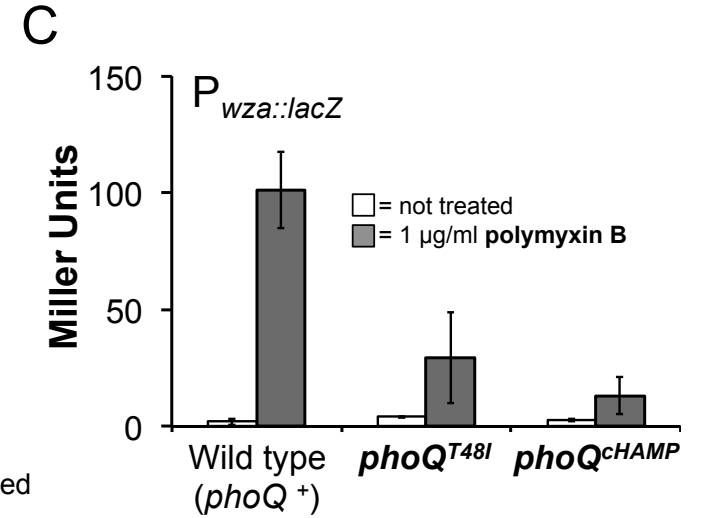
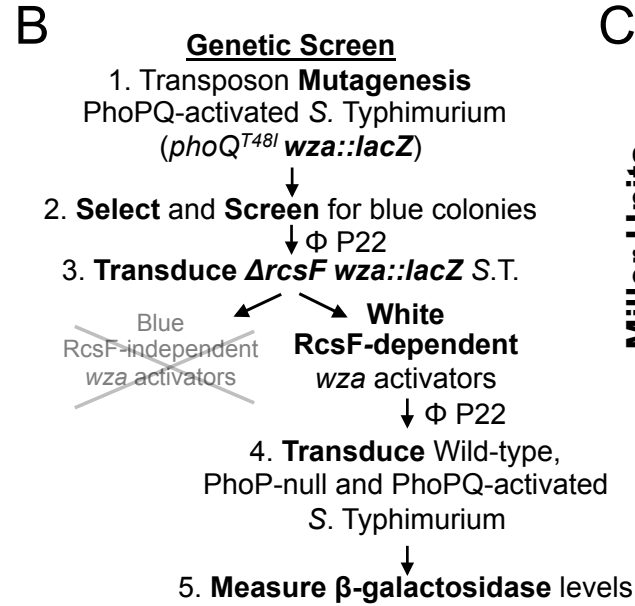
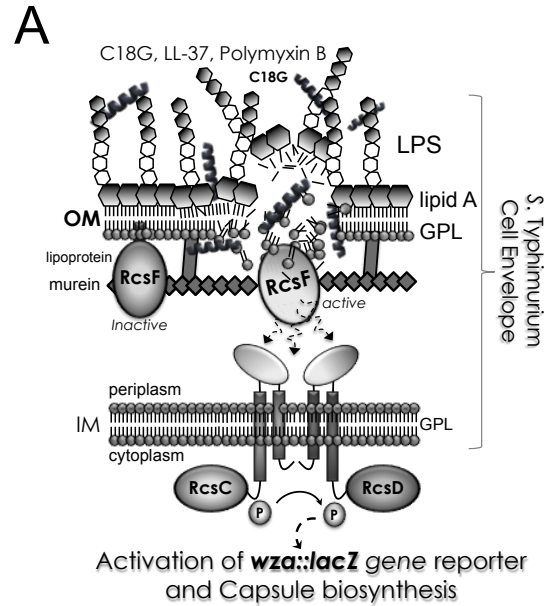


# Supporting Figure 1



## SUPPORTING INFORMATION:

### Experimental Procedures (continued from the main text)

**Strains, growth conditions, and genetics.** For the growth curve in LB (Fig. 3A), bacteria were back diluted to OD<sub>600</sub> 0.05 and monitored until early stationary phase (OD<sub>600</sub> 2.5-3.0). Bacteriophage P22 HT<sub>int</sub> was used for all transductions. Transcomplementation was accomplished by adding 0.002% L-arabinose (Sigma) and 100 µg/ml of ampicillin (Anaspec) to the LB medium (Figs. 3B, 3C, and 5E-G). The *phoQ(cHAMP)* allele was induced by adding 0.2% arabinose to the LB medium in early-exponential phase (OD<sub>600</sub> 0.3) and cells were harvested 1h post-induction. The *phoQ(cHAMP)* allele was repressed by adding 0.2% glucose immediately on back dilution until cells reached the mid-exponential phase.

**Beta-galactosidase activity assays and microscopy.** For Tn10d-*kan* insertion mutants (Fig. 1C), β-gal levels were quantified as Miller-Units (MU) of activity from overnight (OVN) cultures by standard methods. For analysis of the site directed *pbgA*-deletion mutant phenotypes and their transcomplementation, β-gal activity was assessed in bacteria cultured to the mid-exponential phase. *Phase contrast and fluorescence microscopy* were performed on mid-exponential phase bacteria diluted in phosphate buffered saline (PBS), immobilized on 0.2% agarose pads, and sealed beneath coverslips. For fluorescence labeling of bacterial membranes, the bacteria were grown in LB with the FM4-64 membrane dye at 0.5 µg/ml, cultured for 1h, and resuspended in phosphate-buffered saline on agarose pads each containing the dye at the same concentration.

**Ethidium bromide (EtBr) permeability and fluorescence.** Excitation at 530nm and emission at 600nm was measured from aliquots of mid-exponential phase bacteria whose plasma membranes had been depolarized by adding carbonyl cyanide phosphate. Fluorescence readings were taken at one-second time intervals using a plate reader with a monochromatic filter. The average increase in EtBr fluorescence relative to the 1s time point was determined for three wells per bacterial genotype and the data are representative of three independent experiments.

**Macrophage infection.** Differentiated macrophages were re-plated to a concentration  $2.5 \times 10^5$  cells/ml in each well of 24-well tissue culture plates. Macrophages were allowed to re-adhere OVN. Bacterial OVN cultures were diluted in RPMI (Gibco) + fetal-bovine serum and added to the monolayers at  $2.5 \times 10^5$  colony-forming units (CFU)/ml for a multiplicity infection of 1:1. Bacteria were centrifuged onto the monolayers for 10 min at 25°C and the cells were incubated at 37°C for 1h. Infected cells were washed and aspirated three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. RPMI+FBS with 100 µg/ml of gentamycin was added to kill remaining extracellular bacteria. Infected cells were incubated for an additional 1h at 37°C. At 2 h post-infection, PBS+0.1% Triton was added to the macrophages and monolayers were gently scraped. Three wells per bacterial genotype were assessed per time point. Surviving intracellular CFU were enumerated by plating serial dilutions in PBS. At 2h, the wells that would be harvested at twenty-four hours were aspirated and RPMI+FBS with 10 µg/ml of gentamycin was added to kill bacteria that became extracellular during infection. At 24h, macrophages were lysed and surviving bacteria were enumerated.

**Competitive index assay.** Each strain was diluted from cultures grown OVN containing a stable antibiotic marker to allow the strains to be differentiated. The inocula each contained approximately equal concentrations of both strains and the ratios were confirmed by plating onto selective LB agar. Forty-eight hours post-infection, the mice were sacrificed by CO<sub>2</sub> asphyxiation, spleens were dissected, and each spleen was homogenized in 1ml of PBS. Ratios of each strain in each spleen were calculated from bacterial counts produced by plating aliquots of 1:10 dilutions of homogenized spleen on selective media. In the case of the mice infected with the *pbgA*( $\Delta 328-586$ )-deletion mutant *S. Typhimurium*, the 1ml aliquot of the homogenized spleen was pelleted, concentrated in 100µl of PBS, and plated onto selective media. Therefore, in each mouse the limit of detection was one bacterium per spleen. Hence, the spleens of mice infected with  $10^5$  bacteria contained zero bacteria by 48h for nine of the twenty total mice. The competitive index was calculated by dividing the ratio of bacteria isolated from the spleen by the ratio of bacteria inoculated into

the mouse. Competitive index results were determined by calculating the means  $\pm$  SD for five mice in three experiments for a total of fifteen mice for each competition.

**Membrane fractionation.** Spheroplasts were homogenized (Avestin) and the membranes were collected by ultracentrifugation. The SecA and OmpA polyclonal antibodies were obtained from D. B. Oliver at Wesleyan University and used at 1:10,000 and 1:25,000 dilutions, respectively. *Defined sucrose density gradients* were prepared by adding 2ml of 73% sucrose, 4ml of 53%, and 1ml of membranes dounced in 20% sucrose, each in 10mM Tris-HCl pH 7.8 at 0.5mM EDTA. The tubes were filled to volume with 20% sucrose. Both the defined and continuous isopycnic sucrose membrane density gradients were centrifuged for at least 12h at 37k rpm using a Beckman Optima L-90K Ultracentrifuge. Low-density inner membrane (IM) fractions were carefully collected as defined brown upper interfaces using a Pasteur-pipette. High-density outer membranes (OM) were collected as a more diffuse white lower interface by pipetting. The now isolated membranes were washed in 10mM Tris-HCl pH 7.8, resuspended in 1ml of buffer, and stored at -20°C. *Continuous membrane-density gradients* resolving intermediate density membranes that may be membrane contact sites within the envelope. Membranes were collected by ultracentrifugation at 37k-rpm for 1h, dounced in 1ml of 30% sucrose, and applied to the gradients consisting of 0.4ml of 63%, 0.9ml of 55%, 2.2ml of 50%, 2.2ml of 45%, 2.2ml of 40%, 2.2ml of 35% in 10mM Tris-HCl pH 7.8 at 0.5mM EDTA. The ultracentrifuge tubes were filled to volume with the 30%. After ultracentrifugation OVN, the bottoms of the ultracentrifuge tubes were punctured with a 22-gauge needle to fractionate the gradient into 24-28 individual eppendorf tubes each with 400-500 $\mu$ l of total volume. To reduce the number of fractions, every second fraction was pooled 1:1 with the preceding fraction to halve the gradient. Total protein was determined by Bradford assay using Coomassie Plus<sup>TM</sup> and standard curves were generated from bovine-serum albumin. The halved gradients were loaded at 5 $\mu$ g per fraction into each well of two 12% Tris-HCl pH 6.8 polyacrylamide gels containing 1% sodium-dodecyl sulfate (SDS).

**Purification of PbgA-periplasmic peptides.** Bacteria were grown to mid-exponential phase at 37°C, 500mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added, and cells were moved to 20°C and cultured OVN. Cell pellets were resuspended in PBS containing; protease inhibitor (Roche), RNase, DNase, and 1mM MgCl. Bacteria were lysed by homogenization (Avestin). The membranes and insoluble proteins were separated from the soluble material by ultracentrifugation at 37k rpm. The soluble PbgA-containing supernatant was concentrated and applied to a 5ml-HiTrap<sup>TM</sup>Chelating HP Ni-affinity column (GE) equilibrated in PBS. PbgA peptides were eluted over a step-gradient containing 25mM imidazole for 20ml and increasing to 50mM for 20ml before finally eluting with 300mM. Elution was monitored by UV absorption at 280nm. Fractions containing PbgA were concentrated by centrifugation with a 30kD cut-off filter (Millipore) and applied to a 24ml-10/300 Superdex<sup>TM</sup> 200GL size-exclusion column (GE) equilibrated with 20mM HEPES pH 7.5 150mM NaCl. PbgA eluted as homogeneous monomers in fractions that were concentrated and frozen in 5% glycerol at usually 5-25 $\mu$ g/ml. For lipid-binding and enzymatic assays the affinity purified proteins were treated with thrombin to cleave the amino-terminal poly-histidine tag before being concentrated and isolated by size-exclusion chromatography (SEC).

**Preparation of the antisera to the PbgA periplasmic domain.** Aliquots were eluted over a Protein-A column (GE) to isolate the Fc/Fab fragments. The sera were cleared of non-specific *S. Typhimurium* cross-reacting antibodies by incubating aliquots with *pbgA*(*Δ191-586*) mutant cell lysates prepared in 50mM Tris-HCl pH 8.0 10mM EDTA and 5% non-fat dried milk for 4h. After clearing, a 1:20,000 dilution was sufficient to detect PbgA from 1-5 $\mu$ g of total protein.

**Protein-lipid co-sedimentation assays.** The solutions were centrifuged at 13.2k rpm for 10min. Supernatants were collected and the lipid-containing pellet was resuspended in 20 $\mu$ l of buffer. A total of 10 $\mu$ l of the pellet and 10 $\mu$ l of the supernatant were each boiled in SDS-containing sample buffer, loaded onto 12%Tris-HCl gels, and the proteins were visualized by staining with coomassie-blue reagent.

**Purification of full-length PbgA.** Cells were pelleted after the 3h induction period and resuspended in Tris-buffered saline (TBS) containing 5% glycerol, protease inhibitor cocktail, 10µg/ml of RNase A, DNase, and 5mM MgCl<sub>2</sub>. Cells were lysed by homogenization and membranes were pelleted away from supernatants by ultracentrifugation at 37k rpm for 1h. The pellets were resuspended in TBS with 5% glycerol and 1% *n*-dodecyl-β-D-maltoside (DDM) (A.G. Scientific) and the solutions were rocked with the DDM detergent OVN at 4°C. Insoluble protein was removed by ultracentrifugation at 37k rpm for 30min. Supernatants containing DDM and the solubilized membranes were applied to a 5ml-HiTrap<sup>TM</sup> Chelating HP Ni-affinity column equilibrated in TBS, 5% glycerol, 0.02% DDM. A step gradient was used to elute PbgA-6XHis beginning with 25mM imidazol for 20ml, increasing to 30mM for 20ml, 50mM for 10ml, and eluting with 300mM. Elution was monitored by UV-absorption at 280nm. The PbgA-containing fractions were centrifuged and concentrated with a 100kD cut-off filter (Millipore) and used for multiangle light scattering analysis or injected onto a HiLoad 120ml-6/600 Superdex<sup>TM</sup> 200 preparative grade size-exclusion column equilibrated in 20mM HEPES pH 7.5 150mM NaCl with 0.02% DDM at a flow rate of 0.5ml/min. Catalase (232kD) and ferritin (440kD) size standards eluted at 12.39ml and 10.61ml, respectively under these conditions.

**Size-exclusion chromatography multiangle light scattering (SEC-MALS) analysis.** A control injection of 100µl buffer A alone at the flow rate of 0.5ml/min determined the light scattering of DDM micelles at 0.01%. The elution profile was monitored by UV absorption at 280nm, light scattering at 690nm, and differential refractometry using Dawn HELEOS II and OptiLab Rex instruments (Wyatt Technology). Analyses were carried out using Astra software (Wyatt Technology). The differential refractive index increment (dn/dc) value of 0.185 was used in all calculations.

## SUPPORTING FIGURE LEGENDS

### **Supporting Figure 1. Exploiting the outer-membrane lipoprotein, RcsF, to screen for genes necessary**

**for the PhoPQ-activated outer-membrane barrier of *Salmonella Typhimurium*.** (A) RcsF can sense outer-membrane (OM) damage inflicted by cationic antimicrobial peptides inserting into the bilayer. This results in the reorientation of RcsF and signaling at the inner membrane that activates the Rcs-regulon including the *wza*-operon encoding the capsule export machinery (Farris et al., 2010). (B) A comprehensive library of 50,000 independent transposon (TN10d::*kan*) insertion events were packaged as individual P22-bacteriophage virions each containing a single random event marked by a gene cassette conferring Kan<sup>R</sup>. The phage lysates were used to transduce the PhoPQ-activated bacteria carrying a chromosomal copy of the *wza-lacZ* gene reporter. The colonies were screened for their blue color on indicator plates. Blue-colony mutants were re-streaked onto green-indicator plates to remove the remaining lytic and lysogenic phage before reconfirming the blue-colony phenotype. A phage lysate was prepared from each of the blue mutants and was used to transduce the wild type and the *rscF*-deletion mutant *S. Typhimurium* to Kan<sup>R</sup>. Mutants dependent on RcsF for *wza-lacZ* induction were pursued. Candidate lysates were used to transduce wild type, PhoP-null mutant, and a fresh PhoPQ-activated mutant *S. Typhimurium* strain background. Transposon insertion events activating RcsF in the PhoPQ-activated background were pursued as candidate *phoPQ-barrier genes*, or *pbg*'s. (C) Bacteria were treated with polymyxin B for 1h prior to measuring beta-galactosidase levels and thus the activity of the *wza-lacZ* transcriptional gene reporter.

### **Supporting Figure 2. An alignment PbgA-like proteins for select bacteria within the region of PbgA**

**that binds cardiolipins.** The percent identity/positivity for each species was: 87/94 for *Escherichia coli*, 87/94 for *Klebsiella pneumoniae*, 63/76 for *Yersinia pestis*, 41/58 for *Vibrio cholera*<sup>\*</sup>, 25/46 for *Vibrio cholera*<sup>o</sup>, and 25/44 for *Legionella pneumophila*.

### **Supporting Figure 3. The *S. Typhimurium* PhoPQ regulators do not activate increases in the levels of**

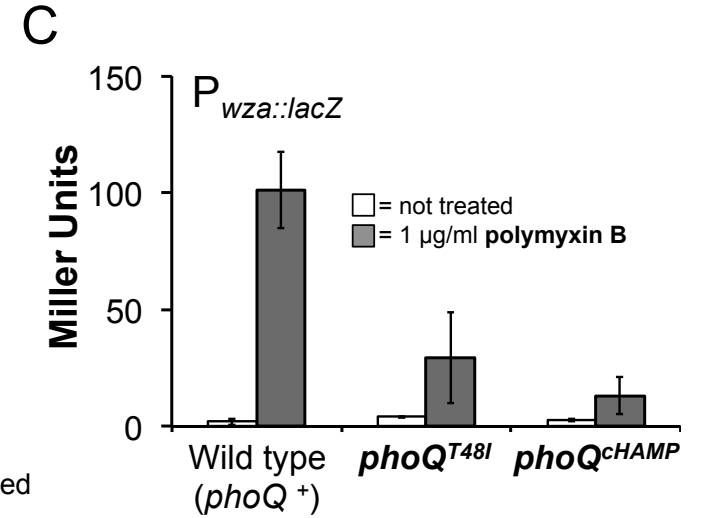
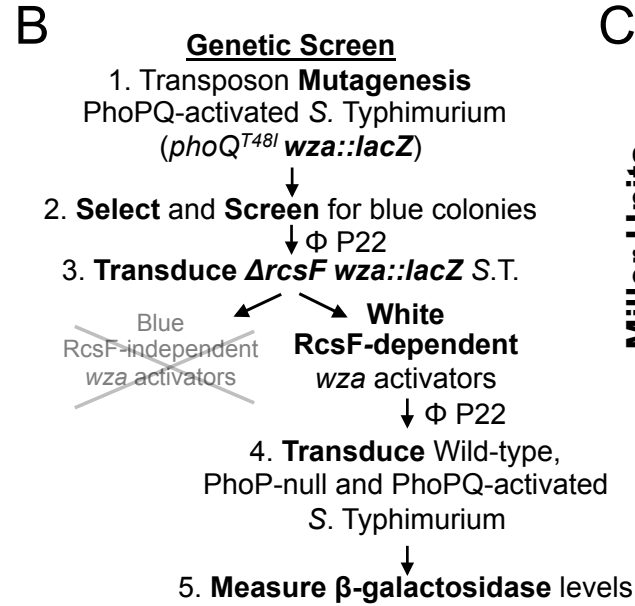
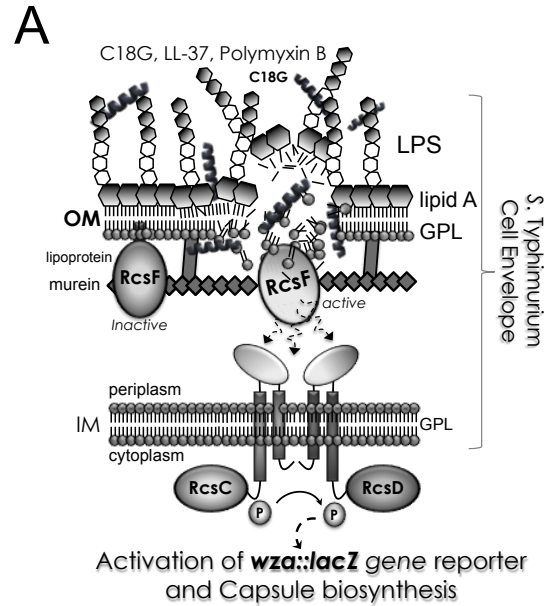
**cardiolipins within the inner membrane.** (A) Glycerophospholipids were extracted from the membranes

of arabinose treated bacteria collected on the defined density gradients. Inner-membrane lipids were assessed by electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) in negative ionization mode  $[M-H]^-$  (Dalebroux et al., 2014) (B) Quantitative small-molecule liquid-chromatography collision-induced-dissociation mass spectrometry (LC-MS/MS) quantified the level of individual GPL molecules from the IM fractions. Shown is the amount of individual CL molecules relative to a PE control ion,  $m/z$  716, that did not vary in these genetic backgrounds under these conditions.

**Supporting Figure 4. Fractionation of the PhoPQ-activated cell envelope of *S. Typhimurium* by continuous isopycnic sucrose-density gradient ultracentrifugation.** The cell envelopes of *phoQ(cHAMP)*-induced *pbgA+* bacteria (Fig. 4A) were fractionated using the more continuous gradients that resolve membranes of intermediate density. Denaturing-gel electrophoresis and Western blotting assessed the proteins from the fractions collected off the gradients.

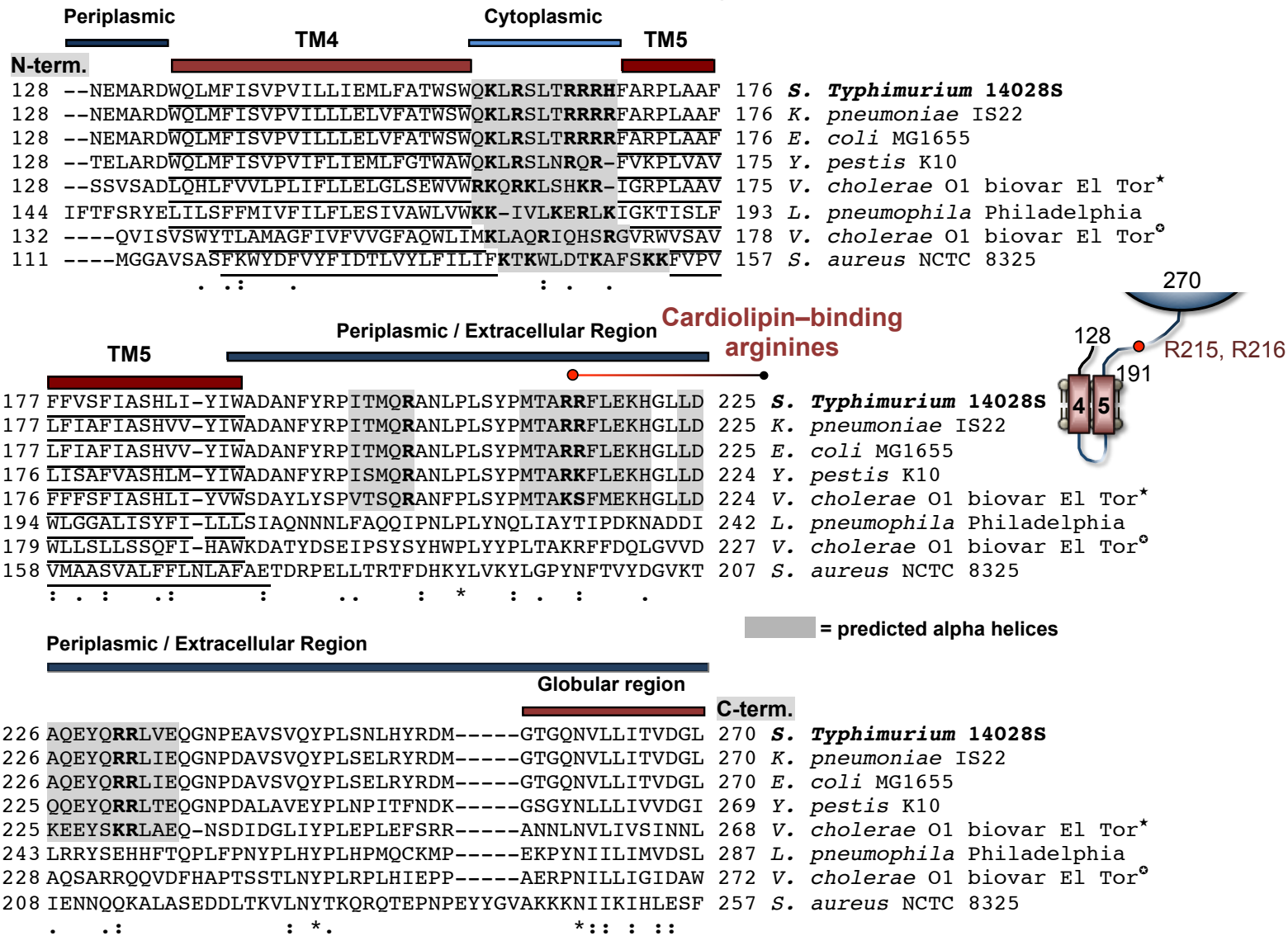


# Supporting Figure 1

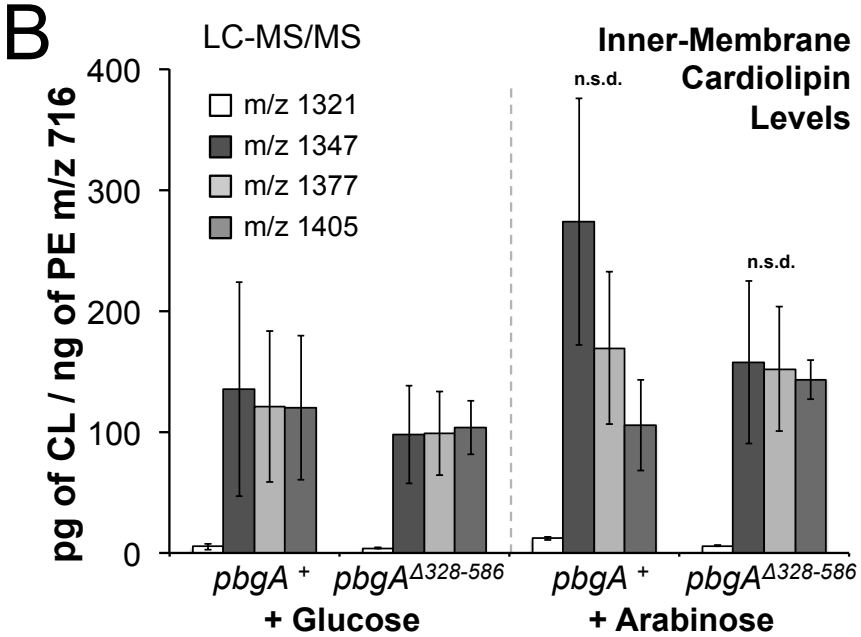
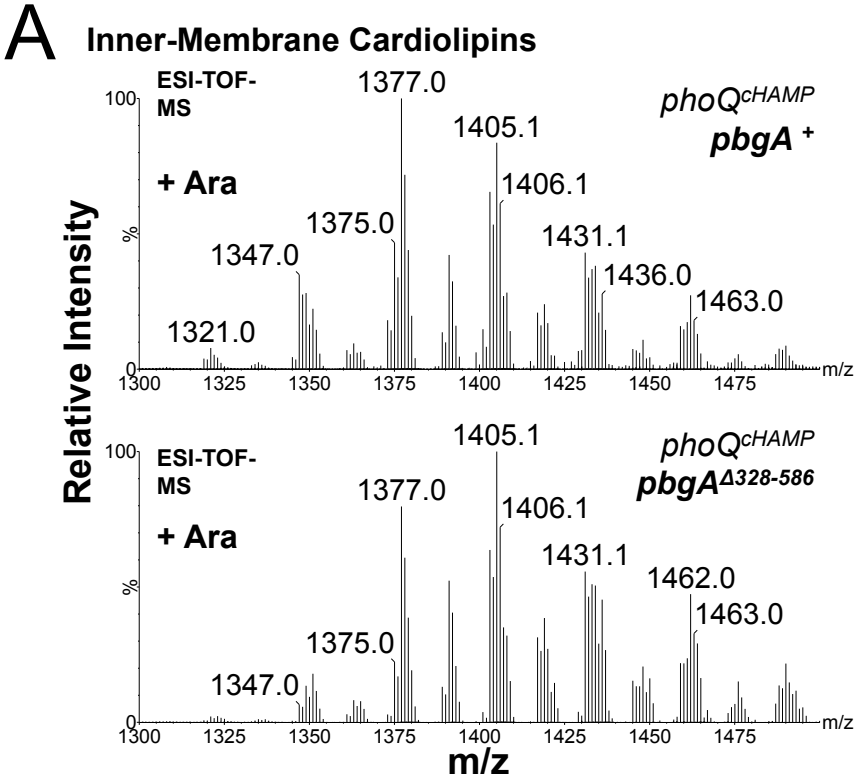


# Supporting Figure 2

## Sequence near the plasma membrane of PbgA-like proteins for bacteria.



# Supporting Figure 3



# Supporting Figure 4

