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Regulation of *Listeria monocytogenes* Virulence

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ABSTRACT Whereas obligate human and animal bacterial pathogens may be able to depend upon the warmth and relative stability of their chosen replication niche, environmental bacteria such as *Listeria monocytogenes* that harbor the ability to replicate both within animal cells and in the outside environment must maintain the capability to manage life under a variety of disparate conditions. Bacterial life in the outside environment requires adaptation to wide ranges of temperature, available nutrients, and physical stresses such as changes in pH and osmolarity as well as desiccation. Following ingestion by a susceptible animal host, the bacterium must adapt to similar changes during transit through the gastrointestinal tract and overcome a variety of barriers associated with host innate immune responses. Rapid alteration of patterns of gene expression and protein synthesis represent one strategy for quickly adapting to a dynamic host landscape. Here, we provide an overview of the impressive variety of strategies employed by the soil-dwelling, foodborne, mammalian pathogen *L. monocytogenes* to straddle diverse environments and optimize bacterial fitness both inside and outside host cells.

A VARIETY OF MULTIFUNCTIONAL GENE PRODUCTS CONTRIBUTE TO *LISTERIA MONOCYTOGENES* ENTRY AND SURVIVAL WITHIN MAMMALIAN CELLS

The move from soil to cytosol requires the interplay of *L. monocytogenes* factors that promote survival in the gut, bacterial invasion, phagosomal escape, replication and movement within the cytosol, and spread to adjacent cells (Fig. 1). Bacterial gene products contributing to many key aspects of host infection continue to be identified, and new factors and novel functions often emerge (1, 2). Among the cast of well-known players are surface proteins that promote bacterial attachment to and invasion of nonprofessional phagocytic cells, such as the internalins InlA and InlB as well as Lap and InlP (which appears to be specific for placental invasion) (3–5). Following cell entry, *L. monocytogenes* escapes from host cell vacuoles via the secretion of the pore-forming cytolysin listeriolysin O (LLO) and two phospholipases, a phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by *plcA* and a broad-range phospholipase C (PC-PLC) encoded by *plcB* (6). Entry into the cytosol requires metabolic adaptation as *L. monocytogenes* shifts from glycolysis to the oxidative pentose phosphate pathway and replicates by scavenging phosphorylated sugars, glycerol, lipoic acid, branched-chain amino acids, and peptides from conquered host cells (7). Bacteria spread to neighboring cells by usurping actin polymerization as a motile force, a process dependent upon expression of the bacterial surface protein ActA (8). The breaking and entering of *L. monocytogenes* into adjacent cells is further

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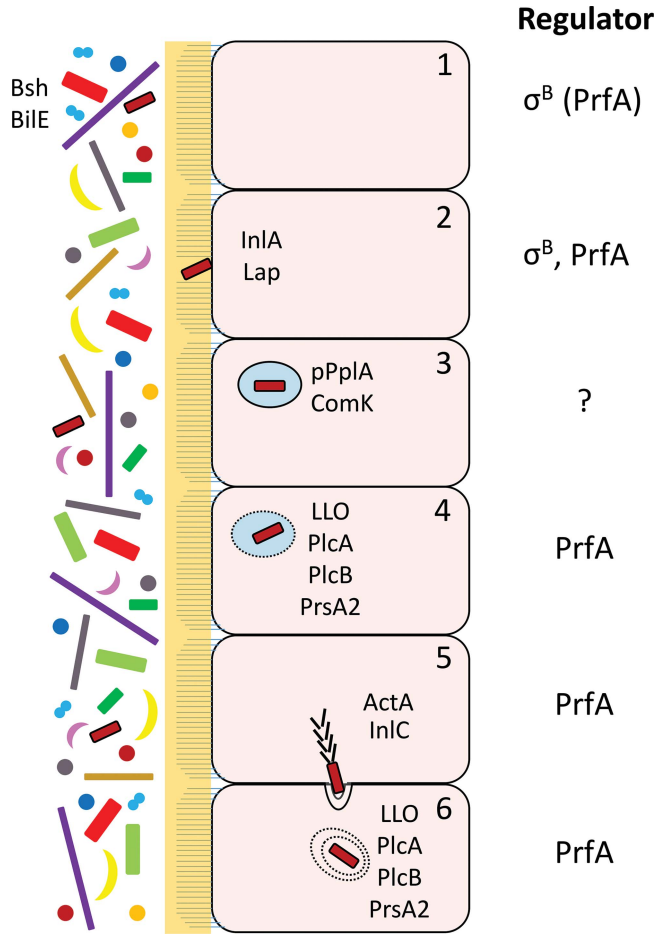


FIGURE 1 Schematic overview of *L. monocytogenes* colonization of the intestine by pathways controlled by σ^B and/or PrfA. The human microbiota is depicted to the left, lying beside the microvilli-containing epithelial cells (1). Survival of *L. monocytogenes* in the gut is mediated by different factors, among them Bsh and BilE (2). The bacterium adheres to cells using different adhesins (e.g., InlA and Lap) (3). Once inside the phagosome, the pPplA and ComK systems become activated, facilitating phagosomal escape (4). Different PrfA-regulated factors mediate lysis of the phagosome (5). Through recruitment of the Arp2/3 complex, ActA allows polymerization of actin at the pole of the bacterium, driving it through the cytoplasm. The entry of *L. monocytogenes* to adjacent cells requires InlC, which relieves cortical tension (6). The escape from the double membrane vacuole requires the action of LLO, PlcA, and PlcB. Regulators (σ^B or PrfA) involved in the different regulatory steps during intestinal passage of *L. monocytogenes* are shown at the right.

facilitated by InlC, which relieves cortical tension to allow the extension of membrane protrusions (9). Escape from the double membrane vacuoles formed as a result of *L. monocytogenes* cell-to-cell spread is once again dependent upon the activities of LLO, PC-PLC, and PI-PLC. Protein secretion of a variety of factors thus promotes bacterial survival and life within host cells, and central to the secretion of active protein products is the presence of the secretion chaperone PrsA2, located at the bacterial membrane-cell wall interface, where it contributes to the folding and activity of translocated polypeptide chains (10). *L. monocytogenes* is thereby able to maintain a complex and multifunctional protein arsenal to increase bacterial survival and replication within mammalian host cells.

To better understand bacterial adaptation to the host environment, a number of studies have focused on the identification of bacterial genes expressed within tissue culture cells, within blood, or within infected animals. Microarray analyses of bacterial transcripts induced during *L. monocytogenes* infection of tissue culture cells revealed that approximately 20% of bacterial genes were differentially expressed, including genes with products that have established roles in bacterial virulence (11). Genes with increased expression in cytosolic bacteria included those with roles in general stress responses, cell division, modification of the cell wall, and the use of carbon sources such as glycerol and phosphorylated sugars. Transcriptional profiling of *L. monocytogenes* genes expressed during *in vivo* growth in mouse spleens also indicated that approximately 20% of bacterial genes were differentially expressed (12). Similar to the findings reported for bacteria grown within tissue culture cells, genes induced *in vivo* included those with defined roles in virulence, stress responses, cell wall metabolism, DNA metabolism, RNA/protein synthesis, and cell division. In contrast to tissue culture-based expression studies, transcripts from genes encoding enzymes involved in glycolysis were induced *in vivo*, while those involved in the nonoxidative phase of the pentose phosphate pathway had decreased levels of expression. These contrasting results may reflect differences observed between growth conditions within tissue culture cells versus growth in whole organs and animal tissues.

PrfA, THE MASTER REGULATOR OF *L. MONOCYTOGENES* INTRACELLULAR SURVIVAL

While life is never simple, a transcriptional activator known as PrfA does appear to suffice as the master virulence regulatory protein that enables *L. monocytogenes* to optimize life within a mammalian host while still managing a saprophytic existence in soil. PrfA is a 27-kDa transcriptional activator that is a member of the Crp/Fnr family of transcriptional regulators, and it regulates its

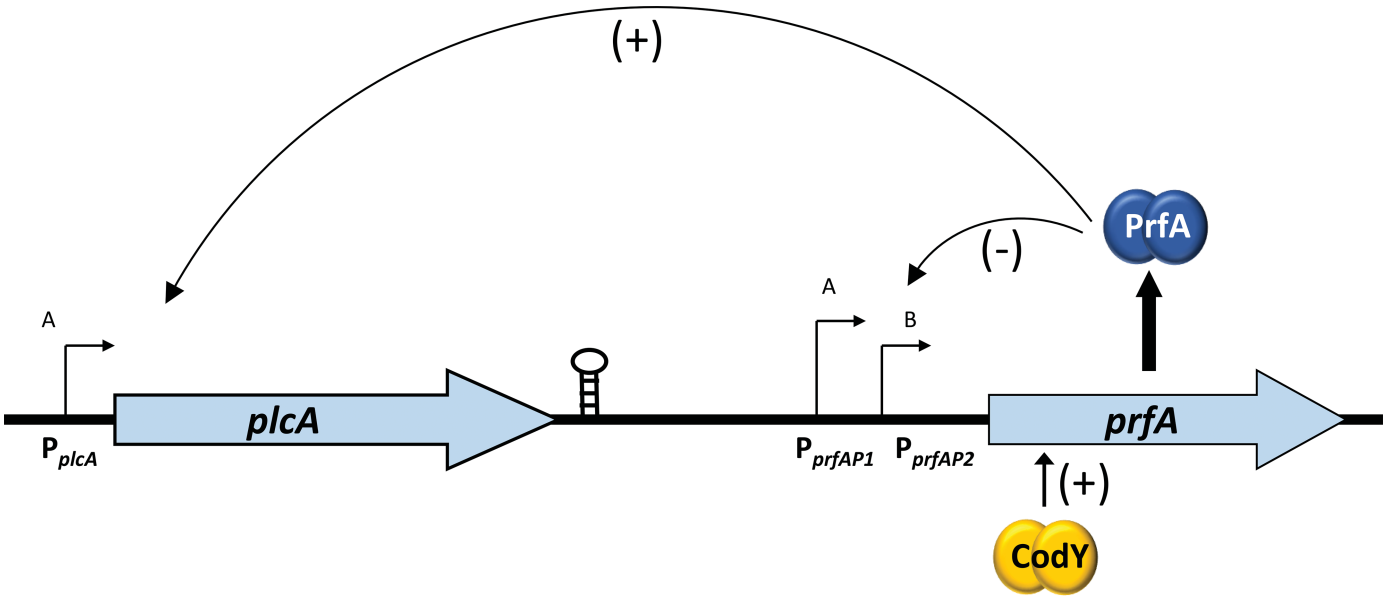
target genes via the binding of a 14-bp palindromic DNA binding site, also known as the PrfA box, located in the –40 region of its target promoters (13–17). PrfA regulates the expression of a large number of gene products directly associated with bacterial virulence in mammals, and mutants lacking *prfA* are severely impaired for intracellular growth and are >100,000-fold less virulent in murine infection models (15). In addition to gene products required for host cell invasion, intracellular replication, and cell-to-cell spread, PrfA induces the expression of gene products required for surviving the journey through the gastrointestinal tract, including a bile salt hydrolase (encoded by *bsh*) and a bile exclusion system (encoded by *bilE*) (18, 19). Full PrfA activation occurs following bacterial entry into the cytosol of host cells and results in the expression of gene products that enable intracellular replication and bacterial spread to adjacent cells. A subset of genes directly regulated by PrfA is located on an *L. monocytogenes* pathogenicity island referred to as LIPI-1 (*hly*, *plcA*, *prfA*, *mpl*, *actA*, and *plcB*), while others (*inlA*, *inlB*, *inlC*, *bsh*, *prsA2*, and *hpt*) have distinct chromosomal locations. Comparison of the profiles of wild-type *L. monocytogenes* grown in brain heart infusion broth with those of a strain harboring a constitutively active PrfA protein suggests that the expression of at least 145 genes may be modulated by PrfA (20).

Given that PrfA is critical for enabling *L. monocytogenes* to mediate the balance between life in soil and life inside an infected host, the activity of PrfA is itself carefully regulated by a variety of mechanisms that include transcriptional, posttranscriptional, and posttranslational control. These critical regulatory circuits work together to tightly control *prfA* expression and PrfA activity as outlined below.

Transcriptional Regulation of *prfA* Expression

Transcriptional regulation of *prfA* expression occurs via three promoter elements (Fig. 2). Two promoters, *prfA*P1 and *prfA*P2, are located immediately upstream of the *prfA* translation initiation codon, while the third promoter lies immediately upstream of *plcA* and results in the generation of a *plcA-prfA* bicistronic transcript (14, 21, 22). The *prfA*P1 and *prfA*P2 promoters direct the synthesis of monocistronic transcripts of *prfA* that generate the initial levels of PrfA protein required to activate expression of *hly* and *plcA*, whose gene products are needed for efficient escape of *L. monocytogenes* from host cell phagosomes (22, 23). The *plcA* promoter, which is activated by PrfA, directs the synthesis of the *plcA-prfA* transcripts, resulting in the high levels of PrfA synthesis that are required to induce *actA* expression for efficient bacterial cell-to-cell spread. The *prfA*P1 promoter con-

FIGURE 2 Transcriptional regulation of *prfA* expression. The *prfA* transcript is expressed from three different promoters, P1 (σ^A regulated), P2 (σ^A and σ^B regulated), and *plcA* (generating a *plcA-prfA* bicistronic messenger). The *plcA* promoter is positively regulated by active PrfA, creating a positive feedback loop of PrfA expression. CodY positively regulates expression of *prfA* by binding within its coding region. The stem-loop structure shows a putative transcriptional terminator located 3′ of the *plcA* gene.



tains characteristics of a σ^A-dependent promoter, which is the vegetative sigma factor determining RNA polymerase specificity required for transcription in actively growing, unstressed bacterial cells. The *prfA*P2 promoter region contains sequences that resemble a PrfA binding box, a σ^A-dependent promoter, and the general stress response sigma factor σ^B-dependent promoters (23–26). σ^B directs RNA polymerase to the promoter regions of a large number of genes involved in adaptation to general environmental stresses, such as conditions of low pH, high osmolarity, oxidative stress, and carbon starvation (see below). A number of genes coregulated by PrfA and σ^B have been shown to contribute to pathogenesis of *L. monocytogenes* (e.g., *bsh* and *inlAB*), suggesting a cross-talk network between these two regulators and possibly other stress response regulators and alternative sigma factors (18, 27, 28). Interestingly, work by Guldimann et al. (29) suggests that activation of PrfA and σ^B occurs stochastically within individual bacterial cells under defined stress conditions, with evidence that PrfA activation appears population-wide, while σ^B activation is restricted to subpopulations.

In addition to stress-responsive promoter elements, Lobel et al. have recently identified CodY to activate transcription of the *prfA* gene (30). CodY is a regulatory protein that links *prfA* expression with the metabolic status of the bacterium. Intriguingly, CodY binds to a region located 15 bases downstream of the *prfA* start codon (30). CodY responds to the presence of branched chain amino acids and GTP, and *L. monocytogenes* appears to regulate CodY activity by controlling the internal isoleucine pool, a mechanism that has been postulated to have evolved to enable isoleucine to serve as a host signal and virulence effector (31, 32). *L. monocytogenes* thus appears to tie in multiple environmental signals that include both stress and metabolism in its quest to regulate virulence gene expression within host cells.

Posttranscriptional Regulation

It has long been recognized that virulence factors as well as PrfA are mainly expressed at body temperature, 37°C, although the mRNA encoding the PrfA protein is present also at low temperatures (below 30°C) (21, 33). Computer prediction and chemical probing experiments showed that a 115-nucleotide (nt)-long 5′ untranslated RNA (UTR) present in front of the *prfA* encoding mRNA could form a long hairpin where the Shine-Dalgarno (SD) site was partially masked by an anti-SD site (34) (Fig. 3). *In vitro* translation of the wild-type *prfA* showed a thermoregulated expression, with PrfA mostly expressed at higher temperatures. Mutational analysis suggested that the actual thermosensing occurred in the middle part of the hairpin since mutations that destabilized the *prfA*-UTR structure abolished much of the thermoregulation (34). These data suggested a simple mechanism of thermosensing: at low temperatures, the SD site is closed due to an interaction with an anti-SD site, and binding of the ribosome is prevented. With increasing temperature, the region surrounding the *prfA*-SD site is destabilized, allowing binding of the ribosome and initiation of translation (34–36) (Fig. 3). However, a more complex mechanism of *prfA* translation initiation has been put forward, involving a ribosomal stand-by site inside the *prfA* coding RNA (37).

In addition, *prfA* translation is controlled by at least two prematurely terminated *S*-adenosyl methionine (SAM) responsive riboswitches (38). Riboswitches are RNA elements that are able to control downstream gene expression by directly binding specific metabolites with a high affinity. The metabolite-riboswitch interaction results in RNA restructuring, usually terminating transcription in Gram-positive bacteria (39, 40). When binding SAM, transcription is terminated and short (150 to 250 nt long) transcripts are generated. Two of the prematurely terminated SAM riboswitches in *L. monocytogenes* (SreA and SreB) can base-pair with the 5′ UTR of *prfA* and thus reduce PrfA expression by an unknown mechanism (38) (Fig. 3). Here, the function of the terminated riboswitches resembles the role played by several *trans*-acting small RNAs (41). Interestingly, SreA is unable to inhibit translation at low temperatures, possibly because its interaction site in the *prfA* thermosensor is in a closed conformation, preventing base-pairing with *trans*-acting RNA elements. Expression of PrfA can therefore be affected by SAM riboswitches only at temperatures encountered during infection. The entire SreA is not required for repression; instead, the central core part of the SreA is sufficient for efficient blocking of PrfA translation (S. Krajewski and J. Johansson, unpublished results). Intriguingly, SreA expression is positively controlled by PrfA, creating a negative feedback loop, where high levels of active PrfA turn off its own expression by increasing the level of the repressive element SreA (38). It therefore seems that the 5′ UTR of the *prfA* transcript can act as a sensor, integrating both the environmental temperature and the metabolic state of the bacterium through SAM sensing.

Posttranslational Regulation

The third critical mechanism for regulating PrfA activity occurs via posttranslational modification (Fig. 4). As stated above, PrfA protein is a member of the cyclic

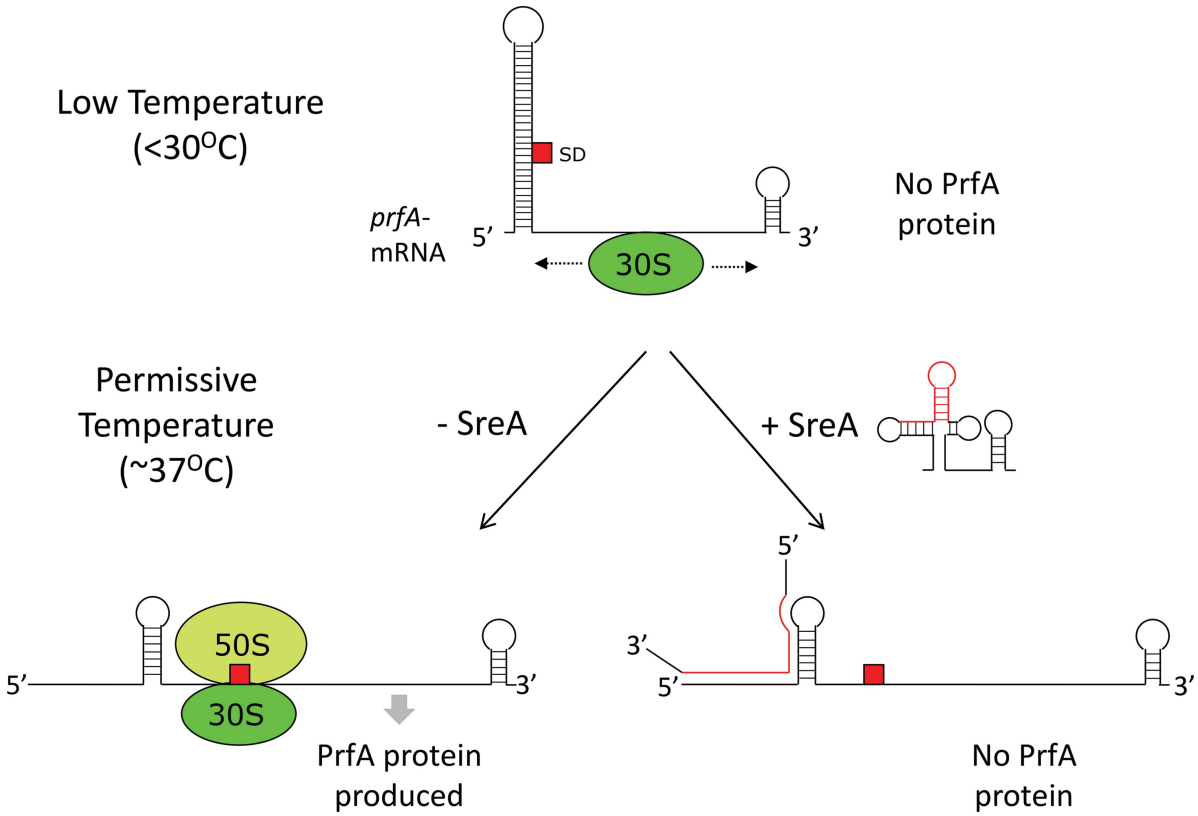


FIGURE 3 Posttranscriptional control of PrfA expression. At low temperatures (<30°C), the Shine-Dalgarno site (SD, red square) is sequestered in an RNA thermosensor, preventing binding of the 30S subunit (green sphere) of the ribosome. Possibly, the binding of the 30S subunit involves a ribosomal standby site, where it primarily binds to the coding region of the *prfA* mRNA, where it scans the transcript. At permissive temperatures (~37°C), the RNA thermosensor dissociates, allowing access of the 30S subunit to the SD, where it can recruit the 50S subunit (light green sphere), and translation can initiate. Under conditions in which a terminated *S*-adenosylmethionine riboswitch is produced (+SreA), it can bind to the RNA thermosensor and by an unknown mechanism prevent PrfA production.

AMP receptor protein (Crp)-Fnr family of transcriptional regulators, of which there are approximately 400 members (42). Proteins in this family generally function as dimers and require the binding of small molecule cofactors (for example, cyclic AMP for Crp) or other forms of posttranslational modification (such as the binding of carbon monoxide by the heme moiety of CooA) for full activity. The PrfA cofactor has recently been identified as glutathione, and PrfA-glutathione cocrystals indicate that the cofactor binding site is located at the intraprotein tunnel site, located between the N- and C-terminal domains of the PrfA monomer (43–46). Working models have suggested that once inside the cytoplasm, both uptake of cytosolic glutathione and, most importantly, upregulation of the glutathione synthase, *gshF*, lead to increased glutathione concentrations in *L. monocytogenes* (43, 45). This is required since glutathione only binds PrfA with a modest affinity (0.5 mM), so the increased glutathione concentration thus signals to the bacterium its arrival into the cytosol. Recently, Krypotou et al. (47) proposed that Cys-containing peptides transported into *L. monocytogenes* via the Opp peptide transport systems provide for the generation of glutathione leading to PrfA activation. Interestingly, the authors further suggested that inhibitory peptides transported by this same system bind to PrfA and inhibit PrfA activation by inhibiting the binding of glutathione. The complexity of PrfA activation continues to increase.

Years prior to the identification of glutathione as the PrfA cofactor, Ripio et al. identified an *L. monocytogenes* strain containing a single mutation within *prfA* coding sequences that resulted in the constitutive expression of

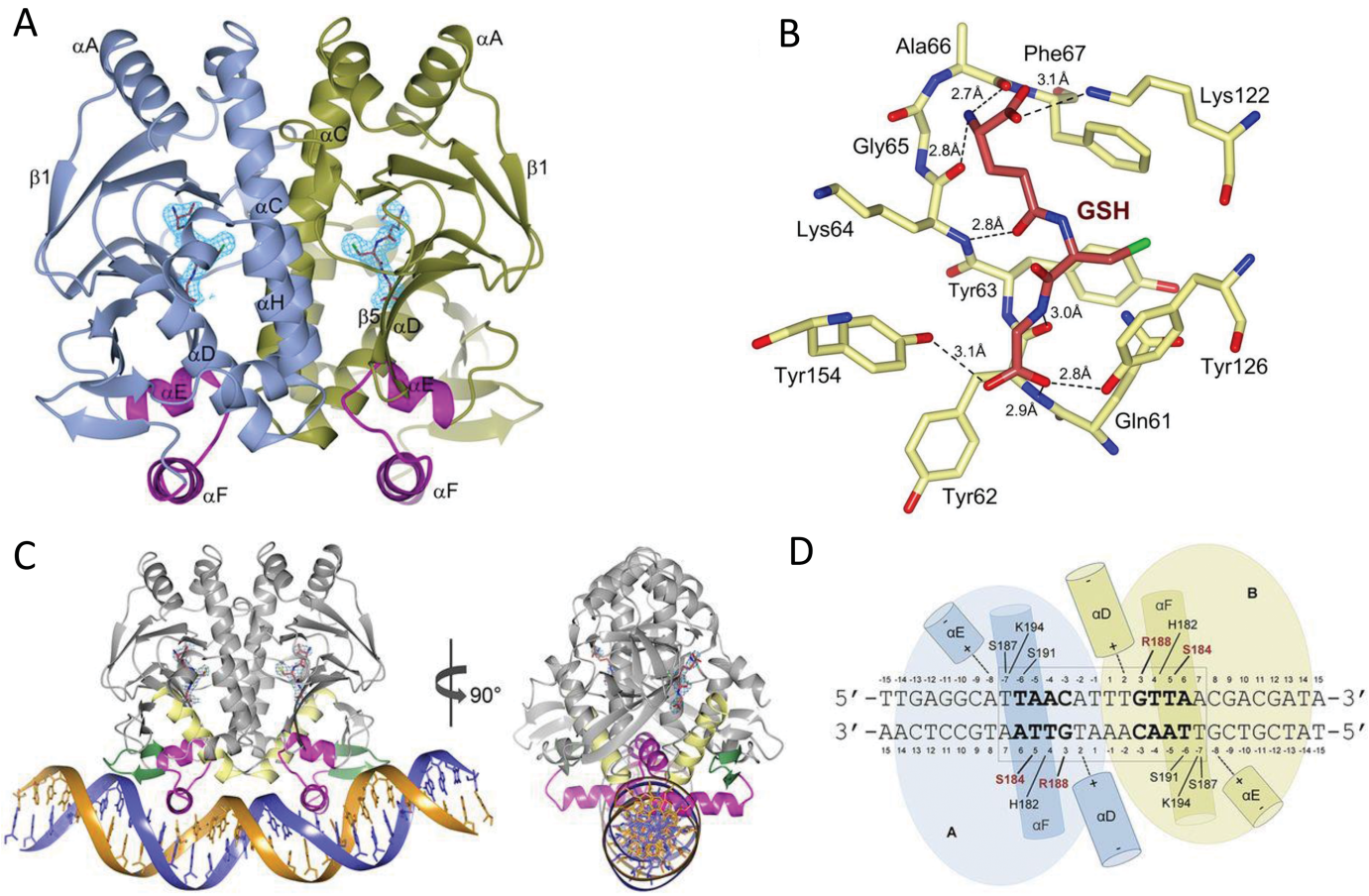


FIGURE 4 Structures of PrfA in complex with glutathione (GSH) and PrfA in complex with GSH and DNA. **(A)** Ribbon representation of the PrfA homodimer with GSH bound at the tunnel site of each monomer. The monomers A and B are colored blue and green, respectively. The helix-turn-helix motif is highlighted in magenta, and GSH has crimson carbon atoms with light blue electron density. **(B)** Local amino acid interactions and structural features of GSH-PrfA binding. Direct hydrogen bonds between amino acids and GSH are shown by dashed lines. **(C)** Ribbon representation of the DNA-bound homodimer in complex with GSH. The monomers A and B are colored gray, the helix-turn-helix motif is magenta, αD is yellow, and the winged β-hairpin is green. GSH is shown as sticks with the carbon atoms in crimson. The 30-bp palindromic operator is shown in dark blue and orange. **(D)** Schematic drawing of the PrfAWT-DNA interactions. Amino acids S184 and R188 make base-specific contacts and are highlighted in red. Other interactions are nonspecific between protein side chains and the DNA phosphate backbone. Figure adapted with permission from Hall et al. (44).

PrfA-dependent virulence genes in broth culture (48). The substitution of a serine for a glycine at position 145 within PrfA was the first identification of a PrfA* mutation, so named because it appeared analogous to an A144T mutation identified within Crp that resulted in the constitutive expression of Crp-dependent gene products in the absence of cofactor (Crp* mutants) (49). Similar to Crp*, the PrfA G145S mutation was demonstrated by structural analyses to alter PrfA protein confirmation and increase the DNA binding affinity of PrfA for its target promoters via the repositioning of a helix-turn-helix DNA binding motif (50, 51).

There have now been a number of additional mutations identified that confer PrfA activation, albeit at differing levels of activation. Reported *prfA** mutations include G145S, Y63C, S71C, E77K, A94T, L140F, Y154C, L148P, G155S, and P219S substitution mutants (52–61). These mutations in some cases map to very different regions of PrfA in comparison to the original G145S PrfA* mutation, and strains containing these dif-

ferent *prfA** alleles exhibit levels of PrfA-dependent gene expression in broth culture that range from 4-fold to >200-fold greater than the levels of expression observed in wild-type bacteria (62, 63). With the exception of PrfA G145S, for which the structure has been solved using X-ray crystallography (51), the mechanisms by which the other *prfA** mutations confer constitutive activation are not clear.

*PRFA** MUTATIONS REVEAL THE DIVERSITY OF GENE PRODUCTS REGULATED BY PrfA ACTIVATION

PrfA* mutants may be considered to phenotypically resemble *L. monocytogenes* under conditions of cytosolic activation (bound to glutathione), and thus the mutant strains have proven useful for the identification of novel virulence factors following transcriptomic and proteomic analyses. Milohanic et al. initially identified substantial overlap between genes whose expression was influenced by PrfA and stress-responsive genes regulated by the stress-responsive alternative sigma factor σ^B (20). However, studies by Ollinger et al. (64) using reverse transcriptase PCR reported that the transcript levels of some of PrfA-associated genes identified by Milohanic et al. (20) were not significantly affected by the presence or absence of PrfA. Discrepancies between these independent studies may reflect disparities between laboratory conditions, variations between strains used for examination (EGDe versus 10403S), or additional undefined complexities associated with PrfA-dependent gene expression.

Secreted proteins are often the first bacterial factors to interact with the host, and a comparison of secreted protein profiles derived from the culture supernatants of wild-type, Δ*prfA*, and *prfA** mutants identified at least 17 proteins that were differentially secreted following PrfA activation (52). The majority of the genes encoding these proteins did not contain recognizable PrfA binding sites in their upstream promoter regions, suggesting that the synthesis and/or secretion of these proteins was indirectly influenced by PrfA activation. Proteins with increased abundance in the supernatants derived from *prfA** cultures included a number of previously identified virulence factors as well as putative ABC transporters, cell wall-modifying enzymes, chitinases, antigenic lipoproteins, and chaperone proteins associated with protein secretion. Many of these PrfA-dependent secreted proteins also depended on the presence of the secretion chaperone PrsA2 for full activity (65). A significant number of secreted gene products that appear indirectly regulated by PrfA have been demonstrated to contribute to *L. monocytogenes* pathogenesis, and these gene products serve as further examples of the expansive influence of PrfA on *L. monocytogenes* life within the host.

THE IMPACT OF CARBON SOURCES ON PrfA ACTIVATION AND THE STIMULATION OF PrfA-DEPENDENT VIRULENCE GENE EXPRESSION

The expression of virulence factors has long been known to respond to the presence of sugars in the medium (66–71). Sugars (e.g., cellobiose and glucose) taken up by the phosphoenolpyruvate):phosphotransferase system (PTS) repress PrfA activity, whereas non-PTS sugars (e.g., glycerol) modestly stimulate PrfA activity. Importantly, the PTS-mediated repression of PrfA activity is not mediated by the catabolite control protein A (CcpA), which controls expression of many genes in different *Firmicutes* in response to PTS sugars (72, 73). Instead, the phosphorylation state of the different PTS permeases appears to dictate the activation level of PrfA (74). In the presence of PTS sugars, the permeases are in an unphosphorylated state as they donate the phosphate group they received from the phosphoenolpyruvate system to the incoming sugar. In the absence of PTS sugars (and the presence of non-PTS sugars), the permeases are phosphorylated. It has been suggested that unphosphorylated permeases might bind and sequester PrfA directly, thereby blocking PrfA-mediated virulence gene expression (74). Glucose can be transported into *Listeria* by several different permeases. One of the mannose permeases, EIIABMan, was suggested to act as a PrfA-binding partner or, alternatively, to interact with a PrfA-activating molecule (75). In addition, the repressive effect of PTS sugars can be relieved by the addition of charcoal or amberlite to the medium during bacterial growth (76, 77). This suggests that a component released by *L. monocytogenes* could repress PrfA activity, either directly or indirectly. Precisely how the action of PTS-permeases or repressive extracellular molecules such as inhibitory peptides affect the level of glutathione (the PrfA cofactor) or PrfA binding of glutathione remains to be determined.

MAINTAINING THE BALANCE BETWEEN LIFE INSIDE AND OUTSIDE THE HOST: CONSTITUTIVE ACTIVATION OF PrfA IS NOT BENEFICIAL FOR *L. MONOCYTOGENES* SURVIVAL

At first glance, *prfA** strains would appear to exhibit a number of advantages over wild-type bacteria. Strains

containing *prfA** are hyperinvasive, mediate more efficient phagosome escape, and initiate bacterial actin-based motility more rapidly. Activation of PrfA also shifts *L. monocytogenes* metabolism toward the preferred use of three carbon sugars and phosphorylated sugars, the principal carbon sources used by *L. monocytogenes* for growth within the cytosol (78). *prfA** mutants are hypervirulent in mouse infection models and exhibit a competitive fitness advantage over wild-type strains during both oral and intravenous mixed infections in mice (78).

The fitness advantage observed for *prfA** strains within the host does not, however, translate into bacterial fitness in the outside environment (78, 79). Constitutively activated *prfA** mutants exhibit impaired flagellum-mediated swimming motility, a defect that would be expected to compromise bacterial fitness in environments where the bacteria must be able to detect and swim toward available nutrient sources and which would additionally impair biofilm formation. *prfA** mutants also exhibit a pronounced fitness defect when grown in the presence of wild-type bacteria in mixed broth culture despite displaying no obvious growth defects in monoculture. Stress conditions such as high osmolarity or low pH exacerbate the competitive defects observed for *prfA** strains in a manner that is independent of the stress-responsive sigma factor σ^B. Lastly, *prfA** mutations appear to negatively impact the ability of *L. monocytogenes* to survive long periods of starvation. Interestingly, a PrfA* strain devoid of most PrfA-regulated genes did not display a fitness cost (80). The multiple mechanisms that thus exist to regulate PrfA activity appear to have evolved to carefully balance gene expression patterns so as to maintain bacterial fitness in the soil as well as in the cytosol.

BEYOND PrfA: OTHER MODES OF *L. MONOCYTOGENES* REGULATION OF GENE EXPRESSION

PrfA is a central factor in the regulation of *L. monocytogenes* virulence gene expression; however, numerous other regulatory factors and mechanisms exist that enable the bacterium to adapt its physiology in response to the diversity of environmental conditions encountered by this ubiquitous organism. A sample of some of these alternative regulatory modalities is presented below.

Posttranscriptional Regulation of Bacterial Gene Expression

Untranslated regions of mRNA (UTRs) have been demonstrated to regulate translation of the associated gene products for multiple genes. The 5′ UTRs located upstream of *inlA*, *actA*, and *hly* have been shown to control expression of their protein products (27, 81, 82). These 5′ UTRs appear, however, to support expression of InlA, ActA, and LLO, respectively, which is in contrast to the previously described *prfA*-UTR, which represses PrfA expression. It has been shown that partial deletions of the 5′ UTRs of *inlA*, *actA*, and *hly* decrease the protein production, but the exact mechanism(s) by which the 5′ UTRs act has not yet been revealed. Interestingly, it was shown that the coding region of *hly* could be important for LLO production (83). Whether this is due to alterations of secondary structures, binding of small regulatory RNAs (sRNAs), or some other factors remains to be investigated.

Regulation by sRNAs

Several hundred sRNAs have been identified in *L. monocytogenes*, ranging in size from less than 100 to more than 500 nt and with different expression patterns (84–88). However, the functions of only a subset of these sRNAs have been revealed, and an even smaller number have been shown to participate in virulence gene expression. Rli27 is an intracellularly induced sRNA able to interact with the 5′ UTR of *lmo0514*, encoding an LPXTG cell wall protein that is also upregulated during intracellular growth (89). Lmo0514 is required for survival of the bacteria in plasma and during infection of mice (90). The 5′ UTR of *lmo0514* contains an inhibitory RNA structure that sequesters the SD region and blocks translation initiation. Binding of Rli27 to the 5′ UTR disrupts the inhibitory structure, thereby allowing intracellular expression of Lmo0514 (89).

Rli38 is a long (514 nt) sRNA that is highly induced when *L. monocytogenes* is exposed to blood (86). A Δ*rli38* mutant strain exhibited reduced bacterial loads in several organs compared to the wild-type strain. The target of Rli38 (RNA or protein) is not yet known, nor is its mechanism of action. Another sRNA induced when *L. monocytogenes* is exposed to blood is RliB (86). In contrast to Rli38, the absence of RliB increased bacterial loads in the liver compared to the wild-type strain. RliB is a substrate for polynucleotide phosphorylase, but it is also a clustered regularly interspaced short palindromic repeat (CRISPR) element, devoid of associated Cas proteins (91). However, presence of *trans*-encoded Cas proteins and polynucleotide phosphorylase allows RliB to exert DNA interference directed against matching protospacers. Why *Listeria* has a degenerated CRISPR system is not clear and deserves further study.

Possibly the most well-known sRNAs in *L. monocytogenes* are the sibling family of LhrC, consisting of seven members, all with varying degrees of conservation (86, 92–95). The LhrCs control expression of at least three virulence factors: *lapB*, encoding an adhesin, *oppA*, encoding an oligopeptide protein, and *tcsA*, encoding a CD4+ T cell-stimulating antigen. The different members of the LhrC family are able to bind to the 5′ UTRs of their targets. For *lapB* and *oppA*, the LhrCs directly interact with their SD region to inhibit translation, whereas the LhrCs destabilizes the *tcsA* transcript by binding a site distally from the SD region (93, 95, 96). Interestingly, two CU-rich binding sites in LhrC4 were identified to be important for *oppA* binding, giving the possibility of two target mRNAs being sequestered by one sRNA. Another exciting aspect of the LhrC family is that the expression of individual members is controlled by exposure to various environmental cues, such as blood and growth in the intestine and in macrophages through at least one bacterial two-component signaling system (93).

The functions of several sRNAs in bacteria are dependent on RNA chaperones. In Gram-negative bacteria, Hfq plays a vital role, stimulating interaction between short complementary sequences of the sRNA and the target mRNA (97). The role of Hfq appears to be more crucial if the level of complementarity between the RNAs is low. Although the phenotypes of a Δ*hfq* strain are less pronounced in *L. monocytogenes* than a Δ*hfq* strain in Gram-negative bacteria, Hfq has been shown to be associated with sRNAs and to contribute to *L. monocytogenes* virulence (84, 98). However, the interaction between the sRNA LhrA and the target mRNA *lmo0850* is the only example so far described in *L. monocytogenes* when Hfq is needed for a functional regulatory outcome (99). Other RNA chaperones could in some cases replace Hfq.

The SpoVG protein of *L. monocytogenes* is an RNA binding protein that regulates lysozyme resistance, virulence, and swarming motility (100). The sRNA Rli31 was shown to interact with both the 5′ UTR of the *spoVG* transcript and the SpoVG protein (100). Surprisingly, Rli31 did not appear to affect the levels of either the *spoVG* transcript or the SpoVG protein, raising the possibility that Rli31 acts as a "sink" to inactivate SpoVG, in analogy with the CsrA/Rsm systems of post-transcriptional regulators in Gram-negative bacteria. Finally, RNA helicases have been shown to contribute to virulence gene expression in *L. monocytogenes* (101, 102). sRNAs are becoming increasingly recognized for their contributions to diverse aspects of *L. monocytogenes* physiology and virulence.

Regulation Induced in Response to Environmental Stress

During its life as a saprophyte and pathogen, *L. monocytogenes* encounters a diversity of stress-conditions, many of them potentially life-threatening. To survive disparate stresses, the bacterium makes use of a number of strategies. As mentioned previously, the expression of several proteins important for stress survival depends on the stress sigma factor σ^B, which competes with the vegetative sigma factor σ^A for binding to the RNA polymerase (103, 104). Activity of σ^B is controlled by a complex relay of protein interactions and phosphorylation events, ultimately freeing σ^B from the anti-sigma factor RsbW. At the top of this relay hierarchy lies a sensory organelle, the "stressosome," which is a large (~1.8 MDa) multiprotein complex, thus far primarily studied in *Bacillus subtilis* (105). The stressosome is able to detect and integrate environmental cues into a signal transduction pathway that eventually allows the liberation of σ^B and initiation of the expression of σ^B-regulated genes.

In *L. monocytogenes*, it has been suggested that there may be interplay between σ^B and PrfA activity, such that σ^B controls genes important for the initial steps of infection (i.e., during survival in the gut and adhesion to intestinal epithelial cells), whereas PrfA controls the expression of genes involved in subsequent steps (i.e., adhesion and intracellular growth as well as further dissemination in the host) (25, 26, 86, 106, 107). Interestingly, some genes, such as the genes encoding the two important invasins, InlA and InlB, are controlled by both σ^B and PrfA (27, 108). This dual regulation allows the expression of the bicistronic *inlA-inlB* message from two promoters, integrating several external cues and thereby permitting bacterial invasion of several host cell types. Both the σ^B- and PrfA-generated *inlA-inlB* transcripts contain unusually long 5′ UTRs (445 and 396 nt, respectively) possibly acting at the posttranscriptional level to sense environmental factors (27). Whether the stressosome is able to act as a central hub to integrate external signals to control virulence gene expression in concert with other regulators (e.g., PrfA) remains to be determined. Very little is known about the mechanisms underlying stressosome sensory perception and signal integration. It has been shown that a blue-light receptor can sense light and activate σ^B through the stressosome, but the exact mechanism is not known (109–111). A small peptide, Prli42, has been demonstrated to bind the stressosome, and its absence reduces σ^B-dependent gene expression while also affecting expression of virulence factors (112). Prli42 harbors a transmembrane domain

and is associated with the membrane, potentially allowing the stressosome to sense stress exerted through membrane disturbance.

Fatty Acids and Regulation of Virulence

Many enterobacteria use fatty acids (FAs) to regulate virulence gene expression. For example, *Vibrio cholerae*, the causative agent of cholera, controls the expression of cholera toxin and toxin coregulated pilus using the transcriptional activator ToxT; unsaturated long-chain FAs were shown to directly bind to and repress activity of ToxT (113). Using the X-ray structure of palmitoleic acid bound to ToxT as a blueprint, highly effective ToxT inhibitors were synthesized that were able to inhibit intestinal colonization in mice (114). FAs have also been shown to affect virulence factor expression in *L. monocytogenes* and have been suggested to interfere with PrfA activity (115, 116). The addition of increasing amounts of non-branched-chain FAs (both saturated and nonsaturated) to bacterial cultures was found to reduce virulence factor expression (116). A similar effect was observed by decreasing the bacterial content of branched-chain FAs (115), possibly because this increases the amount of non-branched-chain FAs. Interestingly, a strain carrying a constitutively active PrfA protein ($PrfA_{G155S}$) also exhibited reduced virulence factor expression in the presence of FA, suggesting that the repressive effect of FAs lies at a level subsequent to PrfA-activation (116). Additional work is required to decipher if FAs interact directly with PrfA or if the effect of the FAs is mediated through another pathway.

Regulation by Two-Component Sensing Systems

To sense and respond to environmental cues, many pathogenic bacteria use two-component systems (TCSs). Typically, the TCS consists of a histidine kinase (HK) that senses an external signal and transfers a phosphor group to activate a response regulator (RR) controlling the expression of genes important for the response of the particular stimulus (117). *L. monocytogenes* harbors 16 TCSs, one of which, DegU, is an orphan RR (118). Several of the *Listeria* TCSs have been implicated in virulence (119–122). One of them is part of the accessory gene regulator (Agr) system of *L. monocytogenes*, consisting of a quorum sensing/TCS system (119). In the Agr system, a small auto-inducing peptide (AIP, derivative of the *agrD* gene product) is processed and transported by AgrB to the outside of the bacterium. The AIP of *L. monocytogenes* was shown to be a cyclic pentapeptide (CFMFV) (123). Once outside, the AIP is recognized by the HK AgrC, which activates the RR AgrA. Exactly how the Agr system contributes to *L. monocytogenes* virulence is unclear, since the bacterium lacks RNAIII, an sRNA expressed divergently from the *agr* locus and which controls the expression of several virulence factors in *Staphylococcus aureus* (124). Another TCS implicated in *L. monocytogenes* virulence is LisrK (120). Loss of LisK (the HK) reduced bacterial growth in the spleens of mice. Interestingly, LisRK has been shown to be important for expression of the serine-protease HtrA and the sRNA LhrC, both of which contribute to *L. monocytogenes* pathogenesis (93, 125). The flagellin-associated TCSs CheY and CheA are important for listerial adhesion and invasion of cultured Caco-2 cells; however, bacteria lacking this system exhibited normal growth within the spleens of mice (121). The DegU RR appears to be of interest, because it lacks its associated HK, and the absence of DegU impairs *L. monocytogenes* growth in mice (126–128). DegU is also the epistatic regulator of motility by positively activating expression of the antirepressor GmaR, which in turn, inactivates the repressor of motility MogR (129). Having no accompanying kinase, DegU appears to be phosphorylated by intracellular acetyl phosphate (130). The VirRS TCS system was originally identified using a signature tagged mutagenesis screen seeking genes important for mouse infection (122). Surprisingly, VirR can be activated independently of the HK VirS, possibly through variations in bacterial intracellular acetyl phosphate concentrations. VirR controls the expression of several virulence-associated genes upregulated during infection (12). Recently, it was shown that the VirAB ABC transporter controls VirR function in response to the antimicrobial Nisin and/or host factors (131).

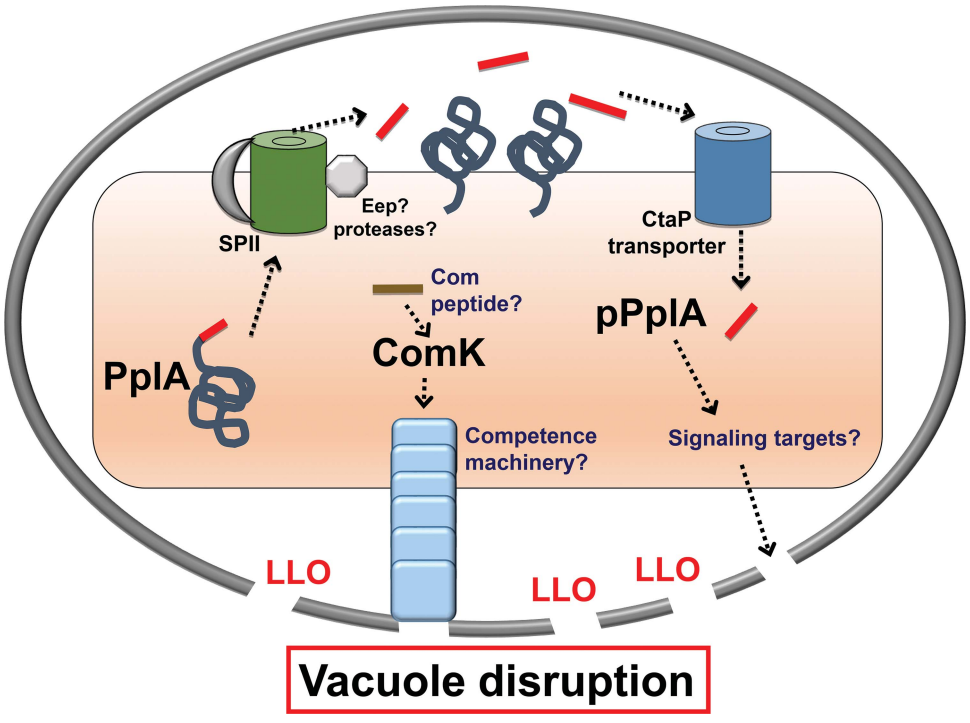
Regulation of Virulence via Peptide Pheromones

In addition to the peptide-responsive Agr system discussed above, an additional peptide pheromone system has been recently shown to contribute to *L. monocytogenes* virulence. A PrfA-inducible secreted peptide pheromone-encoding lipoprotein (PplA) was identified that shares significant homology with the *Enterococcus faecalis* Cad lipoprotein encoding the cAD1 peptide pheromone (132, 133). Similar to the cAD1 pheromone of *E. faecalis*, the pPplA peptide is processed from the released PplA lipoprotein N-terminal signal sequence (SS) peptide following secretion of the lipoprotein through the general secretory pathway. In contrast to *E. faecalis*, where the cAD1 pheromone stimulates a mating response between plasmid-containing and plasmid-free cells, the pPplA peptide pheromone has functionally evolved to enhance

vacuolar escape of *L. monocytogenes* within nonprofessional phagocytic cells. Studies of mutants lacking both the PplA lipoprotein and its signal sequence-encoded peptide pheromone versus the lipoprotein alone have demonstrated that the pPplA peptide pheromone is a critical virulence factor that contributes to both bacterial aggregation in broth culture and survival in mouse models of infection. The PplA lipoprotein has no apparent role in vacuolar escape; however, its function as an extracellular electron transport protein that contributes to bacterial fitness within the gut has recently been described (134).

L. monocytogenes mutants lacking the pPplA peptide exhibit delays in escape from the vacuoles of nonprofessional phagocytic cells but escape with normal kinetics from the phagosomes of macrophage-like cell lines or in bone marrow-derived macrophages (132). Interestingly, loss of the pPplA pheromone did not impair LLO-dependent perforation of the vacuole, only bacterial escape into the cytosol. These data strongly suggest that complete vacuole escape requires an additional mechanism beyond initial pore formation mediated by the activity of the hemolysin LLO. The pPplA peptide was also found to contribute to maintenance of surface-associated and secreted proteins, and it is possible that some of these gene products may contribute to either the stabilization of LLO-induced membrane pores and/or the physical disruption of the vacuole membrane (Fig. 5).

FIGURE 5 *L. monocytogenes* peptide signaling and expression of competence gene products via phage excision within the host vacuole. *pplA* encodes a lipoprotein (PplA) with a peptide pheromone (pPplA) located within the N terminal secretion signal peptide (shown in red). The signal sequence of prePplA is processed by signal peptidase II (SPII), and the released signal peptide is further cleaved by the protease Eep, releasing the pPplA pheromone, while the PplA protein becomes lipid modified and associated with the membrane. The confined space of the vacuole leads to import of the secreted pPplA pheromone, presumably stimulating a signaling cascade that results in the production of an unknown factor(s) that contributes to vacuole lysis. As part of a separate pathway, an unknown signal, potentially also involving a peptide pheromone, leads to the expression of ComK and the expression of a competence-associated pilus that may also aid in vacuole membrane disruption. Select *L. monocytogenes comK* genes harbor a lysogenic phage that must excise to enable ComK expression. The ComK pathway is required for *L. monocytogenes* vacuole escape from professional phagocytic cells; the pPplA system is required for bacterial escape from nonprofessional phagocytic cells.



Interestingly, all bacterial virulence defects associated with the loss of the pPplA peptide could be completely compensated for by the introduction of constitutively active PrfA*, suggesting a possible connection between the pPplA signaling pathway and PrfA activation (132). These studies suggest a model in which *L. monocytogenes* senses the confines of the host vacuoles as a result of the accumulation and import of the pPplA peptide, leading to PrfA activation and enhanced bacterial escape into the cytosol. The *L. monocytogenes* pPplA peptide thus appears to have evolved to enable the bacterium to sense the confined environment of the vacuole so as to induce the expression of gene products required for full membrane disruption and entry of the bacterium into the cytosol.

In addition to the pPplA peptide-signaling pathway, another potential peptide-based signaling system related to competence development has been implicated in enhancing escape of *L. monocytogenes* from host cell vacuoles, specifically, the phagosomes of macrophages (135). Competence is a brief physiological state during which the bacterial cells collectively become primed to transport extracellular DNA across the cell wall and bacterial membrane, at times resulting in integration of the newly acquired DNA into the bacterial genome (136). Several gene products, including those encoded by *com* genes, are required for peptide pheromone signaling and DNA uptake. Although it has not been demonstrated to exhibit natural competence, *L. monocytogenes* possesses a number of *com* genes that share homology with those involved in competence development in other bacteria; however, not all the genes required for competence are present (118). Most but not all of the regulatory components and late genes involved in assembly of the Com apparatus required for DNA transport are present in the *Listeria* genome. Because of these missing components, it is unclear if the competence system is functional in *L. monocytogenes*, and natural competence has not been demonstrated under laboratory conditions.

One competence regulator that is encoded by *L. monocytogenes* is ComK, a master transcriptional regulator that induces the expression of late *com* genes involved in assembly of the Com apparatus for DNA uptake, and it is this system which has been reported to be important for *L. monocytogenes* in vacuole escape of professional phagocytic cells (135). Interestingly, the *comK* gene in some strains of *L. monocytogenes* is inactivated by the presence of an A118-like prophage that integrates at a specific attachment site within the *comK* gene. However, Rabinovich et al. have found that the late competence genes regulated by ComK are highly expressed during intracellular growth as a result of prophage excision (135). In addition, mutants missing the specific components of the pseudopilus or the DNA translocation channel were impaired for vacuole escape in macrophages and were attenuated for bacterial virulence in mice, whereas the DNA binding components were dispensable for these processes. A strain cured of the prophage and containing an intact *comK* gene grew similarly to wild-type *L. monocytogenes* in macrophages, and many naturally occurring strains of *L. monocytogenes* lack this prophage and are capable of growth within macrophages. For strains that do contain prophages, data suggest that prophage integration/excision serves to regulate virulence gene expression and that the ComK system is somehow involved in sensing the presence of *L. monocytogenes* within host cell vacuoles in professional phagocytic cells. Thus, while no peptide pheromone has yet been identified to stimulate the induction of the *L. monocytogenes com* genes within the phagosome, it could be hypothesized that the bacterium has adapted components of the *com* peptide pheromone system for spatial sensing, similar to pPplA (Fig. 5).

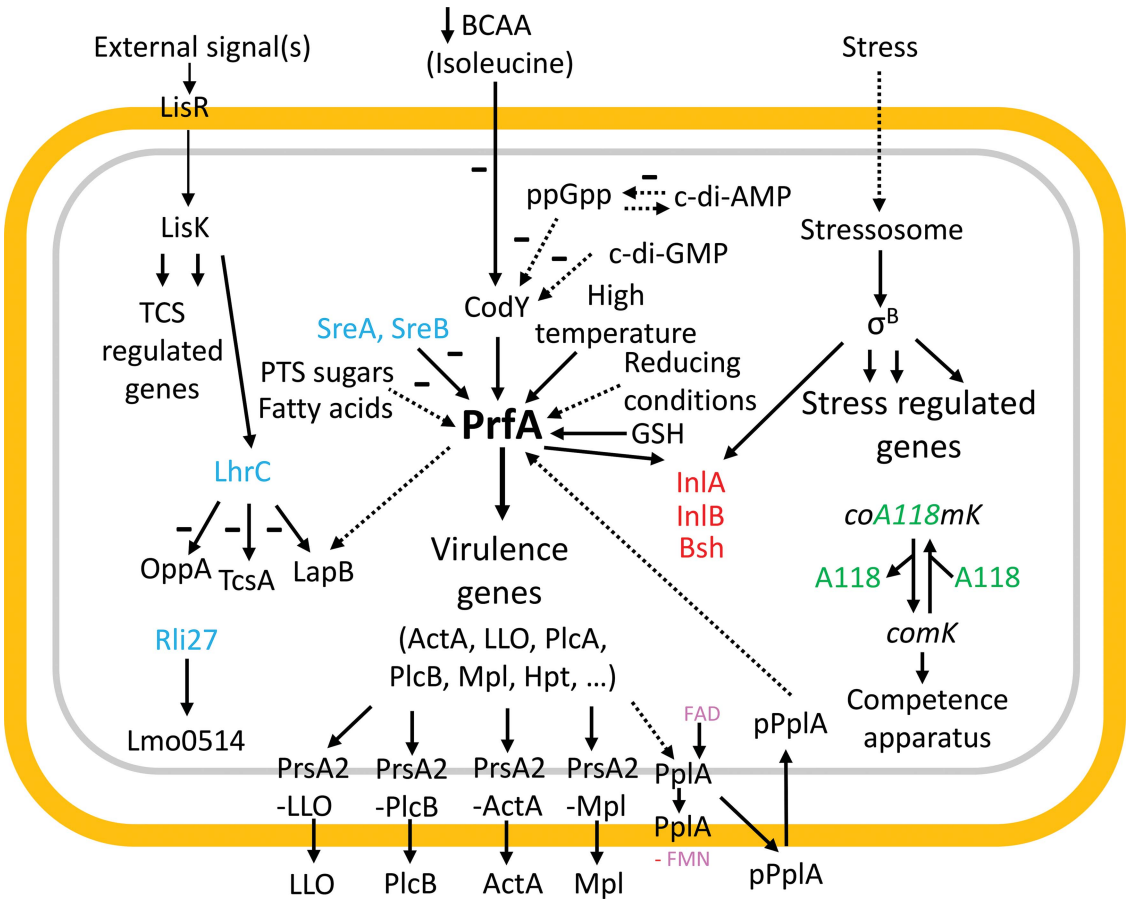
The requirement of selected *com* gene products and the pPplA peptide for vacuole escape in different cell types makes it tempting to speculate that a competence-like pseudopilus may help stabilize the pores initially formed by LLO or that maybe the pseudopilus exerts physical pressure on the vacuole membrane to aid in membrane disruption and bacterial entry into the cytosol. It is possible that the pPplA and ComK systems are interconnected in some way and that fundamental differences in the vacuole membranes of professional phagocytic cells versus nonprofessional phagocytic cells necessitate the requirement for differentially regulated components. Additional characterization of the gene products regulated by pPplA and *com* gene products may clarify how these components function to enhance bacterial entry into the cytosol.

MAKING NEW FRIENDS: REGULATION AND THE EXAMINATION OF *L. MONOCYTOGENES* STRAINS BEYOND THE COMMONLY USED ISOLATES

Most studies of *L. monocytogenes* have been performed using EGDe, EGD, or 10403S strains, all belonging to lineage II. However, the most severe outbreaks of listeriosis are caused by *L. monocytogenes* strains belonging to a subset of lineage I (137, 138). These lineage I strains contain an additional pathogenicity island, LIP-III, which appears crucial for the higher virulence potential of lin-

eage I strains. LIP-III encodes eight proteins involved in the synthesis, modification, and transport of listeriolysin S (LLS) (137). LLS was identified as a hemolysin and cytotoxin contributing to *L. monocytogenes* pathogenicity. This finding was challenged by a study suggesting that LLS was not important for infection or killing of eukaryotic cells (139). Instead, LLS was shown to act as a bacteriocin, potentiating survival of *L. monocytogenes* in the microbiota (140, 141). Bacteriocins are peptides capable of killing bacteria closely related to the bacteriocin-producing strain. It should, however, be noted that bacteriocins produced by another bacterium, *Staphylococcus pseudintermedius*, can act both as a bacteriocin and as a cytotoxin (142). An *L. monocytogenes* lineage I strain overexpressing LLS was shown to kill *Lactococcus lactis* and *S. aureus* as well as *L. monocytogenes* strains of lineage II *in vitro* (140). It was demonstrated that orally administered lineage I strains carrying LLS affected the microbiota in mice, whereas the microbiota remained undisturbed if infected with strains lacking LLS (140). Two of the bacterial genera that were significantly decreased in the gut in the presence of lineage I were *Alloprevotella* and *Allobaculum*. Interestingly, these two genera produced butyric acid, a fatty acid that has been shown to decrease *L. monocytogenes* virulence gene expression, possibly by interfering with PrfA activity (as mentioned earlier in this article). In contrast to LIP-I and LIP-II, expression of genes from LIP-III is not governed by PrfA, thereby allowing expression of LIP-III under conditions that repress PrfA activity (143). It can thus be speculated that LLS-producing *L. monocytogenes* kill bacteria that otherwise would impair PrfA-directed expression of factors needed for passage through the intestinal barrier. The production of LLS in lineage I strains may at least partially explain why this lineage is more often associated with outbreaks than linage II.

FIGURE 6 A summary of regulatory events underlying stress regulation and virulence factor expression in *L. monocytogenes*, especially highlighting the role of the PrfA system, but also TCS (here represented by LisRK), sRNAs (blue), and σ^B. Gene products regulated by both PrfA and σB are shown in red. Consult the text for further details.



The induced expression of LIP-III in lineage II strains might answer if other factors in lineage I strains also contribute to infection.

TARGETING REGULATION: THE SEARCH FOR NOVEL ANTIMICROBIALS EFFECTIVE AGAINST *L. MONOCYTOGENES*

Although resistance to traditional antibiotics is fortunately still limited, the increased occurrence of horizontal gene transfer makes the development of new antibacterial compounds targeting *L. monocytogenes* relevant. Stress survival mechanisms are clearly critical for *L. monocytogenes* existence, and a number of attempts have been undertaken to identify novel antibacterial and/or antivirulence chemical compounds targeting facets of *L. monocytogenes* stress resistance and/or virulence. Through a high-throughput screen, a substance (FPSS) was identified that could block the σ^B regulon and thereby also several genes encoding virulence factors (144). The exact mechanism of action of FPSS remains elusive (for example, direct interaction with σ^B or with another upstream component); however, the prospect of being able to inhibit the general stress response and virulence gene expression is exciting.

A different approach using analogs to metabolites that bind and control purine riboswitches led to the identification of 6-aminohydroxyl purine (6-N-HAP) as being able to exhibit both antibacterial and antivirulence properties (145). The target of 6-N-HAP has not yet been revealed, but addition of 6-N-HAP increased the bacterial mutation rate by almost 10,000-fold. Another screening approach identified a natural flavonoid (fisetin) as an efficient inhibitor of LLO activity (146, 147). Molecular modeling and interaction studies suggested that fisetin directly interacts with loops 2 and 3 of LLO, thereby preventing cholesterol binding and LLO oligomerization. Interestingly, fisetin prolonged the survival of mice infected with *L. monocytogenes*. Yet another screening-based approach was used to identify heterocyclic 2-pyridones as efficient inhibitors of *L. monocytogenes* infection and virulence factor expression; the target of the 2-pyridones was shown to be PrfA (148, 149). A 2-pyridone:PrfA cocrystal revealed that the compound bound in an intraprotein "tunnel" site, pulling the DNA-binding helix-turn-helix motif in an unfavorable position. The possibility of using tailor-made substances (such as fisetin and 2-pyridones) to inactivate specific targets (LLO and PrfA, respectively) is very appealing since it should only impair *L. monocytogenes* while leaving the remainder of the host microbiota untouched.

CONCLUDING REMARKS AND FUTURE OUTLOOK

L. monocytogenes is notable for its ability to survive and thrive both as an extraordinary pathogen of diverse animal hosts and as a saprophyte in the soil; to accomplish this bacterial gymnastic feat, *L. monocytogenes* utilizes an integrated myriad of regulatory systems (Fig. 6). The most centrally important virulence regulator identified to date is the transcriptional activator PrfA, which controls the expression of the majority of virulence factors in response to environmental cues such as redox status, temperature, energy levels, and potentially in combination with diffusion sensing of the limited space of the vacuole. PrfA is itself regulated by an impressive number of mechanisms that include transcriptional and post-transcriptional regulation as well as translational and posttranslational regulation, each combining to integrate environmental signals into an appropriate regulatory output of bacterial gene expression. It will be intriguing to study single bacterial cells to better understand how the PrfA regulatory network integrates into other sensory systems and how these systems collectively contribute to virulence. Despite being the subject of extensive study for the past 30 years, *L. monocytogenes* still appears to hold regulatory secrets that await discovery.

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