The Virulence Regulator PrfA Promotes Biofilm Formation by *Listeria monocytogenes*^{∇†}

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***Listeria monocytogenes* is a food-borne facultative intracellular pathogen. It is widespread in the environment and has several distinct life-styles. The key transcriptional activator PrfA positively regulates *L. monocytogenes* virulence genes to mediate the transition from extracellular, flagellum-propelled cell to intracellular pathogen. Here we report the first evidence that PrfA also has a significant positive impact on extracellular biofilm formation. Mutants lacking *prfA* were defective in surface-adhered biofilm formation. The $\Delta prfA$ mutant exhibited wild-type flagellar motility, and its biofilm defect occurred after initial surface adhesion. We also observed that mutations that led to the constitutive expression of PrfA-dependent virulence genes had a minimal impact on biofilm formation. Furthermore, biofilm development was enhanced in a mutant encoding a PrfA protein variant unable to fully transition from the extracellular form to the virulent, intracellular activity conformation. These results indicate that PrfA positively regulates biofilm formation and suggest that PrfA has a global role in modulating the life-style of *L. monocytogenes*. The requirement of PrfA for optimal biofilm formation may provide selective pressure to maintain this critical virulence regulator when *L. monocytogenes* is outside host cells in the environment.**

Listeria monocytogenes is an opportunistic food-borne pathogen that can be isolated from a variety of environments (5, 14). This Gram-positive bacterium can contaminate foods by persisting on food-processing surfaces, and it has been hypothesized that this persistence is at least partly due to the formation of biofilms, which can be difficult to eradicate (29). Once ingested, *L. monocytogenes* causes life-threatening infections in fetuses, newborns, and immunocompromised people (9, 23). The high case fatality rate of listeriosis (~20%) makes *L. monocytogenes* food contamination, and, thus, biofilms, an important health issue (24). In contrast to its pathogenesis, little is known about *L. monocytogenes* biofilm development.

L. monocytogenes has three distinct life-styles: (i) an intracellular pathogen that utilizes actin-based motility for cytoplasmic movement and cell-to-cell spread; (ii) an extracellular, free-living, flagellum-propelled bacterium in the environment; and (iii) an extracellular bacterial member of a multicellular biofilm community. Biofilm formation and intracellular invasion appear to be mutually exclusive life-styles. The critical virulence transcription factor PrfA regulates the switch from extracellular, flagellum-propelled bacterium to intracellular pathogen (reviewed in references 10, 13, and 39). PrfA binds to a specific DNA sequence (the PrfA box) and positively regulates the expression of the canonical *L. monocytogenes* virulence genes, including itself. During intracellular invasion, the PrfA-dependent transcription of virulence genes increases first

in the phagocytic vacuole. After vacuolar escape into the host cytosol, a subset of PrfA-dependent virulence genes is induced to an even greater extent.

PrfA production is regulated at transcriptional, translational, and protein activity levels (13, 39). Three promoters drive *prfA* transcription (Fig. 1). Two of these promoters, *PprfA_{P1}* and *PprfA_{P2}*, control the expression of a monocistronic *prfA* transcript (11, 12). *PprfA_{P1}* is dependent on the housekeeping sigma factor, σ^A . *PprfA_{P2}* can be driven by either σ^A or the stress response sigma factor, σ^B (30, 36, 38). In addition, the promoter of the upstream gene *plcA*, *PplcA*, contains a canonical PrfA box and is positively autoregulated by PrfA, resulting in the transcription of a bicistronic message containing *plcA* and *prfA* (4, 26). The resulting increased level of PrfA expression from *PplcA* is necessary for wild-type virulence (4, 12). The transcript from *PprfA_{P1}* contains a 5' untranslated region (UTR) that acts as a thermosensor; the translation of this transcript is efficient at the host body temperature but inefficient at 30°C and below (18). In contrast to *PplcA*, transcription from *PprfA_{P2}* is PrfA independent, and in contrast to *PprfA_{P1}*, the translation of this transcript is temperature independent. It has been postulated that this transcript is important for PrfA production outside mammalian hosts; we provide evidence here to support this hypothesis.

PrfA is a member of the cyclic AMP receptor protein-fumarate and nitrate reduction regulator (CRP-FNR) family of transcriptional regulators (20). Small-molecule cofactors regulate the protein activity state of many family members (19, 39). While no such cofactor has been identified yet for PrfA, a variety of point mutations in *prfA* (*prfA** mutants) that result in variant PrfA proteins whose function is locked in the intracellular activity state support the theory that PrfA activity is allosterically regulated (16, 28, 37, 40, 44, 47). These *prfA** mu-

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FIG. 1. Schematic that shows the three promoters that drive the expression of *prfA*. *PplcA* is positively autoregulated by PrfA, which binds to the PrfA box (black box), and produces a bicistronic message. *PprfA_{P1}* is σ^A dependent, and its transcript contains a thermosensor in the 5' untranslated region and is not efficiently translated at 30°C or below. *PprfA_{P2}* can be activated by both σ^A and σ^B , and its transcript does not contain a translational thermosensor.

tants constitutively express levels of the actin-nucleating protein ActA comparable to those observed when bacteria are in the host cytosol (40). These mutations also result in an increased level of production of the PrfA* protein via the activation of *PplcA* (43, 47). Another recently isolated mutation in *prfA* (Y154C) results in a PrfA protein that cannot fully transition from the extracellular activity state to the intracellular activity state (27). The *prfA*(Y154C) mutant demonstrates a 4-fold increase in *actA* expression levels in brain heart infusion (BHI) medium compared to the wild type but fails to increase *actA* expression levels to wild-type levels (100-fold less than the wild type) during intracellular infection (27). It effectively enters the host cytosol but is defective in cell-to-cell spread and is attenuated for virulence (150-fold reduction in a mouse model) (27). Thus, there are PrfA protein variants that are locked in the intracellular activity state (PrfA*) and one [PrfA(Y154C)] that is unable to fully transition to the intracellular activity state.

L. monocytogenes has never been observed to form biofilms in host cells; intracellular bacteria are single cells that move through the cytosol using actin-based motility (34). Extracellularly, *L. monocytogenes* motility is mediated via flagella. Flagellum-mediated motility appears to be important for initial

epithelial cell attachment, and flagellum-minus mutants show an early host invasion defect in a competitive oral infection model (32). Similarly, flagellum-mediated motility is critical for initial abiotic surface attachment, and flagellum-minus mutants are defective in biofilm formation (22, 41). This common need for flagellum-mediated motility to transition from an existence as a single, extracellular cell to either environmental biofilm formation or intracellular invasion led us to hypothesize that there might be regulatory cross talk between PrfA and biofilm formation. Indeed, here we report that *prfA* mutants are defective in biofilm formation at a stage after initial surface attachment. The regulatory connection between PrfA and biofilm formation demonstrates that PrfA has a global role in regulating the life-style of *L. monocytogenes*.

MATERIALS AND METHODS

Strains and media. Media used were tryptic soy broth yeast extract (TSBYE) consisting of 3% Bacto tryptic soy broth (Becton Dickinson and Co.) and 0.6% Bacto yeast extract (Becton Dickinson and Co.), Bacto BHI broth (Becton Dickinson and Co.), and, for biofilm formation, Hsiang-Ning Tsai medium (HTM) with 3% glucose plus an essential amino acid(s), a defined minimal medium (42). Essential amino acids (cysteine and, for 10403S derivatives, methionine) were added to a final concentration of 0.1 mg/ml. Strains used are listed in Table 1. NF-L1124 is a derivative of NF-L100, which is wild-type serotype 1/2a

TABLE 1. *L. monocytogenes* strains used

Strain	Description	Reference(s)
NF-L1124	10403S <i>actA-gus-neo-plcB</i> (serotype 1/2a)	27
NF-L1123	10403S $\Delta prfA$ <i>actA-gus-neo-plcB</i>	27
NF-L1213	NF-L1124 <i>prfA</i> (Y154C)	27
NF-L1166	NF-L1124 <i>prfA</i> (L140F)	28
NF-L1177	NF-L1124 <i>prfA</i> (G145S)	28
NF-L1214	NF-L1124 <i>prfA</i> (Y63C)	28
KPL1712	NF-L1124 $\Delta sigB$	This work
KPL1714	NF-L1124 $\Delta sigB$ <i>prfA</i> ::Tn917-LTV3	This work
KPL1720	NF-L1124 <i>prfA</i> ::Tn917-LTV3	This work
KPL1727	NF-L1124 tRNA ^{Arg} ::pPL2	This work
KPL1733	NF-L1123 tRNA ^{Arg} ::pPL2	This work
KPL1783	NF-L1124 tRNA ^{Arg} ::pHyperSPO1- <i>hly</i> 5'UTR- <i>prfA</i>	This work
KPL1787	NF-L1123 tRNA ^{Arg} ::pHyperSPO1- <i>hly</i> 5'UTR- <i>prfA</i>	This work
M35303A	Wild type, serotype 1/2a (bulk milk container isolate)	3
KPL1676	M35303A <i>prfA</i> ::Tn917-LTV3	This work
H9067	Wild type, serotype 1/2c (cheese isolate)	3
KPL1680	H9067 <i>prfA</i> ::Tn917-LTV3	This work
KPL1018	Wild-type 10403S (serotype 1/2a, human skin lesion)	2, 8
KPL 1672	KPL1018 <i>prfA</i> ::Tn917-LTV3	This work
DP-L1075	10403S <i>prfA</i> ::Tn917-LTV3	12
DP-L4221	10403S tRNA ^{Arg} ::pPL2	21
DH-L1028	10403S $\Delta prfA$ tRNA ^{Arg} ::pHyperSPO1- <i>hly</i> 5'UTR- <i>prfA</i>	A. Shen and D. Higgins, unpublished data

strain 10403S (2, 8, 27). Chloramphenicol was used at a concentration of 5 to 7.5 $\mu\text{g/ml}$ for the integration of pPL2-based constructs and at a concentration of 10 $\mu\text{g/ml}$ for strain construction via allelic exchange in TSBYE. A mix of erythromycin at 1 $\mu\text{g/ml}$ and lincomycin at 25 $\mu\text{g/ml}$ was used for the selection of phage transductants carrying Tn917 insertions.

Mutant strain construction. All mutants containing *prfA*::Tn917-LTV3 were constructed by the transduction of the transposon insertion in *prfA* from DP-L1075 (12) using *L. monocytogenes* phage P35 as described previously (15, 17). Mutants with genes expressed from constructs integrated at the neutral locus tRNA^{Arg} were constructed by using vectors derived from pPL2 (21). For the complementation of $\Delta prfA$, a construct from DH-L1028 that is integrated at tRNA^{Arg} with the HyperSPO1 promoter (35) driving the expression of a transcript containing the full-length 5' UTR of *hly* fused to the first ATG of the *prfA* open reading frame (A. Shen and D. Higgins, personal communication) was transduced into strains NF-L1124 and NF-L1123 by using *L. monocytogenes* phage P35. As controls, pPL2 integrated at tRNA^{Arg} in DP-L4221 (21) was transduced into NF-L1124 and NF-L1123. A mutant with an in-frame deletion of *sigB* was constructed in the NF-L1124 background by using allelic exchange with plasmid pBMB27. pBMB27 carries a *sigB* in-frame deletion identical to pTJA57 (46). This plasmid was previously used to make a *sigB sigL* double mutant (33).

Motility assay. Flagellum-mediated motility was assayed by measuring the diameter of the bacterial swarm in millimeters 24 h after inoculation with a sterile pointed toothpick in 0.3% BHI agar at room temperature (RT) ($\sim 24^\circ\text{C}$), 30°C , or 36°C (22).

Biofilm assay. Surface-adhered biofilm formation was assayed in a 96-well format by using crystal violet (CV) staining as described previously (22). Briefly, a culture of each strain grown overnight at $\sim 20^\circ\text{C}$ in TSBYE was diluted into freshly made biofilm-promoting medium (HTM with 3% glucose plus essential amino acids) to an initial optical density at 600 nm (OD_{600}) of 0.03 to 0.06. One hundred fifty microliters was aliquoted into each of 8 wells of a 96-well polyvinyl chloride (PVC) plate. Biofilms were grown in standing culture for 1 to 4 days at the specified temperature. To quantify biofilm formation, planktonic cells were removed along with any loosely adhered or sedimented cells. Biofilms were washed three times with water and then stained for 30 min with a 0.1% aqueous solution of CV. After staining, wells were washed four times with water and then air dried. Finally, 150 μl of 33% acetic acid was added to each well, mixed well to fully solubilize the CV, and transferred onto a fresh polystyrene 96-well plate; the OD_{590} was then measured by using a SPECTRAMax M2 plate reader equipped with SOFTmax Pro software (Molecular Devices).

Adhesion to glass. Initial surface adhesion to glass coverslips was determined at room temperature as previously described (22). A rolling culture of each strain grown for 16 to 18 h at $\sim 20^\circ\text{C}$ in TSBYE was diluted into freshly made biofilm-promoting medium to a starting OD_{600} of between 0.01 and 0.09. Light microscopy was used to confirm that most cells were swimming, and at time zero, dishes (catalog number P35G-1.5-14-C; MatTek Co., Ashland, MA) were then inoculated with 1.5 ml of each culture. Prior to imaging, the MatTek dish coverslip was washed gently by adding 1 ml of medium to the edge of the dish. Digital images of 5 to 10 high-powered fields (HPF) per dish were captured by using a 100 Plan Apo objective with differential interference contrast optics on an inverted Nikon TE2000-U microscope equipped with a cooled charge-coupled-device camera (ORCA-ER; Hamamatsu) and MetaMorph imaging software (version 6.1; Molecular Devices). Images were converted to 8 bits in MetaMorph and opened using ImageJ (NIH), and the number of cells/HPF were then counted manually.

Statistical analyses. Statistical analyses were performed by using SigmaPlot 11. The *t* test was used for pairwise comparisons except when the data failed the equal variance test, in which case the Mann-Whitney rank sum test was then used. One-way analysis of variance (ANOVA) with Holm-Sidak multiple comparison (H-S) was used for multiple comparisons except where data failed either normality or equal variance testing, in which case the Kruskal-Wallis one-way analysis of variance on ranks with Tukey multiple-comparison testing (KW with T) was used.

RESULTS

***L. monocytogenes prfA* mutants are defective in biofilm formation.** To test if PrfA is involved in biofilm formation, we assayed surface-associated biofilm formation on polyvinyl chloride (PVC) 96-well plates using crystal violet staining. *L. monocytogenes* strain 10403S (serotype 1/2a) was compared to a mutant that contains an in-frame deletion in *prfA* ($\Delta prfA$). Previous work demonstrated that biofilm formation on plastic

corresponds well with attachment to food-processing-grade stainless steel (7, 22). PrfA expression is thought to be optimal at host body temperature, whereas *L. monocytogenes* biofilms are a source of food contamination at or below ambient temperature; therefore, biofilm assays were performed at several temperatures. Compared to wild-type strain 10403S, the $\Delta prfA$ mutant was defective in biofilm formation each day of 4 days at room temperature (RT) ($\sim 24^\circ\text{C}$), 30°C , and 36°C (Fig. 2A). In contrast, the $\Delta prfA$ mutant's growth was comparable to that of the wild type in the same biofilm-promoting medium (HTM with 3% glucose) in a rolling culture (see Fig. S1 in the supplemental material).

To determine if a loss of *prfA* resulted in a biofilm defect in other *L. monocytogenes* strains, we used a previously characterized Tn917 transposon insertion (DP-L1075) that disrupts the *prfA* open reading frame (12). In strain 10403S this insertion results in both a virulence defect (12, 25) and a biofilm defect similar to an in-frame deletion (compare Fig. 2B to A). Mutants were generated by phage transduction of *prfA*::Tn917 into two nondomesticated strains that are robust biofilm formers: M35303A (serotype 1/2a), an environmental isolate from bulk milk, and H9067 (serotype 1/2c), a cheese isolate (3). In both of these strain backgrounds, *prfA* mutants were also defective in biofilm formation (Fig. 2B). This indicates that the disruption of *prfA* results in a biofilm defect that is neither strain nor serotype specific. It is also worth noting that while growths in biofilm-promoting medium in rolling culture were comparable for these strains (see Fig. S2A in the supplemental material), in a clumping assay, the wild-type strains sedimented at a much higher rate than did the corresponding *prfA* mutants (Fig. S2B).

While laboratory strain 10403S was not as robust a biofilm former as the environmental isolates in our biofilm assay, it behaved similarly, and a number of *prfA* mutations were previously generated in this background; therefore, we focused the remainder of our studies on strain 10403S. The expression of PrfA from a heterologous locus restored wild-type levels of biofilm formation to the $\Delta prfA$ mutant (Fig. 2C) while not impacting growth (see Fig. S3A in the supplemental material). Together, the data in Fig. 2 indicate that *L. monocytogenes* needs *prfA* to achieve wild-type levels of biofilm development.

The $\Delta prfA$ mutant displayed wild-type flagellum-mediated motility and initial surface attachment. Similar to most bacteria, *L. monocytogenes* biofilm formation on surfaces proceeds incrementally, starting with initial surface attachment and proceeding to microcolony formation, biofilm maturation, and, finally, dispersion (41). Flagellum-minus mutants are deficient in biofilm formation (22, 41). We have previously shown that flagellum-mediated motility in *L. monocytogenes*, and not the flagella themselves, is critical for initial surface attachment and subsequent biofilm development (22). We therefore investigated the flagellum-mediated motility and surface attachment of the $\Delta prfA$ mutant. Laboratory strain 10403S is one of approximately 20% of *L. monocytogenes* strains that remain motile at close to mammalian physiological temperatures (45). As expected, 10403S formed a biofilm well at 36°C (Fig. 2A). The $\Delta prfA$ mutant (Fig. 3A) displayed flagellum-mediated motility that was comparable to that of wild-type strain 10403S (Fig. 3A) at RT and 30°C and was slightly increased at 36°C . This slight increase was statistically significant ($P < 0.05$ by *t* test)

FIG. 2. The *L. monocytogenes* $\Delta prfA$ mutant is defective in biofilm formation. Shown are bar graphs of the surface-adhered biofilm formed by the wild type (WT) and the $\Delta prfA$ mutant (PrfA⁻) quantified by measuring the OD₅₉₀ of solubilized crystal violet after biofilm staining. (A) Biofilms were formed at RT (~24°C), 30°C, and 36°C for the specified number of days. A pairwise comparison of the difference between the WT (NF-L1124) and the $\Delta prfA$ mutant (NF-L1123) was statistically significant on each day at each temperature ($P < 0.05$ by Mann-Whitney test). Data are from four experiments with seven replicates of each strain per experiment. Error bars represent standard errors of the means (SEM). (B) Biofilms of the wild type of each strain compared to an isogenic *prfA*::Tn917 mutant (PrfA⁻) formed at 30°C and harvested after 3 days. Differences between the wild type and the $\Delta prfA$ mutant were statistically significant for each pair of strains ($P < 0.05$ by Mann-Whitney test). Data are from four or five experiments

FIG. 3. The *L. monocytogenes* $\Delta prfA$ mutant displays wild-type levels of flagellum-mediated motility and initial surface attachment at temperatures below mammalian body temperatures. (A) Motility of wild-type 10403S (WT) (NF-L1124) and the $\Delta prfA$ mutant (PrfA⁻) (NF-L1123) at RT, 30°C, and 36°C measured as the diameter of the bacterial swarm in millimeters 24 h after inoculation. The difference in motility between the wild type and $\Delta prfA$ mutant was statistically significant at 36°C (*, $P < 0.05$ by *t* test) but not at lower temperatures. Data are from 12 independent experiments. Error bars represent SEM. (B) Initial surface adhesions to glass by the wild type (NF-L1124) and the $\Delta prfA$ mutant (NF-L1123) were comparable. There was no statistical difference between the wild type and the $\Delta prfA$ mutant using the Mann-Whitney rank sum test. Data are from three independent experiments with 5 to 10 high-powered fields counted per strain in each experiment. The wild type was counted first during each experiment. Error bars represent SEM.

(Fig. 3A). In addition to having wild-type levels of flagellum-mediated motility, the $\Delta prfA$ mutant also displayed initial surface attachment comparable to that of the wild type in a surface attachment assay (Fig. 3B). These data indicate that the $\Delta prfA$ mutant's defect in biofilm development occurs after initial surface attachment.

SigB is required for wild-type levels of biofilm at 30°C but not at 36°C. PrfA expression is optimal at host physiological temperatures, and the transcription and translation of *prfA* are highly regulated with transcripts from multiple promoters (Fig. 1). As discussed above, transcription from the *PprfA*_{P2} promoter does not rely on PrfA, and the translation of the *PprfA*_{P2}-derived transcript is not temperature dependent. While *PprfA*_{P1} and *PprfA*_{P2} are redundant for virulence in mammalian hosts (11), and *PplcA* is critical (4), it has been postulated that *PprfA*_{P2} might be key for virulence gene expression at lower temperatures, such as in *Drosophila melanogaster* (1, 6, 25). *PprfA*_{P2} contains binding sites for both the housekeeping sigma factor, σ^A , and the stress response sigma factor, σ^B , which overlap and are offset by a few base pairs. We hypothesized that σ^B might influence the production of PrfA at temperatures below mammalian body temperatures and, therefore, biofilm formation in most environments.

with seven replicates of each strain per experiment. Error bars represent SEM. (C) Heterologous expression of *prfA* restores wild-type levels of biofilm formation in the $\Delta prfA$ mutant. For complementation, *prfA* was expressed from the *tRNA*^{Arg} locus by using the pHyper promoter in a transcript containing the 5' UTR from the *hly* gene (cPrfA+) in both the wild type and the $\Delta prfA$ mutant. Biofilms formed at 30°C and 36°C for 3 days. The difference between the $\Delta prfA$ mutant and the complemented *prfA* strain was statistically significant at both temperatures (*, $P < 0.05$ by *t* test). Data are from four experiments with seven replicates of each strain per experiment. Error bars represent SEM.

FIG. 4. *L. monocytogenes* biofilm formation is *sigB* dependent at 30°C but *sigB* independent at 36°C. Quantification of biofilms formed at 30°C and 36°C for 3 days by the wild type (WT) (NF-L1124), the *prfA*::Tn917 mutant (PrfA-) (KPL1720), the Δ *sigB* mutant (SigB-) (KPL1712), and the *prfA*::Tn917 Δ *sigB* double mutant (PrfA- SigB-) (KPL1714) was done by measuring the OD₅₉₀ of solubilized crystal violet. The difference in biofilm formation at 30°C was statistically significant: wild type > *prfA*::Tn917 > both Δ *sigB* and *prfA*::Tn917 Δ *sigB* (i.e., a is different from b, which is different from c, with a *P* value of <0.05 by KW with T). At 36°C, the wild type and the Δ *sigB* mutant were comparable, as were the *prfA*::Tn917 and *prfA*::Tn917 Δ *sigB* mutants, but the two sets of strains differed significantly from each other (i.e., d is different from e, with a *P* value of <0.05 by KW with T). Data are from five experiments with seven replicates of each strain per experiment. Error bars represent SEM.

A mutant containing an in-frame deletion of *sigB* produced wild-type levels of biofilm at 36°C, as measured by a crystal violet assay (Fig. 4, right), but displayed a biofilm defect at 30°C (Fig. 4, left). The Δ *prfA* Δ *sigB* double mutant displayed defective biofilm formation at both temperatures (Fig. 4). Both of these mutants displayed wild-type levels of growth in broth culture (see Fig. S3B in the supplemental material). We postulate that the Δ *sigB* mutant's biofilm defect at 30°C might reflect the importance of PprfA_{P2} for *prfA* expression at lower temperatures and extracellularly.

A PrfA protein activity mutant that is unable to fully transition to the intracellular activity state favors biofilm formation. In addition to transcriptional and translational regulation, PrfA protein activity is also regulated (13, 39). We hypothesized that the conformation of the PrfA protein that positively affects biofilm formation is the extracellular activity form and that it is different from the intracellular form that positively regulates virulence gene expression. To test this hypothesis, we assayed biofilm formation by strains containing *prfA* alleles that produce protein locked in two different activity states: PrfA*, which constitutively expresses virulence genes to levels normally seen only inside host cells, and PrfA(Y154C), which is incapable of completely upregulating virulence gene expression, even inside a host cell (27).

In the biofilm assay, the *prfA*(Y154C) mutant displayed increased biofilm formation at 30°C and with a similar trend at 36°C (Fig. 5A), although it was comparable to that of the wild type at RT (Fig. 5A). The increased level of biofilm formation by the *prfA*(Y154C) mutant was observed by day 2 (data not shown). We observed a slight but statistically significant decrease in the motility of the *prfA*(Y154C) mutant compared to the wild type at 30°C, although this did not appear to impact biofilm formation (Fig. 5B). Growth in broth culture was com-

FIG. 5. The *prfA*(Y154C) mutant forms more biofilm at and above 30°C than the wild type, and the *prfA** mutant forms wild-type biofilm and displays wild-type motility at 30°C and RT. (A) Day 4 biofilm formed by the wild type (WT) (NF-L1124), the Δ *prfA* mutant (P-) (NF-L1123), the *prfA*(Y154C) mutant (Pi) (NF-L1213), and the *prfA** mutant (P*) [*prfA**(L140F)] (NF-L1166) at RT, 30°C, and 36°C. At RT, the wild type, the *prfA*(Y154C) mutant, and the *prfA**(L140F) mutant were comparable, and the difference in biofilms between these strains and the Δ *prfA* mutant was statistically significant (a, *P* < 0.05 by KW with T). At 30°C, the wild type and the *prfA**(L140F) mutant were comparable (b), but the difference with the Δ *prfA* mutant and the *prfA*(Y154C) mutant was statistically significant (c and d, *P* < 0.05 by KW with T). At 36°C, there was a trend toward increased biofilm formation by the *prfA*(Y154C) mutant that did not reach statistical significance (e), but the decrease in biofilm formation by the Δ *prfA* mutant and the *prfA**(L140F) mutant did (f and g, *P* < 0.05 by KW with T). Data are from four experiments with seven replicates of each strain per experiment. Error bars represent SEM. (B) Motility measured as the diameter of the bacterial swarm in millimeters after incubation at RT (~24°C), 30°C, or 36°C for 24 h. At 30°C, the decreased motility of the *prfA*(Y154C) strain compared to the wild type, the Δ *prfA* mutant, and the *prfA**(L140F) mutant was statistically significant (a, *P* < 0.05 by ANOVA with H-S). At 36°C the decreased motility of the *prfA**(L140F) mutant compared to the wild type, the Δ *prfA* mutant, and the *prfA*(Y154C) mutant was statistically significant (b, *P* < 0.05 by KW with T). Data are from 12 independent experiments, and error bars represent SEM.

parable to that of the wild type at all three temperatures (see Fig. S1 in the supplemental material).

PrfA* mutants, which are locked in the intracellular activity form, display wild-type levels of biofilm formation at lower temperatures. In contrast to the *prfA*(Y154C) mutant, three different *prfA** mutants (L140F [44, 47], G145S [37], and Y63C [28]) all displayed both wild-type motility and wild-type levels

of biofilm formation at RT and 30°C [Fig. 5, and see Fig. S1 in the supplemental material for the *prfA*(L140F) mutant; data are not shown for the *prfA*(G145S) or *prfA*(Y63C) mutant]. However, the *prfA** mutants produced less biofilm at 36°C, where they also displayed a motility defect (Fig. 5). The growth of the *prfA** mutant in broth culture was comparable to that of the wild type at all three temperatures (Fig. S1). It is unclear whether the decrease in biofilm formation at 36°C was related to decreased motility or to some other factor(s). However, the wild-type levels of biofilm formation at lower temperatures indicate that the PrfA* form of the protein is not completely lacking in biofilm-promoting activity.

DISCUSSION

In this study, we have demonstrated a new role for the *L. monocytogenes* virulence regulator PrfA in promoting biofilms outside host cells. *L. monocytogenes* has three distinct life-styles: (i) an intracellular pathogen, (ii) a flagellum-propelled extracellular bacterium, and (iii) an extracellular bacterial member of biofilms. The virulence master regulator PrfA is essential for the switch from extracellular flagellum-propelled bacterium to intracellular pathogen. The data presented here are the first to indicate that PrfA also promotes biofilm formation after the transition from extracellular flagellum-propelled bacterium to surface-adhered biofilm former. Thus, PrfA is a global regulator of the life-style of *L. monocytogenes* and provides a regulatory connection between the seemingly mutually exclusive life-styles of biofilm former and intracellular pathogen.

The loss of *prfA* resulted in a biofilm defect in several different strain and serotype backgrounds and was not specific to the commonly used laboratory strain 10403S. The Δ *prfA* mutant displayed wild-type levels of flagellum-mediated motility and initial surface attachment. Based on this finding, PrfA positively impacts biofilm formation at a step in biofilm maturation after surface adhesion.

PrfA expression is highly regulated from multiple promoters. Previous data suggest that extracellularly and at temperatures lower than mammalian body temperatures, the *PprfA*_{P2} promoter is likely to be the primary driver of PrfA expression. *PprfA*_{P2} can be activated by σ^B , and under our conditions, biofilm formation was *sigB* dependent at 30°C but not at 36°C. We speculate that this may be due partly to the effect of σ^B on *prfA* expression. A role for σ^B in biofilm formation at lower temperatures fits with the possibility that biofilm formation might be a response to environmental stress.

In addition to transcriptional regulation, PrfA activity is regulated posttranslationally, and PrfA variants that constitutively mimic the activity of the protein when bacteria are inside host cells have been isolated (PrfA*). While *prfA* is required for wild-type levels of biofilm formation, mutants that produced a PrfA* variant had no impact on biofilm formation at RT and 30°C, although they did demonstrate a modest and statistically significant decrease in biofilm formation at 36°C. This decrease is likely a secondary effect of the decreased motility of the *prfA** mutants at 36°C. *prfA** mutants produce increased levels of the PrfA* protein due to the positive autoregulation of *prfA* transcription (43, 47). Therefore, we propose that the conformation of wild-type PrfA when bacteria

are outside a host cell favors biofilm formation, but either form of PrfA (extracellular or intracellular) is sufficient for biofilm formation.

In contrast to the *prfA** mutant, a mutant with a PrfA variant that is unable to fully transition to the intracellular activity form, PrfA(Y154C), demonstrated a modest but statistically significant increase in biofilm formation at 30°C with a trend in that direction at 36°C. PrfA regulates its own expression, but Western blot analysis with an anti-PrfA antibody showed that, unlike *prfA** mutants, the *prfA*(Y154C) mutant did not produce appreciably more PrfA protein outside host cells than does the wild type (M. Miner and N. E. Freitag, unpublished data). This suggests that PrfA(Y154C) behaves more like the protein conformation that wild-type PrfA assumes when the bacterium experiences biofilm-promoting conditions outside host cells.

While there was an increase in biofilm formation by the *prfA*(Y154C) mutant compared to the *prfA** mutant, it is not nearly as large as the increase in virulence observed for *prfA** mutants in comparison to the *prfA*(Y154C) mutant (27). Therefore, while PrfA clearly has a positive influence on biofilm formation after initial surface attachment, this activity does not appear to be regulated via changes in protein activity in quite the same way, or to quite the same magnitude, as virulence gene expression.

It is not known yet why the loss of *prfA* decreases biofilm maturation. Preliminary data from microarray experiments comparing wild-type 10403S and the Δ *prfA* mutant suggest that the level of expression of *prfA*-dependent virulence genes is significantly increased upon a transition from complex medium (TSBYE) to the defined medium used here to promote biofilm formation (HTM plus 3% glucose) (K. P. Lemon, unpublished data). However, single mutants lacking the virulence gene *actA*, *hly*, *mpl*, or *hpt* and double mutants lacking either *inlA* *inlB* or *plcA* *plcB* did not phenocopy the Δ *prfA* mutant's biofilm defect (Lemon, unpublished). This suggests that none of these virulence factors alone is the PrfA-dependent effector of biofilm formation. However, a subset of these factors (ActA, InlA, and InlB) are cell surface expressed, and a separate subset (listeriolysin O [LLO], encoded by *hly*, and the phospholipases encoded by *plcA* and *plcB*) are secreted proteins; the combined loss of PrfA-dependent cell surface-expressed and secreted virulence factors in the Δ *prfA* mutant might alter the cell surface in a manner detrimental to biofilm formation. Overall, we favor a model in which the extracellular form of the PrfA protein preferentially influences biofilm formation via a hypothetical biofilm-promoting factor(s), whereas the intracellular form preferentially binds to canonical PrfA boxes and massively upregulates the expression of virulence genes in the host cytosol. It will be very interesting to elucidate the molecular mechanism by which PrfA promotes biofilm maturation in future studies.

Individual flagellum-propelled *L. monocytogenes* bacteria appear to be the common precursor to both intracellular invasion and biofilm formation. PrfA has a role in these mutually exclusive life-styles as a critical positive regulator of virulence gene expression and as a promoter of biofilm formation. It is possible that low levels of PrfA in extracellular, motile bacteria would be an advantage and keep the bacteria poised for initial eukaryotic cell adhesion and subsequent invasion as well as for biofilm maturation after adhesion to abiotic surfaces. Similarly,

σ^B , the stress response sigma factor, also has a role in virulence, which is intertwined with that of PrfA (31), and, at temperatures below host body temperature, in biofilm formation (Fig. 4). Thus, under stress-inducing conditions, flagellum-propelled bacteria are prepared for either eukaryotic cell invasion or abiotic surface-associated biofilm formation. This model easily accommodates the role of an intermediate protein activity form of PrfA, characterized by the PrfA(Y154C) mutant, in promoting eukaryotic cell invasion and vacuolar escape as well as biofilm maturation on abiotic surfaces. It may also imply that *L. monocytogenes* in a biofilm has a form of PrfA that is poised for intracellular invasion after ingestion by a mammalian host.

L. monocytogenes is widespread in the natural environment as a saprophyte and can be easily isolated from a number of different sample types (5, 14). It has also been reported to infect a variety of animals (14). We speculate that strains that maintain *prfA* should have a fitness advantage outside host cells in the environment. A role for PrfA in potentiating extracellular biofilm formation may well explain the maintenance of virulence potential in this facultative intracellular pathogen. PrfA's role in both virulence and biofilm formation also suggests that similar key links between virulence regulators and extracellular life-styles might exist for other facultative pathogens.

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REFERENCES

- Agaisse, H., L. S. Burrack, J. A. Philips, E. J. Rubin, N. Perrimon, and D. E. Higgins. 2005. Genome-wide RNAi screen for host factors required for intracellular bacterial infection. *Science* **309**:1248–1251.
- Bishop, D. K., and D. J. Hinrichs. 1987. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. *J. Immunol.* **139**:2005–2009.
- Borucki, M. K., J. D. Peppin, D. White, F. Loge, and D. R. Call. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **69**:7336–7342.
- Camilli, A., L. G. Tilney, and D. A. Portnoy. 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* **8**:143–157.
- Chaturongakul, S., S. Raengpradub, M. Wiedmann, and K. J. Boor. 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol.* **16**:388–396.
- Cheng, L. W., and D. A. Portnoy. 2003. *Drosophila* S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cell. Microbiol.* **5**:875–885.
- Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* **68**:2950–2958.
- Edman, D. C., M. B. Pollock, and E. R. Hall. 1968. *Listeria monocytogenes* L forms. I. Induction maintenance, and biological characteristics. *J. Bacteriol.* **96**:352–357.
- Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* **55**:476–511.
- Freitag, N. E., G. C. Port, and M. D. Miner. 2009. *Listeria monocytogenes*—from saprophyte to intracellular pathogen. *Nat. Rev. Microbiol.* **7**:623–628.
- Freitag, N. E., and D. A. Portnoy. 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential in vitro but are redundant in vivo. *Mol. Microbiol.* **12**:845–853.
- Freitag, N. E., L. Rong, and D. A. Portnoy. 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. *Infect. Immun.* **61**:2537–2544.
- Gray, M. J., N. E. Freitag, and K. J. Boor. 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect. Immun.* **74**:2505–2512.
- Gray, M. L., and A. H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. *Bacteriol. Rev.* **30**:309–382.
- Grundling, A., L. S. Burrack, H. G. Bouwer, and D. E. Higgins. 2004. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. *Proc. Natl. Acad. Sci. U. S. A.* **101**:12318–12323.
- Herler, M., A. Bubert, M. Goetz, Y. Vega, J. A. Vazquez-Boland, and W. Goebel. 2001. Positive selection of mutations leading to loss or reduction of transcriptional activity of PrfA, the central regulator of *Listeria monocytogenes* virulence. *J. Bacteriol.* **183**:5562–5570.
- Hodgson, D. A. 2000. Generalized transduction of serotype 1/2 and serotype 4b strains of *Listeria monocytogenes*. *Mol. Microbiol.* **35**:312–323.
- Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**:551–561.
- Korner, H., H. J. Sofia, and W. G. Zumft. 2003. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol. Rev.* **27**:559–592.
- Lampidis, R., R. Gross, Z. Sokolovic, W. Goebel, and J. Kreft. 1994. The virulence regulator protein of *Listeria ivanovii* is highly homologous to PrfA from *Listeria monocytogenes* and both belong to the Crp-Fnr family of transcription regulators. *Mol. Microbiol.* **13**:141–151.
- Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar. 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. *J. Bacteriol.* **184**:4177–4186.
- Lemon, K. P., D. E. Higgins, and R. Kolter. 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *J. Bacteriol.* **189**:4418–4424.
- Lorber, B. 2005. *Listeria monocytogenes*, p. 2478–2484. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*, 6th ed., vol. 2. Elsevier Churchill Livingstone, Philadelphia, PA.
- Lynch, M., J. Painter, R. Woodruff, and C. Braden. 2006. Surveillance for foodborne-disease outbreaks—United States, 1998–2002. *MMWR Surveill. Summ.* **55**:1–42.
- Mansfield, B. E., M. S. Dionne, D. S. Schneider, and N. E. Freitag. 2003. Exploration of host-pathogen interactions using *Listeria monocytogenes* and *Drosophila melanogaster*. *Cell. Microbiol.* **5**:901–911.
- Mengaud, J., S. Dramsi, E. Gouin, J. A. Vazquez-Boland, G. Milon, and P. Cossart. 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. *Mol. Microbiol.* **5**:2273–2283.
- Miner, M. D., G. C. Port, H. G. Bouwer, J. C. Chang, and N. E. Freitag. 2008. A novel *prfA* mutation that promotes *Listeria monocytogenes* cytosol entry but reduces bacterial spread and cytotoxicity. *Microb. Pathog.* **45**:273–281.
- Miner, M. D., G. C. Port, and N. E. Freitag. 2008. Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA. *Microbiology* **154**:3579–3589.
- Moretro, T., and S. Langsrud. 2004. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. *Biofilms* **1**:107–121.
- Nadon, C. A., B. M. Bowen, M. Wiedmann, and K. J. Boor. 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. *Infect. Immun.* **70**:3948–3952.
- Ollinger, J., B. Bowen, M. Wiedmann, K. J. Boor, and T. M. Bergholz. 2009. *Listeria monocytogenes* sigma B modulates PrfA-mediated virulence factor expression. *Infect. Immun.* **77**:2113–2124.
- O'Neil, H. S., and H. Marquis. 2006. *Listeria monocytogenes* flagella are used for motility, not as adhesins, to increase host cell invasion. *Infect. Immun.* **74**:6675–6681.
- Palmer, M. E., M. Wiedmann, and K. J. Boor. 2009. Sigma(B) and sigma(L) contribute to *Listeria monocytogenes* 10403S response to the antimicrobial peptides SdpC and nisin. *Foodborne Pathog. Dis.* **6**:1057–1065.
- Pizarro-Cerda, J., and P. Cossart. 2006. Subversion of cellular functions by *Listeria monocytogenes*. *J. Pathol.* **208**:215–223.
- Quisel, J. D., W. F. Burkholder, and A. D. Grossman. 2001. In vivo effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. *J. Bacteriol.* **183**:6573–6578.
- Rauch, M., Q. Luo, S. Muller-Altroch, and W. Goebel. 2005. SigB-dependent in vitro transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. *J. Bacteriol.* **187**:800–804.
- Ripio, M. T., G. Dominguez-Bernal, M. Lara, M. Suarez, and J. A. Vazquez-Boland. 1997. A Gly145Ser substitution in the transcriptional activator PrfA causes constitutive overexpression of virulence factors in *Listeria monocytogenes*. *J. Bacteriol.* **179**:1533–1540.
- Schwab, U., B. Bowen, C. Nadon, M. Wiedmann, and K. J. Boor. 2005. The *Listeria monocytogenes prfA*P2 promoter is regulated by sigma B in a growth phase dependent manner. *FEMS Microbiol. Lett.* **245**:329–336.
- Scortti, M., H. J. Monzo, L. Lacharme-Lora, D. A. Lewis, and J. A. Vazquez-Boland. 2007. The PrfA virulence regulon. *Microbes Infect.* **9**:1196–1207.

40. Shetron-Rama, L. M., K. Mueller, J. M. Bravo, H. G. Bouwer, S. S. Way, and N. E. Freitag. 2003. Isolation of *Listeria monocytogenes* mutants with high-level in vitro expression of host cytosol-induced gene products. *Mol. Microbiol.* **48**:1537–1551.
41. Todhanakasem, T., and G. M. Young. 2008. Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. *J. Bacteriol.* **190**:6030–6034.
42. Tsai, H. N., and D. A. Hodgson. 2003. Development of a synthetic minimal medium for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **69**:6943–6945.
43. Vega, Y., C. Dickneite, M. T. Ripio, R. Bockmann, B. Gonzalez-Zorn, S. Novella, G. Dominguez-Bernal, W. Goebel, and J. A. Vazquez-Boland. 1998. Functional similarities between the *Listeria monocytogenes* virulence regulator PrfA and cyclic AMP receptor protein: the PrfA* (Gly145Ser) mutation increases binding affinity for target DNA. *J. Bacteriol.* **180**:6655–6660.
44. Vega, Y., M. Rauch, M. J. Banfield, S. Ermolaeva, M. Scortti, W. Goebel, and J. A. Vazquez-Boland. 2004. New *Listeria monocytogenes* *prfA** mutants, transcriptional properties of PrfA* proteins and structure-function of the virulence regulator PrfA. *Mol. Microbiol.* **52**:1553–1565.
45. Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag, and C. B. Wilson. 2004. Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. *Cell. Microbiol.* **6**:235–242.
46. Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor. 1998. General stress transcription factor sigma B and its role in acid tolerance and virulence of *Listeria monocytogenes*. *J. Bacteriol.* **180**:3650–3656.
47. Wong, K. K., and N. E. Freitag. 2004. A novel mutation within the central *Listeria monocytogenes* regulator PrfA that results in constitutive expression of virulence gene products. *J. Bacteriol.* **186**:6265–6276.