**INTRODUCTION**

Virulence factor secretion is a fundamental aspect of bacterial pathogenesis. In Gram-positive bacteria secreted proteins are translocated in an unfolded state to enter the space that exists between the membrane and cell wall (Sarvas et al., 2004; van Wely et al., 2001). This space is solvent exposed, has a characteristically high cation concentration, and high density of negative charge that presents a challenging environment for protein folding and function (Sarvas et al., 2004). The presence of dedicated secretion chaperones are likely required to maintain optimal secretion homeostasis necessary for protein folding, activity, and correct localization. Chaperones that function to promote bacterial secretion may be required in greater quantities during conditions leading to increased protein secretion such as during host infection and/or under high stress conditions that promote protein denaturation and aggregation.

*Lm* is a Gram-positive bacterium that is ubiquitous in the environment but is capable of transitioning into an intracellular pathogen upon the consumption of contaminated food by the human host (Freitag et al., 2009). In the United States, *Lm* has caused numerous...
multi-state food-borne outbreaks which have resulted in thousands of illnesses and hundreds of deaths with immunocompromised populations being the most at risk, including the elderly, pregnant women and neonates (Hernandez-Milian & Payeras-Cifre, 2014; Lomonaco et al., 2015; Lund, 2015). The bacterium’s transition from life in the outside environment to life within the cytosol of host cells is accompanied by the increased expression and elaboration of a number of secreted virulence factors that facilitate survival by promoting cell entry, bacterial escape from host vacuoles, replication within the cytosol, and spread to adjacent cells (Alonzo & Freitag, 2010; de las Heras et al., 2011; Mostowy & Cossart, 2012; Mueller & Freitag, 2005; Port & Freitag, 2007; Shetron-Rama et al., 2003). The transcriptional activator PrfA regulates the expression of many of these secreted virulence factors required for bacterial survival within the host (Freitag et al., 1992; Leimeister-Wachter et al., 1990; Mengaud et al., 1991).

Lm has two PrsA homologs: PrsA1 and PrsA2 that are members of the parvin peptidyl-prolyl isomerase (PPIase) family that catalyze the cis-trans isomerization of peptide bonds N-terminal to proline residues in polypeptide chains (Alonzo et al., 2011). PrsA1 and PrsA2 are lipid modified and membrane-associated but a significant amount of each is also secreted (Alonzo et al., 2009; Cahoon & Freitag, 2015). Lm mutants lacking PrsA2 exhibit decreased hemolytic and phospholipase activity, are defective for cell-to-cell spread, and are significantly less virulent in mice (Alonzo et al., 2009; Cahoon et al., 2016; Cahoon & Freitag, 2015; Forster et al., 2011; Zemansky et al., 2009). In contrast, while PrsA1 shares 75% amino acid similarity and 58% identity with PrsA2, it makes no detectable contribution in Lm pathogenesis-associated activities or in mouse intravenous infection models suggesting that the protein may have evolved a more general physiological chaperone function (Alonzo et al., 2009; Cahoon et al., 2016). Bacteria lacking prsA2 also exhibit defects associated with a number of physiological activities including swimming motility, growth at acidic and basic pH and growth at high osmolarity (Alonzo et al., 2011; Cahoon et al., 2016; Cahoon & Freitag, 2015; Forster et al., 2011) while once again in contrast, mutants lacking prsA1 have no detectable phenotype for these activities. However, prsA1 mutants are more susceptible to cell wall active antibiotics but this sensitivity is only apparent when prsA2 is also completely absent (Cahoon et al., 2016). Although prsA1 thus far appears to play a minor role in Lm physiology, the allele is well conserved within Lm strains suggesting that selective pressure exists to maintain this gene.

The expression of prsA2 is directly regulated by the transcriptional activator PrfA and increased levels of PrsA2 are necessary to maintain bacterial viability under conditions where PrfA is activated and multiple virulence-associated gene products are secreted (Ahmed & Freitag, 2016; Alonzo & Freitag, 2010). However, deletion of the PrfA binding site within the prsA2 promoter does not significantly influence bacterial survival within the host (Zemansky et al., 2009), an observation that strongly suggests that additional mechanisms exist to increase prsA2 expression. Additional regulatory mechanisms would likewise be consistent with the requirement for PrsA2 activity for flagella-mediated swimming motility, an activity that is negatively regulated following PrfA activation (Cahoon & Freitag, 2015). However, other mechanisms that serve to regulate prsA2 expression as well as mechanisms underlying the regulation of prsA1 expression have thus far not been described. Here we describe the fortuitous identification and subsequent characterization of a novel two-component system (TCS) PieRS that regulates prsA1 and prsA2 as well as additional gene products. Analysis of PieRS and its associated regulon reveals a requirement for PieRS during intragastric infection as well as unique non-overlapping roles for PrsA1 and PrsA2 that contribute to translocation of Lm across the intestinal barrier.

2 | RESULTS

2.1 | Identification of a second site suppressor mutation that partially compensates for the loss of PrsA2 chaperone activity

As part of our original identification and characterization of prsA2 and its encoded gene product, we sought to generate a Lm strain containing a chromosomal in-frame deletion of prsA2 (Alonzo et al., 2009). However, repeated attempts to generate this mutant using the standard approach of allelic exchange by homologous recombination proved unsuccessful (Alonzo et al., 2009). The construction of in-frame deletions in target genes in Lm requires several cycles of bacterial dilution and outgrowth in broth culture, and mutations that confer even subtle growth defects can reduce fitness such that wild type cells out-compete the mutant and take over the culture. We therefore undertook the construction of a prsA2 mutant using Targetron insertions that could be targeted specifically to prsA2 (Alonzo et al., 2009). Once the prsA2 Targetron mutant (prsA2::T) was successfully generated, we used this mutant strain as a starting point for the generation of a prsA2 deletion mutant containing an erythromycin resistance gene (erm) inserted in place of the prsA2 coding sequences; this erythromycin-linked prsA2 mutation could then be transferred by phage transduction into a clean wild type genetic background as well as into additional Lm strains.

Characterization and comparison of the original prsA2::T mutant with prsA2::erm transductants revealed immediately apparent phenotypic differences. Whereas the original prsA2::T mutant exhibited normal swimming motility as well as levels of secreted hemolytic and phospholipase activity that were comparable to wild type strains, the prsA2::erm mutant transductants (∆prsA2) exhibited defects in swimming motility and reduced secreted listeriolysin O (LLO) activity and secreted phospholipase (PC-PLC) activity (Figure 1a–c). The ∆prsA2::erm mutant phenotypes were consistently observed for independently generated transductants and could be complemented by the introduction of prsA2 on a plasmid chromosomal integration vector (Alonzo et al., 2009), indicating that the mutant phenotypes were linked to ∆prsA2::erm. While the Targetron-generated prsA2::T mutant exhibited normal swimming motility and hemolytic and...
phospholipase activity in the absence of prsA2 complementation, it remained highly attenuated in mouse intravenous infection models (Figure 1d). These data strongly suggested that the prsA2::T mutant contained a second site suppressor mutation that partially compensated for the loss of PrsA2 activity but did not restore virulence.

2.2 | Identification of PieRS and its associated gene regulon

Whole genome sequencing analysis of prsA2::T was performed to identify the second site suppressor mutation that partially restored PrsA2 activity. A mutation resulting in the substitution of a valine for a glutamate at position 81 (E81V) in the gene product encoded by lmo1507 was identified within the prsA2::T genome (Figures S1a and 2a). Lmo1507 is predicted to function as the response regulator of a two-component signal transduction system (TCS) for which Lmo1508 is the predicted sensor histidine kinase (Figure 2a). The TCS lmo1507-1508 is flanked by lmo1509, encoding a putative exodeoxyribonuclease and lmo1505-1506 encoding a putative ABC transporter ATP-binding protein and a putative macrolide ABC transporter permease, respectively (Figure 2a).

In silico analysis suggests the response regulator Lmo1507 consists of a receiver domain with a predicted phosphorylation site
at aspartic acid position 51, in addition to a DNA binding domain (Figure S1a,b) (Lu et al., 2020; Mistry et al., 2021). The Lmo1507 receiver domain also contains glutamic acid and aspartic acid at positions 7 and 8, respectively predicted to be important for metal ion-dependent phosphorelay reactions, and threonine at position 78 predicted to interact with the phosphoryl group of phosphorylated D51 (Figure S1a,b) (Lu et al., 2020). Two additional residues in the receiver domain, tyrosine and lysine at positions 97 and 100 respectively, are predicted to be important for phosphorylation mediated conformational changes (Figure S1a,b). A structural prediction of Lmo1507 indicates that E81 is in proximity to Y97 (Figure S1b) (Kelley et al., 2015).

In order to identify genes whose expression could potentially be regulated by the Lmo1507-1508 TCS, we constructed Lm mutants containing the lmo1507 E81V mutation or deletion of lmo1507-1508 in the presence and absence ofprsA2 and compared patterns of bacterial gene expression to the wild-type strain. DNA microarray analysis disregarding fold change differences indicates that 1144 genes are significantly different from wild-type in the ∆prsA2 mutant which is reduced to 259 genes in the ∆prsA2 lmo1507 E81V strain (the ∆prsA2 suppressor mutant) (Figure S2a). Disregarding fold
change differences, the \textit{lmo1507} E81V mutant has 559 genes significantly different than wild-type, whereas the \textDelta \textit{lmo1507-lmo1508} strain and the \textDelta \textit{prsA2} \textDelta \textit{lmo1507-lmo1508} have comparable levels of genes significantly different than wildtype, 228 and 227, respectively (Figure S2a).

Volcano plot analysis shows that four genes are highly upregulated at over 4-fold Log$_2$ in the \textDelta \textit{prsA2} suppressor mutant but not in the \textDelta \textit{prsA2} strain (Figure 2b). These genes: \textit{lmo1505}, \textit{lmo0442}, \textit{lmo0881}, and \textit{prsA1} are also similarly upregulated in the \textit{lmo1507} E81V mutant (Figures 2b and S2b). In addition, \textit{lmo1506} between \textit{lmo1505} and \textit{lmo1507} was modestly upregulated at over 2-fold Log$_2$ in strains containing the \textit{lmo1507} E81V mutation (Figures 2b and S2b). In the absence of the \textit{lmo1507-1508} TCS, \textit{lmo1505} remained upregulated regardless of the presence or absence of \textit{prsA2} (Figure S2c,d). A heat map of the relative expression levels of the \textit{lmo1507-1508} encoded TCS and putative regulon shows that strains containing the \textit{prsA2} suppressor mutation have similar expression patterns which are distinct from strains that are deleted for the TCS (Figure 2c). We used qRT-PCR analysis to confirm the microarray data indicating increased expression of putative Lmo1507 regulated genes (Figure 2d), suggesting that the E81V mutation serves to activate Lmo1507. The function of the \textit{lmo0881} gene product is unknown, whereas \textit{lmo0442} encodes a fructose-specific phosphotransferase transport system (PTS) component permease that has been implicated in virulence (Liu et al., 2017) while \textit{prsA1} encodes a peptidyl-prolyl cis-trans isomerase molecular chaperone that shares 75% similarity and 58% identity to PrsA2 (Alonso et al., 2009). As mentioned previously, \textit{lmo1507-1506} are predicted to encode a putative ABC transporter ATP-binding protein and a putative macrolide ABC transporter permease, respectively. Based on the critical requirement for \textit{prsA1} and \textit{prsA2} for bacterial survival under a variety of stress conditions (Cahoon et al., 2016; Cahoon & Freitag, 2015) and the ability of \textit{lmo1507-1508} to induce the expression of \textit{prsA1} and \textit{prsA2}, we have designated \textit{lmo1507-1508} as PieRS, for the ability of \textit{lmo1507-1508} to induce the expression of a putative molecular chaperone that shares 75% similarity and 58% identity to \textit{prsA1} and \textit{prsA2}.

### 2.3 | Characterization of the PieR response regulator

A protein BLAST search of PieRS revealed that the TCS is orthologous to the YclJ/YclK TCS of \textit{Bacillus subtilis}. PieR is 71% identical and 82% similar to \textit{B. subtilis} response regulator YclJ (Figure 3a), while PieS is 47% identical and 67% similar to \textit{B. subtilis} histidine kinase YclK. Over-expression of the \textit{B. subtilis} YclJ response regulator in a \textDelta \textit{yck} sensor kinase strain was used together with microarray analysis to associate YclJ with the regulation of several genes (Kobayashi et al., 2001) of which two (\textit{yclH} and \textit{yclI}) encode gene products that are homologous with two of those predicted for the PieRS TCS regulon. \textit{B. subtilis} \textit{YclH/YclI} are an ABC transporter ATP-binding protein and a putative macrolide ABC transporter permease, respectively, that share similarity with Lmo1505-1506; the genes encoding YclH and YclI are also similarly located adjacent to the TCS genes (Figure S3).

In \textit{B. subtilis}, the DNA binding site of YcJ has been determined (Ogura et al., 2010); in silico analysis of the YcJ binding site sequence in Lm indicated putative binding sites consisting of the consensus sequence TTCAT-AG-TTTG-TGTATTTT upstream of all genes in the TCS PieRS regulon (Figure 3b). Putative binding sites located 88 base pairs upstream of \textit{prsA1}, 75 base pairs upstream of \textit{lmo1505-1506}, 57 base pairs upstream of \textit{lmo0442}, and 54 base pairs upstream of \textit{lmo0881} were identified based on the YcJ DNA binding consensus sequence. The \textit{prsA2} gene has two potential DNA binding sites located 105 base pairs and 469 base pairs upstream of \textit{prsA2} coding sequences. Following further in silico analysis using a BLAST search (Altschul et al., 1990), one additional putative PieR binding site was identified 52 base pairs upstream of \textit{htrA} which encodes a serine protease/chaperone. However, this appears to be a degenerate PieR binding site with a thymine at position 6 instead of an adenine which may explain why \textit{htrA} was not identified by microarray analysis.

To test whether PieR and PieE81V (hereafter referred to as PieR*, based on the apparent activation of the mutant protein) bound to DNA containing the putative DNA binding consensus sequence present within the upstream regions of target genes of the PieRS regulon, we performed electrophoretic mobility shift assays (EMSA). Purified N-terminal His-tagged PieR or PieR* was incubated with target DNA containing either the \textit{prsA1} or \textit{prsA2} putative PieR binding sites (Figure 3c,d). PieR and PieR* both bound the \textit{prsA1} putative consensus binding site with PieR* observed to bind with an apparent higher affinity based on signal intensity (Figure 3c,d). Binding was specific as PieR* binding was reduced in the presence of unlabeled \textit{prsA1} upstream competitor DNA, and PieR* remained bound to the labeled DNA in the presence of non-specific competitor DNA (Figure 3d). The region upstream of \textit{prsA2} contains two putative PieR binding sites located 105 base pairs (site B) and 469 base pairs (site A) upstream of the \textit{htrA} ATG start site (Figure 3b). The \textit{prsA2} sites A and B are unique when compared to the other putative PieR binding sites in that they are located further upstream from the translation initiation codon and each contains base pair insertions that differ from the consensus sequence (Figure 3b). EMSA analysis of these two sites demonstrated that PieR* had a slightly higher binding affinity for the region upstream of \textit{prsA2} containing site B when compared to the region containing site A (Figure 3e). These data suggest that the expression of both post-translocation secretion chaperones \textit{PrsA1} and \textit{PrsA2} are directly regulated by the TCS PieRS.

### 2.4 | The PieR E81V (PieR*) mutation confers partial suppression for the loss of PrsA2 activity via upregulation of \textit{prsA1}

Given that (1) \textit{PrsA1} is a molecular chaperone of the same family as \textit{PrsA2}, and (2) its expression was up-regulated in the \textDelta \textit{prsA2} strain containing the PieR E81V (PieR*) gain of function mutation, we postulated...
that increased levels of PrsA1 may have provided for the partial suppression of ∆prsA2-associated phenotypes. To determine the extent to which increased abundance of PrsA1 was compensating for the loss of PrsA2, a prsA1 deletion was introduced into the ΔprsA2 pieR* strain (ΔprsA1 ΔprsA2 pieR*). We further examined whether the PieR DNA binding site upstream of prsA1 was required for the partial suppression of the ΔprsA2 mutant phenotypes, and similarly investigated the phenotypes of strains completely lacking the pieRS encoded TCS (ΔpieRS).

The ΔprsA2 pieR* strain recapitulated the partial suppression phenotypes observed for the original prsA2 Targetron insertion mutant (prsA2::T) found to contain the pieR* mutation (Figures 1 and 4). Increased expression of prsA1 resulting from the pieR* mutation restored secreted LLO and PC-PLC activity in ΔprsA2 mutant backgrounds, and PrsA1-dependent restoration was eliminated by substitution of the two conserved cytosines with thymines within the PieR DNA binding site upstream of prsA1 (SD...
The PieR* mutant and the ΔpieRS strain were similar to the wild-type strain in terms of secreted LLO and PC-PLC activity, suggesting that pieRS is not itself required for these activities and that the increased expression of prsA1 resulting from PieR* was less functionally important in the presence of active PrsA2 (Figure 4a,b). All mutant strains lacking prsA2 remained deficient for intracellular growth and cell-to-cell spread as measured by plaque formation in monolayers of L2 fibroblast cells (Figure 4c). The pieR* mutant and the strain containing the full deletion of pieRS both exhibited slightly decreased plaque sizes suggesting that pieRS may have a minor role during the infection of cell monolayers (Figure 4c). However, there was no difference between wild type and the ΔpieRS mutant with respect to bacterial burdens in the livers and spleens of infected animals, a result that

**FIGURE 4** Analysis of bacterial virulence phenotypes. (a) Hemolytic activity. Dilutions of bacterial culture supernatants were assessed for their ability to lyse sheep RBCs. The reciprocal of the supernatant dilution that resulted in 50% RBC lysis (hemolytic units) was determined for 5 independent experiments. Error bars represent the standard error of the mean where *p* ≤ 0.05 by two-tailed Student's T-test when compared to WT as indicated by an asterisks above bars. (b) Phospholipase activity. Strains were incubated on egg yolk agar plates, observation of a zone of opacity surrounding the bacterial streak is indicative of phospholipase activity, a representative is shown of four independent experiments. (c) Intracellular growth and cellular spread as measured by plaque assay. L2 fibroblast monolayers were infected with the indicated bacterial strain and plaque formation was determined in the presence of gentamicin 72h post-infection. At least 20 plaques were measured in 3 independent experiments for all strains. Measurements represent plaque size with respect to WT (set at 100%). Error bars represent the standard error of the mean where *p* ≤ 0.05 by two-tailed Student’s T-test when compared to WT as indicated by an asterisks above bars. (d) Mouse bacterial burdens. Mice were infected with 2 × 10^4 colony forming units (CFUs) intravenously with the indicated strain. At 72h post infection, bacterial burdens were determined in livers and spleens. Box plots are shown where each point represents one animal (N = 7) and a dotted line indicates the limit of detection. Asterisks above plots indicate statistical significance of *p* ≤ 0.05 by two-tailed Wilcoxon rank-sum test when compared to WT.
indicates that pieRS is not required during intravenous infection of mice (Figure 4d).

To further delineate possible roles for the pieRS encoded TCS in Lm physiology, we examined bacterial growth under a number of conditions for which a requirement for PrsA2 activity has been previously demonstrated (Cahoon et al., 2016; Cahoon & Freitag, 2015). Growth of bacteria under acidic pH indicated that PrsA1 can compensate for the lack of PrsA2 in the presence of the pieR* mutation (Figure 5a). Interestingly, the ΔprsA2 ΔprsA1 pieR* mutant exhibited less of a growth defect under low pH than the ΔprsA2 ΔprsA1 mutant suggesting that other genes within the PieRS regulon may be capable of compensating for the lack of PrsA2 (Figure 5a). pieRS is not itself required for bacterial survival under conditions of acidic pH (Figure 5a), suggesting that there may be redundant systems that contribute to resistance to acid stress. Under conditions of high osmolarity, the pieR* mutation did not suppress the ΔprsA2 sensitive phenotype nor was pieRS required for survival (Figure 5b). Bacterial motility was also not affected by the absence of pieRS, however PrsA1 was able to compensate for the lack of PrsA2 in the pieR* background providing that the PieR DNA binding site upstream of prsA1 was intact (Figure 5c).

Strains lacking PrsA2 exhibit increased sensitivity to antibiotics that target the bacterial cell wall (Alonzo et al., 2011; Cahoon & Freitag, 2015; Forster et al., 2011). We examined whether pieRS contributes to cell wall integrity by examining the minimum inhibitory concentrations of ΔpieRS mutants to penicillin and lysozyme. The ΔpieRS mutant exhibited similar patterns of growth to wild type strains in the presence of both penicillin and lysozyme (Figure 6a,b). PrsA1 contributes a minor role to penicillin resistance as the ΔprsA2 ΔprsA1 mutant was more sensitive to the drug than the ΔprsA2 mutant (Figure 6a). However, increased expression of prsA1 resulting from the presence of the pieR* mutation did not compensate for the lack of PrsA2 in the presence of penicillin, although the suppressor mutation did enable PrsA1 to completely compensate for the lack of PrsA2 during growth in the presence of lysozyme (Figure 6a,b). We conclude that the pieRS encoded TCS is not required for bacterial survival in the presence of penicillin or lysozyme, however the pieR* mutation provides sufficient PrsA1 to confer lysozyme resistance to ΔprsA2 strains. The failure of increased PrsA1 expression to completely compensate for the lack of PrsA2 in the presence of penicillin suggests that PrsA2 may be uniquely required for the function of penicillin binding proteins.

**FIGURE 5** Analysis of cellular physiology phenotypes. (a) Growth at acidic pH. Growth is shown as optical density (OD 600nm) of strains inoculated from a saturated culture into liquid broth at pH 6 and grown overnight. Error bars represent the standard error of the mean for 3–6 experiments where p ≤ .05 by two-tailed Student’s T-test when compared to WT as indicated by an asterisks. (b) Growth at high osmolarity. Growth is shown as optical density (OD 600nm) of strains inoculated from a saturated culture into liquid broth containing 5% NaCl and grown overnight. Error bars represent the standard error of the mean for 6 experiments where p ≤ .05 by two-tailed Student’s T-test when compared to WT as indicated by an asterisks. (c) Swimming motility. The diameters of the swimming colonies are expressed as the average of 6–8 colonies from 3 independent experiments. Error bars represent the standard error of the mean where p ≤ .05 by two-tailed Student’s T-test when compared to WT as indicated by an asterisks.
2.5 The PieRS TCS is required for ethanol resistance

An alternative form of cell stress can be manifested by exposure to ethanol which is known to modify lipid bilayers and cellular proteins (Konopasek et al., 2000; Silveira et al., 2004). Given that the secretion chaperones PrsA1 and PrsA2 reside at the membrane-cell wall interface and that the gene products are also part of the PieRS regulon, we assessed the effects of 4% ethanol exposure on strains lacking pieRS, prsA1, or prsA2 (Figure 6c). Mutant strains lacking pieRS, prsA1,
or prsA2 were all restricted for growth in the presence of ethanol, and each mutant could be successfully complemented by the introduction of the wild-type gene (Figure 6c). In addition, PrsA1 was able to fully compensate for the absence of PrsA2 in the presence of the pieR* gain of function mutation and this suppression of the ΔprsA2 defect required the presence of the PieR DNA binding site upstream of prsA1 (Figure 6c). Analysis of the combined pieR* ΔprsA2 ΔprsA1 mutant suggests that the other gene products encoded within the PieRS regulon at best have very minor roles during ethanol exposure when compared to PrsA1 and PrsA2 (Figure 7). In addition, the ΔpieR ΔprsA2 and the ΔpieRS ΔprsA1 mutants were found to be as sensitive to ethanol exposure as a ΔprsA2 ΔprsA1 double mutant indicating that activation of the pieRS TCS and the encoded chaperone activity are essential for bacterial survival following ethanol exposure (Figure 6c).

2.6 | The PieRS TCS is involved in prsA1 and prsA2 dependent survival in the gut

Ethanol exposure is a condition that interferes with protein folding and requires heightened chaperone activity at the membrane-cell wall interface; similar stresses on protein folding and cell membrane integrity are anticipated to be encountered by Lm during bacterial transit through the gastrointestinal tract. We therefore examined whether the prsA1 and prsA2 and possibly the pieRS gene products contribute to Lm survival following intragastric inoculation of mice, the natural route of Lm infection (Figure 7). Bacterial burdens in the intestine at 72h post intragastric inoculation indicated that prsA2 is essential for survival in the gut, whereas ΔprsA1 and ΔpieRS-associated defects were only evident when combined (ΔpieRS ΔprsA1), indicating roles for PieRS, PrsA1 and PrsA2 during oral infection (Figure 7). In comparison to the ΔpieRS TCS mutant and the ΔprsA1 single mutant, the bacterial burdens of ΔpieRS in combination with ΔprsA1 were reduced during intragastric infection, indicating an additive effect. Following intestinal colonization, Lm translocates across the intestinal epithelium to distal organs such as the liver and spleen. As expected, bacterial burdens in the livers and spleens of infected animals were significantly reduced for ΔprsA2 mutants as well as for ΔprsA1 ΔpieRS mutants and ΔprsA1 or ΔpieRS in combination with a prsA2 deletion. Notably, ΔprsA1 mutants were also significantly reduced in numbers in the liver but not within the intestine, suggesting that bacterial survival within the intestine was not significantly affected while bacterial translocation from the intestine to the liver was impaired (Figure 7). Taken together, these results suggest that pieRS TCS, prsA1, and prsA2 contribute distinct and important roles during intragastric infection.

3 | DISCUSSION

The PrsA2 secretion chaperone has been shown to contribute to multiple aspects of Lm physiology and virulence, enabling Lm to survive under multiple stress conditions that likely interfere with protein folding at the membrane-cell wall interface. While PrsA2 activity is critical for Lm cell wall integrity, swimming motility, and the secretion of multiple virulence factors, the contributions of the related Lm secretion chaperone, PrsA1, have been less apparent. Here we show that PrsA2 and PrsA1 function together under the control of a two-component signal transduction system PieRS to enhance bacterial survival during intragastric infection and translocation across the intestinal epithelium to distal organs of mice. PrsA1 and PrsA2 occupy distinct roles during intragastric infection in that PrsA2 is necessary for intestinal colonization whereas PrsA1 is required for bacterial translocation across the intestine to reach the liver. The contributions of PieRS are somewhat more subtle, in that a mutant lacking the PieRS regulators is at best modestly compromised for bacterial virulence during intragastric infection. We interpret these findings to suggest that prsA2 and prsA1 are not exclusively regulated by PieRS during intragastric infection and that other regulatory mechanisms must exist for the induction of these critical chaperones in the GI tract.

The pieR E81V (pieR*) mutation was fortuitously identified as gain of function mutation that led to the suppression of some but not all ΔprsA2-associated phenotypes. The presence of the pieR* mutation resulted in the up-regulation of genes within the pieRS regulon including prsA1 and prsA2 (Figure 2b-D). We found that the suppression of many of the ΔprsA2-associated phenotypes was dependent on the presence of PrsA1 and required an intact PieR DNA binding site upstream of prsA1. With respect to PieRS regulon members outside of prsA1 and prsA2 and under the conditions tested thus far, only growth under acidic pH appeared to require the activity of other PieRS-regulated gene products in the absence of prsA2 (Figure 5a). In addition, we identified one additional degenerate PieR binding site upstream of htrA which may account for the modest upregulation of htrA, ~1.44 Fold Log2 and ~1.98 Fold Log2 in the ΔprsA2/pieR* and pieR* strains, respectively. It is likely that the serine protease/chaperone HtrA may be induced and/or compensating for the loss of prsA2 under acidic conditions as htrA has been previously shown to be required under conditions of acidic pH (Stack et al., 2005). It is also possible that other PieRS regulon members contribute to bacterial survival in the acidic gastric environment of the host following oral ingestion of Lm, however this remains to be determined. Based on the increased levels of gene expression of the PieRS regulon resulting from the PieR* mutation (Figure 2c,d) and the location and proximity of the PieR E81V mutation between the predicted phosphorylation site and residues important for phosphorylation mediated conformational changes (Figure S1b), we speculate that the PieR* mutation likely results in a conformational change that may mimic that which occurs following PieR phosphorylation, thereby increasing the affinity of PieR for its target DNA binding sites.

Given the importance of PrsA2 for multiple aspects of Lm physiology and virulence, it is perhaps not surprising that multiple regulators control prsA2 expression. In addition to PieRS, prsA2 expression is regulated by PrfA, the central regulator of Lm virulence gene
expression (Chatterjee et al., 2006; Port & Freitag, 2007) however binding of PrfA to the prsA2 promoter does not appear to be required for virulence (Zemansky et al., 2009). The region upstream of prsA1 does not have a recognizable PrfA binding site and loss of prsA1 has no detectable effect on Lm bacterial virulence following intravenous infection of mice (Alonso et al., 2009). Overall, our data indicate that PrsA2 acts as a multifaceted chaperone required for both intravenous and oral infections, whereas PrsA1 is required more selectively for bacterial transit from the intestine into distal organs such as the liver.

The regulation of prsA1 and prsA2 expression by PieRS represents the first genetic linkage identified between these two secretion chaperones. Based on in silico analysis and microarray data, the serine protease/chaperone encoded by htrA may also be modestly regulated by PieRS. Other Gram-positive prsA homologs have been shown to be activated by TCSs, such as prsA of Streptococcus pneumoniae (designated ppmA) which is activated by the TCS CiaRH that similarly activates the expression of htrA (Halfmann et al., 2007) and protects the cell from a variety of lysis-inducing conditions (Dagkessamanskaia et al., 2004; Mascher et al., 2006). The prsA of Staphylococcus aureus is regulated by the TCS VraRS and induced by cell wall stress (Jousselin et al., 2012). S. pneumoniae and S. aureus both have one prsA homolog whereas Streptococcus pyogenes, similar to Lm, has two prsA homologs; other Gram-positive bacteria have more (Cahoon & Freitag, 2014). Thus far the regulation of more than one prsA homolog by a single signaling system appears novel.

PieR appears to have a similar consensus DNA binding sequence to the B. subtilis orthologue YcIJ (Ogura et al., 2010). B. subtilis TCS ycIJ-K is induced under anaerobic conditions (Ye et al., 2000) whereas pieRS was not observed to be required under anaerobic conditions (data not shown). The response regulators, PieR and YcIJ are more conserved than the sensor kinases, PieS and YcIK, having 71% identity and 82% similarity versus 47% identity and 67% similarity, respectively. It seems likely that the amino acid differences in the sensor kinases may account for the difference in responses to external stimuli. The YcIJ/YcIK TCS is similar to the PieRS TCS in that both activate adjacent operons that have similar putative functions. YcIJ/YcIK activates yclH/yclI whereas PieRS activates lmo1505-1506, with each of these gene pairs encoding putative ABC-type transporter and permease proteins of an unknown substrate. Lmo1505 is 69% identical and 81% similar to YcIH, whereas Lmo1506 is 58% identical and 72% similar to YcII. Whether these putative transporter proteins have similar functions and how function might contribute to stress resistance is unknown.

Previously, we observed that constitutive expression of prsA1 in a ΔprsA2 strain complemented many ΔprsA2-associated phenotypes (Cahoon & Freitag, 2015). Just as PrsA1 in the presence of the PieR* mutation suppressed the ΔprsA2 associated swimming motility defect (Figure 5c), sensitivity to lysozyme (Figure 6b) and acidic pH (Figure 5a), so too could constitutive expression of PrsA1 complement these phenotypes in a ΔprsA2 strain (Cahoon & Freitag, 2015). However constitutive expression of PrsA1 could also complement ΔprsA2 sensitivity to penicillin and growth at high osmolality (Cahoon & Freitag, 2015) whereas PrsA1 in the presence of the PieR* mutation did not (Figures 5b and 6a). There are many possible scenarios that may account for these differences: plasmid-associated constitutive expression of prsA1 may be greater than the expression of prsA1 in pieR* background, penicillin and high osmolality conditions may decrease the amount of PieR protein levels, other systems may regulate prsA1 expression or PrsA1 protein translation under these conditions, or potentially the over-expression of the other gene products within the PieRS regulon in the absence of PrsA2 is somehow detrimental to the cell. Clarification awaits further experimentation.

Lm has 14 TCSs which are thought to respond to various stimuli to coordinate the expression of multiple genes (Glaser et al., 2001). There are likely redundancies among these systems that respond to specific signals or stresses. For example, similar to the TCS PieRS, the TCSs LisRK and CesRK and associated regulons are ethanol responsive (Nielsen et al., 2012). In contrast to PieRS, the absence of lisRK or cesRK resulted in enhanced growth of Lm in the presence of ethanol (Gotte et al., 1999; Kallipolitis et al., 2003; Kallipolitis & Ingmer, 2001; Williams et al., 2005). It is possible that other mechanisms compensate for the loss of either lisRK or cesRK, such as perhaps increased expression of the TCS PieRS regulon. There is only one gene product shared between the TCS PieRS and LisRK regulons, as both systems may regulate the expression of the serine protease/chaperone encoded by htrA (Nielsen et al., 2012; Stack et al., 2005). PieRS also regulates the expression of lmo0442 encoding a fructose-specific PTS permease subunit IA (Liu et al., 2017), whereas interestingly CesRK regulates the expression of the two genes surrounding lmo0442 (Gottschalk et al., 2008; Kallipolitis et al., 2003). CesRK responsive elements have been identified upstream of lmo0443 and lmo0441 (Gottschalk et al., 2008) which encodes a fructose specific PTS permease subunit IIB (Liu et al., 2017) and a penicillin binding protein (Guinan et al., 2006), respectively. The lmo0441 CesRK responsive element (Gottschalk et al., 2008) is within 37 base pairs of the PieR binding site upstream of lmo442 (Figure 3b); whether the location of these sites relative to each other affects the expression of CesRK-regulated lmo441 and PieRS-regulated lmo442 is unknown.

We propose the following working model for the PieRS TCS (Figure 8). In the presence of ethanol or under forms of gastric stress, the sensor kinase PieS activates the response regulator PieR which in turn leads to the activation of prsA1, prsA2, and potentially the other genes of the pieRS regulon. Ethanol attacks lipid bilayers and causes the unfolding of proteins (Konopasek et al., 2000; Silveira et al., 2004); the up-regulation of the molecular chaperones PrsA1 and PrsA2 is a likely stress response necessary to maintain proper protein folding at the cell membrane-cell wall interface as well as normal protein secretion (Figure 8). Since ethanol elicits protein unfolding, we hypothesize that similar stress conditions very likely exist in the host gastrointestinal tract that result in protein unfolding.
and require the apparently distinct activities of both PrsA secretion chaperones, thus enhancing bacterial survival and the ability to translocate across the intestinal epithelium to deeper tissues and distal organs.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains, plasmids, and media

Bacterial strains used in this study are listed in Supplemental Table S1. Lm 10403S is the wild-type (WT) strain and ΔprsA2 is Lm 10403S containing an erythromycin resistance gene (erm) in place of the prsA2 coding sequence (Alonzo et al., 2009), while ΔprsA1 transduced with ΔprsA2:erm is ΔprsA2 ΔprsA1 (Alonzo & Freitag, 2010). The original prsA2 suppressor mutant (prsA2::T) contains a targeted insertion disruption of prsA2 that was generated by using the Targetron gene knockout system (Sigma) (Alonzo et al., 2009). Escherichia coli One Shot TOP10 (Invitrogen), SM10, and S17 (a kind gift from N. Cianciotto, Northwestern University) were used as host strains for recombinant plasmids, whereas E. coli BL21-CodonPlus (DE3)-RIL cells was used for protein expression. E. coli and Lm were grown in Luria broth (LB) or terrific broth (TB) and brain heart infusion (BHI) medium, respectively. The integration plasmid pPL2 (Lauer et al., 2002) was used for genetic complementation of lmo1507 E81V ΔprsA2 ΔprsA1 with prsA1 or SDprsA1 (Figure 3c) while the integration plasmid pMK2 (Monk et al., 2008) (a kind gift from C. Hill, Cork College) was used for complementation of Δlmo1507/1508 with lmo1507/1508, Δlmo1507/1508 with lmo1507 E81V/1508, ΔprsA1 with prsA1, and ΔprsA2 with prsA2 (Cahoon & Freitag, 2015). The N-terminal His-tagged plasmid pQE30 (Qiagen) was used for protein expression of Lmo1507 and Lmo1507 E81V in E. coli.

4.2 | Whole genome sequencing of the prsA2::T strain

Genomic DNA from the prsA2::T strain was isolated (Qiagen) and sequenced using the SOLiD DNA sequencing system at the University of Oklahoma Health Sciences Center. Reads were aligned to the genome of Listeria monocytogenes 10,403s using CLC Genomics Workbench to determine nucleotide differences.

4.3 | Construction of the lmo1507/1508 mutant strains

To generate lmo1507 E81V, a fragment containing the encoded E81V mutation was PCR amplified from prsA2::T genomic DNA using primers designed with a BamHI and SacI restriction site, lmo1507F2-BamHI and lmo1507R2-SacI, respectively (Supplemental Table S2). The fragment was cloned into the shuttle vector pKSV7 and used for allelic exchange in strains WT, ΔprsA2, and ΔprsA2 ΔprsA1 as previously described (Camilli et al., 1993). The Δlmo1507/1508 in frame deletion was constructed by splicing by overlap extension (SOE) PCR (Horton, 1993). Two DNA fragments were generated by PCR using primers 1507F1BamHIpA/1507KpnI1508pB and 1508R1SacIpD/1507KpnI1508pC (Supplemental Table S2) with genomic DNA from Lm 10403S. The two fragments were gel purified (Qiagen) and PCR amplified using primer pair 1507F1BamHIpA/1508R1SacIpD to generate a ~1520 bp product encompassing the upstream and downstream region of lmo1507/1508. This fragment was cloned into the shuttle vector pKS7V7 and used for allelic exchange in strains WT, ΔprsA2, and ΔprsA2 ΔprsA1 as previously described (Camilli et al., 1993). To generate prsA1 complemented strains, the region encompassing 195bp upstream and 66bp

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**FIGURE 8** Model of PieRS regulation. An external stimulus activates the sensor kinase PieS that activates the cognate response regulator PieR which induces expression of the PieRS regulon. Genes associated with the PieRS regulon include prsA1, prsA2, lmo1505-lmo1506, lmo0442, and lmo0881. PrsA1 and PrsA2 are PPIase secretion chaperones. Lmo1507 and Lmo1506 are a putative ABC transporter ATP-binding protein and a putative macrolide ABC transporter permease, respectively. Lmo0442 is a putative fructose specific phosphotransferase transport system (PTS) component permease whereas the function of Lmo0881 is unknown. The localization of the PieRS regulon members is indicated.
downstream of prsA1 was amplified with primers PrsA1UPSacF1/PrsA1XmaR2 (Supplemental Table S2) from Lm 10,403 s genomic DNA and cloned into the integration plasmid pPL2 (Lauer et al., 2002); to generate the SDprsA1 strain, this construct was mutated with a site directed mutagenesis kit (Agilent Technologies) and primers listed in Supplemental Table S2. Each plasmid was transformed into SM10 cells and allowed to integrate into Lm 1507 E81V ∆prsA2 ∆prsA1 by conjugation. The Lmo1507/1508 and Lmo1507 E81V 1508 complementation strains were generated by amplification of a region 257 bp upstream of lmo1507 and 180 bp downstream of lmo1508 from Lm 10,403 s or Lm1507 E81V genomic DNA, respectively, and cloned into the integration plasmid pIMK2 (Monk et al., 2008). These constructs were transformed into S17 cells and delivered to integrate into ∆lmo1507/1508 by conjugation.

4.4 | Construction and purification of recombinant Lmo1507 and Lmo1507 E81V

The DNA sequence corresponding to Lmo1507 and Lmo1507 E81V was amplified from Lm 10,403 s or prsA2::T genomic DNA with primers designed with a SacI and XmaI restriction site, lm1507F-1SacEx and lm1507R1XmaEx, respectively. The fragment was cloned into the N-terminal His-tagged pQE30 expression system (Qiagen). E. coli BL21-CodonPlus (DE3)-RIL cells (Stratagene) were inoculated with a 1:100 dilution of Two liters of TB medium was inoculated with a 1:100 dilution of the appropriate overnight culture. Cells were grown at 37 °C until reaching an OD600 of 0.9. The temperature was then lowered to 25 °C and Lmo1507 expression was induced by the addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.5 mM. The next morning, cells were collected by centrifugation and resuspended in sample buffer [10 mM Tris–HCl pH 8.3, 500 mM NaCl and 1 mM tris(2-carboxyethyl)phosphine] supplemented with 10% glycerol. Cells were lysed by sonication and the cellular lysate was cleared by centrifugation. The soluble fraction of the lysate was loaded onto a 5 ml HisTrap FF nickel column (GE Healthcare Life Sciences) and washed with wash buffer (sample buffer supplemented with 25 mM imidazole). His-tagged Lmo1507 or Lmo1507 E81V was eluted with elution buffer (sample buffer supplemented with 500 mM imidazole). The eluant was immediately injected onto a HiPrep 26/60 Superdex size-exclusion column (GE Healthcare Life Sciences) that had been pre-equilibrated with sample buffer. A single peak eluted from the size-exclusion column and was confirmed to contain homogenous Lmo1507 or Lmo1507 E81V by SDS-PAGE.

4.5 | Electrophoretic mobility shift assay (EMSA)

Putative binding sites from prsA1 and prsA2 were PCR amplified from the Lm genomic DNA with primers containing a biotin tag at the 3’ end using Phusion HF DNA polymerase (Biolabs). The resulting PCR product was run on a 0.8% agarose gel and purified using Qiagen gel extraction kit (Qiagen). The gel shift assay was performed according to the instructions in the LightShift EMSA Optimization and Control Kit (Thermo Scientific) with the contents added in the following order to a final reaction volume of 10 μl: ultrapure water (variable), 10X binding buffer (1X), 50% glycerol (2.5%), 100 mM MgCl2 (5 mM), 1 μg/μl Poly (dI.dC) (50 ng/μl), 1% NP-40 (0.05%), unlabeled DNA, respective protein and labeled DNA (40 fmol). The reactions were then incubated for 20 min at room temperature (RT) before loading the entire volume onto a pre-run (30 min in chilled 0.5X TBE, 100 V) 5% PAGE gel and ran in chilled 0.5X TBE at 100 V. The binding reactions were then transferred onto a nylon membrane (Millipore) at 280–300 mA for 60 min. The transferred DNA was then crosslinked to the membrane using UV-light (120 mJ/cm²) for 45–50 s followed by detection of the biotin labeled DNA by using the Chemiluminescent Nucleic Acid detection module (Thermo Scientific). In experiments where unlabeled DNA was used, the reaction was first incubated with protein and unlabeled DNA for 20 min at RT, followed by incubation with labeled DNA for 20 min at RT.

4.6 | Swimming motility assay

To assess swimming motility, mid-log phase (OD600nm ~0.8) cultures (2 μl) were inoculated into soft BHI agar (0.3%) plates and grown at 37°C for 24 h and subsequently at 25°C for 24 h. Motility was measured as the diameter of the spreading colony in at least 5 independent experiments.

4.7 | Hemolysin assays

Hemolytic activity was measured as previously described (Cahoon & Freitag, 2015). Briefly, bacterial cultures were grown overnight in LB, diluted 1:10 in fresh media and grown for 5 h. Culture supernatants were normalized to equivalent OD600 values and serially diluted into PBS pH 5 containing 1 mM DTT (PBS-DTT) and incubated at 37°C for 30 min. Subsequently, 100 μl of a 1:5 dilution of PBS-DTT washed sheep red blood cells (RBCs) was added and incubated for 30 min at 37°C. Bacterial supernatant/RBCs mixtures were pelleted and the supernatant dilution resulting in 50% RBC lysis was determined by visual inspection of the pellet.

4.8 | Detection of phospholipase activity

Phospholipase activity was detected using Brilliance Listeria Agar with differential supplement containing lecithin (Oxoid). An opaque halo around the bacterial streak is produced upon Lm phospholipase hydrolysis of lecithin in the medium. Single colonies were struck onto the medium and incubated for 24 h at 37°C followed by visual inspection of the zone of opacity surrounding the bacterial streaks.
4.9  Intravenous and oral mouse infections

Animal procedures were approved by the UIC Animal Care Committee and were conducted in the Biological Resources Laboratory following AAALAC approved procedures and guidelines. Overnight cultures were diluted 1:20 in BHI broth and grown to mid-log phase and normalized based on OD\textsubscript{600} values, washed twice with PBS pH 7, diluted, and re-suspended in PBS pH 7. For intravenous infections, female 7–9 week old Swiss Webster mice (Charles River Laboratories) were injected with 200\(\mu\)l containing 2\times10\textsuperscript{4} CFU of bacteria by tail vein injection, whereas for oral infections, mice were infected with 200\(\mu\)l containing 1\times10\textsuperscript{5} CFU of bacteria by gastric gavage. For competitive intravenous and oral infections, a 1:1 mixture or reference strain DP-3903 (Auerbuch et al., 2001) to test strain was used totaling 2\times10\textsuperscript{4} CFU and 1\times10\textsuperscript{9} CFU, respectively. At 72 h post infection, organs of infected animals were collected, homogenized, and 10-fold serial dilutions were plated for total CFUs.

4.10  L2 plaque assays

Plaque assays were conducted as previously described (Sun et al., 1990). Briefly, in 6-well culture dishes monolayers of L2 mouse fibroblasts were infected at an MOI of 30:1 for 1 h. Subsequently, infected monolayers were washed three times with PBS pH 7 and overlaid with DMEM/agarose containing 10 \(\mu\)g/ml gentamicin to kill extracellular bacteria. At 72 h, plaques were measured with a micrometer.

4.11  Growth assays under conditions of acidic pH, high osmolarity, and in ethanol

For growth assays, 2 \(\mu\)l of a saturated overnight culture was inoculated into 2 ml BHI liquid broth at pH 6 (acidic pH), containing 5% NaCl w/v (high osmolarity), or 4% ethanol. Cultures were grown overnight at 37\(^\circ\)C with agitation and growth was measured as a function of optical density (OD\textsubscript{660nm}).

4.12  Determination of antibiotic minimum inhibitory concentration

To determine the MIC, 2 \(\mu\)l of a mid-log phase (OD\textsubscript{600nm} ~0.8) culture was inoculated into 2 ml BHI broth in 4 ml polypropylene tubes containing dilutions of penicillin G or lysozyme. Cultures were grown with agitation at 37\(^\circ\)C for 16 h followed by visual inspection where the MIC was noted as the complete inhibition of bacterial growth.

4.13  RNA extraction, microarray and qRT-PCR analysis

\(Lm\) overnight cultures were diluted 1:20 in BHI broth and grown to an OD\textsubscript{600nm} ~0.6. Bacteria were normalized based on OD\textsubscript{600} values and ~7 ml was collected for each strain then 1 volume of RNAProtect Bacteria Reagent (Qiagen) was added. Bacteria were recovered by centrifugation and treated with 20mg/ml lysozyme and 50mg/ml \(Lm\) endolysin in TE pH 8 in a total volume of 1 ml for 15 min at 37\(^\circ\)C, vortexing every 5 min. Then pellets were sonicated on ice for 30s followed by incubation on ice for 30s; this was repeated an additional 4 times. RNA extraction was performed using the RNeasy Mini Kit (Qiagen) with on-column DNAse digestion. DNA microarrays were performed using extracted RNA by Microarrays Inc. Three independent microarrays were performed for each strain where each microarray contained 3 spots per gene; microarray values were averaged and normalized with RpoB. For qRT-PCR, both cDNA synthesis and qPCR was performed using the IDT Prime Time Custom qPCR Probes application and purchased from the manufacturer premixed in a 20\(\mu\)l volume at a ratio of 1:1:0.5 nanomoles of forward primer to reverse primer to probe, respectively (Integrated DNA Technologies). For qPCR reactions, TaqMan gene expression master mix (Applied Biosystems) was used as specified by the manufacturer with 2 \(\mu\)l of a 1:10 dilution of the premixed primer/probe in a final reaction volume of 20\(\mu\)l. Fold changes were determined using the comparative \(2^{\Delta\DeltaCT}\) method (Schmittgen & Livak, 2008).

4.14  Statistical analyses

A two-tailed Student’s T-test was used for statistical analysis where \(p \leq 0.05\) and error bars represent the standard error of the mean for data represented as a bar graph. A two-tailed Wilcoxon Rank-Sum Test was used where \(p \leq 0.05\) for data represented as box plots.

ACKNOWLEDGEMENT

We thank members of the Freitag laboratory and the UIC Positive Thinking group for helpful discussions. This work was supported by NIH grants R01 AI083241 and AI083241-03S1 to NEF and NRSA F32 AI115954-01 to LAC. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the funding source.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in https://doi.org/10.6084/m9.figsh are.19723630.v1.

ORCID

Avinash N. Gururaja https://orcid.org/0000-0002-7500-9670
Nancy E. Freitag https://orcid.org/0000-0003-1322-3978

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Cahoon, L. A., Alejandro-Navarreto, X., Gururaja, A. N., Light, S. H., Alonzo, F., Anderson, W. F. & Freitag, N. E. (2022). *Lysteria monocytogenes* two component system PieRS regulates secretion chaperones PrsA1 and PrsA2 and enhances bacterial translocation across the intestine. *Molecular Microbiology*, 118, 278–293. [https://doi.org/10.1111/mmi.14967](https://doi.org/10.1111/mmi.14967)