Delivery of cardiolipins to the bacterial outer membrane to promote virulence

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SUMMARY

Gram-negative bacteria generate an outer-membrane (OM) barrier to their environment. The PhoPQ-gene regulators coordinate OM-barrier remodeling mechanisms for bacterial resistance to innate-immune killing strategies. A screen for genes necessary for the PhoPQ-regulated OM-barrier of *Salmonella* Typhimurium has identified an inner-membrane (IM) protein, PbgA that binds glycerophospholipids known as cardiolipins and relies upon its periplasmic domain for their PhoPQ-regulated delivery to the OM. Purified PbgA forms tetramers *in vitro* and binds acidic cardiolipin head-groups using periplasmic arginines near the IM that are necessary for PbgA to function *in vivo*. The periplasmic-globular region of PbgA interacts with the OM in a PhoPQ-dependent manner suggesting PbgA forms a complex that may bridge the envelope to allow cardiolipin movement. Deleting portions of the periplasmic domain severely attenuates Salmonellae in their pathogenesis supporting that regulated delivery of cardiolipins to the OM is necessary for their survival within host tissues that activate PhoPQ.

ETOC

Gram-negative bacteria produce an outer membrane (OM) that can act as a barrier to antibiotics and the innate-immune system. We define a conserved transmembrane protein that binds and traffics acidic glycerophospholipids known as cardiolipins to the OM to promote OM-barrier function for intracellular survival of *Salmonella* within host macrophages.

INTRODUCTION

The cell envelope of Gram-negative bacteria comprises an inner and an outer membrane (OM) separated by a periplasmic space containing a thin layer of peptidoglycan, which is

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bound to the OM by lipoproteins (Nikaido, 2003). The OM itself is an asymmetric lipid bilayer of outer-leaflet lipid A molecules and inner-leaflet glycerophospholipids (GPL). The fatty-acyl chains of lipid A and GPL engage in hydrophobic interactions that anchor long negatively charged polar lipopolysaccharides (LPS) and capsular exopolysaccharides to the microbial surface (Whitfield and Trent, 2014). Lipids, proteins, and sugars interact to form a barrier that allows the bacterium to control the passage of ions, solutes, metabolites, and antibiotics into and out of the cell (Nikaido, 2003). During infection, pathogenic Gram-negative bacteria remodel the molecular landscape and chemical properties of the OM to promote their survival and replication within host tissues during infection (Needham and Trent, 2013; Pages et al., 2008). Mechanisms for the transport and assembly of lipopolyaccharides, proteins, and capsular exopolysaccharides have been defined (Dong et al., 2006; Dong et al., 2014; Hagan et al., 2011; Whitfield and Trent, 2014). However, how glycerophospholipids are transported across the periplasm and inserted into the inner-leaflet of the OM remains unknown. Therefore, much remains to be learned about the biogenesis and the regulation of the OM.

Cell-envelope regulation is perhaps best understood for the Enterobacteriaceae and the Salmonellae in particular. Both *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Typhi rely on the PhoPQ two-component gene regulators to survive within the acidified phagolysosome vacuoles of host macrophages and their mutants are attenuated in their pathogenesis for mammals including humans (Fields et al., 1989; Galan and Curtiss, 1989; Haraga et al., 2008; Hohmann et al., 1996; Miller et al., 1989). The *S.* Typhimurium PhoPQ regulators activate genes encoding proteins and enzymes that increase OM-lipid hydrophobicity and decrease OM-lipid negative charge to prevent cationic antimicrobial peptides (CAMP) from binding and inserting into the membrane to ultimately kill the microbe (Dalebroux and Miller, 2014). To promote the barrier function of the OM, the PhoPQ-gene regulators coordinate changes in lipopolysaccharide structure, alterations in the levels of specific OM proteins, and palmitoylation of lipid A and phosphatidylglycerol within the asymmetric bilayer (Dalebroux and Miller, 2014). Previously our laboratory provided data to support that the PhoPQ-gene regulators may promote specific increases in the levels of cardiolipins within the OM, though the molecular mechanism and biological significance were not yet known (Dalebroux et al., 2014).

Cardiolipins are acidic diphosphatidylglycerols found within the membranes of bacteria and mitochondria where they contribute unique bioactivities for cell physiology. Their tetra-acylated dimeric structure and chirally distinct phosphates allows cardiolipins to self-associate and form non-bilayer hexagonal phases (Schlame, 2008). Cardiolipins can generate negative-membrane curvature, initiate membrane hemifusion, and become concentrated at membrane-contact sites (Schlattner et al., 2014). Bacterial proteins that bind to cardiolipins function in membrane fission for sporulation of *Bacillus subtilus* and in cytoskeletal arrangement for cell division of *Escherichia coli* (Doan et al., 2013; Renner and Weibel, 2012). Mitochondrial cardiolipins form protein-lipid signaling platforms that activate cell apoptosis and directly bind and activate the Nlr3p inflammasome (Iyer et al., 2013; Schug and Gottlieb, 2009).

Given their importance for cell physiology and immunity, we sought to determine the molecular mechanism and biological significance for the regulated increase in cardiolipins within the OM for *S.* Tyhimurium. A screen for genes necessary for the PhoPQ-regulated OM barrier has identified a conserved IM protein, PbgA that binds cardiolipins near the IM and traffics them to the OM by a mechanism that involves the periplasmic-globular region and its PhoPQ-dependent interaction with the OM. The globular region of PbgA is necessary for *S.* Typhimurium to survive within host tissues providing evidence to support that regulated increases in OM cardiolipins are required during infection.

RESULTS

Identification of a gene required for the PhoPQ-regulated outer-membrane barrier of Salmonella Typhimurium

Damage to the OM inflicted by CAMP molecules causes RcsF-dependent activation of the Rcs regulon, which is a *S.* Typhimurium multicomponent gene-regulatory network controlling the synthesis, export, and assembly of the Group-1 enterobacterial exopolysaccharide capsule (Fig. S1A) (Farris et al., 2010; Majdalani and Gottesman, 2005). The *wza* promoter drives expression of a three-gene operon encoding the capsule-export machinery that becomes activated when the RcsF-sensor lipoprotein is altered in its localization by physical damage to the OM (Fig. S1A) (Farris et al., 2010). Accordingly, the *wza* promoter can serve as an effective gene reporter of OM perturbation as long as the mutations inducing its activity require RcsF (Fig. S1B). On receiving environmental cues that include CAMP, Salmonellae use the PhoPQ-gene regulators to increase their OM barrier and resist the binding and killing mechanisms for host-derived CAMP molecules (Dalebroux and Miller, 2014). Therefore, we predicted that the chemically remodeled OM structure for the PhoPQ-activated bacteria would diminish the Rcs-regulon activation by CAMP. To test this hypothesis, we treated bacteria experiencing low, moderate, and high levels of PhoPQ-signaling activity with sub-minimal inhibitory concentrations of polymyxin B and measured the levels of *wza-lacZ* gene-reporter activity (Dalebroux et al., 2014). Indeed, bacteria with higher PhoPQ signaling had decreased gene-reporter levels compared to bacteria with lower signaling (Fig. S1C) (Dalebroux et al., 2014). Accordingly, we screened for strains with transposon insertions in genes that conferred activation of the *wza-lacZ* reporter when the PhoPQ regulators were also genetically activated (Fig. S1B).

Hypothetical genes with transposon insertions were termed *phoPQ-barrier genes* (*pbg*). Five insertions inducing the *wza* reporter mapped to a gene, *PbgA* that was predicted to encode an IM protein with a carboxyl-terminal periplasmic domain (Fig. 1A). *S.* Typhimurium PbgA is a homologue of an *E. coli* hypothetical protein, YejM whose transmembrane (TM) domain is essential for bacterial viability (De Lay and Cronan, 2008; Gerdes et al., 2003). The periplasmic-globular region of PbgA is dispensable for bacterial viability and shares predicted structure homology to aryl-sulfatase enzymes and lipotechoic-acid synthase (LtaS) enzymes, the latter found in most Gram-positive bacteria (Lu et al., 2009). Similar to LtaS, PbgA has five-TM helices (Fig. 1B). The final helix is flanked on both sides of the IM by two basic regions, a short cytoplasmic helix (pI: 12.48) and a longer periplasmic region of three consecutive helices (pI: 9.52) that links the TM segments to an unstructured region,

followed by the sulfatase-like globular region at the carboxyl-terminus (Fig. 1B and S2). Insertions within the globular region of *pbgA* caused the greatest levels of RcsF activity in the PhoPQ-activated bacteria (Fig. 1A and 1C). The screen had indicated that the globular region of PbgA might play an important function in the stability of the PhoPQ-activated OM-barrier for *S.* Typhimurium.

PbgA is an inner membrane protein that forms tetrameric oligomers when purified

The periplasmic domain of PbgA(191–586) was affinity purified and antisera were raised against it so that the full-length PbgA polypeptide could be localized within the envelope of *S.* Typhimurium. Membranes for the wild type, the PhoP-null, and the PhoPQ-activated bacteria were separated on the defined sucrose-density gradients that partition the envelope into two distinct fractions, a low-density IM fraction enriched in SecA and a high-density OM fraction enriched in OmpA (Cornet et al., 2006; Dalebroux et al., 2014). For all three bacterial genotypes, the 67 kilodalton (kDa) PbgA polypeptide was in similar abundance for the low-density IM fractions (Fig. 2A). This suggested that PbgA synthesis was not regulated by the PhoPQ system. PbgA was also detected within the OM fractions for the wild type and the PhoPQ-activated bacteria albeit at much lower levels than for within the IM. By contrast, the PhoP-null mutants were diminished in their levels of PbgA within the OM (Fig. 2A), suggesting that PbgA is predominantly localized to the IM but may interact with the OM in a manner that involves PhoPQ.

Bacterial membranes were solubilized in detergent so that full-length PbgA could be purified. Affinity purification and size-exclusion chromatography (SEC) were used. The PbgA polypeptide eluted at a greater than predicted molecular size by SEC, between 230 and 440 kDa (Fig. 2B). Since PbgA elution could be impacted by the 0.02% dodecylmaltoside within the SEC buffers, multi-angle light-scattering (MALS) analysis was performed to confirm that the absolute molecular mass for the PbgA oligomers was indeed 268 kDa (Fig. 2C). Thus, PbgA forms tetramers *in vitro*.

The periplasmic domain of PbgA is necessary for PhoPQ-regulated increases in outer-membrane barrier function and survival of S. Typhimurium during infection

A variety of prior and current analyses including the inability to isolate insertions or to delete *pbgA* sequence indicated that the *yejM/pbgA*-transmembrane region is essential for both *E. coli* and *S.* Typhimurium viability. The insertions isolated in our genetic screen were predicted to disrupt ~ 85 to 233 amino acids for the carboxyl-terminus of PbgA (Fig. 1A). Similarly, others found the periplasmic domain was dispensable (Canals et al., 2012). Furthermore, site-directed *S.* Typhimurium deletions removing a substantial portion of the PbgA-globular region, *pbgA(328–586)*, grew at rates comparable to the wild type in Luria-broth (LB) growth medium (Fig. 3A). Similarly, RcsF was not activated in the *pbgA(328–586)*-mutant bacteria unless PhoPQ signaling was induced (Fig. 3B). Likewise, outer-membrane permeability to ethidium bromide was increased for the PhoPQ-activated *pbgA(328–586)* mutants relative to the activated *pbgA+* control cells (Fig. 3C). Therefore, the periplasmic-globular region of PbgA is only required for bacterial barrier integrity during bacterial-PhoPQ signaling. To genetically compliment the *pbgA*-deletion mutant defects, two plasmids were constructed. The first plasmid encoded full-length PbgA. The

second encoded the periplasmic-domain as a peptide, PbgA(191–586), that was targeted and released into the periplasm by use of the PagC-signal export sequence and peptidase cleavage site, which was encoded in the gene so as to be expressed at the amino terminus (Fig. 3D) (Farris et al., 2010). When the PhoPQ-activated *pbgA(328–586)*-mutant bacteria were induced to express either full-length PbgA or the periplasmic peptide, RcsF activity and the rate of increase in EtBr fluorescence within the cells were each fully restored to levels determined for the PhoPQ-activated *pbgA+* bacteria (Fig. 3B). These results indicated that the phenotypes for the mutants were not a result of second-site mutations or polar effects. Additionally, the periplasmic peptide was functionally sufficient for rescue of the PhoPQ-activated OM-barrier defects, by a mechanism that is independent of a *cis*-encoded sequence for the TM domain. Therefore, the carboxyl terminus of PbgA specifically functions in the PhoPQ-activated OM-barrier for *S*. Typhimurium.

Acidic pH and CAMP within the phagolysosomes of macrophages activate the *S*. Typhimurium PhoPQ-gene regulators to coordinate OM-remodeling mechanisms that promote bacterial survival within host tissues (Dalebroux and Miller, 2014). Therefore, the contribution of the PbgA-periplasmic domain to intracellular survival of *S*. Typhimurium was tested. Mouse bone-marrow-derived macrophages were infected with *pbgA+* and *pbgA(328–586)*-mutant *S*. Typhimurium. At two and twenty four hours-post infection bacterial colonies were enumerated to reveal dramatic 10^3–10^4 log decreases in the number of surviving intracellular bacteria for *pbgA(328–586)* mutants compared to wild type (Fig. 3E). Since *S*. Typhimurium survives and replicates within the vacuoles of macrophages to colonize the spleens of inbred susceptible mice, we used an intraperitoneal infection model to test PbgA's contribution to systemic infection of mammals. Consistent with the their defective survival in macrophages, the *pbgA(328–586)* mutants were severely attenuated in their competition with wild type for splenic colonization and survival in mice and their competitive indices were highly significantly different (Fig. 3F). For nine of the twenty mice, zero surviving *pbgA(328–586)* mutants were recovered from animals infected with >10^5 microbes. These mice were not used for the statistics, so the *pbgA*-mutant attenuation is highly severe. In conclusion, the globular region of PbgA is necessary for bacteria to increase the OM barrier that promotes survival within host tissues that activate PhoPQ.

Independent of the PhoPQ-gene regulators, the TM domain and periplasmic basic region of PbgA are required for bacterial cell growth

Despite repeated efforts and several strategies neither the TM domain of *pbgA* nor the polycistronic gene locus upstream, *yejL*, could be deleted from the chromosome (Fig. 1A). Therefore, both might be essential for bacterial viability. Alternatively, mutations in *yejL* might exert polar effects on *pbgA*. The initial deletion, *pbgA(328–586)*, retained the periplasmic-basic region near the IM (Figs. 1B and S2). To assess the contribution of the basic region to PbgA function, a more severe deletion mutant was generated. The *pbgA(191–586)* mutants were viable but grew at a slightly reduced rate in LB-growth medium (Fig. 3A). These mutants were increased in their *wza-lacZ* reporter levels independent of the PhoPQ-activation state (Fig. 3B). The phenotypes of the *pbgA(191–586)* bacteria could be rescued by expression of the full-length PbgA polypeptide *in trans*. However, targeted expression of the periplasmic-domain peptide to the periplasm was not

functionally sufficient (Fig. 3B). Thus, the periplasmic-basic region likely participates in a function of PbgA that is required for cell growth and OM integrity independent of PhoPQ. Bacteria were next assessed for their morphology using phase contrast and fluorescence microscopy and a lipophilic dye, FM4-64 that stains biological membranes. Morphologic and fluorescence analysis of the *pbgA(328–586)*-mutant cells and their membranes revealed no obvious defects consistent with their near wild-type growth rate in LB medium (Fig. 3G). For the *pbgA(191–586)* mutants, the reduced rate of growth was likely caused by a defect in septation, since although the membranes appeared to be constricted at the midcell of the dividing-mutant bacteria, the cells grew as chains that could not properly separate from one another (Fig. 3G). Their morphology resembled that of classical *S.* Typhimurium cell-division mutants with specific defects in OM constriction (Fung et al., 1980; Weigand et al., 1976). Therefore, PbgA may generally function for *S.* Typhimurium cell growth and division by a mechanism that involves septation and constriction of the OM.

The periplasmic-globular region of PbgA is required for PhoPQ-regulated increases in outer-membrane cardiolipins

S. Typhimurium cells that are highly activated for PhoPQ signaling contain four-to-five times greater levels of OM cardiolipins than bacteria that are not though no specific mechanism for this increase has been identified (Dalebroux et al., 2014). Given the importance of the PbgA-periplasmic globular region for the PhoPQ-activated OM-barrier of *S.* Typhimurium, we tested whether it was necessary for the regulated increase in OM cardiolipins. We devised a genetic strategy to regulate PhoPQ activity levels within the cell (Fig. 4A). This approach allowed us to measure the glycerophospholipid content of the membranes in a dynamic manner. The highly-activated *phoQ(cHAMP)* allele was cloned behind a regulatable promoter *in trans* (Dalebroux et al., 2014). The *pbgA(328–586)*-mutant cells that were cultured with glucose to repress *phoQ(cHAMP)* showed minimal *wza-lacZ* reporter activity consistent with their low levels of PhoPQ signaling (Fig. 4B). By contrast, the arabinose-induced *pbgA(328–586)* mutants rapidly increased their levels of reporter activity. Therefore, OM-cardiolipins were determined at a time point when the mutants were experiencing the breach in their barrier.

Cardiolipins were quantified by liquid-chromatography collision-induced-dissociation mass spectrometry (Dalebroux et al., 2014). Consistent with previous work, induction of *phoQ(cHAMP)* in the *pbgA+* bacteria increased the levels of four uniquely acylated cardiolipins within the OM to levels that were greater than and statistically different from those of the repressed *pbgA+* bacteria (Fig. 4C–D). By contrast, the levels within the IM did not vary (Fig. S3). Consistent with the globular region playing a role in the regulated delivery of OM cardiolipins, induction of *phoQ(cHAMP)* in the *pbgA(328–586)* mutants did not result in a significant increase in any of the OM cardiolipin molecules relative to repression in bacteria of the same genotype (Fig. 4D). Importantly, deleting the periplasmic-globular region specifically impacted the PhoPQ-regulated increases in OM cardiolipins and did not generally perturb cardiolipin trafficking or impact cardiolipin synthesis, since the repressed *pbgA(328–586)* mutants still measured cardiolipin levels that were not statistically different from those of the repressed *pbgA+* bacteria in either membrane (Fig.

4D and S3). Therefore, the globular region of PbgA is necessary for PhoPQ-regulated increases in OM cardiolipins.

PbgA binds cardiolipins using arginines near the inner membrane

Primary sequence implicated the basic region near the IM in possible electrostatic interactions with the acidic head-group phosphates for cardiolipins (Fig. S2). Persistent non-specific glycerophospholipid-interacting detergent molecules made it difficult to assess the cardiolipin-binding activity for the full-length PbgA oligomers. Instead, soluble periplasmic-domain encoding peptides with or without the basic region were assayed for their glycerophospholipid-binding activity *in vitro* (Fig. 5A). Protein-lipid co-sedimentation assays indicated that the PbgA(191–586) peptide specifically interacted with the acidic glycerol-1,3-diphosphate head-groups for cardiolipins but not with the zwitterionic head-groups for phosphatidylethanolamines or the more mildly acidic head-groups for phosphatidylglycerols (Fig. 5B). Binding was electrostatic, since millimolar levels of divalent metal cations and removal of the periplasmic basic region, in the case of the truncated PbgA(245–586) peptide, each reduced the ability of PbgA to co-sediment with cardiolipins (Fig. 5C). Mutating Arg215 and Arg216 to alanine also reduced the cardiolipin-binding activity *in vitro* suggesting that the interaction may be specific with these two amino acids (Fig. 5D).

The cardiolipin-binding arginine residues of PbgA are required for cell growth and barrier function for S. Typhimurium

To determine whether the binding to cardiolipins was related to the *in-vivo* function of PbgA, the wild type and PbgA(R215A R216A) substitution-mutant proteins were expressed *in trans* to rescue the phenotypes of *pbgA(191–586)* bacteria, which were defective in maintaining OM integrity and cell growth (Fig. 3A–B, 3G). To first test for their equivalent expression, stability, and localization within the membranes of the cell, the envelopes were collected and fractionated on a continuous isopycinic sucrose-density gradients that resolve membranes of intermediate density that exist (Zhou et al., 1998). Denaturing-gel electrophoresis and immunoblotting were used to assess the localization of the proteins. For both the wild type and the substitution mutants, the proteins were equivalently expressed and localized throughout the envelopes of the bacteria (Fig. 5E). The cardiolipin-binding arginine residues were indeed required for the *in-vivo* function of PbgA since unlike the wild-type protein, which fully restored the barrier and growth defects of the *pbgA(191– 586)* mutants (Figs. 5F–G), the double arginine-to-alanine substitution-mutant protein failed to rescue the phenotypes. Therefore, the arginine repeat within the basic region near the IM is required for PbgA to function in cell growth and OM integrity independent of PhoPQ.

The PhoPQ-gene regulators promote interactions between PbgA and the OM

The globular region of PbgA shares predicted structural homology with ary-sulfatase and LtaS enzymes. However, neither the periplasmic domain nor the full-length protein was active toward a plethora of substrates including sulfate/phosphate-containing reporter molecules and glycerophospholipids. Thus, PbgA is likely not an enzyme. Sulfatases bind metal and require a cysteine for catalysis. Site-directed mutants predicted to disrupt the residues for PbgA-metal binding were fully functional *in vivo*. Additionally, PbgA contains

no cysteine. Therefore, it was possible that the periplasmic-globular region was predominantly providing a structural role in promoting PbgA's physical interaction with the OM when PhoPQ were activated. Membranes for the wild type and the PhoP-null mutants were each fractionated on the more continuous sucrose-density gradients capable of resolving the intermediate-density membranes. For both bacterial genotypes, SecA was more abundant within the low-density fractions relative to the high-density fractions, while the opposite was true for OmpA (Fig. 6A–B). Thus, the membrane proteins used to mark the IM and the OM, respectively, fractionated similarly for the wild type and the PhoP-null mutants. For wild type, PbgA was most highly abundant within the intermediate-density membrane fractions relative to the high-density and low-density fractions, which each also contained full-length PbgA but in slightly lower abundance (Fig. 6A). For the PhoP-null mutants, PbgA was more shifted in its localization towards the lower-density IM fractions compared to the wild type (Fig. 6B). In fact, the levels of the full-length PbgA polypeptide were severely diminished within the highest-density OM fraction of the PhoP-null mutants compared to the wild type, though their OmpA levels remained comparable (Fig. 6B). Perhaps most revealing, the antisera routinely detected a smaller PbgA peptide associated with the highest-density OM fractions of the PhoP-null mutants. Therefore, the PhoPQ-gene regulators may promote stable interactions between PbgA and the OM.

For further analysis, we decided to test the fractionation of PbgA within the membranes for the envelope of the arabinose-induced *phoQ(cHAMP)* bacteria using the continuous sucrose-density gradients (Fig. 4A). For these highly PhoPQ-activated bacteria, the full-length PbgA polypeptide was more uniformly distributed throughout all the membranes within the envelope compared to the other two bacterial genotypes (Fig. S4 and 6A–B). However, since the IM-SecA protein marker was also shifted in its localization towards the more intermediate-density fractions, it seemed possible that the PhoPQ regulators were inducing more general changes to the envelope that impacted the fractionation of the IM. Similar to the PhoP-null mutants, a smaller possible PbgA peptide was also specifically detected within the highest-density OM fractions for the PhoPQ-activated bacteria (Fig. S4). However, by contrast to the PhoP-null mutants (Fig. 6B), the PhoPQ-activated bacteria maintained equivalent levels for the full-length protein and the smaller peptide. Therefore, it seems that perhaps additional functional units of the PbgA-periplasmic domain might exist and be regulated for the functions *in vivo*. These data support the possibility that the PhoPQ-gene regulators are involved in promoting stable PbgA interactions with the OM that allow *S.* Typhimurium to traffic cardiolipins for barrier function and survival during infection.

DISCUSSION

A screen for genes necessary for the PhoPQ-regulated OM-barrier for *S.* Typhimurium revealed a conserved enterobacterial IM protein, PbgA that binds glycerophospholipids known as cardiolipins and promotes their regulated trafficking to the OM. The PbgA-TM segments are essential for *S.* Typhimurium viability, but the periplasmic domain can be deleted. The full-length protein can be purified from membranes as tetramers predicted to assemble within the IM with their twenty-TM segments and four-soluble periplasmic domains. The periplasmic domains consist of a basic region near the IM and a carboxyl-terminal globular region. The PhoPQ-gene regulators promote PbgA's fractionation with the

OM and increase the cardiolipin levels by a mechanism(s) that involve(s) the globular region. Regulated increases in OM cardiolipins are likely necessary for Salmonellae barrier function and survival during infection, since deleting the globular region attenuates the bacteria in their pathogenesis for macrophages and mice. Additionally, PbgA binds cardiolipins near the IM using periplasmic arginines that are required for *S*. Typhimurium cell growth and OM-barrier function independent of PhoPQ. Therefore, *S*. Typhimurium and the PhoPQ-gene regulators have co-opted PbgA to maintain OM integrity and barrier properties necessary for bacteria to survive during their pathogenesis for mammals.

What are the mechanisms by which increasing cardiolipin trafficking to the outer membrane promotes bacterial survival within host tissues?

S. Typhimurium cells with high PhoPQ-signaling activity increase their OM cardiolipins and CAMP resistance to levels that are much greater than for cells experiencing lower levels of signaling (Dalebroux et al., 2014). This motivated the screen for genes necessary for the PhoPQ-regulated OM barrier for *S*. Typhimurium. Deletions truncating the terminal globular region of PbgA caused specific defects in PhoPQ-activated bacteria. These included increased activation of RcsF (Fig. 3B), increased OM permeability to EtBr (Fig. 3C), decreased trafficking of cardiolipins to the OM (Fig. 4D), and decreased bacterial survival during infection (Figs. 3E–F). The definition of PbgA has provided a more mechanistic understanding and biological significance for the PhoPQ-regulated increases in OM cardiolipins that must occur during Salmonellae infection. Cardiolipins may increase the local barrier properties of the OM by forming specific interactions with other PhoPQ-regulated lipid-A molecules, proteins, or glycerophospholipids.

Hexa-acylated and hepta-acylated lipid-A structures predominate the landscape of the OM outer-leaflet when Salmonellae are within the host environments that activate PhoPQ (Gibbons et al., 2005; Guo et al., 1997). Hepta-acylated lipid A molecules contain palmitate and are generated by the PhoPQ-activated enzyme, PagP (Bishop et al., 2000). PagP is a bi-functional palmitoyltransferase and its induction by PhoPQ also results in the synthesis of tri-acylated glycerophospholipids known as palmitoyl-PG within the OM (Dalebroux et al., 2014). Perhaps tetra-acylated cardiolipins and tri-acylated palmitoyl-PG molecules assemble with hepta-acylated lipid-A structures to form a tripartite asymmetric barrier unit for *S*. Typhimurium. These tightly packed hydrophobic lipid microdomains may promote local barrier properties of the OM to increase the resistance of bacteria to the killing mechanisms deployed against them by their hosts.

What are the mechanisms by which cardiolipin-binding activity of PbgA contributes to cell growth?

Cardiolipins establish negative-membrane curvature for cell division and membrane fission for bacteria (Doan et al., 2013; Oliver et al., 2014; Renner and Weibel, 2011, 2012). It is possible that PbgA binds cardiolipins and either concentrates or orients within the envelope in order for the fission and division machinery to bind and function. The carboxyl-terminal globular region of *S*. Typhimurium PbgA was dispensable for growth and barrier function when PhoPQ were not highly activated (Figs. 3A–B). By contrast, bacteria deleted for the entire periplasmic domain including the basic region were attenuated for their growth and

barrier function in all states of PhoPQ activation. Likewise, the cardiolipin-binding arginine residues within the basic region were also required for growth and barrier function independent of PhoPQ (Fig. 5F). Therefore, the TM segments and cardiolipin-binding residues within the basic region are sufficient for PbgA to function in environments that do not activate PhoPQ. In this regard, PbgA was predominantly localized to intermediate-density membrane fractions within the envelopes for the wild-type bacteria (Fig. 6A). These membranes may represent membrane-contact sites between the two bilayers (Bayer, 1991; Tatsuta et al., 2014). It is conceivable that PbgA either establishes or becomes localized to sites within the envelope where the membranes are fused or brought into close proximity. Perhaps the PhoPQ-gene regulators induce the formation of these junctions as suggested by the fractionation of SecA for the PhoPQ-activated *S*. Typhimurium using the continuous gradients (Fig. S4). At these sites, the TM and IM-proximal basic regions of PbgA might promote sufficient cardiolipin trafficking to the OM for bacteria to grow and maintain integrity of the OM within environments that do not activate PhoPQ.

It seems likely that PbgA will require additional protein partners to function. For example, YejL now PbgX is a small cytoplasmic protein encoded upstream of *pbgA* suggesting a related function. PbgX could bind or regulate PbgA activity, control PbgA insertion or multimerization within the plasma membrane, or function in complex with PbgA to target the cardiolipins or facilitate their binding. It is tempting to speculate that tetrameric PbgA oligomers will form a cardiolipin-binding complex within the envelope that interacts with other cell-division components, peptidoglycan-synthesis machinery, or other proteins that may function for constriction of the OM for bacterial septation towards cell division (Typas et al., 2012).

What is the mechanism by which PhoPQ-regulates cardiolipin trafficking to the outer membrane?

Fractionation of the cell envelope for wild-type *S*. Typhimurium showed that PbgA associated with the OM in a PhoP-dependent manner (Fig. 6A–B). Even at low levels of PhoPQ activation PbgA was detectable within the highest-density OM fractions (Fig. 6A). The PhoPQ-gene regulators induce the synthesis of number of OM proteins that could bind PbgA for final delivery and assembly of cardiolipins within the OM inner-leaflet including periplasmic proteases (Guina et al., 2000). Though this is an attractive hypothesis, PbgA may also bind to a variety of PhoPQ-regulated OM-lipid species. Perhaps chemically remodeled lipids themselves promote conformational changes in specific OM proteins that enable PbgA to bind and deliver cardiolipins. It is also possible that other PhoPQ-regulated changes to the periplasm or even the cytoplasm could alter the conformation of the PbgA to promote its association with the OM.

The IM of dividing *S*. Typhimurium contains 4–7% cardiolipin in nutrient-rich broth media, while by contrast the OM contains only 1–2% (Osborn et al., 1972). This indicates that a potential cardiolipin gradient can exist across the cell envelope for enterobacteria. Cells highly activated for PhoPQ signaling quadruple their OM-cardiolipin concentrations to 4–8% (Dalebroux et al., 2014), but do not significantly alter their levels of cardiolipins within the IM (Fig. S3). Our working model predicts that when *S*. Typhimurium enounter host

environments that activate PhoPQ, PbgA is induced to bind the OM. Binding bridges envelope to generate a gradient that allows cardiolipins to traffic to the OM by way of the globular region (Fig. 7). If the functional unit of PbgA is tetrameric, than the three-basic helices near the IM (Fig. S2) may provide an amphipathic surface that sufficiently shields or orients cardiolipins during their movement across the periplasmic space (Fig. 7). Regulated trafficking of cardiolipins to the inner-leaflet of the OM promotes the barrier and is necessary for intracellular survival of *S*. Typhimurium. Within host environments that do not activate PhoPQ, PbgA is mostly unbound and cardiolipin levels within the OM remain at baseline (Fig. 7). In addition, it is plausible that the PbgA complex might determine the fate of IM cardiolipins by controlling whether they are trafficked to the OM for barrier function or used toward essential cell division and membrane fission processes that occur upon the surface of the IM or within the periplasmic space at the bacterial septum (Typas et al., 2012).

Is membrane glycerophospholipid remodeling for barrier stability a common function of PbgA-like proteins in bacteria?

Enterobacteria like *Shigella*, *Klebsiella*, and *Yersinia spp.* encode PbgA homologs that are highly similar and in some cases nearly identical to PbgA of *S*. Typhimurium (Fig. S2). Non-enteric gammaproteobacteria including *Vibrio*, *Pseudomonas*, and *Legionella spp* also encode PbgA orthologs though their sequence is more highly divergent. Perhaps in these bacteria the PbgA-like proteins evolved to bind different glycerophospholipids, proteins, sugars, or lipid A molecules as part of their function within the cell envelope. For example, *V. cholera* encode at least two PbgA-like proteins with five TM segments and a periplasmic globular region with homology to sulfatases, but with only 41% and 25% identity to *S*. Typhimurium PbgA, repectively (Fig. S2). Therefore, *Vibrio spp.* may have evolved multiple PbgA-like proteins to traffic multiple kinds of glycerophospholipids, or that these orthologs retain their catalytic activity toward glycerophospholipids and promote their use in the synthesis of other lipid-based structures that exist within the envelopes of Gram-negative bacteria. Therefore, it seems possible that PbgA-like proteins might commonly function for glycerophospholipid metabolism and trafficking in bacteria for their pathogenesis.

EXPERIMENTAL PROCEEDURES

Strains, growth conditions, and genetics

S. Typhimurium (Table S1) were grown in Luria-broth (LB) medium at 37°C to the mid-exponential phase (OD_{600} 0.6–0.8) typically after a 1:100 back dilution of an overnight culture. Further methods for culturing *S*. Typhimurium are provided in Supporting Information. The Tn*10*d-*kan* transposon mutant library was prepared as described (Kleckner et al., 1991) and the genetic screen is described (Fig. S1C).

Beta-galactosidase activity assays and microscopy were performed according to standard methods described in the Supporting Information.

Macrophage infections

Bone-marrow-derived macrophages were prepared from the femurs of female C57/Blk6 mice (Jackson Laboratories) by standard methods provided in Supporting Information.

Competitive index assays

Six- to eight-week-old female BALB/c mice (Jackson) were inoculated intraperitoneally with a mixture of 5×10^4 organisms for each of two strains for a total of 10^5 bacteria per mouse. Further information is provided in Supporting Information.

Membrane fractionation

Cell envelopes of *S.* Typhimurium strains were collected by an osmotic spheroplasting method involving lysozyme-ethylenediaminetetraacetic acid (EDTA) treatment (Osborn and Munson, 1974). Additional details are provided in Supporting Information.

Glycerophospholipid analysis

Lipids were extracted and assessed by mass spectrometry as described previously (Dalebroux et al., 2014).

Purification of PbgA-periplasmic peptides

The full-length PbgA-periplasmic domain peptide (191–586), the amino-terminal truncation mutant peptide (245–586), and the double arginine-to-alanine substitution mutant peptide were cloned into pET28a using *Nhe*I and *Bam*HI restriction enzyme sites to generate an amino-terminal polyhistdine tag and thrombin cleavage site for the removal of the 6XHis-tag. Remaining details are found in Supporting Information.

Preparation of the antisera to the PbgA periplasmic domain

Antisera to PbgA(191–586) were raised in rabbits (Pocono Farms) and the remaining details are provided in Supporting Information.

Protein-lipid co-sedimentation assays

Chloroform solutions containing mixed acyl combinations of purified phosphatidylethanolamines (PE), phosphatidylglycerols (PG), and cardiolipins (CL) (Avanti) were dried under nitrogen, resuspended in 20mM HEPES pH 7.5 150mM NaCl, sonicated, and vortexed to form a suspension of micelles at the desired concentration. Lipids were incubated with proteins for 1h at 37°C at a 10:1 molar ratio of lipid-to-protein (20mM: 2mM). Further details are provided in Supporting Information.

Purification of full-length PbgA

A carboxyl-terminal 6XHis tag was engineered after residue 586 of the full-length PbgA protein. The tagged allele was ligated collinear with the pBAD24 promoter using *Bam*HI and *Eco*RI. *E. coli* DH5-alpha expressing the PbgA-6XHis protein were cultured to mid-exponential phase and induced for 3h with 0.2% arabinose at 37°C before harvest. Further details are found in the Supporting Information.

Size-exclusion chromatography multiangle light scattering (SEC-MALS) analysis was performed by injecting the affinity purified full-length PbgA protein (5mg/ml) in solution with 25mM Tris pH 8, 200mN NaCl, 0.01% DDM (buffer A) on a MP050S5 column (Wyatt

Technology) at a flow rate of 0.5ml/min in buffer A. Further details are provided in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Bayer ME. Zones of membrane adhesion in the cryofixed envelope of *Escherichia coli*. J Struct Biol. 1991; 107:268–280. [PubMed: 1807357]

Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, Raetz CR. Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. EMBO J. 2000; 19:5071–5080. [PubMed: 11013210]

Canals R, Xia XQ, Fronick C, Clifton SW, Ahmer BM, Andrews-Polymenis HL, Porwollik S, McClelland M. High-throughput comparison of gene fitness among related bacteria. BMC Genomics. 2012; 13:212. [PubMed: 22646920]

Castanie-Cornet MP, Cam K, Jacq A. RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia* coli. J Bacteriol. 2006; 188:4264–4270. [PubMed: 16740933]

Dalebroux ZD, Matamouros S, Whittington D, Bishop RE, Miller SI. PhoPQ regulates acidic glycerophospholipid content of the *Salmonella* Typhimurium outer membrane. Proc Natl Acad Sci USA. 2014; 111:1963–1968. [PubMed: 24449881]

Dalebroux ZD, Miller SI. Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. Curr Opin Microbiol. 2014; 17:106–113. [PubMed: 24531506]

De Lay NR, Cronan JE. Genetic interaction between the *Escherichia coli* AcpT phosphopantetheinyl transferase and the YejM inner membrane protein. Genetics. 2008; 178:1327–1337. [PubMed: 18245839]

Doan T, Coleman J, Marquis KA, Meeske AJ, Burton BM, Karatekin E, Rudner DZ. FisB mediates membrane fission during sporulation in *Bacillus subtilis*. Genes Dev. 2013; 27:322–334. [PubMed: 23388828]

Dong C, Beis K, Nesper J, Brunkan-Lamontagne AL, Clarke BR, Whitfield C, Naismith JH. Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. Nature. 2006; 444:226–229. [PubMed: 17086202]

Dong H, Xiang Q, Gu Y, Wang Z, Paterson NG, Stansfeld PJ, He C, Zhang Y, Wang W, Dong C. Structural basis for outer membrane lipopolysaccharide insertion. Nature. 2014; 511:52–56. [PubMed: 24990744]

Farris C, Sanowar S, Bader MW, Pfuetzner R, Miller SI. Antimicrobial peptides activate the Rcs regulon through the outer membrane lipoprotein RcsF. J Bacteriol. 2010; 192:4894–4903. [PubMed: 20675476]

Fields PI, Groisman EA, Heffron F. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science. 1989; 243:1059–1062. [PubMed: 2646710]

Fung JC, MacAlister TJ, Weigand RA, Rothfield LI. Morphogenesis of the bacterial division septum: identification of potential sites of division in *lkyD* mutants of *Salmonella typhimurium*. J Bacteriol. 1980; 143:1019–1024. [PubMed: 7009540]

Galan JE, Curtiss R 3rd. Virulence and vaccine potential of *phoP* mutants of *Salmonella typhimurium*. Microb Pathog. 1989; 6:433–443. [PubMed: 2671582]

Gerdes SY, Scholle MD, Campbell JW, Balazsi G, Ravasz E, Daugherty MD, Somera AL, Kyrpides NC, Anderson I, Gelfand MS, et al. Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. J Bacteriol. 2003; 185:5673–5684. [PubMed: 13129938]

Gibbons HS, Kalb SR, Cotter RJ, Raetz CR. Role of Mg^{2+} and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. Mol Microbiol. 2005; 55:425–440. [PubMed: 15659161]

Guina T, Yi EC, Wang H, Hackett M, Miller SI. A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar Typhimurium promotes resistance to alpha-helical antimicrobial peptides. J Bacteriol. 2000; 182:4077–4086. [PubMed: 10869088]

Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. Science. 1997; 276:250–253. [PubMed: 9092473]

Hagan CL, Silhavy TJ, Kahne D. β-Barrel membrane protein assembly by the Bam complex. Annu Rev Biochem. 2011; 80:189–210. [PubMed: 21370981]

Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. Nat Rev Microbiol. 2008; 6:53–66. [PubMed: 18026123]

Hohmann EL, Oletta CA, Miller SI. Evaluation of a *phoP/phoQ*-deleted, *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. Vaccine. 1996; 14:19–24. [PubMed: 8821644]

Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, Sadler JJ, Knepper-Adrian V, Han R, Qiao L, et al. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. Immunity. 2013; 39:311–323. [PubMed: 23954133]

Kleckner N, Bender J, Gottesman S. Uses of transposons with emphasis on Tn*10*. Method Enzymol. 1991; 204:139–180.

Lu D, Wormann ME, Zhang X, Schneewind O, Grundling A, Freemont PS. Structure-based mechanism of lipoteichoic acid synthesis by *Staphylococcus aureus LtaS*. Proc Natl Acad Sci USA. 2009; 106:1584–1589. [PubMed: 19168632]

Majdalani N, Gottesman S. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol. 2005; 59:379–405. [PubMed: 16153174]

Miller SI, Kukral AM, Mekalanos JJ. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc Natl Acad Sci USA. 1989; 86:5054–5058. [PubMed: 2544889]

Needham BD, Trent MS. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol. 2013; 11:467–481. [PubMed: 23748343]

Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003; 67:593–656. [PubMed: 14665678]

Osborn MJ, Gander JE, Parisi E, Carson J. Mechanism of assembly of the outer membrane of *Salmonella typhimurium. Isolation and characterization of cytoplasmic and outer membrane*. J Biol Chem. 1972; 247:3962–3972. [PubMed: 4555955]

Osborn MJ, Munson R. Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. Methods Enzymol. 1974; 31:642–653. [PubMed: 4608978]

Pages JM, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol. 2008; 6:893–903. [PubMed: 18997824]

Renner LD, Weibel DB. MinD and MinE interact with anionic phospholipids and regulate division plane formation in *Escherichia coli*. J Biol Chem. 2012; 287:38835–38844. [PubMed: 23012351]

Schlame M. Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes. J Lipid Res. 2008; 49:1607–1620. [PubMed: 18077827]

Schlattner U, Tokarska-Schlattner M, Rousseau D, Boissan M, Mannella C, Epand R, Lacombe ML. Mitochondrial cardiolipin/phospholipid trafficking: the role of membrane contact site complexes and lipid transfer proteins. Chem Physics Lipids. 2014; 179:32–41.

Schug ZT, Gottlieb E. Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. Biochimica et biophysica acta. 2009; 1788:2022–2031. [PubMed: 19450542]

Tatsuta T, Scharwey M, Langer T. Mitochondrial lipid trafficking. Trends Cell Biol. 2014; 24:44–52. [PubMed: 24001776]

Typas A, Banzhaf M, Gross CA, Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol. 2012; 10:123–136. [PubMed: 22203377]

Weigand RA, Vinci KD, Rothfield LI. Morphogenesis of the bacterial division septum: a new class of septation-defective mutants. Proc Natl Acad Sci USA. 1976; 73:1882–1886. [PubMed: 778849]

Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem. 2014; 83:99–128. [PubMed: 24580642]

Willis LM, Whitfield C. Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. Carbohyd Res. 2013; 378:35–44.

Zhou Z, White KA, Polissi A, Georgopoulos C, Raetz CR. Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. J Biol Chem. 1998; 273:12466–12475. [PubMed: 9575204]

HIGHLIGHTS

- PbgA is a tetrameric inner-membrane protein that binds cardiolipins.

- PbgA traffics cardiolipins to the outer membrane (OM) for barrier function.

- The PhoPQ-gene regulators promote PbgA interaction with the OM.

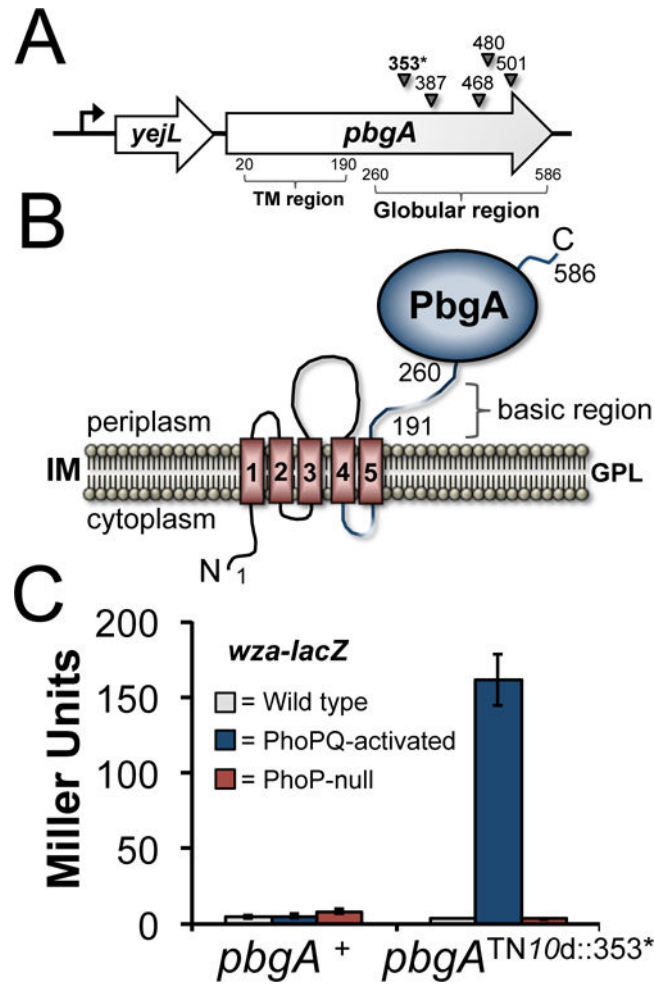- To survive within host tissues, *Salmonella* delivers cardiolipins to the OM.

Figure 1. Transposon mutagenesis and genetic screening identify, *pbgA* as a gene required for the PhoPQ-regulated outer-membrane barrier of *Salmonella* Typhimurium

(A) Schematic of the *yejL-pbgA* operon with numbers above the inverted triangles indicating the approximate amino-acid codons disrupted by the insertion events. These sites were mapped in our screen for mutations inducing the *wza-lacZ* secondary gene reporter of RcsF activity, specifically within bacterial cells experiencing high levels of PhoPQ-signaling activity (Fig. S1A–B). (B) The predicted topology of PbgA within the plasma membrane of *S.* Typhimurium and approximate boundaries for its tertiary structure. The periplasmic-basic region comprises a single short helix followed by two longer helices that ultimately become connected to the globular region by an unstructured region (Fig. S2) (C). The *wza-lacZ* reporter levels were determined by measuring beta-galactosidase activity. Shown is the average ± standard deviation (SD) from three independent experiments.
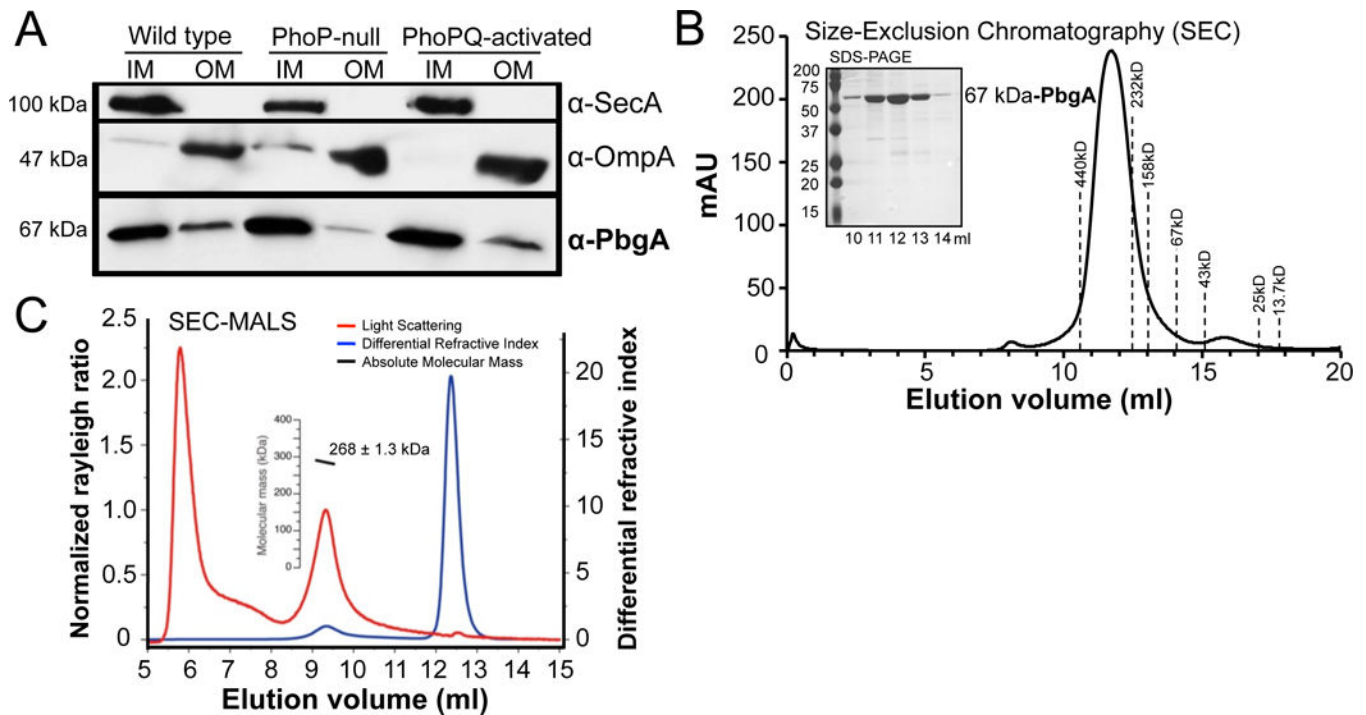
Figure 2. PbgA is an inner-membrane protein that forms tetrameric oligomers when purified
(A) Cell envelopes were fractionated on the defined sucrose-density gradients. Denaturing-gel electrophoresis and Western blotting were used to detect the proteins. (B) Detergent-solubilized and affinity-purified PbgA was concentrated and isolated by size-exclusion chromatography (SEC). Dashed lines indicate the elution volumes for the size standards. (C) The absolute molecular mass was determined by coupling SEC with multiangle-light scattering (MALS). The LS signal (normalized Rayleigh ratio) is in red, the differential refraction index is in blue, and the absolute molecular mass of the peak between 9.0 and 9.5ml is in the black inset.
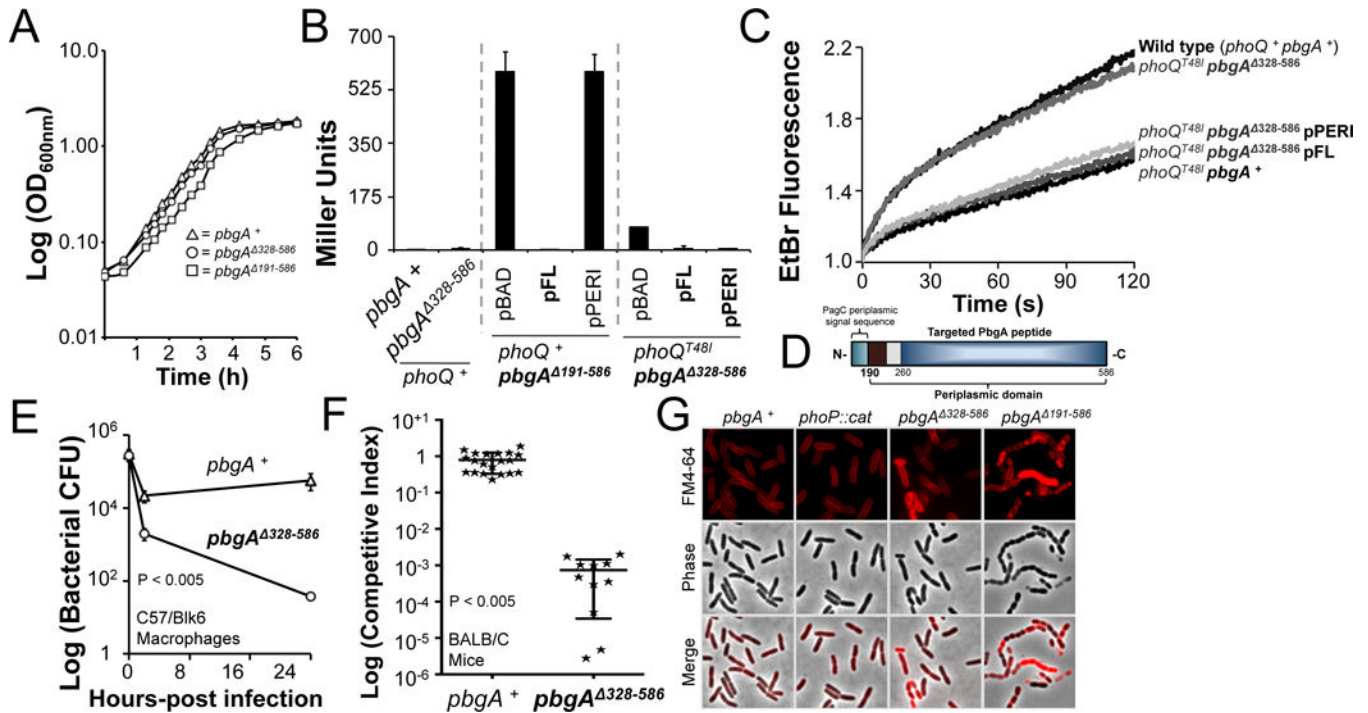
Figure 3. *S*. Typhimurium require the PbgA globular region for their regulated increases in OM-barrier function and virulence

(A) Bacterial growth in Luria-broth (LB) medium was measured by optical density at 600nM as function of time. (B) The levels of the *wza-lacZ* gene reporter were quantified for wild type and *pbgA*-mutant genotypes carrying either the plasmid-borne full-length PbgA protein (pFL), an empty control vector (pBAD), or the periplasmic domain of PbgA targeted and released into the periplasm (pPERI). (C) Ethidium bromide (EtBr) uptake and fluorescence was measured to assess the permeability across the OM as a function of time and is further described in the Supporting Information. (D) The targeted periplasmic peptide is illustrated. (E) Intracellular survival was determined as the average colony forming units (CFU)/ml from macrophage lysates collected in triplicate wells for each strain in three independent experiments. Shown is the average ± SD from the three experiments and the p-value is provided (F) Bacterial survival after intraperitoneal infection of mice with 10^5 organisms per mouse containing a 1:1 ratio of wild type/wild type, or wild type/*pbgA*-mutant *S*. Typhimurium. The competitive indices were determined for five mice in each of four independent infections for a total of twenty mice per competition. In the case of the wild type/wild type competition the average competitive index for all twenty mice is shown. For the wild type/*pbgA*-mutant competition, the average and p-value for only eleven of the twenty mice is shown, since for nine of the mice zero-surviving *pbgA*-mutant CFU were recovered for which our limit of detection was one bacterium per spleen per mouse from an innoculum of $>10^5$ microbes. (G) Phase-contrast and fluorescence microscopy of mid-exponential phase bacteria at 100× magnification.
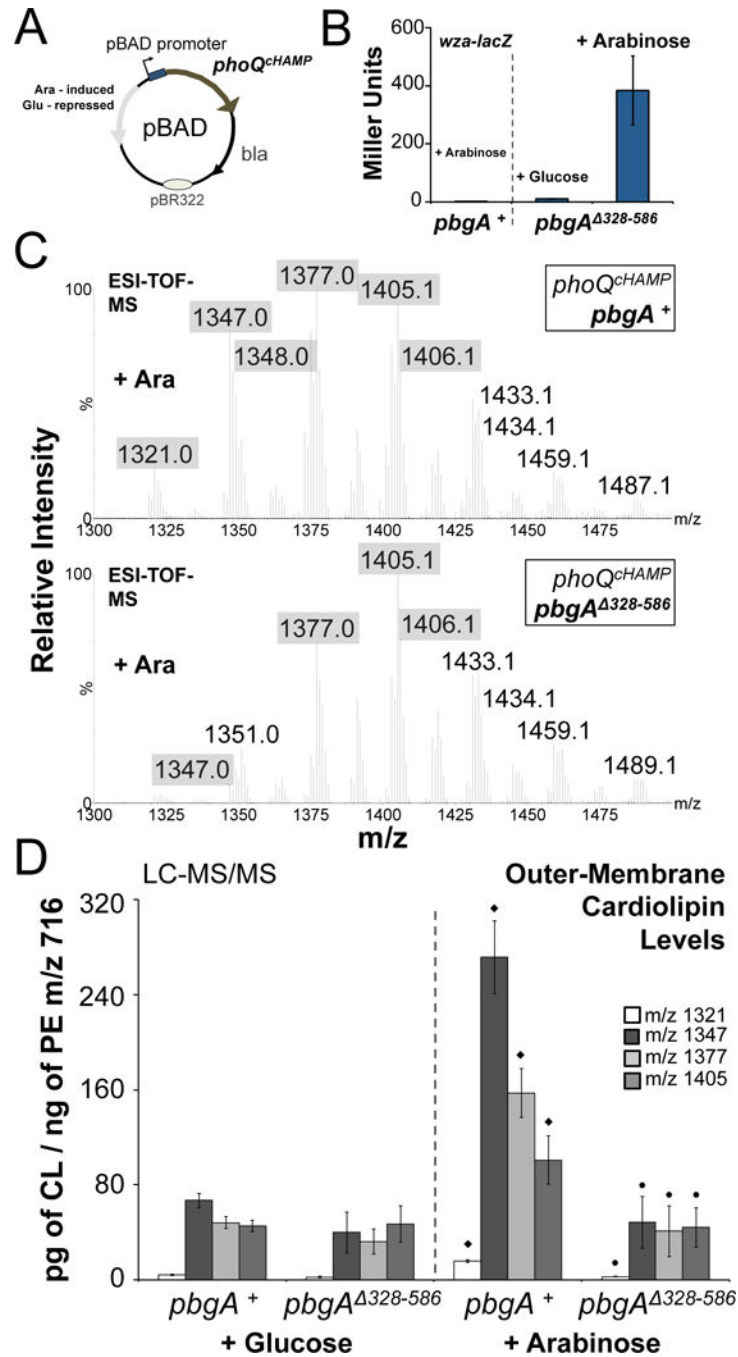
Figure 4. The PbgA-periplasmic domain is required for PhoPQ-regulated increases in outer-membrane cardiolipins

(A) The plasmid system was built to regulate the expression of *phoQ(cHAMP)*, or *phoQ(E232K)* that encodes a constitutively-activated mutant enzyme (Dalebroux et al., 2014). (B) The *wza-lacZ* reporter levels were determined for bacteria at 1h post-arabinose addition, or in the case of glucose-treated cells when bacteria had reached the mid-exponential growth phase. (C) Membranes were collected and glycerophospholipids were extracted from the higher-density OM fractions (Fig. 2A) and assessed by electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) in negative ionization mode [M-

H]⁻. Gray-shaded ions in the spectra represent cardiolipins with varying acyl-chain combinations and molecular protonation state (Dalebroux et al., 2014). (D) Quantitative liquid-chromatography collision-induced-dissociation mass spectrometry (LC-MS/MS) determined the levels of the individual lipids. The cardiolipins shown in the graph were quantified and normalized to a PE control ion, m/z 716 that did not vary under these conditions. Shown is average of three independent experiments ± SD. ◆ and ● indicate that the average pg/ng value for the arabinose-induced *pbgA+* bacteria was significantly greater than the value obtained for either the glucose-repressed *pbgA+* or the arabinose-induced *pbgA(328–586)*-mutant bacteria, respectively by an unpaired Student *t* test p value <0.05, which were used for all statistics.
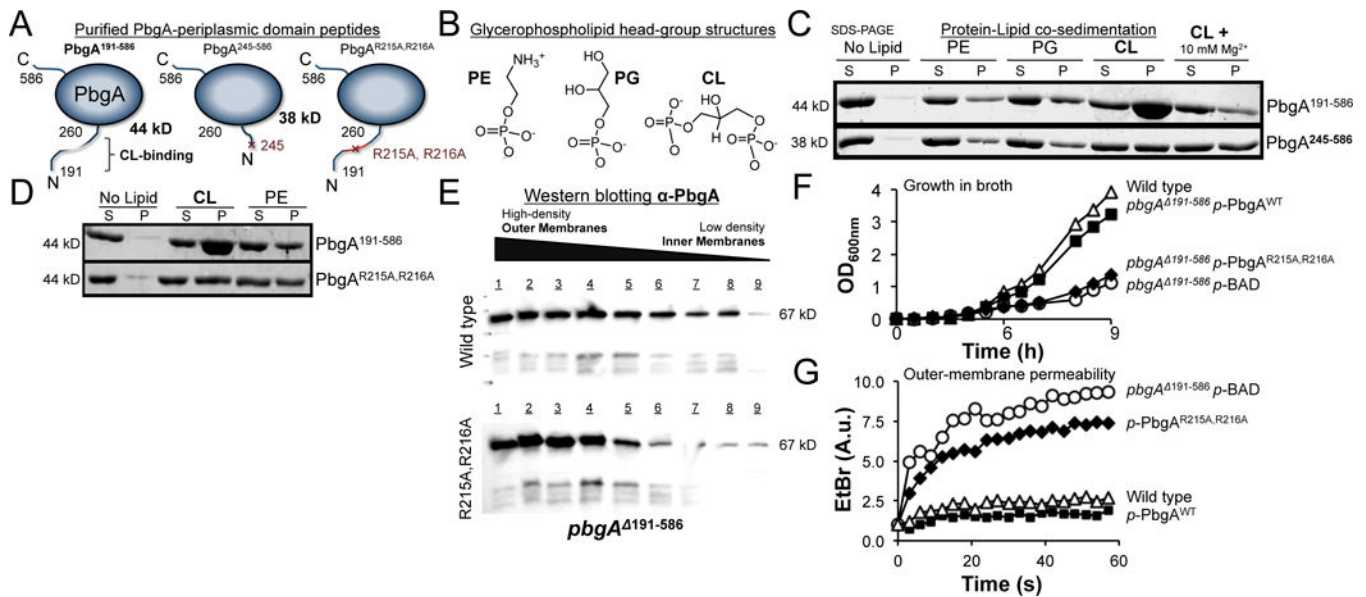
Figure 5. The cardiolipin-binding arginines of PbgA are needed for optimal bacterial growth and barrier function *in vivo*

(A) Schematics for the PbgA peptides purified and used for the protein-lipid binding assays. (B) Chemical structures for the common glycerophospholipid head-groups for enterobacteria including; ethanolamine phosphate (PE), glycerol-3-phosphate (PG), and glycerol-1,3-phosphate (CL). (C) Protein-lipid solutions were incubated and centrifuged to determine whether the lipids could bind the peptides and cause them to sediment. Denaturing-gel electrophoresis and coomassie-blue staining assessed the protein abundance within the supernatents (S) and the pellets (P). (D) Protein-lipid co-sedimentation was also used to assess the cardiolipin-binding activity for the double-arginine substitution mutant peptides. (E) Continuous isopycnic sucrose-density gradients, denaturing-gel electrophoresis, and Western blotting were used to assess the levels and localization for the wild type and mutant proteins within the membrane fractions. (F) Optical density to measure bacterial growth in LB medium. (G) Ethidium bromide uptake and fluorescence to assess the permeability across the OM of the bacteria as a function of time as described in (Fig. 3C).
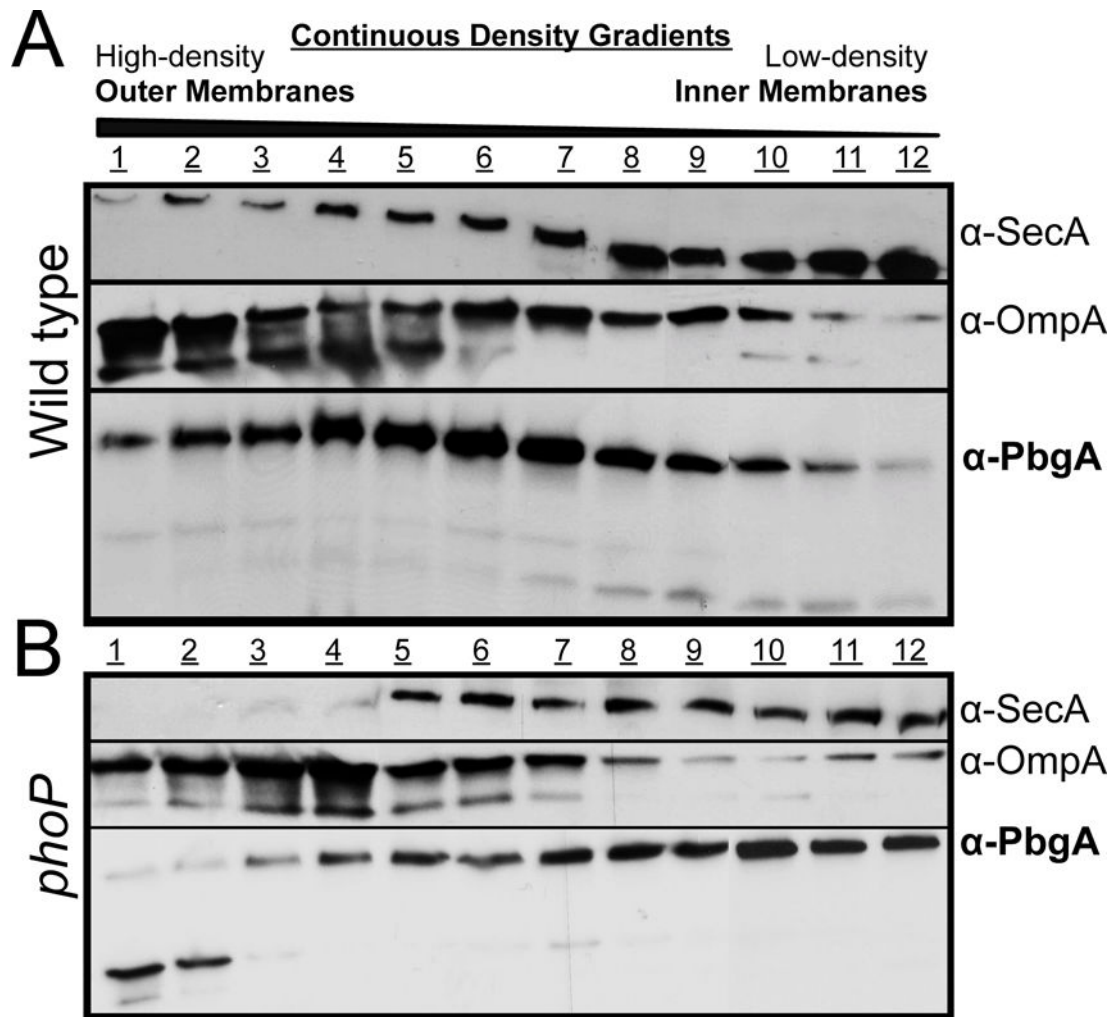
Figure 6. PhoPQ is necessary for PbgA to fractionate with the *S*. Typhimurium OM
The cell envelopes for the (A) wild type and the (B) *phoP* mutants were fractionated using the more continuous sucrose-density gradients that resolve the membranes of intermediate density within the envelope. Denaturing-gel electrophoresis and Western blotting assessed the proteins from the fractions.
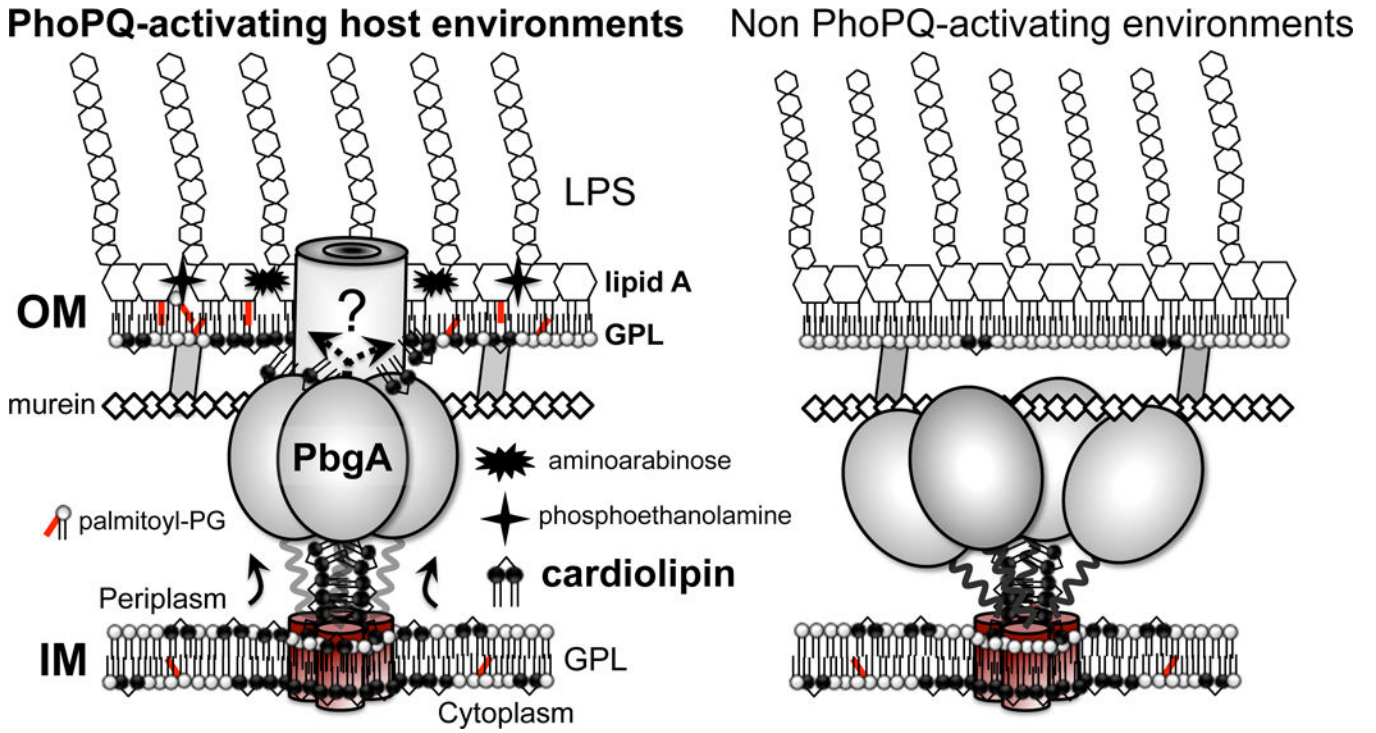
Figure 7. PbgA traffics cardiolipins to the OM for bacterial survival within host environments that activate PhoPQ

The working model for how *S.* Typhimurium regulate trafficking of cardiolipins to the OM. Host environments that activate PhoPQ (left panel) induce PbgA to bind the OM and deliver cardiolipins that promote the barrier necessary for bacteria to survive and replicate during infection. When PhoPQ are not activated (right panel), PbgA is mostly unbound and the OM-cardiolipin levels remain at baseline.