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A PhoP-Regulated Outer Membrane Protease of *Salmonella enterica* Serovar Typhimurium Promotes Resistance to Alpha-Helical Antimicrobial Peptides

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The outer membrane protein contents of *Salmonella enterica* serovar Typhimurium strains with PhoP/PhoQ regulon mutations were compared by two-dimensional gel electrophoresis. At least 26 species of outer membrane proteins (OMPs) were identified as being regulated by PhoP/PhoQ activation. One PhoP/PhoQ-activated OMP was identified by semiautomated tandem mass spectrometry coupled with electronic database searching as PgtE, a member of the *Escherichia coli* OmpT and *Yersinia pestis* Pla family of outer membrane proteases. *Salmonella* PgtE expression promoted resistance to alpha-helical cationic antimicrobial peptides (α-CAMPs). Strains expressing PgtE cleaved C18G, an 18-residue α-CAMP present in culture medium, indicating that protease activity is likely to be the mechanism of OmpT-mediated resistance to α-CAMPs. PhoP/PhoQ did not regulate the transcription or export of PgtE, indicating that another PhoP/PhoQ-dependent mechanism is required for PgtE outer membrane localization. PgtE is a posttranscriptionally regulated component of the PhoP/PhoQ regulon that contributes to *Salmonella* resistance to innate immunity.

Innate immunity is a mechanism by which animals sense and control invading microbes, including bacteria. Cationic antimicrobial peptides (CAMPs) are a major component of innate immunity (55). CAMPs are released at mucosal and skin surfaces and are part of the phagocytic vacuole microbicidal mechanism. CAMPs have a variety of amphipathic structures that function to kill bacteria by permeabilization of lipid bilayers.

Upon host colonization, gram-negative bacteria can increase their resistance to innate host defenses, including CAMPs, by changing the structure, immunogenic properties, and permeability of their surfaces. The PhoP/PhoQ regulatory system of *Salmonella enterica* serovar Typhimurium is a host defense mechanism by which bacteria respond to environmental signals and induce changes in the bacterial outer membrane that promote CAMP resistance (20). These changes include addition of aminoarabinose and palmitate to the lipid A moiety of lipopolysaccharide (LPS) (16, 19, 21), which promotes resistance to polymyxin (15, 19), alpha-helical antimicrobial peptides, and protegrin, a β-sheet antimicrobial peptide (21). Besides regulating the expression of enzymes involved in LPS modifications, PhoP/PhoQ regulates the expression of several secreted and membrane proteins (4, 44) that could be important for resistance to bactericidal agents. To identify additional members of the PhoP/PhoQ regulon that are involved in resistance to CAMPs, outer membrane protein (OMP) profiles of *Salmonella* strains with *phoP/phoQ* mutations were compared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Bacterial cultures were grown at 37°C with aeration in Luria broth (LB) (31), tryptic soy broth (Difco), Mueller-Hinton broth (Difco), or N minimal medium supplemented with 0.1% casamino acids, 38 mM glycerol, and 8 μM MgCl$_2$ (13). Antibiotics were used at the following concentrations: kanamycin, 45 μg/ml; ampicillin, 50 to 100 μg/ml; and streptomycin, 1,000 μg/ml.

Cloning of *pgtE* and construction of mutant strains. DNA sequences flanking *pgtE* were amplified by PCR using appropriate oligonucleotide primers and cloned into the *Xba*I and *Hin*dIII sites of pBluescript KS+. The resulting plasmid contained DNA sequences deleted for most of the *pgtE* open reading frame (nucleotides 3912 to 4785 and 5594 to 5916; GenBank accession no. M21279). A kanamycin resistance cassette was then inserted into the *Eco*RI site within Δ*pgtE*. The Δ*pgtE*::*Kan* cassette was then cloned into the pKAS32 suicide vector (41) digested with *Kpn*I and *Sac*I (pTG51), and SM10λpir cells were transformed. Allelic exchange was performed by a conjugative transfer of pTG51 into CS401, a wild-type strain of serovar Typhimurium containing a recessive streptomycin sensitivity allele. Single homologous recombination derivatives were isolated by selection for ampicillin and kanamycin resistance and then selected on streptomycin for loss of plasmid sequences. The presence of the Δ*pgtE*::*Kan* cassette on the chromosome was determined by PCR amplification. The mutated locus was transferred into other *Salmonella* strains by P22HT*int* phage-mediated transduction.

Isolation of bacterial outer membranes and analysis of OMPs by 2-D PAGE. Outer membranes were isolated by a modified protocol of Osborn et al. (36). One liter of LB medium was inoculated from an overnight culture at a dilution factor of 1:100. Cultures were grown until late log phase (optical density at 600 nm [OD$_{600}$] = 0.8 to 1.0), when cells were collected by centrifugation at 8,000 × *g* for 15 min at 4°C. Bacterial spheroplasts were generated by cold osmotic shock in 0.5 M sucrose–10 mM Tris-Cl (pH 7.8)–60 μg of lysozyme per ml and subsequent addition of an equal volume of ice-cold 1 μM EDTA. Spheroplasts were broken by French press at 16,000 lb/in^2, unbroken cells were removed by centrifugation at 6,000 × *g* for 15 min at 4°C, and the bacterial extract was separated into fractions by centrifugation at 200,000 × *g* for 1 h at 4°C. The pellet fraction containing total bacterial membranes was homogenized in 20% sucrose and subjected to sucrose density gradient centrifugation at 180,000 × *g* for 12 to 16 h at 4°C. The outer membrane fraction was separated as the band of highest buoyant density in the sucrose gradient.

For more efficient separation during isoelectric focusing (IEF), aliquots containing 250 μg of total OMP were washed in deionized water and solubilized by boiling for 10 minutes in 1% (wt/vol) sodium dodecyl sulfate (SDS). Protein was subsequently precipitated in 10 volumes of ice-cold acetone. Removal of the bulk of the outer membrane lipid and LPS aggregates by this technique significantly improved protein separation by IEF. The protein precipitate was resuspended in solubilization buffer containing 9 M urea, 2% Triton X-100, 2% Pharmalyte pH 3-10 (Pharmacia), 2% β-mercaptoethanol, and the protease inhibitors pepstatin (2 μg/ml), aprotinin (2 μg/ml), and leupeptin (2 μg/ml). After 2 h of incubation at 37°C, insoluble material was removed by sedimentation at 14,000 × *g* for 10 min at room temperature. Solubilized protein was first separated by IEF using a Pharmacia Multiphor II electrophoresis unit with immobilized pH gradients (pH

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or property[a]	Source or reference[b]
Serovar Typhimurium		
CS015	*phoP102*::Tn*10d*-Cam	32
CS401	*phoN2 zxx-6251*::Tn*10d*-Cam *rpsL* (Str^r)	21
CS093	14028s; wild type	ATCC
CS022	*pho-24* (PhoP constitutive); a mutation in *phoQ* that results in increased net phosphorylation of PhoP and unregulated expression of PhoP-activated genes (18)	33
TG59	CS401 Δ*pgtE*::Kan	This work
TG61	CS022 Δ*pgtE*::Kan	This work
TG71	TG61 with pBluescript KS+	This work
TG73	TG61 with pTG73	This work
CS435	*pho-24 pagP*::Tn*10d*-Tet *phoN2 zxx-6251*::Tn*10d*-Cam (also LG069)	21
TG66	CS435 Δ*pgtE*::Kan	This work
TG172	CS401 with pTG171	This work
TG173	CS015 with pTG171	This work
TG174	CS022 with pTG171	This work
TG200	CS022 *pagA*::MudJ	This work
CS404	CS022 *pagA*::MudJ *pagP*::Tn*10d*-Tet	This work
E. coli		
SM10 λpir	*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu	
DH5α	F⁻ φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE441 gyrA96 relA1*	Gibco-BRL
CC118	*araD139* Δ(*ara,leu*)7697 Δ*lacX74 phoA*Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	28
Plasmids and transposons		
pKAS32	Amp^r Str^s (*rpsL*); a low-copy allelic exchange vector	41
pTG51	Amp^r Kan^r; *pgtE* with an inserted Kan^r cassette (39) replacing a portion of *pgtE* is ligated to *Kpn*I-*Sac*I fragment of pKAS32	This work
pBluescript KS+	Amp^r; a high-copy cloning vector	Stratagene
pTG73	Amp^r; contains *pgtE* and 904 bp of upstream region (nt 671 to 2671; GenBank accession no. M21279) cloned into *Xba*I site of pBluescript KS+	This work
pGPLFR03	Amp^r; firefly (*f-luc*) and vanilla (*v-luc*) double luciferase suicide vector	30
pTG171	Amp^r; contains a transcriptional fusion of *pgtE* (nt 5266 to 5641; GenBank no. M21279) to *f-luc*; ligated with an *Eco*RI-*Kpn*I fragment of pGPLFR03	This work
pWSK129	Kan^r; low-copy cloning vector	51
pTG82	Kan^r; contains *pgtE* and 904 bp of upstream region (nt 671 to 2671; GenBank no. M21279) cloned into *Xba*I site of pWSK129	This work
pTG85	Kan^r; contains Tn*phoA*/in insertion at codon 112 of *pgtE* in pTG82	This work
pTG86	Kan^r; contains Tn*phoA*/in insertion at codon 152 of *pgtE* in pTG82	This work
pTG87	Kan^r; contains Tn*phoA*/in insertion at codon 190 of *pgtE* in pTG82	This work
Tn*phoA*/in	Tn*5* derivative; generates translational fusions to alkaline phosphatase (*phoA*)	27

[a] nt, nucleotide.
[b] ATCC, American Type Culture Collection.

4 to 7) and then on SDS–12% polyacrylamide gels. Protein spots were visualized by staining with Coomassie brilliant blue. Gels were scanned using an UMAX Astra 1200S scanner, and two-dimensional (2-D) profiles of outer membrane proteomes were compared.

Peptide isolation, sequencing by tandem mass spectrometry, and identification of PgtE. Protein spots of interest were excised from Coomassie-stained 2-D SDS-polyacrylamide gels and digested in situ with trypsin as described below (obtained as a personal communication from Michael Kinter, University of Virginia). Polyacrylamide gel slices were fragmented, destained during an overnight incubation in 50% (vol/vol) methanol, and dehydrated by incubation in acetonitrile for 10 min. Excess liquid was removed under vacuum, and gel fragments were rehydrated in 50 μl of sequencing-grade modified trypsin (Promega) at the concentration of 20 μg/ml. After a 30-min incubation on ice, excess trypsin was removed, 20 μl of 50 mM ammonium bicarbonate was added, and the mixture was incubated overnight at 37°C. Resulting peptides were eluted from the gel with several changes of extraction buffer (200 μl of 50% acetonitrile–5% formic acid) and dried by evaporation. Peptides were solubilized in 5% acetonitrile–0.5% acetic acid for further analysis.

LC/MS/MS analysis (liquid chromatography combined with tandem mass spectrometry) of tryptic peptides was carried out with a Finnigan/Thermoquest TSQ 7000 triple quadrupole mass spectrometer (San Jose, Calif.), coupled with a microcapillary high-pressure liquid chromatography (HPLC) apparatus built in-house. The details of our modifications of a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, Md.) for use with capillary HPLC have been described previously (50). The Finnigan API (atmospheric pressure ionization) electrospray source was modified with a capacitive sprayer assembly

(49). A linear binary gradient of solvents at a flow rate of 200 ml/min (Shimadzu LC-10AD pumps) was split precolumn to give a 1:1,000 split ratio, as measured at the beginning of the gradient. A fused-silica capillary column was packed in-house, 75 μm inner diameter by 12 cm (Magic C18; 5-μm packing; 100 Å pore size; Michrom Bioresources, Auburn, Calif.), and connected to the splitter. The column was eluted with a gradient of 0 to 75% acetonitrile in the presence of 0.4% acetic acid over 25 min. The temperature of the heated capillary inlet to the mass spectrometer was set at 180°C. When the main beam ion peaks for a peptide reached a preset threshold (40,000 counts), the ion of interest was automatically selected for collision-induced dissociation (CID) using the data-dependent scanning capabilities built into the TSQ, which are accessed using ICL (instrument control language) procedures (11). Protein identification was accomplished by use of the SEQUEST computer program (53).

Bacterial sensitivity to antimicrobial peptides. Standard MICs of CAMPs were determined as described (45), except that bacterial cultures were grown overnight in N minimal medium with low (8 μM) magnesium or in tryptic soy broth and diluted to 2 × 10⁵ bacteria per ml in N minimal medium with low magnesium. Complementation experiments were performed in Mueller-Hinton broth. Test peptides were assayed at final concentrations of 0.15 to 40.0 μg/ml in 96-well polypropylene microtiter plates (Costar). The MIC was determined as the lowest concentration of the peptide that did not allow visible bacterial growth after 24 h (for assays performed in rich medium) or after 48 h (for assays that were performed in minimal medium). C18G was a gift of Richard Darveau; LL-37 and CRAMP were a gift of Robert Lehrer. Resistance to defensins HNP-1 (a gift of Thomas Ganz), cryptidin 2 (a gift of Michael Selsted), and NP-1 and protegrin PG-1 (a gift of Robert Lehrer) were analyzed as above and in radial
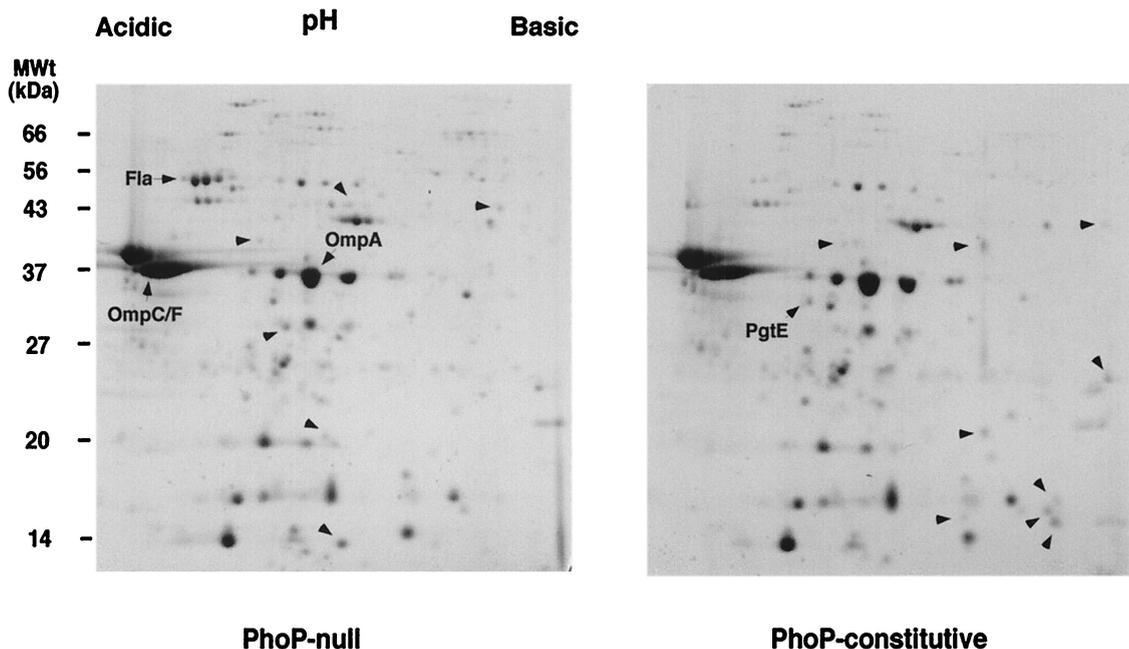
FIG. 1. 2-D PAGE map of PhoP/PhoQ-regulated OMPs. OMPs were separated by IEF on a linear pH gradient of 4 to 7 and on SDS–12% PAGE gels. Proteins were visualized by staining in Coomassie brilliant blue. 2-D map positions of the porins OmpA, OmpC/OmpF, and PhoP-regulated phase I flagellin (Fla) were determined by comparison with previous studies (38). Unlabeled arrowheads point to protein species present exclusively in the absence (PhoP null) or presence (PhoP constitutive) of an active PhoP/PhoQ regulatory system. PgtE is a protein that was sequenced and analyzed in this study.

diffusion assays as described previously (23). Peptide-killing assays were performed with mid-log-phase bacterial cultures grown in rich medium (LB) as described in Miller et al. (34). All experiments were performed two or three times.

To determine the specificity of the protease action in *pgtE*-mediated CAMP resistance, 2×10^5 bacterial cells were incubated with 2.5 µg of C18G peptide per ml for 16 h in 0.5% tryptone. Cells were sedimented by centrifugation, and peptide-containing supernatants were collected. Relative amounts of cleaved and uncleaved peptide were determined by separating the supernatants on a reversed-phase column (250-mm by 1.00-mm Jupiter column; 5-µm particle size; 300-Å pore size; C-18 bonded silica; Pharmacia, Inc., Kalamazoo, Mich.) used with the Shimadzu LC-10AD VP liquid chromatography system. A linear gradient of 5 to 95% acetonitrile in H_2O was applied over a period of 30 min. The aqueous and organic buffers contained 0.1 and 0.08% trifluoroacetic acid, respectively. The sensitivity of the UV detector at 214 nm was set at 0.1 absorbance unit full scale.

DNA techniques. Bacterial chromosomal DNA was isolated as previously described (17). Plasmid DNA was isolated using kits from Promega and Qiagen. PCR was performed with Pfu Turbo DNA polymerase (Stratagene) and *Taq* DNA polymerase (Gibco-BRL) according to the manufacturers' instructions.

Luciferase assays. A fusion of the *pgtE* promoter region to the transcriptional reporter *f-luc* was integrated into the chromosome of different *Salmonella phoP* strains using the suicide vector pGPLFRO3 (30) (Table 1). Correct chromosomal localization of the *pgtE-f-luc* fusion was confirmed by Southern blotting and hybridization and also genetically by allelic replacement of a kanamycin resistance marker inserted into *pgtE*. Bacteria were grown in LB medium or in N minimal medium containing different concentrations of Mg^{2+}. Luciferase assays were performed throughout the growth curve of each strain as previously described (30). Aliquots (20 µl) of bacterial cultures were lysed by freezing and thawing, followed by sonication for 20 s. Luciferase activity of cell lysates was determined using the Luciferase Reporter assay system (Promega). Units were recorded in a Berthold LB9501 luminometer.

Nucleotide sequence accession numbers. The revised nucleotide sequence of *pgtE* has been deposited in GenBank under accession number AF239770. *Salmonella* genome sequence data were produced by the *Salmonella typhi* Sequencing Group at the Sanger Centre, Cambridge, U.K., and by the Genomes Sequencing Center at Washington University, St. Louis, Mo., and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/st/ and ftp://genome.wustl.edu/pub/gsc1/sequence/st.louis/bacterial/salmonella/, respectively.

RESULTS

PhoP/PhoQ regulates OMPs. A variety of studies have identified PhoP/PhoQ-mediated transcriptional regulation of genes that encode predicted envelope proteins and alter outer membrane structure through LPS modifications (4, 16, 20, 44). To further define PhoP/PhoQ-regulated changes in the protein composition of the *Salmonella* envelope, a method for high-resolution analysis of gram-negative OMPs by 2-D polyacrylamide gel electrophoresis (2-D PAGE) was developed. *Salmonella* outer membranes were purified by separation on a sucrose gradient. Then OMPs were separated from LPS aggregates by heating in the presence of an anionic detergent. Next, protein separation was accomplished by IEF (described in Materials and Methods). A large number of *Salmonella* OMPs were detected by Coomassie staining of 2-D polyacrylamide gels.

Twenty-six spots corresponding to OMPs that are members of the PhoP/PhoQ regulon were detected by comparing the 2-D PAGE profiles of strains with PhoP-null and PhoP-constitutive phenotypes (Fig. 1 and data not shown). Twelve OMP spots were unique to the PhoP-null strain (CS015), and 14 spots were unique to the strain with increased PhoQ kinase activity (PhoP constitutive, CS022). Thus, PhoP/PhoQ mediates significant alteration in the protein content of the outer membrane in addition to LPS structural changes.

Identification of PgtE protease as a PhoP-regulated OMP. One abundant protein species of approximately 32 kDa and isoelectric point 5.2 localized to the outer membrane of strains that expressed activated PhoP/PhoQ but not to the membrane of the PhoP-null strain (Fig. 1). To confirm that this protein was localized to the outer membrane as a result of PhoP/PhoQ activation, OMPs isolated from wild-type serovar Typhimurium grown in a PhoP-repressing (1 mM) or PhoP-inducing
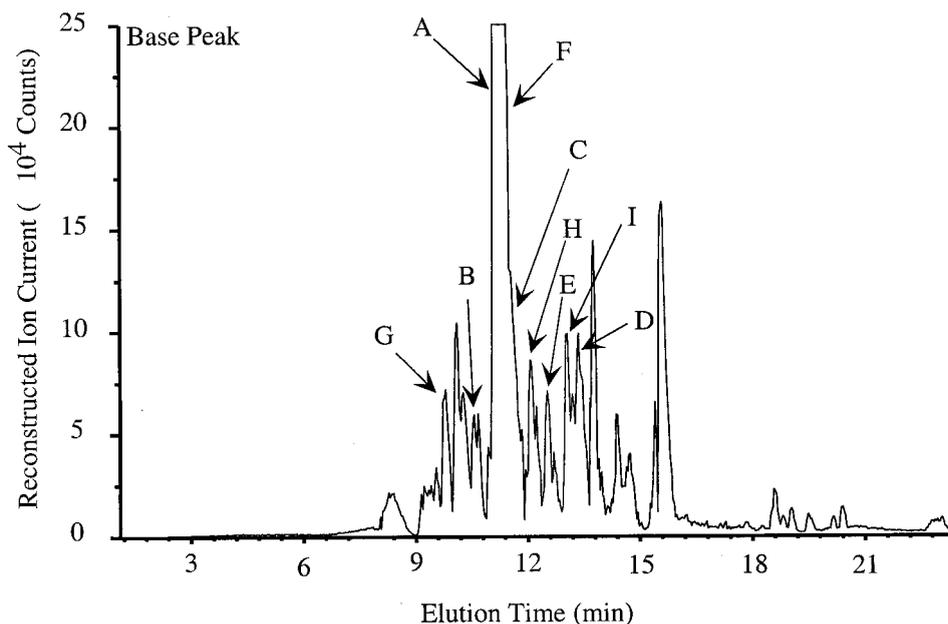
FIG. 2. Base peak capillary HPLC mass chromatogram from the tryptic digest of *Salmonella* PgtE. In this type of HPLC trace, the signal intensity of the most abundant ion is plotted for each scan. Each scan of the mass spectrometer takes about 1 s. Peptides identified by SEQUEST are indicated by arrows and contain the following partial sequences: (A) ELVYDTDTGR; (B) ELVYDTDGRK; (C) KLSQLDWK; (D) GWLLQGDNYK; (E) FSWTAR; (F) YIGNFPHGVR; (G) GIGYSQR; (H) YSDWVNAHDNDEHYMR; and (I) IFAEFAYSK.

(8 μM) concentration of Mg^{2+} (13) were separated by 2-D PAGE. A 32-kDa protein of pI 5.2 was detected in the outer membranes of salmonellae that were grown in PhoP-activating conditions (8 μM Mg^{2+}), but not in the outer membranes of salmonellae grown in the presence of a high concentration of Mg^{2+} (data not shown), further indicating that this protein was regulated by PhoP/PhoQ-activating conditions and was not specific to strains with this *phoQ* mutation.

To begin the characterization of PhoP-regulated OMPs, this protein was excised from the polyacrylamide gel and digested in situ with trypsin, and the resulting peptide mixture was eluted from the gel. An aliquot of this tryptic peptide mixture was analyzed by on-line microcapillary HPLC coupled with electrospray ionization-mass spectrometry (Fig. 2). Sequences of isolated tryptic peptides were determined by semiautomated tandem mass spectrometry coupled with protein database searching. Raw-product ion (MS^2 or MS/MS) data were searched using the SEQUEST computer program, which is capable of identifying peptides from protein amino acid sequence data coded in FASTA format, such as that found in the OWL database (5), from uninterpreted MS/MS spectra (53). Three peptides (LSQLDWK, AGVTAGYQETR, and SIHPD TSVNYANEYDLN) had high SEQUEST cross-correlation scores (2.96, 3.80, and 7.18, respectively) with peptides originating from PgtE, the *Salmonella* OmpT protease homologue (14). The SEQUEST search results suggested that all three peptides originated from the same protein, based on the gene sequence found in the SWISS-PROT database (2), accession number P06185. Fragmentation of selected peptide ions by CID (24) resulted in sufficient partial sequence information to characterize the protein as PgtE (Fig. 3). *Salmonella pgtE* was originally identified as an open reading frame located downstream of the inducible *pgtBCA* operon, which is involved in phosphoglycerate transport (54).

Nucleotide sequence analysis and correction of PgtE sequence. The genomic region of serovar Typhimurium that contains *pgtE* was cloned into a plasmid vector (pTG73, Table 1), and its nucleotide sequence was analyzed and compared to sequences in GenBank and to sequences available from the *Salmonella* genome sequencing projects. Plasmid-derived *pgtE* sequence was identical to regions of two *S. enterica* LT2 genomic fragments (gnl/WUGSC 99287/stmlt2-.Contig1497 and gnl/WUGSC 99287/stmlt2-A2A.Contig104, respectively) and highly similar to genomic fragments of serovars Typhi (gnl/Sanger 601/S. typhi Contig427) and Paratyphi (gnl/WUGSC 32027/spara B SPA.0.1882). The nucleotide sequences derived from plasmid pTG73 and from the above-mentioned *Salmonella* genome fragments contain several frameshifts within the *pgtE* open reading frame with respect to a previously reported *pgtE* sequence (GenBank accession number M21279). The newly assigned PgtE sequence exhibits higher similarity to other OmpT-like proteases than the previously reported one (72% identity and 83% similarity to *Y. pestis* Pla, 46% identity and 65% similarity to *E. coli* OmpT and OmpP, and 38% identity and 59% similarity to *Shigella flexneri* SopA) throughout the length of the protein. Revised PgtE contains a C-terminal phenylalanine residue that is essential for outer membrane insertion of trimeric OMPs (46). *E. coli* contains two highly homologous genes encoding OmpT-like proteases: *ompT* on the chromosome (15) and *ompP* (25) on plasmid F (29). OmpP and OmpT have 87% sequence identity (25). A *pgtE*-specific probe hybridized to a single *Salmonella* genomic fragment on a Southern blot (data not shown).

PhoP/PhoQ does not regulate *pgtE* transcription and export into the periplasm. Adams et al. (1) have reported the activity of a PhoP-regulated OmpT-like protease in serovar Typhimurium cellular extracts (1). The 2-D PAGE analysis described in this study suggested that PhoP/PhoQ might regulate the transcription of *pgtE*. Single-copy transcriptional fusions of *pgtE* to the firefly luciferase (*f-luc*) gene were constructed by using the pGPLFR03 suicide vector (Table 1). The resulting gene fusion contained *f-luc* inserted into the last third of the

(A)

$$b_n$$

58 **244 357 470** 598 655 770 884 **1047** 1175

G---W---L---L---Q---G---D--N---Y---K

1194 1136 **950 837 724 596 539 424 310 147**

$$y_n$$

(B)

$$b_n$$

114 **261 332 461** 608 679 842 929 1058

I---F---A---E---F---A---Y---S---K

1076 **963 815 744 615 468 397 234** 147

$$y_n$$

(C)

$$b_n$$

148 **235** 421 522 593 749

F---S---W---T---A---R
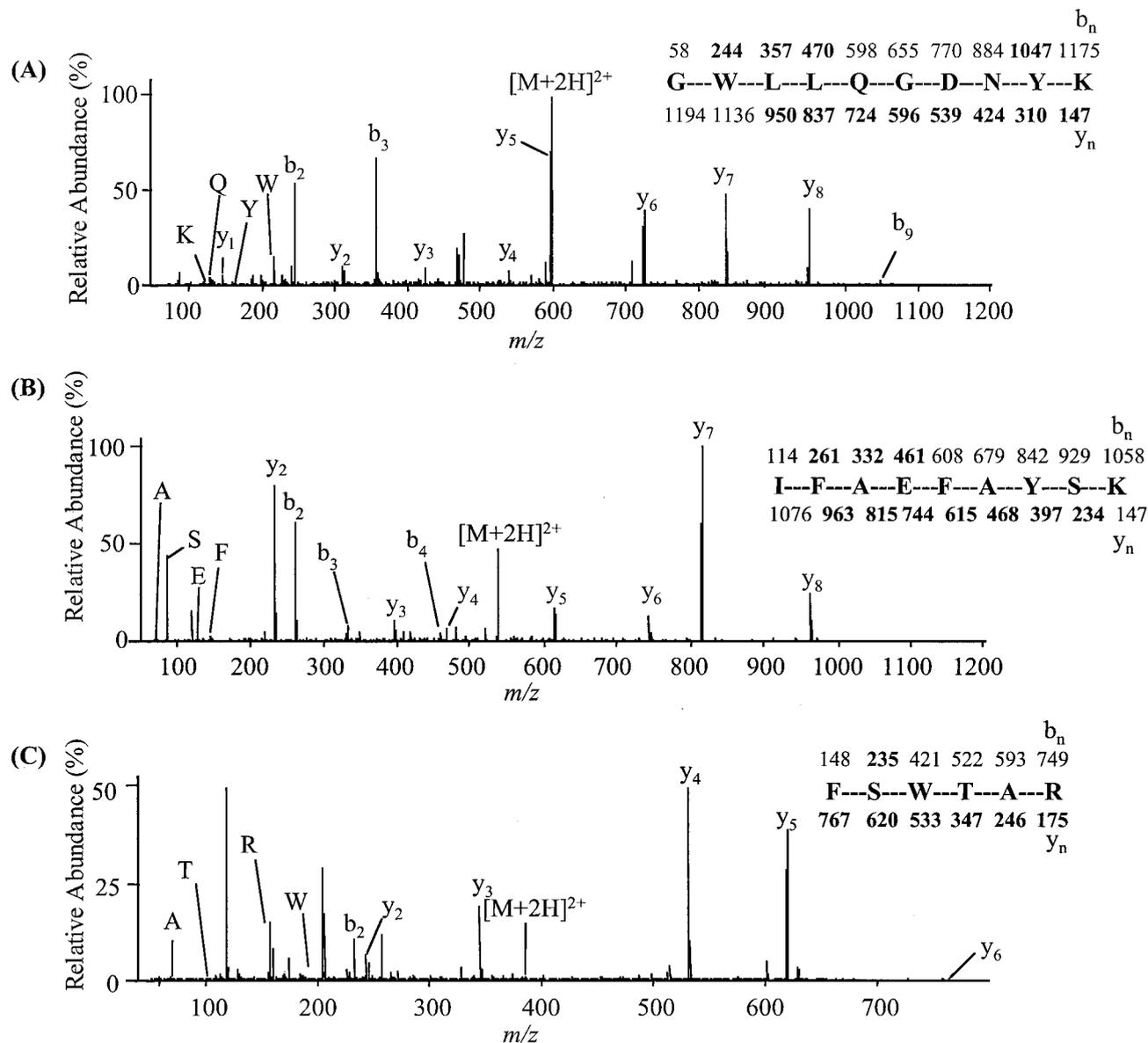
767 620 533 347 246 175

$$y_n$$

FIG. 3. Peptide sequencing by tandem mass spectrometry and identification of *Salmonella* PgtE. Fragment ions observed are indicated in boldface above (b-type ions) and below (y-type ions) the peptide sequence. (A) CID mass spectrum of GWLLQGDNYK. (B) CID mass spectrum of IFAEFAYSK. (C) CID mass spectrum of FSWTAR. The nomenclature used for b and y peptide fragment ions has been described by Biemann (4a). Low-mass ions that are indicative of amino acid composition but not sequence are described by the amino acid single-letter code.

pgtE. Expression of *f-luc* was measured throughout the growth curve in bacterial cultures grown in LB and in minimal medium supplemented with a high (PhoP-repressing) or low (PhoP-inducing) concentration of Mg^{2+}. Surprisingly, similar levels of *pgtE-f-luc* expression were detected in the PhoP-null (TG172), wild-type (TG173), and PhoP-constitutive (TG174) strains. Expression of the *pgtE-f-luc* fusion increased steadily in all strains during logarithmic growth (Fig. 4). These results indicated that transcription of *pgtE* is constitutive and it is not dependent on PhoP/PhoQ. Therefore, localization of PgtE to the outer membrane is mediated by a PhoP/PhoQ-dependent mechanism that acts after the transcription of the *pgtE* gene.

To further analyze PhoP-mediated regulation, *pgtE* and a 904-bp upstream region were cloned downstream of the inducible *lac* promoter on a low-copy-number plasmid (pTG82, Ta-

ble 1). Random translational fusions of *pgtE* to the alkaline phosphatase gene (*phoA*) were generated in *E. coli* by utilizing transposon Tn*phoA*/in delivered by the method of Manoil and Bailey (27). Plasmids containing transposon insertions in *pgtE* were isolated and analyzed. Three plasmids, pTG85, pTG86, and pTG87, contained exported PhoA fusions to residues 112, 152, and 190 of PgtE, respectively. These plasmids were transferred to appropriate *Salmonella* strains, and the alkaline phosphatase activity of the fusion proteins in the presence and absence of *phoP* expression was measured as previously described (6). Rates of PgtE-PhoA synthesis and export were similar in all strains (data not shown), indicating that the translation initiation and likely Sec-dependent translocation of PgtE across the inner membrane were independent of PhoP/PhoQ.

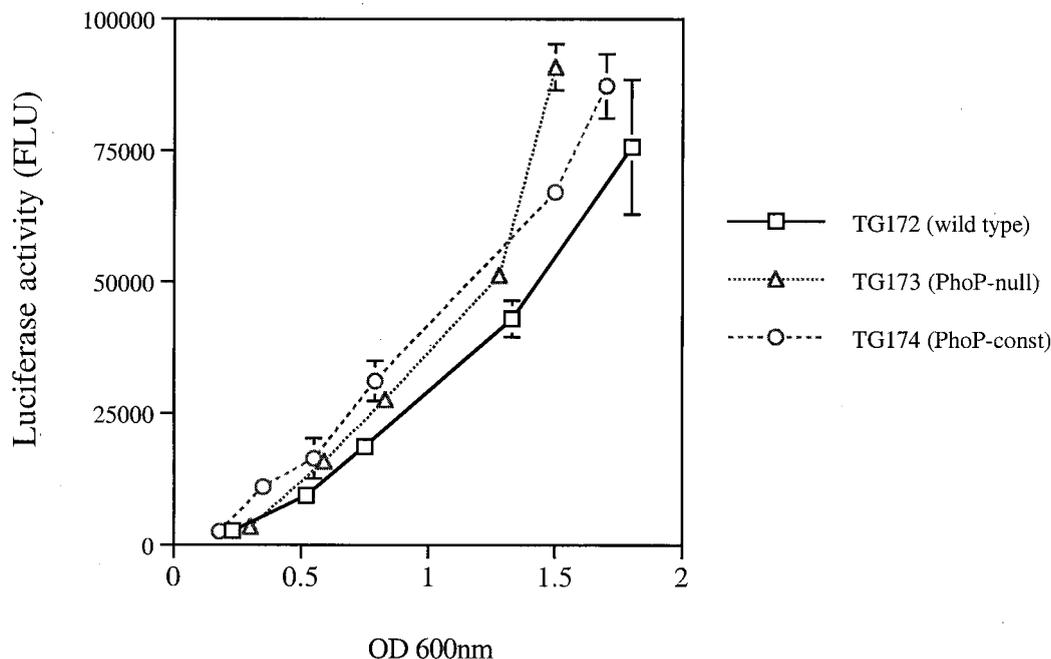The results of this study suggested that localization at the

FIG. 4. *pgtE* expression does not require PhoP. The expression of *pgtE* was measured throughout the growth of bacterial cultures by quantitating the amount of luciferase activity produced by strains containing the *pgtE-f-luc* transcriptional fusion. Bacterial cultures were grown in rich medium (LB). The graph depicts one experiment performed in triplicate and is representative of several experiments. Error bars represent the standard deviation (SD); no bars indicate that the SD is insignificant. FLU, firefly luciferase light units. const, constitutive.

outer membrane could be important for the protease activity of PgtE. It has been demonstrated that proper folding, oligomerization, and insertion of some *E. coli* OMPs into the outer membrane are dependent on interaction with LPS molecules or specific LPS structures (9, 10, 40). Therefore, it is possible that PhoP/PhoQ-mediated modifications of LPS could affect the insertion and localization of some *Salmonella* OMPs, including PgtE. To explore this possibility, OMPs from PhoP-constitutive Typhimurium strains containing a mutation in *pagP*, *pagA*, or both genes (CS435, TG200, and CS404, respectively), were analyzed by 2-D PAGE for the presence of PgtE. PhoP-regulated *pagP* and *pagA* mediate additions of palmitate and aminoarabinose, respectively, to *Salmonella* lipid A (19, 21). The absence of these two lipid A modifications did not affect the localization of PgtE to the outer membrane, as determined by 2-D PAGE (data not shown), indicating that these modifications are not essential for localization of PgtE to the outer membrane.

PgtE promotes resistance to antimicrobial peptides. PgtE belongs to the family of outer membrane endopeptidases (42) that specifically cleave between paired basic residues and after a basic residue that is followed by a nonpolar amino acid (48). Therefore, CAMPs are potential targets of PgtE. Recently, Stumpe et al. (48) have demonstrated that expression of OmpT increases survival of *E. coli* grown in the presence of protamine, a CAMP isolated from salmon sperm (47). To test whether PgtE plays a similar role in serovar Typhimurium, *pgtE* mutants were generated by allelic exchange and their ability to survive in the presence of CAMPs that contain predicted OmpT (PgtE) cleavage sites was determined. The ability of *pgtE* strains to resist the bactericidal action of alpha-helical peptide C18G (8) was determined in a growth inhibition (MIC) assay. The *pgtE* deletion strain TG61 showed increased sensitivity to C18G (Table 2). The sensitivity of this mutant was increased when bacterial cultures were grown in N minimal medium prior to their incubation with C18G peptide. A pre-

TABLE 2. MICs of cationic peptides[a]

Strain[a]	MIC (µg/ml)					
	C18G		LL-37, N-minimal	CRAMP, N-minimal	NP-1, N-minimal	PG-1, N-minimal
	N-minimal	TSB				
CS015 (PhoP null)	ND	0.62	ND	ND	ND	ND
CS022 (PhoP^C)	2.50	2.50	2.50	2.50	0.62	0.31
TG61 (PhoP^C *ompT*)	0.31	1.25	1.25	1.25	0.62	0.31
CS435 (PhoP^C *pagP*)	0.62	1.25	2.50	1.25	0.62	0.31
TG66 (PhoP^C *pagP ompT*)	0.15	0.62	1.25	0.62	0.62	0.31

[a] PhoP^C, PhoP constitutive. Bacterial strains were grown either in N minimal medium containing 8 µM Mg^{2+} or in tryptic soy broth (TSB) prior to incubation with the peptides. Bacterial cells were diluted in N minimal medium when incubated with alpha-helical peptides C18G, LL-37, and CRAMP and in 1% TSB in 10 mM sodium phosphate buffer (pH 7.4) when incubated with defensin NP-1 and protegrin PG-1. ND, not determined. N minimal medium (13) does not support growth of CS015.

TABLE 3. High-copy expression of PgtE protease increases
Salmonella survival upon exposure to C18G and LL-37
(strains were grown in Mueller-Hinton broth)[a]

Strain	MIC (µg/ml)	
	C18G	LL-37
CS022 (PhoP[C])	5.00	5.00
TG71 (PhoP[C] *pgtE* + vector)	1.25	2.50
TG73 (PhoP[C] *pgtE* + pTG73)	40.0	40.0

[a] See Table 2 footnote *a*.

viously described *pagP* mutant (CS435) that is sensitive to
alpha-helical CAMPs (α-CAMPs) (21) had a higher survival
rate than TG61 under the same assay conditions. A strain
containing mutations of *pagP* and *pgtE* (TG66) showed greater
sensitivity to C18G than strains containing single *pagP* or *pgtE*
mutations (Table 2). *pgtE* mutants also displayed increased
sensitivity to human CAMP LL-37 (26) and mouse CRAMP
(3), other naturally occurring alpha-helical CAMPs that con-
tain predicted OmpT cleavage sites (Table 2).

To observe if the phenotype conferred by deletion of *pgtE*
could be complemented, a high-copy-number plasmid express-
ing *pgtE* (pTG73) or a control vector was introduced into
mutant strain TG61. Bacterial cultures were grown in rich
medium prior to incubation with CAMP to ensure high levels
of *pgtE* expression. As shown in Table 3, high-level expression
of *pgtE* greatly increases the survival of serovar Typhimurium
in the presence of C18G compared to strain CS022 (PhoP
constitutive). In a peptide-killing assay, the number of surviv-
ing CFU was determined after 2×10^5 bacteria were exposed
to varied amounts of C18G for 2 h. Although the *pgtE* mutant
(TG61) did not display sensitivity to C18G under these condi-
tions, expression of *pgtE* from a high-copy vector increased the
resistance of serovar Typhimurium to C18G (Fig. 5).

The effect of *pgtE* deletion on resistance to several beta-
sheet CAMPs was also examined by peptide-killing assays,
MIC assays, and radial diffusion assays. Strains deleted of *pgtE*
did not exhibit increased sensitivity or resistance to HNP-1,
NP-1, cryptidin-2, or protegrin PG-1 under any of the condi-
tions tested (Table 2 and data not shown). Therefore, *pgtE* is
essential for *Salmonella* resistance to alpha-helical CAMPs but
not to peptides of beta-sheet structure. A *pagP* mutant (CS435)
did not exhibit sensitivity to protegrin PG-1 in MIC assays
(Table 2), although it was shown to be sensitive to PG-1 in a
peptide-killing assay (22).

**Evidence that protease activity is the mechanism of PgtE-
mediated CAMP resistance.** To determine if peptide cleavage
promotes resistance to CAMP, *Salmonella* strains expressing
pgtE were assayed for cleavage of C18G. C18G is an 18-residue
CAMP (ALYKKLLKKLLKSAKKLG) that contains at least
three putative *PgtE* cleavage sites. In this experiment, super-
natants of cultures grown in the presence of C18G were col-
lected, and their contents were separated on a reversed-phase
HPLC column. C18G was completely degraded when incu-
bated with strain TG73, which expresses high levels of PgtE,
while no peptide degradation was observed when it was incu-
bated with the *pgtE*-null strain TG61 (Fig. 6). An intermediate
amount (approximately 40%) of C18G was cleaved when in-
cubated with parental strain CS022 (PhoP constitutive) (data
not shown). These results indicated that peptide cleavage cor-
relates with the serovar Typhimurium CAMP resistance phe-
notype.

DISCUSSION

Previous studies have demonstrated that PhoP/PhoQ regu-
lates the transcription of over one dozen genes encoding *S.
enterica* envelope and secreted proteins, including ones such as
PagC that were predicted to locate to the outer membrane (4,
37). PhoP/PhoQ also regulates changes in structure of the lipid
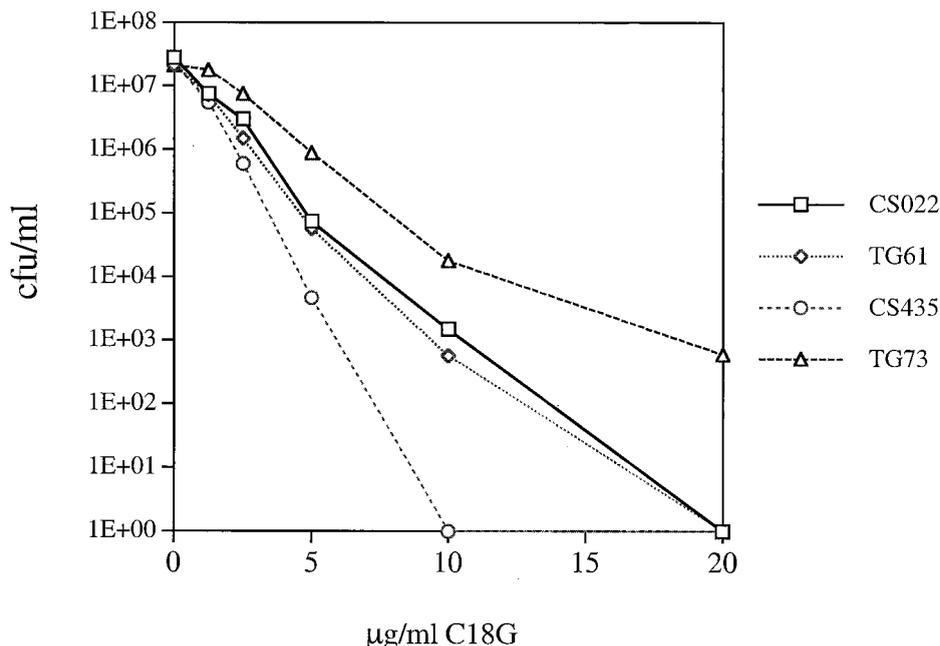

FIG. 5. High levels of *pgtE* expression increase *Salmonella* survival in the presence of C18G. Mid-log-phase bacterial cultures were incubated with the indicated
concentrations of C18G, and the number of CFU was determined. Expression of *pgtE* from a high-copy plasmid (in TG73) increased survival in the presence of C18G
compared to the parental strain (CS022), while the strain with the *pgtE* deletion (TG61) did not display significant sensitivity to C18G. A *pagP* mutant (CS435) was
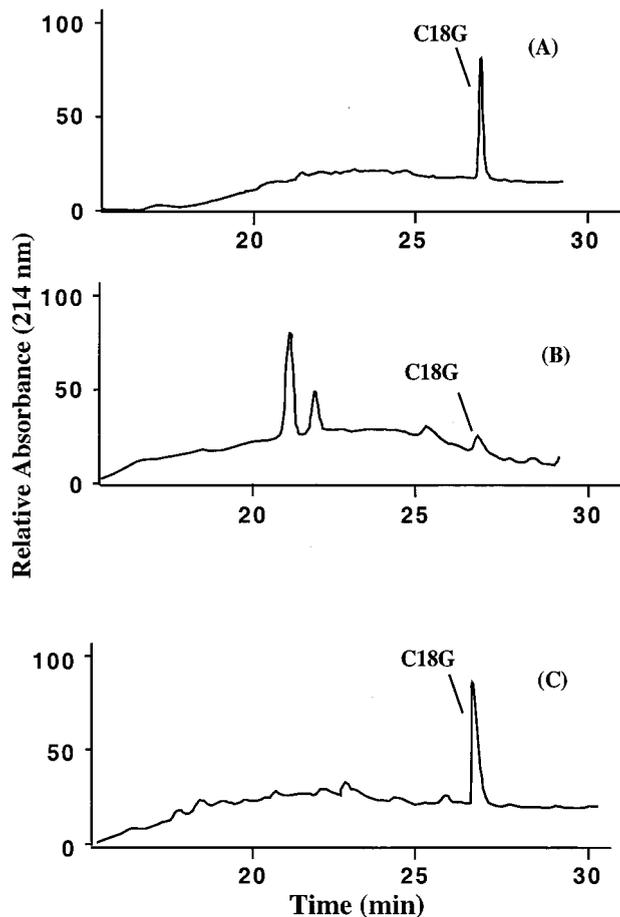sensitive to C18G.

FIG. 6. *Salmonella* cells expressing PgtE cleave C18G peptide present in the culture supernatants. Culture supernatants were collected after an MIC experiment, and their contents were analyzed by reversed-phase HPLC as described in Materials and Methods. A strain expressing *pgtE* from a high-copy plasmid (TG73) efficiently cleaved C18G (B), while a strain carrying a mutation in *ompT* (TG61) did not cleave C18G (C). The control sample contained C18G in buffer without bacteria present (A).

A component of LPS, which constitutes the outer leaflet of the *Salmonella* outer membrane (16, 19, 20). In recent years, analyses of proteomes by 2-D gel electrophoresis and tandem mass spectrometry have allowed characterization of complex biological processes (52). In this work the technique of 2-D proteome mapping was utilized to study PhoP/PhoQ regulation of *Salmonella* outer membrane proteins. This work indicates that PhoP/PhoQ regulates a significant number of OMP species. Fourteen species of OMPs that are positively regulated by PhoP/PhoQ were detected, and 12 species were repressed. Therefore, it appears that in addition to alteration of LPS, a major function of PhoP/PhoQ is to regulate extensive structural changes in both the lipid and protein components of the outer membrane.

A surprising observation of this work was that PgtE is an abundant component of the outer membrane upon PhoP/PhoQ activation, even though *pgtE* transcription and PgtE export into the bacterial periplasm are not regulated by PhoP/PhoQ. This finding indicates the utility of searching for post-transcriptionally regulated factors localized to the bacterial envelope. The results of this study suggested that PgtE insertion into the outer membrane is dependent upon transcription of another PhoP/PhoQ-activated factor(s). It is possible that

PhoP/PhoQ-mediated modifications of LPS could affect the insertion and localization of some OMPs. Though localization of PgtE was not affected by mutations in *pagP* and *pagA*, which mediate some *Salmonella* PhoP-activated LPS modifications, other regulated LPS modifications or factors might be important for localization of PgtE.

In this study, maximal resistance to CAMPs was observed in strains expressing PgtE, indicating that PgtE is part of the resistance to innate immunity regulated by PhoP/PhoQ. The PgtE contribution to inducible resistance was significant when bacteria were exposed to peptides with an alpha-helical structure, such as C18G and LL-37. Such resistance correlated with the ability of bacteria to digest this peptide in the culture medium, indicating that PgtE cleavage of such peptides is the likely mechanism of resistance. The sensitivity of the *pgtE* mutant was the most obvious in the microbroth dilution (MIC) assays, when the bacteria were incubated with the peptide for a longer period of time (24 to 48 h). The rate of PgtE-mediated peptide hydrolysis is likely to be the limiting factor for the survival of the bacteria. Expression of PgtE is likely advantageous for a small number of bacteria that survive the initial exposure to α-CAMPs. Bacteria expressing PgtE can slowly digest the remaining unbound α-CAMP that was not bound to the bacterial membranes and replicate more efficiently than the bacteria lacking the protease. In support of this, the contribution of PgtE to bacterial resistance in a peptide-killing assay was significant only in the presence of high-copy *pgtE*. The PgtE protease was not demonstrated to contribute to *Salmonella* resistance to defensins or protegrin, CAMPs with an amphipathic beta-sheet structure stabilized through intramolecular disulfide bonds (35). Defensin structure could prevent access of PgtE to cleavage sites predicted by amino acid sequence of defensins. In contrast to α-CAMPs, which are produced throughout the animal kingdom, defensins have been found only in higher vertebrates, mammals (22), and birds (7). Higher vertebrates might have evolved defensins as an additional component of innate immunity to combat microbial pathogens which acquired the ability to resist peptides of a less complex structure through PgtE (OmpT) production.

In this work, mutation of both *pgtE* and *pagP* resulted in greater α-CAMP sensitivity than in strains containing a single mutation in either gene. Though mutation of *pagP* has a minor effect on the beta-sheet CAMP protegrin, it also has been found to have a greater effect on antimicrobial α-CAMP resistance. *pagP* encodes a PhoP/PhoQ-activated acyltransferase which catalyzes the addition of palmitate to lipid A and promotes a decrease in the permeability of the *Salmonella* outer membrane (21). The envelope-preserving function of *pagP* could contribute to the survival of salmonellae exposed to a variety of environmental stresses or to antimicrobial compounds and CAMPs within the intestinal lumen or phagocyte vacuoles. The action of a surface protease such as PgtE could further protect bacteria by lowering the concentration of α-CAMP to sublethal doses prior to insertion of the cationic peptide into the bacterial envelope. Synergistic action of PgtE and PagP could significantly decrease the amount of α-CAMP inserted into the cytoplasmic membrane and allow increased survival of bacteria during exposure to CAMPs.

Interestingly, the *E. coli* and *Y. pestis* homologues of PgtE have been implicated in virulence. *E. coli ompT* has been associated with the ability to cause urinary tract infections (12). Additionally, the *Y. pestis* homologue Pla is essential for virulence in mice when injected subcutaneously but not intravenously (43). It is possible that these effects are a result of OmpT-mediated resistance to innate immunity.

ACKNOWLEDGMENTS

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