Identification of *Listeria monocytogenes* Genes Contributing to Oxidative Stress Resistance under Conditions Relevant to Host Infection

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ABSTRACT The Gram-positive bacterium *Listeria monocytogenes* survives in environments ranging from the soil to the cytosol of infected host cells. Key to *L. monocytogenes* intracellular survival is the activation of PrfA, a transcriptional regulator that is required for the expression of multiple bacterial virulence factors. Mutations that constitutively activate *prfA* (*prfA*^{*} mutations) result in high-level expression of multiple bacterial virulence factors as well as the physiological adaptation of *L. monocytogenes* for optimal replication within host cells. Here, we demonstrate that *L. monocytogenes* *prfA*^{*} mutants exhibit significantly enhanced resistance to oxidative stress in comparison to that of wild-type strains. Transposon mutagenesis of *L. monocytogenes* *prfA*^{*} strains resulted in the identification of three novel gene targets required for full oxidative stress resistance only in the context of PrfA activation. One gene, *Imo0779*, predicted to encode an uncharacterized protein, and two additional genes known as *cbpA* and *ygbB*, encoding a cyclic di-AMP binding protein and a 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, respectively, contribute to the enhanced oxidative stress resistance of *prfA*^{*} strains while exhibiting no significant contribution in wild-type *L. monocytogenes*. Transposon inactivation of *cbpA* and *Imo0779* in a *prfA*^{*} background led to reduced virulence in the liver of infected mice. These results indicate that *L. monocytogenes* calls upon specific bacterial factors for stress resistance in the context of PrfA activation and thus under conditions favorable for bacterial replication within infected mammalian cells.

KEYWORDS CbpA, *Listeria monocytogenes*, PrfA, YgbB, oxidative stress, peroxide

Listeria monocytogenes is a Gram-positive bacterium that is widespread in the outside environment as well as in habitats where survival depends on the organism's ability to mitigate a variety of stresses, including fluctuations in temperature, salinity, and pH (1, 2). One important and medically relevant habitat occupied by *L. monocytogenes* is the cytosol of infected mammalian cells, where intracellular replication of bacteria occurs within susceptible hosts and can result in devastating disease (3). *L. monocytogenes* is primarily a foodborne pathogen, and to reach its intracellular replication niche the bacterium must survive passage through the gastrointestinal (GI) tract and translocation across the intestinal barrier before reaching additional tissues for replication (4, 5). As a result, *L. monocytogenes* encounters numerous stress conditions that span large variations in pH and osmolarity as well as bacterial exposure to degradative enzymes (such as lysozyme) and reactive oxygen and nitrogen species. Importantly, a number of bacterial gene products associated with stress resistance in *L. monocytogenes* have been identified and linked to successful survival in the environment and to intragastric survival; however, less is known regarding how the bacterium manages stresses encountered during systemic infection (2, 6).

Oxidative stress is often considered to represent a significant host defense against

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pathogen invasion of host cells and tissues (7, 8). Professional phagocytes such as macrophages limit pathogen viability via the oxidative burst delivered following the assembly of the NADPH oxidase complex on the phagosome; however, *L. monocytogenes* has been reported to be capable of antagonizing complex assembly (9). In addition to oxidative stress encountered during host infection, *Listeria*, like all aerobes, generates its own sources of oxidative stress via aerobic metabolism and has evolved mechanisms to protect itself. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are generated during oxidative respiration and *L. monocytogenes* employs superoxide dismutase (SOD) and catalase, respectively, to neutralize these reactive species. Both O_2^- and H_2O_2 can react further with free heavy metals to form the highly potent hydroxyl ($OH^\cdot$) radical, capable of reacting with most macromolecules (10). Other gene products reported to contribute to *L. monocytogenes* oxidative stress resistance include the metalloregulatory protein PerR and 2-Cys peroxiredoxin (Prx) (11–13).

Recent evidence suggests that *L. monocytogenes* uses redox-responsive transcription factors to coordinate virulence factor expression within the host and that the redox environment of bacteria within host cells differs significantly from the environment for bacteria grown in broth culture (14, 15). The transition of *L. monocytogenes* from life as a saprophyte to life within host cells is primarily mediated by the virulence regulator PrfA, which activates the expression of core virulence factors required for growth in response to increased glutathione levels within the host cell (14). PrfA coordinates virulence factor gene expression while also modulating *L. monocytogenes* physiology to adapt bacteria to conditions favorable for intracellular replication (16–19). PrfA activity is maintained at a low basal level in the outside environment, but the protein becomes highly activated upon cytosol access (14, 16–22). Ripio et al. (23) identified a mutation within *prfA* (*prfA*^{*}) that resulted in the constitutive activation of PrfA and high-level expression of PrfA-regulated genes in broth-grown cultures; since this original description, a number of other amino acid substitutions have been identified that confer differing levels of PrfA activation (21, 24–28). The isolation of these various *prfA*^{*} mutant strains has enabled detailed characterization of the effects of PrfA activation on both patterns of *L. monocytogenes* gene expression and bacterial physiology (16, 17, 29, 30). The constitutive activation of PrfA increases the susceptibility of *L. monocytogenes* to selected forms of stress, such as high salinity and low pH, while simultaneously optimizing growth on host-relevant carbon sources (16, 31). As a result of these physiological changes, *prfA*^{*} strains exhibit reduced fitness in comparison to that of wild-type bacteria in broth culture but are hypervirulent and have a competitive advantage over wild-type bacteria in mouse models of infection (16, 32).

Here, we demonstrate that *L. monocytogenes prfA*^{*} strains are significantly more resistant to oxidative stress than wild-type strains, a phenotype that may contribute to their competitive advantage within the infected host. The increased resistance to oxidative stress appears to be due at least in part to gene products that uniquely contribute to resistance during PrfA activation, with little to no contribution detected in the absence of PrfA activation. Our results suggest that *L. monocytogenes* relies on multiple mechanisms for stress resistance in a manner dependent on environmental status, with subsets of gene products contributing to aspects of stress resistance specific to conditions of PrfA activation.

RESULTS

Activation of PrfA via *prfA*^{*} confers increased resistance of *L. monocytogenes* to oxidative stress. During the course of host infection, *L. monocytogenes* is anticipated to encounter multiple exposures to oxidative stress (11, 15, 33). Given that activation of the central virulence regulator PrfA has been shown to increase bacterial fitness during host infection (16), we sought to determine whether constitutively activated *prfA*^{*} strains exhibited any changes in resistance to oxidative stress in comparison to wild-type bacteria. An assay designed to assess killing by peroxide exposure *in vitro* was used to compare the oxidative stress resistance of wild-type, *prfA*^{*}, and $\Delta prfA$ strains. Strains were incubated in brain heart infusion (BHI) medium containing 110 mM H_2O_2 ,

FIG 1 A *prfA** strain is more resistant to H_2O_2 -mediated killing *in vitro*. Strains were grown to mid-log phase and adjusted to an OD_{600} of 0.2. A total of $890 \mu l$ of culture was transferred to a 1.5-ml microcentrifuge tube and combined with $110 \mu l$ of freshly diluted 1 M H_2O_2 solution in BHI. Tubes were incubated statically for 2 h at room temperature and plated on LB agar for viable bacteria at 30-min intervals (A) or after 2 h (endpoint assays) (B). Data are representative of those from four independent experiments.

and aliquots were removed at 30, 60, 90, and 120 min to determine bacterial viability by plating for CFU on LB agar. *L. monocytogenes prfA** strains were significantly more resistant to H_2O_2 exposure than wild-type or $\Delta prfA$ strains, with up to 90% of bacteria remaining viable after 2 h of exposure to H_2O_2 (Fig. 1A). In contrast, no viable CFU were observed following 90 min of peroxide exposure for wild-type cells, and the $\Delta prfA$ mutant was even more susceptible, with no viable colonies recovered after 1 h. Resistance to peroxide exposure was influenced by culture density, such that wild-type 10403S required a nearly 2-fold-greater cell density in the initial inoculum to withstand peroxide exposure than did the *prfA** mutant (Fig. 1B). Culture density is relevant given that molecules and/or enzymes that scavenge or neutralize reactive oxygen species (ROS) (such as secreted catalase) would be anticipated to confer increased resistance to a population of cells at a high cell density. It is therefore of interest that *prfA** strains remain resistant to ROS at cell densities that are approximately half of those observed for wild-type strains. Despite differential susceptibilities to peroxide exposure, wild-type and *prfA** strains exhibited no difference in catalase activity (see Fig. S1A in the supplemental material). These data indicate that activation of PrfA results in an enhancement of the ability of *L. monocytogenes* to survive exposure to oxidative stress.

Identification of transposon insertion mutations that reduce the resistance of *L. monocytogenes prfA strains to oxidative stress *in vitro*.** The ability of *prfA** strains to better withstand exposure to H_2O_2 suggested that PrfA activation influenced the expression of gene products contributing to oxidative stress resistance. We therefore sought to identify gene products that contribute to bacterial survival following H_2O_2 exposure by screening for transposon insertions mutants with reduced oxidative stress resistance in the *prfA** background (Fig. 2). Mariner transposon random insertion libraries were constructed in *L. monocytogenes prfA** strains containing transcriptional fusions of *gus* and *neo* to the PrfA-dependent *actA* gene (*actA-gus-neo*) (24). The *actA-gus-neo* fusion allows selection of PrfA* activity based on increased expression of *gus*-encoded β -glucuronidase activity and *neo*-encoded neomycin resistance. Individual

FIG 2 Screening of *L. monocytogenes* *prfA** transposon insertion mutant libraries for increased sensitivity to hydrogen peroxide. Two independently generated *prfA** transposon libraries were generated and randomly screened for increased sensitivity to 40 mM H₂O₂ in BHI medium. Individual mutants were picked into 200 μ l of BHI medium in 96-well plates and grown at 37°C statically overnight. The following day, cell OD₆₀₀s were recorded and 10 μ l of each culture was transferred in duplicate to new 96-well plates containing 190 μ l of 40 mM H₂O₂ in BHI. These plates were grown statically at 37°C for 48 h, and mutants that failed to grow were rescreened for susceptibility. Transposon insertion sites were determined with inverse PCR and Sanger sequencing.

transposon insertion mutants were isolated on BHI plates containing 2 μ g ml⁻¹ of erythromycin to select for the transposon insertion and 10 μ g ml⁻¹ of neomycin to select for the retention of PrfA* activity. Isolated mutants were then inoculated into BHI medium with 10 μ g ml⁻¹ of neomycin in 96-well plates and grown statically at 37°C overnight, then subcultured into BHI and neomycin and 40 mM H₂O₂, and grown at 37°C for 48 h (the reduced concentration of H₂O₂ for the screen permitted growth of the *prfA** mutants but not the wild-type *L. monocytogenes* strains). Transposon insertion mutants that failed to replicate in the presence of H₂O₂ were selected and retested to confirm increased sensitivity to H₂O₂ while maintaining PrfA* activity as assessed by neomycin resistance and blue colony color on beta-glucuronidase indicator plates.

Twenty-five mutants with increased sensitivity to hydrogen peroxide *in vitro* were identified after screening of more than 7,500 transposon insertion mutant colonies. After the elimination of candidate mutants that exhibited growth defects in BHI broth with no H₂O₂ selection, three mutants were identified for further characterization and transposon mapping.

Identification of three genes associated with *prfA-dependent resistance to oxidative stress.** Transposon insertion sites were identified within the three individual mutants (Fig. 3A). Two mutants contained insertions within predicted open reading frames: *LMRG_00235* (or *Imo0553*, using EGDe notation) and *LMRG_02671* (*Imo0236*), and one in the immediate upstream region and predicted promoter region shared by *LMRG_00467* (*Imo0779*) and *LMRG_00468* (*Imo0780*) (Fig. 3A). *Imo0236*, also known as *ygbB* or *ispF*, encodes the enzyme 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC or MECPP) synthase, part of the alternative 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid synthesis in bacteria (34). The MEP pathway has been associated with *L. monocytogenes* bile resistance (35), and loss of some pathway enzymes has been reported to reduce bacterial virulence in a mouse model of systemic infection (34, 36). The functions of the *Imo0779/Imo0780* predicted gene products are not known; however, the *Imo0553* gene product, also known as CbpA, has been identified as a binding receptor for the signaling molecule cyclic di-AMP (c-di-AMP),

FIG 3 Phage transduction to introduce transposon insertions into clean genetic backgrounds and confirm mutant phenotypes. (A) Location of each transposon insertion. Transposon insertions in *cbpA* (B), *lmo0779* (C), and *ygbB* (D) were introduced into both 10403S and *prfA** background strains as described in Materials and Methods. Strains were tested for peroxide sensitivity by incubation in BHI containing 110 mM H₂O₂ over 2 h. Viable CFU were enumerated at the indicated time points by plating on BHI agar. Standard growth curves in BHI at 37°C with shaking were performed (E). The data are representative of those from three independent experiments.

associated with *L. monocytogenes* metabolism and cell wall synthesis (37, 38). CbpA contains a cystathionine- β -synthase domain which binds c-di-AMP and an ACT small-molecule binding domain of unknown specificity (37). The function of this protein *in vivo* remains unclear. Structural predictions using the Phyre2 protein fold prediction server (39) predicted that the *lmo0779* gene product is membrane bound, with potential nitrite/sulfite reductase activity in the C-terminal cytoplasmic domain, and that the *lmo0780* gene product may have DNA binding activity. All three transposon insertion mutants demonstrated a marked increase in sensitivity to H₂O₂ in the context of *prfA**

FIG 4 Gene complementation restores oxidative stress resistance to transposon insertion mutants *in vitro*. Transduced transposon mutants were complemented in single copy as described in Materials and Methods. The transposon insertion mutants and the complemented strains were incubated statically at room temperature for 2 h and viable CFU were enumerated via plating on LB agar. Data are representative of those from three independent experiments.

(Fig. 3B to D) while exhibiting similar patterns of growth in BHI medium (Fig. 3E) and no significant change in catalase activity (Fig. S1B). The phenotype of each individual transposon insertion was confirmed following transduction into a clean *prfA** *actA-gus-neo* genetic background. In addition, to assess the insertion mutant phenotypes in the absence of PrfA activation, each mutant was also transduced into the 10403S parent strain. All of the transposon insertions were found to recapitulate the original H_2O_2 -sensitive phenotype when introduced back into *prfA** (Fig. 4). Interestingly, none of the transposon insertions increased wild-type sensitivity to H_2O_2 , indicating that the enhanced sensitivity to oxidative stress was observable only in the context of PrfA activation. Complementation of mutant phenotypes was achieved via the introduction of the wild-type copy of each gene (*lmo0779* for the promoter insertion), confirming the role of each in enhancing *prfA** sensitivity to oxidative stress (Fig. 4). Interestingly, an

FIG 5 PrfA activation does not alter expression of candidate peroxide resistance genes or catalase. $prfA^*$, $\Delta prfA$, and wild-type strains were grown to mid-log phase and RNA was extracted. qRT-PCR was performed using primers listed in Table 1 to determine the transcript levels of *cbpA*, *lmo0779*, *ygbB*, *kat*, *prfA*, and PrfA-regulated gene *actA* relative to the 16S rRNA. ****, $P < 0.0001$ (two-factor ANOVA with the Tukey MCT). Data are representative of those from three independent experiments.

assessment of *cbpA*, *lmo0779*, and *ygbB* transcript levels indicates that the gene products are not subject to PrfA regulation, nor is the gene encoding catalase (*kat*) (Fig. 5).

Loss of peroxide resistance reduces *L. monocytogenes prfA** virulence in mice.

Resistance of *L. monocytogenes* to oxidative stress is predicted to contribute to bacterial viability during host infection (11, 15, 33, 40, 41). We have previously demonstrated that $prfA^*$ strains are hypervirulent in mouse models of infection (16); therefore, we sought to determine how the transposon insertions that increase the sensitivity of $prfA^*$ strains to oxidative stress might potentially influence bacterial virulence. Female 6- to 8-week-old Swiss Webster mice were infected via tail vein injection with either $prfA^*$ or the transposon insertion mutants in the $prfA^*$ background. Virulence *in vivo* was assessed by the measurement of bacterial burdens in target organs at 72 h postinfection (Fig. 6, top row). All strains exhibited comparable splenic burdens at this time point. In contrast, strains containing the transposon insertion within *cbpA* or *lmo0779* exhibited significantly lower bacterial burdens within the liver than did the $prfA^*$ strain, with an approximately 10-fold drop in median CFU per organ. The strain containing an insertion in *ygbB* showed a similar trend, but the reduction in bacterial numbers was not statistically significant.

The defects in virulence observed for strains containing transposon insertions within *cbpA* and *lmo0779* could be linked to the reduced resistance of $prfA^*$ strains containing these mutations to oxidative stress or could play an independent role in *L. monocytogenes* virulence regardless of the *prfA* mutation. We therefore examined the virulence of strains containing transposon insertions in *cbpA*, *lmo0779*, or *ygbB* in the context of the wild-type *prfA* allele (Fig. 6, bottom row). In contrast to findings in the $prfA^*$ strains, the disruption of these genes in a wild-type background had no significant effect on bacterial burdens in either the spleen or the liver in comparison to wild-type *L. monocytogenes*. The loss of *cbpA* and *lmo0779* therefore appeared to reduce the virulence of $prfA^*$ strains in the context of liver infection while having little to no impact of bacterial colonization of $prfA^*$ mutants within the spleen or within either organ in the context of the wild-type *prfA* allele. The loss of *cbpA*, *lmo0779*, and/or *ygbB* can therefore dramatically reduce the enhanced oxidative resistance of $prfA^*$ strains without a substantial impact on the virulence of wild-type strains during mouse infection.

DISCUSSION

The activation of the *L. monocytogenes* virulence regulator PrfA is central to the ability of this bacterium to transition from a saprophyte into an intracellular pathogen within mammalian hosts (1, 21). PrfA is required for the expression of multiple gene

FIG 6 Loss of peroxide resistance impacts virulence in *PrfA*^{*} strains but not wild-type strains. (A) Six- to-8-week-old female Swiss Webster mice were infected via tail vein injection with 1×10^4 CFU of either *prfA*^{*} or each *prfA*^{*} transposon insertion mutant as indicated. Burdens were assessed 72 h postinfection. *, $P < 0.05$ Kruskal-Wallis test with Dunn's MCT. (B) Six- to-8-week-old female Swiss Webster mice were infected via tail vein injection with 1×10^4 CFU of either 10403S or each 10403S transposon insertion mutant as indicated. Burdens were assessed 72 h postinfection. Kruskal-Wallis test identified no significant differences. Data are representative of those from two independent experiments.

products that promote bacterial invasion of host cells, replication within the cytosol, and spread to adjacent cells during infection (19). The characterization of constitutively activated *prfA*^{*} mutants has revealed the scope and breadth of this regulator's influence on virulence factor expression, bacterial metabolism, and adaptation to periods of nutrient starvation (16, 17, 23–25, 27, 28, 30, 32, 42). Here, we demonstrate that *PrfA* activation adds an additional facet to *L. monocytogenes* survival by conferring enhanced resistance to oxidative stress. This enhanced resistance relies on gene products whose activity was most notable under conditions of *PrfA* activation, as loss of these products in wild-type strains resulted in no significant changes in peroxide resistance. It thus appears that specific gene products may uniquely contribute to *L. monocytogenes* stress resistance under conditions of *PrfA* activation without themselves being *PrfA* regulated.

L. monocytogenes resistance to oxidative stress was influenced by bacterial density for both wild-type and *prfA*^{*} strains (Fig. 1B). Approximately 40% fewer *prfA*^{*} bacteria than wild-type bacteria were required to survive peroxide challenge, suggesting that *prfA*^{*} strains are better able to tolerate exposure to specific concentrations of reactive

oxygen species. The basis for this resistance is not known, but *prfA** strains have been shown to exhibit altered surface properties leading to bacterial aggregation, a situation that may increase local concentrations of bacteria and perhaps limit the exposure of certain members of the population to oxygen radicals.

We identified the sites of transposon insertion for three selected mutants that demonstrated increased sensitivity to peroxide exposure, implicating the gene products of *lmo0779*, *cbpA*, and *ygbB* in oxidative stress resistance under conditions of PrfA activation. None of these three gene products have been previously implicated in oxidative stress resistance, most likely because none of the gene products appear to contribute to the oxidative stress resistance of wild-type strains. The insertion of the transposon in the 5' upstream region of *lmo0779* has the potential to also influence the expression of the divergently transcribed gene *lmo0780*; however, complementation studies indicated that the introduction of a wild-type copy of *lmo0779* was sufficient to restore resistance to *prfA** levels. The predicted *lmo0779* gene product has no known homologues; however, structural predictions using Phyre2 (39) suggest that Lmo0779 is a putative membrane protein that may feature a cytosolic C-terminal nitrite or sulfite reductase domain, making it likely to be a metal binding enzyme important for nitrogen or sulfur assimilation, respectively. Lmo0779 has a single cysteine residue, and it is possible that this membrane protein may be part of a signal relay that coordinates oxidative stress resistance. While highly conserved among *Listeria* species with >80% identity among sequenced isolates, *lmo0779* gene product homologues are not found among other bacteria.

The *cbpA* gene product was recently identified as a *L. monocytogenes* high-affinity cyclic di-AMP (c-di-AMP) receptor protein (43). The protein contains a CBS domain, which binds c-di-AMP, and an ACT domain, which can bind a variety of small molecules (37, 44). The function of CbpA and the ligand of its ACT domain remain unstudied, but CbpA may be involved the sensing or regulation of c-di-AMP. It has been suggested that PrfA activation leads to elevated c-di-AMP levels through inhibition of c-di-AMP phosphodiesterases, though this relationship has not been directly demonstrated (45). c-di-AMP has been shown to regulate *L. monocytogenes* metabolism, resistance to cell wall-targeting antibiotics, and osmoregulation. Intriguingly, one of the principal functions of c-di-AMP signaling is to control the flux of pyruvate into the citric acid cycle through allosteric inhibition of pyruvate carboxylase (37). Since pyruvate is also the starting substrate for MEP isoprenoid synthesis, increased c-di-AMP might favor increased activity of that pathway, which includes another identified gene, *ygbB* (34).

The *ygbB* gene product has also been associated with bacterial metabolism, as it encodes the fifth of seven enzymes in the nonmevalonate (MEP) pathway of isoprenoid biosynthesis (34). *L. monocytogenes* is exceptional among pathogens in possessing both the classical and nonmevalonate pathways for the end product isopentenyl pyrophosphate (IPP), which is essential for growth and survival (46). Isoprenoids and their biosynthetic pathways have only recently begun to be appreciated in bacteria (47, 48) and their derivatives have been implicated in oxidative stress resistance (49). Ostrovsky et al. (49) reported the accumulation of a MEP pathway intermediate, 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC or MECPP), in response to benzyl viologen-induced oxidative stress, and stress-resistant mutants accumulated more MEC than sensitive strains. YgbB is the enzyme that generates MEC (34); thus, it is possible that the reduction in *L. monocytogenes* resistance to peroxide is due to a lack of MEC accumulation resulting from the loss of YgbB. The MEP pathway has been previously shown to be required for *L. monocytogenes* virulence in mice (34), and the current study confirms an association of the MEP pathway with oxidative stress and with virulence; whether these phenotypes are linked or distinct from one another remains to be determined.

Perhaps notable is the absence of insertions detected within the gene encoding catalase (*kat*), an enzyme associated with detoxifying peroxide (8). Our finding that PrfA activation does not alter catalase activity further supports the notion that it is not

involved in PrfA-mediated peroxide resistance. We also note a recent report that other redox enzymes, such as superoxide dismutase and glutathione peroxidase, but not *kat* are upregulated in wild-type *L. monocytogenes* subjected to peroxide stress (50). In addition, the loss of catalase does not appear to affect *L. monocytogenes* virulence in mouse models of infection, and catalase-negative strains have been isolated from human infections (51). These findings suggest a model in which catalase detoxifies peroxide produced by normal respiration but during other periods of peroxide stress, such as potentially found during infection, other mechanisms promote bacterial survival.

The *in vivo* observation that the transposon insertion mutants exhibited reduced virulence in the liver in the presence of the *prfA** allele (Fig. 6) may provide some insight into the role of peroxide resistance during infection. Neutrophils, which produce large amounts of ROS and RNS, have been found to be particularly important for defense against *L. monocytogenes* in the liver (52–54, 63), a finding that may explain why PrfA-mediated peroxide resistance is more important for bacterial infection within the liver than within the spleen. Mice infected with *prfA** strains have previously been shown to have increased bacterial burdens in the liver in comparison to those of mice infected with wild-type *L. monocytogenes* (16); thus, the contributions of *cbpA*, *Imo0779*, and *ybgB* to oxidative stress resistance may be visible only in this context. The enhanced resistance of *prfA** strains to oxidative stress may thus explain the increased fitness of these mutants within the liver, given that mutations that reduce peroxide resistance of *prfA** strains to wild-type levels resulted in bacterial numbers of *prfA** strains in the liver that were comparable to those of wild-type strains.

Taken together, our results reveal yet another facet of *L. monocytogenes* physiology that is influenced by the central virulence regulator PrfA, that being enhanced bacterial resistance to oxidative stress under conditions of PrfA activation. Given the recent evidence suggesting that *L. monocytogenes* uses redox-responsive transcription factors to coordinate virulence factor expression within the host, and that PrfA itself binds a reducing agent (14, 15), it would appear that this bacterium is well positioned for both redox sensing and survival during the course of host infection.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (55). Female outbred Swiss Webster mice, 6 to 8 weeks of age (Charles River Labs, Boston, MA), were subjected to a 12-h light/dark cycle with free access to food and water. Animals were carefully monitored twice daily for signs of distress (unkempt appearance, hunched posture, and lethargy), and humane endpoints were used in all experiments such that any animal exhibiting severe discomfort, an inability to move around or attain food, water, etc., was euthanized immediately. Euthanasia was carried out via CO₂ inhalation from a bottled source followed by cervical dislocation. Animal suffering and distress were minimized by monitoring the animals as described above and in that tail vein injections were carried out with only brief periods (<5 min) of physical restraint.

Bacterial strains, media, and culture conditions. *L. monocytogenes* 10403S (NF-100) (56), 10403S *prfA* L140F (*prfA** *actA-gus-neo*, NF-L1166) (24), and 10403S Δ *prfA* (NF-L890) (57) were used in this study. All bacterial strains were grown overnight at 37°C with shaking in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI). Antibiotic concentrations were as follows: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 5 μ g ml⁻¹; erythromycin, 2 μ g ml⁻¹; lincomycin, 25 μ g ml⁻¹; neomycin, 10 μ g ml⁻¹; and streptomycin, 200 μ g ml⁻¹.

Oxidative stress assay. Strains were grown overnight at 37°C with shaking in BHI broth, then subcultured by diluting cultures 1:20 into fresh BHI broth the following day, and grown to mid-log phase. The optical density at 600 nm (OD₆₀₀) for each strain was adjusted to 0.2. Hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) was diluted in fresh BHI broth to create a 1 M working solution. In a 1.5-ml microcentrifuge tube, 890 μ l of culture was mixed with 110 μ l of BHI plus 1 M H₂O₂ and incubated at room temperature for 2 h. Viable bacteria were enumerated by serial dilution in 1 $\times$ sterile phosphate-buffered saline (PBS), plated on LB agar, and incubated at 37°C overnight.

Quantitative catalase assay. Quantitative catalase assay was adapted from the procedure described by Iwase et al. (58). Strains were grown overnight at 37°C with shaking in BHI broth, then subcultured by diluting cultures 1:20 into fresh BHI broth the following day, and grown to mid log phase. The OD₆₀₀ for each stain was adjusted to 1 by centrifugation of an appropriate volume of culture and resuspension of the pellet in PBS. To measure enzyme activity, 10 μ l of bacterial suspension was mixed with 10 μ l of 1% Triton X-100 and 10 μ l of 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) in a 13-mm by 100-mm borosilicate test tube. After 5 min, the height of the column of bubbles produced in

the tube by the generation of O_2 from H_2O_2 was measured. To enable relative comparison of enzyme activity, a standard curve was generated using 2 to 20 μ l of the wild-type suspension.

Construction of a random Mariner transposon insertion library. The *L. monocytogenes* *prfA** strain (NF-L1166) was made electrocompetent as previously described (59). *Escherichia coli* (NF-E1130) containing the Mariner transposase driven by the *Bacillus subtilis* *mrgA* promoter on pMC38 (60) was grown on BHI agar containing 5 μ g ml^{-1} of chloramphenicol overnight and then grown in LB broth. Plasmid pMC38 was purified using the Qiagen (Frederick, MD) plasmid midi-kit. Plasmid pMC38 was introduced into electrocompetent *L. monocytogenes* 10403S *prfA** using a chilled 1-mm electroporation cuvette (Bio-Rad, Hercules, CA) and pulsed at 10 kV/cm, 400 Ω , and 25 μ F. Cells were immediately resuspended in 1 ml of fresh BHI broth containing 500 mM sucrose and incubated statically at 30°C for 1.5 h. Transformants were selected on BHI agar containing 2 μ g ml^{-1} of erythromycin. Two independent transformations were carried out to generate two separate independent libraries. Transformants were then grown in fresh BHI broth overnight at 30°C while shaking. Cultures were diluted 1:200 in fresh BHI broth, grown with shaking at 30°C for 1 h, and then shifted to 40°C with shaking for an additional 6 h until the OD_{600} registered between 0.3 and 0.5. Cultures were then plated on BHI agar plus 2 μ g ml^{-1} of erythromycin, with the remainder frozen in 1-ml aliquots with 20% sterile glycerol.

Screening of transposon libraries. Frozen library aliquots were thawed and plated on BHI agar containing 2 μ g ml^{-1} of erythromycin and 10 μ g ml^{-1} of neomycin (to select for *PrfA* activation as measured by *actA-gus-neo* expression) and grown overnight at 37°C. Individual colonies were picked into 96-well plates containing 200 μ l of BHI broth and grown overnight at 37°C without shaking. The following day, optical densities were measured and 10 μ l of each isolate culture was transferred in duplicate to a new 96-well plate containing 190 μ l of BHI broth with 40 mM H_2O_2 . Cultures were grown for 48 h at 37°C statically and assessed for growth. Isolates failing to grow were retested under the same conditions before being verified individually. For GUS indicator media plates, a filtered solution of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (XG; Inalco) was added just before pouring to a final concentration of 50 mg ml^{-1} .

Phage transduction. Bacteriophage U153 was used for phage transduction experiments. Susceptible mutants were grown overnight at 37°C without shaking in 2 ml of BHI plus 2 μ g ml^{-1} of erythromycin and 10 μ g ml^{-1} of neomycin and then subcultured 1:10 in 2 ml of fresh BHI broth. Cultures were grown for 3 h at 30°C with shaking. In sterile 12-by-75-mm culture tubes, *Listeria* mutant cultures were mixed with phage in several ratios from 1:1 to 100:1 and incubated at room temperature statically for 40 min. Three milliliters of liquid LB soft agar (0.75% agar, 10 mM $CaCl_2$, 10 mM $MgSO_4$) was added to each tube, mixed, and then poured over a prewarmed LB plate. Plates were grown overnight at room temperature. Two milliliters of TM buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgSO_4$) was added to the plate with confluent plaques for each mutant. After 20 min, the soft agar with TM buffer was scraped into a 15-ml centrifuge tube (Denville Scientific, South Plainfield, NJ) with a flame-sterilized scoop and centrifuged at 8,000 rpm for 15 min. The supernatant was collected into a sterile microcentrifuge tube and 1/5 volume of chloroform was added. The solution was incubated at room temperature with rocking for 30 min. The recovered phage suspensions were incubated with either the 10403S or NF-L1166 (*prfA**) strain for the recovery of transductants containing the selected transposon insertion as follows. *Listeria* strains for phage transduction were grown overnight at 37°C with shaking, subcultured 1:40 in fresh BHI the following day, and grown at 30°C shaking to an OD of ~ 0.19 . In separate tubes, 200 μ l of *Listeria* was mixed with 100 μ l of the phage library, plus 10 mM $CaCl_2$ and 10 mM $MgSO_4$, and incubated at room temperature for 40 min with gentle shaking every 10 min. Each tube was then supplemented with 200 μ l of fresh BHI and incubated at 37°C with shaking for 2 h. The cultures were plated on BHI plus 2 μ g ml^{-1} of erythromycin and grown overnight at 37°C. Finally, each transductant was confirmed for the transposon-encoded erythromycin resistance by streaking each colony on plates with BHI plus 25 μ g ml^{-1} of lincomycin.

Inverse PCR to identify transposon insertion sites. Genomic DNA of sensitive mutants was isolated with the GenElute bacterial DNA genomic DNA kit (Sigma-Aldrich). Genomic DNA was restriction digested with DpnI (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. Digests were ligated with T4 DNA ligase (New England BioLabs) overnight at 16°C. Ligated loops were PCR amplified with primers p255-1 and Marq255, gel purified on a 0.8% Tris-acetate-EDTA (TAE) agarose gel with the QIAquick gel extraction kit (Qiagen), and then Sanger sequenced with primer TnSeq (Table 1).

Complementation of LMRG_00235 (*lmo0553* or *cbpB*), LMRG_00467 (*lmo0779*), and LMRG_02671 (*lmo0236* or *ygbB*). Each gene was PCR amplified from the 10403S genome using primers listed in Table 1, restriction digested with BamHI overnight at 16°C, and then treated with calf intestinal alkaline phosphatase (CIP) for 1 h at 37°C. The digested fragment was ligated into plasmid pPL2 (61) and sequence verified. The constructs were electroporated into *E. coli* SM10 cells and subsequently mated into *L. monocytogenes* *prfA** to generate *prfA** *cbpB::tnHimar1*, *prfA** *lmo0779::tnHimar1*, or *prfA** *ygbB::tnHimar1* mutants and selected for on BHI agar containing 200 μ g ml^{-1} of streptomycin and 5 μ g ml^{-1} of chloramphenicol.

RNA extraction and cDNA preparation. Isolated colonies for each strain were picked from BHI agar plates into 5 ml of BHI broth and incubated with shaking at 37°C overnight. The following day, each culture was diluted 1:10 into fresh BHI broth and incubated with shaking at 37°C for 1.5 h. Two milliliters of each culture was centrifuged at 13,000 rpm for 5 min and supernatant was discarded. Each pellet was resuspended in 1 ml of TRIzol (Invitrogen, Carlsbad, CA). RNA was isolated per the manufacturer's protocol and dissolved in 100 μ l of Tris-EDTA buffer (pH 8.0; Millipore-Sigma). RNA was treated with the Turbo DNase kit (Ambion, Carlsbad, CA) and quantified on a NanoDrop OneC spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized with a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Carlsbad, CA) and diluted in diethyl pyrocarbonate (DEPC)-treated water.

TABLE 1 Strains, plasmids, and primers used in this study

Strain or plasmid	Feature(s) ^a	Reference
Strains		
NF-100	10403S parent strain	56
NF-1166	10403S <i>prfA</i> L140F <i>actA-gus-neo-plcB</i> (<i>prfA</i> * strain)	18
NF-890	10403S Δ <i>prfA</i>	27
NF-4239	<i>prfA</i> * <i>ygbB::tnHimar1</i>	This study
NF-4238	10403S <i>ygbB::tnHimar1</i>	This study
NF-4241	<i>prfA</i> * <i>cbpA::tnHimar1</i>	This study
NF-4240	10403S <i>cbpA::tnHimar1</i>	This study
NF-4242	<i>prfA</i> * <i>lmo0779::tnHimar1</i>	This study
NF-4241	10403S <i>lmo0779::tnHimar1</i>	This study
NF-4245	<i>prfA</i> * <i>ygbB::tnHimar1</i> + pPL2- <i>ygbB</i>	This study
NF-4247	<i>prfA</i> * <i>cbpA::tnHimar1</i> + pPL2- <i>cbpA</i>	This study
NF-4249	<i>prfA</i> * <i>lmo0779::tnHimar1</i> + pPL2- <i>lmo0779</i>	This study
Plasmid		
pPL2	Single-copy integration vector	61
pPL2- <i>ygbB</i>	<i>ygbB</i> complementation vector	This study
pPL2- <i>cbpA</i>	<i>cbpA</i> complementation vector	This study
pPL2- <i>lmo0779</i>	<i>lmo0779</i> complementation vector	This study
Primer		
p255-1	5'-TCT TTT AGC AAA CCC GTA TTC CAC G-3'	This study
Marq255	5'-CAG TAC AAT CTG CTC TGA TGC CGC ATA GTT-3'	60
TnSeq	5'-ACA ATA AGG ATA AAT TTG AAT ACT AGT CTC GAG TGG GG-3'	This study
<i>ygbB</i> _BamHI_F2	5'-CAC <u>GGG ATC</u> CTT GAG CGA ATT CCT TGT CCT-3'	This study
<i>ygbB</i> _BamHI_R3	5'-CAG <u>CGG ATC</u> CGT ACA CGC ACT CGT TTT GT-3'	This study
<i>cbpA</i> _BamHI_F1	5'-CGG <u>GAT CCT</u> CATTTC CAA GCT GTT TCA-3'	This study
<i>cbpA</i> _BamHI_R2	5'-GCG <u>GAT CCC</u> GTT CTA CAC TTC CAC CAC CA-3'	This study
<i>lmo0779</i> _BamHI_F2	5'-CGG <u>GAT CCG</u> GAA GTA AGC GTG GCG TTT-3'	This study
<i>lmo0779</i> _BamHI_R2	5'-GCG <u>GAT CCT</u> TTT GTT AAC CAA CCG TGT C-3'	This study
RT-PCR primer		
<i>ygbB</i> _F	5'-CAT AGG CGC AAT TGG TGC TG-3'	This study
<i>ygbB</i> _R	5'-ACC ATC CGC CTC TAC CTT CT-3'	This study
<i>cbpA</i> _F	5'-ACA CGA AGT CCT TTG CGT TC-3'	This study
<i>cbpA</i> _R	5'-CAT TCG CCG CGT ACT TGT TT-3'	This study
<i>lmo0779</i> _F	5'-CGA ACT GCG GAA GAA GCA GA-3'	This study
<i>lmo0779</i> _R	5'-GTG GTC GTT GGG ACG GTT AT-3'	This study
<i>prfA</i> _F	5'-GCT TGG CTC TAT TTG CGG TC-3'	This study
<i>prfA</i> _R	5'-AAC AGC TGA GCT ATG TGC GA-3'	This study
<i>actA</i> _F	5'-GCG AAG GAA GAA CCA GGG AA-3'	This study
<i>actA</i> _R	5'-ACG CCC CTA AAG AGA ACA CG-3'	This study
<i>kat</i> _F	5'-AGA AGC TTG CTC CGT CGA AA-3'	This study
<i>kat</i> _R	5'-GGG CTC TAG TGT TCA TGC GT-3'	This study
16S_F	5'-GAG TAC GAC CGC AAG GTT GA-3'	This study
16S_R	5'-CCT GGT AAG GTT CTT CGC GT-3'	This study

^aUnderlined nucleotides indicate inserted restriction sites used for cloning.

qRT-PCR. Primers for reverse transcription-quantitative PCR (qRT-PCR) were designed using PrimerBLAST (NCBI) and synthesized by Millipore-Sigma and are listed in Table 1. Samples were prepared using Fast SYBR green master mix (Applied Biosystems) with 200 nM final primer concentrations in 10- μ l reaction volumes and run in 96-well plates on a ViiA 7 quantitative PCR machine (Applied Biosystems). Gene expression was assessed in triplicate for each sample and normalized to 16S rRNA.

Animal infections. All animal procedures were approved by the University of Illinois at Chicago Animal Care Committee and were conducted in the Biological Resources Laboratory. A total of 1×10^4 CFU of each strain were injected via tail vein into 6- to 8-week-old Swiss Webster mice (Charles River) as previously described (62). After 72 h, mice were sacrificed and livers and spleens isolated, homogenized, and plated on LB agar for enumeration of bacterial burdens in each organ.

Statistical analyses. Gene expression data were analyzed using two-factor analysis of variance (ANOVA) with the Tukey multiple-comparison test (MCT). *In vivo* infection assays were tested for significance using the nonparametric Kruskal-Wallis test with Dunn's multiple-comparison test as appropriate. All statistical tests were performed using Prism (GraphPad, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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