Molecular Microbiology (1998) **27**(6), 1171–1182

PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance

John S. Gunn,[1,2] Kheng B. Lim,[3] Jackie Krueger,[1] Kevin Kim,[1] Lin Guo,[1] Murray Hackett[3] and Samuel I. Miller[1]*

[1]*University of Washington, Departments of Medicine and Microbiology, HSB K-140, Box 357710, Seattle, WA 98195, USA.*

[2]*University of Texas Health Science Center at San Antonio, Department of Microbiology, 7703 Floyd Curl Dr, San Antonio, TX 78284-7758, USA.*

[3]*University of Washington, Department of Medicinal Chemistry, HSB K-140, Box 357710, Seattle, WA 98195, USA.*

Summary

Antimicrobial peptides are distributed throughout the animal kingdom and are a key component of innate immunity. *Salmonella typhimurium* regulates mechanisms of resistance to cationic antimicrobial peptides through the two-component systems PhoP–PhoQ and PmrA–PmrB. Polymyxin resistance is encoded by the PmrA–PmrB regulon, whose products modify the lipopolysaccharide (LPS) core and lipid A regions with ethanolamine and add aminoarabinose to the 4′ phosphate of lipid A. Two PmrA–PmrB-regulated *S. typhimurium* loci (*pmrE* and *pmrF*) have been identified that are necessary for resistance to polymyxin and for the addition of aminoarabinose to lipid A. One locus, *pmrE*, contains a single gene previously identified as *pagA* (or *ugd*) that is predicted to encode a UDP-glucose dehydrogenase. The second locus, *pmrF*, is the second gene of a putative operon predicted to encode seven proteins, some with similarity to glycosyltransferases and other complex carbohydrate biosynthetic enzymes. Genes immediately flanking this putative operon are also regulated by PmrA–PmrB and/or have been associated with *S. typhimurium* polymyxin resistance. This work represents the first identification of non-regulatory genes necessary for modification of lipid A and subsequent antimicrobial peptide resistance, and provides support for the hypothesis that lipid A aminoarabinose modification promotes resistance to cationic antimicrobial peptides.

Received 15 September, 1997; revised 1 December, 1997; accepted 16 December, 1997. *For correspondence. E-mail millersi@u.washington.edu; Tel. (206) 616 5107; Fax (206) 616 4295.

Introduction

Bacterial pathogens encounter a wide range of host microenvironments, within which they must survive to successfully colonize and cause disease. Salmonellae encounter numerous such environments during the course of infection. After passage through the stomach and into the small intestine, these organisms transcytose the intestinal barrier and interact with macrophages and lymphocytes. Pathogenic salmonellae are able to survive and replicate within the harsh environment of the phagocytic cell (Fields *et al*., 1986), which contains a potent group of cytotoxic agents, antimicrobial peptides (APs).

APs are an important component of innate immunity (Zasloff, 1992; Bevins, 1994; Stolzenberg *et al*., 1997). These peptides are ubiquitous in nature and are found in various human organs or organ systems besides the reticuloendothelial system including the lung, skin and digestive tract (Jones and Bevins, 1992; Diamond *et al*., 1993; Goldman *et al*., 1997; Stolzenberg *et al*., 1997). APs are cationic, amphipathic and have a broad spectrum of killing, which involves pore formation or solubilization of membranes. These peptides bind to the outer membrane of Gram-negative organisms such as *Salmonella typhimurium* through electrostatic interactions with the negatively charged groups of lipopolysaccharide (LPS) core and lipid A, and traverse to the inner membrane where pore formation occurs.

S. typhimurium has developed mechanisms of resistance to AP. The PhoP–PhoQ two-component regulatory system is required for virulence of *Salmonella* spp. (Miller *et al*., 1989) and controls resistance of *S. typhimurium* to a number of APs, including defensin NP-1, magainin-2, cecropin P1, melittin, mastoparan, as well as crude neutrophil granule extracts (Fields *et al*., 1989; Miller *et al*., 1990; Groisman *et al*., 1992; Hohmann *et al*., 1996). The actual mechanism of resistance controlled by PhoP–PhoQ is not known; however, as PhoP–PhoQ has been shown to regulate LPS modifications (Guo *et al*., 1997), these alterations may be, in part, responsible for peptide resistance.

PmrA–PmrB governs resistance to antimicrobial compounds including polymyxin, polylysine, protamine and neutrophil antimicrobial peptides CAP37 and CAP57 (Vaara, 1981a,b; Shafer *et al*., 1984; Spitznagel, 1990; Roland *et al*., 1993). The genes encoding this two-component system have been shown to be activated *in vivo* (Alpuche-Aranda *et al*., 1992; Heithoff *et al*., 1997) and are regulated by PhoP–PhoQ (Gunn and Miller, 1996), but can also be activated independent of PhoP–PhoQ by growth in mild acidic conditions (Soncini and Groisman, 1996). PmrA–PmrB activation results in the modification of phosphate groups of the LPS core and lipid A with ethanolamine, and modification of the 4′ phosphate of lipid A with aminoarabinose (Vaara *et al*., 1981; Helander *et al*., 1994; Guo *et al*., 1997). These covalent modifications reduce electrostatic interactions and, hence, binding between the peptide and the cell surface. Other bacteria, including *Yersinia enterocolitica*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pnemoniae and Burkholderia* (*Pseudomonas*) *cepacia*, add similar covalent modifications to LPS (Cox and Wilkinson, 1991; Boll *et al*., 1994; Nummila *et al*., 1995; Helander *et al*., 1996).

Several PmrA–PmrB-regulated genes have been identified, which include *pagA* (UDP-glucose dehydrogenase) (Soncini and Groisman, 1996), the *pmrA-pmrB* operon itself (Gunn and Miller, 1996; Soncini and Groisman, 1996) and other undefined loci (Soncini and Groisman, 1996). However, no PmrA–PmrB-regulated gene (or any gene in any Gram-negative organism) has been identified that is involved in LPS modification and subsequent AP resistance. In this work, we report the identification of two PmrA–PmrB-regulated loci required for polymyxin resistance and for the addition of aminoarabinose to lipid A.

Results

Identification of loci required for resistance to polymyxin

To identify PmrA–PmrB-regulated genes necessary for resistance of *S. typhimurium* to polymyxin, JSG435 was mutagenized with the transposon Tn*10*d. JSG435 carries a mutant *pmrA* locus allele (*pmrA505*) that results in high-level polymyxin resistance, probably due to constitutive expression of PmrA–PmrB-activated genes. Transposon mutants were replica plated on LB plates with or without 12 μg ml^{-1} polymyxin. From plating of 20 000 total colonies from three separate Tn*10*d mutagenesis experiments, three PM^s clones were obtained. Southern hybridization experiments using a Tn*10*d-specific probe showed that two of the isolates had identical fragment hybridization patterns (data not shown). Strains containing the two unique transposon insertions exhibiting a PM^s phenotype were called JGS485 and JSG486. Using standard MIC testing, each of these mutants was shown to have a 63-fold reduction in MIC compared with the parental strain, JSG435 (Table 1).

Insertion of the Tn*10*d in the *pmrA-pmrB* (93.5 centisomes) or *phoP-phoQ* (25 centisomes) loci could have resulted in a PM^s phenotype. However, chromosomal mapping of the transposon insertions of the two PM^s strains showed them to be located at 50–52 centisomes (JSG485) and 43–45 centisomes (JSG486), thus eliminating the possibility that mutations in these regulatory loci were responsible for the observed phenotypic change. Therefore, two unlinked loci, designated *pmrE* and *pmrF*, were identified as necessary for resistance of *S. typhimurium* to PM.

Aminoarabinose is missing from the lipid A of pmrE *and* pmrF *mutants*

Because modification of LPS, and specifically aminoarabinose addition to the 4′ phosphate of lipid A, is associated with PM^r in Gram-negative bacteria (Helander *et al*., 1994), we examined JSG485 and JSG486 for the presence of aminoarabinose by mass spectrometry (MALDI-TOF). Mass spectra of lipid A from *S. typhimurium* grown in LB show two major structures: a hexa- or hepta-acylated form (*m/z* 1798 or 2036 respectively) (Guo *et al*., 1997). As seen in Fig. 1, the two peaks in the parental strain, JSG435 that correspond to hexa- or hepta-acyl forms of lipid A with aminoarabinose (which adds 131 mass units to the base hexa- or hepta-acylated lipid A to make the ions observed at *m/z*, 1929 and 2167 respectively), are both absent in JSG485 and JSG486. Therefore, *pmrE* and *pmrF* (or downstream genes if located in an operon) encode proteins necessary for the production of aminoarabinose or the addition of aminoarabinose to lipid A. These data provide further support for the hypothesis that aminoarabinose is necessary for resistance of *Salmonella* to PM and not simply

Table 1. Activity (MIC) of polymyxin against *S. typhimurium* strains.

Strain	Plasmid	MIC[a]
14028s (WT)	NA[b]	1.9
JSG435 (PmrA[c])	NA	6.3
JSG421 (PmrA$^-$)	NA	0.1
JSG485	NA	0.1
JSG486	NA	0.1
JSG485	pKK011	6.3
14028s	pKK011	3.1
JSG421′	pKK011	0.1
JSG486	pKK011	0.1
JSG485	pKK012	0.1
JSG485	pKK013	6.3
JSG486	pJG02	4.4
14028s	pJG02	3.1
JSG485	pJG02	0.1
JSG421	pJG02	0.1

a. MIC, minimum inhibitory concentration in μg ml^{-1}.
b. NA, not applicable (no plasmid is present in these strains).

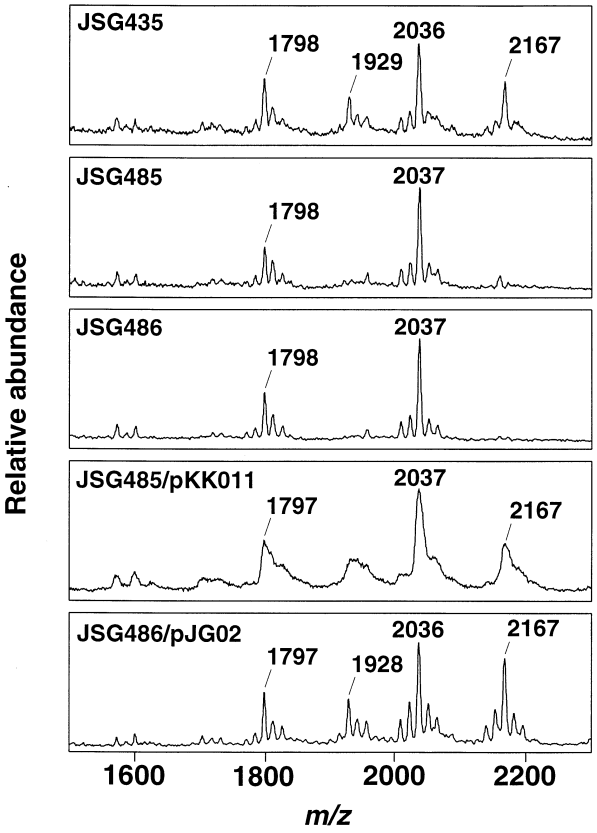

Fig. 1. MALDI-TOF mass spectra of lipid A from various strains. In the parental strain (JSG435), in addition to the peaks representing hexa- (*m/z* 1798) and hepta- (*m/z* 2036) acyl forms of lipid A, the corresponding aminoarabinose-modified lipid A forms were also present (*m/z*, 1929 and *m/z* 2167). In the *pmrF1*::Tn*10*d and *pmrE1*::Tn*10*d JSG435 mutant strains (JSG485 and JSG486 respectively), the aminoarabinose modifications were missing. However, the modifications were restored upon complementation with plasmids containing the respective wild-type loci (JSG485/pKK011 and JSG486/pJG02). All mass spectra were internally calibrated and assigned masses to the nearest whole mass unit. The poor resolution of the JSG485/pKK011 spectrum was probably caused by incomplete removal of detergent from the sample.

an associated finding related to regulatory mutations that promote polymyxin resistance.

Cloning of the loci defined by JSG485 and JSG486 transposon insertions

To perform complementation analysis and to determine if the defined loci were regulated by PmrA–PmrB, it was necessary to clone the DNA from wild-type *S. typhimurium* corresponding to locations of the Tn*10*d insertions. Limited DNA sequence (≈200 bp) adjacent to the *pmrF* transposon insertion was obtained from a clone containing the Tn*10*d and flanking DNA. This clone was identified by construction of a *Pst*I recombinant DNA library from JSG485 and selection for the Tn*10*d encoded tetracycline resistance. A PCR fragment was generated specific to the DNA directly 5′ to the Tn*10*d using primers derived from the DNA sequence. This PCR fragment was labelled and used as a probe to identify a hybridizing clone from a 14028s (wild type) *Pst*I gene bank (pKK011). This clone complemented the defect in PM^r of JSG485 (Table 1) and resulted in the restoration of the *m/z* 1929 and 2167 peaks, and, therefore, aminoarabinose to lipid A (Fig. 1). The addition of aminoarabinose to the lipid A from JSG485 complemented with pKK011 was further confirmed by a CAD (collision-activated dissociation) experiment using electrospray ionization with a triple quadropole mass spectrometer. The presence of pKK011 also slightly increased the MIC of wild type (14028s) to PM, but did not result in an increase in PM^r of JSG486 or a PmrA-null strain (Table 1).

To further define the gene(s) present on pKK011 necessary for resistance to PM, plasmids pKK012 and pKK013 were constructed using the restriction enzymes *Hin*dIII (located approximately in the centre of the insert) and *Pst*I (Fig. 2). Plasmid pKK013 complemented the PM^s phenotype of JSG485 to the same level as pKK011, whereas the presence of pKK012 (insert in either orientation with respect to *lacZ*) provided no increase in resistance to PM. This localized DNA essential to PM^r to a 7 kb region between the *Pst*I and *Hin*dIII sites (Fig. 2). The location of the *pmrF* locus was further defined within this 7 kb region by the fact that a *Cla*I site was found in the DNA sequence adjacent to the Tn*10*d, and only one *Cla*I site exists in the pKK013 insert. Therefore, *pmrF* probably overlaps this *Cla*I site (see Fig. 1).

Surprisingly, although introduction of pKK013 complemented the PM^s phenotype of the JSG485, the aminoarabinose modification was not reacquired (data not shown). An alternate lipid A structure is present in these strains that may contain unconventional ethanolamine substitutions (data not shown). These data may suggest that aminoarabinose is not absolutely essential for resistance to PM under conditions of multicopy expression of genes associated with the *pmrF* locus, and that other LPS modifications can also promote and/or contribute to resistance. These data also suggests the possibility that this locus could contain genes encoding proteins necessary for synthesis (or addition to LPS) of ethanolamine as well as aminoarabinose.

A clone containing *pmrE* (defined by the JSG486 Tn*10*d insertion) was identified by complementation. A *S. typhimurium* gene bank was transformed into JSG486, and plasmid-containing colonies were selected on LB ampicillin plates. Transformants were pooled and plated onto LB polymyxin (12 μg ml^{-1}) plates, and several PM^r colonies were identified. Six colonies were selected and found to contain two different recombinant plasmids. Using Southern blot analysis, these two plasmids (pJG01 and pJG02) contained overlapping DNA (see Fig. 1) and contained

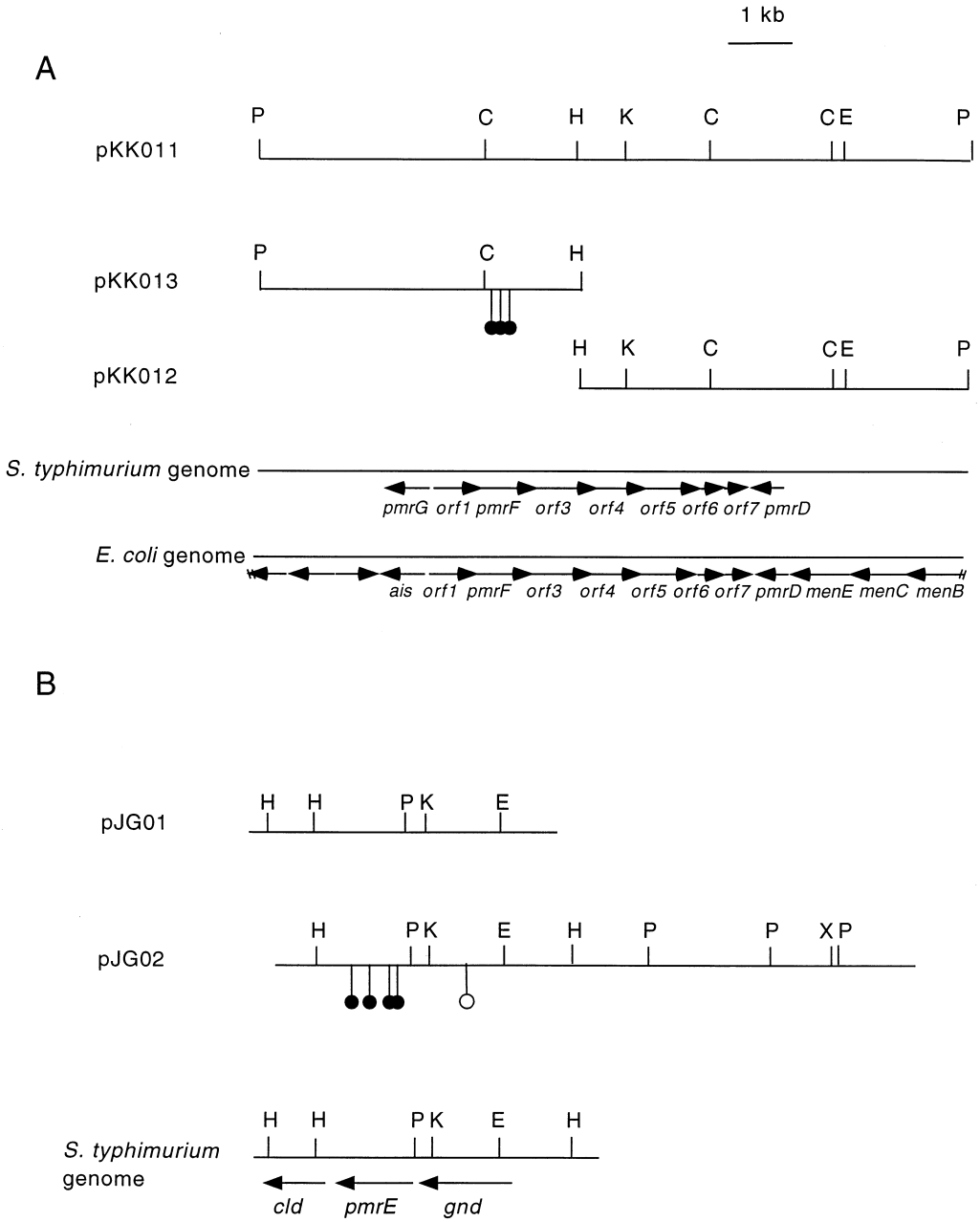

Fig. 2. Restriction maps of clones containing loci necessary for polymyxin resistance and for the addition of aminoarabinose to lipid A.
A. Restriction map of the chromosomal insert in pKK011 and two *Pst*I–*Hin*dIII subclones of this plasmid (pKK012 and pKK013). Solid circles show the location of Mu*d*J insertions that eliminate the ability of pKK013 to complement the polymyxin-sensitive phenotype of JSG485. Arrows represent open reading frames and show the direction of transcription. The names of the genes that have been defined, or that we have defined in the *S. typhimurium* or *E. coli* regions, are shown below the maps. The *S. typhimurium* genomic region sequenced corresponds to that between *pmrG* and *pmrD*.
B. Restriction maps of the chromosomal inserts in pJG01 and pJG02 and comparison of the maps of these clones to that of the chromosomal DNA to which they are homologous. Solid circles show the location of Mu*d*J insertions that eliminate the ability of this clone to complement the polymyxin-sensitive phenotype of JSG486. The white circle is an insertion not affecting the ability of this clone to complement the polymyxin sensitive phenotype of JSG486. The names of the genes that have been defined, or that we have defined in this region, are shown below the map. Restriction sites: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; X, *Xho*I.

DNA adjacent to the *pmrE1*::Tn*10*d (data not shown). Mass spectral analysis of lipid A from JSG486 containing pJG02 showed the reacquisition of those peaks containing the aminoarabinose modification (Fig. 1). pJG02 was unable to complement JSG485 or a PmrA-null strain, but as with pKK011 could increase the level of PM^r in 14028s (wild type) (Table 1).

pKK013 and pJG02 contain PmrA–PmrB-regulated genes

To determine if the identified genes were regulated by PmrA–PmrB and the direction of transcription of these genes, pKK013 and pJG02 were mutagenized with Mu*d*J, a transposon containing a promoterless *lacZ* gene at the 5′ end. A transcriptional fusion to β-galactosidase is created upon insertion into a transcribed region in the correct orientation. JSG485 (with pKK013) and JSG486 (with pJG02) were mutagenized with Mu*d*J, and transposon mutants were selected on agar plates. Approximately 40 000 transposon mutants (from four independent transductions) were pooled, and plasmid DNA isolated from this pool was used to retransform either JSG485 or JSG486. Transformants containing both the plasmid (ampicillin) and transposon (kanamycin) markers were selected on agar plates also containing the chromogenic substrate for β-galactosidase, X-Gal, so that blue or white colonies could be identified. Colonies were replica plated onto plates containing polymyxin to identify those plasmid Mu*d*J insertions that disrupted a gene necessary for resistance to PM.

A colony was selected for each mutated plasmid with a β-galactosidase-positive, PM^S phenotype, which must contain a correctly oriented Mu*d*J insertion in a gene or operon necessary for PM^r. Strains phenotypically PmrA-constitutive (JSG435), $PmrA^-$ (JSG421), PhoP-constitutive (CS022) and $PhoP^-$ (CS015) were transformed with these plasmids (pKK0135 and pJG0251) and assayed for β-galactosidase activity. Both fusions were highly regulated by PmrA–PmrB, with the pKK0135 fusion exhibiting a 464-fold, and the pJG0251 fusion a 58-fold increase in expression between JSG435 and JSG421 (Fig. 3). Not unexpectedly, both pKK0135 and pJG0251 fusions were also regulated by PhoP–PhoQ as PhoP–PhoQ can activate the transcription of *pmrA–pmrB* to result in LPS modifications and resistance to PM. However, the levels of regulation were reduced from that observed with PmrA–PmrB directly (41- and 15-fold respectively). In addition, little expression was observed in a $PhoP^c$ strain carrying a PmrA-null mutation, whereas a $PmrA^c$ strain carrying a PhoP-null mutation showed high-level expression similar to that of a $PmrA^c$ strain (Fig. 3). β-Galactosidase positive insertions not affecting the ability of the plasmid to complement the PM^S phenotype were used as controls. Expression of the control fusions did not vary when these plasmids were examined in the different *pmrA* and *phoP* mutant strains used in this experiment (data not shown). Collectively, these data demonstrate that these two loci are regulated by PmrA–PmrB and by PhoP–PhoQ through its regulation of PmrA–PmrB.

DNA sequence analysis of pmrE *and* pmrF*::Mu*d*J insertions*

To characterize further the PmrA–PmrB-regulated genes defined by transposon mutagenesis, the DNA sequence (≈100–200 bp) adjacent (5′) to the Mu*d*J insertions was determined with a primer specific for the left end of Mu*d*J. From this DNA sequence, the locations of several of the Mu*d*J transposon insertions in pKK013 ($n = 3$) and pJG02 ($n = 5$) that resulted in the various observed phenotypic classes (β-galactosidase positive, PM^S; β-galactosidase negative, PM^S; β-galactosidase positive, PM^r; and β-galactosidase negative, PM^r) were determined. All of the examined transposon insertions in each plasmid (β-galactosidase negative or β-galactosidase positive) that eliminated resistance to PM were found to be clustered within 1 kb, with most sequences showing some overlap (Fig. 2). Sequences adjacent to those transposon insertions not affecting PM resistance were unique and did not overlap

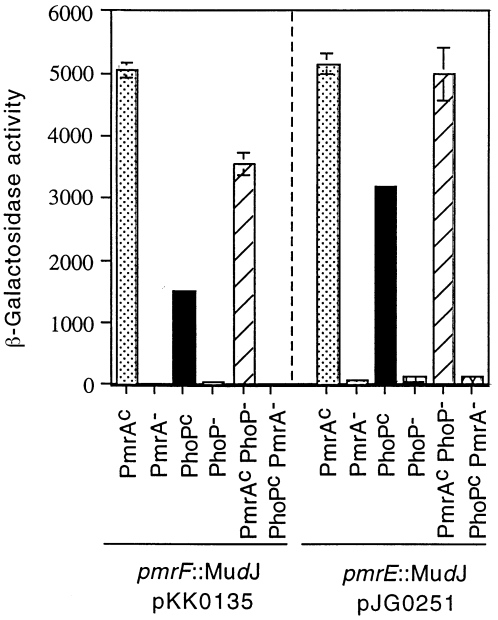

Fig. 3. Examination of PmrA–PmrB and PhoP–PhoQ regulation of *pmrE* and *pmrF*. Mu*d*J insertions in *pmrF* and *pmrE* (plasmids pKK0135 and pJG0251 respectively) producing active β-galactosidase fusions were examined in various PmrA–PmrB and PhoP–PhoQ mutant backgrounds. Both fusions were highly regulated by both two-component systems, but expression of each required PmrA–PmrB, suggesting that these loci were regulated directly by PmrA–PmrB.

the sequences of the insertions affecting the PM phenotype (data not shown).

PmrE *is predicted to be a UDP-glucose dehydrogenase*

The predicted translations of the sequences derived from the Mu*d*J insertions in pJG02 showed significant similarity to protein sequences in the database. The insertions in pJG02 that affected the PM phenotype were located in *pagA*, a PmrA–PmrB-activated gene originally identified as an acid-regulated and PhoP–PhoQ-activated gene (Miller *et al*., 1989; Soncini and Groisman, 1996; Valdivia and Falkow, 1996). We propose that this gene be renamed *pmrE*. *pmrE* encodes a putative UDP glucose dehydrogenase. The map position of *pmrE* (44.9 cs) agrees with the map position determined for the Tn*10*d insertion in JSG486 (43–45 cs).

A β-galactosidase-positive Mu*d*J insertion not affecting the ability of pJG02 to complement JSG486 to PM^r was located in the gene upstream of *pmrE*, *gnd*, a 6-phosphogluconate dehydrogenase. The gene downstream of *pmrE* is *cld*, which is involved in determining the LPS oligosaccharide chain length. To determine if *cld* was co-transcribed with *pmrE*, an internal fragment of the *cld* gene was amplified by PCR and cloned into pGPL01 (Gunn *et al*., 1996), a suicide vector used to construct chromosomal, single-copy transcriptional fusions to the firefly luciferase gene. This fusion was recombined into strains JSG421 ($PmrA^-$), JSG435 ($PmrA^c$), CS015 ($PhoP^-$), and CS022 ($PhoP^c$) by P22-mediated transduction, and these strains were grown and assayed. No differences were observed in the amount of luciferase activity between the strains (data not shown). Therefore, the *cld* gene is not regulated by PmrA–PmrB or PhoP–PhoQ. These data demonstrate that *pmrE* is transcribed as a individual, PmrA–PmrB-activated gene.

PmrF *is encoded within a putative multicistronic operon*

The sequence from the Mu*d*J insertions in pKK013 that affected the PM phenotype were found to be similar to the second gene of an uncharacterized *E. coli* chromosomal region predicted to be a six or seven gene operon (Sharma *et al*., 1996; Blattner *et al*., 1997). The sequence derived from the original clone containing the JSG485 Tn*10*d, and flanking DNA indicated that this insertion was also located in this gene. The DNA sequence of the corresponding *S. typhimurium* region was derived from plasmids pKK012 and pKK013. The region sequenced is 71% identical (DNA) to the corresponding *E. coli* region with no insertions or deletions. Our sequence analysis predicts this region to contain seven genes transcribed unidirectionally with no more than 5 bp separating any two ORFs (open reading frame) (0 bp separate most). All ORFs are preceded by a region with similarity to the consensus ribosome-binding site. The genes of this putative operon (orf1, *pmrF*, orf3-orf7 respectively) are predicted to encode proteins of 42 kDa (385 aa), 36.5 kDa (327 aa), 73.6 kDa (660 aa), 33 kDa (299 aa), 62 kDa (548 aa), 12 kDa (111 aa) and 13 kDa (146 aa). Orf1 is similar to several aminotransferases, perosamine (4-amino-4,6-dideoxy-D-mannose) synthetases, and carbohydrate biosynthetic proteins (highest score: 41% identical, 59% similar to a *Bacillus* spore coat polysaccharide biosynthesis protein [P39623]). Orf2 (PmrF) is most similar to dolicol-phosphate mannosyltransferases (23% identical, 45% similar, 12 gaps). Orf3 is similar to methionyl-tRNA formyltransferases (highest score: 29% identical, 50% similar over a 274 aa stretch, Bacillus {Y13937}) and to carbohydrate biosynthetic enzymes (23% identical, 41% similar with numerous gaps to a putative Methanococcus UDP glucose 4-epimerase {Q57664}). The remaining ORFs contain no significant overall similarities to proteins in the database.

Immediately downstream and also divergently transcribed from this putative operon is *pmrD*, which has been mapped in both *E. coli* and *S. typhimurium*. The presence of the *pmrD* gene on a high-copy plasmid increases the resistance of *S. typhimurium* to PM (Roland *et al*., 1994). The map position of *pmrD* (50.1 cs) in *S. typhimurium* agrees with the map position determined for the Tn*10*d insertion in JSG486 (50–52 cs).

From comparison of the DNA sequence of this chromosomal region to that of previously identified PhoP-activated genes, *pagH* is located immediately 5′ to and divergently transcribed from this proposed operon. Because the *pagH* gene was physically linked with this putative PmrA–PmrB-regulated operon, we assayed a strain carrying a *pagH*1::T*nphoA* fusion (Belden and Miller, 1994) to determine if this gene was regulated by PmrA–PmrB. Genes encoding the PmrA–PmrB two-component system are transcriptionally activated by PhoP–PhoQ, therefore several PhoP-activated genes have been found to be directly regulated by PmrA–PmrB instead of PhoP–PhoQ. As shown in Fig. 4, *pagH*–alkaline phosphatase fusion activity showed a 12-fold decrease in a $PmrA^-$ background compared with a $PmrA^C$ background. In addition, a similar decrease in expression was observed in a $PhoP^CPmrA$-null background when compared with a $PmrA^CPhoP$-null background. Therefore, this data demonstrated that this gene (which we have renamed *pmrG*) is not regulated directly by PhoP–PhoQ but by PmrA–PmrB.

Discussion

The PmrA–PmrB regulon is necessary for resistance to several antimicrobial compounds including polymyxin (Vaara, 1981a,b; Shafer *et al*., 1984; Spitznagel, 1990;

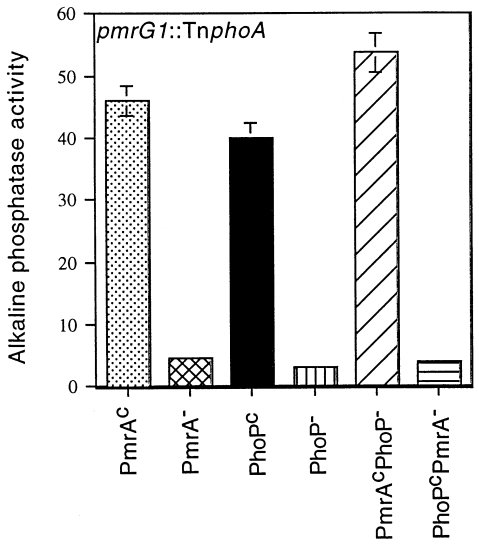

Fig. 4. Examination of PmrA–PmrB regulation of *pagH* (*pmrG*). Alkaline phosphatase activity of strains containing a *pagH1*::Tn*phoA* fusion were examined in various PhoP and PmrA mutant strain backgrounds. The results demonstrate that this fusion is not regulated directly by PhoP-PhoQ, but by PhoP–PhoQ through PmrA–PmrB.

Roland *et al*., 1993). Transcription of *pmrA-pmrB* is activated by PhoP–PhoQ (Gunn and Miller, 1996; Soncini and Groisman, 1996), and this cascade of two-component regulatory system expression can activate genes of the PmrA–PmrB regulon to result in resistance to PM. Alternatively, PmrA–PmrB can be activated independent of PhoP–PhoQ by growth in mild acidic conditions (Soncini and Groisman, 1996). The products of PmrA–PmrB-activated genes are responsible for LPS modifications (ethanolamine and aminoarabinose), which are thought to mask core and lipid A negative charges and reduce the affinity of the positively charged AP to the cell surface.

We have identified two *S. typhimurium* loci that are necessary for resistance to PM by transposon mutagenesis of a PmrA^c strain (which exhibits high level PM^r). These loci map to different chromosomal regions that are distinct from those of the regulators PhoP–PhoQ and PmrA–PmrB. The DNA defined by these insertions was cloned from ATCC14028s (pKK011 from JSG485 and pJG02 from JSG486), and each locus was able to complement the PM^s phenotype of its corresponding strain. These plasmids were not able to cross-complement (pKK011 could not complement JSG486 and pJG02 could not complement JSG485), nor were they able to complement a PmrA-null strain. Therefore, it is unlikely that these loci produce proteins of similar function and do not alone encode products sufficient to provide resistance to PM. These plasmids could, however, increase the MIC of wild-type *S. typhimurium* to PM, probably due to increased copy number of the genes involved in the PM^r phenotype.

The complementing plasmids were mutagenized by Mu*dJ*, and a subset of insertions abolished the ability of the plasmid to complement the PM^s phenotype. Mu*dJ* fusions (those affecting or not affecting the ability of the plasmid to complement the PM^s defect, and those producing or not producing an active β-galactosidase fusion) were used to assay these loci for PmrA–PmrB and PhoP–PhoQ regulation and as a template to sequence the DNA adjacent to the site of the Mu*dJ* insertions. Both loci were highly regulated by PmrA–PmrB and PhoP–PhoQ (although to a lesser extent than PmrA–PmrB), but expression of both genes required PmrA–PmrB.

Analysis of the DNA sequence extending from the transposon insertions that affected resistance to PM in pJG02 showed the insertions to be located in *pagA* (now called *pmrE*), a previously identified acid-regulated PhoP–PhoQ and PmrA–PmrB-activated gene predicted to be a UDP glucose dehydrogenase (Miller *et al*., 1989; Soncini and Groisman, 1996; Valdivia and Falkow, 1996). UDP glucose dehydrogenases in other organisms such as *Streptococcus pneumoniae*, *Vibrio cholerae* and *Shigella flexneri* are involved in capsule or complex carbohydrate synthesis (Morona *et al*., 1994; Dillard *et al*., 1995; Comstock *et al*., 1996). Mass spectrometry of a *S. typhimurium pmrE* mutant showed that this strain was unable to add aminoarabinose to LPS, and that the inability to add this modification could be complemented with the plasmid carrying the wild-type gene. It is likely that this enzyme is involved in the production of aminoarabinose as UDP glucose dehydrogenase catalyses the formation of UDP glucuronic acid, which is a precursor of UDP arabinose.

Transcription of *pmrE* is activated during growth in conditions of mild acidic pH (≈5.0), that is independent of PhoP–PhoQ and is therefore probably a direct signal affecting PmrA–PmrB activation (Soncini and Groisman, 1996). Indeed, a limited number of PmrA–PmrB-regulated fusions have been identified, and activation in the presence of mild acidic conditions appears common to all (Soncini and Groisman, 1996; J. S. Gunn, unpublished). The gene located upstream and downstream of *pmrE* are *gnd* (6-phosphogluconate dehydrogenase) and *cld* (chain length determinant) respectively. Data presented here show that neither gene is regulated by PmrA–PmrB, so under the conditions examined, *pmrE* is transcribed as an individual unit.

Analysis of the DNA sequence extending from *pmrF1*::Tn*10*d, as well as complementing DNA clones, showed the insertion to be located in the second gene of what appears to be a seven-gene operon. The gene arrangement in this region suggested that it may comprise an operon as each of the genes are separated by no more than 5 bp. The predicted products of three of the genes within this putative operon, one of which is similar to *pmrF*, show similarity to glycosyl- and aminotransferases

from a variety of organisms. These similarities include those to spore coat polysaccharide biosynthetic genes, perosamine (4-amino-4,6-dideoxy-D-mannose) synthetases and dolichol-phosphate mannosyltransferases. Therefore, the *S. typhimurium* homologues of these genes may encode sugar transferases or enzymes involved in sugar biosynthesis that are necessary for the production of aminoarabinose and the addition of aminoarabinose to the lipid A 4′ phosphate.

The *S. typhimurium* and *E. coli* putative operons containing *pmrF* are flanked at the 5′ end by a the homologous genes *ais* (*E. coli*) and *pmrG* (*S. typhimurium*). *pmrG* (previously known as *pagH*) was shown here to be regulated by PmrA–PmrB. Located at the 3′ end of this putative operon in both *S. typhimurium* and *E. coli* is *pmrD* (Roland *et al*., 1994). Expression of *pmrD* from a multicopy plasmid results in polymyxin resistance, but it is unclear if this gene is regulated by PmrA–PmrB. Therefore, this chromosomal region contains numerous known and putative PmrA–PmrB-regulated genes and may be a region of antimicrobial peptide-resistance genes common to many enterobacteriaciae.

Modification of the 4′ phosphate of Gram-negative LPS appears to be necessary for polymyxin resistance. In support of this, *Proteus mirabilis*, *Chromobacteruim violaceum* and *Burkholderia* (*Pseudomonas*) *cepacia* are resistant to PM and are nearly completely substituted at the 4′ phosphate of lipid A with aminoarabinose (Hase and Rietschel, 1977; Sidorczyk *et al*., 1983; Cox and Wilkinson, 1991; Vaara, 1992). In addition, *Bacteroides fragilis* lacks the 4′ phosphate and is polymyxin resistant (Weintraub *et al*., 1989). We have presented data here that *S. typhimurium* strains lacking the aminoarabinose modification are susceptible to PM. These data, however, do not eliminate the possibility that other modifications, such as ethanolamine modifications in the core region, are also affected by the transposon insertions in JSG485 and JSG486 and are important for resistance to polymyxin. However, given the probable function of PmrE as a UDP glucose dehydrogenase, it seems unlikely that this enzyme is involved in the addition of the two-carbon ethanolamine moiety to the LPS core.

Despite the weight of evidence in support of the hypothesis that aminoarabinose modification of lipid A is the main mechanism of resistance to PM, our own data show that a plasmid (pKK013) containing part of the putative operon was able to complement the PM^S defect of JSG485 without the reacquisition of aminoarabinose on lipid A. Although there are several possible explanations for this result, one possibility is that aminoarabinose addition to lipid A is not the only mechanism for promoting resistance to PM. Interestingly, the lipid A of this strain contains ions consistent with ethanolamine modification. A detailed analysis of the core region of *pmrF* locus mutants will be necessary to conclude that the aminoarabinose addition to the 4′ phosphate of lipid A is necessary and/or sufficient for resistance to PM, or if other modifications participate in resistance to PM.

S. typhimurium LPS, and LPS of other Gram-negative organisms, is highly modified in response to environmental conditions including those that occur *in vivo* (Guo *et al*., 1997; L. Guo and S. Miller, unpublished). These intracellular LPS modifications probably promote both resistance to cationic antimicrobial peptides and assist bacterial survival by lowering cytokine and chemokine production. Although the PmrA–PmrB dependent modifications do not appear to be those that affect cytokine production (Guo *et al*., 1997), it is probable that the PmrA–PmrB regulon plays an important role in salmonellae pathogenesis by providing resistance to AP within macrophages or at other anatomic sites encountered during infection.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are described in Table 2. Cultures were grown at 37°C with aeration in Luria broth (Miller, 1972). Antibiotics were used at the following concentrations: kanamycin, 45 μg ml^{-1}; tetracycline, 8–25 μg ml^{-1}; and ampicillin, 50 μg ml^{-1}. The chromogenic substrate for β-galactosidase, X-Gal, was used in LB agar plates at a concentration of 40 μg ml^{-1}. The plasmid library used to identify pJG01 and pJG02 was kindly provided by Dr Jim Slauch, University of Illinois, Champagne-Urbana. This library contains ≈8 kb *Sau*3AI fragments of *S. typhimurium* ATCC 14028s cloned into the low copy vector, pWSK30 (Fu and Kushner, 1991).

Bacterial genetics and enzyme assays

Southern blotting and detection of hybridization was accomplished as previously described (Gunn and Miller, 1996). P22HT*int* bacteriophage was used in all transductional crosses (Chan *et al*., 1972; Davis *et al*., 1980), and all strains resulting from transductional crosses were routinely confirmed to be non-lysogens of P22. Mapping of the Tn*10*d insertions was accomplished by hybridization of labelled probes to blots of digested DNA separated by pulsed-field gel electrophoresis, or by a combination of Bochner selection and phage transduction as described by Benson and Goldman (1992). A brief explanation of the latter mapping technique is as follows. Approximately 10^8 CFU of JSG485 or JSG486 were spread on the surface of a Bochner plate. A set Mud-P22 phage was spotted onto the surface of these plates. These phages exist as prophages inserted at random locations along the genome, but when induced generate transducing lysates that contain bacterial DNA flanking one side of the insertion. Because cells containing the Tn*10*d transposon expressing tetracycline resistance are unable to grow on these plates, those phage spots containing numerous colonies after overnight growth are a result of recombination

Table 2. Strains and plasmids.

Strain	Description	Reference
JSG421	pmrA::Tn*10*d	Gunn and Miller (1996)
JSG435	pmrA505 zjd::Tn*10*d-cam (PmrA-constitutive)	Gunn and Miller (1996)
JSG485	JSG435 *pmrF1*::Tn*10*d	This work
JSG486	JSG435 *pmrE1*::Tn*10*d	This work
JSG158	*pmrG1*::Tn*phoA* (previously *pagH1*::Tn*phoA*)	Belden and Miller (1994)
CS015	*pho*P*102*::Tn*10*d-cam	Miller *et al*. (1989)
CS022	*pho*-24 (PhoP-constitutive)	Miller and Mekalanos (1990)
14028s	Wild type	ATCC
TT*10*288	Mu*dJ* donor strain	Hughes and Roth (1988)
TT*10*604	Tn*10*d donor strain	Elliott and Roth (1988)
DH5a	F^- Ø 80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169*endA*1*recA*1*hsdR*17deoR *thi*-1*supE*44l$^-$*gyrA*96*rel*A1	BRL
Plasmids		
pGPL01	Firefly luciferase recorder, suicide vector	Gunn *et al*. (1996)
pCLD1	pGPL01 containing a fragment internal to *cld*	This work
pNK272	Tn*10*d transposase donor	Elliott and Roth (1988)
pJK01	JSG485 ≈16 kb *Pst*I fragment containing Tn*10*d and flanking DNA	This work
pKK011	14028s ≈13 kb *Pst*I fragment containing the wild-type DNA region defined by th Tn*10*d in JSG485	This work
pKK012	*Pst*I–*Hin*dIII subclone of pKK011	This work
pKK013	*Pst*I–*Hin*dIII subclone of pKK011	This work
pJG01	≈5.0 kb *Sau*3AI partial fragment containing the wild-type DNA region defined by the Tn*10*d in JSG486	This work
pJG02	≈9.0 kb *Sau*3AI partial fragment containing the wild-type DNA region defined by the Tn*10*d in JSG486	This work
pKK0135	pKK013 Mu*dJ* insertion (*pmrF1*::Mu*dJ*)	This work
pKK0251	pJG02 Mu*dJ* insertion (*pmrE*1::Mu*dJ*)	This work
pWSK29	Low copy cloning vector	Fu and Kushner (1991)
pWSK30	Low copy cloning vector	Fu and Kushner (1991)
pBluescript II sk+	High copy cloning vector	Stratagene

between the phage DNA and the chromosomal DNA, which displaces the Tn*10*d. β-Galactosidase and alkaline phosphatase assays, reported as Miller units, and firefly luciferase assays of sonicated culture lysates were performed as previously described (Gunn and Miller, 1996).

Mutagenesis

Mutagenesis of JSG435 was accomplished with Tn*10*d (Elliott and Roth, 1988). Plasmid pNK972, the transposase donor, was transformed into JSG435. This strain was infected with P22 phage propagated on TT10604, the Tn*10*d donor. After mutagenesis, ≈20 000 tetracycline-resistant transductants were picked and replica plated onto LB plates or LB plates with polymyxin (12 μg ml^{-1}). Two unique PM^s transductants (JSG485 and JSG486) were identified. These strains were infected with P22 phage, and the resulting lysates were used to retransduce JSG435, which confirmed that the PM^s phenotype was linked to the transposon insertion.

Mu*dJ* mutagenesis of JSG485 with pKK013 and JSG486 with pJG02 was accomplished as follows. TT10288, the Mu*dJ* donor strain, was infected with P22 phage (at 30°C), and the resulting lysate was used to infect JSG485 and JSG486 carrying the complementing plasmids. Approximately 40 000 ampicillin (vector) and kanamycin (Mu*dJ*) colonies from four independent transductions were pooled for each strain infected, and plasmid DNA was isolated from the pooled colonies. JSG485 or JSG486 were transformed with their corresponding plasmid pools, and transformants were selected on agar plates containing ampicillin, kanamycin and the chromogenic substrate for β-galactosidase, X-Gal. Numerous blue and white colonies were identified and replica plated onto LB (ampicillin, kanamycin, X-Gal) or LB polymyxin plates. Colonies were identified in all expected phenotypic classes: β-galactosidase+, PM^r; β-galactosidase–, PM^r; β-galactosidase+, PM^s; β-galactosidase–, PM^s.

The *cld* firefly luciferase recorder fusion was generated as follows. Primers flanked by restriction sites (*Eco*RI and *Kpn*I) were constructed internal to the *cld* ORF (JG65, 5′-GGAATTCACGTCTTCCGGGCGTGGG-3′; JG66, 5′-GGG-GTACCAGCACAATCCCGGCACCG-3′). The PCR fragment generated from these primers was cloned into pGPL01 and

transformed into SM10λpir. After identification of a correct recombinant (pCLD1), this plasmid was mobilized into various *S. typhimurium* strains and single-colony chromosomal integrants were identified.

Cloning of pmrE *and* pmrF

Two different methodologies were used to clone the wild-type loci corresponding to the locations of the Tn*10*d insertions in JSG485 and JSG486. To identify the desired DNA defined by the JSG485 Tn*10*d insertion, a clone was obtained from a *Pst*I gene bank of JSG485 DNA. This enzyme does not cut within the Tn*10*d, therefore *E. coli* containing the *Pst*I fragment of interest was identified by selection on LB tetracycline plates. This DNA was confirmed to be adjacent to the Tn*10*d by Southern hybridization using a piece of chromosomal DNA from the Tn*10*d clone as a probe. Approximately 200 bp of sequence was generated from this clone using a primer located at the 5′ end of Tn*10*d. Using this sequence information, primers were made and a fragment was amplified by PCR specific to this DNA. *E. coli* containing a *S. typhimurium* 14028s *Pst*I gene bank in pWSK29 was probed with the labelled PCR fragment and a hybridizing colony was identified. The insert of this clone was ≈13 kb and the plasmid was called pKK011. Subclones pKK012 and pKK013 both are pWSK29 containing a *Hin*dIII–*Pst*I fragment of the pKK011 insert. pKK013 was able to complement the PM^s phenotype of JSG485, whereas pKK012 was not.

The chromosomal region containing the desired wild-type DNA defined by the JSG486 Tn*10*d insertion was identified by complementation of the PM^s phenotype of JSG486. A *Sau*3AI *S. typhimurium* gene bank constructed in pWSK30 was transformed into JSG486 by electroporation. Transformants (≈8000) were pooled and various dilutions were plated onto LB polymyxin plates. Six of the resulting PM^r colonies were picked and the plasmids were retransformed into JSG486 to confirm that the PM^r phenotype was associated with the plasmid. Of these six, two clones (pJG01 and pJG02) having unique restriction banding patterns were identified. This restriction mapping in combination with Southern blot analysis demonstrated that these plasmids contained overlapping DNA.

LPS and lipid A isolation

LPS was isolated using Mg^{2+}–ethanol precipitation as described by Darveau and Hancock (1983), and lipid A by hydrolysis in 1% SDS at pH 4.5 (Caroff *et al*., 1988). Lipid A purity was assessed by GC analysis of fatty acid methyl esters (Darveau *et al*., 1995; Somerville *et al*., 1996).

DNA sequencing

DNA was sequenced from plasmids pKK012 and pKK013 using primer extension and fluorescent-labelled dye-terminator chemistry on an Applied Biosystems (Perkin-Elmer) model 373XL automated sequencer. Primers were synthesized using an Oligo 1000M multicolumn synthesizer (Beckman Instruments). Both DNA strands were sequenced. Each strand was sequenced a minimum of two times. DNA assembly and analysis was conducted on computer software SEQUENCHER (Gene Codes Corporation) and GCG (Genetics Computer Group). DNA sequencing was performed with the assistance of the Center for Advanced DNA Technologies in the Department of Microbiology at the University of Texas Health Science Center at San Antonio. The 8051 bp *S. typhimurium* sequence between *pmrG* and *pmrD* has been deposited in GenBank (no. AF036677).

Polymyxin-resistance assays

Polymyxin (US Biochemicals, 8040 U mg^{-1}) was used at concentrations of 0.05–12 μg ml^{-1} in both plates and broth assays. Standard minimal inhibitory concentration (MIC) testing of susceptibility to polymyxin was accomplished as described by Steinberg *et al*. (1997).

Mass spectrometry

MALDI experiments were conducted as follows. The sample preparation procedure was modified from the published Fast Evaporation Method (Vorm and Roepstorff, 1994). Three layers of matrix/sample were applied consecutively to the sample plate, allowing each layer to dry completely before applying the next one. The bottom layer contained 1 ml of saturated super DHB (5-methoxysalicylic acid/2,5-dihydroxybenzoic acid, 1:10 w/w) prepared in chloroform–methanol 3:1 (v/v) and 0.002% triethylamine. The middle layer was the lipid A sample (1 ml, ≈500 pmol) prepared in chloroform–methanol 3:1 (v/v). And for internal standard calibration purposes, the top layer contained a 1 ml mixture of 1 pmol of angiotensin and 8 pmol of insulin A prepared in saturated DHB and 50% acetonitrile.

Negative ion spectra were acquired from a delayed extraction matrix-assisted laser desorption ionization time-of-flight (DE-MALDI-TOF) mass spectrometer (PerSeptive Biosystems Voyager-Elite biospectrometry Workstation), using 25 KV accelerating voltage, 92% grid voltage, 0.1% guide wire voltage and 200 ns delay time between the firing of laser and ion extraction. A total of 100–150 scans were averaged for each acquisition.

For electrospray experiments, the lipid A sample (saturated solution, prepared in chloroform–methanol, 3:1 v/v) was introduced into an electrospray tandem quadrupole mass spectrometer (Finnigan TSQ 7000) by infusion at 300–500 nl min^{-1}. The spray voltage was 2500 V and the capillary temperature was 150°C. For MS^2 experiments, the collision-activated dissociation (CAD) analysis was obtained by first selecting the precursor ions in the first mass filter, fragmenting the precursor ions in the second mass filter (which contained argon gas at a pressure of 3 mT), with the product ions analysed in the third mass filter. The collision energy was 30–50 eV.

Acknowledgements

This work was supported by grant AI30479 from the National Institutes of Health to S.I.M., the School of Pharmacy and the Department of Medicinal Chemistry at the University of Washington (M.H.), and the Department of Microbiology at The University of Texas Health Science Center at San Antonio (J.S.G.).

References

Alpuche-Aranda, C.M., Swanson, J.A., Loomis, W. P., and Miller, S.I. (1992) *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. *Proc Natl Acad Sci USA* **89:** 10079–10083.

Belden, W.J., and Miller, S.I. (1994) Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. *Infect Immun* **62:** 5095–5101.

Benson, N.R., and Goldman, B.S. (1992) Rapid mapping in *S. typhimurium* with Mu*d*-P22 Prophages. *J Bacteriol* **174:** 1673–1681.

Bevins, C.L. (1994) Antimicrobial peptides as agents of mucosal immunity. *Ciba Found Symp* **186:** 250–260.

Blattner, F.R., Plunkett, G. III, Bloch, C.A., Perna, V., Burland, N.T., Riley, M., Collado-Vides, J. *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453–1462.

Boll, M., Radziejewska-Lebrecht, J., Warth, C., Krajewska-Pietrasik, D., and Mayer, H. (1994) 4-Amino-4-deoxy-L-arabinose in LPS of enterobacterial R-mutants and its possible role for their polymyxin reactivity. *FEMS Immunol Med Microbiol* **8:** 329–341.

Caroff, M., Tacken, A., and Szabo, L. (1988) Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the ''isolated lipid A'' fragment of the *Bordetella pertussis* endotoxin. *Carbohyd Res* **175:** 273–282.

Chan, R.K., Botstein, D., Watanabe, T., and Ogata, Y. (1972) Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high-frequency-transducing lysate. *Virology* **50:** 883–898.

Comstock, L.E., Johnson, J.A., Michalski, J.M., Morris, J.G.J., and Kaper, J.B. (1996) Cloning and sequence of a region encoding a surface polysaccharide of Vibrio cholerae O139 and characterization of the insertion site in the chromosome of Vibrio cholerae O1. *Mol Microbiol* **19:** 815–826.

Cox, A.D., and Wilkinson, S.G. (1991) Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. *Mol Microbiol* **5:** 641–646.

Darveau, R.P., Cunningham, M.D., Bailey, T., Seachord, C., Ratcliffe, K., Bainbridge, B., Dietsch, M., Page, R.C., and Aruffo, A. (1995) Ability of bacteria associated with chronic inflammatory disease to stimulate E-selectin expression and promote neutrophil adhesion. *Infect Immun.* **63:** 1311–1317.

Darveau, R.P., and Hancock, R.E. (1983) Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J Bacteriol* **155:** 831–838.

Davis, R.W., Botstein, D., and Roth, J.R. (1980) *Advanced Bacterial Genetics: A Manual for Genetic Engineering*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Diamond, G., Jones, D.E., and Bevins, C.L. (1993) Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene. *Proc Natl Acad Sci USA* **90:** 4596–4600.

Dillard, J.P., Vandersea, M.W., and Yother, J. (1995) Characterization of the cassette containing genes for type 3 capsular polysaccharide biosynthesis in Streptococcus pneumoniae. *J Exp Med* **181:** 973–983.

Elliott, T., and Roth, J.R. (1988) Characterization of Tn*10*d-Cam: a transposition-defective Tn*10* specifying chloramphenicol resistance. *Mol Gen Genet* **213:** 332–338.

Fields, P.I., Groisman, E.A., and Heffron, F. (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243:** 1059–1062.

Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83:** 5189–5193.

Fu, R., and Kushner, S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100:** 195–199.

Goldman, M.J., Anderson, G.M., Stolzenberg, E.D., Kari, U.P., Zasloff, M., and Wilson, J.M. (1997) Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88:** 553–560.

Groisman, E.A., Parra-Lopez, C., Salcedo, M., Lipps, C.J., and Heffron, F. (1992) Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci USA* **89:** 11939–11943.

Gunn, J.S., Hohmann, E.L., and Miller, S.I. (1996) Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation Results in increased net phosphotransfer to PhoP. *J Bacteriol* **178:** 6369–6373.

Gunn, J.S., and Miller, S.I. (1996) PhoP/PhoQ activates transcription of *pmrA/B*, encoding a two-component system involved in *Salmonella typhimurium* antimicrobial peptide resistance. *J Bacteriol* **178:** 6857–6864.

Guo, L., Lim, K., Gunn, J.S., Bainbridge, B., Darveau, R., Hackett, M., and Miller, S.I. (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* **276:** 250–253.

Hase, S., and Rietschel, E.T. (1977) The chemical structure of the lipid A component of the lipopolysaccharides from *Chromobacterium violaceum* NCTC 9694. *Eur J Biochem* **75:** 23–34.

Heithoff, D.M., Conner, C.P., Hanna, P.C., Julio, S.M., Hentschel, U., and Mahan, M.J. (1997) Bacterial infection as assessed by in vivo gene expression. *Proc Natl Acad Sci USA* **94:** 934–939.

Helander, I.M., Kato, Y., Kilpelainen, I., Kostiainen, R., Lindner, B., Nummila, K. *et al.* (1996) Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. *Eur J Biochem* **237:** 272–278.

Helander, I.M., Kilpelainen, I., and Vaara, M. (1994) Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a ^{31}P-NMR study. *Mol Microbiol* **11:** 481–487.

Hohmann, E.L., Oletta, C.A., Killeen, K.P., and Miller, S.I. (1996) *phoP*/*phoQ*-deleted *Salmonella typhi* (TY800) is a safe and immunogenic single dose typhoid fever vaccine in volunteers. *J Infect Dis* **173:** 1408–1414.

Hughes, K.T., and Roth, J.R. (1988) Transitory *cis* complementation: a method for providing transposon functions to defective transposons. *Genetics* **119:** 9–12.

Jones, D.E., and Bevins, C.L. (1992) Paneth cells of the

human small intestine express an antimicrobial peptide gene. *J Biol Chem* **267:** 23216–23225.
Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.
Miller, S.I., Kukral, A.M., and Mekalanos, J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* **86:** 5054–5058.
Miller, S.I., and Mekalanos, J.J. (1990) Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. *J Bacteriol* **172:** 2485–2490.
Miller, S.I., Pulkkinen, W.S., Selsted, M.E., and Mekalanos, J.J. (1990) Characterization of defensin resistance phenotypes associated with mutations in the *phoP* virulence regulon of *Salmonella typhimurium*. *Infect Immun* **58:** 3706–3710.
Morona, R., Mavris, M., Fallarino, A., and Manning, P.A. (1994) Characterization of the rfc region of Shigella flexneri. *J Bacteriol* **176:** 733–747.
Nummila, K., Kilpelainen, I., Zahringer, U., Vaara, M., and Helander, I.M. (1995) Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol Microbiol* **16:** 271–278.
Roland, K.L., Esther, C.R., and Spitznagel, J.K. (1994) Isolation and characterization of a gene, *pmrD*, from *Salmonella typhimurium* that confers resistance to polymyxin when expressed in multiple copies. *J Bacteriol* **176:** 3589–3597.
Roland, K.L., Martin, L.E., Esther, C.R., and Spitznagel, J.K. (1993) Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* **175:** 4154–4164.
Shafer, W.M., Martin, L.E., and Spitznagel, J.K. (1984) Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. *Infect Immun* **45:** 29–35.
Sharma, V., Hudspeth, M.E., and Meganathan, R. (1996) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the *menE* gene from *Escherichia coli*. *Gene* **168:** 43–48.
Sidorczyk, Z., Zahringer, U., and Reitschel, E.T. (1983) Chemical structure of the lipid A component of lipopolysaccharide from a *Proteus mirabilis* Re-mutant. *Eur J Biochem* **137:** 15–22.
Somerville, J.E.J., Cassiano, L., Bainbridge, B., Cunningham, M.D., and Darveau, R.P. (1996) A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J Clin Invest* **97:** 359–365.
Soncini, F.C., and Groisman, E.A. (1996) Two-component regulatory systems can interact to process multiple environmental signals. *J Bacteriol* **178:** 6796–6801.
Spitznagel, J.K. (1990) Antibiotic proteins of human neutrophils. *J Clin Invest* **86:** 1381–1386.
Steinberg, D.A., Hurst, M.A., Fujii, C.A., Kung, A.H., Ho, J.F., Cheng, F.C. *et al.* (1997) Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. *Antimicrob Agents Chemother* **41:** 1738–1742.
Stolzenberg, E.D., Anderson, G.M., Ackermann, M.R., Whitlock, R.H., and Zasloff, M. (1997) Epithelial antibiotic induced in states of disease. *Proc Natl Acad Sci USA* **94:** 8686–8690.
Vaara, M. (1981) Effect of ionic strength on polymyxin resistance of *pmrA* mutants of *Salmonella. FEMS Microbiol Lett* **11:** 321–326.
Vaara, M. (1981) Increased outer membrane resistance to ethylenediaminetetraacetate and cations in novel lipid A mutants. *J Bacteriol* **148:** 426–434.
Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56:** 395–411.
Vaara, M., Vaara, T., Jenson, M., Helander, I., Nurminen, M., Rietschel, E.T., and Makela, P.H. (1981) Characterization of the lipopolysacharride from the polyxyxin-resistant *pmrA* mutants of *Salmonella typhimurium*. *FEBS Lett* **129:** 145–149.
Valdivia, R.H., and Falkow, S. (1996) Bacterial genetics by flow cytometry: rapid isolation of Salmonella typhimurium acid-inducible promoters by differential fluorescence induction. *Mol Microbiol* **22:** 367–378.
Vorm, O., and Roepstorff, P. (1994) Peptide sequence information derived by partial acid hydrolysis and matrix-assisted laser desorption/ionization mass spectrometry. *Biol Mass Spectrom* **23:** 734–740.
Weintraub, A., Zahringer, U., Wollenweber, H.W., Seudel, U., and Rietschel, E.T. (1989) Structural characterization of the Lipid A component of Bacteroides fragilis strain NCTC 9343 lipopolysaccharide. *Eur J Biochem* **183:** 425–431.
Zasloff, M. (1992) Antibiotic peptides as mediators of innate immunity. *Curr Opin Immunol* **4:** 3–7.