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MINIREVIEW

How the Bacterial Pathogen *Listeria monocytogenes* Mediates the Switch from Environmental Dr. Jekyll to Pathogenic Mr. Hyde

Michael J. Gray,[1]† Nancy E. Freitag,[2] and Kathryn J. Boor[1]*

Department of Food Science, Cornell University, Ithaca, New York,[1] and Seattle Biomedical Research Institute and the Departments of Pathobiology and Microbiology, University of Washington, Seattle, Washington[2]

Listeria monocytogenes is a gram-positive bacterium with a Jekyll and Hyde personality (108): it is well adapted as a saprophyte for peaceful survival in soil and decaying vegetation (Dr. Jekyll) (36), but it has a second life as an intracellular bacterial pathogen capable of causing serious infection in humans and in many animal species (Mr. Hyde) (28, 96, 115). In its Mr. Hyde phase, the bacterium is a significant public health hazard, responsible for an estimated 28% of deaths attributable to known food-borne pathogens in the United States (75). How does *L. monocytogenes* manage the switch between mild-mannered environmental bacterium and potentially deadly human pathogen? The transformation appears to be mediated through complex regulatory pathways that modulate the expression of virulence factors in response to environmental cues. This review will summarize the current understanding of *L. monocytogenes* virulence gene regulation and will put forth a model that depicts how a humble soil-grown bacterium might transform into a deadly invader.

LIFE IN THE SOIL: THE PEACEFUL EXISTENCE OF A BACTERIAL DR. JEKYLL

L. monocytogenes is a ubiquitous bacterium that sets up home in a variety of environmental locations. *L. monocytogenes* has been isolated from soil, ground water, silage, and decaying vegetation (reviewed in reference 36); however, relatively little is known about the bacterium's potentially peaceful Dr. Jekyll existence. Genome sequencing indicates the presence of multiple gene products that may facilitate the utilization by *L. monocytogenes* of a variety of carbon sources, including plant sugars (48, 84). To access nutrient sources, *L. monocytogenes* expresses flagella and exhibits swimming motility at temperatures below 30°C; in many strains (but not all) swimming motility is repressed at 37°C (51, 87, 117). Although *L. monocytogenes* does not form spores, the bacterium is well known for its ability to withstand a variety of environmental stresses, including low temperature and high osmolarity (99), thus making it a hardy environmental organism.

It is possible and, perhaps, probable that the existence of *L. monocytogenes* outside of mammalian host cells is not entirely a quiet and sedate country life but, rather, a constant territorial battle with other single-cell and multicellular organisms that are lurking nearby. Although it is commonly isolated from environmental sources (36), *L. monocytogenes* maintains an arsenal of gene products that appear to be designed to facilitate survival within mammalian host cells. Maintenance of this arsenal in an organism that is broadly present in the environment suggests the possibility that these gene products may be utilized not only in mammals but also against other eukaryotic organisms in the environment. For example, while protozoa have not been reported as a reservoir for *L. monocytogenes*, as is the case with *Legionella pneumophila*, *L. monocytogenes* does survive and replicate within amoebae (49, 70, 111). *L. monocytogenes* is an efficient pathogen of at least one insect species (*Drosophila melanogaster*), although infections must be systemically induced (73). It is likely that further studies will identify additional nonmammalian organisms that serve as hosts for *L. monocytogenes*.

L. MONOCYTOGENES WITHIN MAMMALIAN HOSTS: A BACTERIAL MR. HYDE

Although *L. monocytogenes* is well adapted to persistence in the environment (36), the majority of studies focused on *L. monocytogenes* have investigated infection of mammalian hosts, or the Mr. Hyde phase of the organism: the invasion and survival within mammalian host cells and the immune response to bacterial infection (reviewed in references 28, 66, and 86). *L. monocytogenes* is capable of invading and replicating within a wide range of animal cell types, including macrophages and nonprofessional phagocytes (45, 64, 71, 89). A number of bacterial gene products have been identified that facilitate the intracellular growth and spread of the bacterium to adjacent host cells (88, 90), and the functions of these gene products have been discussed in several excellent recent reviews (20, 28, 57, 63, 115). Briefly, these gene products include the invasion-associated surface proteins internalin A and B (InlA and InlB), gene products associated with escape from the host cell vacuole (the *hly*-encoded cholesterol-dependent cytolysin listeriolysin O [LLO] as well as phospholipases encoded by *plcA* and *plcB*), and ActA, a protein required for actin-based intracellular bacterial motility and cell-to-cell spread. Additional gene products, such as Mpl, a zinc-dependent metalloprotease that processes PlcB to its mature form, and Hpt, a hexose phosphate transporter that allows bacteria to utilize phosphorylated

* Corresponding author. Mailing address: Department of Food Science, Cornell University, 413 Stocking Hall, Ithaca, NY 14853. Phone: (607) 255-3111. Fax: (607) 254-4868. E-mail: kjb4@cornell.edu.

† Present address: Department of Bacteriology, University of Wisconsin, Madison, WI 53706.

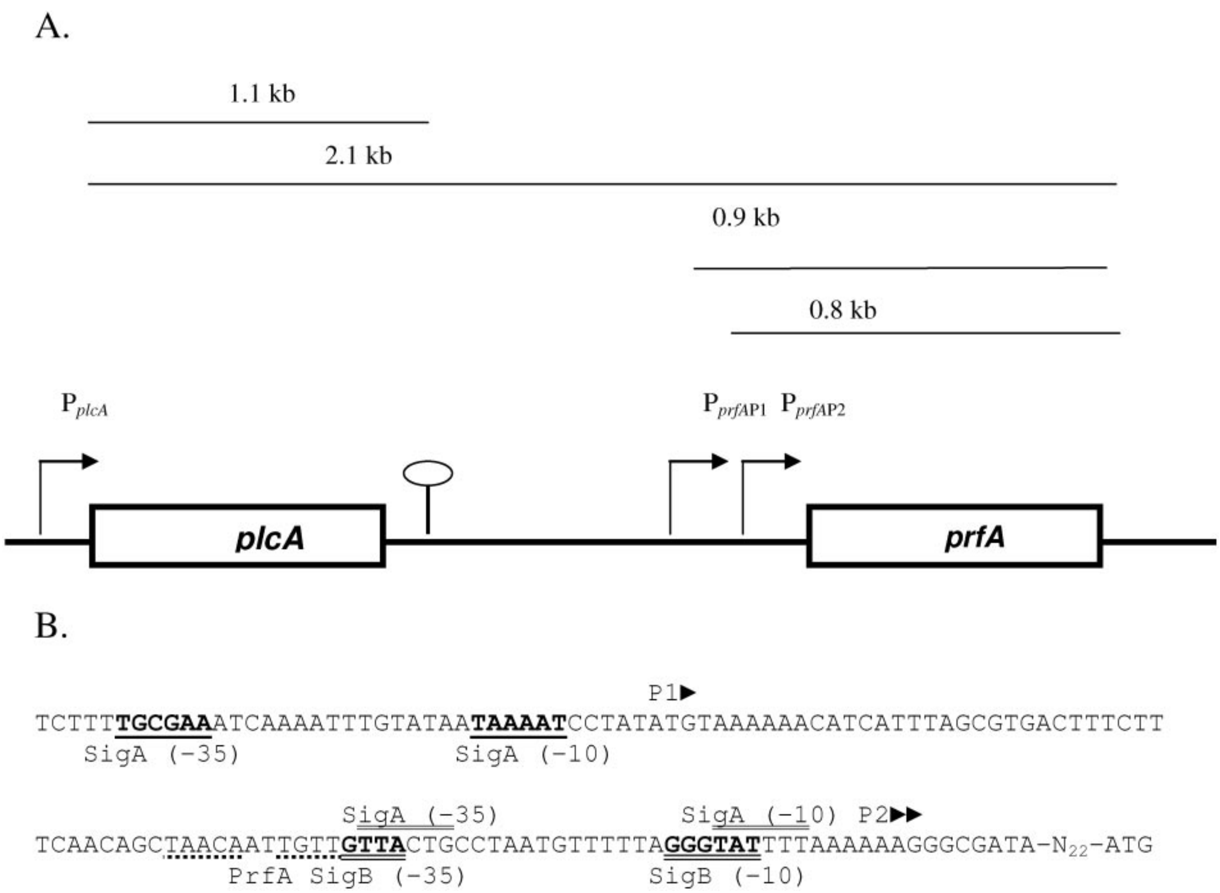

FIG. 1. (A) Map of the *L. monocytogenes plcA-prfA* region, not drawn to scale. The heavy line at the bottom of the panel represents the DNA sequence, with gene coding regions indicated as boxes; the four lines above the gene coding region represent possible mRNA transcripts, including a 1.1-kb *plcA* transcript, a 2.1-kb *plcA-prfA* bicistronic transcript, and 0.9- and 0.8-kb *prfA* transcripts (11, 13, 42, 67, 73, 91). Transcriptional start sites are indicated by bent arrows, and the *plcA* transcription terminator is indicated by a stem-loop. (B) DNA sequence of the *prfA* promoter region (46, 91). Triangles indicate transcriptional start sites identified for P1*prfA* and P2*prfA*. The σ^A-dependent P1*prfA* promoter is in boldface and underlined once. Two adjacent transcriptional start sites, possibly reflecting transcription from either the σ^A-dependent or σ^B-dependent promoter comprising the P2*prfA* region, are marked by triangles (46). In the P2*prfA* region, the σ^B-dependent promoter is in boldface and underlined twice; the proposed σ^A-dependent promoter is marked by double lines above the sequence, and the PrfA binding box (100), which is immediately upstream of the P2 promoter region, is marked by a dotted line beneath the sequence.

sugars such as glucose-1-phosphate within the host cell cytosol, also contribute to bacterial life within the mammalian host cell. Other gene products, such as the bile salt hydrolase encoded by *bsh* (4, 27) and the bile exclusion locus *bilE* (104), may function to promote bacterial survival in the liver or in extracellular environments within the mammalian host, such as the small intestine or within the gall bladder (52). For nearly every gene product identified thus far as contributing to *L. monocytogenes* survival within the host, gene expression is regulated by a transcriptional activator known as positive regulatory factor A (PrfA) (12, 40, 76, 78). Strains lacking functional PrfA are highly attenuated in animal models of infection and are forever locked into a docile and nonthreatening state.

As *L. monocytogenes* is clearly capable of adapting to multiple environments, including those outside as well as inside host cells, it is important to ask what the mechanisms are that control the switch that changes *L. monocytogenes* from a quiet soil bacterium to a ruthless invader. At least some of the answers appear to lie in the regulation of the key regulatory protein PrfA and include both the regulation of *prfA* transcription and PrfA protein activity.

INITIAL CONTROL OF THE BACTERIAL BEAST: REGULATION OF *prfA* EXPRESSION

Transcriptional control of *prfA* is the first mechanism used by *L. monocytogenes* to regulate the expression of its virulence gene products. Three promoter regions have been identified that contribute to the regulation of *prfA* expression (Fig. 1A). Two promoter regions, P*prfA*$_{P1}$ and P*prfA*$_{P2}$, are located just upstream of the *prfA* coding sequence and direct the expression of monocistronic *prfA* transcripts. The upstream *plcA* promoter (P*plcA*) directs both a monocistronic *plcA* transcript and a bicistronic transcript encoding *plcA* and *prfA* (11).

Transcription of DNA in bacteria is driven by RNA polymerase, whose specificity is determined by regulatory proteins known as sigma (σ) factors (reviewed in reference 50). The primary sigma factor determining RNA polymerase specificity in actively growing, unstressed cells is σ^A (50, 81). The P*prfA*$_{P1}$ promoter has characteristics of a σ^A-dependent promoter (83). Transcripts are produced from this promoter by actively growing *L. monocytogenes* in broth culture. The RNA transcript of *prfA* directed by P*prfA*$_{P1}$ contains a thermosensitive structure that inhibits translation of PrfA at temperatures lower than 30°C but melts at higher temperatures, allowing translation (56). The reduced efficiency of PrfA translation at low temperatures may explain the reduced transcription of PrfA-dependent genes observed at low temperatures in broth culture. The production of monocistronic *prfA* transcript is independent of temperature, while bicistronic *plcA-prfA* transcript, which is dependent on PrfA activation, is only produced at higher temperatures (67). The presence of a pool of untranslated *prfA* transcripts may allow rapid synthesis of PrfA following infection of warm-blooded mammalian and avian host organisms, which generally have temperatures higher than the surrounding environment. Temperature regulation of bacterial protein levels is not unique to *L. monocytogenes* PrfA. To illustrate, the *Escherichia coli* heat shock response associated with σ^{32} is dependent upon the presence of a pool of untranslated *rpoH* mRNA to achieve rapid increases in σ^{32} under increased temperature conditions (82, 109).

A second *prfA* promoter region, P*prfA*$_{P2}$, also directs monocistronic *prfA* transcripts (42) (Fig. 1A and B). The P2*prfA* region contains a putative PrfA binding box, which provides an autoregulatory loop (42, 100). The P2*prfA* region comprises both a σ^A- and a σ^B-dependent promoter (91). σ^B-Dependence of the P*prfA*$_{P2}$ promoter has been demonstrated (83, 91, 98). RNA polymerase complexed with σ^B recognizes the promoters of a number of genes whose products contribute to the ability of *L. monocytogenes* to withstand environmental stresses including low pH, high osmolarity, oxidative stress, and carbon starvation (3, 15, 37, 38, 39, 58, 118, 119). The products of a number of stress response genes have been implicated in virulence; these genes include *bsh*, whose product is important for resisting the stresses imposed by exposure to bile salts (27), the *gad* system, involved in resisting acid shock (22), and *hfq*, a general stress response gene involved in resistance to osmotic and ethanol stress (15). Transcription of the invasion-associated internalin genes *inlA* and *inlB* is also influenced by σ^B (59, 60). *L. monocytogenes* cells exposed to environmental stress conditions (specifically, 0.3 M NaCl or growth to stationary phase) show a relative increase in monocistronic *prfA* transcripts initiated from P*prfA*$_{P2}$ (M. Kazmierczak, M. Wiedmann, and K. J. Boor, submitted for publication). As the P*prfA*$_{P2}$-directed message does not contain the thermosensitive RNA secondary structure present in P*prfA*$_{P1}$-directed messages (56), translation of the P*prfA*$_{P2}$ transcript may thus account for the observed expression of PrfA in some low-temperature environments, such as the cytosol of insect cells, where PrfA-dependent gene products are expressed and functional (13, 26, 73).

Finally, bicistronic *plcA-prfA* transcripts are produced from the upstream PrfA-dependent P*plcA* promoter (Fig. 1A) (11, 13, 73). PrfA thereby upregulates its own production, and this autoregulation is required for bacterial cell-to-cell spread within tissue culture cells and for bacterial virulence in animal models of infection (11, 41).

ADDITIONAL CONTROL OF THE BACTERIAL BEAST: REGULATION OF PrfA ACTIVITY

In addition to the existence of transcriptional and posttranscriptional mechanisms that control *prfA* expression and translation, PrfA activity is controlled on a posttranslational level. PrfA is a member of the Crp/Fnr transcription regulator family (65, 112). As a group, Crp/Fnr regulators respond to a broad array of signals, both intracellular and exogenous, such as the presence of small molecular cofactors (e.g., cyclic AMP for Crp) (53), as well as changes in redox potential, oxygen availability, or temperature (reviewed in reference 62). Mutants of Crp, known as Crp*, have been identified that contain amino acid substitutions that appear to lock the protein into a constitutively active form, even in the absence of the signal molecule, cyclic AMP (53). Ripio et al. (95) were the first to describe a similar mutation in PrfA (PrfA G145S, or PrfA*), which was identified in an *L. monocytogenes* strain (NCTC 7973) that constitutively expressed high levels of PrfA-dependent gene products. Recent evidence suggests that the PrfA G145S mutation may stabilize the helix-turn-helix motif relative to that of the wild-type PrfA to enhance the protein's DNA-binding affinity (29). Since the identification of PrfA G145S, additional PrfA mutations have been identified that also appear to result in a constitutively activated form of the protein (PrfA I45S, PrfA E77K, PrfA L140F, and PrfA G155S) (54, 103, 116, 121). Interestingly, recent data suggest that strains containing PrfA* mutations may be locked into a Mr. Hyde state that can increase bacterial virulence in animal models (103). For example, strains containing the PrfA G155S mutation were approximately fivefold more virulent than wild-type strains following intravenous injection of mice (103).

It is clear that PrfA exists in high- and low-activity states, with the transitions between activation states occurring in response to environmental signals; however, the nature of the potential small molecule cofactor bound by PrfA (or PrfA posttranslational modification) that triggers PrfA activation is not yet known (65, 92). A number of environmental conditions influence the expression of PrfA-dependent gene products (106). Growth in rich medium or in medium supplemented with readily metabolized carbohydrates (such as glucose, fructose, maltose, or cellobiose) inhibits transcription of PrfA-dependent virulence genes (*hly*, *plcA*, *plcB*, *mpl*, and *actA*) without affecting PrfA protein levels (32, 77). Repression of virulence gene expression by cellobiose, a common carbohydrate in plant materials but not in animal hosts, appears to be mediated by at least three different mechanisms (5, 6, 9, 55, 69, 77). In contrast to the repression of virulence gene expression by these readily metabolized sugars, the presence of phosphorylated sugars, such as glucose-1-phosphate, supports bacterial growth with no repression of PrfA-dependent virulence gene expression (94). Phosphorylated sugars present within the cytosol of mammalian host cells are postulated to serve as molecular cues signaling the opportunity for rapid intracellular *L. monocytogenes* growth (14).

Other environmental signals are known to influence virulence gene expression in *L. monocytogenes*. PrfA-dependent LLO production and *actA* expression are both activated in iron-depleted medium (17, 23). As free iron levels are extremely low in mammalian host cells ($\sim 10^{-18}$ M) (68), available iron may serve as a cue used by *L. monocytogenes* to assess its location. It is well established that expression of PrfA-dependent genes increases following treatment of the culture medium with activated charcoal (31, 32, 47, 93). Ermolaeva et al. (33) have presented evidence to suggest that activated charcoal acts by absorbing a small diffusible autorepressor molecule which *L. monocytogenes* produces during exponential growth. This strategy is reminiscent of quorum sensing mechanisms used in other bacteria to regulate genes in a bacterial cell concentration-dependent fashion (1, 34), but whether this form of virulence gene repression occurs in *L. monocytogenes* remains undetermined. In summary, several environmental conditions have been shown to influence virulence gene expression, presumably by influencing the state of PrfA activation, but the molecular mechanism responsible for the conversion of PrfA to its fully active state remains unknown.

THE TRANSITION TO MR. HYDE FOLLOWING BACTERIAL INVASION OF THE MAMMALIAN HOST

Animals have a wide array of defense mechanisms specifically designed to prevent pathogenic bacteria from settling in and making themselves at home. Once *L. monocytogenes* is ingested by a mammalian host organism, its survival within that host depends upon the bacterium's ability to withstand a number of defense mechanisms. Exposure to stresses imposed by host defense mechanisms may actually help prepare *L. monocytogenes* for its Mr. Hyde existence. Specifically, accumulating evidence suggests that environmental stress conditions encountered during passage through the stomach to the gut contribute to the infectious life cycle of *L. monocytogenes* (18, 19, 21, 74, 85, 97). For example, one early host defense encountered by *L. monocytogenes* following ingestion is the low pH environment of the stomach. A clear connection has been established between acid tolerance and virulence in *L. monocytogenes*, in that mutants with increased acid tolerance show increased virulence in mice (85), and decreased acid tolerance is correlated with decreased virulence (21, 74). The genetic mechanism(s) of this effect is not well understood, but preadaptation of *L. monocytogenes* (by exposure to pH 4.5 to 5.5, similar to the pH found in the stomach after eating [22]) increases the invasiveness of the bacteria in cell culture (18) as well as bacterial survival following macrophage infection (18, 19, 44) and following intragastric inoculation of mice (97).

The acid tolerance response of *L. monocytogenes* is at least partially dependent on σ^B (37, 38, 119), and at low pH, the production of monocistronic *prfA* transcript is strongly increased; this transcript accumulation may serve to prime the bacterium for its responses to subsequent host environments (19). As expression of a variety of stress response genes and invasion-associated internalins is regulated by the stress-responsive σ^B (58, 59, 60), the predicted net effect of *L. monocytogenes* passage through the stomach and intestine may be an increase in the production of a variety of proteins important for invasion and infection. Indeed, recent data indicate that σ^B plays a critical role during the gastrointestinal stage of listeriosis in guinea pigs (46). While passage of *L. monocytogenes* through the gut clearly is not essential for virulence, as infections in animals can be established by intraperitoneal or intravenous injection (11, 14, 27, 30, 44, 71, 119), it may increase the efficiency of infection under natural conditions.

THE MAMMALIAN CYTOSOL AND THE FULL UNLEASHING OF MR. HYDE

When *L. monocytogenes* leaves the lumen of the intestine and enters a host cell, it once again encounters several changes in its immediate environment. In contrast to the relatively high available iron and carbohydrate levels in the intestinal lumen, the phagocytic vacuole is postulated to have low quantities of available iron and carbohydrates. Low iron and low carbohydrate concentrations activate transcription from some PrfA-dependent virulence gene promoters (8, 16, 17, 32, 77). Exposure of *L. monocytogenes* to H_2O_2 increases transcription of *prfA* and *hly*, suggesting that the presence of reactive oxygen intermediates, such as those generated in activated macrophages, also may up-regulate virulence gene expression (72). The phagocytic vacuole of a macrophage rapidly becomes acidified to a pH of approximately 5.5 to 6 (2). Hence, following engulfment, the *L. monocytogenes* invader is subjected to multiple rapid environmental changes, including exposure to oxygen radicals, reduced pH, and reduced nutrient density.

Some PrfA-dependent gene products have clearly targeted roles within specific cellular locations and are differentially expressed depending upon their cellular location (10). For example, *actA* expression is primarily confined to the host cell cytosol, where it directs actin polymerization (10, 43, 80). Differential expression of PrfA-dependent promoters is influenced by sequence variations within a promoter region's PrfA box, with relative activation reflecting the similarity of a given promoter's PrfA box to the PrfA-box consensus sequence (24, 100, 120). The promoters with perfect PrfA-box sequences, P*hly* and P*plcA*, are the most efficiently transcribed and produce transcripts at relatively low PrfA concentrations (100).

Activation of transcription from the *plcA* promoter by PrfA initiates an important regulatory circuit within the host by which PrfA upregulates its own production (11, 76) and produces an increase in PrfA concentrations to enable the bacteria to establish themselves in a host cell and to move to infect new cells. Mutants that produce very small amounts of PrfA are capable of escaping from vacuoles but not of polymerizing actin or spreading between host cells (41). Cell-to-cell spread is mediated by the actin nucleating protein ActA (61, 87, 113), which is transcribed from two promoters, P*actA* and P*mpl* (61, 114). These promoters each have a single mismatched base in their PrfA boxes; therefore, transcription activation from these promoters requires an increased concentration of PrfA, such as that produced by *L. monocytogenes* present in host cytosol (100). PC-PLC, the product of the *plcB* gene, is also produced by transcription from P*actA* and P*mpl* (61, 114), and its production is important for efficient bacterial cell-to-cell spread, as it permits bacterial escape from the secondary vacuoles created when an *L. monocytogenes* cell moves into a neighboring host cell (105, 114). Two additional PrfA-dependent genes that contribute to virulence, *inlC* and *hpt*, also have single mismatches in their PrfA boxes (14, 30, 74). *inlC* encodes a small, secreted protein called internalin C (30). Expression of *inlC* is enhanced in the cytoplasm of mammalian cells (10, 30), and Δ*inlC* mutants have reduced virulence in mice (30). The function of internalin C has not yet been fully established, although recent results show that it supports internalin A in stimulating invasion of mammalian cells (7). The *hpt* gene encodes a hexose phosphate transporter which allows *L. monocytogenes* to grow using phosphorylated sugars such as glucose-1-phosphate as a carbon source (14). Deletion of the *hpt* gene results in bacteria with a significantly reduced intracellular growth rate and attenuated virulence in mice (14), suggesting that hexose phosphates serve as important carbon sources for growth of *L. monocytogenes* in the cytoplasm.

PrfA HELPS MEDIATE THE *L. MONOCYTOGENES* SWITCH FROM ENVIRONMENTAL DR. JEKYLL TO PATHOGENIC MR. HYDE

Increasing evidence suggests that PrfA is a key part of the potion that transforms Dr. Jekyll into pathogenic Mr. Hyde. Overall, if one were to generate a model (or write a novel) describing the fateful Jekyll-and-Hyde transition of *L. monocytogenes*, it might be best put forward as follows: in response to environmental signals outside of a host, *L. monocytogenes* maintains its Dr. Jekyll persona by repressing both PrfA production and activity through transcriptional (promoter expression), posttranscriptional (RNA thermosensor), and posttranslational mechanisms (PrfA activation), thereby cloaking the expression of its primary virulence factors except for the internalins, which appear to be produced in advance of infection (12, 59). Once the bacteria are ingested by a mammalian host, the increase in temperature and exposure to reduced pH in the stomach stimulates increased production of stress response proteins, internalins, and PrfA, thus beginning the transition to virulence. In the intestine, internalin A mediates attachment and invasion of host epithelial cells with the support of other internalin proteins. Once within the cell phagosome, low iron and low carbohydrate concentrations repress internalin production while PrfA-dependent activation of the P*hly* and P*plcA* promoters allows production of LLO and PlcA to promote lysis of the phagocytic vacuole, thereby enabling entry of the bacteria into the cytosol. Within the cytosol, the full transformation of the *L. monocytogenes* Dr. Jekyll into Mr. Hyde is completed when high levels of active PrfA protein activate transcription from the P*actA* and P*mpl* promoters. The resulting production of ActA and PlcB enables spread of the bacteria to adjacent cells.

While this story in progress features PrfA as the protagonist controlling the *L. monocytogenes* transition from the outside environment to the inside of the host, additional characters are clearly required. Full induction of ActA expression, for example, seems to require additional unknown steps or factors beyond what can be explained by PrfA binding (102, 103). Secondary structure of the 150-bp 5′ untranslated region of the *actA* mRNA has recently been shown to be important in full ActA expression, but the detailed mechanism is as yet unknown (122). Posttranscriptional mechanisms also contribute to synthesis of internalin A and B (110) and LLO (101). Mutations mapping outside of the PrfA locus that affect virulence gene expression in *L. monocytogenes* have been identified (69, 103), suggesting the potential presence of other transcription factors, regulatory elements, and signaling molecules required for the regulation of virulence in *L. monocytogenes*.

THE FINALE: THE GOOD NEWS AND THE BAD NEWS

While the bad news is that *L. monocytogenes* is capable of undergoing the dangerous transition from an environmental Dr. Jekyll to a pathogenic Mr. Hyde within the host, the good news may be that the Mr. Hyde form seems to suffer a competitive disadvantage outside the host. *L. monocytogenes prfA* mutants that contain constitutively activated alleles of *prfA* (and are thus locked into the Mr. Hyde phase) are fully virulent, and in some cases hypervirulent, in mouse models of infection; however, these mutants are severely compromised for flagellum-mediated swimming motility and therefore may be hindered in nutrient acquisition in environments outside the host. It therefore appears that *L. monocytogenes* must maintain a balance between life in the outside environment and life within the host; thus, bacteria that can undergo the switch back to the humble Dr. Jekyll form may be favored over the evolution of increasingly dangerous Mr. Hydes.

The last decade has seen an enormous expansion in our understanding of how *L. monocytogenes* regulates the transition from peaceful saprophyte to deadly pathogen. The switch from environmental microbe to pathogen is mediated by a diverse array of microorganisms encompassing both bacteria and fungi. In addition to *L. monocytogenes*, the organisms able to make the transition from the outside environment to inside a mammalian host include important pathogens such as *Vibrio cholerae* (107), *Bacillus anthracis* (25), *Cryptosporidium parvum* (35), and *L. pneumophila* (79). In most cases there is limited understanding of what molecular mechanisms serve to mediate the switch from life outside the host to life within a host, and, thus, the more we know of the strategies used by one environmental pathogen, *L. monocytogenes*, the better we may understand whether similar strategies might exist and be used by other pathogens to mediate deadly transitions.

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