Starvation- and Stationary-Phase-Induced Resistance to the Antimicrobial Peptide Polymyxin B in *Salmonella typhimurium* Is RpoS (σ^S) Independent and Occurs through Both *phoP*-Dependent and -Independent Pathways

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A common stress encountered by *Salmonella* serovars involves exposure to membrane-permeabilizing antimicrobial peptides and proteins such as defensins, cationic antibacterial proteins, and polymyxins. We wanted to determine if starvation induces cross-resistance to the membrane-permeabilizing antimicrobial peptide polymyxin B (PmB). We report here that starved and stationary-phase (Luria-Bertani [LB] medium) cells exhibited ca. 200- to 1,500-fold-higher (cross-)resistance to a 60-min PmB challenge than log-phase cells. Genetic analysis indicates that this PmB resistance involves both *phoP*-dependent and -independent pathways. Furthermore, both pathways were σ^S independent, indicating that they are different from other known σ^S -dependent cross-resistance mechanisms. Additionally, both pathways were important for PmB resistance early during C starvation and for cells in stationary phase in LB medium. However, only the *phoP*-independent pathway was important for P-starvation-induced PmB resistance and the sustained PmB resistance seen in 24-h-C-starved (and N-starved) or stationary-phase cells in LB medium. The results indicate the presence of an *rpoS*- and *phoP*-independent pathway important to starvation- and stationary-phase-induced resistance to membrane-permeabilizing antimicrobial agents.

Polymyxins (e.g., polymyxin B [PmB] and polymyxin E or colistin) are members of a group of small cationic amphipathic antimicrobial peptides that are bactericidal for most gram-negative bacteria. Other members of this group include maganins found in frog skin and defensins and cationic antibacterial proteins (CAP) found in the granules of mammalian phagocytic cells (39). Polymyxins are bactericidal to gram-negative bacteria via a two-step mechanism of action. In the first step, they bind to the outer membrane (OM) through interactions with the lipopolysaccharide of the bacteria. This initial step makes the OM permeable but is not in itself lethal to the bacteria; however, it does provide access for the polymyxin to the cytoplasmic membrane where permeabilization results in cytoplasmic leakage and eventually cell death (38). Studies with defensins show that they form voltage-gated channels in lipid bilayers and are thought to kill the bacterial target by depolarizing the cytoplasmic membrane (12). Polymyxins and the defensins and CAP of mammalian phagocytic cells appear to be similar in overall structure and mechanisms of action, sharing a common binding site at the OM, indicated by the fact that polymyxin can inhibit the binding of the human cationic antibacterial proteins CAP37 and CAP57 to the cell surface of *Salmonella typhimurium* (3, 39). Cationic antimicrobial peptides (e.g., defensins and polymyxins) and proteins (e.g., CAP) are present both in nature and mammalian hosts. Thus, they can be important agents of environmental stress to bacterial pathogens spending time in both natural and host microenvironments.

Salmonella serovars, as a result of their life style, face a variety of potentially lethal environmental stresses. These

stresses can include nutrient starvation and extremes in temperature, pH, and/or osmolarity, as well as the presence of reactive oxygen species (e.g., H_2O_2) and small cationic antimicrobial peptides or proteins (6). Thus, in order for the salmonellae to be successful as pathogens, they must be able to sense, respond to, and survive exposures to potentially lethal environmental extremes and hazards encountered in natural microcosms and the arsenal of antimicrobial systems initiated within the infected host.

A prominent stress encountered by *Salmonella* serovars is nutrient starvation. *S. typhimurium* and other bacteria upon starvation undergo a dramatic physiological alteration, the starvation stress response that not only protects the cell against the harmful effects of prolonged starvation but can also provide cross-resistance to other environmental stresses such as oxidative and thermal challenges (6–8, 14, 18, 20, 25–27, 32–37). Because starved cells are generally more resistant to environmental stress than growing or log-phase cells and because starvation is a common stress encountered by *S. typhimurium* in its journeys, we wanted to examine the possibility that starved or stationary-phase cells are more resistant to the membrane-permeabilizing effects of the antimicrobial peptide PmB than actively growing log-phase cells.

MATERIALS AND METHODS

Bacterial strains used. The virulent wild-type *S. typhimurium* SL1344 (*hisG46*) (11), an *rpoS* mutant derivative (SMS438) (*hisG46 rpoS::Amp^r*) (2, 26), and a *phoP* mutant derivative (SMS559) (*hisG46 phoP105::Tn10*) (this study; J. Roth) were used in this study.

Media and chemical reagents. The minimal media used in these studies were morpholinepropanesulfonic acid (MOPS)-salts based (MS) medium containing nonlimiting concentrations of phosphate (P), carbon (C), and nitrogen (N) sources or limiting P, C, or N source as previously described in detail (26, 35). Since the concentrations of divalent cations can affect PmB activity (39), the final concentrations of divalent cations present in both the growth and challenge media were as follows: 1 mM $MgCl_2$, 10 μ M $CaCl_2$, 100 nM $MnCl_2$, and 10 nM $ZnCl_2$. The rich medium used in these studies was Luria-Bertani (LB) complex broth or agar (22). The minimal media were supplemented with histidine at a

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FIG. 1. C starvation, P starvation, and LB-stat induce cross-resistance to a 60-min PmB challenge. *S. typhimurium* SL1344 was carbon (C) starved, phosphate (P) starved, or grown to stationary phase in LB medium and then challenged with 100 μg of PmB ml^{-1} for up to 60 min as described in Materials and Methods. Dashed lines are used for cells in various stages of log-phase growth, while solid lines are used for cells at various times during starvation or LB-stat. The data presented are from a representative experiment from at least three separate trials.

final concentration of 0.2 mM. Antibiotics were used as needed at the following final concentrations: ampicillin at 30 μg ml^{-1} and tetracycline at 20 μg ml^{-1} (rich medium) or 10 μg ml^{-1} (minimal media).

PmB challenge protocol. The appropriate PmB concentration used in this study was determined by exposing log-phase wild-type cells to a range of PmB (specific activity, 8,702 USP units/mg; AMRESCO, Solon, Ohio; United States Pharmacopoeia XXII) concentrations (0 to 125 μg ml^{-1}) for 60 min. The concentration of 100 μg (870.2 USP units) of PmB ml^{-1} was determined to produce significant loss of viability in log-phase cells over the 60-min challenge time period (data not shown) and thus was selected as the concentration to be used in subsequent PmB challenge experiments.

To compare the PmB sensitivity of log-phase and starved or stationary-phase cells, cells were challenged with PmB during log-phase growth in MS medium with nonlimiting concentrations of P, C, and N sources or LB broth and after a predetermined time interval of phosphate (P), carbon (C), or nitrogen (N) source starvation or stationary phase in LB medium up to a maximum of 24 h. Cells were starved as a result of exhaustion of the limiting nutrient (C, P, or N) as previously described (26, 35). At desired time intervals, cells were challenged by diluting the cultures 1:100 into MS buffer with 100 μg of PmB ml^{-1} and incubating at 37°C with shaking. To determine the kinetics of the development of PmB resistance, cells were challenged at various predetermined time intervals during growth and starvation in minimal media or log and stationary phase in LB broth. Growth was monitored by optical density at 600 nm (OD_{600}). For the purposes of definition, early-, mid-, and late log-phase cells came from cultures at OD_{600} s of 0.1 to 0.2, 0.4 to 0.5, and 0.75 to 0.85, respectively. Cells were considered to be starved or in stationary phase when the OD_{600} of the culture ceased to increase.

At predetermined time intervals following dilution into the MS buffer-PmB challenge medium, aliquots were removed, serially diluted, and plated onto LB agar plus antibiotics, as needed, to determine viable cell counts in CFU milliliter^{-1} . Percent survival was calculated as CFU milliliter^{-1} at each time point divided by the CFU milliliter^{-1} at time zero (maximum survival) multiplied by 100. One hundred percent survival was typically between 5×10^5 and 3×10^6 CFU ml^{-1} .

RESULTS AND DISCUSSION

Starved cells, but not log-phase cells, are resistant to PmB challenge. Figure 1A illustrates that wild-type *S. typhimurium* SL1344 cells, carbon starved for as little as 30 min, were up to 1,200-fold more resistant (as measured by the percent survival of the challenged cells) to the effects of a 60-min challenge with 100 μg of PmB ml^{-1} than cells in early log phase. Maximum resistance to PmB challenge was achieved within the first 30 min of C-starvation-induced stationary phase. This maximum level of C-starvation-induced cross-resistance gradually declined about 40% over the next 4 h (Fig. 1A) and remained at this lower level for the next 6 h of C starvation (data not shown). Interestingly, between 10 and 15 h of C starvation, the level of PmB resistance decreased further to about 15% of the maximum level of resistance seen at 30 min of C starvation (data not shown). It remained at this lower level of (cross-) resistance for at least the next 9 h of C starvation (total of 24 h starvation). However, it should be noted that 24-h-C-starved cells were still ca. 200- to 250-fold more resistant to PmB challenge than log-phase cells (Fig. 1A). Cells starving for N source also induced PmB cross-resistance with kinetics and levels similar to those observed for C-starving cells (data not shown).

The kinetics of phosphate-starvation-induced PmB resistance were different from that of C-starvation-induced PmB resistance. As seen in Fig. 1B, P-starved cells were up to ca. 1,500-fold more resistant than cells in early log phase to PmB challenge. P-starvation-induced PmB resistance was seen to

FIG. 2. Effect of a *phoP* null mutation on carbon (C)-starvation-induced cross-resistance to PmB challenge. Strains SL1344 (wild type) and SMS559 (*phoP::Tn10*) were starved for carbon source. Cells were challenged with 100 μ g of PmB ml⁻¹ for up to 60 min at mid-log phase or 5 or 24 h of C starvation as described in Materials and Methods. The data presented are from a representative experiment from at least three separate trials.

increase over the first 4 h of starvation, reaching a maximum level of cross-resistance (ca. 100% survival) at 4 h of P starvation. Furthermore, this level of cross-resistance was sustained over the next 20 h of P starvation (Fig. 1B). This is in contrast to C starvation where maximum resistance was achieved after only 30 min of C starvation and where the level of resistance then gradually declined to a lower sustained level by 15 h of C starvation. Thus, P starvation appears to induce a greater and more sustainable cross-resistance to PmB challenge than either C or N starvation.

Stationary phase in complex medium (LB) also induces PmB resistance. Figure 1C illustrates that wild-type *S. typhimurium* SL1344 cells also develop PmB resistance upon entry into stationary phase in LB broth (LB-stat). In contrast to early log-phase cells in minimal MS medium, early log-phase cells in LB medium were hypersensitive to PmB challenge, as indicated by the fact that no viable cells were detected after just 10 min of PmB challenge (Fig. 1C). Interestingly, late log-phase cells growing in LB medium acquired increased resistance to PmB challenge relative to early log-phase cells. However, it should be noted that they were still very sensitive to PmB challenge compared with stationary-phase cells. The level of PmB resistance increased as the cells entered stationary phase, reaching a maximum level after only 1 h in LB-stat (Fig. 1C). As with C starvation, this maximum level of LB-stat-induced PmB resistance gradually decreased over the next 23 h to a level equivalent to that of early stationary-phase cells (com-

pare 30 min LB-stat cells to 24 h LB-stat cells [Fig. 1C]), i.e., to about 10% of the maximum level of LB-stat-induced PmB cross-resistance. Because LB medium is a complex medium, it is difficult to determine exactly why cells enter stationary phase in this medium. We have some data suggesting that C-source limitation is a major reason (data not shown), although it is probably a combination of factors such as C-source limitation, the accumulation of weak acids (31), reduced oxygen availability, high cell density, N-source limitation, or additional factor(s). However, on the basis of similarity to the kinetics of C-source (and N-source) starvation-induced PmB resistance, these two conditions or conditions that generate similar signals may play some role in the development of LB-stat-induced PmB resistance.

Starvation- and LB-stat-induced PmB resistance is *rpoS* independent. The *rpoS* (*katF*) gene encodes an alternative σ transcription factor (σ^S or σ^{38}) that controls gene expression during starvation and the transition to stationary phase in complex medium (16). Many of the gene products whose expression is regulated by σ^S are required for long-term survival during nutrient starvation, cross-resistance to thermal, oxidative, and osmotic stresses, and *Salmonella* virulence (1, 2, 10, 15–18, 21, 32, 35, 41, 42). Therefore, we wanted to determine if σ^S might play a role in starvation- or LB-stat-induced cross-resistance to PmB challenge.

Our results indicated that the absence of a functional *rpoS* gene product does not impair the establishment of (C, P, or N) starvation- or LB-stat-induced PmB resistance (data not shown). There was virtually no difference between an *rpoS* null mutant (2, 26) and the parent *rpoS*⁺ strain (Fig. 1) in terms of the PmB resistance generated during P, C, or N starvation or LB-stat (data not shown). It should be noted, however, that this same *rpoS* null mutant is defective in starvation survival, as well as starvation- or stationary-phase-induced cross-resistance to oxidative and thermal challenge (2, 18, 26, 32). This indicates that the development of PmB cross-resistance is independent of σ^S and σ^S -dependent genes. Thus, starvation- and LB-stat-induced cross-resistance to PmB appears to involve different mechanisms from those for starvation-induced cross-resistance to environmental stresses such as thermal or oxidative challenge.

Starvation- and LB-stat-induced PmB resistance involves both *phoP*-dependent and -independent mechanisms. The *phoP* locus was originally identified as a positive regulator of a nonspecific acid phosphatase activity (the *phoN* gene product) in *S. typhimurium* (13). Later, Fields et al. (4, 5) identified the *phoP* locus as a virulence locus for *S. typhimurium*, being required for its intracellular survival in macrophages. The *phoP* locus encodes two proteins, one a transmembrane sensor or transmitter protein (PhoQ) and the other a transcriptional regulator (PhoP). The PhoPQ regulon includes, at least in part, genes and proteins involved in resistance to defensins and other cationic antimicrobial peptides (9). Strains carrying null mutations in *phoP* or *phoQ* exhibit about a 1,000-fold-higher susceptibility to defensins. In addition, Groisman et al. (9) demonstrated that resistance to host antimicrobial peptides is also necessary for *Salmonella* virulence. However, the increased susceptibility of *phoP* mutants to various antimicrobial peptides was measured only in log-phase cells (4). Therefore, we wanted to determine if the *phoP* locus is involved in the starvation and stationary-phase induction of PmB resistance that is reported here.

As expected, on the basis of previous studies with defensins and other similar antimicrobial peptides, log-phase cells carrying a *phoP* null mutation were extremely sensitive to PmB challenge compared with isogenic wild-type strains (Fig. 2A,

FIG. 3. Effect of a *phoP* null mutation on phosphate (P)-starvation-induced cross-resistance to PmB challenge. Strains SL1344 (wild type) and SMS559 (*phoP*::Tn10) were starved for phosphate source. Cells were challenged with 100 μg of PmB ml^{-1} for up to 60 min at mid-log phase or 4 or 24 h of P starvation as described in Materials and Methods. The data presented are from a representative experiment from at least three separate trials.

3A, and 4A). This supports the hypothesis that PmB is a good (and less expensive) model for antibacterial peptides or proteins and that the results presented here may be extrapolated to the in vivo situation.

The results presented in Fig. 2, 3, and 4 demonstrate that there are at least two mechanisms involved in the development of C-starvation- and LB-stat-induced PmB resistance. One is *phoP* dependent and established early in starvation or stationary phase but appears to decline in importance the longer cells remain starved or in stationary phase. It may also be argued from our findings that the *phoP*-dependent pathway is needed for the basal-level PmB resistance observed in log-phase cells in our minimal medium (but not LB medium), since there was a significant difference between log-phase *phoP* mutant and wild-type cells under these conditions (Fig. 2, 3, and 4). This model is supported by the findings that 5-h-C-starved and 1-h-LB-stat *phoP* mutants were more sensitive to PmB challenge than equivalently treated wild-type cells (Fig. 2B and 4B). This *phoP*-dependent mechanism appears to be especially important for cells early in stationary phase in LB medium, since a *phoP* mutant only 1 h into LB-stat (normally the point where maximum PmB resistance was achieved) (Fig. 1C) was approximately 200 times more sensitive to a 60-min PmB challenge than the equivalently treated isogenic wild-type strain (Fig. 4B). The importance of the *phoP* regulon in log-phase, early C-starved, and LB-stat cells is reflected by the differences between the wild-type and mutant strains in Fig. 2B and 4B.

Interestingly, the *phoP* mutation had virtually no effect on the development of PmB resistance in 4-h-P-starved cells. There was no difference between the wild-type and *phoP* mutant strains over the first 40 min of PmB challenge; it was not until the 60-min time point that cell viability was found to decline (ca. fivefold) (Fig. 3B).

The second starvation- or stationary-phase-induced PmB resistance mechanism(s) is *phoP* independent. This PmB resistance mechanism(s) also appears to function early during starvation or LB-stat. However, unlike the *phoP*-dependent system, this mechanism(s) of resistance is sustained for at least 24 h of starvation or LB-stat and does not appear to be important in log-phase cells. This hypothesis is supported by three findings: (i) 5-h-C-starved, 4-h-P-starved, and 1-h-LB-stat *phoP* mutant cells were more resistant to PmB challenge than log-phase *phoP* mutant cells; (ii) the fact that 24-h-C-starved, -P-starved, or -LB-stat *phoP* mutant and wild-type strains exhibited little to no significant difference in PmB resistance; and (iii) a *phoP* null mutation alone was sufficient to knock-out basal PmB resistance in log-phase cells (Fig. 2C, 3C, and 4C). Thus, any PmB resistance observed in starved or stationary-phase *phoP* mutant cells is *phoP* independent.

In summary, the results presented here indicate that C (and N)-starved, P-starved cells, as well as cells in LB-stat are 200- to 1,500-fold more resistant to the lethal effects of the cationic antimicrobial peptide PmB. Furthermore, this cross-resistance induced by starvation or stationary phase is independent of the

FIG. 4. Effect of a *phoP* null mutation on LB-stat-induced cross-resistance to PmB challenge. Strains SL1344 (wild type) and SMS559 (*phoP*::Tn10) were grown to stationary phase in LB broth. Cells were challenged with 100 μg of PmB ml^{-1} for up to 60 min at mid-log phase or 1 or 24 h of LB-stat as described in Materials and Methods. The data presented are from a representative experiment from at least three separate trials.

rpoS-encoded σ factor, σ^S . Thus, the mechanisms of PmB cross-resistance reported in this study are different from the σ^S -dependent mechanisms described for cross-resistance to thermal, oxidative, and osmotic stresses (2, 7, 15–18, 21, 25, 32).

Furthermore, our results indicate that the increased PmB resistance observed in starved or stationary-phase cells is a dynamic process that involves at least two separate pathways, a *phoP*-dependent pathway and more intriguingly a *phoP*-independent pathway(s). The dynamic aspect of this process is illustrated by the findings that the level of PmB resistance can change over at least the first 24 h of starvation or LB-stat and the importance of the two PmB resistance pathways can also change over this period. Moreover, the growth conditions can also influence the level of PmB resistance and the relative importance of the *phoP*-dependent and -independent pathways. For example, both the *phoP*-dependent and -independent mechanisms are important early during C starvation or LB-stat. However, as the cells continue to C starve or continue in LB-stat, the *phoP*-dependent mechanism becomes less necessary while the *phoP*-independent mechanism remains integral, providing a sustained albeit lower level of cross-resistance (compare the maximal levels of PmB resistance seen in 5-h-C-starved and 1-h-LB-stat cells to that observed in 24-h-C-starved and LB-stat cells; Fig. 2 and 4). However, this is not the case for P-starvation-induced PmB resistance, since there was little to no difference between the P-starved wild-type and *phoP* mutant PmB resistance profiles (Fig. 3). Moreover, P starvation induces a consistently higher and more sustainable level of cross-resistance (ca. 100% survival after 60 min of PmB challenge).

The reasons why starved or LB-stat cells are more resistant to PmB are unclear at this time. However, clues to potential mechanisms involved in PmB resistance can be gleaned from reports from several laboratories that have examined resistance to other cationic antimicrobial peptides such as defensins, polymyxin E, and protamine. It should be noted, however, that the resistance or sensitivity to antimicrobial peptides reported for these various mutants has been characterized in log-phase cells but not in starved or stationary-phase cells. Nonetheless, similar mechanisms may be involved in the PmB resistance pathways described in this report.

It is still unknown as to which PhoPQ-regulated proteins are important for resistance to host antimicrobial peptides, i.e., defensins. Several PhoP-activated genes or *pag* genes have been identified, although none have been reported to be involved specifically in defensin resistance. However, many *pag* genes, do appear to be starvation inducible (9, 13, 23, 24). Mäkelä et al. (19) isolated mutants exhibiting constitutive resistance to the antimicrobial peptide polymyxin E. One of these mutants was designated *pmrA* (*pmr* for polymyxin resistant). Roland et al. (30) later isolated spontaneous polymyxin E-resistant mutants, some of which mapped at the *pmrA* locus. The *pmrA* locus was found to define a two-component regulatory system, encoded by the *pmrA* and *pmrB* genes, that also controls resistance to antimicrobial peptides (30). One characteristic of the *pmrA* mutants is increased 4-aminoarabinose substitutions at the lipid A phosphoryl moieties which in turn reduce the net negative charge of the bacterium's lipopolysaccharide and, thus, decrease the ability of cationic antimicrobial peptides to bind to the OM (40). Interaction with the OM is the first step in polymyxin (as well as other similar antimicrobial peptides) microbicidal activity (38, 39). Therefore, a logical conclusion is that C, N, or P starvation and LB-stat may result in structural changes in the lipopolysaccharide and/or other OM components, rendering the cell more resistant to the

action of PmB. Such a mechanism may also explain the findings that P starvation induces a greater and more sustained PmB resistance than C or N starvation, since carbon and nitrogen are required for the synthesis of the 4-aminoarabinose lipid A modifying groups (40).

In contrast, Groisman and coworkers searched for mutants that were hypersensitive to the antimicrobial peptide protamine (9). Five of their mutants (designated *sap* mutants for sensitivity to antimicrobial peptides) were found to possess mutations in five separate genes, *sapABCDF*, encoding components of a new member of the ABC transporter family that shares homology with an *S. typhimurium* oligopeptide permease (28). These researchers hypothesize that (i) this transporter may transport small cationic antimicrobial peptides into the cell where they can be degraded by cellular peptidases or proteases or (ii) this ABC-type transporter senses the presence of these antimicrobial peptides in the environment and leads to the induction of resistance mechanisms. Thus, starvation- and LB-stat-induced PmB resistance may involve such antimicrobial peptide transporter, degradative, and/or sensor mechanisms. Two additional *sap* mutations are in the *sapG* and *sapJ* loci. Analysis of the products of these two genes reveals two proteins, SapG and SapJ. These proteins are very similar to the TrkA and TrkH protein components of a low-affinity K^+ transporter in *Escherichia coli* (29). Thus, starvation- and/or LB-stat-induced PmB resistance may also involve the synthesis of or activation of ion transporters that may alleviate some of the detrimental effects of increased membrane permeability that can occur as a result of PmB action.

These and other possibilities are currently under investigation. However, since log-phase and starved or stationary-phase cells are physiologically very different, it is likely that there are unique mechanisms that make starved or stationary-phase cells more resistant to PmB.

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