zhou et al., 2011; Tschauner et al., 2014). It is thought that CpxP, in the presence of misfolded envelope proteins, will be titrated away from CpxA, and subsequently degraded by DegP/HtrA (Buelow and Raivio, 2005; Isaac et al., 2005; Tschauner et al., 2014). However, the role of CpxP in Cpx signaling is, like NlpE, not essential for most studied inducing cues, as inducers such as NlpE overexpression and alkaline pH do not require CpxP to activate the response (DiGiuseppe and Silhavy, 2003). Interestingly, although induction via alkaline pH doesn't require CpxP, *cpxP* mutants are hypersensitized to alkaline pH (Danese and Silhavy, 1998; DiGiuseppe and Silhavy, 2003). Overall, NlpE and CpxP, under the conditions in which they have been studied, rather than acting as integral players in signal transduction, appear to allow for finer regulation of the response, both as a damper of activation, as in the case of CpxP or as an enabler for other sensory inputs, as in the case of NlpE.

Interestingly, recent work has revealed the existence of a conserved, RNase E and Hfq-dependent sRNA encoded in the 3' untranslated region (UTR) of the *cpxP* mRNA, aptly named CpxQ (Chao and Vogel, 2016). In *Salmonella,* CpxQ targeted several envelope proteins, including the sodium-proton antiporter NhaB, the major subunit of the type I pilus FimA, and the periplasmic chaperone protein Skp (Chao and Vogel, 2016). CpxQ was needed for optimal survival in the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a reagent that disrupts the proton motive force (PMF) at the IM (Chao and Vogel, 2016). This, combined with the regulation of NhaB by CpxQ suggests that its role is connected to preserving the PMF at the IM. In *E. coli,* CpxQ was shown to repress CpxP production by decreasing translation of *cpxP* mRNA (Grabowicz et al., 2016). Furthermore, CpxQ was shown to help combat stress due to a mutant allele of the OM protein LamB [*lamB*(*A23D*)], that aberrantly tethers it to the IM, a lethality that is suppressed by Cpx activation (Cosma et al., 1995; Grabowicz et al., 2016). Interestingly, it was not the regulation of CpxP by CpxQ but repression of the periplasmic chaperone Skp that was responsible for alleviating the toxicity of LamB(A23D) (Grabowicz et al., 2016). Skp is thought to facilitate the aberrant insertion of LamB(A23D) into the IM, creating a pore and disrupting the PMF at the IM, a stress that is relieved by the repression of Skp by CpxQ (Grabowicz et al., 2016). This is consistent with the finding that CpxQ is involved in alleviating stress due to CCCP (Chao and Vogel, 2016).

Overall, these results point to a growing body of evidence for the role of sRNAs in mediating the stress-alleviating effects of ESRs. It had previously been shown that the Cpx response regulates the expression of several sRNAs, such as *cyaR, omrA, omrB,* and *rprA* and that these sRNA are involved in a regulatory network that not only regulates CpxRA but also links it to other 2CST systems such as EnvZ/OmpR (Raivio et al., 2013; Vogt et al., 2014). In the σ^E response, σ^E-regulated sRNAs can directly target and repress porins and OM lipoproteins that are potential sources of envelope stress (Gogol et al., 2011; Guo et al., 2014). Given the demonstrated relevance of sRNAs to envelope stress adaptation, further study of CpxQ presents an opportunity to better understand the mechanism of how the Cpx response alleviates envelope stress.

The Cpx Response and Bacterial Pathogenesis

Escherichia coli

The relevance of the Cpx response to the physiology and pathogenesis of various strains of *Escherichia coli* is well-documented (**Figure 5A**). Studies in MP1, a commensal strain of *E. coli* isolated from mice, found that deleting *cpxR* caused a severe colonization defect in mice (Lasaro et al., 2014). Further, *cpxRA* deleted strains of UPEC are attenuated in colonization and virulence in both mouse and zebrafish models (Debnath et al., 2013).

At the molecular level, multiple studies have focused on envelope-localized protein machineries such as the UPEC P-pilus and the enteropathogenic *E. coli* (EPEC) type IV bundle-forming pilus (BFP), which are assembled at the envelope and play key roles in adhesion to host cells (Wullt et al., 2000; Tobe and Sasakawa, 2001). Overexpressing PapE and PapG subunits of the P pilus activates the Cpx response but the activation of the Cpx response is not due to non-specific protein misfolding stress but rather the presence of a specific N-terminal extension on PapE involved in pilus subunit polymerization, suggesting that the Cpx response specifically monitors pilus assembly (Jones et al., 1997; Lee et al., 2004). In support of this, in the absence of *cpxR*, K-12 strains containing P pili genes produce shorter P pili, suggesting that the Cpx response is needed for efficient pilus expression and assembly (Hung et al., 2001). CpxR is also able to repress the expression of the *pap* pilin genes directly (Hernday et al., 2004). Similar roles for the Cpx response in both efficient assembly and inhibition of the elaboration of pili have been demonstrated in

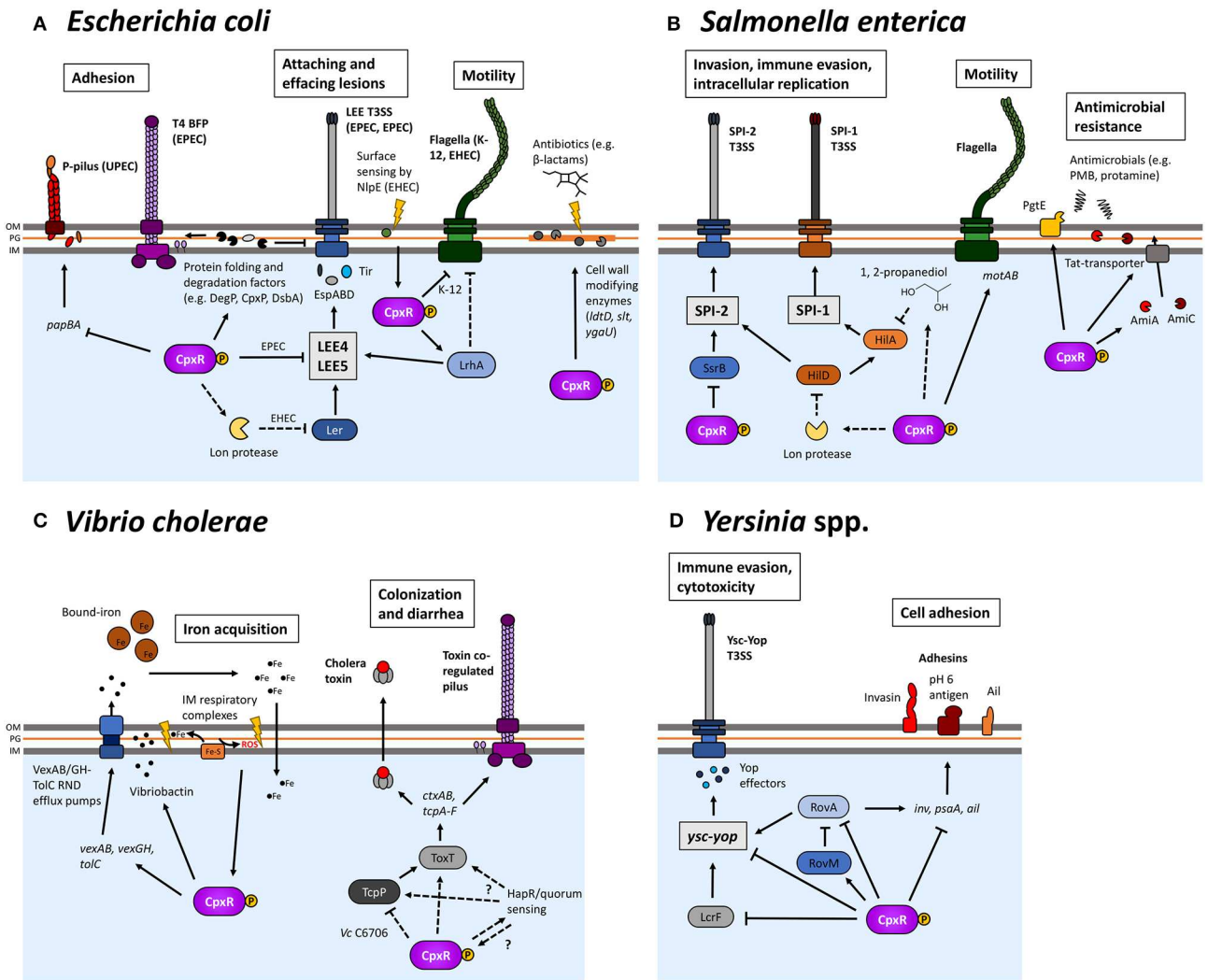

FIGURE 5 | The Cpx response is involved in the pathogenesis of several Gram-negative bacteria. The Cpx response regulates many different processes related to the pathogenesis of several different organisms, such as *Escherichia coli* **(A)**, *Salmonella enterica* **(B)**, *Vibrio cholerae* **(C)**, and *Yersinia* spp. **(D)**. Cpx regulation not only regulates the assembly of envelope-localized virulence determinants but also several other processes that impact envelope integrity and affect the ability of bacteria to colonize hosts. Proposed positive regulation is indicated by regular arrows, whereas blunt arrows represent negative regulation. Dashed lines indicate where regulation is indirect.

relation to the BFP of EPEC, despite their differing assembly and structural composition compared to P-pili (Nevesinjac and Raivio, 2005; Humphries et al., 2010; Vogt et al., 2010). In particular, efficient expression of the BFP was dependent on the Cpx-regulated periplasmic protein folding factors CpxP, DsbA, and DegP/HtrA (Vogt et al., 2010). The regulation of multi-protein envelope complexes by the Cpx response is not limited to pili, as flagella in several strains of *E. coli* are shown to be negatively regulated (De Wulf et al., 1999; Price and Raivio, 2009; Shimizu et al., 2016) and the Cpx response also regulates secretion machinery in several pathogenic *E. coli* (see below).

EPEC and EHEC are known to cause attaching and effacing (A/E) lesions on host intestinal tissue that are dependent on the locus of enterocyte effacement (LEE) T3SS (McDaniel et al., 1995; McDaniel and Kaper, 1997). Activation of the Cpx response represses LEE T3S in EPEC (Macritchie et al., 2008). This regulation occurs through the repression of the transcription of several LEE operons and post-transcriptionally by the action of the periplasmic chaperone and protease DegP/HtrA (Macritchie et al., 2008; MacRitchie et al., 2012). The regulation of the LEE T3SS does have common features between EPEC and EHEC, namely the transcriptional regulator Ler (Mellies et al., 1999; Abe et al., 2008). However, recent studies in enterohaemorrhagic *E. coli* (EHEC) have led to contrasting conclusions as to the involvement of the Cpx response in regulating the LEE (De la Cruz et al., 2016; Shimizu et al., 2016). De la Cruz et al. (2016) found that increased levels of phosphorylated CpxR in the absence of CpxA repressed levels of LEE5-encoded EspABD

translocator proteins as well as transcription of *ler,* in a manner dependent on the protease Lon. Overexpressing NlpE lead to a reduction in *espA* transcript as quantified by qPCR. However, Shimizu et al. (2016) found that *espA-luxE* reporter activity was activated in response to adhesion to hydrophobic glass beads and undifferentiated Caco-2 cells in a manner dependent on NlpE and CpxA. In adhered cells, NlpE activated *espA* transcription (Shimizu et al., 2016). This led to the suggestion that activating the Cpx response after surface sensing by NlpE leads to an upregulation of LEE T3SS genes, specifically by CpxR regulation of the positive regulator LhrA (Shimizu et al., 2016). It is possible that the difference between these two studies is due to the fact that in most experiments, surface-adhered EHEC cells were used in Shizimu and colleagues' study, whereas the study of De la Cruz et al. was conducted mostly on cells grown in liquid media. More work should be conducted to elucidate the impact that surface sensing has on modulating Cpx signaling, as it appears that adhesion significantly alters the Cpx regulation of virulence in EHEC.

A growing body of evidence highlights the relevance of the Cpx response to pathogenesis beyond protein quality control in the envelope. Studies of the Cpx regulon in *E. coli* have shown that several genes involved in cell wall modifications are upregulated by Cpx activation (Raivio et al., 2013; Bernal-Cabas et al., 2015). These include D-alanyl-D-alanine carboxypeptidase DacC (penicillin binding protein (PBP) 6) (Pedersen et al., 1998), L,D-transpeptidase LdtD (Magnet et al., 2008), lytic transglycosylase Slt (Höltje et al., 1975) and YgaU, a hypothetical protein with a LysM domain predicted to be involved in cell wall degradation (Buist et al., 2008). Accordingly, the Cpx response is activated when *ygaU* and/or *ldtD* are deleted and in strains that lack PBPs 4, 5 7 and AmpH (Evans et al., 2013; Bernal-Cabas et al., 2015). Activating the Cpx response increased diaminopimelic acid (DAP)-DAP crosslinks formed by LdtD, suggesting that Cpx activation has direct ramifications on the structural composition of PG (Bernal-Cabas et al., 2015).

It was recently shown that deleting *cpxR* increased susceptibility to the β-lactam antibiotic mecillinam, but constitutive activation led to defects in cell shape, growth, and division (Delhaye et al., 2016). Moreover, these defects were dependent on the expression of LdtD (Delhaye et al., 2016). These results point to a role for the Cpx response in monitoring not only protein quality control in the envelope, but also as a key regulatory factor for proper cell wall integrity. It has been observed that the Cpx response regulates amidases in *P. aeruginosa* and *S.* Typhimurium, suggesting that Cpx regulation of cell wall homeostasis may be a conserved function across Gram-negative organisms (Weatherspoon-Griffin et al., 2011; Yakhnina et al., 2015). The cell wall is essential for maintaining cell shape and preventing lysis during shifting osmotic conditions and is an important target for many antibiotics and innate immune strategies. Upregulation of PG-modifying enzymes may serve to reinforce the envelope during stress in other compartments of the envelope so as to increase its overall stability. Interestingly, it was recently shown that increased levels of DAP-DAP crosslinkages increases resistance to lysozyme (Stankeviciute et al., 2019). It is possible that Cpx-regulation of *ldtD* and *ygaU* may be a strategy for dealing with envelope stress arising from host innate immunity. Although the full picture remains to be seen, these studies point to the Cpx response as a fine-tuning regulator of PG structure that serves to mitigate potential stresses to the cell wall, both in and out of hosts.

Citrobacter rodentium

The presence of shared virulence determinants, most notably the LEE, in the murine pathogen *Citrobacter rodentium,* has led to its use as a model to study EPEC and EHEC infections *in vivo* (reviewed in Collins et al., 2014). Accordingly, the *in vivo* contribution of the Cpx response to virulence is arguably best demonstrated by recent studies in *C. rodentium.* The role of the Cpx response in promoting *in vivo* fitness and virulence has also previously been demonstrated in other organisms, such as in uropathogenic *E. coli* (Debnath et al., 2013), *Salmonella* (Fujimoto et al., 2018), and gonococcal *Neisseria*, where MisRS, a 2CST system suggested to be the *Neisseria* CpxRA homolog, was needed to establish infection in the genital tract of mice (Kandler et al., 2016; Gangaiah et al., 2017).

Deleting *cpxRA* attenuates the ability of *C. rodentium* to colonize and cause death in mice, in some cases completely (Thomassin et al., 2015, 2017; Vogt et al., 2019). *cpx* gene expression is induced during infection suggesting that the response is activated during host colonization (Thomassin et al., 2015, 2017). Deleting *cpxP* or *nlpE* had no significant impact on colonization, virulence or ability to bind to HeLa cells unlike the *cpxRA* null mutant which was attenuated in all these aspects (Giannakopoulou et al., 2018). Attenuation of virulence was found to be independent of type III secretion and growth rate, as secretion profiles and growth rates remain relatively the same as compared to wildtype in *cpxRA* mutants (Thomassin et al., 2015; Vogt et al., 2019).

Interestingly, it was recently reported that deleting the Cpx-regulated genes *degP/htrA* and *dsbA* led to a reduction in secreted protein levels of the T3SS translocator protein EspB in *C. rodentium* (Vogt et al., 2019). Furthermore, deleting *degP* or *dsbA* caused *C. rodentium* to become completely avirulent, unlike *cpxRA,* which in this study resulted in less attenuation of virulence compared to previous studies (Thomassin et al., 2015, 2017; Vogt et al., 2019). The reason for the difference between these studies is currently unknown. However, mutating the promoter of *degP* and *dsbA* such that they are no longer under the control of CpxR does not attenuate virulence, suggesting that Cpx regulation of *degP* or *dsbA* is not the entire reason why these mutants are avirulent (Vogt et al., 2019). Overall, these studies point to a key role for the Cpx response in promoting *in vivo* fitness and virulence, but further work characterizing the mechanism of how this occurs is needed.

Salmonella

The Cpx response has been strongly implicated in the regulation of virulence in *Salmonella enterica*, which include *S. enterica* serovar Typhi and Typhimurium (*S.* Typhi and *S.* Typhimurium, respectively; **Figure 5B**). In both *S.* Typhi and Typhimurium,

virulence is heavily dependent on two pathogenicity islands SPI-1 and SPI-2, both of which encode T3SS that facilitate invasion, immune invasion and intracellular survival (reviewed in McGhie et al., 2009; Que et al., 2013; Jennings et al., 2017). *cpx* genes in *Salmonella* are organized as they are in *E. coli* (Nakayama et al., 2003). The sequences of *cpxRA* and *cpxP* have 97, 96, and 88% identity, respectively, to their corresponding homologs in *E. coli* (Nakayama et al., 2003). As in *E. coli*, overexpression of the OM lipoprotein NlpE activates the Cpx response (Humphreys et al., 2004). CacA (Cpx-activating connector-like factor A), a small protein conserved in several Gram-negative species including *E. coli*, is induced by RpoS and is needed for full expression of Cpx-dependent genes such as *cpxP* and *spy* in *Salmonella*, suggesting that fine-tuning of Cpx regulation occurs by input from other regulatory systems, for example, in response to growth phase (Kato et al., 2012). Similarly, in *E. coli*, RpoS positively regulates *cpxRA*, further suggesting that Cpx regulation occurs in conjunction with other regulatory systems (De Wulf et al., 1999).

Transposon insertions in *cpxA* were identified in a screen of mutants of *S.* Typhi with lowered ability to invade INT407 cells (LeClerc et al., 1998). In *S.* Typhimurium, deletion of *cpxA* but not *cpxR* at low pH (pH 6.0) strongly represses the expression of a *hilA-lacZ* reporter, leading to a loss of invasion-effector protein expression and a severe defect in ability to invade INT407 cells. HilA is a key activator for SPI-1, which encodes T3SS responsible for secreting SipBCD translocator proteins and facilitating invasion (Que et al., 2013). These observations prompted the suggestion that CpxA repression of SPI-1 was CpxR-independent (Nakayama et al., 2003). However, several recent lines of evidence show that the regulation of SPI-1 by CpxA is not independent of CpxR. Deleting *cpxRA* mimics the phenotype of *cpxR* not *cpxA* mutants and overexpressing NlpE represses the expression of SPI-1 (De la Cruz et al., 2015; Subramaniam et al., 2019). Further, deleting the AckA-Pta (acetate kinase, phosphotransacetylase) pathway responsible for generating acetyl phosphate mitigates the effect of deleting *cpxA*, suggesting that in the absence of CpxA phosphatase activity, non-specific phosphodonors activate CpxR, a phenomenon previously noted (McCleary and Stock, 1994; Danese and Silhavy, 1998; Wolfe et al., 2008; De la Cruz et al., 2015). The repression of SPI-1 by phosphorylated CpxR was dependent on Lon, a protease that degrades HilD, a positive regulator of HilA, suggesting that Cpx regulation of SPI-1 occurs by regulating the stability of HilA (De la Cruz et al., 2015). Recently, a study of the Cpx regulon in *S.* Typhimurium suggested that *pocR*, a negative regulator of the *pdu-cob* cluster that encodes for genes involved in the breakdown of 1,2-propanediol (PDO), is CpxR-regulated (Subramaniam et al., 2019). PDO has been shown to repress *hilA* (Nakayama and Watanabe, 2006), suggesting that Cpx regulation of SPI-1 may occur with inputs from metabolism, although the precise mechanism of how this occurs is not clear.

Activation of the Cpx response was also shown to repress SsrB, a key activator for SPI-2 found on SPI-2 itself (Yoon et al., 2009; De la Cruz et al., 2015). It appears that this repression of SPI-2 may occur through two mechanisms, by CpxR directly binding the promoter of *ssrB* (Subramaniam et al., 2019) and by the repression of HilD, which has been shown to activate both SPI-1 and SPI-2 (Bustamante et al., 2008; De la Cruz et al., 2015). Interestingly, CpxR positively regulates motility in *Salmonella*, a finding that stands in contrast to observations in *E. coli* (Subramaniam et al., 2019).

Antimicrobial peptides (AMPs) are among the many challenges enteric pathogens face as they transition to the intestinal environment. These AMPs cause stress primarily by disrupting the envelope, and in particular, disrupt important processes such as ATP generation (reviewed in Li et al., 2017). It makes sense, then, that the Cpx response is important for mediating resistance to various antimicrobial compounds. The Cpx response is activated by polymyxin B (Fujimoto et al., 2018). CpxR was found to regulate *pgtE,* an OM protease implicated in cleaving and inactivating antimicrobial peptides and deleting *cpxR* accordingly increased sensitivity to polymyxin B (Subramaniam et al., 2019). NlpE overexpression increased resistance to antimicrobial peptides protamine, magainin 2 and melittin by upregulating two twin-arginine transport (Tat)-dependent N-acetyl muramoyl-L-alanine amidases AmiA and AmiC in a CpxR-dependent manner (Weatherspoon-Griffin et al., 2011). As these AMPs are an important component of innate immunity, overcoming this barrier is key for pathogens attempting to establish an infection. It appears that the Cpx response may play an important role in overcoming this challenge, a suggestion supported by other studies that have implicated the Cpx response in mediating resistance to AMPs, such as studies of MisRS, a CpxRA homolog in *Neisseria* spp. (Kandler et al., 2016).

It was initially reported that deleting *cpxR* did not affect colonization of mice organs relative to WT (Humphreys et al., 2004). Interestingly, a recent *in vivo* study in a streptomycin-treated mouse model found that while CpxRA was not needed to cause colitis, it was needed for colonization (Fujimoto et al., 2018). It is likely that the differences between these studies are due to the mouse model used in their experiments. While streptomycin-treated mice infected with *S.* Typhimurium cause symptoms analogous to the gastroenteritis caused by *S.* Typhimurium in humans, non-cleared mice infected with *S.* Typhimurium display a typhoid-like illness (Barthel et al., 2003). Further *in vivo* work characterizing the contribution of the Cpx response to *Salmonella* virulence is needed to elucidate the causes of the differences between these models of infection.

Vibrio cholerae

Vibrio cholerae is a Gram-negative enteric pathogen and the causative agent of the diarrheal disease cholera (reviewed in Clemens et al., 2017). In recent years, a number of studies have examined the impact of the Cpx response in *V. cholerae*. The basic genomic organization of *cpx* genes resembles that of *E. coli* and *S. enterica*: *cpxRA* is encoded as an operon with *cpxP* encoded upstream and transcribed divergently (Slamti and Waldor, 2009). The sequences of *cpxA, cpxR* and *cpxP* possess 43.6, 60.3, and 21.6% identity, respectively, to their *E. coli* counterparts (Slamti and Waldor, 2009). Importantly, the degree of conservation in the predicted periplasmic sensing domain of *cpxA* is far lower (20.7%) compared to the cytoplasmic region responsible for

CpxR phosphorylation (54.3%), suggesting potential differences in CpxA-activating signals (Slamti and Waldor, 2009). Unlike in *E. coli,* NlpE in *V. cholerae* is type II (lacking a C-terminal domain) (Hirano et al., 2007) and NlpE overexpression does not activate the Cpx response in *V. cholerae* (Slamti and Waldor, 2009). Similarly, alkaline pH is also not a Cpx pathway inducer in *V. cholerae* (Acosta et al., 2015b). The Cpx response in *V. cholerae* is activated in response to $CuSO_4$, chloride ions, aberrant disulfide bond formation, iron chelation, the absence of RND (resistance-nodulation-division) efflux pumps and ROS (Slamti and Waldor, 2009; Taylor et al., 2014; Acosta et al., 2015b; Kunkle et al., 2017). Mutations in DsbD, which is involved in the folding of secreted proteins by mediating disulfide bond formation (Ito and Inaba, 2008) and TolC, the OM component of efflux pumps (Koronakis et al., 2004), were enriched in a screen of mutants that activated the Cpx response, suggesting a conserved role for Cpx regulation of envelope homeostasis in *V. cholerae* (Slamti and Waldor, 2009).

The Cpx response has been implicated in regulating several processes that are important for the survival and growth of *V. cholerae* as it infects its host (**Figure 5C**). An important innate immune strategy is the sequestration of important minerals to limit bacterial growth. Iron, in particular, is key to several metabolic processes and is normally limited in hosts as it is sequestered in heme groups or iron-carrying proteins such as ferritin. As such, iron uptake presents an important challenge for enteric pathogens to overcome to establish infection (reviewed in Hood and Skaar, 2012). The Cpx response has been implicated in adapting to the stresses caused by low iron. The Cpx response in *V. cholerae* is activated in response to the chelation of iron and the Cpx regulon in *V. cholerae* O1 El Tor C6706 is enriched in genes involved in iron acquisition and metabolism, such as those involved in biosynthesis of the siderophore vibriobactin, ferrichrome transport and heme uptake (Acosta et al., 2015b). Furthermore, supplementing growth media with an excess of $FeSO_4$ was able to decrease activation of the response not only in iron-limiting conditions by 2,2'-bipyridyl, but also in response to diamide and RND efflux pump deletions suggesting that these Cpx-activating cues are related in some way to iron uptake (Acosta et al., 2015b).

V. cholerae possess six RND efflux systems, VexAB, CD, EF, GH, IJK, and LM, all of which likely utilize the OM protein TolC as their OM pore (Bina et al., 2008). These efflux pumps are responsible for the efflux of a variety of potentially harmful substances, including antibiotics such as polymyxin B, erythromycin and ampicillin and detergents such Triton X-100 and sodium dodecyl sulfate (SDS) (Bina et al., 2008; Taylor et al., 2012). Loss of these RND efflux pumps leads to a reduction in cholera toxin (CT) and toxin coregulated pilus (TCP) production and abolishes *V. cholerae*'s ability to colonize in an infant mouse model (Bina et al., 2008). Deletions of *tolC* and genes encoding RND efflux pumps VexAB and VexGH activate the Cpx response and likewise, activating the Cpx response induces expression of TolC and VexAB and VexGH (Slamti and Waldor, 2009; Taylor et al., 2014; Acosta et al., 2015b). These results strongly link the Cpx response to *V. cholerae*'s efflux machinery. However, although inducing the Cpx response by KCl or by a *cpxA** mutation was able to increase growth on thiosulfate-citrate-bile sucrose (TCBS) agar, a medium which requires the action of RND efflux to allow for growth, deleting *cpxR* did not negatively affect growth on TCBS agar relative to WT suggesting that the Cpx response is not normally required for growth in efflux-requiring conditions (Taylor et al., 2014). Thus, although efflux and the Cpx response are strongly linked genetically, the extent to which the Cpx response is linked to innate antimicrobial resistance in *V. cholerae* remains to be determined.

Interestingly, the significance of Cpx regulation of RND efflux pumps was shown to be connected to iron acquisition and transport (Kunkle et al., 2017). Mutations in genes involved in the synthesis of vibriobactin, a catechol siderophore (Griffiths et al., 1984), were found to suppress Cpx activation in efflux-deficient mutants (Kunkle et al., 2017). Levels of extracellular vibriobactin were reduced in RND efflux negative mutants, suggesting RND efflux pumps function to transport vibriobactin out of the cell and that in their absence, vibriobactin aberrantly accumulates in the periplasmic space (Kunkle et al., 2017). As iron is an essential component of several IM complexes of the electron transport chain (ETC) (Friedrich et al., 2016), it was suggested that the activation of the Cpx response in the RND-deficient mutant was due to the chelation of iron away from these complexes, leading to aberrant protein folding at the IM and/or the production of ROS during respiration (Kunkle et al., 2017). Consonant with this hypothesis is the observation that activation in efflux-negative mutants was abolished in anaerobic growth and in a *sdhA* mutant, which encodes for a subunit of succinate dehydrogenase (Kunkle et al., 2017). Studies in EPEC have shown that respiratory complexes found at the IM, such as NDH-I and cytochrome bo_3 are negatively regulated by the Cpx response, suggesting that monitoring respiratory complexes at the IM may be a conserved function of the Cpx response, although it remains to be seen how the Cpx response senses these stresses (Raivio et al., 2013; Guest et al., 2017).

Elucidating the precise role of the Cpx response in regulating virulence in *V. cholerae* is complicated by contrasting results in different, albeit closely related strains. The primary diarrheagenic effect of *V. cholerae* stems from the ADP-ribosylating action of CT, which leads to changes in intracellular signaling and fluid secretion from intestinal epithelial cells (Field et al., 1972; and Gill et al., 1978). RND efflux pumps have been shown to be needed for the optimal production of CT and TCP in O1 El Tor strain N16961, but deleting *cpxR* in a strain lacking all six RND efflux pumps did not affect decreased levels of CT and TCP, suggesting a Cpx-independent mechanism for the regulation of CT and TCP production (Bina et al., 2008; Taylor et al., 2014). Furthermore, neither *cpxA** (constitutively active) or *cpxR* mutants changed CT or TCP levels relative to WT (Taylor et al., 2014). Accordingly, neither deletions of *cpxR, cpxA* or *cpxP* or activation of the response via a *cpxA** allele affected the ability of N16961 to colonize infant mice (Slamti and Waldor, 2009).

In contrast to these results, mutating *cpxR* in a related O1 El Tor strain, C6706, led to an increase in *ctxB, tcpA,* and *tcpP* transcription (Acosta et al., 2015a). Correspondingly, overexpressing CpxR led to a complete abrogation of the expression of CT and TCP both at the transcriptional and protein

level (Acosta et al., 2015a). CpxR overexpression repressed the transcription of ToxT, the direct regulator of TCP and CT (DiRita et al., 1991) and TcpP, a regulator involved in promoting the expression of ToxT (Häse and Mekalanos, 1998), suggesting that Cpx regulation of virulence occurs through established virulence regulators (Acosta et al., 2015a). It was suggested that the discrepancy in results between C6706 and N16961 could be due to a known defect in the ability of N16961 to quorum sense, another process closely involved in regulating virulence in *V. cholerae* (Zhu et al., 2002). Nonetheless, these contrasting results underscore the need to be cognizant of strain differences when conducting work on even closely related strains and future studies investigating these differences may help elucidate the precise mechanisms of Cpx regulation of key *V. cholerae* virulence determinants.

Yersinia spp.

The genus *Yersinia* include a number of Gram-negative pathogens including *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterolitica*. Studies of the Cpx response have focused on the latter two species, *Y. pseudotuberculosis* and *Y. enterolitica*, which, while not as well-known as the infamous cause of the bubonic and pneumonic plagues, are nonetheless significant pathogens of humans (Drummond et al., 2012). However, a study in *Y. pestis* has implicated the Cpx response in promoting survival in neutrophils (O'Loughlin et al., 2010), and as such it is certainly possible that the findings in other species of *Yersinia* are applicable to *Y. pestis*.

The virulence of *Yersinia* spp. depends heavily on the action of the virulence plasmid-encoded Ysc-Yop (*Yersinia* secretion-*Yersinia* outer protein) T3SS. Ycs proteins form the machinery responsible for the translocation of a collection of different Yop effectors that block phagocytosis into host cells directly and allow for extracellular replication of *Yersinia* (reviewed in Cornelis, 2002). *Yersinia* spp. are thought to primarily be extracellular pathogens, although they possess the ability to invade and survive intracellularly (Grabenstein et al., 2004). Several ESRs contribute to virulence factor regulation in *Yersinia*, including the Psp and Rcs responses (Flores-Kim and Darwin, 2012; Li et al., 2015b).

In the last two decades, several studies have examined the Cpx response in *Y. enterolitica* and *Y. pseudotuberculosis*, particularly in relation to type III secretion and adhesion to host cells (**Figure 5D**). The sequence identity of *cpxR* and *cpxA* to their *E. coli* homologs are high in both *Y. pseudotuberculosis* and *Y. enterolitica* (89 and 80%, respectively in *Y. pseudotuberculosis* and 81 and 74%, respectively in *Y. enterolitica*) (Heusipp et al., 2004; Carlsson et al., 2007a). As in *E. coli,* the Cpx regulon in *Yersinia* spp. includes envelope protein folding factors and proteases such as *htrA*/*degP*, *dsbA* and *ppiA* (Heusipp et al., 2004; Carlsson et al., 2007a,b; Liu et al., 2011).

Several studies have shown that deleting *cpxA* represses the production of the Ysc secretion machinery, Yop effectors as well as adhesins such as invasin (*inv*) in *Y. pseudotuberculosis* (Carlsson et al., 2007a,b; Liu et al., 2011, 2012). Furthermore, deleting *cpxA* abrogates the ability of *Y. pseudotuberculosis* to cause cytotoxicity in HeLa cells caused by YopE, a type III secretion secreted cytotoxin (Carlsson et al., 2007a,b; Vlahou et al., 2009; Liu et al., 2012). This repression of virulence occurs in the *cpxA* mutant as a result of non-specific phosphorylation of CpxR in the absence of CpxA phosphatase activity, as constitutively active *cpxA** mutants produce similar results to *cpxA* knock-out mutants, and removing acetyl-phosphate by introducing a Δ*ackA, pta* mutation abolishes the effects of deleting *cpxA* (Carlsson et al., 2007a,b; Liu et al., 2011, 2012). Cytotoxicity was not affected in a *cpxR* null mutant and this mutation actually increased cell adhesion and invasion (Carlsson et al., 2007a,b). Furthermore, ectopically expressing CpxR, but not CpxR D51A (unable to be phosphorylated), repressed cytotoxicity, indicating that phosphorylated CpxR is responsible for the repression of cell adhesion and type III secretion (Carlsson et al., 2007a; Liu et al., 2011). These results suggest that the Cpx response functions to repress T3SS assembly and secretion and are corroborated by transcriptional data which shows that *cpxR* is downregulated during conditions that induce the expression of the Ysc-Yops T3SS (Carlsson et al., 2007a).

The precise mechanism of regulation of these processes appears to require a multitude of different regulatory approaches. Although Ysc structural protein levels are reduced in membrane fractions in *cpxA* null mutants, the promoters of these genes lack CpxR binding sites, suggesting indirect regulation at the transcriptional level or potential post-transcriptional regulation by other Cpx-induced factors (Carlsson et al., 2007a; Liu et al., 2012). In contrast, the transcription of several *yop* effector genes and *syc* effector chaperones are directly regulated by phosphorylated CpxR (Liu et al., 2012). In addition, LcrF, a transcription factor which induces the expression of *yop* genes when the temperature shifts from 26 to 37°C (Cornelis et al., 1989), is also directly regulated by CpxR suggesting that regulation of Yop effectors occurs not only through directly modifying transcription but also by modulating expression of other transcriptional activators (Liu et al., 2012).

A similar story can be told in regards to cell adhesion. Phosphorylated CpxR can directly modulate transcription of known adhesins invasin and pH 6 antigen (*psaA*), as well as proposed adhesins such as the OM protein Ail, suggesting direct regulation (Carlsson et al., 2007b; Liu et al., 2011). However, regulation of these factors also occurs through other regulators. RovA, a global transcriptional regulator that activates the expression of invasin, is directly regulated by CpxR (Carlsson et al., 2007b). A further level of control was recently discovered when it was demonstrated that RovM, a regulator of RovA that represses its expression during nutrient limitation, was found to be directly CpxR-P regulated (Heroven and Dersch, 2006; Heroven et al., 2008; Thanikkal et al., 2019). Thus, RovA expression is not only modulated by CpxR at its own promoter, but also through CpxR upregulation of RovM. The *in vivo* significance of this result may be related to the finding that the RovM regulatory cascade is implicated in causing a lifestyle switch from acute to persistent infection in mice (Avican et al., 2015). This is also consistent with findings in *S.* Typhimurium, where the Cpx response was dispensable for causing acute virulence in mice, but was needed to colonize the mouse gut over longer spans of time (Fujimoto et al., 2018). The sum of these results points to a network of regulatory connections that control

the expression of several key *Yersinia* virulence determinants. An area for further investigation is elucidating the precise inducing cues of the Cpx response in *Yersinia* spp. so as to understand why the Cpx response negatively regulates key envelope localized virulence determinants.

The Cpx response in *Y. enterolitica* has not been explored as thoroughly as in its sister species. Nonetheless, studies point to some key differences. Unlike in *Y. pseudotuberculosis,* to our knowledge, no studies have been published successfully deleting *cpxA* in *Y. enterolitica,* and attempts to do so even in the presence of CpxA expression in *trans* have not been successful (Ronnebaumer et al., 2009). Furthermore, overexpression of CpxA is deleterious even in normal growth conditions, suggesting that the effect of deleting and overexpressing CpxA is related to the phosphorylation of CpxR (Ronnebaumer et al., 2009). In *Y. pseudotuberculosis,* however, deleting *cpxA* only causes a minor growth defect (Carlsson et al., 2007a). At this point, it is unclear what is responsible for the apparent differences between *Y. pseudotuberculosis* and *Y. enterolitica*. However, as the Cpx regulon remains relatively uncharacterized in both of these species, its further characterization may yield insights into the root of these differences.

Summary

It should be noted that studies of the Cpx response are not limited to the organisms we discuss in this review. Unfortunately, given restraints on space, it is impossible to discuss all of the relevant work in other organisms. As such, we wish to point readers to the following studies for further reading: *Shigella* spp. (Nakayama and Watanabe, 1995, 1998; Mitobe et al., 2005), *Legionella pneumophila* (Gal-Mor and Segal, 2003; Vincent et al., 2006; Altman and Segal, 2008), *Haemophilus ducreyi* (Labandeira-Rey et al., 2009, 2010; Gangaiah et al., 2013), and *Neisseria* spp. (Tzeng et al., 2004, 2008; Kandler et al., 2016; Gangaiah et al., 2017).

The work conducted in a diverse array of different organisms highlights the contribution of the Cpx response to the virulence of several Gram-negative pathogens. Although the exact details can differ between organisms, or even closely related strains, there are a few important motifs that we see repeated throughout these studies. The Cpx regulon across species typically includes envelope-localized protein folding and degradation factors, such as DegP/HtrA. There exists a consistent link between envelope-localized, multi-protein complexes, such as pili or secretion machinery, and regulation by the Cpx response. These patterns support an overarching paradigm where the Cpx response functions to maintain envelope integrity by monitoring protein quality control. However, the specific effect of the Cpx response in different organisms is not uniform, despite the fact that essentially all of the organisms surveyed in this review are enteric pathogens that, generally speaking, share a similar niche in the host. In some organisms (such as *C. rodentium*), the Cpx response is indispensable to colonization and virulence *in vivo*, while in other organisms, activation of the Cpx response has a predominantly negative impact on key virulence determinants. Related to this, while in general, the Cpx response negatively regulates key virulence determinants, the presence of the Cpx response tends to be positively associated with colonization or long-term persistence. Given this general observation, it appears that the role of the Cpx response in pathogenesis tends not toward promoting virulence factor expression, but rather ensuring that the cell can survive in the midst of the multitude of stresses faced during the course of infection, whether that be due to the expression of the virulence factors themselves or from external factors, such as host immune strategies.

What is responsible for the observed differences between organisms? It is almost certain that phylogenetic differences can explain aspects of the observed diversity. For example, remarkable similarities exist in the mechanism of Cpx regulation of the LEE T3SS in pathogenic *E. coli* and the SPI-1 T3SS in *S.* Typhimurium (see **Figures 4**, **5** and the corresponding sections). But when comparing more distantly related species (as is reflected in greater sequence dissimilarity in *cpx* genes), such as *E. coli* and *V. cholerae*, more disparities tend to be seen in inducing cues, mechanisms of signaling or regulon members. It is also highly likely that differences in life-cycle and infection strategies influence how the Cpx response functions in different species. For example, while phylogenetically, *E. coli* may be more closely related to *Salmonella*, than to *V. cholerae*, the extracellular lifestyle adopted by EPEC and EHEC is more analogous to *V. cholerae* than to the invasive lifestyle of *Salmonella*. And so, commonalities, such as the regulation of pilus expression, can be seen in *E. coli* and *V. cholerae*. Perhaps part of the issue is that the Cpx response is best characterized in various strains of *E. coli* and studies in other pathogens have yet to be conducted to the same level of depth. If this is the case, further study in these organisms will only yield expanded insight into our understanding of the Cpx response and its function in the physiology and pathogenesis of a variety of different organisms.

CONCLUDING REMARKS

It is clear from the literature that ESRs such as the σ^E response and the Cpx response play key roles in the physiology and pathogenesis of several Gram-negative bacteria. Nonetheless, it is also clear that many questions remain about these ESRs, such as those about precise mechanisms of signaling and regulation of target genes and processes, and their relevance to *in vivo* fitness. Particularly of interest is exploring the precise points at which these ESRs are relevant to colonization and infection. When and where are these ESRs active? What precise cues *in vivo* induce these responses? Do ESRs predominantly mitigate encountered stresses or do ESRs play more proactive roles in promoting colonization or virulence? Exploring these questions further will not only increase our basic understanding of these Gram-negative bacteria but also may help provide potential strategies for the prevention and treatment of the diseases caused by these organisms.

AUTHOR CONTRIBUTIONS

CH and TC reviewed the literature, co-wrote the manuscript, and prepared the figures. GR and TR conceived the idea and co-edited the drafts.

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Cannabidiol Is a Novel Modulator of Bacterial Membrane Vesicles

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Membrane vesicles (MVs) released from bacteria participate in cell communication and host-pathogen interactions. Roles for MVs in antibiotic resistance are gaining increased attention and in this study we investigated if known anti-bacterial effects of cannabidiol (CBD), a phytocannabinoid from *Cannabis sativa*, could be in part attributed to effects on bacterial MV profile and MV release. We found that CBD is a strong inhibitor of MV release from Gram-negative bacteria (*E. coli* VCS257), while inhibitory effect on MV release from Gram-positive bacteria (*S. aureus* subsp. *aureus* Rosenbach) was negligible. When used in combination with selected antibiotics, CBD significantly increased the bactericidal action of several antibiotics in the Gram-negative bacteria. In addition, CBD increased antibiotic effects of kanamycin in the Gram-positive bacteria, without affecting MV release. CBD furthermore changed protein profiles of MVs released from *E. coli* after 1 h CBD treatment. Our findings indicate that CBD may pose as a putative adjuvant agent for tailored co-application with selected antibiotics, depending on bacterial species, to increase antibiotic activity, including via MV inhibition, and help reduce antibiotic resistance.

Keywords: bacterial membrane vesicles (MVs), cannabidiol (CBD), antibiotic resistance, gram-negative, gram-positive, *E. coli* VCS257, *S. aureus* subsp. *aureus* Rosenbach

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INTRODUCTION

Outer membrane vesicles (OMVs) and membrane vesicles (MVs) are released from Gram-negative and Gram-positive bacteria and participate in inter-bacterial communication, including via transfer of cargo molecules (Dorward and Garon, 1990; Li et al., 1998; Fulsundar et al., 2014; Jan, 2017; Toyofuku et al., 2019). MVs are released in greater abundance from Gram-negative, than Gram-positive bacteria and their production seems crucial for bacterial survival and forms part of the stress response (McBroom and Kuehn, 2007; Macdonald and Kuehn, 2013; Jan, 2017). Gram-negative bacteria generate, in addition to common one-bilayer vesicles (OMV), also double-bilayer vesicles (O-IMVs), and in some stress conditions other types of MVs (Pérez-Cruz et al., 2016) and therefore we will use the umbrella term "membrane vesicles" (MVs) hereafter. MVs are important in biofilm formation and dissemination of toxins in the host (Wang et al., 2015; Cooke et al., 2019).

MVs participate in host-pathogen interactions (Gurung et al., 2011; Koeppen et al., 2016; Bitto et al., 2017, 2018; Codemo et al., 2018; Turner et al., 2018; Cecil et al., 2019) and may also be involved in antibiotic resistance, for instance by protecting biofilms from antibiotics via increased vesiculation (Manning and Kuehn, 2011). Furthermore, MVs from *Porphyromonas gingivalis* have been linked to metabolic remodeling in the host (Fleetwood et al., 2017), while MVs from *Neisseria gonorrhoeae* have been shown to target host mitochondria and to induce macrophage death (Deo et al., 2018). Besides roles for cellular and bacterial communication, the use of MVs as nano-carriers for various compounds, including for antibiotic and vaccine delivery, has also raised considerable interest in the research community (Gnopo et al., 2017; Rüter et al., 2018; Tan et al., 2018; Wang et al., 2018).

The regulation of bacterial MV biogenesis and release may therefore be of great importance, both in relation to inter-bacterial communication, including biofilm formation, their host interactions as commensals, as well as in host-pathogen interactions and in antibiotic resistance.

Cannabidiol (CBD) is a phytocannabinoid from *Cannabis sativa* with anti-inflammatory (Martin-Moreno et al., 2011), anti-cancerous (Pisanti et al., 2017; Kosgodage et al., 2018) and anti-bacterial activity (Hernández-Cervantes et al., 2017). While immunoregulatory roles for cannabinoids have been reported in infectious disease (reviewed in Hernández-Cervantes et al., 2017), and *C. sativa* has been identified as a natural product with a capability of controlling bacterial infections, including a strong anti-bacterial activity against antibiotic resistant strains (Appendino et al., 2008), a link between CBD and bacterial MV release has hitherto not been investigated.

As our recent work identified CBD as a potent inhibitor of extracellular vesicle (EV) release in eukaryotes (Kosgodage et al., 2018; Gavinho et al., 2019), we sought to investigate whether CBD may work via phylogenetically conserved pathways, involving bacterial MV release from bacteria. As we, and other groups, have previously shown that cancer cells can be sensitized to chemotherapeutic agents via various EV-inhibitors (Jorfi et al., 2015; Koch et al., 2016; Muralidharan-Chari et al., 2016; Kosgodage et al., 2017), including CBD (Kosgodage et al., 2018, 2019), we sought to establish whether in bacteria, similar putative MV modulatory effects could be utilized to sensitize bacteria to antibiotics.

MATERIALS AND METHODS

MV Isolation From *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

E. coli (VCS257, Agilent, La Jolla, CA) and *S. aureus* subsp. *aureus* Rosenbach (ATCC 29247, USA) static cultures were grown in Luria-Bertani (LB) broth for 24 h at 37°C. The growth phase before vesicle isolation was exponential; the volume of the cultures was 20 ml. For MV isolation, ultracentrifugation and nanoparticle tracking analysis (NTA) were used based on previously established methods by other groups (McCaig et al., 2013; Klimentova and Stulik, 2015; Roier et al., 2016).

E. coli and *S. aureus* cultures were maintained by plating on Mueller-Hinton agar plates and weekly sub-culturing was performed according to previously established methods (Iqbal et al., 2013).

Before MV isolation, all bacterial growth medium (LB broth) was pre-treated before use by ultracentrifugation at 100,000 g for 24 h to ensure minimum contamination with extracellular vesicles (EVs) from the medium (Kosgodage et al., 2017).

For MV isolation, bacteria were grown in EV-free medium (as described above) for 24 h at 37°C, the culture medium was collected and centrifuged once at 400 g for 10 min for removal of cells, followed by centrifugation at 4,000 g for 1 h at 4°C to remove cell debris. The resultant supernatant was then centrifuged for 1 h at 100,000 g at 4°C and the isolated MV pellet was resuspended in Dulbecco's phosphate buffered saline (DPBS; ultracentrifuged and sterile filtered using a 0.22 μm filter) and centrifuged again at 100,000 *g* for 1 h at 4°C. The resulting MV pellet was sterile filtered (0.45 μm) once and then resuspended in sterile filtered DPBS. The quantitative yield of vesicles was $\sim 6.5 \times 10^9$ MVs per liter of culture. The isolated MV pellets were then either used immediately, or stored at −80°C for further experiments.

Transmission Electron Microscopy (TEM) Imaging of Bacterial MVs

A suspension of isolated MVs (1.4×10^8 MVs/ml) was used for TEM imaging. MV samples (10 μL) were applied to mesh copper grids, prepared with glow discharged carbon support films, and incubated for 2 min. The grids were then washed five times with 50 μl of 1 % aqueous uranyl acetate. Grids were left to dry for 5 min before being viewed. Micrographs were taken with a JEOL JEM 1230 transmission electron microscope (JEOL, Japan) operated at 80 kV at a magnification of 80,000 to 100,000. Digital images were recorded using a Morada CCD camera (EMSIS, Germany) and processed via iTEM (EMSIS).

Western Blotting

Protein was isolated from MV pellets using Bacterial Protein Extraction Reagent (B-PER, ThermoFisher Scientific, U.K.), pipetting gently and shaking the pellets on ice for 2 h, where after samples were centrifuged at 16,000 g at 4°C for 20 min and the resulting supernatant collected for protein analysis. Samples were prepared in 2x Laemmli buffer, boiled at 95°C for 5 min, electrophoresed by SDS-PAGE on 4–20 % TGX gels (BioRad, U.K.), followed by semi-dry Western blotting. Approximately 10 μg of protein was loaded per lane and even protein transfer was assessed by Ponceau S staining (Sigma, U.K.). Blocking of membranes was performed for 1 h at room temperature (RT) in 5 % BSA in TBS-T. The membranes were then incubated with the anti-OmpC (Outer-membrane protein C antibody; orb6940, Biorbyt, U.K.; diluted 1/1000 in TBS-T) overnight at 4°C, followed by washing in TBS-T and incubation for 1 h in anti-rabbit-HRP conjugated secondary antibody at RT. Visualization was performed using ECL (Amersham, U.K.) and the UVP BioDoc-ITTM System (U.K.).

Nanoparticle Tracking Analysis for Assessment of MV Release From *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

MVs were isolated from control and CBD-treated bacterial cultures as described above. Nanoparticle tracking analysis (NTA) was performed using the Nanosight LM10 (Malvern, U.K.), equipped with a 405 nm diode laser and a sCMOS camera. MV pellets were resuspended in equal volumes (100 μl) of DPBS before NTA analysis to ensure comparable analysis of quantification. Before application, samples were diluted 1:50 in sterile-filtered EV-free DPBS and applied at a constant flow rate, maintaining the number of particles in the field of view in the range of 20–40 with a minimum concentration of samples at 5×10^7 particles/ml. Camera settings were according to the manufacturer's instructions (Malvern), five 60 s videos per sample were recorded and replicate histograms averaged. Each experiment was repeated three times.

CBD-Mediated MV Release Inhibition in *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

E. coli and *S. aureus* cultures were cultivated using EV-free Müeller-Hinton broth for 24 h. An inoculate of 0.1 ml of bacteria, in a 20 ml culture volume of bacterial growth medium (Luria-Bertani (LB) broth), were grown at exponential phase overnight, as assessed by OD600. The bacterial cells were then washed using DPBS at 4,000 g for 10 min and seeded in 1.5 ml triplicates in micro centrifuge tubes. For treatment with CBD, CBD (GW research Ltd) was applied at concentrations of 1 or 5 μM and incubated with the bacterial cultures for 1 h at 37°C. Treatments were performed in triplicates, including DMSO as a control. MV isolation following CBD and control treatment was carried out using step-wise centrifugation and ultracentrifugation as before. Changes in MV release were assessed by quantifying numbers of MVs by NTA analysis as described above, with each experiment repeated three times. Cell viability was assessed before the start of every experiment and after treatment with CBD compared to controls determined by colony forming unit (CFU) measurement.

Disc Diffusion Test for Assessment of CBD-Mediated Enhancement of Antibiotic Treatment

Discs were impregnated with the following antibiotics (all from Sigma-Aldrich): colistin (10 μg/ml), rifampicin (15 μg/ml), erythromycin (50 μg/ml), kanamycin (1,000 μg/ml) and vancomycin (5 μg/ml). Concentration of the antibiotics used was based on previously published and established MIC values (Maclayton et al., 2006; Moskowitz et al., 2010; Kshetry et al., 2016; Rojas et al., 2017; Goldstein et al., 2018). *E. coli* and *S. aureus* agar plates were prepared for the disc diffusion test (Iqbal et al., 2013) by soaking a sterile paper disc in 5 μM CBD and placing it in the middle of the agar plate, while the impregnated antibiotic discs were placed equidistant to the CBD disc. Zones of inhibition were assessed after 24 h using the Kirby-Bauer test.

Proteomic Analysis of MVs Released From CBD Treated and Control Untreated *E. coli* VCS257

To assess differences in *E. coli* VCS257 MV protein composition in response to CBD treatment, MVs were isolated as before, after 1 h treatment with 1 μM or 5 μM CBD treatment or control untreated, respectively. MVs were assessed by SDS-PAGE (using 4–20 % gradient TGX gels, BioRad, U.K.) and silver staining using the BioRad Silver Stain Plus Kit (1610449, BioRad, U.K.), according to the manufacturer's instructions (BioRad). For assessment of proteomic changes, MVs were subjected to liquid chromatography-mass spectrometry (LC-MS/MS) analysis. MVs from CBD treated, vs. non-treated *E. coli* were run 1 cm into a SDS-PAGE gel and the whole protein lysate cut out as one band, whereafter it was processed for proteomic analysis (carried out by Cambridge Proteomics, U.K.). Peak list files were submitted to Mascot (in-house, Cambridge Center for Proteomics) using the following database: Uniprot_Escherichia_coli_20180613 (4324 sequences; 1357163 residues).

Statistical Analysis

Histograms and graphs were prepared and statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, U.S.A.). One-way ANOVA and Student's *t*-test analysis were performed, followed by Tukey's *post-hoc* analysis. Histograms represent mean of data, with error bars representing standard error of mean (SEM). Significant differences were considered as $p \leq 0.05$.

RESULTS

Characterization of MVs From *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

Isolated MVs were assessed by morphology using transmission electron microscopy (TEM), revealing a poly-dispersed population in the size range of mainly 20–230 nm in diameter for *E. coli*, including MVs showing inner and outer membranes (**Figure 1A.1**), and characteristic one layer membranes for *S. aureus* MVs, which were in the 37–300 nm range (**Figure 1A.2**). Nanoparticle tracking analysis (NTA) verified that the majority of the vesicle population fell in a similar size range under standard culture conditions (mode 143.3 nm; SD ± 72.3 nm for *E. coli* (**Figure 1A.1**) and 141.4 nm; SD ± 7.3 nm for *S. aureus* (**Figure 1A.2**). Furthermore, Western blotting showed positive for the MV specific marker OmpC (**Figure 1A**).

Effects of CBD on Membrane Vesicle Release From *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

CBD changed the MV release profile from *E. coli* compared to control treatment (**Figures 1B–D**). Modal size of MVs released

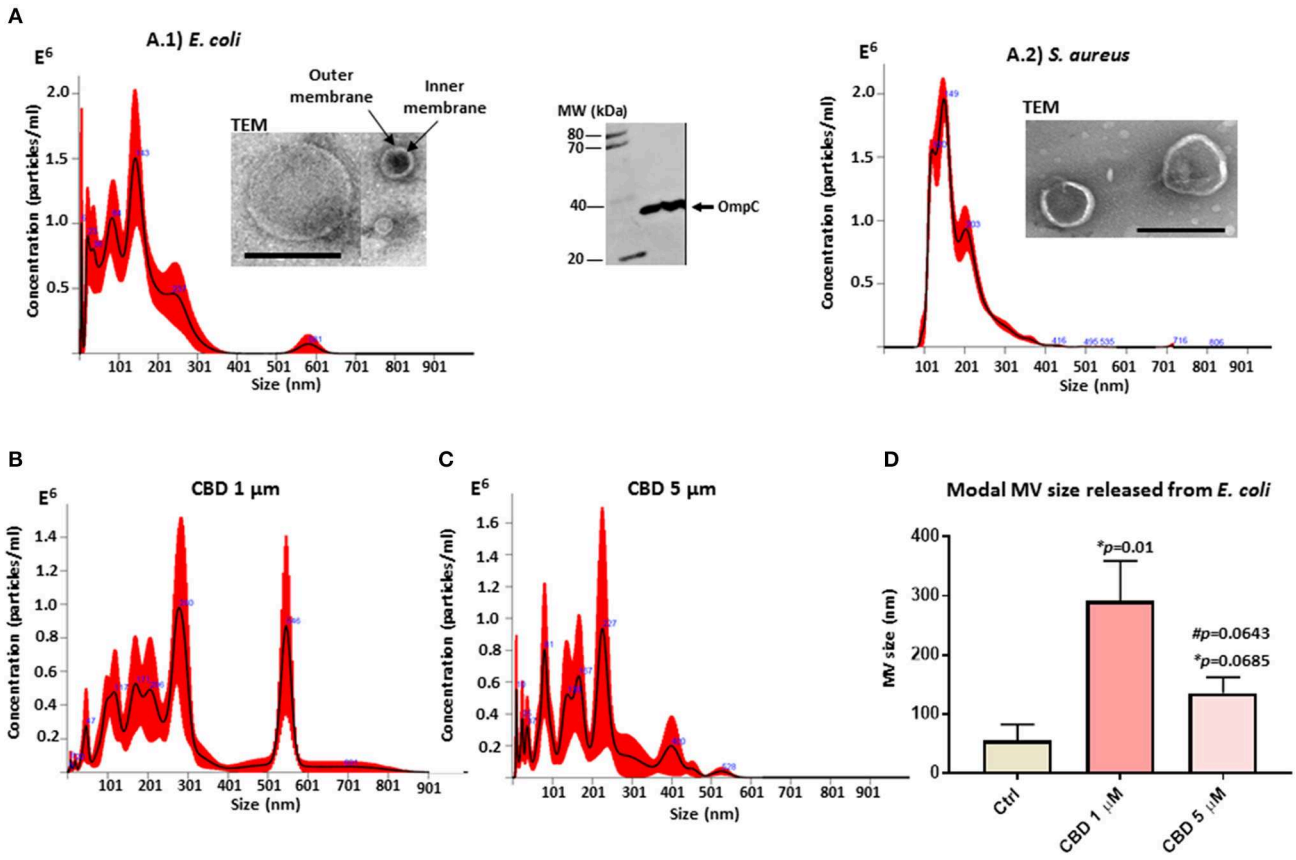

FIGURE 1 | Bacterial MV profile under standard conditions and after CBD treatment. **(A)** MVs released from untreated *E. coli* VCS257 **(A.1)** and *S. aureus* subsp. *aureus* Rosenbach **(A.2)**, shown by NTA analysis (Nanosight); Transmission electron microscopy (TEM, scale bar = 200 nm) and Western blotting with the MV-specific marker OmpC. **(B)** NTA analysis showing MV release from *E. coli* after 1 h CBD treatment (1 µM). **(C)** NTA analysis showing MV release from *E. coli* after 1 h CBD treatment (5 µM). **(D)** Modal size of MVs released from *E. coli* under normal culture conditions compared to CBD treatment. Error bars indicate SEM; **p* represents *p*-values compared to control (ctrl) while #*p* represents *p*-values compared to 1 µM CBD treatment.

from *E. coli* was significantly increased ($p = 0.01$) after 1 µM CBD treatment, compared to control treated cells, while 5 µM CBD treatment did not have statistically significant effects on MV size ($p = 0.0685$). Effects on modal size of vesicles released from *E. coli* between the two doses of CBD was also not statistically significant ($p = 0.0643$; **Figure 1D**).

CBD had a significant inhibitory effect ($p < 0.0001$) on total MV release from *E. coli* VCS257 at both concentrations tested (1 and 5 µM, respectively; **Figure 2A**). In addition, the lower dose of CBD (1 µM) had stronger MV-inhibitory effects (73 % reduction, $p < 0.0001$) than 5 µM CBD (54 % reduction, $p < 0.0001$; **Figure 2A**) and resulted in a markedly increased peak at 500 nm (**Figure 1B**), which otherwise was negligible in the control (**Figure 1A**) and 5 µM CBD (**Figure 1C**) treated *E. coli*.

Effects of CBD on *E. coli* VCS257 MVs was furthermore assessed by TEM, verifying the presence of fewer vesicles per field and showing some change in vesicle size and morphology after CBD (**Supplementary Figures 2A–C**).

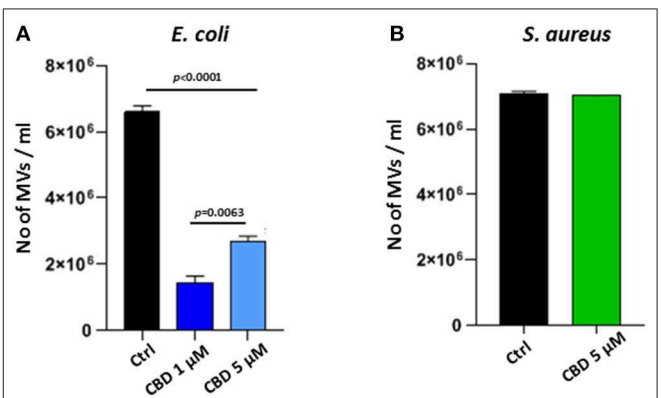

FIGURE 2 | CBD affects MV-release from the Gram-negative bacteria *E. coli* VCS257 but not Gram-positive *S. aureus* subsp. *aureus* Rosenbach. **(A)** MV release from *E. coli* was significantly reduced after CBD treatment, with lower dose of CBD being more effective ($p = 0.0063$). **(B)** MV release from *S. aureus* was not significantly affected by CBD treatment. Exact *p*-values are shown.

Contrary to what was observed for the Gram-negative *E. coli*, CBD treatment (5 μM) had no significant effect on MV release from the Gram-positive bacterium *S. aureus* subsp. *Aureus* Rosenbach ($p > 0.1$; **Figure 2B**).

Effects of CBD on Bacterial Viability of *E. coli* VCS257 and *S. aureus* subsp. *aureus* Rosenbach

CBD had negligible effect on *E. coli* cell viability after 24 h incubation with the lower 1 μM dose, while an 11 % ($p = 0.0161$) reduction in cell viability was observed in response to 5 μM CBD, but no significant effect was observed on *S. aureus* cell viability, as assessed by disk diffusion test (**Supplementary Figure 1**).

CBD Treatment Affects Antibiotic Sensitivity in *E. coli* VCS257

CBD treatment (5 μM), when applied in combination with a range of antibiotics tested, was found to sensitize *E. coli* VCS257 to selected antibiotics, as assessed by an increase in the radius of zone of inhibition, using the disk diffusion test (**Figure 3**). Significantly enhanced antibacterial effects were found for erythromycin (35 % increase; $p = 0.006$), rifampicin (50 % increase; $p = 0.0007$) and vancomycin (100 % increase; $p < 0.0001$), when combined with CBD treatment (5 μM), compared to antibiotic treatment alone. Notably, vancomycin alone did not have bactericidal effects on *E. coli*, but only in the presence of CBD. Antibacterial effects of kanamycin were increased by 18 % but this was not statistically significant compared to antibiotic alone ($p = 0.09$). Zone of inhibition with CBD treatment only was also observed in the *E. coli* plates (**Figure 3**), but this was significantly lower than when CBD was combined with antibiotics, except for vancomycin. The zone of inhibition for *E. coli* caused by antibiotic treatment only, vs. CBD alone, differed also significantly for erythromycin ($p = 0.0010$), vancomycin ($p = 0.0158$), rifampicin ($p = 0.0003$) and kanamycin ($p = 0.0008$), but not for colistin ($p = 0.224$). Therefore, while CBD showed some anti-bacterial activity against *E. coli* when applied in isolation, this was significantly lower than observed for the antibiotics alone (except for vancomycin which did not show antibacterial activity while CBD did). However, when applied in combination, CBD increased bactericidal effects of all antibiotics tested, except for colistin.

CBD-Mediated Effects on Antibiotic Sensitivity in *S. aureus* subsp. *aureus* Rosenbach

When added to *S. aureus* subsp. *aureus* Rosenbach, 5 μM CBD increased the antibiotic activity of kanamycin (30 %; $p = 0.0028$), as assessed by increased radius of zone around the diffusion disk (**Figure 4**). CBD did not enhance anti-bacterial activity for the other antibiotics tested and reduced antibacterial effects of both erythromycin and rifampicin ($p = 0.0325$ and $p = 0.0001$, respectively). Importantly, there was no halo observed around the diffusion disk containing CBD alone in the *S. aureus* plates, indicating no bactericidal effects of CBD on this strain of *S. aureus*.

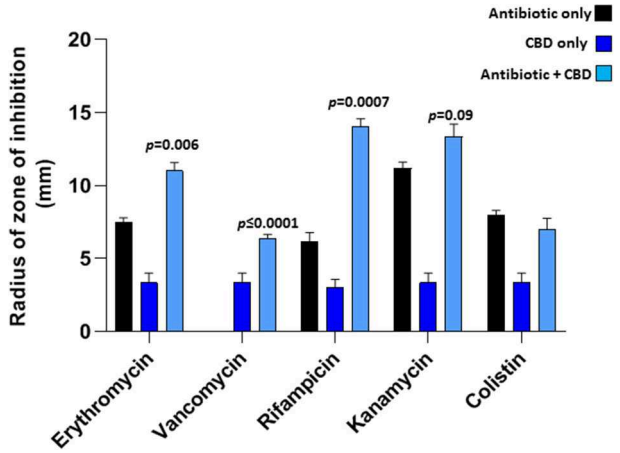

FIGURE 3 | CBD sensitizes Gram-negative bacteria *E. coli* VCS257 to selected antibiotics. Combinatory treatment of CBD with a range of antibiotics (24 h treatment) showed enhanced CBD-mediated antibacterial effects on *E. coli* VCS257, as assessed by increased radius of zone around the diffusion disks. CBD was most effective in combination with rifampicin ($p = 0.0007$), vancomycin ($p \leq 0.0001$) and erythromycin ($p = 0.006$). CBD in isolation also had bactericidal effects on *E. coli*, while combinatory treatment with the antibiotics was most effective. Exact *p*-values are shown.

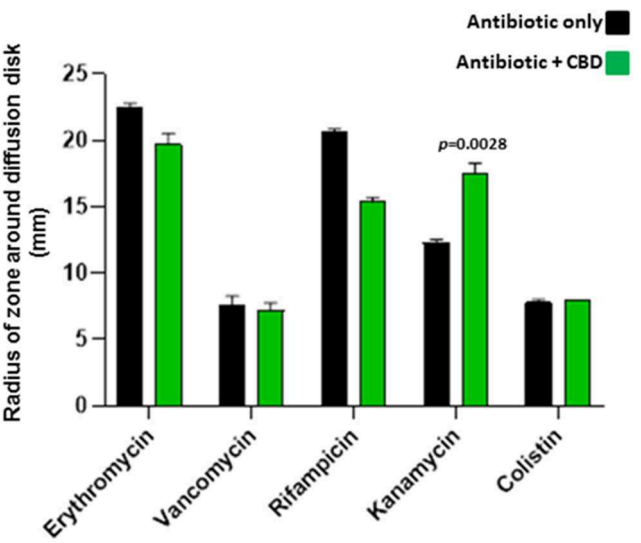

FIGURE 4 | CBD sensitizes Gram-positive bacteria *S. aureus* subsp. *aureus* Rosenbach to kanamycin. Combinatory treatment of CBD with a range of antibiotics showed enhanced antibacterial effects of kanamycin only on *S. aureus*, as assessed by an increased radius of zone around the diffusion disk ($p = 0.0028$). CBD did not enhance bactericidal activity for the other antibiotics tested and reduced bactericidal effects of both erythromycin ($p = 0.0325$) and rifampicin ($p = 0.0001$). CBD application in isolation did not form a halo around the diffusion disk in the *S. aureus* plates, opposed as to what was observed in *E. coli*, and CBD treatment in isolation is therefore not included in the histogram. Exact *p*-values are shown.

Effects of CBD Treatment on Protein Profiles of MVs Released From *E. coli* VCS257

Protein composition of MVs was assessed in MVs isolated from *E. coli* VCS257 after 1 h treatment with 1 μM and 5 μM CBD, respectively, compared to non-treated *E. coli* MVs, using SDS-PAGE silver stained gels and LC-MS/MS analysis. Silver stained gels revealed some band differences between the three conditions (**Figure 5A**). Proteins were further analyzed by LC-MS/MS and peak list files submitted to Mascot (in-house, Cambridge Center for Proteomics, Uniprot_Escherichia_coli_20180613). Hits are listed in **Tables 1**–**3**. Compared to untreated MVs, five protein hits were absent in MVs released from the 1 μM CBD treated *E. coli* and four protein hits were absent in MVs released from the 5 μM CBD treated *E. coli*, respectively (**Table 1** and **Figure 5B**). When comparing the two CBD treatments, 26 protein hits were specific to the *E. coli* MVs following 1 μM CBD treatment (**Table 2** and **Figure 5B**) while 68 protein hits were unique to the MVs released from *E. coli* treated with 5 μM CBD (**Table 3** and **Figure 5B**).

DISCUSSION

To our knowledge this is the first study to evaluate the putative effects of CBD on the release of membrane vesicles (MVs) from bacteria and effects of CBD on MV profile, including protein composition. In eukaryotic cells, CBD was recently identified as an effective inhibitor of extracellular vesicle (EV) release both in human cancer cells (Kosgodage et al., 2018, 2019) as well as in the intestinal parasite *Giardia intestinalis* (Gavinho et al., 2019). Therefore, our present findings may indicate phylogenetically conserved pathways of membrane vesicle release from bacteria to mammals that can be modulated via CBD. Moreover, CBD could enhance the anti-bacterial effect of certain antibiotics in some bacterial types, but also inhibit it in others. This indicates that inhibition of MV release and anti-bacterial action are likely linked, as previously suggested (Tashiro et al., 2010). Indeed, a recent study using indole derivatives has revealed a role for MVs in antibiotic resistance/persistence, in particular in Gram-negative bacteria tested (Agarwal et al., 2019).

Here we report that CBD significantly reduced MV release in *E. coli* VCS257, a Gram-negative bacterium, but had negligible effects on membrane vesicle release in *S. aureus* subsp. *aureus* Rosenbach, a Gram-positive bacterium, as assessed here by *in vitro* analysis. In addition, we also found that lower doses of CBD had a stronger MV inhibitory effect in *E. coli* VCS257 than a higher 5 μM dose ($p = 0.0063$), and such an effect has also previously been observed for EVs in certain cancer cell types (Kosgodage et al., 2018). Biphasic effects of CBD are indeed recognized (Bergamaschi et al., 2011) and may be reminiscent of "hormesis," an effect we have suggested could explain its more general medical benefits as well as effects on mitochondrial dynamics (Nunn et al., 2013). Interestingly, at the lower 1 μM concentration, CBD significantly increased the release of a 500 nm peak of MVs, as observed by NTA analysis, while this peak was negligible both in the control treated bacteria and those treated with 5 μM CBD. Such an effect of CBD on MV profile, and protein MV profile as observed by proteomic analysis here, may be relevant in the light of recent recognition of the importance of MV size for cellular entry and uptake (Turner et al., 2018) and in line with an increased interest in the research community for the identification and characterization of MV sub-populations (Pérez-Cruz et al., 2016; Turner et al., 2018; Cooke et al., 2019; Toyofuku et al., 2019; Zavan et al.,

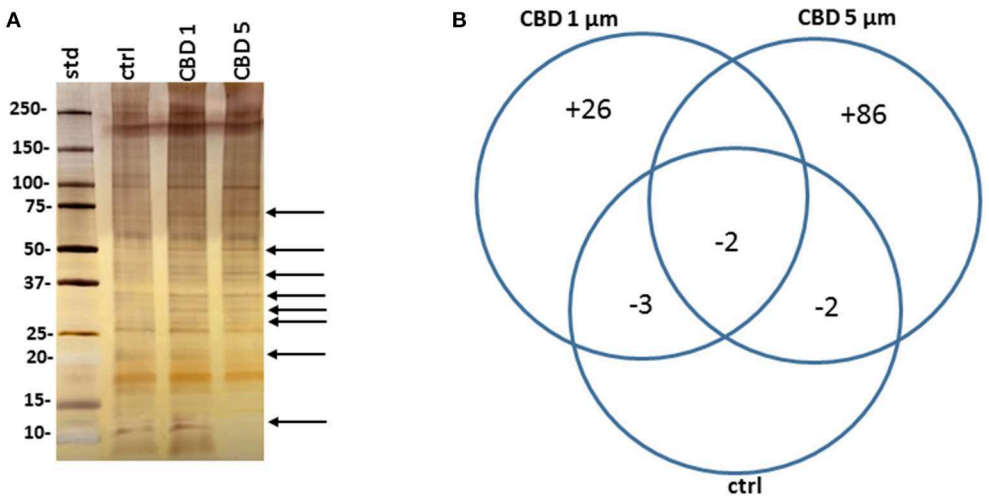

FIGURE 5 | CBD affects protein composition of *E. coli* VCS257 MVs. **(A)** A SDS-PAGE silver stained gel reveals banding differences between the CBD treated and non-treated *E. coli* derived MVs (see arrows highlighting some present and absent bands). **(B).** Venn diagram showing protein changes in MVs released from CBD treated compared to untreated control *E. coli* VCS257. Plus ("+") indicates hits unique to MVs following CBD 1 or 5 μM treatment, respectively; minus ("−") indicates number of proteins absent in the respective CBD treated MVs, compared to control untreated MVs. For specific protein hits see **Tables 1**–**3**.

TABLE 1 | Proteins identified as present in *E. coli* VCS257 control untreated MVs only and absent in MVs from CBD treated *E. coli*.

Protein name	Symbol	Score ($p < 0.05$)‡	CBD 1 μM	CBD 5 μM
Glutamate decarboxylase alpha	P69908|DCEA_ECOLI	37	–	+
2-oxoglutarate dehydrogenase E1 component	P0AFG3|ODO1_ECOLI	36	+	–
RNA chaperone ProQ	P45577|PROQ_ECOLI	32	–	+
Uncharacterized protein YffS	P76550|YFFS_ECOLI	29	–	+
Serine transporter	P0AAD6|SDAC_ECOLI	26	+	–
Fumarate and nitrate reduction regulatory protein	P0A9E5|FNR_ECOLI	26	–	–
Uncharacterized protein YcaQ	P75843|YCAQ_ECOLI	22	–	–

Proteins were isolated from E. coli derived MVs and analyzed by LC-MS/MS. Peak list files were submitted to Mascot (in-house, Cambridge Center for Proteomics, Uniprot_Escherichia_coli_20180613; 4324 sequences; 1357163 residues).

*‡Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 19 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits.*

2019). The approximately 6.5-fold and 2.5-fold decreases in MV release observed after CBD (1 and 5 μm, respectively) treatment from *E. coli*, compared to non-treated controls, also correlated with a trend in shift toward proportionally larger vesicles released according to NTA analysis and change in protein profile. The exact mechanism for packaging proteins and other reagents in MVs is not fully understood and given the plethora of targets for CBD (Ibeas Bih et al., 2015; Hernández-Cervantes et al., 2017; Pisanti et al., 2017), the exact mechanism of this cannabinoid on MV formation remains subject to further extensive studies. In the current study we have indeed identified a range proteins, including proteins involved in metabolism and antibiotic metabolic processing, which differ in MVs released from *E. coli* VCS257 treated with CBD, compared to MVs released from non-treated *E. coli*. Previous studies have discussed the use of MVs for example as drug delivery vehicles (Ellis and Kuehn, 2010; Gujrati et al., 2014; Gerritzen et al., 2017; Jain and Pillai, 2017; Jan, 2017; Wang et al., 2018), while MVs have also been tested as delivery vehicles for targeted gene silencing using siRNA-packaged MVs (Alves et al., 2016). Whether CBD may be utilized for combinatory application with such approaches may also be of putative interest, in addition to its observed effects in this study, in effectively reducing MV release.

In relation to antibiotic activity, cannabinoids including CBD, have been widely studied for their anti-bacterial activity (Wasim et al., 1995; Bass et al., 1996; Appendino et al., 2008; Hernández-Cervantes et al., 2017). For example, *C. sativa* extracts have previously been shown to have microbicidal activity on various Gram-positive bacteria, including several strains of *S. aureus*, as well as some Gram-negative bacteria (Wasim et al., 1995; Elphick, 2007; Nissen et al., 2010), with the minimum inhibitory concentrations (MIC) for the main phytocannabinoids, such as CBD, being in the 0.5–5 μM range, which is similar to many modern antibiotics (Van Klingeren and Ten Ham, 1976; Appendino et al., 2008). How precisely CBD may be working as an anti-bacterial agent is still not entirely clear (Appendino et al., 2008), particularly in the light of a plethora of targets for CBD (Ibeas Bih et al., 2015; Hernández-Cervantes et al., 2017), while structure-activity studies indicate that the ability of plant-derived phenolic compounds to interact with membranes and the existence of electrophilic functional groups are important (Miklasinska-Majdanik et al., 2018). Hitherto though, no association has been made into a putative regulatory effect of cannabinoids on bacterial membrane vesicle release. Furthermore, as the current study has revealed changes in proteomic profile of MVs released from *E. coli* VCS257 following CBD treatment, such findings may inform anti-bacterial effects of CBD. Using LC-MS/MS analysis to assess changes in protein profile of MVs from CBD treated and untreated *E. coli,* respectively, five proteins were found to be absent in the 1 μM CBD treated MVs and 4 proteins were absent in the 5 μM CBD treated MVs, compared to control untreated *E. coli* MVs. Out of these, 2 proteins overlapped between the two CBD treatments. In addition, comparing 1 and 5 μM CBD treated *E. coli* MVs, 26 protein hits were unique to MVs released following the 1 μM CBD treatment and 68 protein hits to MVs released following the 5 μM CBD treatment. Using STRING analysis, PPI enrichment *p*-value was found to be $p = 0.0204$ for proteins identified as unique to MVs from the 1 μM CBD treatment and $p = 1.56 \times 10^{-6}$ for proteins identified as unique to MVs from the 5 μM CBD. This indicates that for both treatments these proteins have significantly more interactions among themselves, than what would be expected for a random set of proteins of similar size, drawn from the genome. Such enrichment indicates that the proteins are at least partially biologically connected, as a group. Protein networks are represented showing biological GO pathways and KEGG pathways, respectively, in **Supplementary Figures 3**, **4** for proteins specific to EVs from *E. coli* after 1 and 5 μM CBD treatment, respectively. Proteins identified are related to metabolic processes, cellular respiration and antibiotic functions (**Supplementary Figures 3A,B**, **4A,B**).

When assessing the effectivity of CBD to enhance susceptibility of Gram-positive and Gram-negative bacterial species to a range of antibiotics, CBD-mediated MV inhibition rendered *E. coli* VCS257 significantly more sensitive to erythromycin, vancomycin and rifampicin and somewhat to kanamycin, but did not augment the bactericidal effects observed for colistin. This was somewhat unexpected, given a previous study showing that MVs isolated from the *E. coli* strain MG1655 could protect bacteria against membrane-active

TABLE 2 | Proteins identified as present only in MVs released from *E. coli* VCS257 following 1 h treatment with 1 μM CBD.

Protein name	Symbol	Score ($p < 0.05$)‡
Glutamate decarboxylase beta	P69910|DCEB_ECOLI	230
Tryptophan synthase alpha chain	P0A877|TRPA_ECOLI	85
2-oxoglutarate dehydrogenase E1 component	P0AFG3|ODO1_ECOLI	70
Uncharacterized protein YgaU	P0ADE6|YGAU_ECOLI	67
Spermidine/putrescine-binding periplasmic protein	P0AFK9|POTD_ECOLI	67
Serine transporter	P0AAD6|SDAC_ECOLI	57
Inorganic pyrophosphatase	P0A7A9|IPYR_ECOLI	56
Succinate dehydrogenase flavoprotein subunit	P0AC41|SDHA_ECOLI	54
NADH-quinone oxidoreductase subunit A	P0AFC3|NUOA_ECOLI	53
Periplasmic dipeptide transport protein	P23847|DPPA_ECOLI	49
Uncharacterized protein YqiC	Q46868|YQIC_ECOLI	48
Formate dehydrogenase, nitrate-inducible, major subunit	P24183|FDNG_ECOLI	47
Acyl carrier protein	P0A6A8|ACP_ECOLI	45
Maltose/maltodextrin-binding periplasmic protein	P0AEX9|MALE_ECOLI	44
Septum site-determining protein MinD	P0AEZ3|MIND_ECOLI	42
Phosphate-specific transport system accessory protein PhoU	P0A9K7|PHOU_ECOLI	40
Ribosome-associated inhibitor A	P0AD49|YFIA_ECOLI	36
DNA-binding protein H-NS	P0ACF8|HNS_ECOLI	35
RNA-binding protein Hfq	P0A6X3|HFQ_ECOLI	33
Phosphate transport system permease protein PstA	P07654|PSTA_ECOLI	32
Galactoside transport system permease protein MglC	P23200|MGLC_ECOLI	32
Sec translocon accessory complex subunit YajC	P0ADZ7|YAJC_ECOLI	31
Isoform Beta of Translation initiation factor IF-2	P0A705-2|IF2_ECOLI	30
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	P62707|GPMA_ECOLI	30
Peptidoglycan D,D-transpeptidase FtsI	P0AD68|FTSI_ECOLI	28
Inner membrane protein YjcH	P0AF54|YJCH_ECOLI	27
HTH-type transcriptional regulator GntR	P0ACP5|GNTR_ECOLI	27
Histidinol-phosphate aminotransferase	P06986|HIS8_ECOLI	26
SsrA-binding protein	P0A832|SSRP_ECOLI	25
2-dehydro-3-deoxyphosphooctonate aldolase	P0A715|KDSA_ECOLI	25
Deoxyribose-phosphate aldolase	P0A6L0|DEOC_ECOLI	25
Ribosome hibernation promoting factor	P0AFX0|HPF_ECOLI	24
Ribokinase	P0A9J6|RBSK_ECOLI	24
Probable ATP-dependent helicase l hr	P30015|LHR_ECOLI	22
Membrane-bound lytic murein transglycosylase B	P41052|MLTB_ECOLI	21
Uncharacterized protein YjaA	P09162|YJAA_ECOLI	21
Adenylate kinase	P69441|KAD_ECOLI	21
Fructose-1,6-bisphosphatase 2 class 2	P21437|GLPX2_ECOLI	20
Transcription termination/antitermination protein NusA	P0AFF6|NUSA_ECOLI	20

Proteins were isolated from CBD treated (1 μM) E. coli MVs and analyzed by LC-MS/MS. Peak list files were submitted to Mascot (in-house, Cambridge Center for Proteomics, Uniprot_Escherichia_coli_20180613; 4324 sequences; 1357163 residues).

‡Ions score is $-10^{*}Log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 18 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits.

antibiotics such as colistin (Kulkarni et al., 2015). Our finding, that CBD did not sensitize *E. coli* further to colistin, when applied in combination with this antibiotic, may arise from the fact that a different strain of *E. coli* (VCS257) was used in the current study, compared to in the study by Kulkarni et al. (2015). It has also been previously shown that the presence of calcium decreases the bactericidal effect of colistin on *Paenibacillus polymyxa*, suggesting a role for Ca^{2+} in generating a protective barrier against colistin (Yu et al., 2015). As CBD is known to modulate calcium (Rimmerman et al., 2013) it can be postulated that this may interfere with the mode of action of colistin. Our findings also indicate that combinatory application of CBD is not effective for all antibiotics, which may possibly be explained by their different modes of action. Importantly, zones of inhibition were observed in the plates which were only treated with the CBD discs in the presence

TABLE 3 | Proteins identified as present only in *E. coli* VCS257 derived MVs following 1 h treatment with 5 μM CBD.

Protein name	Symbol	Score ($p < 0.05$)‡
Glutamate decarboxylase alpha	P69908|DCEA_ECOLI	189
ATP-dependent zinc metalloprotease FtsH	P0AAI3|FTSH_ECOLI	128
Rod shape-determining protein MreB	P0A9X4|MREB_ECOLI	109
Uncharacterized protein YibN	P0AG27|YIBN_ECOLI	101
Outer membrane protein X	P0A917|OMPX_ECOLI	99
Galactitol 1-phosphate 5-dehydrogenase	P0A9S3|GATD_ECOLI	91
UPF0381 protein YfcZ	P0AD33|YFCZ_ECOLI	85
50S ribosomal protein L31	P0A7M9|RL31_ECOLI	83
Biotin carboxylase	P24182|ACCC_ECOLI	83
GMP synthase [glutamine-hydrolyzing]	P04079|GUAA_ECOLI	82
Cytochrome bd-I ubiquinol oxidase subunit 1	P0ABJ9|CYDA_ECOLI	74
Galactokinase	P0A6T3|GAL1_ECOLI	74
RNA chaperone ProQ	P45577|PROQ_ECOLI	71
Protein GrpE	P09372|GRPE_ECOLI	68
Purine nucleoside phosphorylase	P0ABP8|DEOD_ECOLI	61
50S ribosomal protein L21	P0AG48|RL21_ECOLI	59
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	P0AFG6|ODO2_ECOLI	58
Sec-independent protein translocase protein TatA	P69428|TATA_ECOLI	56
Bifunctional protein GlmU	P0ACC7|GLMU_ECOLI	56
PTS system mannose-specific EIIAB component	P69797|PTNAB_ECOLI	55
Anaerobic glycerol-3-phosphate dehydrogenase subunit C	P0A996|GLPC_ECOLI	54
Proline/betaine transporter	P0C0L7|PROP_ECOLI	52
Pyruvate formate-lyase 1-activating enzyme	P0A9N4|PFLA_ECOLI	52
Pyruvate/proton symporter BtsT	P39396|BTST_ECOLI	52
Protein translocase subunit SecY	P0AGA2|SECY_ECOLI	49
Penicillin-binding protein activator LpoB	P0AB38|LPOB_ECOLI	49
Signal peptidase I	P00803|LEP_ECOLI	45
Thiol peroxidase	P0A862|TPX_ECOLI	45
UPF0307 protein YjgA	P0A8X0|YJGA_ECOLI	45
Peptidyl-prolyl cis-trans isomerase D	P0ADY1|PPID_ECOLI	44
3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase	P0A6Q3|FABA_ECOLI	44
ATP-dependent protease subunit HslV	P0A7B8|HSLV_ECOLI	43
Inosine-5'-monophosphate dehydrogenase	P0ADG7|IMDH_ECOLI	42
Peptide chain release factor RF2	P07012|RF2_ECOLI	41
Nucleoside diphosphate kinase	P0A763|NDK_ECOLI	40
Inositol-1-monophosphatase	P0ADG4|SUHB_ECOLI	40
Respiratory nitrate reductase 1 gamma chain	P11350|NARI_ECOLI	40
Succinate dehydrogenase hydrophobic membrane anchor subunit	P0AC44|DHSD_ECOLI	39
Outer membrane protein assembly factor BamB	P77774|BAMB_ECOLI	36
Signal recognition particle receptor FtsY	P10121|FTSY_ECOLI	36
Anaerobic C4-dicarboxylate transporter DcuB	P0ABN9|DCUB_ECOLI	34
Glucans biosynthesis protein	P33136|OPGG_ECOLI	34
Adenine phosphoribosyltransferase	P69503|APT_ECOLI	34
Maltoporin	P02943|LAMB_ECOLI	34
NADH-quinone oxidoreductase subunit C/D	P33599|NUOCD_ECOLI	32
ATP-dependent protease ATPase subunit HslU	P0A6H5|HSLU_ECOLI	32
CDP-diacylglycerol–serine O-phosphatidyltransferase	P23830|PSS_ECOLI	32
PTS system trehalose-specific EIIBC component	P36672|PTTBC_ECOLI	31
Transcription termination/antitermination protein NusG	P0AFG0|NUSG_ECOLI	31

(Continued)

TABLE 3 | Continued

Protein name	Symbol	Score ($p < 0.05$)‡
Protein translocase subunit SecF	P0AG93|SECF_ECOLI	30
Oligopeptide transport system permease protein OppB	P0AFH2|OPPB_ECOLI	30
Uncharacterized protein YffS	P76550|YFFS_ECOLI	29
NADH-quinone oxidoreductase subunit J	P0AFE0|NUOJ_ECOLI	29
Glucosamine-6-phosphate deaminase	P0A759|NAGB_ECOLI	29
Uncharacterized protein YiaF	P0ADK0|YIAF_ECOLI	28
Tol-Pal system protein TolQ	P0ABU9|TOLQ_ECOLI	28
Multidrug export protein EmrA	P27303|EMRA_ECOLI	27
UPF0246 protein YaaA	P0A8I3|YAAA_ECOLI	25
DNA-directed RNA polymerase subunit omega	P0A800|RPOZ_ECOLI	24
ATP-binding/permease protein CydD	P29018|CYDD_ECOLI	24
Glycine betaine-binding protein YehZ	P33362|YEHZ_ECOLI	23
NADP-dependent malic enzyme	P76558|MAO2_ECOLI	23
Multiphosphoryl transfer protein	P69811|PTFAH_ECOLI	23
Ribose-5-phosphate isomerase A	P0A7Z0|RPIA_ECOLI	22
Disulfide bond formation protein B	P0A6M2|DSBB_ECOLI	22
Uncharacterized protein YbjD	P75828|YBJD_ECOLI	22
NADH-quinone oxidoreductase subunit L	P33607|NUOL_ECOLI	21
Pyridoxine 5'-phosphate synthase	P0A794|PDXJ_ECOLI	21

Proteins were isolated from CBD treated (5 μM) E. coli MVs and analyzed by LC-MS/MS. Peak list files were submitted to Mascot (in-house, Cambridge Center for Proteomics, Uniprot_Escherichia_coli_20180613; 4324 sequences; 1357163 residues).

‡Ions score is $-10^{*}Log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 19 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20.

of *E. coli*, and this clearly revealed the antibacterial property of CBD.

Interestingly, CBD did increase antibacterial effects of vancomycin on *E. coli*, in spite of vancomycin's limited effectiveness on Gram-negative species, also seen here by the fact that vancomycin alone did not result in a halo around the diffusion disk for *E. coli*. Therefore, CBD seems to overcome previously established resistance of *E. coli* to vancomycin, which has reported to partly be due to its inability to significantly penetrate the outer membrane (Zhou et al., 2015). It may also be important to note that erythromycin, rifampicin and kanamycin inhibit protein synthesis, whereas vancomycin is a glycopeptide that inhibits cell biosynthesis in Gram-positive bacteria, while colistin binds to the outer membrane of Gram-negative bacteria, disrupting it. Thus, these antibiotics display very different modes of action.

In the Gram-positive bacterium *S. aureus* subsp. *aureus* Rosenbach, CBD increased bactericidal activity of kanamycin only. The reduced ability of CBD to sensitize this Gram-positive bacterium to antibiotics, compared to the significantly higher effects in the Gram-negative bacterium, tallied in with CBD's ability to regulate MV-release, indicating a relevant contribution of MVs to antibiotic resistance. Roles for MVs in protecting biofilms via adsorption of antimicrobial agents have indeed been previously recognized (Schooling and Beveridge, 2006; Manning and Kuehn, 2011; Toyofuku et al., 2019). This also indicates that MV-inhibitors that target membrane vesicles from specific bacteria species, such as CBD here, could be applied in combination with selected antibiotics for tailored antibiotic treatment to tackle antibiotic resistance.

CONCLUSIONS

CBD effectively inhibited MV release from the Gram-negative bacterium *E. coli* VCS257, exhibiting a stronger MV-inhibiting effect at lower dose. In addition, CBD modulated MV protein profiles of *E. coli* following 1 h treatment. CBD did not have significant effects on MV release in the Gram-positive bacterium *S. aureus* subsp. *aureus* Rosenbach. When applied in combination with a range of antibiotics, CBD increased antibacterial effects of selected antibiotics, depending on bacteria type. CBD, in combination with specific antibiotics, may thus possibly be used as an adjuvant to selectively target bacteria to sensitize them to antibiotic treatment and reduce antibiotic resistance.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

UK, PM, BA, IK, PW, and SL performed the experiments. UK, JB, AN, JI, and SL analyzed the data. PM, GM, GG, IK, SL, and JI

provided resources. UK, SL, and JI designed the study. SL, UK, and AN wrote the manuscript. All authors critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00324/full#supplementary-material

Supplementary Figure 1 | Effects of CBD on bacterial growth in **(A)** *E. coli* VCS257 and **(B)** *S. aureus* subsp. *aureus* Rosenbach, after 24 h incubation, as assessed by disk diffusion test. Exact *p*-values are shown.

Supplementary Figure 2 | TEM of MV released from *E. coli* VCS257 following 1 or 5 μM CBD treated for 1 h, compared to MVs isolated from control, untreated *E. coli*. **(A)** A composite image showing MVs released from control, untreated *E. coli*. **(B)** A composite image showing MVs released from *E. coli* treated with 1 μM CBD for 1 h. **(C)** A composite image showing MVs released from *E. coli* treated with 5 μM CBD for 1 h. Scale bars indicate 100 nm, respectively, and are included in the individual figures.

Supplementary Figure 3 | Protein-protein interaction networks of protein hits identified in MVs from 1 μM CBD treated *E. coli* VCS257. Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis. Colored nodes represent query proteins and first shell of interactors; white nodes are second shell of interactors. **(A)** Biological GO processes are highlighted as follows: red, citrate metabolic process; green, antibiotic metabolic process; yellow, regulation of cellular amide metabolic process; purple, carboxylic acid metabolic process; dark green, regulation of phosphate metabolic process; light blue, cellular respiration; orange, small molecule metabolic process; dark red, negative regulation of translational elongation; dark blue, generation of precursor metabolites and energy. **(B)** KEGG pathways are highlighted as follows: dark green, oxidative phosphorylation; dark red, citrate cycle (TCA cycle); red, biosynthesis of antibiotics; purple, butanoate metabolism; dark blue, biosynthesis of secondary metabolites; light blue, carbon metabolism; orange, phenylalanine, tyrosine and tryptophan biosynthesis; light green, Microbial metabolism in diverse environments; yellow, Metabolic pathways; violet, glycine, serine, and threonine metabolism. Colored lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighborhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see color key for connective lines).

Supplementary Figure 4 | Protein-protein interaction networks of protein hits identified in MVs from 5 μM CBD treated *E. coli* VCS257. Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis. Colored nodes represent query proteins and first shell of interactors; white nodes are second shell of interactors. **(A)** Biological GO processes are highlighted as follows: red, cellular respiration; green, purine-containing compound metabolic process; yellow, electron transport chain; purple, ribose phosphate metabolic process; dark green, purine ribonucleotide metabolic process; light blue, generation of precursor metabolites and energy; orange, nucleobase-containing small molecule metabolic process; dark red, purine ribonucleoside metabolic process; dark blue, organophosphate metabolic process. **(B)** KEGG pathways are highlighted as follows: red, bacterial secretion system; light green, metabolic pathways; yellow, oxidative phosphorylation; purple, butanoate metabolism; dark green, quorum sensing; light blue, amino sugar and nucelotide sugar metabolism; dark blue, protein export; violet, purine metabolism. Colored lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighborhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see color key for connective lines).

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frontiers in Cellular and Infection Microbiology

REVIEW
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RNA-Dependent Regulation of Virulence in Pathogenic Bacteria

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During infection, bacterial pathogens successfully sense, respond and adapt to a myriad of harsh environments presented by the mammalian host. This exquisite level of adaptation requires a robust modulation of their physiological and metabolic features. Additionally, virulence determinants, which include host invasion, colonization and survival despite the host's immune responses and antimicrobial therapy, must be optimally orchestrated by the pathogen at all times during infection. This can only be achieved by tight coordination of gene expression. A large body of evidence implicate the prolific roles played by bacterial regulatory RNAs in mediating gene expression both at the transcriptional and post-transcriptional levels. This review describes mechanistic and regulatory aspects of bacterial regulatory RNAs and highlights how these molecules increase virulence efficiency in human pathogens. As illustrative examples, *Staphylococcus aureus*, *Listeria monocytogenes,* the uropathogenic strain of *Escherichia coli, Helicobacter pylori,* and *Pseudomonas aeruginosa* have been selected.

Keywords: regulatory RNA, riboregulation, UPEC, *S. aureus*, *L. monocytogenes*, *H. pylori*, *P. aeruginosa*

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INTRODUCTION

Numerous bacterial species are infamous for their role in causing human diseases (Kusters et al., 2006; Gellatly and Hancock, 2013; Dayan et al., 2016; Terlizzi et al., 2017; Radoshevich and Cossart, 2018). These bacterial pathogens possess certain key distinguishing features. First, they can efficiently sense environmental cues presented by the host such as changes in nutrient availability, pH, osmolarity, and temperature (Fang et al., 2016). Second, pathogenic organisms quickly adapt their metabolic physiology accordingly, thereby switching between their free-living lifestyles and that within the host (Groisman and Mouslim, 2006; Fuchs et al., 2012). Finally, they are characterized by an arsenal of virulence attributes, which they robustly modulate to survive and proliferate during host infection (Pettersson et al., 1996). For example, bacteria harbor potent toxins and toxin delivery systems (Green and Mecsas, 2016). One major function of bacterial toxicity is to kill surveilling immune cells such as neutrophils (do Vale et al., 2016). While this is an immune-evading mechanism employed by pathogenic bacteria, the proteins constituting these toxins and their delivery conduits are highly immunostimulatory (Miao et al., 2010; Gall-Mas et al., 2018). Additionally, toxin expression and secretion are energy intensive (Lee and Rietsch, 2015; Joo et al., 2016). Thus, pathogenic bacteria cannot afford to constitutively express toxin genes but must modulate their expression according to the site and stage of the infection.

A second important virulence property of pathogenic organisms is their ability to form biofilms. A biofilm lifestyle, as markedly opposed to a single organismic one, is characterized by bacteria

clustered with each other and attached to a foreign surface such as the host epithelium (Costerton et al., 1995). Another important feature includes encapsulation of the bacterial community inside of an extracellular matrix consisting of polymeric substances synthesized by the bacteria themselves such as polysaccharides (Sutherland, 2001). This abiotic outer layer adopts distinct three-dimensional structures. For example, it can form water channels, critical for efficient nutrient mobilization and uptake by biofilm bacteria (Stewart, 2003; Wilking et al., 2013). Additionally, this protective matrix provides a barrier against host immune responses (Leid et al., 2005; Begun et al., 2007; Toska et al., 2018; Tseng et al., 2018) and antimicrobial therapy (Goltermann and Tolker-Nielsen, 2017; Hall and Mah, 2017; Singh et al., 2017). Because of this, and due to altered gene expression, biofilm-associated bacteria are highly recalcitrant to antibiotics (Mah and O'Toole, 2001; Stewart, 2002; Hall and Mah, 2017). Consequently, bacterial biofilm formation represent a huge clinical burden, being widely implicated in the establishment and maintenance of chronic infections (James et al., 2008; Calo et al., 2011; Chen and Wen, 2011; Omar et al., 2017). A classic example is the formation of highly antibiotic resistant biofilms in the airways of Cystic Fibrosis (CF) patients (Lopez-Causape et al., 2015) (described later in detail). Other clinically relevant biofilm infections include otitis media (Bakaletz, 2007), and biofilms frequently found on medical devices such as catheters dwelling inside the patient (Donlan, 2008).

Another community-associated behavior contributing to bacterial virulence is quorum sensing (QS) (Antunes et al., 2010). QS is a bacterial cell-to-cell communication mechanism dependent on the abundance of signaling molecules, known as autoinducers (AI), in the extracellular milieu (Miller and Bassler, 2001). AIs are regulators of bacterial gene expression (Rutherford and Bassler, 2012; Banerjee and Ray, 2016, 2017). Each bacterial cell is capable of synthesizing and secreting AI molecules. Thus, the magnitude of AI accumulation hinges upon both bacterial cell-density as well as gene expression profile (i.e., whether the AI production is on or off) of the whole bacterial community. At an adequate cell density, when the AI levels reach a certain threshold, they are detected by receptors located in the bacterial cell membrane or in the cytoplasm. Some of these receptors comprise the membrane-associated sensor histidine kinase of bacterial two-component signal transduction systems. Binding of the AI to the receptor activates its kinase activity thus autophosphorylating it, followed by transmission of the phosphate group to the corresponding response regulator, thereby facilitating regulation of genes in that particular QS regulon. The second mechanism of QS mediated regulation starts with secretion of the inactive AI. In the extracellular environment, it is processed to its active form, and either diffuses freely or is transported back into bacterial cells. There, the AI binds its cognate cytoplasmic receptor, which is characteristically a global transcription factor controlling the whole QS regulon (Rutherford and Bassler, 2012).

Altered gene expression is key to a pathogen's optimization of its virulence attributes. For example, significant changes exist in both transcript and proteome profiles of the same bacterial species existing as a free-floating single bacterium vs. in a biofilm (Oosthuizen et al., 2002; Nigaud et al., 2010; Chavez-Dozal et al., 2015; Charlebois et al., 2016; Jia et al., 2017; Favre et al., 2018). Bacterial regulatory RNAs are now established as pivotal players in facilitating these coordinated changes in gene expression, acting at all levels, starting from transcription to protein translation and protein activity (Romby et al., 2006; Toledo-Arana et al., 2007; Svensson and Sharma, 2016; Westermann, 2018). These RNA regulators can be classified in different groups, as detailed below (**Figure 1**).

The first type of regulatory RNA elements includes those present in the 5′ untranslated regions (UTR) of their cognate mRNA. 5′UTRs can contain complex structures that undergo alterations depending on environmental conditions (Winkler and Breaker, 2005; Waters and Storz, 2009). These 5′UTR structure are known as riboswitches and are important regulators of gene expression at the transcriptional or the translational level. Riboswitches respond to changes in abundance of small metal ions, small molecules, or metabolites (Winkler and Breaker, 2005; Waters and Storz, 2009). Another example of 5′UTR regulatory elements are RNA thermometers, which responds to temperature changes during infection (Loh et al., 2018). For instance, the start codon of an mRNA might be embedded in the 5′UTR region, which adopts a stem loop structure at lower temperatures, thus preventing translation. Upon colonization of the host, this stem loop loosens as the temperature increases (>37°C), facilitating translation. Finally, another type of 5′UTR regulatory elements respond to pH changes, by forming inaccessible structures at one pH and opening up at a different one (Nechooshtan et al., 2009).

The second type of regulatory RNAs are encoded in *cis* and are known as anti-sense RNAs (asRNA). They regulate (i) transcription efficiency, by transcription interference, (ii) RNA stability, by forming RNA-RNA double stranded complex which may be degraded, and (iii) translation initiation, by interacting with and sequestering the ribosome binding site (RBS) (Svensson and Sharma, 2016; Westermann, 2018).

The third type of regulatory RNAs are expressed in *trans*, that is, at a different genomic site than the genes they regulate (Svensson and Sharma, 2016; Westermann, 2018). They are known as small regulatory RNAs (sRNA). The first mechanism employed by sRNAs involves binding of the sRNA to a regulatory protein to titrate it away from its target. For example, the 6S RNA is expressed upon entry in stationary phase of growth and acts by titrating the RNA polymerase holoenzyme containing the sigma70 (σ^{70}) specificity factor, preventing transcription dependent on σ^{70} (Wassarman, 2007). The second mechanism employed by sRNAs involves direct RNA-RNA base-pairing of the sRNA to its target mRNA. In instances of inhibition of gene expression, the sRNA base-pairs with the RBS of the target mRNA, thus occluding translation. In cases of positive regulation, the sRNA have been reported to act by binding to sequences in the 5′UTR of the target mRNAs and preventing the formation of inactivating stem-loop structures (Morfeldt et al., 1995).

Additionally, proteins commonly interact with the regulatory RNAs mentioned above in facilitating the observed regulatory roles. For example, the hybridization of sRNAs with their target mRNAs in Gram negative bacteria is often mediated by the RNA chaperone Hfq (Updegrove et al., 2016). Furthermore,

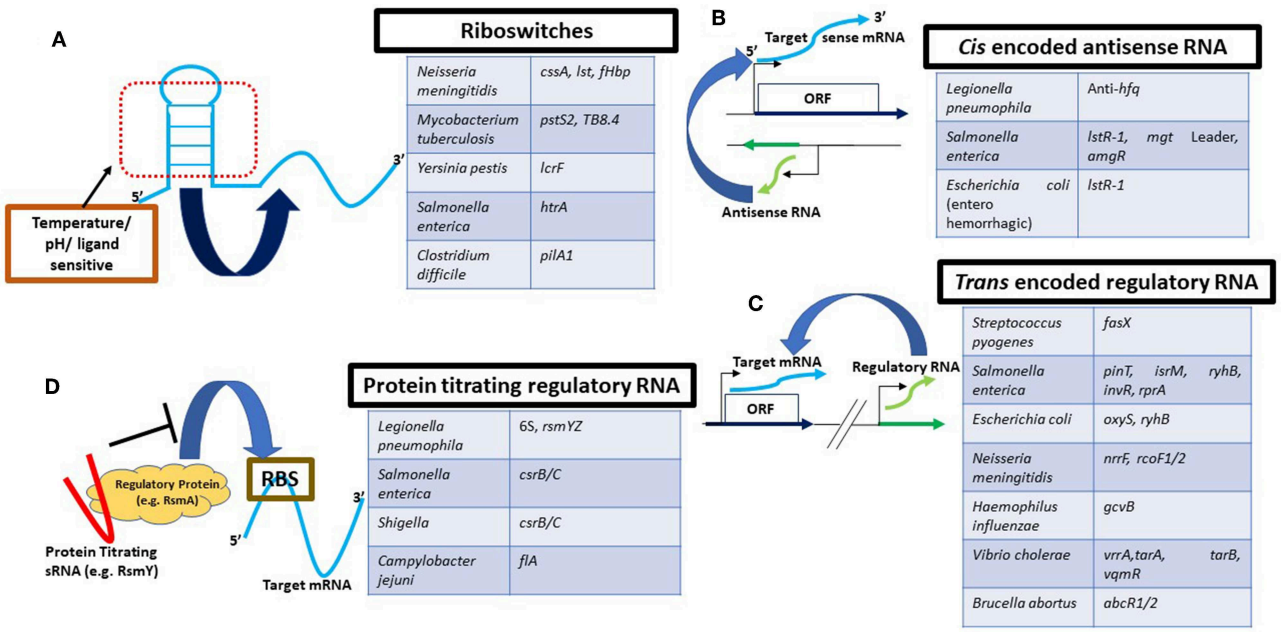

FIGURE 1 | Schematic representation of major types of RNA-based regulatory mechanisms in pathogenic bacteria. **(A)** Riboswitches are most commonly part of the 5′UTR of the corresponding target mRNA. They are responsive to chemical ligands or environmental signals such as temperature (as in an RNA thermometer) for structural rearrangements leading to gene expression changes. sRNAs can be expressed from the complimentary strand **(B)** (as antisense RNA) or from a different genomic location **(C)**. Blue: transcript under regulation; green: regulatory RNA. **(D)** Regulatory proteins (yellow) typically bind their target mRNAs (blue) at the RBS or the SD sequence to modulate their stability and/or activate/inhibit translation. Protein binding sRNAs (red) on the other hand can sequester these regulatory proteins by direct binding and titrate them away from their targetome. Pathogenic organisms not described in the main text are referred in this figure to highlight different RNAs as examples. For more details, refer to the following articles that have been extensively referred to for construction of this figure (Svensson and Sharma, 2016; Westermann, 2018).

downstream degradation of mRNAs targeted by sRNAs is mostly attributed to RNases (Matos et al., 2017).

Despite different types of bacterial regulatory RNAs and mechanisms of action, certain paradigms can be deduced (Westermann, 2018). One striking feature is that while some regulatory RNAs do have specific stray targets, they mostly target the mRNA encoding a central regulatory player such as a global transcription factor, thereby acting on a large number of indirect targets at once. This determines major virulence transitions such as switching over from a planktonic lifestyle to a biofilm lifestyle (Williams McMackin et al., 2019). Secondly, in multiple pathogenic bacteria, reshaping of metabolism, and virulence by the action of regulatory RNAs appear to be intertwined. A prominent example is the sugar-phosphate stress (thus related to metabolism) associated sRNA SgrS, which also regulates the pathogenesis effector SopD (Papenfort and Vogel, 2014) in *Salmonella*. A second example is the TarA sRNA in *Vibrio*, which links virulence with glucose acquisition (Richard et al., 2010). Finally, it appears that functional redundancy among multiple regulatory RNAs exists, with more than one of them facilitating the same regulation, albeit to different intensities (Deng et al., 2012, 2014; Heidrich et al., 2017; Pannekoek et al., 2017).

In light of the major virulence attributes and RNA based regulatory mechanisms described previously, this review aims to describe specific pathways of riboregulation of virulence factors in prominent human pathogens (**Table 1**). For this purpose, we will focus on pathogens affecting different niches of infection; *Staphylococcus aureus* for disseminated systemic infections and those associated with prosthetic implants; *Listeria monocytogenes* as a model intracellular bacterial pathogen; UPEC (uropathogenic *Escherichia coli*) for urinary tract infections; *Helicobacter pylori* as an enteric pathogen and *Pseudomonas aeruginosa* as a major causative agent of airway infections in cystic fibrosis patients.

Staphylococcus aureus

The Gram-positive bacteria *S. aureus* is often present among the normal human skin microbiome (Becker and Bubeck Wardenburg, 2015). However, it is also one of the most common pathogens implicated in bacterial infections of all areas of the body including skin (McCaig et al., 2006), bones (Olson and Horswill, 2013), heart (Fernandez Guerrero et al., 2009), respiratory tract (Parker and Prince, 2012), and bloodstream (Corey, 2009). Additionally, it is well-known to form highly persistent biofilms on prosthetic devices and implants (Lister and Horswill, 2014). *S. aureus* is one of the primary causative agents of nosocomial infections, a majority of which are antibiotic resistant (Wang and Ruan, 2017). This pathogen is listed in the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa,*

TABLE 1 | List of riboregulatory molecules described in the text.

Pathogen	Regulatory RNA/Protein	Mechanism of Action
Staphylococcus aureus	RNAIII	Trans acting sRNA
	RsaA	Trans acting sRNA
	SprD	Trans acting sRNA
Listeria monocytogenes	Rli27	Trans acting sRNA
	LhrC	Trans acting sRNA
	Rli55	Riboswitch
	AspocR	Riboswitch
	PfrA	Riboswitch
	SreA	Riboswitch
	SreB	Riboswitch
	Anti0677	Antisense RNA (Excludon)
UPEC	PapR	Trans acting sRNA
	RyhB	Trans acting sRNA
Helicobacter pylori	IsoA1	Trans acting sRNA
	RepG	Trans acting sRNA
	5′*ureB*	Trans acting sRNA
Pseudomonas aeruginosa	RsmA	RNA binding protein
	RsmF	RNA binding protein
	RsmV	Trans acting sRNA
	RsmW	Trans acting sRNA
	RsmY	Trans acting sRNA
	RsmZ	Trans acting sRNA
	CrcZ	Trans acting sRNA
	ReaL	Trans acting sRNA
	PhrS	Trans acting sRNA
	PrrF1	Trans acting sRNA
	PrrF2	Trans acting sRNA
	ErsA	Trans acting sRNA
	Sr0161	Trans acting sRNA

and *Enterobacter* sp.) group of bacteria, which represent the most antibiotic resistant species (Santajit and Indrawattana, 2016). *S. aureus* possesses a myriad of virulence mechanisms including expression of toxins, surface adhesins, immune-evading molecules, quorum sensing, and biofilm formation (Powers and Bubeck Wardenburg, 2014). These pathogenic determinants are intricately regulated, and sRNAs play a significant role in that regulatory network (Fechter et al., 2014; Tomasini et al., 2014). Key sRNAs of *S. aureus* are described below.

RNAIII

The best characterized sRNA in *S. aureus* is RNAIII (Boisset et al., 2007; Bronesky et al., 2016). RNAIII is under control of the *agr* QS system. The *agr* locus comprises of two ORFs (open reading frames), transcribed by promoters P2 and P3 in opposite directions. P2 drives the transcription of a four-cistron mRNA, RNAII. Among these four gene products, AgrD is an autoinducer peptide (AIP) synthesized in its inactive form. AgrB is a membrane associated AIP transporter, which matures the precursor AgrD AIP to its active form and exports it out of the cell. The remaining two cistrons *agrC* and *agrA* form the sensor histidine kinase and its cognate response regulator, respectively, in a two-component signaling (TCS) cascade. At high cell density, the autoinducer peptide AgrD is detected by the sensor AgrC and the signal is globally transmitted intracellularly by the now phosphorylated response regulator, AgrA. AgrA, in turns, upregulates transcription of RNAIII (from promoter P3) that will exert pleiotropic roles in *S. aureus*.

First, the 5′ region of RNAIII encodes the δ-hemolysin, conferring hemolytic activity to the bacterium. Then, RNAIII can act as a regulatory RNA, regulating multiple target mRNAs. RNAIII has a long half-life of 45 min and is structurally characterized by 14 stem-loops and two lengthy helical structures. Specific hairpins are involved in base-pairing to target mRNAs, with more than one stem-loop acting in concert to achieve regulation. To repress translation, RNAIII may bind at the RBS (e.g., *lytM* mRNA), both at the RBS and the 5′UTR (e.g., *rot* mRNA), using multiple stem loops, or at the coding region (e.g., *coa* mRNA) (Felden et al., 2011). RNAIII can also positively regulate targets. The only two targets known to be upregulated by RNAIII are *hla* mRNA, encoding the α-hemolysin, and *eap*, encoding the extracellular adherence protein (Guillet et al., 2013).

While it has numerous mRNA targets to which it directly binds, RNAIII also modulates a large indirect regulon, for example, by inhibiting translation of the key transcriptional repressor *rot* (*r*epressor *o*f *t*oxins) (Geisinger et al., 2006). Of note, as in most cases of regulatory RNA binding events in Gram-positive pathogens, RNAIII does not require the RNA chaperone Hfq, even though it has been shown to bind to RNAIII *in vitro* (Liu et al., 2010).

The overarching feature of RNAIII-mediated regulation is that it represses translation of genes encoding for surface proteins or those associated with high peptidoglycan turnover, which are typically required at primitive stages of infection marked by low cell numbers to facilitate and consolidate early events in bacterial colonization. Conversely, it activates synthesis of secreted exotoxins, which are required for bacterial dissemination at later time points of infection when bacterial cell density is high. Indeed, RNAIII is reported to assist *S. aureus* switch from a biofilm mode of growth (colonization and persistence in a new niche) to a more invasive one, required for dispersal to new host tissues (Boisset et al., 2007). Observations that *S. aureus* isolates from antibiotic resistant chronic bacteremia, like those associated with prosthetic implants, are commonly defective in both *agr* locus and RNAIII expression, further bolster this view. RNAIII holds a pivotal position in *S. aureus* regulation of virulence. Evidently, apart from a few exceptions, all downstream effects of *S. aureus* QS signaling are mediated through RNAIII. RNAIII is therefore versatile as a regulator, cascading both direct and indirect pathways.

Methicillin resistant *S. aureus* (MRSA) are considered the most dangerous of *S. aureus* isolates. The mobile genetic element SCCmec was shown to confer resistance to methicillin (Noto et al., 2008). Interestingly, the mRNA of one of the genes in this region, *psm-mec*, binds to and inhibits translation of the previously described *agrA* gene. Consistent with reports that

the *agr* system and its effectors, such as RNAIII, produce a pronounced invasive character in *S. aureus*, *psm-mec* mutations in community-acquired MRSA isolates account for their acute virulence nature (Qin et al., 2016).

RsaA

Contrarily to RNAIII, the sRNA RsaA promotes chronic persistence, biofilm formation, and expression of cell surface proteins (Romilly et al., 2014). The *rsaA* gene is under positive transcriptional control of the specialized factor SigmaB (σ^B) and is minimally expressed at exponential phase and highly expressed at stationary phase (Geissmann et al., 2009). Furthermore, both endoribonucleases RNase III and RNase Y regulate its degradation (Romilly et al., 2014). The primary target of RsaA is the *mgrA* mRNA, encoding the global transcriptional regulator MgrA, whose translation is inhibited by base-pairing of the sRNA. Rate constant for this binding indicates rapid association, which is important for impeding the formation of ribosomal initiation complex (Romilly et al., 2014). The translationally repressed *mgrA* mRNA is then likely degraded. RsaA can bind to two distinct regions of the *mgrA* mRNA: a cytosine-rich motif targets the Shine-Dalgarno (SD) and two hairpin loops in the 5′ region of RsaA interact with the coding sequence of *mgrA*.

MgrA has ∼350 genes in its regulon, including its own mRNA that is positively autoregulated (Luong et al., 2006). The culminating effect of MgrA is activation of capsule synthesis and inhibition of biofilm formation by repressing surface proteins expression and releasing extracellular DNA (Trotonda et al., 2008). Intriguingly, through *mgrA* modulation, RsaA is connected with RNAIII and the *agr* QS system, as MgrA activates transcription of the *agr* locus (Ingavale et al., 2005).

It is postulated that presence of a functional RsaA may have been evolutionarily favored in *S. aureus*. Given that it is primarily a commensal organism in the human host, it is possible that RsaA prevents the bacteria from being hyperinvasive at all times, and this regulation is critical for normal colonization fitness (Romilly et al., 2014). Using MAPS (a technology developed to affinity purify RNA-RNA complexes *in vivo* and identify the targets by sequencing), a recent study has further validated the interaction of *mgrA* mRNA with RsaA and has further extended its RNA targetome (Tomasini et al., 2017).

SprD

SprD is a sRNA transcribed from a pathogenicity island (region on the chromosome predominately harboring virulence associated genes) whose main target elucidated thus far is the *sbi* mRNA, encoding the immune evading effector Sbi (Second binder of *i*mmunoglobulins) (Chabelskaya et al., 2010). Sbi inhibits opsonization, which is usually followed by phagocytosis and action of the complement system (Haupt et al., 2008). Specifically, Sbi binds IgG, the C3b, and H complement factors for these purposes. While these functions of Sbi are important in the survival of *S. aureus* in the host, it is to be noted that Sbi nevertheless elicits a major pro-inflammatory response by activating multiple immune-signaling cascades that results in the production of major cytokines (IL-6 for example) and leukocytes chemotaxis to the site of infection. Thus, for success of the organism, Sbi expression needs to be optimally fine-tuned rather than constitutively expressed. The regulatory RNA SprD facilitates this by keeping Sbi levels in check through impeding its translation. Indeed, part of the 5′UTR, the SD sequence and the start codon of *sbi* have been reported to be critical for the regulation by SprD. Consistent with that seen with RNAIII, SprD does not require the RNA chaperone Hfq in binding its target. Though blocking translation of *sbi*, SprD does not facilitate degradation of the mRNA. The fact that SprD is a major regulator of *S. aureus* pathogenesis is evident from the fact that its deletion severely decreases morbidity and mortality of the mouse model of infection. Interestingly, RNAIII also binds to and represses *sbi* translation by a similar mechanism, further illustrating the need for only modest synthesis of the Sbi protein.

Listeria monocytogenes

L. monocytogenes is an especially interesting model to describe the role of regulatory RNAs in shaping pathogenesis during the intracellular lifestyle of a bacterium. In addition, *L. monocytogenes* represents a group of uncommon bacterial pathogens. Indeed, in contrast to other pathogens described in this review, *L. monocytogenes* infections (listeriosis) are relatively scarce. Nevertheless, listeriosis mortality rates remain in the high range of 20–30% (Radoshevich and Cossart, 2018), particularly for children, the elderly, immunocompromised patients, and during pregnancy. The primary mode of transmission is via contaminated food, and for people in vulnerable groups, the minimum infective dose can be as low as 100 bacteria (Radoshevich and Cossart, 2018). As a gastro-intestinal pathogen, this bacterium breaches the intestinal epithelial barrier and spreads via blood and lymphatic system to the whole body, colonizing especially the liver and spleen (Cossart, 2011). Importantly, it resides intracellularly in both phagocytic and non-phagocytic cells, helped by an arsenal of virulence factors modulating host cell processes (Pizarro-Cerda et al., 2012). Higher than 150 sRNAs have been reported in *L. monocytogenes* (Lebreton and Cossart, 2016). Some of those involved in intracellular pathogenesis are described in this section.

Rli27

Lm0514 is a protein in *L. monocytogenes* that is recognized by sortase enzymes through its LPXTG motif and is tethered to peptidoglycan located on the cell surface (Garcia-del Portillo et al., 2011; Mariscotti et al., 2012). Importantly, Lm0514 protein level is highly augmented during intracellular growth of the bacteria, where it significantly facilitates survival (Pucciarelli et al., 2005; Garcia-del Portillo et al., 2011). Interestingly, *lm0514* mRNA level increases only by 6-fold during intracellular vs. extracellular growth, while the rise in protein level is 200 times higher (Garcia-del Portillo et al., 2011). This is strongly indicative of posttranscriptional regulation, and indeed, during infection, the *lm0514* mRNA transcript is upregulated by the sRNA Rli27. Rli27 binds to the 5′UTR of *lm0514*, exposing the RBS and enhancing translation (Quereda et al., 2014).

LhrC

Listeria Hfq-binding RNA C (LhrC) is a small non-coding (nc) RNA first discovered amongst a pool of RNAs that co-immunoprecipitated with the RNA chaperone Hfq in *L. monocytogenes* (Christiansen et al., 2006; Sievers et al., 2014). However, later studies have established that, as in most cases of RNA based regulation in Gram-positive species, Hfq is not required for LhrC stability or its interaction with mRNA targets. There are five copies of the *lhrC gene* ranging from 111 to 114 nucleotides in size (Sievers et al., 2014). Initial observations of LhrC expression during *L. monocytogenes* intracellular growth in macrophages and its putative role in facilitating pathogenesis have been substantiated by three reports of Kallipolitis and colleagues (Sievers et al., 2014; Dos Santos et al., 2018; Ross et al., 2019). LhrC RNAs 1-5 (LhrC1-5) were found to be upregulated during conditions of cell envelope stress such as those generated by the antibiotic cephalosporin or bile salts (Sievers et al., 2014). A later study reported that heme, commonly encountered by *L. monocytogenes* during systemic dissemination in human hosts, is also a trigger for LhrC expression (Dos Santos et al., 2018).

The first LhrC target characterized was the mRNA *lapB*, encoding a cell wall-tethered adhesin (Sievers et al., 2014). LhrC contains three regions with high concentration of cytosine, resulting in a UCCC motif which is critical in mediating interaction with the AG rich SD sequence of the target mRNA to repress translation. Downregulation of *lapB* expression probably benefits the organism in evading host immune response while spreading through blood, as bacterial surface proteins are well-known immune stimulators (Toledo-Arana et al., 2009). Five copies of LhrC and three redundant CU-rich target binding sites indicate the need and the potential of LhrC to transform a low input signal into a magnified output response. Further, the five copies of LhrC appear to act additively rather than redundantly, corroborating this hypothesis. Subsequent work has established that LhrC, by using its UCCC motifs, binds to and represses mRNAs of genes involved in heme influx into the cell and its subsequent metabolism (Dos Santos et al., 2018). Thus, it is not surprising that LhrC plays an important role in survival of the pathogen in heme-rich niche containing lysed erythrocytes.

By using the same motif and mechanism, LhrC also represses mRNA translation of another membrane protein-coding mRNA, *oppA*, involved in oligopeptide binding (Sievers et al., 2015). Recent work has demonstrated the inhibitory mechanism of LhrC on another mRNA target, the T cell stimulating antigen (TcsA) (Ross et al., 2019). Here, the repressive mechanism involves LhrC base pairing at a site upstream of the SD region of *tcsA*, which does not affect translation but rather induces rapid transcript turnover.

Riboswitch-Regulated Nutrient Utilization

During gastro-intestinal infection in vertebrate hosts, *L. monocytogenes* commonly encounters ethanolamine, and metabolizes it by expressing the *eut* (*e*thanolamine *ut*ilization) genetic locus (Garsin, 2010; Archambaud et al., 2012). Transcription of *eut* is under RNA-based regulation (Freitag, 2009).

The first level of regulation is mediated by the sensor histidine kinase EutW and its cognate response regulator EutV, which are activated by presence of ethanolamine in the extracellular milieu. Phosphorylated EutV serves as an ANTAR antiterminator that prevents *eut* gene transcription cessation by binding to stem-loop structures in the nascent mRNA (Fox et al., 2009; Lebreton and Cossart, 2016). Located upstream of the *eut* locus is the gene Rli55, acting as the second level of RNA-based *eut* regulation. The 5′ region of Rli55 harbors a vitamin B12 riboswitch.

In absence of vitamin B12, Rli55 is transcribed as a 450 nt-long transcript, which sequesters EutV, thus leading to transcriptional attenuation of *eut* genes. Conversely, in presence of vitamin B12, the riboswitch binds its ligand, resulting in transcription of a much shorter Rli55 transcript (200 nt), incapable of sequestering EutV, leading to *eut* transcriptional antitermination and ultimately, *eut* expression (Mellin et al., 2014). Both ethanolamine (for EutV phosphorylation) and vitamin B12 (for Rli55 inhibition) are therefore essential to activate the ethanolamine utilization pathway in *L. monocytogenes.*

Another example where a vitamin B12 riboswitch controls nutrient utilization in *L. monocytogenes* is AspocR, which regulates propanediol usage by *L. monocytogenes* during infection (Mellin et al., 2013). Expression of the *pdu* (*p*ropane*d*iol *u*tilization) operon is driven by the transcription factor PocR (Kim et al., 2014). The complementary strand of *pocR* mRNA region encodes the riboswitch-controlled asRNA AspocR. In the absence of vitamin B12, this riboswitch serves as an antiterminator to the asRNA of *pocR* (AspocR), located downstream. Thus, under these conditions, the asRNA AspocR blocks expression of *pocR* and hence of *pdu* genes. In presence of vitamin B12, the riboswitch conformation promotes transcriptional attenuation of AspocR, and the resultant truncated asRNA is unable to bind the *pocR* mRNA transcript, allowing utilization of propanediol.

Regulation of *PrfA* Expression

Translational control of *pfrA*, a master transcriptional activator of virulence genes in *L. monocytogenes* (de las Heras et al., 2011) is a notable example of RNA-based regulation. The temperature-sensitive thermoswitch located in its 5′UTR forms a hairpin at 30°C, thereby occluding the RBS and hindering *prfA* translation. Conversely, at the host infection temperature of 37°C, this secondary RNA structure is not favored resulting in upregulation of *prfA* translation. With PrfA activating expression of a myriad of toxins, lytic enzymes and actin-remodeling proteins, this mechanistic control ensures that virulence factors are produced by the bacteria when in the host (37°C) but not otherwise (30°C) (Lebreton and Cossart, 2016). A second control mechanism is associated with nutrient availability and is exerted by sRNAs SreA and SreB (Loh et al., 2009). In the presence of the ligand S-adenosylmethionine (SAM), premature transcriptional termination occurs in riboswitches SreA and SreB facilitating expression of smaller non-coding transcripts which bind to *prfA* mRNA RBS, preventing translation.

Excludon-Mediated Control of Flagellar Motility

Excludon is a gene locus wherein the transcript serves as both an antisense RNA (asRNA) to block expression of the mRNA transcribed in the opposite direction as well as being

the mRNA of adjacent gene(s) (Schultze et al., 2015; Lebreton and Cossart, 2016). Thus, an excludon negatively regulates its complementary gene but promotes expression of the neighboring gene in the same DNA strand. A classic example in *L. monocytogenes* is regulation of flagella biosynthesis, the cellular appendages facilitating bacterial swimming in liquid milieu and swarming on semisolid surfaces (Sesto et al., 2012). In *L. monocytogenes*, loci *lmo0675-0689* encodes the flagella-related *fli* operon. *lmo0676* and *lmo0677* encode proteins FliP and FliQ, which are integral parts of the flagellar export apparatus. On the opposite strand, a promoter drives the expression of a long RNA, named Anti0677, harboring full sequence complementarity to *lmo0675*, *lmo0676,* and *lmo0677*. Anti0677 acts as an antisense RNA, downregulating expression of the flagellar export apparatus. Additionally, transcription of Anti0677 also reads through *mogR*, the motility gene repressor. Anti0677 therefore acts as a mRNA, increasing MogR levels in the cell. This excludon regulates flagella synthesis from two angles: an asRNA mechanism leads to decrease in flagellar export apparatus and expression of *mogR* from two promoters (*anti0677* promoter and *mogR* promoter) increases MogR production, which also downregulates flagellar expression.

UROPATHOGENIC *Escherichia coli* (UPEC)

In the human host, pathogenic strains of *E. coli* cause infection in a plethora of sites, including the urinary tract (Kaper et al., 2004; Terlizzi et al., 2017). The uropathogenic *E. coli* (UPEC) is the primary cause of urinary tract infection (UTI), affecting both the urinary bladder (cystitis) and the kidney (nephritis), with widespread morbidity and even mortality. UPEC bacteria are armed with a variety of toxins, adhesins, and iron scavenging molecules called siderophores (Terlizzi et al., 2017). They also have excellent stress response systems and have the capability to form biofilms, even intracellularly (Anderson et al., 2003).

Regulators are essential determinants of UPEC virulence. The RNA chaperone Hfq has been reported to be important for UPEC colonization of mouse urinary tract (Kulesus et al., 2008). Intracellular microcolony formation, a hallmark of UPEC infections, as well as biofilm formation, which increases UPEC persistence, will not be as efficient upon Hfq deletion. Additionally, Hfq maintains lipopolysaccharide homeostasis, mediates tolerance to cell envelope stress, cationic antimicrobial polymyxin B, reactive free radicals and acidic conditions, on top of facilitating motility.

Commonly associated to the chaperone Hfq in *E. coli* are small regulatory RNAs. sRNAs play major roles in coordinating UPEC's virulence. Discussed below are the specific roles played by two important regulatory RNAs in this pathogenic bacterium.

PapR

As described above, Hfq is a major regulator of virulence in UPEC. Because of its well-known RNA chaperone role, a group aimed at co-immunoprecipitating RNAs with Hfq to try and identify novel Hfq-associated sRNAs expressed during infection (Khandige et al., 2015). Hfq-bound sRNA profiles varied greatly depending on whether they were obtained from UPEC growing under lab conditions or within host cells. Particularly, envelope stress related sRNAs were found to increasingly co-precipitate during infection conditions.

The same screen uncovered the novel *trans* acting sRNA PapR, which negatively regulates *papI* mRNA, encoding a regulator of the adhesin P-fimbriae, a critical pathogenic factor aiding bacterial attachment to renal tissue (Lane and Mobley, 2007; Khandige et al., 2015). PapI is an activator of P-fimbriae biosynthesis, which turns on transcription of the P-fimbriae associated *pap* operon. PapR has been found to base-pair within the coding sequence of *papI* mRNA, ∼80 nt downstream of its translational start site, to achieve negative translational regulation.

RyhB

In non-pathogenic *E. coli*, the 90 nt-long sRNA RyhB regulates iron usage and uptake (Massé and Gottesman, 2002). Congruently, in UPEC, it promotes synthesis of iron-scavenging siderophores enterobactin, aerobactin, and salmochelin, critical for pathogenesis in the host environment (Porcheron et al., 2014). RyhB facilitates siderophore biosynthesis by base-pairing with mRNAs of the precursor molecules thereby stabilizing them and enhancing translation. Further supporting its role as a virulence mediator, in animal models of UTI, deletion of *ryhB* leads to defects in colonization of urinary bladder. RyhB also regulates infection in various other pathogens such as *Shigella* (Murphy and Payne, 2007) and *Vibrio* (Oglesby-Sherrouse and Murphy, 2013).

Helicobacter pylori

H. pylori is an important pathogen using RNA-based virulence regulation to infect the gastric mucosa. Stomach of 50% of the total human race is believed to be colonized by this Gram-negative organism, which will remain in the gastric mucosa unless treated with persistent antimicrobial therapies (Testerman and Morris, 2014). Manifestations of *H. pylori* infections range from mild inflammation of the gastric tissue to severe and chronic peptic ulceration and finally to malignancies, the pathology with the worst prognosis (Wroblewski et al., 2010). *H. pylori* is known to modulate expression of micro RNAs (miRNAs) in the gastric tissue, altering the human immune response to its advantage (Libânio et al., 2015). This pathogen also possesses an array of other virulence factors, such as sRNAs, to survive and proliferate in the host (Pernitzsch and Sharma, 2012). *In silico* analysis has indeed revealed that multiple sRNAs might have important effects on its infectivity. Below are described three major *H. pylori* ncRNAs and their modes of action.

IsoA1

Type 1 toxin-antitoxin systems are mechanistically represented by the mRNA of the toxin gene being inhibited by binding of the antitoxin sRNA (Unterholzner et al., 2013). In *H. pylori*, synthesis of the toxic 30 aa polypeptide AapA1 is stalled by base pairing of the IsoA1 sRNA, which serves as the corresponding antitoxin in this system (Arnion et al., 2017). During log phase growth, both *aapA1* and *isoA1* are constitutively transcribed.

However, the 250 nt full length *aapA1* transcript is translationally inactive because of internal secondary structures occluding the RBS. It is only after the 3′end is processed, leading to a truncated 225 nt mRNA, that the RBS becomes available for translational initiation. However, this active structure also facilitates IsoA1 base-pairing, which leads to quick degradation of the sRNA-mRNA complex by RNase III. Thus, AapA1 toxin synthesis is repressed at two posttranscriptional levels; first by its own 5′UTR secondary structure, and second by the IsoA1 sRNA and RNase III, ensuring that the toxin is not formed during exponential growth of *H. pylori*.

RepG

Implicated in *H. pylori* infections of animal models, TlpB is a chemotaxis receptor positively responding to quorum sensing signals and negatively responding to low pH (Croxen et al., 2006). *tlpB* mRNA has a characteristic 6–16 guanine repeat, termed simple sequence repeats (SSR), in its 5′ leader region. Variation in *tlpB* transcript SSR length is observed between *H. pylori* isolated from different patients, and sometimes even from the same patient.

This G repeat sequence is targeted by the highly conserved RepG sRNA (Regulator of polymeric G repeats) (Pernitzsch et al., 2014). The span of the G repeat determines the interaction of *tlpB* mRNA with RepG. Regulation is at the level of translation and is a fine-tuning system rather than being a binary on/off decision. Base-pairing of RepG to *tlpB* mRNA occurs in the 5′UTR, upstream of the RBS. Thus, translational attenuation is probably conferred by structural rearrangements and/or occlusion of ribosome stand-by sites. In addition, RepG is reported to diminish *tlpB* mRNA stability, indicating that the dimerization event enhances degradation. In addition to *tlpB*, RepG likely has a larger targetome, with which it probably interacts via its C/U laden terminator loop.

5′*ureB*-Regulatory RNA

Copious production of urease is a key mechanism that enables *H. pylori* to survive in the acidic gastric environment (Mobley, 1996; Graham and Miftahussurur, 2018). Urease metabolizes the available urea to ammonia and bicarbonate, both of which serve as buffer to maintain a healthy pH in the bacterial cell. Expressed from the same operon, UreA and UreB are the two subunits of the precursor urease apoenzyme. At the transcription level, *ureAB* is positively regulated by the acid-activated TCS ArsRS, ensuring plentiful synthesis (constituting ∼8% of total cell protein) at low pH (Pflock et al., 2005).

Additionally, restricted urease synthesis is also required at neutral or high pH (Wen et al., 2013). This is facilitated by the 5′*ureB*-sRNA, an antisense RNA transcribed from the *5′ureB* non-coding strand.

While the phosphorylated (in acidic conditions) response regulator ArsR promotes sense *ureAB* transcription, the unphosphorylated (in neutral/alkaline conditions) protein upregulates the antisense 5′*ureB*-sRNA expression. It is observed that when the 5′*ureB*-sRNA is expressed (i.e., at neutral to high pH), the sense *ureAB* dicistronic mRNA is shortened to only 1400 nt instead of the regular 2700 nt transcript found at low pH, capable of synthesizing both subunits of the apoenzyme. Mechanistic explanation is that the antisense 5′*ureB*-sRNA base-pairs with the *ureAB* transcript, to promote transcription termination of the sense *ureAB* mRNA. A characteristic YUNR motif is essential for the initial annealing of the sense and antisense transcripts. Interestingly, this attenuation of transcription does not involve binding of Rho or even characteristic Rho-independent structures. As reported elsewhere, the asRNA binding leads to structural reassignments that ultimately destabilizes the RNA polymerase (Stork et al., 2007). At the same time, the termination of transcription is reported to be bona fide, rather than being caused by transcriptional interference of the sense and antisense transcripts being expressed at the same time (Shearwin et al., 2005). Finally, a low amount of the antisense 5′*ureB*-sRNA is enough to repress even high quantities of *ureAB* transcripts.

Pseudomonas aeruginosa

Whenever it finds its host innate immunity weak, *P. aeruginosa* can cause opportunistic infections in virtually every human tissue (Lang et al., 2004; de Bentzmann and Plesiat, 2011). The most notable among these infection sites are the lungs of CF patients, where *P. aeruginosa* forms persistent biofilms (Hoiby et al., 2010; Smith et al., 2017). Once established, usually by late adolescence of the patient, these infections are almost impossible to eradicate and contribute largely to the decreased quality of life and life expectancy of these patients (Emerson et al., 2002; Bjarnsholt et al., 2009).

Multiple host environmental factors promote *P. aeruginosa* biofilm infections in the CF lungs. Among these is the viscous mucus, a hallmark of CF airway environment, which provides a favorable niche for microbial pathogens to thrive (Matsui et al., 2006; Moreau-Marquis et al., 2008). Additionally, the microorganisms induce a huge immune response by recruiting leucocytes to the area. The leucocytes however are failing to eliminate the infection and rather cause extensive tissue damage. This "frustrated phagocytosis" (Conese et al., 2003; Alexis et al., 2006; Simonin-Le Jeune et al., 2013; Okkotsu et al., 2014) further contributes to disease by providing extracellular DNA, aiding in the biofilm formation process (Tolker-Nielsen and Høiby, 2009; Fuxman Bass et al., 2010).

The other host-associated factors that will favor biofilm formation by *P. aeruginosa* include altered iron and oxygen availability in the CF airways (Moreau-Marquis et al., 2008). *P. aeruginosa* senses and responds adequately to such environmental cues. For example, motility and Type 3 Secretion System (T3SS), a machinery to produce and inject toxins directly into the host cytoplasm by a multiprotein syringe complex (Hauser, 2009), are downregulated while production of exopolysaccharides is increased (Furukawa et al., 2006; Folkesson et al., 2012; Winstanley et al., 2016). These behavioral changes are facilitated by modulation of gene expression, mainly carried out by sRNAs at the post-transcriptional level (Vakulskas et al., 2015). Therefore, it is not surprising that more than 570 sRNAs are reported to be expressed by *P. aeruginosa* (Pita et al., 2018).

Below, we explore some of the regulatory RNAs that play major roles in the switch from the planktonic (free swimming) acute lifestyle of *P. aeruginosa* to its biofilm lifestyle, characteristic of CF lung infections.

Rsm Signaling

Almost a tenth of *P. aeruginosa* transcriptome is part of the Rsm regulon. This Rsm (regulator of secondary metabolites) regulon acts as a posttranscriptional regulatory system that controls multiple virulence determinants, ultimately governing the transition between the acutely toxic planktonic and the chronic biofilm growth modes (Vakulskas et al., 2015; Janssen et al., 2018).

The key components of the Rsm system are two RNA binding proteins, RsmA, and RsmF (also known as RsmN), which are orthologs to the *E. coli* CsrA (Carbon storage regulator A) protein. RsmA and RsmF share 31% identity and a conserved arginine is critical for their RNA binding activity (Janssen et al., 2018). Both proteins can directly bind target mRNAs and positively or negatively affect transcript stability and/or translation.

Presence of one (RsmA) and two (RsmF) conserved GGA motifs is important for target recognition (Romero et al., 2018). Both RsmA and RsmF activates the acute phenotype (T3SS, pilus, etc.) and represses the biofilm features (Pel and Psl exopolysaccharides, T6SS expression etc.).

These RNA binding proteins are tightly regulated by sRNAs RsmV, RsmW, RsmY, and RsmZ, also part of the Rsm system (Janssen et al., 2018). The sRNAs RsmV, RsmW, RsmY, and RsmZ can bind RsmA and RsmF by their GGA consensus sequence and sequester them away from their mRNA targets. Thus, these sRNAs are activators of the chronic biofilm-lifestyle phenotype while being repressors of the acute one. It is noteworthy that despite the apparent redundancy among the four similar acting sRNAs constituents of the Rsm system, variations do exist in their binding affinities. For example, RsmY and RsmZ have 10 times stronger affinity for RsmA than for RsmF. Moreover, their expression patterns will differ (Janssen et al., 2018).

Further, some differences have been observed in the regulons of RsmA vs. RsmF, despite their overall similar phenotypic regulatory patterns (**Table 1**) (Brencic and Lory, 2009; Romero et al., 2018). This highlights the critical need for fine-tuning mechanisms alongside major decision-making on-off switches, given the heterogeneity in the CF lung environment (Wei and Ma, 2013; Janssen et al., 2018).

Environmental regulation of the Rsm cascade, at least in part, is facilitated by the GacAS TCS (Brencic et al., 2009). The GacA response regulator, when phosphorylated by the sensor histidine kinase GacS, directly binds to the promoters of *rsmY* and *rsmZ* and activates their transcription. Two other membrane associated proteins LadS and RetS activate and inhibit GacA phosphorylation, respectively (Williams McMackin et al., 2019). LadS is stimulated by high calcium in the extracellular milieu (Broder et al., 2016). Further, recent work (Chakravarty et al., 2017) has reported that the inner membrane magnesium transporter MgtE, whose own expression is augmented during antibiotic pressure (Redelman et al., 2014) and low magnesium (both signals present in CF airway) (Coffey et al., 2014; Santi et al., 2016), signals through GacS to increase *rsmYZ* transcription. GacAS thus represents a hub of environmental regulation of the Rsm signaling. Contrary to RsmYZ, the sRNAs RsmW, and RsmV are not upregulated by GacAS (Janssen et al., 2018).

To add to the complex regulation of the Rsm regulon, another protein, the polynucleotide phosphorylase (PNPase) stabilizes both RsmY and RsmZ (Chen et al., 2016). Furthermore, *rsmYZ* transcription is inhibited by TspR, which acts through RetS (Williams McMackin et al., 2019). *rsmZ* transcription is also regulated by MvaT, and by BswR which thwarts the negative effects of MvaT (Williams McMackin et al., 2019). Another important sRNA implicated in T3SS repression is CrcZ, which sequesters both Crc and RsmF and activate T3SS gene expression (Sonnleitner et al., 2009; Williams McMackin et al., 2019).

RNA Based Regulation of Quorum Sensing

ReaI

The conserved 100 nt sRNA ReaL (Regulator of alkyl quinolone) is involved in QS regulation in *P. aeruginosa* (Carloni et al., 2017). It is under the negative regulation of the Las QS system and activates translation of the *pqsC* transcript, thereby connecting the two QS systems important for modulating pleiotropic virulence phenotypes. ReaL is also under RpoS regulation and is therefore expressed strongly in stationary growth phase. Consistent with the phenotypes generally observed in isolates from CF lungs, ReaL downregulates swarming but increases biofilm formation and secretion of pyocyanin and pyoverdine (Meyer et al., 1996; Lau et al., 2004).

PhrS

The stationary phase of growth, characteristic of CF airway infections, sees another sRNA being upregulated: PhrS (Folsom et al., 2010; Sonnleitner et al., 2011). PhrS transcription is also activated through the oxygen responsive DNA binding ANR protein in low oxygen conditions, typical of the CF lungs (Zimmermann et al., 1991). Interestingly Hfq is required for steady state levels of PhrS, not because Hfq promotes its stability, but because it is required for ANR production. Consistent with its expression in CF lung-like conditions, it increases pyocyanin production. Finally, PhrS facilitates synthesis of the QS-related transcriptional regulator protein PqsR (Brouwer et al., 2014) by directly binding to it and activating translation of a short ORF element located upstream of the *pqsR* mRNA. This ORF is translationally joined with the *pqsR* transcript (Sonnleitner et al., 2011).

PrrF1 and PrrF2

A major sRNA regulatory system is encoded by the *prrF* locus in *P. aeruginosa* (Reinhart et al., 2017). The genes *prrF1* and *prrF2* are 95% identical in sequence and are located adjacent to each other in the genome, separated just by 95 bases (Djapgne et al., 2018). They are functional homologs of *E. coli* RyhB sRNA, and as such, both these sRNAs play key roles in iron homeostasis and in virulence (Nelson et al., 2019). PrrF sRNAs are transcriptionally upregulated under low iron conditions, during which they

repress synthesis of non-essential iron-requiring proteins like SodB (Reinhart et al., 2015). Both PrrF1 (116 nt) and PrrF2 (114 nt) regulate QS in *P. aeruginosa* by base pairing with the *antR* mRNA and blocking its translation (Oglesby et al., 2008). AntR is a transcription factor that activates transcription of loci *antABC* and *catBCA*, responsible for breakdown of anthanilate, a precursor compound of the alkyl quinolone signaling molecule (Pita et al., 2018).

The apparent redundancy in structure and function of the two PrrF sRNAs can be rationalized by the finding that different signaling cascades, other than iron availability, may differentially regulate these two genes, thereby fine-tuning gene expression in response to slight variations in the extra and intracellular environment. For example, the AlgZR TCS, very important in rendering the CF lung mucoid phenotype in *P. aeruginosa* (Williams McMackin et al., 2019), activates the *prrF2* promoter but not the *prrF1* one (Little et al., 2018). Also, the tandem repeat structure of the *prrF* genes facilitate expression of another sRNA, PrrH, known to be involved in heme metabolism (Reinhart et al., 2015, 2017).

ErsA

The envelope stress responsive sRNA A (ErsA) is activated by the envelope stress responsive factor Sigma22 (σ^{22}) (AlgT/U) (Falcone et al., 2018). Additional CF lung signals like scarce iron and low oxygen both drives its transcription.

The first role of ErsA is direct base-pairing to the RBS of *algC* and repressing its translation in a Hfq-dependent manner. AlgC provides sugar residues for downstream synthesis of exopolysaccharides of the biofilm matrix (Okkotsu et al., 2014). Thus, through AlgC, ErsA is part of a feed forward cycle involved in biofilm polysaccharide formation.

The second role of ErsA is to directly bind the 5′UTR of the *oprD* mRNA and negatively regulate its translation (Li et al., 2012). Result of this regulation includes, but is not limited to, reducing influx of carbapenem antibiotics into the cell. This is important, given the widespread antibiotic resistance of CF airway-associated *P. aeruginosa*.

Interestingly, another sRNA called Sr0161 was also reported to repress *oprD* expression by the same mechanism (Zhang et al., 2017). Additionally, Sr0161 represses T3SS, consistent with its role in shaping *P. aeruginosa* phenotypes suited for the CF lung environment. The same study identified yet another sRNA, Sr006, which increases bacterial recalcitrance to polymyxin as well as decreases the pro-inflammatory profiles of the lipopolysaccharide, which can be considered important adaptations to the CF lung niche.

CONCLUSION

Robust approaches for characterizing sRNA targetomes in bacteria have revolutionized our understanding of the gene regulatory patterns facilitated by these regulatory RNAs and their associated chaperones such as Hfq (Santiago-Frangos and Woodson, 2018). Development of techniques such as MAPS (Lalaouna et al., 2017), RilSeq (Melamed et al., 2018), ClipSeq (Andresen and Holmqvist, 2018), and Grad-seq (Smirnov et al., 2016) techniques are important milestones in the field. Additionally, obtaining information about sRNA-based regulation even at the single cell level is now possible, but nevertheless needs improvement (Saliba et al., 2014). We now have significant knowledge about RNA mediated regulation in a wide range of bacterial pathogens (**Table 1**, **Figure 1**) that has the potential to tremendously bolster the development of therapeutic approaches targeting these signaling pathways. This is critical, given the rapid expansion of antibiotic resistance in bacteria (Ventola, 2015; Hofer, 2019) and increasing ineffectiveness of existing antimicrobial treatments.

TABLE 2 | Riboswitches explored as targets.

Riboswitch	References
FMN	Lee et al., 2009; Howe et al., 2015
glmS	Mayer and Famulok, 2006; Fei et al., 2014
Guanine-binding riboswitch	Kim et al., 2009; Mulhbacher et al., 2010
Cyclic di-GMP riboswitch	Furukawa et al., 2012
T-box riboswitch	Means et al., 2006; Anupam et al., 2008
Thiamine pyrophosphate riboswitch	Sudarsan et al., 2005
Lysine riboswitch	Sudarsan et al., 2003

There are certain considerations when targeting sRNAs for developing antimicrobial therapeutics. First, sRNAs and their mechanisms of action are often not conserved (Richter and Backofen, 2012; Colameco and Elliot, 2017) and thus antibiotics targeting a certain sRNA might have limited spectrum. Secondly, lack of defined structural configurations in sRNAs like that of rRNAs and tRNAs (both of which are targets of numerous known antibiotics Chopra and Reader, 2014; Hong et al., 2014), makes the design of small molecule inhibitors challenging. In this regard, as certain studies (El-Mowafi et al., 2014) have already addressed this, sRNA chaperones such as Hfq might be a more lucrative target because of its conserved three-dimensional structure across bacterial species. Finally, most sRNAs act as fine tuning regulators of gene expression rather than as a binary on/off switch. This limits their promise in being a molecule target that can decisively clear an infection. Rather, targeting sRNAs could likely be a potential way to bolster conventional antibiotic strategies. Nevertheless, studying mechanisms of sRNA action, give us information on gene regulation right at the nucleotide resolution. This has been exploited in studies exploring "nucleotide-based antimicrobials" (Nikravesh et al., 2007).

The prospects of antimicrobial development demonstrate more potential with riboswitches. On the one hand, often riboswitches dictate major metabolic transitions (for example, see ethanolamine utilization by a riboswitch in *L. monocytogenes* described previously), rather than functioning only as a fine tuner of gene expression as sRNAs do. On the other hand, riboswitches, by nature, are excellent binders of small ligands. Another major advantage with riboswitches is that they have so far been never found in humans (Colameco and Elliot, 2017), and thus greatly reduce the chances of host toxicity. Though still in its infancy, there have been a few studies

on targeting riboswitches for antimicrobial development. Some of them are summarized in **Table 2**. Such attempts should continue to grow and improve, as our knowledge about the occurrence and mode of action of more riboregulatory agents increases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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A Fly on the Wall: How Stress Response Systems Can Sense and Respond to Damage to Peptidoglycan

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The envelope of Gram-negative bacteria is critical for survival across a wide range of environmental conditions. The inner membrane, the periplasm and the outer membrane form a complex compartment, home to many essential processes. Hence, constant monitoring by envelope stress response systems ensure correct biogenesis of the envelope and maintain its homeostasis. Inside the periplasm, the cell wall, made of peptidoglycan, has been under the spotlight for its critical role in bacterial growth as well as being the target of many antibiotics. While much research is centered around understanding the role of the many enzymes involved in synthesizing the cell wall, much less is known about how the cell can detect perturbations of this assembly process, and how it is regulated during stress. In this review, we explore the current knowledge of cell wall defects sensing by stress response systems, mainly in the model bacterium *Escherichia coli*. We also discuss how these systems can respond to cell wall perturbations to increase fitness, and what implications this has on cell wall regulation.

Keywords: stress response, ESRS, cell wall, Cpx, RCS, Psp, sigmaE, BAE

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THE CELL WALL OF GRAM-NEGATIVE BACTERIA

In the environment, bacteria face an ever-changing range of conditions to which they have to adapt in order to survive and thrive. To overcome the many challenges that they face, Gram-negative bacteria have evolved a complex envelope made out of two membranes, the inner membrane (IM) and the outer membrane (OM) surrounding a soluble chamber, the periplasm. The OM is asymmetric, composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet (Silhavy et al., 2010). In the periplasm lies the cell wall, the determinant of cell shape, and essential for resistance to osmotic stress (Höltje, 1998; Vollmer et al., 2008). The cell wall is composed of a single-layered biopolymer, the peptidoglycan (PG), composed of repeating units of a disaccharide (N-acetylglucosamine-N-acetylmuramic acid, or GlcNac-MurNac) crosslinked with short peptides, forming a mesh-like structure (the main architecture of the PG and its assembly are summarized in **Figure 1**). The synthesis of PG proceeds in 3 major steps, all of which can be inhibited by antibiotics (Zhao et al., 2017): (1) the generation of the key intermediate lipid II, the lipid-linked disaccharide-pentapeptide precursor, in the cytoplasm; (2) the translocation of lipid II across the cytoplasmic membrane; and (3) the assembly of the cell wall in the periplasm (Typas et al., 2012; Ruiz, 2015). During growth, elongation is the process by

which cells increase their size, and division is the process by which one bacterial cell separates into two daughter cells. In *E. coli* and other Gram-negative bacteria, both these processes rely on complex PG remodeling involving both PG synthesis and PG degradation (van Teeffelen and Renner, 2018). PG synthesis requires the polymerization of new glycan strands by transglycosylases and the crosslinking of their peptide side-chains by transpeptidases. To this end, multiple PG synthases are required. Monofunctional glycosyltransferases of the shape, elongation, division and sporulation (SEDS) family polymerize GlcNac-MurNac disaccharides from lipid II subunits into long glycan strands. These strands are crosslinked together mostly between the fourth (D-ala) and the third (diaminopimelic acid, DAP) amino acid of their peptide side chains, resulting in 4–3 D,D crosslinks whose formation is catalyzed by Penicillin-Binding Proteins (PBPs). The broad family of PBPs consists of two different classes: the class B PBPs are monofunctional and can only carry out the transpeptidase reaction, while the bifunctional class A PBPs also exhibit a transglycosylase activity. The main class A PBPs in *E. coli* are PBP1a and PBP1b. Neither is essential in normal conditions, but a double mutant lacking both is nonviable (Sauvage et al., 2008). In addition to the 4–3 D,D crosslinks, non-canonical 3–3 L,D crosslinks between two DAP residues of peptide side chains are synthesized by L,D-transpeptidases that are mostly active during stationary phase (Pisabarro et al., 1985; Magnet et al., 2007, 2008). These 3–3 crosslinks are also required when defects in the LPS transport pathway occur, to strengthen the PG and avoid lysis (Morè et al., 2019). Additionally, cell wall homeostasis during both elongation and division requires enzymes that digest the PG to allow the insertion of newly synthesized material (Uehara and Bernhardt, 2011). PG fragments (muropeptides) are thus continually extracted from the PG mesh by the action of lytic transglycosylases and endopeptidases, transported back to the cytoplasm through a permease, and recycled predominantly as precursors (although they can be used as an energy source as well) (Park and Uehara, 2008), making the cell wall a highly adaptable entity. In fact, there is mounting evidence that cell wall synthesis is adapted depending on the extracellular environment. Indeed, the activity of cell wall modifying enzymes (such as *E. coli* PBP6b, MltA or *Salmonella* Typhimurium PBP3) changes depending on the chemical properties (pH) of the environment (van Straaten et al., 2005; Peters et al., 2016; Castanheira et al., 2017). A striking example is the requirement of PBP1a for maximal fitness in alkaline conditions and of PBP1b under acidic conditions (Mueller et al., 2019), consistent with the idea that PG synthesis machinery and the general structure of the PG itself change with the environmental conditions to optimize fitness (Pazos et al., 2017).

Furthermore, the cell wall is not an isolated entity: it must be constructed and remodeled within the envelope, a compartment home to many delicate and essential processes. The biogenesis of the envelope is a never-ending ballet in which membrane-anchored lipoproteins, integral membrane proteins, β-barrels (outer membrane proteins, OMPs), phospholipids and LPS have to be correctly sorted, transported and inserted in the right membrane (Silhavy et al., 2010; Rollauer et al., 2015; Okuda et al., 2016; Szewczyk and Collet, 2016). Elongation and division of the cell wall must happen without any loss of integrity and in exquisite coordination with both membranes (Gray et al., 2015). It is thus very important for the cell to monitor the state of the envelope to avoid lethal prejudice following changes in the environment, such as variations in pH and osmolarity or the use of antibiotics. Constant surveillance by envelope stress response systems (ESRS) is necessary: these systems transduce distress signals from the envelope, across the IM, into the cytoplasm in order to elicit a reaction to damages in the envelope by modifying gene expression. One of the objectives of this review is to gather evidence that ESRS can monitor cell wall related processes and react to potential problems. Therefore, in the next section, we briefly detail the major ESRS of *E. coli*, schematized in **Figure 2**.

SIGNAL TRANSDUCTION SYSTEMS

Two-component systems (TCS) are a universal solution employed to transduce signals across membranes. An archetypal envelope-associated TCS relies on a membrane-embedded sensor histidine kinase and a cytoplasmic response regulator. Upon activation by a specific signal, the histidine kinase autophosphorylates, then transfers the phosphoryl group to the response regulator, which becomes active and proficient for DNA binding to regulate the expression of a particular set of genes (Zschiedrich et al., 2016). We will now briefly introduce the main TCS that are involved in sensing and responding to envelope defects in *E. coli*, i.e., the Cpx, Bae and Rcs responses (**Figure 2A**).

The Cpx pathway is a classical TCS, consisting of the histidine kinase CpxA and the response regulator CpxR, with two accessory proteins: CpxP, which is a negative regulator of the response, and NlpE, an OM lipoprotein involved in the sensing of lipoprotein maturation and sorting defects (Delhaye et al., 2019; May et al., 2019). Cpx is usually seen as an envelope quality control system detecting the presence of misfolded proteins in the periplasm (Raivio and Silhavy, 1997; Hunke et al., 2011) and activating the expression of folding and degradation factors in response (chaperones and proteases) (Pogliano et al., 1997; Raivio et al., 2013; Surmann et al., 2016). Because of its broad role in protein maintenance and repair, Cpx is considered to be one of the main ESRS. Another classical TCS that has been pegged as an ESRS is the Bae system, composed of the histidine kinase BaeS and the response regulator BaeR (Raffa and Raivio, 2002). Bae mainly regulates the expression of multidrug efflux pumps as well as the expression of *spy*, encoding a periplasmic chaperone (Leblanc et al., 2011; Quan et al., 2011). In contrast to the Cpx and Bae pathways, the Rcs phosphorelay exhibits more complexity (Wall et al., 2018). Instead of the signal being transferred directly from the IM sensor histidine kinase RcsC to the response regulator RcsB, it must first transit through another transmembrane protein at the IM, RcsD (Takeda et al., 2001). RcsD and RcsC are maintained in an inactive state by IgaA, an IM inhibitor of the phosphorelay (Cho S.H. et al., 2014; Hussein et al., 2018). When active, the response regulator RcsB can bind either to itself or to other regulators, such as RcsA

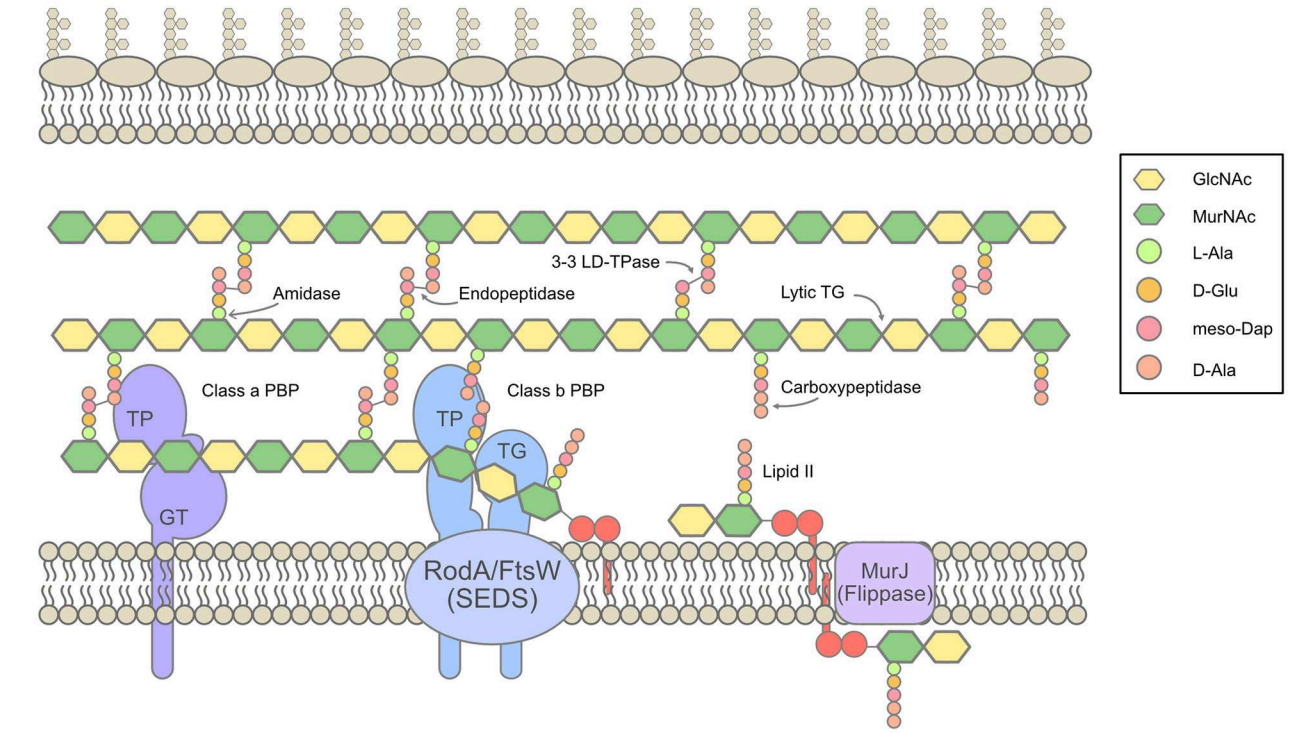

FIGURE 1 | Overview of cell wall synthesis in *E. coli.* **(A)**. The synthesis of PG in the periplasm from lipid II precursors in the cytoplasm, with indications of the main synthetic, and lytic activities involved. Figure inspired by Typas et al. (2012), Cho S.H. et al. (2014), and Zhao et al. (2017).

to control the expression of distinct sets of genes (Wall et al., 2018). Most Rcs-inducing cues require the presence of an OM lipoprotein, RcsF, for transducing the signal across the periplasm (reviewed in Laloux and Collet, 2017). In inducing conditions, RcsF interacts with IgaA, which relieves its inhibiting effect on Rcs and turns the system on Cho S.H. et al. (2014) and Hussein et al. (2018). The targets of the Rcs phosphorelay include genes involved in important cell surface structures such as flagella, LPS and fimbriae as well as acid resistance and virulence (Bury-Moné et al., 2009; Clarke, 2010). Of note, the expression of the colanic acid capsular polysaccharide genes, i.e., the capsule or *cps* genes which will be mentioned further in this review, is dependent on Rcs and the RcsA/RcsB heterodimer.

There are at least two other systems that monitor the state of the envelope of *E. coli* and that do not rely on a TCS machinery. We introduce these systems here, namely the σ^E-dependent signaling cascade and the phage shock response (Psp) (**Figure 2B**). σ^E is an alternative sigma factor that is normally sequestered on the cytoplasmic side of the IM by the membrane-bound anti-sigma factor RseA. Under inducing conditions, a cascade of proteolytic reaction degrades RseA and releases σ^E in the cytoplasm (Ades et al., 1999). σ^E-bound RNA polymerase then promotes transcription of genes encoding periplasmic chaperones involved in the transport of unfolded β-barrels across the periplasm, components of the β-barrel assembly machinery (BAM) required for β-barrel insertion in the OM, and proteins involved in LPS assembly (Rhodius et al., 2005; Grabowicz and Silhavy, 2016). On the other hand, the Psp response is induced by many signals, all having in common the fact that they result in severe IM perturbation and disrupt the proton motive force (PMF) (Brissette et al., 1990). During non-inducing conditions, PspF, the transcriptional regulator of the Psp response, is bound to PspA in the cytoplasm, which prevents it from regulating transcription. When induced, the IM proteins PspB and PspC, which are thought to be the sensors of the system, bind PspA, thus freeing PspF to activate the transcription of the *psp*A operon (Darwin, 2005). PspA is also able to bind membrane phospholipids and repair proton leakage of the damaged membranes that set off the response in the first place (Kleerebezem et al., 1996; Kobayashi et al., 2007). Overall, the Psp response seems to help maintaining PMF and thus the energy state of the cell during various envelope stresses such as growth in alkaline pH or bile salts while in the stationary phase (Joly et al., 2010).

Together the Cpx, Bae, Rcs, σ^E, and Psp systems are usually considered to be the main ESRS of *E. coli*, the "watchdogs of the envelope" (Ruiz and Silhavy, 2005). Important envelope biogenesis processes have been described to be monitored by these systems. For instance, the σ^E response directly reacts to problems in the assembly of β-barrels: unfolded OMPs are sensed

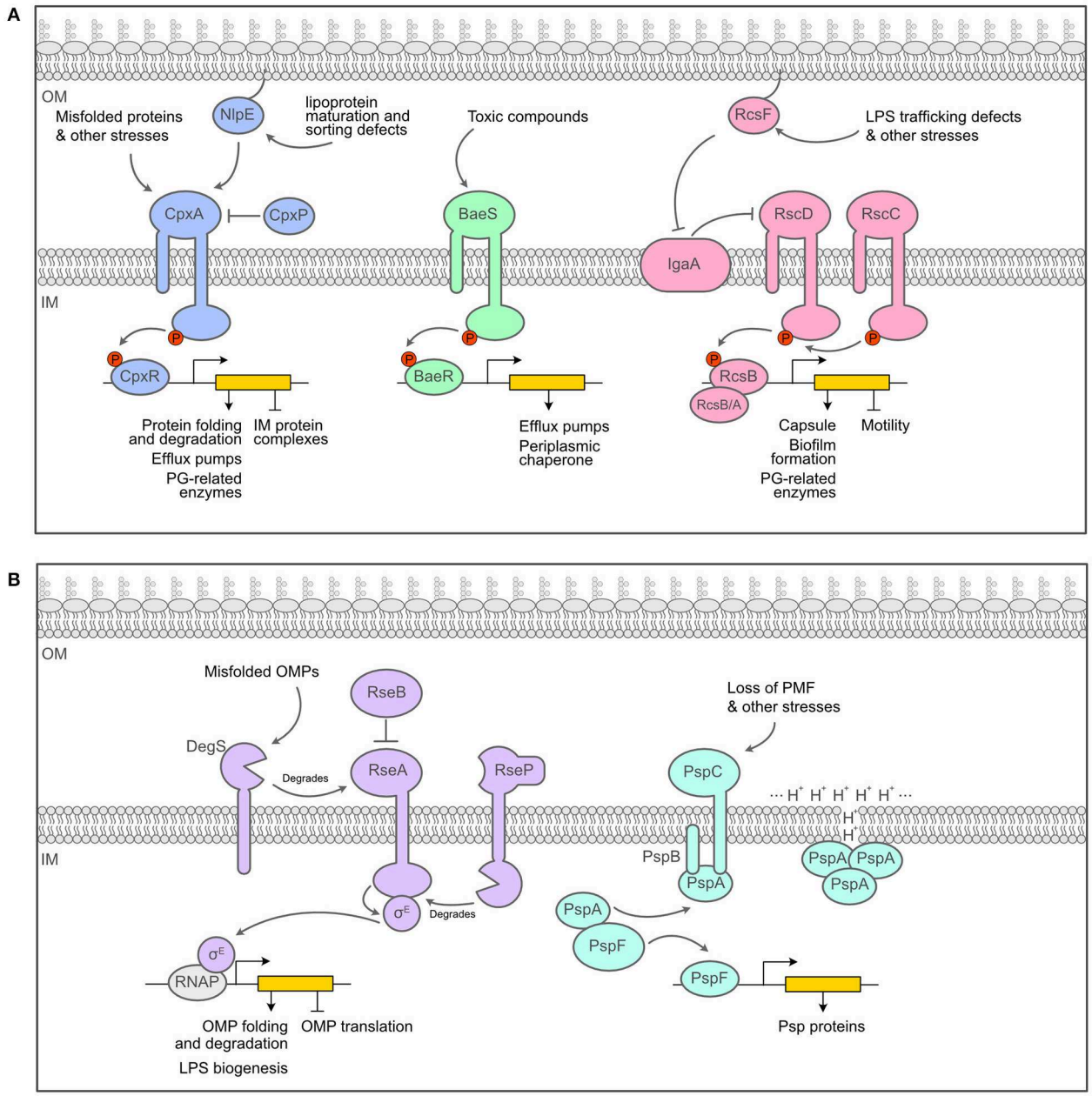

FIGURE 2 | Overview of the major envelope stress response systems of *E. coli.* **(A)**. Schematics of the two-component systems of *E. coli* that act as ESRS, the Cpx, Bae and Rcs systems. **(B)** Schematics of alternative response systems that act as ESRS, the σ^E and the Psp responses. Figure inspired by Guest et al. (2017) and Mitchell and Silhavy (2019).

by the essential IM serine protease DegS, which recognizes and binds a motif in their C-terminal sequence. Binding activates DegS (Walsh et al., 2003), which is then able to degrade RseA and start the proteolytic cascade activating the σ^E response, relieving the initial stress. Such an elegant mechanism, in which failures in a process are sensed directly and subsequently activate a response that deals with the damage, has yet to be described for the synthesis and maintenance of the cell wall of *E. coli*. Indeed, while cell wall synthesis and its inhibition by antibiotics have been the subject of a vast amount of research, very little is known about how the cell senses and responds to damage to the PG, or even alter PG composition as a means of adaptation to a dynamic environment. In this review, we will cover the sensing of PG defects by the main ESRS presented above, focusing on *E. coli* but also pointing to some insights obtained with other Gram-negative bacterial species. We will also assess how these systems can help the cell to survive attacks to their cell wall and how other signal transduction pathways, which were not

previously thought to be specifically related to envelope stress sensing, can also detect and react to the loss of PG integrity. **Table 1** summarizes the different types of PG stress and responses that we are reviewing here.

TRANSCRIPTOMIC STUDIES SHOWED THAT THE MAIN ESRS OF *E. COLI* CAN BE INDUCED BY PG-TARGETING ANTIBIOTICS

Transcriptional and transcriptomic studies investigate the effect of a specific stimulus on gene expression. These types of studies were the first to demonstrate that PG-related stress could set off the main ESRS of *E. coli*. An early transcriptional study found a link between the Rcs phosphorelay and inhibition of cell wall synthesis (Sailer et al., 2003). Indeed, treatment with β-lactams induced the expression of the genes involved in the synthesis of colanic acid, a polysaccharide found in the capsule of *E. coli* (the *cps* genes), known to be regulated by the Rcs phosphorelay (Bury-Moné et al., 2009; Clarke, 2010). In contrast, antibiotics that targeted DNA replication or protein synthesis had no such effect. Surprisingly, some β-lactams were effective in triggering capsule synthesis (such as cephaloridine), while others were not (such as penicillin G), indicating that the Rcs phosphorelay could potentially sense the inhibition of a specific step in PG synthesis, and not a general inhibition of all growth-related processes (Sailer et al., 2003). In a subsequent study using transcriptomics, ampicillin, a non-specific β-lactam antibiotic that targets several PBPs, was shown to upregulate not only the colanic acid synthesis genes but also members of the Psp regulon, hinting that cell wall damage could potentially elicit multiple responses (Kaldalu et al., 2004). In a third study, the authors disrupted the twin-arginine transport (Tat) pathway, a secretion system that transports folded proteins across the IM and showed that this led to the upregulation of genes of the Rcs and Psp regulons. Although the mechanism remains unknown, it is possible that Rcs and Psp induction was triggered by the inhibition of cell division and PG hydrolysis that occurs when Tat is perturbed. Tat is indeed required for the export of the cell wall amidases AmiA and AmiC involved in division (Bernhardt and De Boer, 2003; Ize et al., 2004; Uehara et al., 2010). In a different study though, inhibiting cell division with aztreonam, a drug that specifically inhibits the septal PG synthase PBP3, resulted in very little changes in gene expression, apart from one upregulated gene involved in colanic acid production (*wcaE*) and known to be under the control of Rcs (Arends and Weiss, 2004). The last transcriptomic study reviewed here found that treatment with different combinations of β-lactams elicited as many as 4 of the 5 main ESRS of *E. coli*. Indeed, β-lactams specifically targeting the bifunctional PBPs (PBP1a and PBP1b, cefsulodin) or the monofunctional PBP2 (mecillinam), used in combination, increased the expression of genes regulated by the Rcs, Cpx, σ^E, and Bae systems. Interestingly, Rcs was the only response that was activated in all conditions tested (multiple combinations of the drugs), suggesting that it may have an especially important role to play during PG stress (Laubacher and Ades, 2008). This is a striking example that the main ESRS of *E. coli* are turned on when PG synthesis is perturbed.

Transcriptomic studies are very informative, as they reveal a broad scope of the bacterial response to a specific stimulus. However, when it comes to the main question of this review, i.e., how do Gram-negative bacteria sense and respond to PG-related stress, they have a few shortcomings. First, these studies typically generate large amounts of data that often need to be confirmed individually (Rockett and Hellmann, 2004; Dallas et al., 2005). Second, experimental conditions such as the type and concentration of drug used, time of treatment and type of growth medium tend to vary between studies, which sometimes leads to divergent conclusions. For example, one study concluded that ampicillin did not affect capsular synthesis (Sailer et al., 2003), while another showed that ampicillin was effective in triggering the expression of the *cps* genes (Kaldalu et al., 2004). This discrepancy can easily be explained by the fact that these studies used different concentrations of ampicillin (3.75 μg mL^{-1} vs. 100 μg mL^{-1}). Third, the expression of many genes is controlled by more than one stress response. For instance, *degP*, encoding the primary periplasmic protease, is induced both upon triggering of either Cpx or σ^E (Bury-Moné et al., 2009; Grabowicz and Silhavy, 2016) and therefore increased expression of that gene could ambiguously reflect the activation of either or both ESRS. To address this issue, it is possible to use specific reporters for different ESRS. Here a reporter protein such as β-galactosidase, luciferase or a fluorescent protein is fused to the promoter of a gene whose expression is strictly controlled by a single regulator. For example, a P*cpxP-lacZ* fusion is a specific reporter of Cpx activation (DiGiuseppe and Silhavy, 2003; Hunke et al., 2011), while a P*rprA-lacZ* fusion is a specific reporter of Rcs activation (Majdalani et al., 2002). In the next section, we cover data that result from the use of more targeted approaches to dissect the sensing of PG-related stress.

ESRS CAN BE INDUCED SPECIFICALLY BY DIFFERENT TYPES OF CELL WALL ATTACKS

Using a specific reporter, some of the large-scale transcriptomic studies could be validated (Laubacher and Ades, 2008). The activation of the Rcs phosphorelay was verified using a P*rprA-lacZ* fusion after treatment with cefsulodin, mecillinam or both. The Rcs system was shown to be active in all 3 conditions, and this activation was dependent on the presence of the accessory lipoprotein RcsF (Laubacher and Ades, 2008). Another work with the same reporter additionally found that A22, a drug that targets MreB, an essential component of the elongation process, could also specifically elicit the Rcs response in an RcsF-dependent manner (Cho S.H. et al., 2014). Similar results were also obtained for the Cpx system using a P*cpxP-lacZ* reporter. Here, mecillinam, A22, and cephalexin (a drug targeting PBP3, essential for division) led to a 2-fold increase in Cpx activation (Delhaye et al., 2016). In addition to external stimuli, endogenous signals, such as genetically blocking a step in PG synthesis, can also set off ESRS. In *E. coli,* the deletion of a

TABLE 1 | List of PG stresses, their effect on stress responses, and the benefits of stress responses on the overall fitness of the cells.

Source of PG stress	Target	Stress response activated	Evidence	Benefits for the cell
BETA-LACTAMS				
A22	MreB	Rcs	P*rprA-lacZ* induction (Cho H. et al., 2014), P*cpxP-lacZ* induction (Delhaye et al., 2016)	
Cephaloridine	PBPB1a	Rcs	*cps* induction (Sailer et al., 2003)	/
Mecillinam	PBP2	Rcs, Cpx, SigmaE	Rcs, Cpx, and sigmaE regulons (microarray), P*rprA-lacZ* induction (Laubacher and Ades, 2008), P*cpxP-lacZ* induction (Delhaye et al., 2016)	Δ*rcsF* and Δ*rcsB* more sensitive than WT, constitutive activation of Rcs leads to enhanced fitness (Laubacher and Ades, 2008), Δ*cpxR* more sensitive than WT, constitutive activation of Cpx leads to enhanced fitness (Delhaye et al., 2016)
Aztreonam	PBP3	Rcs	*wcaE* (Rcs regulon) induction (microarray) (Arends and Weiss, 2004)	/
Cephalexin	PBP3	Cpx, Dpi	P*cpxP-lacZ* induction (Delhaye et al., 2016), *dpiBA* operon induction (Miller et al., 2004)	Δ*cpxR* is slightly more sensitive than WT (Delhaye et al., 2016)
Pipericillin	PBP3	Dpi	*dpiBA* operon induction (Miller et al., 2004)	
Cefsulodin	PBP1a and PBP1b	Rcs	Rcs regulon (microarray), P*rprA-lacZ* induction (Laubacher and Ades, 2008)	Δ*rcsF* and Δ*rcsB* more sensitive than WT (Laubacher and Ades, 2008)
Mecillinam + cefsulodin	PPB1a and PBP1b, PBP2	Rcs, Cpx, SigmaE, Bae	Rcs, Cpx, sigmaE and Bae regulons (microarray), P*rprA-lacZ* induction (Laubacher and Ades, 2008)	Δ*rcsF* and Δ*rcsB* more sensitive than WT. constitutive activation of Rcs leads to enhanced fitness (Laubacher and Ades, 2008)
Ampicillin	Multiple PBPs	Rcs, Psp, Dpi	Rcs and psp regulon induction (microarray) (Kaldalu et al., 2004), *dpiBA* operon induction (Miller et al., 2004)	Δ*dpiA* displays lower survival rates than WT (Miller et al., 2004), Δ*cpxR* is more sensitive than WT (Delhaye et al., 2016)
Penicillin G	Multiple PBPs	*Vc*Wig	Wig operon (microarray) (Dörr et al., 2016)	Δ*wigK,* Δ*wigR* lead to reduced fitness in *V. cholerae* (Dörr et al., 2016)
Multiple	Multiple	/	/	Overexpression of BaeR, RcsB, CpxR, EvgA and DcuR (and others) conferred intermediate to high level resistance to multiple beta-lactams (Hirakawa et al., 2003)
Lyzozyme	Glycan strands of PG	Rcs	P*rprA-lacZ* induction (Callewaert et al., 2009)	Δ*rcsB* and Δ*rcsF* show growth inhibition (Callewaert et al., 2009)
MUTANTS				
Δ*tatC*	Protein secretion and indirectly cell division	Rcs, Psp	Rcs and psp regulon induction (microarray) (Ize et al., 2004)	/
ftsI^{ts}	PBP3	Dpi	*dpiBA* operon induction (Miller et al., 2004)	/
Δ*PBP4,* Δ*PBP5,* Δ*PBP7,* Δ*ampH*	Carboxypeptidase and endopeptidases	Rcs, Cpx	P*rprA-sfgfp* induction, P*cpxP-luxCDABE* induction (Evans et al., 2013)	/

In blue, stresses that mostly target elongation processes, in green, stresses that mostly target division processes. /, no data available.

precise combination of PBPs, one carboxypeptidase (PBP5) and 3 endopeptidases (PBP4, PBP7, and AmpH) led to a reduction in motility that was dependent on the activation of both the Cpx and Rcs systems (measured with specific reporters) (Evans et al., 2013). Surprisingly the activation of the Rcs system was dependent on the activation of the Cpx system, but not *vice versa*, highlighting the existence of complex interconnections between stress responses that remain to be investigated. The reduction in motility was only observed when this specific set of genes was missing out of the 60 mutants (lacking between 1 and 7 PBPs) that were tested. This high specificity of sensing suggests that these ESRS might respond to subtle changes in PG structure and/or composition (Evans et al., 2013). Interestingly, endogenous signals do not always corroborate results obtained with the use of antibiotics. For example, whereas antibiotics with a high affinity for PBP1a led to a high induction of the *cps* genes, this was not observed in a strain in which the gene encoding PBP1a was deleted (Sailer et al., 2003). Antibiotics may thus have other effects beyond the simple inhibition of a specific enzymatic activity, such as causing a futile cycle of PG synthesis and degradation, as suggested previously (Cho H. et al., 2014). Conversely, full gene deletions may lead to polar effects or phenotypes due to the absence of the protein itself, beyond the loss of its activity. Thus, it is important to combine experiments using antibiotic treatment and genetics to firmly conclude that problems in PG synthesis are sensed by signal transduction systems. Another interesting feature is that the sensing of PG stress by ESRS is not limited to the inhibition

of PBPs, as it was found that lysozyme-treated cells specifically induced the Rcs reporter (Callewaert et al., 2009). The fact that the Rcs phosphorelay is activated by antibiotics targeting PBPs, which synthesizes the PG, and lysozyme, which degrades the disaccharide backbone of the PG, is an intriguing example that this system can respond to different types of PG stress. Of note, copper ions have recently been shown to specifically inhibit L,D-transpeptidases, leading to increased sensitivity to β-lactams (Peters et al., 2018). It is well-known that treatment with high concentration of metal ions such as copper, zinc or tungstate can induce the Cpx and/or Bae response (Guest and Raivio, 2016), but a functional link between metal ions, PG damage, and activation of ESRS is still missing and could be the focus of future research.

In summary, transcriptomic studies have revealed that antibiotics targeting PBPs lead to the expression of genes controlled by the main ESRS of *E. coli*. Some of these results have since then been confirmed by more targeted approaches using specific reporters of stress responses. Although the Rcs and the Cpx systems appear as the most responsive ESRS, PG stress seems to elicit a global response through multiple regulators. It is still unclear if specific steps of PG synthesis are sensed by specific ESRS, or if all ESRS can sense a global inhibition of cell wall synthesis. Some results suggest that the former hypothesis is more likely since antibiotics that target different PBPs have a different effect on gene expression. Systematic studies of the effect of disrupting each step of cell wall synthesis and remodeling on specific reporters of the main ESRS are necessary to dissect the complicated issue of cell wall defects sensing by ESRS.

ESRS CONTRIBUTE TO FITNESS IN PG DAMAGING CONDITIONS

If the main ESRS of *E. coli* can sense damages to the cell wall, it seems reasonable to assume that these ESRS can provide a beneficial response and ensure cellular fitness when damage occurs. There are indeed a few occurrences in the literature that can clearly link the activation of a stress response to improved fitness during PG-related damage. For instance, genetically blocking Rcs induction led to increased sensitivity to lysozyme (Callewaert et al., 2009). Likewise, strains that are unable to elicit the Rcs response (Δ*rcsB*) could not grow on medium containing sublethal concentrations of cefsulodin, mecillinam or both (Laubacher and Ades, 2008). Moreover, a strain with a constitutively active Rcs phosphorelay survived better on mecillinam, or mecillinam and cefsulodin together (but not on cefsulodin alone), than the wild-type control (Laubacher and Ades, 2008). In these conditions, the survival of the cells (along with stress-sensing by Rcs), was dependent on the presence of RcsF, like most Rcs-inducing cues, but independent of RcsA (Laubacher and Ades, 2008). Since RcsA is required to activate capsule production, the increased survival was thus not dependent on the presence of a potentially protective capsule, but on other factors controlled by the Rcs response. Analogous results were obtained for the Cpx system: the growth inhibition in presence of mecillinam (as measured by a disk diffusion assay) was stronger for a strain deficient in Cpx activation (Δ*cpxR*) and slightly lower for a strain in which Cpx was turned on at moderate level. Curiously, high Cpx activation led to increased growth inhibition, indicating that the Cpx system may regulate components that are essential for cell wall homeostasis and that the extent of the Cpx response is associated with distinct effects on the PG (Delhaye et al., 2016).

To put these results in perspective, it is interesting to mention an earlier study that reported comparable findings on a larger scale. The 32 putative response regulators of all TCS in *E. coli* were overexpressed to elicit their cognate responses, and then the susceptibility of these cells against multiple β-lactams was assayed (Hirakawa et al., 2003). While there are some caveats to this method, because overexpressing the response regulator does not always lead to full activation of the TCS (Bury-Moné et al., 2009), this work helps to understand the degree at which signal transduction systems can impact resistance to β-lactams. This study revealed that the overexpression of 13 response regulators led to increased resistance to several β-lactams, as indicated by a higher minimal inhibitory concentration (MIC). Those response regulators included BaeR and RcsB, which conferred high to intermediate resistance and CpxR, which conferred low-level resistance. The other response regulators that provided high-level resistance were EvgA and DcuR (Hirakawa et al., 2003). While the EvgAS system controls the expression of a multidrug transporter (Nishino and Yamaguchi, 2002) and can therefore logically be linked to survival in the presence of β-lactams, the relationship is more cryptic in the case of the DcuRS system, which controls the expression of genes related to the intake and metabolism of external C4-dicarboxylates (Golby et al., 1999). Clearly resistance to (and most probably sensing of) cell wall defects is also dependent on other, perhaps less studied, signal transduction systems that have previously not been linked with envelope quality control and monitoring.

THE REGULON OF SELECT ESRS INCLUDE GENES ENCODING CELL WALL MODIFYING ENZYMES

One would expect that when the wall is attacked, the cell responds either by increasing the amount of the building machineries that are inhibited or by diverting resources to increase the expression of alternative machineries to reinforce the cell wall. Yet while the main ESRS seem to be able to sense PG stress, and at least two of them (the Rcs and the Cpx responses) have been shown to increase *E. coli* survival during cell wall targeting antibiotic treatment (as elaborated in earlier sections), their response is usually thought to deal with general quality control of the envelope and thus with effects that are not directly involved in PG synthesis, its regulation or its protection. In other words, the presence of a feedback loop that induces the production of new PBPs or other cell wall altering enzymes or protective agents in response to sensing cell-wall defects by ESRS has seldom been demonstrated. Nevertheless, other examples have been reported. First, the Rcs phosphorelay is active during treatment with lysozyme and induces the expression of two lysozyme inhibitors, *ivy* and *ydhA*, which are responsible for better survival during lysozyme treatment (Callewaert et al., 2009). A more noteworthy

instance concerns the Cpx system. Relatively recent studies found that genes encoding 3 predicted cell wall modification proteins were part of the Cpx regulon: Slt, a lytic transglycosylase, LdtD, a L,D transpeptidase that catalyzes non-canonical 3–3 L,D crosslinks between glycan strands in the PG, and YgaU, a conserved protein with a LysM domain found in enzymes that interact with and degrade the cell wall (Raivio et al., 2013; Bernal-Cabas et al., 2015). The Cpx-dependent expression of *ldtD* was later shown to substantially influence PG-related processes such as elongation and division as well as sensitivity to β-lactams, as *ldtD* was largely responsible for cell wall defects observed in conditions that induce a very high level of Cpx response (Delhaye et al., 2016). In contrast, a moderate Cpx-induced expression of *ldtD* may also explain the Cpx-dependent increased survival during PG stress (Delhaye et al., 2016) (summarized in **Table 1**), as it has been shown recently that production of LdtD along with surprisingly few additional factors lead to a complete bypass of D,D transpeptidase activity of PBPs for cell wall synthesis, and broad-spectrum β-lactam resistance (Hugonnet et al., 2016). While it seems that depending on the condition (expression levels for exemple), LdtD may have either a beneficial or detrimental effect, it is clear that its expression impacts PG synthesis. These data showed that at least one of the main ESRS of *E. coli* can modulate the expression of cell wall acting enzymes, and the integrity of the PG itself. As the Cpx system is known to be active during late exponential and stationary phase, this has important implications for the regulation of cell wall structure in response to stress, but also for housekeeping purposes. It should be noted that Rcs has been reported to modestly increase (around 2-fold) the expression of *mrcA*, *mrcB* (the genes encoding PBP1a and PBP1b) and *minD* (a cell division inhibitor) in a transcriptomic study (Ferrières and Clarke, 2003). As far as we know, it is the only mention of these enzymes being in the regulon of the Rcs response. It also has not been tested whether activation of Rcs could lead to cell wall alterations *via* these enzymes, although it was shown that *E. coli* requires Rcs to regenerate its cell wall *de novo* after it was completely removed with lysozyme (Ranjit and Young, 2013). Additionally, Rcs was shown to promote the expression of both *ftsA* and *ftsZ*, which are genes that are crucial for cell division (Carballès et al., 1999). However, here again, no functional link was established between the activation of Rcs and alterations in cell division. These data suggest that, similarly to Cpx, Rcs may influence cell wall synthesis and housekeeping, but more research is necessary to understand how this function may be accomplished.

UNEXPECTED SIGNAL TRANSDUCTION SYSTEMS CAN MONITOR GROWTH PROCESSES

There are additional clues in the literature that sensing and responding to PG stress is not an activity limited to the main ESRS described above. For instance, several lines of evidence

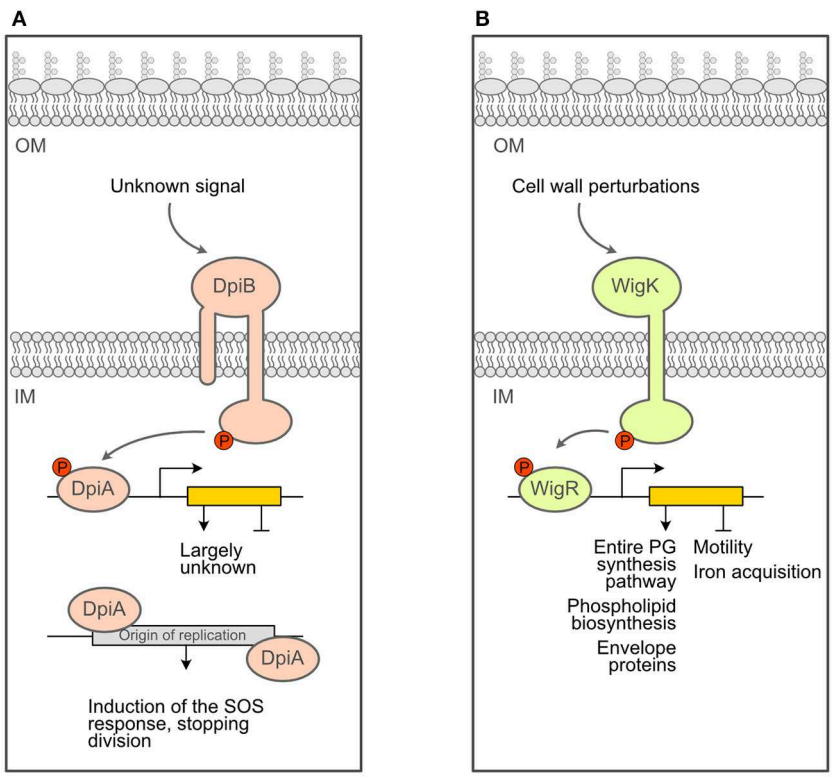

FIGURE 3 | Schematics of additional response systems that deal with PG stress. **(A)**. Schematics of the DpiBA two-component system. **(B)** Schematics of the WigKR two-component system of *Vibrio cholerae*.

connect PG synthesis during cell division with the DpiBA two-component system (schematized in **Figure 3A**), although this system was primarily associated with DNA replication and induction of the SOS response: when overexpressed, DpiA, the effector protein (or response regulator) of the TCS, binds replication origin sequences on the *E. coli* chromosome and certain plasmids, which interferes with DNA replication and triggers the SOS response (Huisman et al., 1984; Ingmer et al., 1998; Miller et al., 2003). First, it was found that treating cells with ampicillin, cephalexin or pipericillin (which targets PBP3) turned on the expression of both the *dpiBA* operon and *pabA*, a gene regulated by DpiA. Second, inactivation of PBP3 by shifting an *ftsI*^{ts} (encoding PBP3) strain to non-permissive temperature and therefore blocking cell division also resulted in induced *dpiBA* expression. Interestingly, no effect was observed when PBP2 and FtsW were inactivated, indicating that the lack of PBP3 activity is a specific stimulus for *dpiBA* expression (Miller et al., 2004). Third, treatment with ampicillin and inactivation of PBP3 both activated the expression of *sfiA*, an SOS response-induced gene that prevents FtsZ polymerization and thus cell division in a *dpiA*-dependent manner. Taken together, these data suggest that interfering with PG assembly, in particular during cell division, triggers the DpiBA two-component system. Supporting the physiological relevance of these findings, *dpiA* null mutants display markedly reduced cell survival when exposed to ampicillin for a short time (<4 h) (Miller et al., 2004). However, it remains unclear whether DpiA acts alone as an effector protein to set off the SOS response during treatment with β-lactams, or if genes present in the DpiBA regulon are also necessary for resistance to β-lactams.

Another example of non-canonical ESRS being able to sense and respond to cell wall defects is the newly characterized TCS WigRK described in *Vibrio cholerae* (schematized in **Figure 3B**). It was identified in a Tn-Seq screen for *V. cholerae* factors that are required for recovery from penicillin exposure (Dörr et al., 2016). Mutants that lack *wigK, wigR* or *wigRK* exhibit lower (2–3 orders of magnitude) colony-forming units after treatment with penicillin G. Extraordinarily, the regulon of this TCS includes the full set of genes required for cell wall biosynthesis (Dörr et al., 2016). The increased expression of these genes leads to a higher cell wall content and markedly increased resistance to hypo-osmotic shock (Dörr et al., 2016). Interestingly, disrupting cell wall synthesis with penicillin induces the expression of *mraY* (involved in lipid II biosynthesis), *murJ* (lipid II flippase) and genes encoding PBP1A and PBP1B (the major *V. cholerae* cell wall synthases) in a *wigR*-dependent manner. In contrast, compounds targeting envelope processes unrelated to cell wall synthesis, such as cerulenin (inhibitor of fatty acid synthesis) and crude bile (general membrane perturbator), did not result in induction of *pbp1a*, suggesting that WigKR is turned on in response to cell wall damaging agents and not cell envelope damage in general. In addition to the important role of this system in surviving treatment with cell wall inhibitors, WigKR also affects cell wall homeostasis during normal growth. Indeed, mutants lacking *wigR* had a larger diameter and cell volume, whereas strains overexpressing WigR had a significantly reduced cell width, indicating a fundamental role of this TCS in maintaining cell wall homeostasis (Dörr et al., 2016).

THE MECHANISMS OF SENSING CELL WALL DAMAGE BY ESRS ARE LARGELY UNKNOWN

There is convincing evidence that ESRS can sense and respond to cell wall damage, yet the molecular signals that trigger these responses remain mostly unknown. One possibility is that ESRS actually sense downstream effects of cell wall impairment, such as membrane perturbations (known to trigger the Rcs response, Farris et al., 2010) due to cell shape defects (Huang et al., 2008). However, there is some evidence that suggests that the signal sensed could also be direct and specific, notably the fact that β-lactams with different targets elicit different responses (Sailer et al., 2003; Arends and Weiss, 2004; Kaldalu et al., 2004; Laubacher and Ades, 2008). Some studies suggest that the main candidates for a direct and specific sensing are the pool of PG precursors and PG fragments, destined for recycling (Sailer et al., 2003; Evans et al., 2013; Dörr et al., 2016). Such a signal has already been described for the regulation of the production of β-lactamase (Jacobs et al., 1994, 1997). In many Gram-negative bacteria, the expression of the β-lactamase AmpC is induced by the AmpR regulator after activation by β-lactams (Lindberg et al., 1985; Vadlamani et al., 2015). The activity of AmpR is modulated by PG intermediates: it is maintained in its inactive form by a UDP-MurNAc-pentapeptide, a PG precursor whose concentration decreases during treatment with β-lactams, and activated by a anhMurNAc-tripeptide, a product of PG recycling that accumulates in the cytoplasm during treatment with β-lactams (Jacobs et al., 1997). So not only does treatment with β-lactams lead to changes in the pool of different PG species and intermediates, but these changes have been demonstrated to influence the activity of a very specific response system that directly deals with the initial stress. This is an elegant mechanism, and there is supporting evidence that a similar process could be responsible for the activation of ESRS during cell wall stress. For instance, an *E. coli* mutant strain that lacks 4 specific PBPs has constitutively active Rcs and Cpx systems (as discussed previously), and the amount of pentapeptides and different species of cross-linked muropeptide was shown to rise and fall along with the activity of the Cpx and Rcs systems (Evans et al., 2013). Still, a detailed mechanistic understanding of how the main ESRS, as well as other signal transduction systems, can sense cell wall damage, remains elusive.

CONCLUSION

E. coli and other Gram-negative bacteria are equipped with sophisticated systems (including ESRS) to monitor and convert a stress stimulus into remodeling their gene expression pattern, thereby rewiring the cell physiology to match the new environmental state. While important envelope biogenesis processes have been shown to be monitored by precise signal transduction systems, the question of how cell wall related

processes, such as elongation and division, are tracked to avoid lethal malfunctioning, remains unresolved. Extensive research efforts were focused on identifying the players required for PG synthesis and its control in *E. coli* and other species. For example, post-translational regulators were discovered, such as the lipoproteins LpoA and LpoB, which modulate the activity of the PG synthases PBP1a and PBP1b, respectively (Paradis-Bleau et al., 2010; Typas et al., 2010). However, few transcriptional regulators of PG synthesis are actually described in *E. coli*. This review attempts to shed light on these two issues: how can stress responses sense the correct or incorrect synthesis of the cell wall (sensing) and how do they modulate gene expression to respond to any defects detected (response)?

Concerning the sensing, the body of work presented here clearly outlines the fact that the main ESRS of *E. coli* can sense a compromised cell wall. Both exogenous factors (such as treatment with β-lactams or lysozyme) and endogenous factors (such as the deletion of a specific set of PBPs) can act as a trigger to set off the Rcs, Cpx, Bae, σ^E, or Psp response. Depending on the stimulus, one or multiple responses can be fired off simultaneously. Likewise, a specific stress response can be triggered by one or multiple stimuli. This highlights a major lack of knowledge: whereas the sensing of PG stress by major ESRS has been documented numerous times in the literature, the mechanistic details of such sensing by the different stress responses are often missing. Moreover, as this has not been the focus of intense research, there are probably many occurrences of sensing of cell wall defects by signal transduction systems that remain to be revealed, both by well-known ESRS and by other, less-studied systems.

Regarding the response, a few signal transduction systems have been shown to increase survival when the integrity of the PG is challenged. These include some of the main ESRS, Rcs, Cpx, and Bae, as well as other TCS, such as EvgA and DcuR. In most cases, it is still unclear how activation of these responses helps cells with cell wall defects. Do they deal with side-effects of PG synthesis inhibition by stabilizing other components of the envelope? Do they directly regulate growth to adapt to certain PG stress? Or is it a combination of both? So far, only two of the main ESRS, Rcs and Cpx, have been shown to not only detect cell wall perturbations but also to control the expression of genes involved in PG remodeling in *E. coli*. While a functional link between the activation of Cpx and growth-related processes could be described, this is not the case for Rcs.

Future research should focus on these shortcomings (concerning the mechanisms of sensing and response), to elucidate how cells react to harsh stresses such as cell wall-targeting antibiotic treatment, but also how they adjust their cell wall to different growth conditions, for example when switching to stationary phase or during infection. To this end, complementary approaches could be envisioned. First, a global, high throughput approach may help to thoroughly define the stimuli triggering each ESRS and other signal transduction systems. For instance, a library of strains could be engineered to carry specific reporters of these systems; the activity of these reporters could be quantified when cells are grown in a vast array of conditions known to perturb PG integrity, hence providing a systematic overview of which PG stress induces which pathway. Besides, a more directed genetic approach could help identifying novel factors involved in sensing PG stress, for example by screening a mutagenized library to identify genes that are required for the activation of a given ESRS by a specific PG-damaging condition. A follow-up biochemical characterization of the newly identified factors would be needed to uncover the molecular mechanism(s) of PG stress sensing by ESRS. Moreover, these strategies can be combined with the analysis of PG species released under different stress conditions to potentially identify PG fragments that could act as inducers or repressors of stress responses.

An as yet largely untapped resource for insights might be found in the many stress responses that were not previously thought to deal with envelope perturbation, including those that are not as extensively investigated as the main ESRS of *E. coli*, as it is likely that those systems still conceal interesting secrets. A better knowledge of how stress responses can sense and mitigate PG stress can lead to a better understanding of both the functioning of stress responses and the regulation of cell wall synthesis.

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AD, GL, and J-FC wrote the manuscript.

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Switching Lifestyles Is an *in vivo* Adaptive Strategy of Bacterial Pathogens

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Gram-positive and Gram-negative pathogens exist as planktonic cells only at limited times during their life cycle. In response to environmental signals such as temperature, pH, osmolality, and nutrient availability, pathogenic bacteria can adopt varied cellular fates, which involves the activation of virulence gene programs and/or the induction of a sessile lifestyle to form multicellular surface-attached communities. In *Salmonella*, SsrB is the response regulator which governs the lifestyle switch from an intracellular virulent state to form dormant biofilms in chronically infected hosts. Using the *Salmonella* lifestyle switch as a paradigm, we herein compare how other pathogens alter their lifestyles to enable survival, colonization and persistence in response to different environmental cues. It is evident that lifestyle switching often involves transcriptional regulators and their modification as highlighted here. Phenotypic heterogeneity resulting from stochastic cellular processes can also drive lifestyle variation among members of a population, although this subject is not considered in the present review.

Keywords: acid stress, lifestyles, biofilms, SsrB, CsgD, Spo0A, chronic infections, virulence

INTRODUCTION

Pathogenic bacteria constantly face a multitude of chemical and physical stresses associated with external environments and host-specific niches. In order to survive and grow as parasites, they have evolved molecular mechanisms for altering their lifestyles in response to changes in environmental conditions. For example, free-living bacteria can switch their lifestyle to a virulent form inside hosts or undergo development to form matrix-encased aggregates called biofilms on different abiotic and biotic surfaces (**Figure 1A**). The virulent form rapidly colonizes and disseminates in host tissues to cause acute infections for a limited period of time. However, a prolonged association of pathogens with hosts enables carriage or persistence leading to chronic infections, which may be associated with clinical symptoms (for example, when *Pseudomonas aeruginosa* persists in the lungs of cystic fibrosis patients) or not (for example, when *Salmonella* Typhi forms biofilms on gallstones of asymptomatic carriers). We define the ability to shift from a planktonic lifestyle to a multicellular community as a "lifestyle switch," as observed in a majority of chronic bacterial infections (Bjarnsholt, 2013). The regulation of lifestyle switches in bacterial pathogens is important to enable successful pathogenesis.

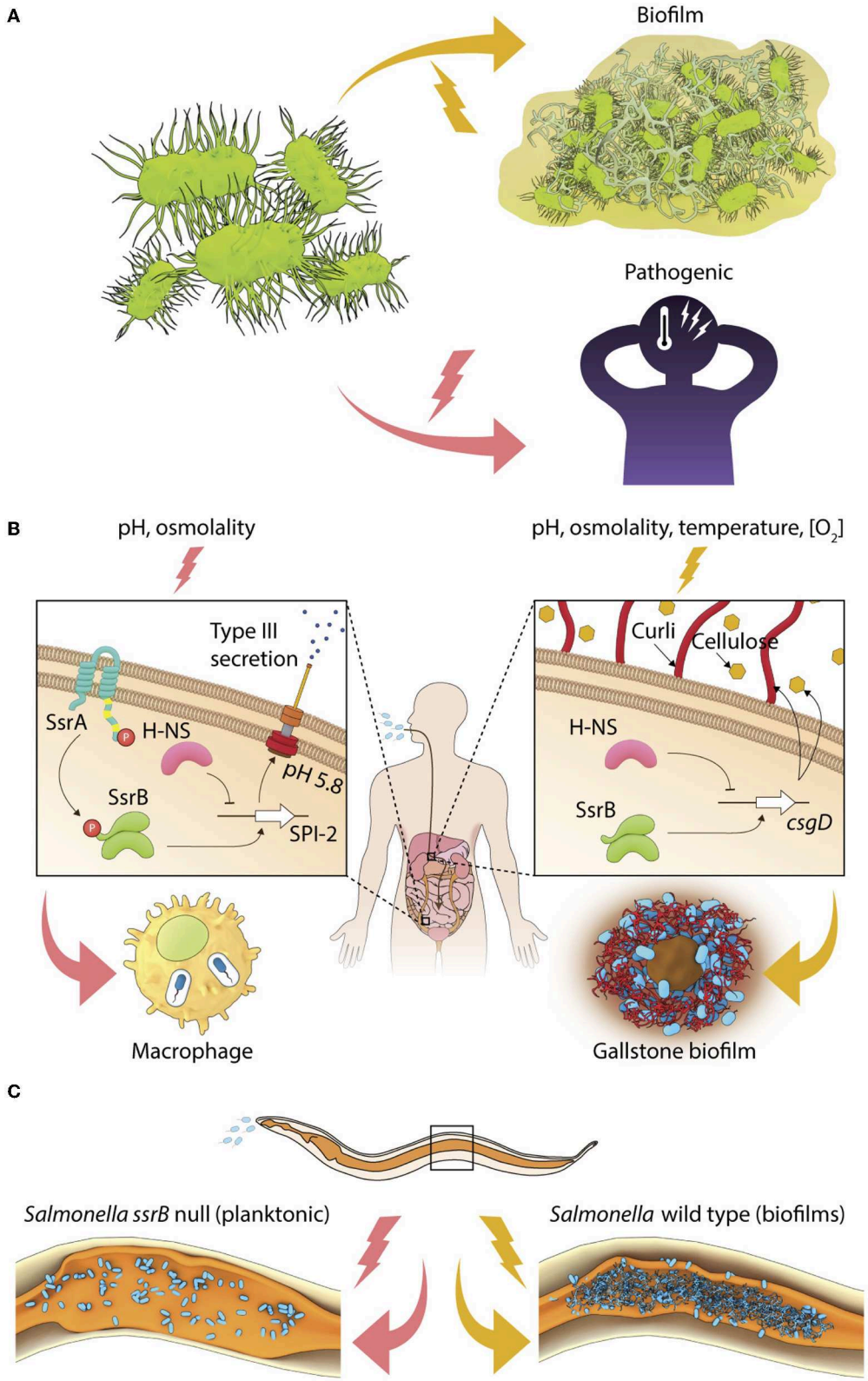

FIGURE 1 | Environmental regulation of bacterial lifestyles. **(A)** A general scheme depicting lifestyle switches in pathogenic bacteria to favor virulence or biofilm formation. **(B)** In *Salmonella*, SsrB∼P regulates the intracellular lifestyle and SsrB favors the formation of the carrier state and **(C)** *Salmonella* forms SsrB-dependent multicellular aggregates during persistent infections in *C. elegans*.

Phenotypic variations that occur in a subset of a population in the absence of any genetic or environmental drivers are classified as phenotypic heterogeneity. When this occurs, subpopulations in a clonal group adopt distinct life forms, such as planktonic or sessile, or express different functional markers, for example toxins or cell surface proteins. In this review, we address lifestyle transitions in response to changes in environmental conditions as a population trait, and do not focus on sub-populations that arise due to phenotypic or genetic heterogeneity. The exception is in the case of *Acinetobacter baumanii*, where we describe how a molecular switch gives rise to phenotypic sub-types. The adaptive significance of phenotypic heterogeneity has been superbly described in a recent review (Ackermann, 2015). In the present review, we use *Salmonella* as an example to compare with other bacterial pathogens that are known to undergo lifestyle switches.

AN OVERVIEW OF *SALMONELLA* PATHOGENESIS

The enteric pathogen *Salmonella enterica* is typically ingested from contaminated food or water. Most bacteria are killed in the extreme acid pH of the stomach, but those bacteria that survive have to traverse the intestinal mucosal layer before transiting the intestinal epithelium to eventually survive inside macrophages. *Salmonella* exploits host-associated environmental cues such as acidic pH and high osmolality to form a *Salmonella*-containing vacuole (SCV), enabling intracellular replication (Lee et al., 2000; Feng et al., 2003, 2004, see Kenney, 2018, for a review). The virulence genes of *Salmonella* are encoded on horizontally acquired AT-rich segments of the genome called *Salmonella* Pathogenicity Islands (SPIs), which are tightly regulated by two-component regulatory systems (TCRSs). For example, the SsrA/B TCRS is essential for activation of the SPI-2 regulon genes encoding a type-three secretory needle and effectors that are involved in the maintenance of the SCV (Shea et al., 1996; Cirillo et al., 1998; Lee et al., 2000). Intracellular replication of *Salmonella* ultimately causes gastroenteritis (serovar Typhimurium) or systemic typhoid fever (human-restricted serovar Typhi).

Transcriptional activation of the SsrA/B system is tightly regulated by the action of upstream TCRSs, EnvZ/OmpR, and PhoP/Q, which respond to environmental changes in pH, osmolality, phosphate, and Mg^{2+} (Groisman et al., 1989; Feng et al., 2003, 2004; Liew et al., 2019). When planktonic or invasive *Salmonellae* encounter acidic pH or high osmolality, their cytoplasm acidifies, activating the membrane-bound sensor kinase EnvZ, by increasing intra-helical hydrogen bonding in its cytoplasmic domain (Wang et al., 2012; Chakraborty et al., 2015). Downstream activation of the response regulator OmpR orchestrates transcriptional activation of the SPI-2 genes, and other stress-protective mechanisms involving RpoS, the stationary-phase sigma factor, oxidoreductases, outer membrane porins, etc. (reviewed in Chakraborty et al., 2017; Kenney, 2018). SPI-2 genes are expressed when SsrB∼P mediates direct transcriptional activation at SPI-2 promoters, and SsrB also functions to relieve H-NS repression (Walthers et al., 2007). Thus, coordinated activation of the sensor kinase SsrA, by mechanism(s) not precisely understood, leads to the phosphorylation of the response regulator SsrB, and enables the intra-vacuolar lifestyle of *Salmonella* in infected epithelial cells or macrophages (**Figure 1B**).

THE LIFESTYLE SWITCH IN *SALMONELLA* INVOLVES NON-CANONICAL SIGNALING

Salmonella also alters its genetic program to switch to a multicellular lifestyle, or biofilms, in the presence of several abiotic (for example, temperature, nutrient availability, osmolality, etc.) and biotic (for example, bile and gallbladder inflammation) stresses (reviewed in Steenackers et al., 2012). On host tissues such as gallstones and intestinal epithelial cells, individual *Salmonella* cells become encased in an intricate network of three-dimensional extracellular matrix to form mature biofilms (Boddicker et al., 2002; Crawford et al., 2010). This ability to switch to a sessile lifestyle is essential for maintaining the carrier state, allowing *Salmonella* to persist in asymptomatic patients, as well as in non-host reservoirs (Crawford et al., 2010). Studies of Typhoid carriage using the mouse model in which mice were fed a lithogenic diet to induce the formation of gallstones, have failed to provide clear insights regarding the signal transduction pathways that regulate the formation of biofilms *in vivo* or drive the switch in lifestyle from free-living cells to surface-attached communities (see Gunn et al., 2014, for a review). The transcriptional regulator CsgD in the unphosphorylated state activates the expression of biofilm matrix genes to allow the formation of *Salmonella* biofilms *in vitro* (Römling et al., 1998; Zakikhany et al., 2010; MacKenzie et al., 2015). SsrB acts non-canonically in biofilm formation (**Figure 1B**), in a manner that is distinct from its classical function of regulating pathogenicity island genes (reviewed in Desai et al., 2016; Desai and Kenney, 2017).

During neutral pH conditions, unphosphorylated SsrB binds to the *csgD* regulatory region and DNA binding and bending is sufficient to relieve H-NS-mediated repression, favoring formation of *S.* Typhimurium biofilms (Desai et al., 2016). Thus SsrB, a response regulator that was acquired during the evolution of *Salmonella* as a pathogen, sits at a pivotal position in governing *Salmonella* lifestyle fate: to either exist inside the host (in the SCV) as a promoter of virulence, or to drive surface-attached multicellular biofilms, which serves to maintain the carrier state (**Figure 1B**).

THE ADAPTIVE SIGNIFICANCE OF LIFESTYLE SWITCHING IN *SALMONELLA*

The SsrB-driven molecular switch also functions during persistent infections *in vivo*. During *Salmonella* infection of the heterologous host *Caenorhabditis elegans*, sessile communities of *Salmonella* were clearly visible in the intestinal lumen (Desai et al., 2019 and see **Figure 1C)**. Although the size of *Salmonella* aggregates was smaller *in vivo* (10–20 μm^2) than a typical *in*

vitro flow cell biofilm (at least 2 mm^2), SsrB was still required, but phosphorylation of SsrB was not. The quintessential biofilm components were present, including: the master regulator CsgD, and the extracellular matrix components, curli, cellulose, and O-antigen that enabled the formation of *Salmonella* biofilms during long-term infections. Interestingly, biofilm formation enhanced the lifespan of worms, indicating a reciprocal relationship between virulence activation and the existence of biofilms. The lifestyle switch to form biofilms *in vivo* inhibited pathogenesis genes encoded on the SPI-1 pathogenicity island, and activated a mitogen-activated protein kinase (MAPK)-driven innate immunity pathway (Desai et al., 2019 and see **Figure 1C)**. In the future, it will be important to understand the host-associated environmental cues and signal transduction pathways that activate the formation of *Salmonella* biofilms. Although we have a detailed understanding of how the SsrA/B TCRS responds to acidic pH (Liew et al., 2019), it will be germane to understand the regulation of SsrA/B expression and activity in biofilm favoring conditions.

LIFESTYLE SWITCHING IN SPORE-FORMING BACTERIA- *B. subtilis*

The non-pathogenic Gram-positive bacterium *Bacillus subtilis* is an important model to understand environment driven lifestyle changes in pathogenic Gram-positive bacteria. *B. subtlis* also forms biofilms in the intestines of worms and biofilm formation increases lifespan by ∼25% (Donato et al., 2017). Lifespan extension occurs when nitric oxide (NO) and Competence Sporulation stimulating Factor (CSF) produced by *B. subtilis* biofilms programs Insulin-like signaling (ILS) and MAPK innate immunity pathways of *C. elegans*. In response to starvation, the master response regulator Spo0A orchestrates elaborate genetic changes in development and differentiation pathways in *B. subtilis* (Hamon and Lazazzera, 2001, reviewed in Vlamakis et al., 2013). In this scenario, the intracellular level of Spo0A∼P controls the lifestyle decision in *Bacillus*. Intermediate levels of Spo0A∼P favor biofilm formation, while a higher accumulation of Spo0A∼P leads to sporulation (Fujita et al., 2005). The mature *B. subtilis* biofilm is a fine example of how heterogeneity in Spo0A∼P levels leads to a division of labor, as only the matrix-producing cells differentiate to form spores (see Vlamakis et al., 2013, for a review). Such a Spo0A∼P driven lifestyle switch could also be governing cell fates in the closely related anaerobe, *Clostridia*, which causes notorious nosocomial infections (**Figure 2A**) (see below). This is in contrast to what we observed with *Salmonella*, where unphosphorylated SsrB drove the biofilm pathway and SsrB∼P was responsible for activation of virulence (Desai et al., 2016).

Spo0A REGULATES BIOFILMS AND SPORULATION IN *CLOSTRIDIA*

Chronic infections by the Gram-positive pathogens *C. difficile* and *C. perfringens* are highly antibiotic-tolerant and transmissible due to their remarkable ability to form hardy spores. Germination to vegetative cells leads to the production of toxins, TcdA and TcdB, adhesin, fibronectin-binding protein A, and different cell wall proteins (CWPs), which drives host colonization and disease (Waligora et al., 2001; Calabi et al., 2002; Kuehne et al., 2010; Barketi-Klai et al., 2011). In response to possible changes in temperature and nutrient levels, these free-living vegetative cells also come together and form matrix-encased biofilms (Dapa and Unnikrishnan, 2013; Obana et al., 2014). Apart from contributing to environmental persistence, biofilms of *C. difficile* have been observed on intestinal mucosal membranes of patients suffering from irritable bowel syndrome and as components of mixed species biofilms in the intestines of infected mice (Swidsinski et al., 2005; Semenyuk et al., 2015). This observation raises the question then, as to what regulates the transitions from the vegetative stage to multicellular communities and ultimately to sporulation?

Interestingly, Spo0A, the master regulator of *B. subtilis* lifestyles, is conserved in *C. difficile* with a 56% sequence identity (Deakin et al., 2012). Inactivation of Spo0A reduces biofilm formation and sporulation in *C. difficile in vitro* (Dawson et al., 2012; Dapa et al., 2013). However, complementation by Spo0A *in trans* leads to a complete recovery of the ability to form biofilms, but only to a partial rescue of sporulation (Dawson et al., 2012). Drawing parallels with what is known in *B. subtilis*, it is possible that in *C. difficile*, the activation of sporulation genes requires much higher levels of Spo0A∼P than that required for biofilm formation (**Figure 2A**). In order to gain a clear understanding of the lifestyle switch in *C. difficile*, it would be worthwhile to examine the effect of a point mutation in the Asp66 residue (Spo0A phosphorylation site), as well as to identify the upstream kinases of Spo0A (possible homologs of *B. subtilis* KinA/B/C/D). These studies would provide insights regarding the differential regulation of Spo0A in response to environmental stresses associated with biofilms and sporulation in *C. difficile*.

DUAL STATES OF PrfA REGULATES LIFESTYLES OF *Listeria monocytogenes*

Listeria monocytogenes is another Gram-positive gastrointestinal pathogen that invades and survives intracellularly in epithelial cells and macrophages to cause dangerous listeriosis in humans. The virulence program is well-characterized and requires the master transcriptional regulator PrfA. PrfA belongs to the cyclic adenosine monophosphate (cAMP) receptor protein (CRP)/fumarate nitrate reductase (FNR) family. The CRP/FNR superfamily of transcriptional regulators have evolved in several lineages of *Firmicutes*, *Actinobacteria*, *Proteobacteria,* and Cyanobacteria, and perform essential physiological functions such as catabolite repression, oxygen sensing, nitrogen fixation, and survival in stationary phase (refer to Körner et al., 2003, for a review). PrfA activates expression of the invasion factors, InlA and InlB (Gaillard et al., 1991; Dramsi et al., 1995), and the pore-forming toxin listeriolysin (LLO), enabling vacuolar escape and cytosolic replication of *L. monocytogenes* in host cells (Goebel et al., 1988; Cossart et al., 1989). The

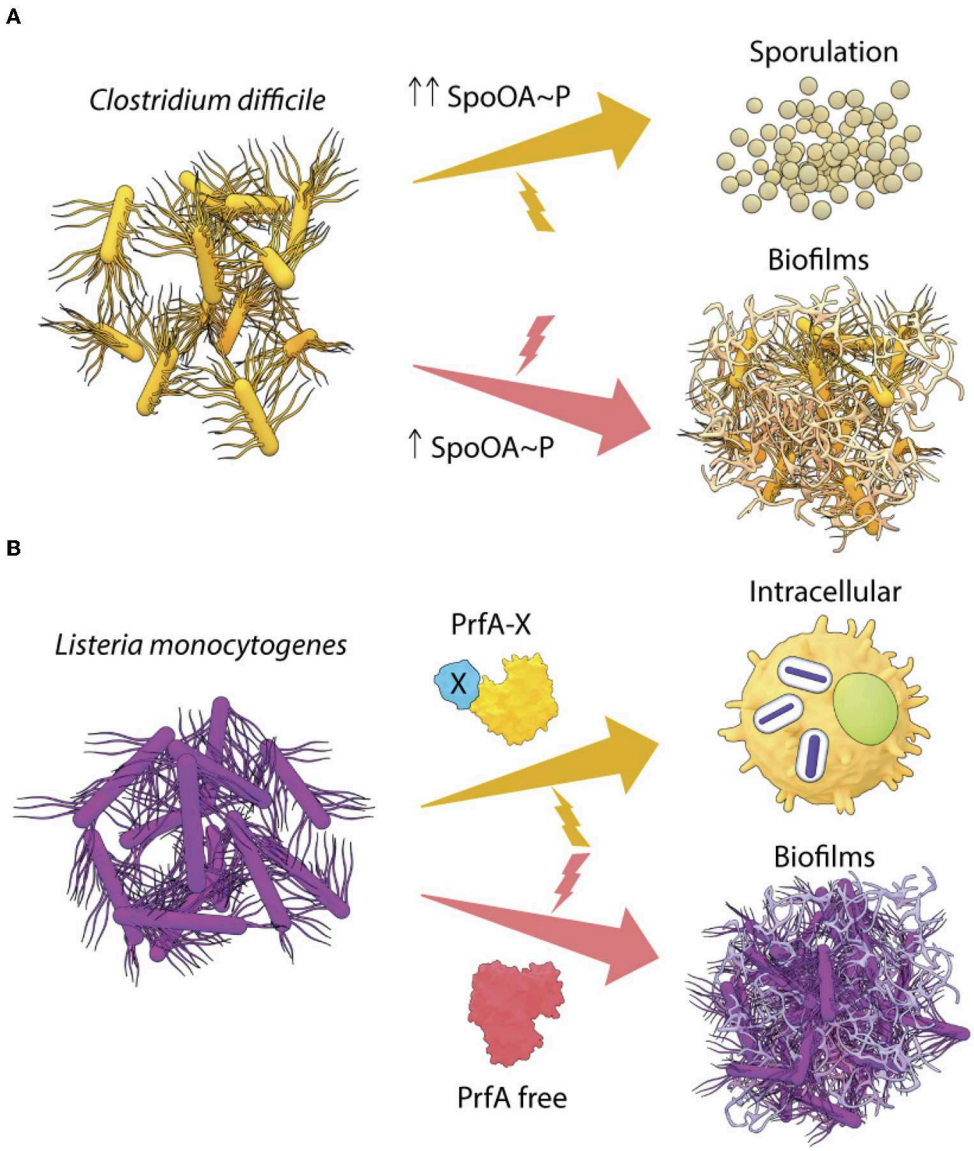

FIGURE 2 | Transcriptional regulators drive lifestyle changes in Gram-positive pathogens. **(A)** In *C. difficile*, the intracellular levels of Spo0A~P regulate the lifestyle switch to form spores or biofilms and **(B)** two different forms of the transcriptional regulator PrfA in *L. monocytogenes,* are required to activate the intracellular lifestyle or to form *in vivo* aggregates.

intracellular levels of PrfA are tightly controlled by feedback loops, involving post-transcriptional regulation and stress-responsive alternative sigma factors in order to ensure optimum expression during the switch from the free-living/saprophytic phase to the intracellular virulent lifestyle (see de las Heras et al., 2011, for a review). Active PrfA forms a homodimer with the C-terminus harboring a DNA-binding helix-turn-helix motif (HTH) and its N-terminus forms a β-barrel structure that was predicted to bind cyclic nucleotide(s), based on its high level of homology to the N-termini of other members of the CRP/FNR family of transcriptional regulators. However, PrfA lacks the critical cAMP-binding residues, emphasizing that sequence homology does not always predict conservation of key residues (Eiting et al., 2005). *In vivo*, transcriptional activation by PrfA is enabled by glutathione (GSH) binding (Reniere et al., 2015). Although GSH is not essential for PrfA binding to DNA *in vitro*, *in vivo*, the binding of GSH to each PrfA monomer stabilizes its HTH motif, and increases the probability of binding to promoters (Hall et al., 2016). Allosteric regulation of PrfA activity is also indicated by PrfA* mutants that are "locked" in an active state, leading to hyper-virulence *in vivo* and a constitutive over-expression of the PrfA regulon outside the host (Ripio et al., 1996; Wong and Freitag, 2004).

Surprisingly, PrfA expression was also crucial for *L. monocytogenes* to switch to a sessile lifestyle as aggregates or biofilms (Lemon et al., 2010). The PrfA-driven pathway for formation of biofilms *in vitro* has not been worked out in detail, but studies showed that PrfA regulated biofilm maturation and growth. Interestingly, biofilms formed by the hyper-virulent PrfA* mutants were similar to the wild type, however, an avirulent PrfA mutant (Y154C) formed greater biofilms *in vitro* than the wild type (Lemon et al., 2010), indicating that PrfA can exist in multiple forms with differing activities. A simple model of PrfA-mediated lifestyle switching in *L. monocytogenes* is described in **Figure 2B**, involving a GSH-bound form of PrfA (PrfA-X) that activates the virulence program and a free form of PrfA that drives biofilms. However, it is also possible that biofilm genes are regulated by an intermediate conformation of PrfA or an activated state bound to a different allosteric effector.

In addition, a key PrfA-regulated virulence factor ActA, also enabled *in vivo* aggregation of *L. monocytogenes* by cell-cell mediated contact in the murine infection model (Travier et al., 2013). ActA has three distinct domains, the N-terminus and P-domains are required for actin polymerization, while ActA homodimer interactions that mediate aggregate formation require N-, P-, and C-terminal domains. Thus, different active conformations of ActA might exist *in vivo* and play a role in deciding the fate of *L. monocytogenes*. An ActA-mediated lifestyle switch may also be driven by its binding to the peptidoglycan layer during the intracellular phase, although the signal(s) that enable such an association with the cell wall remain unknown (García-del Portillo et al., 2011). Interestingly, a decrease in ActA levels was also found to be correlated with the persistence of *L. monocytogenes* in vacuoles of non-phagocytic host cells (Kortebi et al., 2017). In the future, a combination of biochemical, genetic and cell biological approaches will be required to clearly delineate the structure-function relationship and regulation of expression of PrfA and ActA for favoring intracellular survival (acute phase) or persistence in hosts.

A NOVEL TetR FAMILY REGULATOR SWITCHES LIFESTYLES IN *Acinetobacter baumannii*

Phenotypic heterogeneity is the basis for several bacterial functions in specific sub-populations, including: the expression of virulence factors, quorum sensing, antibiotic resistance, and persister formation. Recent studies revealed that clinical strains of *A. baumannii* are characterized by sub-populations that differ in their cell surface properties and virulence gene expression (Chin et al., 2018). This is unlike *Listeria* (see above), in which the entire population adopts a similar morphology to become virulent or avirulent in response to niche-specific signals. *A. baumannii* cells from avirulent transparent colonies (AV-T) failed to colonize and cause disease in mice, while infections with the virulent opaque cells (VIR-O) resulted in 100% death within 2 days post infection. Transcriptomic analysis revealed that a gene encoding a TetR-type transcriptional regulator (ABUW_1645) was highly expressed in AV-T cells compared to VIR-O cells. Over-expression of ABUW_1645 in VIR-O cells reversed the phenotypic switch, leading to a loss of virulence *in vivo* (Chin et al., 2018). Since AV-T cells retained the ability to form biofilms at 25°C (a non-host temperature), biofilms and virulence might be mutually exclusive in *A. baumannnii*. How ABUW_1645 expression is regulated or whether its behavior is modified by small molecule effectors is presently not known.

HYBRID SENSOR KINASES CONTROL THE FATE OF *Pseudomonas aeruginosa*

In the opportunistic Gram-negative pathogen *Pseudomonas aeruginosa*, signaling pathways that regulate virulence and biofilm lifestyles have been extensively studied. RetS is a hybrid sensor kinase/response regulator that regulates the switch between virulence and biofilms (Goodman et al., 2004). Activation of *retS* during acute infections (in response to as yet uncharacterized environmental signals), leads to the inhibition of downstream biofilm-favoring GacS/GacA/*rsmZ* signaling pathways. Since a typical DNA-binding domain has not been identified in RetS, it is not clear how RetS mediates the activation of virulence genes. Recent studies suggest that calcium may play a discriminating role. Calcium activates the periplasmic domain of LadS, a hybrid sensor kinase harboring both histidine kinase and response regulator domains, and LadS∼P relays through GacS/GacA to activate the biofilm pathway (Broder et al., 2016). A similar periplasmic domain is also present in RetS, raising the possibility that calcium might inhibit the kinase activity of RetS while selectively stimulating LadS∼P formation.

An interesting aspect of biofilm formation in *P. aeruginosa* is the involvement of chemosensory-type signaling by the Wsp system. Wsp signaling involves a membrane-bound methyl-accepting protein (WspA), a methyltransferase (WspC), and a methyl-esterase (WspF) which regulate phosphorylation of the response regulator, WspR, to catalyze the synthesis of cyclic-di-guanosine monophosphate (c-di-GMP) (Hickman et al., 2005). However, it is not known whether there is any cross-regulation of the Wsp system with the homologous Che proteins. It is possible that chemical stimuli might activate the sessile lifestyle in bacteria through the Che signaling system, as has been observed in the regulation of *Comamonas testosteroni* biofilms by the FlmD-CheA axis (Huang et al., 2019).

WHAT REGULATES THE SWITCH IN LIFESTYLES IN *Vibrio cholera*?

The ability of *V. cholerae* to enter into a non-culturable state is a major factor for environmental persistence and forms the basis of periodic cholera epidemics in endemic regions. Interestingly, aggregates of these non-culturable coccoid cells have been isolated from aquatic environments and stool samples of infected patients as matrix-encased biofilms (Alam et al., 2007). Using the rabbit ileal loop model, *Vibrio* aggregates were discovered to be hyper-virulent (Faruque et al., 2006). Although the components of *V. cholerae* biofilms are well-characterized, the signaling mechanisms

that trigger biofilm formation remain unknown (reviewed in Teschler et al., 2015; Silva and Benitez, 2016). Moreover, what regulates the switch to a non-culturable state and how does temperature and salinity activate the formation of dormant cells? Detailed investigations of the TCRSs and cyclic-di-guanosine monophosphate (c-di-GMP) signaling pathways regulating *Vibrio* lifestyles in the host and outside environments will be informative.

CONCLUDING REMARKS

A critical step for the effective targeting of bacterial pathogens is to unravel the regulatory mechanisms that govern their transitions from a free-living non-pathogenic state to a virulent state to cause disease. This is especially relevant in the present day due to rising antibiotic resistance in bacteria, frequent nosocomial infections, and a lack of novel antibiotics. In the future, the signaling mechanisms that drive the development of biofilms or multicellular communities need to be determined in actual hosts, in order to devise strategies for controlling the spread of pathogenic bacteria and eradicating chronic persistence.

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SD and LK wrote the review.

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