Molecular Microbiology (2004) **53**(1), 229–241 doi:10.1111/j.1365-2958.2004.04107.x

PhoP-regulated *Salmonella* resistance to the antimicrobial peptides magainin 2 and polymyxin B

Yixin Shi,[1,2] Michael J. Cromie,[1] Fong-Fu Hsu,[3] John Turk[3] and Eduardo A. Groisman[1,2]*

[1]*Department of Molecular Microbiology,* [2]*Howard Hughes Medical Institute, and* [3]*Mass Spectrometry Resource, Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8230, St Louis, MO 63110, USA.*

Summary

In *Salmonella enterica*, the PhoP–PhoQ two-component system governs resistance to structurally different antimicrobial peptides including the alpha-helical magainin 2, the β-sheet defensins and the cyclic lipopeptide polymyxin B. To identify the PhoP-regulated determinants mediating peptide resistance, we prepared a plasmid library from a *phoP* mutant, introduced it into a *phoP* mutant and selected for magainin-resistant clones. One of the clones harboured the PhoP-activated *ugtL* gene, deletion of which rendered *Salmonella* susceptible to magainin 2 and polymyxin B, but not defensin HNP-1. We established that *ugtL* encodes an inner membrane protein that promotes the formation of monophosphorylated lipid A in the lipopolysaccharide. Inactivation of both *ugtL* and the regulatory gene *pmrA*, which controls lipid A modifications required for resistance to polymxyin B (but not to magainin 2) and is post-transcriptionally activated by the PhoP–PhoQ system, resulted in a strain that was as susceptible to polymyxin B as a *phoP* mutant. The most frequently recovered clone harboured the *yqjA* gene, which we show is PhoP regulated and required for resistance to magainin 2 but not to polymyxin B or defensin HNP-1. Our results indicate that different PhoP-mediated modifications in lipid A are necessary for resistance to different antimicrobial peptides.

Introduction

Cationic antimicrobial peptides have been isolated from phylogenetically diverse animal, plant and bacterial species (Zasloff, 1992). Based on features of their secondary structure, antimicrobial peptides have been classified into two major groups: one forming amphipathic alpha helixes and another adopting β-sheet structures that are cross-linked by disulphur bridges (Nicolas and Mor, 1995). Although antimicrobial peptide resistance has been correlated with virulence (Groisman *et al*., 1992a; Peschel, 2002; Poyart *et al*., 2003), the mechanisms used by pathogens to resist and/or to avoid antimicrobial peptide action are poorly understood (for reviews, see Ernst *et al*., 2001; Peschel, 2002).

The PhoP–PhoQ regulatory system is required for resistance to a variety of structurally different antimicrobial peptides in several Gram-negative species including *Salmonella enterica* (Groisman *et al*., 1992a), *Shigella flexneri* (Moss *et al*., 2000), *Escherichia coli* (Groisman *et al*., 1992b), *Erwinia chrysanthemi* (Llama-Palacios *et al*., 2003) and *Pseudomonas aeruginosa* (Ernst *et al*., 1999). *Salmonella phoP* mutants are hypersensitive to the alpha-helical peptides magainin 2 from frog skin (Groisman *et al*., 1992a) and cecropin A from the cecropia moth (Groisman *et al*., 1992a), to the β-sheet defensins from different mammalian species (Fields *et al*., 1989; Miller *et al*., 1990) and to the cyclic lipopeptide polymyxin B from the soil bacterium *Paenibacillus polymyxa* (McLeod and Spector, 1996; Wosten *et al*., 2000). Two PhoP-activated outer membrane proteins have been implicated in resistance to C18G, an alpha-helical peptide derived from the carboxy-terminus of platelet factor IV (Darveau *et al*., 1992): PagP, which mediates the palmytoylation of the lipid A in the lipopolysaccharide (Guo *et al*., 1998); and PgtE, a protease that can cleave C18G (Guina *et al*., 2000). Expression of PhoP-activated genes is induced in response to low Mg^{2+} sensed by the PhoQ protein (Garcia Vescovi *et al*., 1996).

Resistance to polymyxin B is primarily controlled by the PmrA–PmrB regulatory system (Roland *et al*., 1993), inactivation of which increases susceptibility several orders of magnitude (Groisman *et al*., 1997; Kox *et al*., 2000). This system is necessary for transcription of the *ugd* gene and *pbgP* operon (Groisman *et al*., 1997; the *pbgP* locus is referred to as *pmrF* by Gunn *et al*., 1998), which mediate the synthesis and incorporation of 4-aminoarabinose into lipid A (Gunn *et al*., 1998). Transcription of PmrA-activated genes is induced in low Mg^{2+} in a process that requires the PhoP and PhoQ proteins, the

Accepted 1 March, 2004. *For correspondence. E-mail groisman@borcim.wustl.edu; Tel. (+1) 314 362 3692; Fax (+1) 314 747 8228.

PhoP-activated *pmrD* gene as well as the PmrA and PmrB proteins (Kox *et al*., 2000). In addition, PmrA-activated genes are turned on in response to Fe^{3+} sensed by the PmrB protein in a PhoP-, PhoQ- and PmrD-independent fashion (Wosten *et al*., 2000). A *phoP* mutant is >20 times more polymyxin B sensitive than a *pmrA* mutant when bacteria are grown in low Mg^{2+} and >20 times more sensitive than wild-type *Salmonella* when bacteria are grown in the presence of Fe^{3+} (Wosten *et al*., 2000), indicating that a PhoP-regulated PmrA-independent gene(s) participate(s) in polymyxin B resistance. Candidate genes for this activity include *mig-14*, *virK* and *somA* because inactivation of these PhoP-activated genes results in strains displaying increased susceptibility to polymyxin B (Brodsky *et al*., 2002; Detweiler *et al*., 2003).

In this paper, we report the identification of PhoP-regulated loci mediating resistance to magainin 2 and polymyxin B. We establish that the PhoP-activated *ugtL* gene is required for resistance to magainin 2 and polymyxin B, and determine that it encodes an inner membrane protein needed for the modification of the lipid A. Moreover, we are able to recapitulate the susceptibility of the *phoP* mutant to magainin 2 and polymyxin B by constructing strains defective in different combinations of PhoP-regulated genes. Our findings indicate that diverse lipid A modifications are required for resistance to different antimicrobial peptides.

Results

A survival enrichment strategy for recovering peptide resistance genes

The *Salmonella phoP* mutant strain MS7953s exhibits 10^5 times lower survival than the isogenic wild-type parent when incubated for 90 min in LB broth containing 100 μg ml^{-1} magainin 2 (Garcia Vescovi *et al*., 1996). However, when *phoP* and wild-type *Salmonella* are streaked onto magainin 2-containing LB agar plates, >500 μg ml^{-1} magainin 2 is required to detect growth differences between the two strains. Because such a high concentration of magainin 2 made a genetic screening using plates cost prohibitive, we developed a survival enrichment selection using liquid medium. We determined that, when incubated overnight in the presence of magainin 2 (50 μg ml^{-1}), the OD_{600} of the culture of the *phoP* mutant was <0.05 (corresponding to <200 cfu ml^{-1}) whereas that of the wild-type strain was >1.2. Thus, we reasoned that it should be possible to recover resistant clones from a plasmid library by conducting serial incubations in the presence of magainin 2.

We focused on *Salmonella* genes conferring resistance to magainin 2 (Zasloff, 1987) because, first, this frog peptide has been widely used to investigate the mechanism of action of cationic alpha-helical peptides (Ludtke *et al*., 1996; Bechinger, 1997), secondly, a derivative of magainin 2 was the first antimicrobial peptide tested in human clinical trials (Zasloff, 2001) and, thirdly, *Salmonella* has been isolated from frogs (Sarvamangala Devi and Shivananda, 1983). Thus, understanding resistance to magainin 2 might illuminate how alpha-helical cationic peptides exert their microbicidal activities and how *Salmonella* colonizes non-mammalian hosts.

To identify PhoP-regulated genes mediating magainin 2 resistance, we prepared a genomic library from the *phoP* mutant strain MS7953s in the multicopy number plasmid pUC19 (Norrander *et al*., 1983), introduced the library into the same *phoP* mutant and exposed the transformants to magainin 2 (see *Experimental procedures*). This strategy was based on the premise that PhoP-regulated genes might be expressed from the *lac* promoter in pUC19 and confer magainin 2 resistance upon the *phoP* mutant. By preparing the library from a *phoP* mutant, we ensured that the recovered clones would not include a wild-type copy of the *phoP* gene, which would obviously complement the *phoP* mutant. The first round of selection resulted in a >1000-fold enrichment for magainin 2-resistant clones: there were 2300–2500 cfu in the wells corresponding to the *phoP* mutant harbouring the plasmid library and only 0–2 cfu in the wells corresponding to the *phoP* mutant harbouring the vector. After two additional enrichment rounds, we isolated plasmid DNA from 211 individual colonies and used it to retransform the original *phoP* mutant, selecting for ampicillin resistance and checking the magainin 2 susceptibility of the resulting transformants. Thirty-four out of the 211 plasmids retained the ability to confer magainin 2 resistance upon the *phoP* mutant (i.e. survival rates >5%) and were investigated further.

Molecular analysis of plasmid clones conferring magainin 2 resistance upon the phoP *mutant*

Sequence analysis of the 34 plasmids conferring magainin 2 resistance revealed the presence of DNA originating from 10 regions in the *Salmonella* chromosome (Fig. 1). Three of the plasmids harboured two complete *Salmonella*-specific open reading frames (ORFs): *ugtL*, a PhoP-activated gene of unknown function (Hilbert *et al*., 1999); and *STM1600*, an uncharacterized ORF. Fifteen of the 34 plasmids carried the *yqjA* and *yqjB* genes, also of unknown function. One additional clone harboured two complete ORFs: STM0725 and STM0726, which exhibit sequence similarity to glycosyltransferases. Some clones contained single complete ORFs: the O-antigen polymerase gene *rfc* (recovered once), the L-rhamnose/H^+ symporter gene *rhaT* (recovered once), the methylglyoxal synthase gene *mgsA* (recovered three times) and the

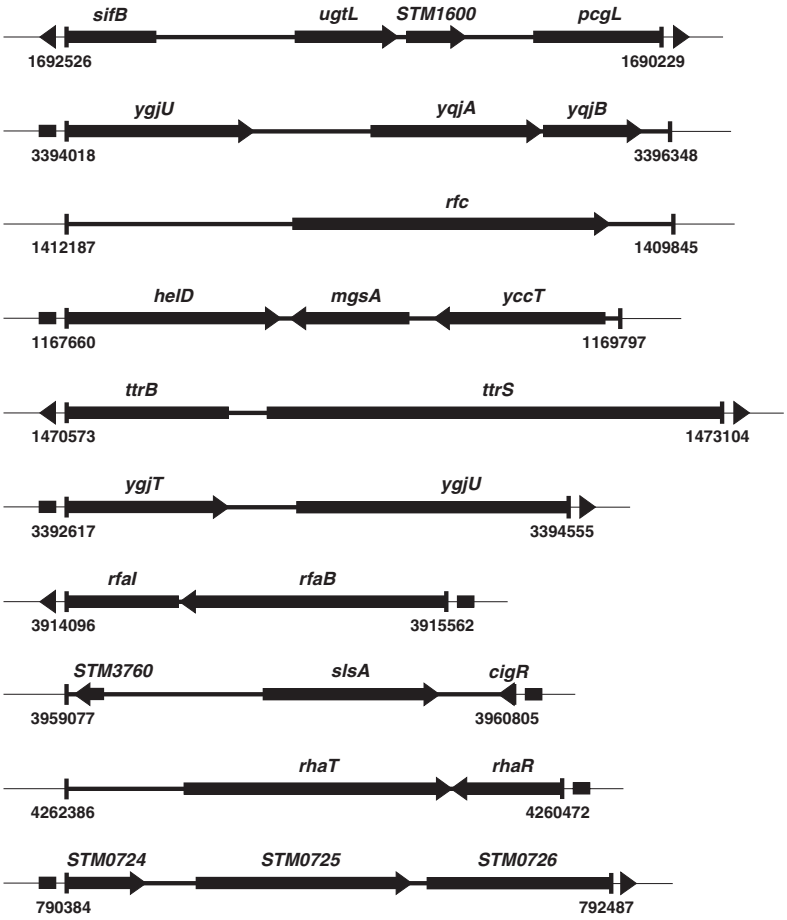

Fig. 1. Genomic regions of the inserts present in the plasmids recovered in the survival enrichment selection based on their ability to confer magainin 2 resistance upon *phoP* mutant MS7953s. Numbers correspond to location in the *S. enterica* serovar Typhimurium genome (McClelland *et al*., 2001). Thick lines correspond to complete or partial ORFs. Lines of intermediate thickness correspond to intergenic regions. Thin lines correspond to plasmid sequences. Vertical lines of intermediate thickness indicate the borders of the inserts in the plasmids. Small boxes and arrowheads indicate that parts of the ORFs are absent from the insert. The *lac* promoter in pUC19, which was used to make the plasmid library, was located to the left of the DNA segments listed and could transcribe sequences from left to right.

Salmonella-specific *slsA* that is part of the SPI-3 pathogenicity island and encodes a putative isochorismatase (recovered three times). Yet, other clones contained incomplete ORFs implicated in tetrathionate respiration (recovered once), lipopolysaccharide (LPS) biosynthesis (recovered four times) and unknown function (recovered twice), suggesting that sequences other than complete ORFs can confer magainin 2 resistance, perhaps by titrating a regulatory protein or by creating an antisense RNA. Here, we will focus on the *ugtL* and *yqjA* genes.

The PhoP-regulated ugtL *gene is required for resistance to magainin 2 and other alpha-helical antimicrobial peptides*

To examine whether the *ugtL* and *STM1600* genes were required for resistance to magainin 2, we constructed strains completely deleted for the chromosomal copies of these genes (see *Experimental procedures*). The *ugtL* mutant displayed increased susceptibility to magainin 2 whereas the *STM1600* mutant behaved like the wild-type parent (Fig. 2A). The magainin 2 susceptibility of the *ugtL* mutant was solely due to lack of *ugtL* gene function (as opposed to polarity on the downstream *STM1600* gene) because resistance could be restored by a plasmid containing a wild-type copy of the *ugtL* gene (Fig. 2B). Having demonstrated previously that *ugtL* is a PhoP-activated gene (Hilbert *et al*., 1999), these results validated our strategy for the recovery of the PhoP-regulated peptide resistance determinants.

To determine whether the *ugtL* gene was involved in resistance to other antimicrobial peptides known to kill *phoP Salmonella* preferentially (Groisman *et al*., 1992a), we examined the susceptibility of the *ugtL* mutant towards structurally different peptides. The *ugtL* mutant displayed increased susceptibility towards cecropin A (Fig. 2D) and melittin (data not shown), which can adopt amphipathic alpha-helical structures like magainin 2 (Bechinger, 1997), but it exhibited wild-type resistance to the β-sheet defensin HNP-1 (Fig. 2E) and to protamine (data not shown).

The ugtL *gene mediates polymyxin B resistance independently of the PmrA–PmrB system*

phoP Salmonella are >20 times more polymyxin sensitive than a *pmrA* mutant when bacteria are grown in low

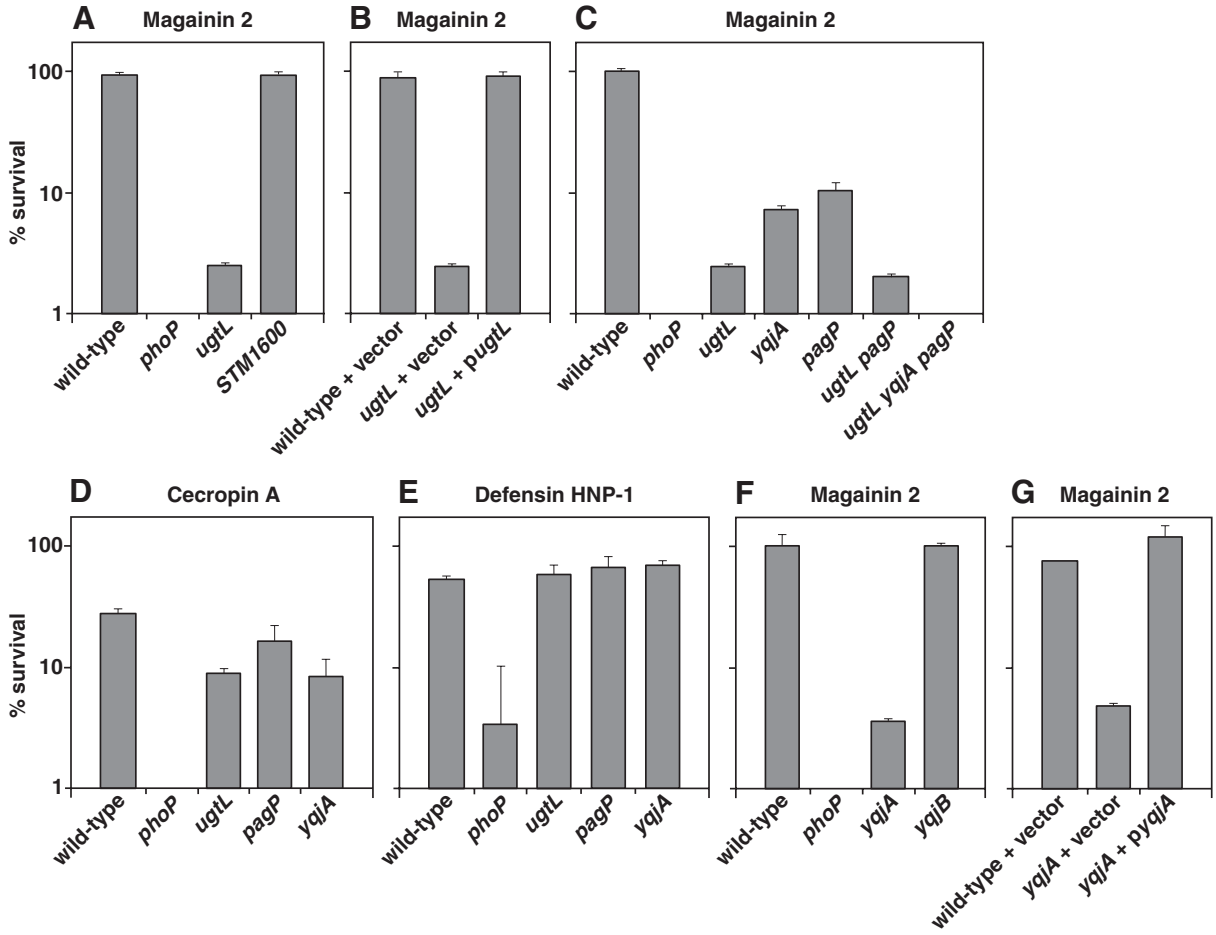

Fig. 2. The PhoP-activated *ugtL*, *pagP* and *yqjA* genes are required for resistance to the alpha-helical peptides magainin 2 and cecropin A but not to the β-sheet defensin HNP-1.
A. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *ugtL* (EG13682) and *STM1600* (EG14077) strains after incubation with magainin 2 (50 μg ml^{-1}).
B. Plasmid pUHE-*ugtL* (p*ugtL*), harbouring a wild-type copy of the *ugtL* gene, restores resistance to magainin 2 (50 μg ml^{-1}) to the *ugtL* strain (EG13682), whereas the plasmid vector pUHE21-2 (vector) does not.
C. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *ugtL* (EG13682), *yqjA* (EG13233), *pagP* (EG13678), *ugtL pagP* (EG14084) and *ugtL yqjA pagP* (EG13700) strains after incubation with magainin 2 (50 μg ml^{-1}).
D. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *ugtL* (EG13682), *pagP* (EG13678) and *yqjA* (EG13233) strains after incubation with cecropin A (0.6 μg ml^{-1}).
E. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *ugtL* (EG13682), *pagP* (EG13678) and *yqjA* (EG13233) strains after incubation with defensin HNP-1 (100 μg ml^{-1}).
F. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *yqjA* (EG13233) and *yqjB* (EG13679) strains after incubation with magainin 2 (50 μg ml^{-1}).
G. Plasmid pUC-*yqjA* (p*ugtL*), harbouring a wild-type copy of the *yqjA* gene, restores resistance to magainin 2 (50 μg ml^{-1}) to the *yqjA* strain (EG13233), whereas the plasmid vector pUC18 (vector) does not. Bacteria were grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$ in the absence (A and C–G) or presence (B) of IPTG (0.5 mM). Data correspond to mean values from at least three independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are shown only if bigger than the resolution of the figure).

Mg^{2+} (Wosten *et al*., 2000), indicating that a PmrA-independent PhoP-regulated gene(s) is necessary for polymyxin resistance. We determined that *ugtL* is such a gene because the *ugtL* mutant was ≈20 times more susceptible to polymyxin B than the wild-type strain (Fig. 3A), and because a *ugtL pmrA* double mutant was as susceptible to polymyxin B as a *phoP* mutant (Fig. 3C). The polymyxin B susceptibility phenotype of the *ugtL* mutant results solely from the absence of *ugtL* gene function because resistance could be restored to wild-type levels by a plasmid containing a wild-type copy of the *ugtL* gene (Fig. 3B). These results demonstrate that PhoP-regulated polymyxin B resistance is mediated by the *ugtL* gene and by some of the targets of PmrA–PmrB regulation (Groisman *et al*., 1997; Gunn *et al*., 1998).

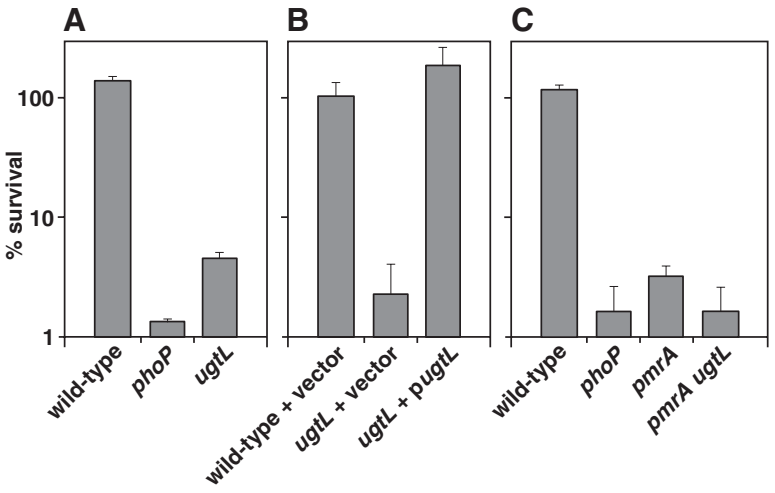

Fig. 3. The *ugtL* and *pmrA* genes mediate PhoP-controlled resistance to polymyxin B. A. Percentage survival of wild-type (14028s), *phoP* (MS7953s) and *ugtL* (EG13682) strains after incubation with polymyxin B (1.0 μg ml^{-1}). B. Plasmid pUHE-*ugtL* plasmid (p*ugtL*), harbouring a wild-type copy of the *ugtL* gene, restores resistance to polymyxin B (1.0 μg ml^{-1}) to the *ugtL* strain (EG13682), whereas the plasmid vector pUHE21-2 (vector) does not. C. Percentage survival of wild-type (14028s), *phoP* (MS7953s), *pmrA* (EG7139) and *pmrA ugtL* (EG13689) strains after incubation with polymyxin B (1.0 μg ml^{-1}). Bacteria were grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$ in the absence (A and C) or presence (B) of IPTG (0.5 mM). Data correspond to mean values from at least three independent experiments performed in duplicate. Error bars correspond to the standard deviation (and are shown only if bigger than the resolution of the figure).

PhoP-regulated magainin 2 resistance is mediated by the ugtL*,* pagP *and* yqjA *genes*

Because the *ugtL* mutant was not as susceptible to magainin 2 as the *phoP* mutant (Fig. 2A), we reasoned that another PhoP-regulated gene(s) must be involved in magainin 2 resistance. Thus, we investigated the role of the PhoP-activated *pagP* and *pgtE* genes, because they had been implicated in resistance to the alpha-helical peptide C18G (Guo *et al*., 1998; Guina *et al*., 2000). We determined that the *pagP* mutant displayed increased susceptibility to magainin 2 (Fig. 2C), whereas the *pgtE* mutant exhibited wild-type resistance (data not shown). A *ugtL pagP* double mutant was more sensitive than either the *ugtL* or *pagP* single mutants but still more resistant than the *phoP* mutant (Fig. 2C), indicating that the *ugtL* and *pagP* genes participate in different pathways of magainin 2 resistance and that additional PhoP-regulated genes contribute to magainin 2 resistance.

Nearly half the plasmid clones that conferred magainin 2 resistance upon the *phoP* mutant harboured the *yqjA* and *yqjB* genes (Fig. 1; see above). This raised the possibility that the *yqjA* and/or *yqjB* genes might be required for magainin 2 resistance and regulated by the PhoP protein. Thus, we first constructed strains deleted for the *yqjA* and/or *yqjB* genes and examined their susceptibility to magainin 2. We determined that the *yqjA* mutant was ≈10-fold more susceptible to magainin 2 than the wild-type strain (Fig. 2F). The increased susceptibility of the *yqjA* mutant results solely from lack of *yqjA* gene function because magainin 2 resistance could be restored to wild-type levels by a plasmid containing a wild-type copy of the *yqjA* gene (Fig. 2G), and because deletion of the downstream *yqjB* had no effect on magainin 2 resistance (Fig. 2F). The *yqjA* mutant exhibited wild-type resistance towards polymyxin B (data not shown) and defensin HNP-1 (Fig. 2E) but increased sensitivity towards protamine (data not shown), which also preferentially kills the *phoP* mutant (Groisman *et al*., 1992). And secondly, we investigated *yqjA* expression using isogenic wild-type and *phoP* strains encoding a YqjA–FLAG protein from its normal chromosomal location and promoter. When bacteria were grown under PhoP-inducing conditions (i.e. 10 μM Mg^{2+}), the levels of the *yqjA* gene product were lower in the *phoP* mutant than in the wild-type strain (Fig. 4A). Taken together with the reduced YqjA–FLAG expression displayed by the *phoP*$^+$ strain grown under PhoP-repressing conditions (i.e. 10 mM Mg^{2+}) (Fig. 4A), these results indicate that PhoP controls YqjA expression. This control is exerted at the transcriptional level because the β-galactosidase activity was greatly diminished when a *phoP* strain harbouring a chromosomal *yqjA–lac* transcriptional fusion was grown in low Mg^{2+} (Fig. 4B).

We established that a *ugtL pagP yqjA* triple mutant exhibited similar magainin 2 susceptibility to the *phoP* mutant (Fig. 2C). Cumulatively, these results demonstrate that *yqjA* is a PhoP-activated gene required for magainin 2 resistance and that *yqjA* confers magainin resistance by a pathway that is different from those mediated by the *ugtL* and *pagP* genes.

UgtL is an inner membrane protein that promotes the formation of monophosphorylated lipid A

We examined the lipid A structure of the *ugtL* mutant because the PhoP–PhoQ system controls the modification of several positions in lipid A (Ernst *et al*., 2001) and because PmrA-mediated polymyxin B resistance requires the incorporation of 4-aminoarabinose into lipid A (Gunn *et al*., 1998). Matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) analysis of lipid A prepared from wild-type *Salmonella* grown under PhoP-inducing conditions (i.e. 10 μM Mg^{2+}) revealed the presence of a

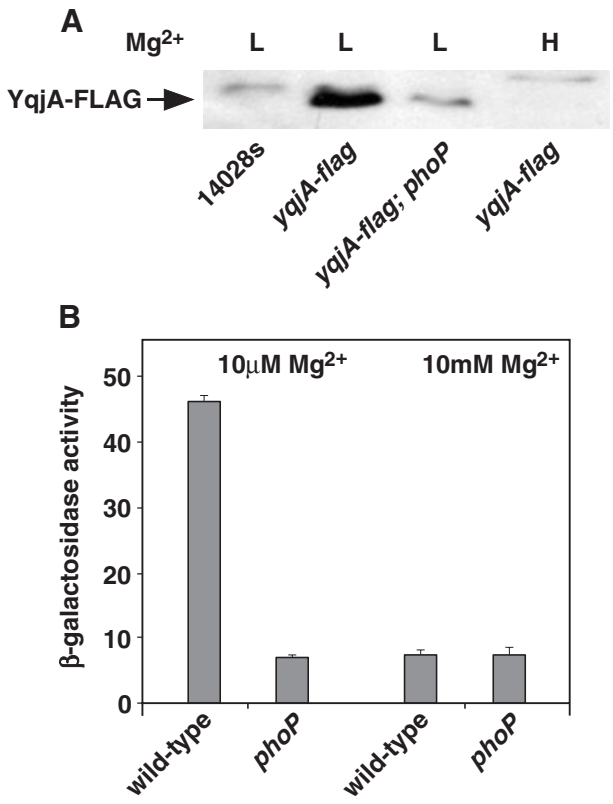

Fig. 4. Expression of the *yqjA* gene is controlled by the PhoP protein at the transcriptional level.
A. Western blot of YqjA–FLAG protein from a strain harbouring a chromosomally encoded YqjA–FLAG protein (EG13690) and its isogenic *phoP* mutant (EG13706) grown in N-minimal medium, pH 7.4, with either 10 μM or 10 mM $MgCl_2$, using anti-FLAG antibodies. Wild-type 14028s was used as a negative control for FLAG antibody.
B. β-Galactosidase activity (Miller units) from a chromosomal *ΔyqjA–lac* transcriptional fusion expressed by wild-type (EG14488) and *phoP* (EG14489) strains grown in N-minimal medium, pH 7.4, with either 10 μM or 10 mM $MgCl_2$.

peak (i.e. *m/z* 1955), which appears to represent hepta-acyl monophosphorylated lipid A (Johnson *et al*., 1990; Chan and Reinhold, 1994), and its hydroxyl form (i.e. *m/z* 1971) (Fig. 5), the production of which is mediated by the PhoP-activated PagQ protein (Ernst *et al*., 2001). These peaks were absent from the *phoP* and *ugtL* mutants and from wild-type *Salmonella* grown under PhoP-repressing conditions (i.e. 10 mM Mg^{2+}) (Fig. 5). The absence of these peaks from the *ugtL* mutant results solely from lack of *ugtL* gene function because their presence was restored by a plasmid harbouring a wild-type copy of the *ugtL* gene (Fig. 5).

The UgtL protein is predicted to be an integral membrane protein (http://psort.nibb.ac.jp). To examine the location of the UgtL protein, we purified outer and inner membranes from a strain expressing a chromosomally encoded FLAG epitope-tagged UgtL protein and performed Western blot analysis with anti-FLAG antibodies. [This strain displayed wild-type resistance to both magainin 2 and polymyxin B (data not shown), indicating that the FLAG epitope did not interfere with UgtL function.] We detected a band in the inner membrane fraction (and not in the outer membrane fraction) of an apparent molecular weight consistent with that calculated for the UgtL–FLAG protein (Fig. 6A). Cross-linking experiments revealed the formation of several oligomeric forms of the UgtL protein (Fig. 6B). Cumulatively, these results demonstrate that UgtL is an oligomeric inner membrane protein that is necessary for the formation of monophosphorylated lipid A.

Discussion

The PhoP–PhoQ regulatory system controls resistance to a variety of structurally different antimicrobial peptides (Groisman *et al*., 1992), but the identity of the PhoP-regulated determinants mediating peptide resistance has remained largely unknown. We have now used a novel enrichment strategy to recover peptide resistance loci based on their ability to restore partial magainin 2 resistance to a *phoP* mutant when present in a multicopy number plasmid. This strategy was validated with the recovery of two genes – *ugtL* and *yqjA* – that are both required for magainin 2 resistance (Fig. 2A–C, F and G) and transcriptionally regulated by the PhoP–PhoQ system (Fig. 4; Hilbert *et al*., 1999). This suggests that similar genetic screenings may be used for the identification of PhoP-regulated determinants mediating resistance to additional peptides or other PhoP-controlled phenotypes.

The *ugtL* gene was first identified as part of a *Salmonella*-specific DNA region harbouring the PhoP-activated *pcgL* gene, which encodes a D-Ala-D-Ala dipeptidase (Hilbert *et al*., 1999). However, despite being co-regulated by the PhoP–PhoQ system, the *ugtL* and *pcgL* genes appear to participate in different cellular functions because the *ugtL* mutant retains D-Ala-D-Ala dipeptidase activity (Hilbert *et al*., 1999), and the *pcgL* mutant exhibits wild-type resistance to antimicrobial peptides (our unpublished results). We have now established that *ugtL* encodes an oligomeric inner membrane protein (Fig. 6) that mediates the presence of hepta-acyl monophosphorylated lipid A in the LPS (Fig. 5). Because the lipid A is normally phosphorylated at two positions, this low-Mg^{2+} PhoP-dependent lipid A modification (Fig. 5) would decrease the overall negative charge of the bacterial cell, thus reducing the opportunity for electrostatic interactions with cationic antimicrobial peptides. This could explain the increased susceptibility of the *ugtL* mutant towards the alpha-helical peptides magainin 2 (Fig. 2A), cecropin A (Fig. 2D) and melitin (data not shown) and the cyclic peptide polymyxin B (Fig. 3). Consistent with this notion, the

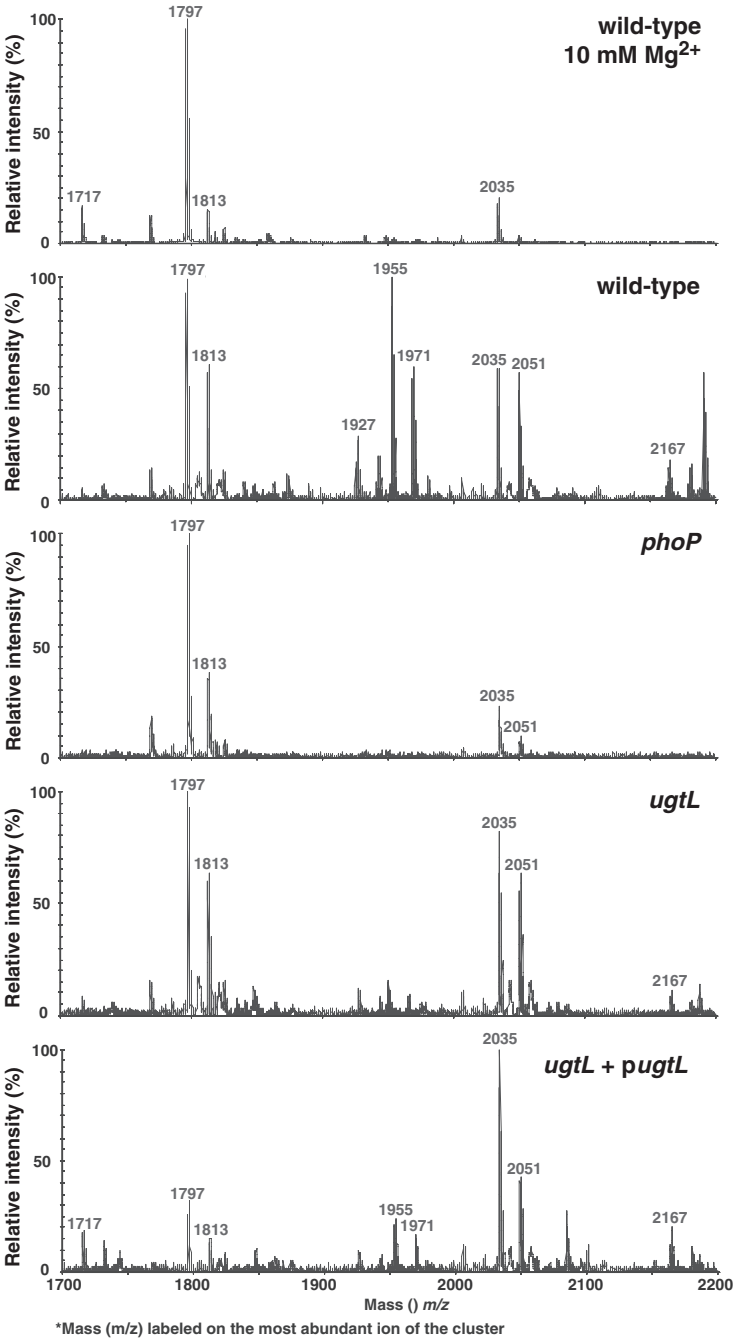

Fig. 5. The PhoP-regulated *ugtL* gene mediates the formation of a monophosphorylated lipid A. MALDI-TOF mass spectra analysis of lipid A from wild-type *Salmonella* (14028s) grown in N-minimal medium, pH 7.4, with 10 mM $MgCl_2$, or from wild-type (14028s), *phoP* (MS7953s) and *ugtL* (EG13682) strains grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$; or from *ugtL* (EG13682) strain harbouring a wild-type copy of the *ugtL* gene in plasmid p*ugtL* grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$ and 0.5 mM IPTG.

formation of monophosphorylated lipid A is essential for polymyxin B resistance in *Rhizobium leguminosarum* (Karbarz *et al*., 2003). The mechanism by which the *ugtL* gene product generates monophosphorylated lipid A is presently unclear as it does not exhibit sequence identity to the *Rhizobium* lipid A 1-phosphatase LpxE or to other proteins in the sequence databases.

In addition to *ugtL*, PhoP-regulated magainin 2 resistance requires the *pagP* and *yqjA* genes (Fig. 2C). The *pagP* gene has been implicated previously in resistance to the alpha-helical peptide C18G (Guo *et al*., 1998), and we now established that, like *ugtL* and *yqjA* mutants, a *pagP* mutant exhibits increased susceptibility to the alpha-helical cecropin A (Fig. 2D) but not to the β-sheet defensin HNP-1 (Fig. 2E). The *pagP* gene encodes an outer membrane protein (Bishop *et al*., 2000) that mediates the palmytoylation of lipid A (Guo *et al*., 1998). A *ugtL pagP* double mutant was more susceptible to magainin 2 than either *ugtL* or *pagP* single mutants (Fig. 2C), consistent with their respective gene products participating in sepa-

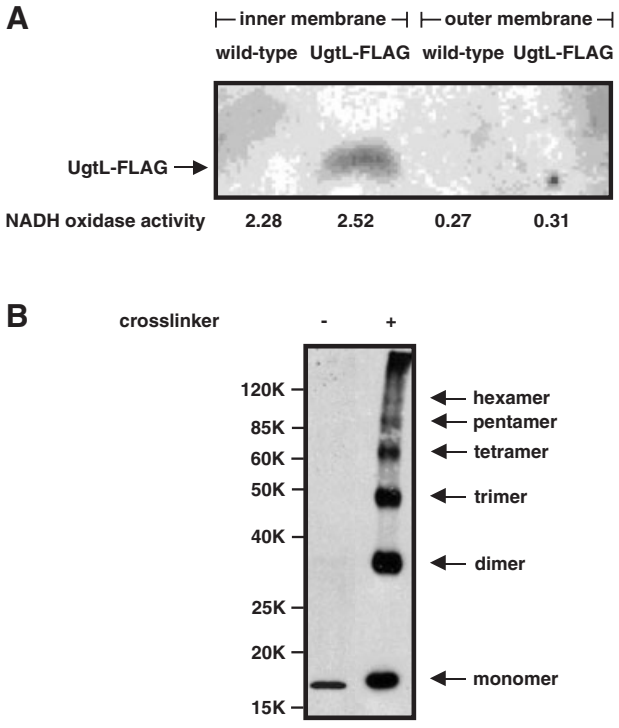

Fig. 6. UgtL is an oligomeric inner membrane protein.
A. Western blot of inner and outer membranes prepared from a strain harbouring a chromosomally encoded UgtL–FLAG protein (EG13685) grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$, using anti-FLAG antibodies. The UgtL protein localizes to the inner membrane. NADH oxidase activity (ΔOD_{340} β-NADH μg^{-1} protein min^{-1}) confirmed the purity of the membrane preparations.
B. Cross-linking and Western blot analysis of a whole-cell lysate prepared from a strain harbouring a chromosomally encoded UgtL–FLAG protein (EG13685) grown in N-minimal medium, pH 7.4, with 10 μM $MgCl_2$, using anti-FLAG antibodies. The UgtL protein forms oligomers.

rate resistance pathways (even when both affect the lipid A). Yet, the *ugtL pagP* mutant was not as susceptible as the *phoP* mutant (Fig. 2C), whereas the *ugtL pagP yqjA* triple mutant was (Fig. 2C). Despite its hypersusceptibility towards magainin 2 (Fig. 2F and G) and cecropin A (Fig. 2D), the *yqjA* mutant exhibits a wild-type lipid A profile under PhoP-inducing conditions (our unpublished results). The *E. coli yqjA* gene is transcriptionally controlled by the alternative sigma factor RpoE (Dartigalongue *et al*., 2001), suggesting that the YqjA protein may help the bacterial cell to cope with envelope stress.

Our experiments demonstrate that more than one type of lipid A modification and/or cell envelope change may be necessary for full resistance to certain antimicrobial peptides (Fig. 7). This is true even when these modifications appear have the same effect: decreasing the overall negative charge of the bacterial cell surface. For example, PhoP-controlled polymyxin B resistance requires not only the UgtL-mediated presence of hepta-acyl monophosphorylated lipid A (Fig. 3A and B) but also the PmrA–PmrB regulatory system (Fig. 3C), which governs the biosynthesis and incorporation of 4-aminoarabinose into lipid A (Groisman *et al*., 1997; Gunn *et al*., 1998) and is post-transcriptionally regulated by the PhoP–PhoQ system (Soncini and Groisman, 1996; Kox *et al*., 2000). We determined that a *ugtL pmrA* double mutant was as susceptible to polymyxin B as a *phoP* mutant (Fig. 3C), which suggests that both types of lipid A modifications may operate at the same time and argues against the participation of other PhoP-regulated genes in resistance to polymxyin B. Thus, the increased polymyxin B susceptibility reported for mutants defective in the PhoP-activated *mig-14*, *virK* and *somA* genes may be characteristic of the SL1344 genetic background used in those studies (Brodsky *et al*., 2002; Detweiler *et al*., 2003) because derivatives of the 14028s strain deleted for the *mig-14* gene or harbouring a MudJ transposon insertion in the *virK* gene retained wild-type resistance to both polymxyin B and magainin 2 (our unpublished results).

When organisms experience Mg^{2+}-limiting environments, the PhoP–PhoQ system is activated (Garcia Vescovi *et al*., 1996), which promotes the expression of a variety of proteins resulting in remodelling of the bacterial cell surface (Groisman *et al*., 1997; Ernst *et al*., 2001). That these cell surface modifications are not equivalent is demonstrated by the distinct phenotypes displayed by mutants in different PhoP-regulated genes. For example, a *pmrA* mutant displays wild-type resistance to magainin 2 (our unpublished results) despite being exquisitely sen-

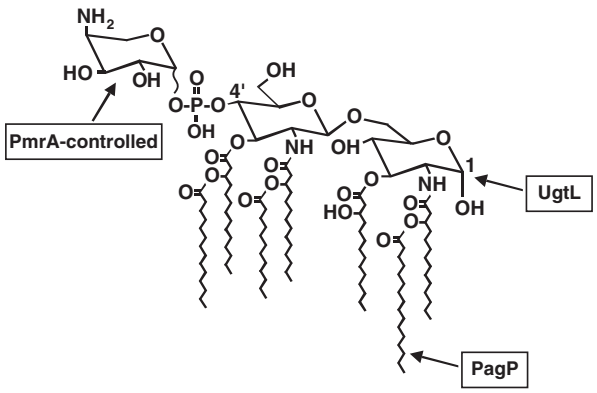

Fig. 7. PhoP-regulated lipid A modifications associated with resistance to antimicrobial peptides. The formation of monophosphorylated lipid A is mediated by the UgtL protein, which is required for resistance to both magainin 2 and polymyxin B. Although UgtL is shown as acting at the 1 position of lipid A, it is presently unclear whether it affects the 1 and/or 4′ positions. The PagP-mediated transfer of a palmitate from phospholipids to lipid A is required for resistance to magainin 2 but not to polymyxin B. PmrA-controlled loci encode enzymes involved in the biosynthesis and incorporation into lipid A of 4-aminoarabinose, which is necessary for resistance to polymyxin B but not magainin 2. Although the 4-aminoarabinose modification is shown as acting at the 4′ position of lipid A, it is presently unclear whether it affects the 1 and/or 4′ positions.

sitive to polymyxin B (Fig. 3C) (Groisman *et al*., 1997; Gunn *et al*., 1998). Likewise, the *pagP* gene is dispensable for polymyxin B resistance (our unpublished results) even though it is required for resistance to magainin 2 (Fig. 2C) and other peptides (Guo *et al*., 1998). Finally, characterization of *yqjA* and other peptide resistance determinants recovered in our selection (Fig. 1) may uncover other regulatory systems in the control of bacterial cell envelope changes that affect susceptibility to the ubiquitous antimicrobial peptides.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *Salmonella enterica* serovar Typhimurium strains used in this study are derived from strain 14028s. Phage P22-mediated transductions were performed as described previously (Davis *et al*., 1980). Bacteria were grown at 37°C in Luria–Bertani (LB) broth or in N-minimal medium, pH 7.4 (Snavely *et al*., 1991), supplemented with 0.1% casamino acids, 38 mM glycerol, 10 μM or 10 mM $MgCl_2$. When necessary, antibiotics were added at final concentrations of 50 μg ml^{-1} for ampicillin, 20 μg ml^{-1} for chloramphenicol, 50 μg ml^{-1} for kanamycin and 12.5 μg ml^{-1} for tetracycline. *E. coli* DH5α was used as host for the preparation of plasmid DNA.

Plasmid constructions

Plasmid pUHE21-2*lacI*^{q}-*ugtL* (p*ugtL*) was constructed by cloning between the *Bam*HI and *Hin*dIII sites of pUHE21-2*lacI*^{q} a polymerase chain reaction (PCR) fragment containing the *ugtL* coding region generated with primers 2240 (5′-CGGGATCCAGGAGGCTCAAAATGAAGAAATCAG-3′) and 2241 (5′-CCCAAGCTTATCACGGGCGTGAAGAAACAT CCTG-3′) and strain 14028s chromosomal DNA as template, and digesting with *Bam*HI and *Hin*dIII. Plasmid pUC18-*yqjA* was constructed by cloning between the *Bam*HI and *Hin*dIII sites of pUC18 a PCR fragment containing the *yqjA* coding region generated with primers 1943 (5′-CGGGATCCCCAG GAATACTGAATGGAAC-3′) and 1944 (5′-CCCAAGCT TCGTGGTTTAAGCATAAGTTA-3′) and wild-type strain 14028s chromosomal DNA as template, and digesting with *Bam*HI and *Hin*dIII. Sequence analysis demonstrated the presence of wild-type *ugtL* and *yqjA* sequences in the two plasmids.

Construction of chromosomal mutations, lac *gene fusions and epitope-tagged proteins*

Deletion of the coding region of a gene was generated as described previously (Datsenko and Wanner, 2000). Inactivation of the *ugtL* gene was carried out using primers 1772 (5′-ATGAAGAAA TCAGATGGTGA AATTCACGAAGTGTAGGCT GGAGCTGCTTC-3′) and 1773 (5′-CGGGCCTGAAGAAA CATCCTGTGCCCCAAATCATATGAATATCCTCCTTA-3′) to

Table 1. Bacterial strains used in this study.

Strain or plasmid	Description[a]	Reference or source
S. enterica serovar Typhimurium		
14028s	Wild type	Fields *et al*. (1989)
MS7953s	*phoP7953*::Tn*10*	Fields *et al*. (1989)
EG13682	Δ*ugtL*::Km^r	This work
EG14077	Δ*STM1600*::Cm^r	This work
EG13678	Δ*pagP*::Cm^r	This work
EG13690	*yqjA-flag*::Cm^r	This work
EG13706	*yqjA-flag*::Cm^r *phoP7953*::Tn*10*	This work
EG13233	Δ*yqjA*::Cm^r	This work
EG14488	*yqjA*$^-$-*lacZY* Km^r	This work
EG14489	*yqjA*$^-$-*lacZY* Km^r *phoP7953*::Tn*10*	This work
EG13679	Δ*yqjB*::Cm^r	This work
EG14084	Δ*ugtL*::Km^r Δ*pagP*::Cm^r	This work
EG13685	*ugtL-FLAG*::Cm^r	This work
EG7139	*pmrA*::Cm^r	Soncini and Groisman (1996)
EG13689	*pmrA*::Cm^r Δ*ugtL*::Km^r	This work
EG13700	Δ*ugtL* Δ*ygjA* Δ*pagP*::Cm^r	This work
E. coli		
DH5α	F$^-$ *supE*44 Δ*lacU*169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan (1983)
Plasmids		
pUC19	rep$_{pMB1}$ Ap^r	Norrander *et al*. (1983)
pKD3	rep$_{R6K}$γ Ap^r FRT Cm^r FRT	Datsenko and Wanner (2000)
pKD46	rep$_{pSC101}^{ts}$ Ap^R p$_{araBAD}$ γ β exo	Datsenko and Wanner (2000)
pCP20	rep$_{pSC101}^{ts}$ Ap^r Cm^r c*I857* λp$_R$	Cherepanov and Wackernagel (1995)
pCE36	rep$_{R6K}$γ Km^r FRT *lacZY* t$_{his}$	Ellermeier *et al*. (2002)
pUHE21-2*lac*^{q}	rep$_{pMB1}$ Ap^r *lacI*^{q}	Soncini *et al*. (1995)
p*ugtL*	rep$_{pMB1}$ Ap^r *lacI*^{q} *ugtL*	This work
p*yqjA*	rep$_{pMB1}$ Ap^r *yqjA*	This work

a. Gene designations are as described by Sanderson et al. (1995).

amplify the Km^r cassette from pKD4 and integrate the resulting PCR product into the chromosome. The *ugtL* chromosomal deletion was confirmed by Southern blot analysis using as probes a PCR fragment generated with primers 2201 (5′-CGTGTAGAAATTGTCTTATAAGAAG-3′) and 2202 (5-TTGTCGCCTTCCATAACTAAATAAC-3′) and strain 14028s chromosomal DNA as a template, as well as a Km^r cassette-specific DNA fragment (data not shown). Inactivation of the *yqjA* gene was carried out using primers 1776 (5′-ATGGAACTACTGACTCA AT TGCTGA ATGCCGTGTAGGCT GGAGCTGCTTC-3′) and 1777 (5′-TCCCCGGCTCCC ATACTTCTTTTTCCACAGCCATATGAATATCCTCCTTA-3′) to amplify the Cm^r cassette from pKD3 and integrate the resulting PCR product into the chromosome. The *yqjA* chromosomal deletion was confirmed by Southern blot analysis using as probes a PCR product generated with primers 2040 (5′-GTTGACCTTTGTTACAATTAGATG-3′) and 2041 (5′-CAT GTCATTTTTAGGCGTAATGCTT-3′) and strain 14028s chromosomal DNA as a template, as well as a Cm^r cassette-specific DNA fragment (data no shown). An analogous strategy was used to inactivate several genes as follows: *STM1600*, using primers 2373 (5′-GCCCGTGATCCGC CGCTGCA ACCTG CGGTCT TACTGAATAGTGTAGGCTG GAGCTGCTTC-3′) and 2374 (5′-TCAGGAGCACAGATAAC GATGTGTTTTCACAAAGGCGAGTA TATGAATATCCTCCTT AGT-3′); *yqjB*, using primers 1895 (5′-AGAAGTATGGGAGC CGGGGAT AACTT ATGCGTGT AGGCTGGAGCTGCTTC-3′) and 1896 (5′-TGAGTGATATGATTCGGATTCCTGGAGATTA CATATGAATATCCTCCTTA-3′); *pagP*, using primers 2203 (5′-GGTT AA TGTTGTT ATTATCACAGTCGAATTTTTGAACGGT GTGTAGGCTGGAGCTGCTTC-3′) and 2204 (5′-TAAG ACTT T TTAATTCACAACTGAAGCATACCCTTCCCCACATAT GAATACCTCCTTAGT-3′). Deletion of *STM1600*, *yqjB* and *pagP* genes was confirmed by Southern blot analysis using as probes PCR fragments generated with the following primer sets: for *STM1600*, 2370 (5′-AATCATGCTTCGCT TGATTTATGGC-3′) and 2371 (5′-CGGACACCGCTTAAA CAGATGGTTA-3′); for *yqjB*, 2042 (5′-CAACTGGATGAGCG GCTTGTTGTG-3′) and 2043 (5′-CCTGACAAAGCGAATT AGCGTAGC-3′); for *pagP*, 2276 (5′-CGAATTTTCTGTAGCA CTATAAG-3′) and 2277 (5′-CATTAAACCACTTAACGTTA CCT-3′) (data not shown). To create a strain expressing a UgtL protein with a FLAG tag from the chromosomal *ugtL* gene, we used a previously described method (Uzzau *et al*., 2001) with primers 2242 (5′-GTCCAAAAAATTTGGGG CACAGGATGT TTCTTC ACGCCCGACT ACAAGGACGACG ATGACAAGTAACATATGAA TATCCTCCTTA G-3′), which is designed to have the FLAG sequence immediately before the stop codon of the *ugtL* gene, followed by priming site 2 sequence of pKD3 (Datsenko and Wanner, 2000), and 2243 (5′-ATAGCCAT TATTCAGT AAGACCGCAGGT TGCAGCGGC GGAGTGTAGGCTGGAGCTGCTTC-3′), which harbours the sequence downstream of the *ugtL* gene followed by the priming site 1 of pKD3. The Cm^r cassette of pKD3 was amplified using these primers and integrated at the 3′ end of *ugtL* on the chromosome. To incorporate a FLAG tag into the chromosomally encoded YqjA protein, the same steps were used as above with primers 2050 (5′-ACTGGTCATGCTGTGGA AAAAGAAGTATGGGAGCCGGGGAGA CTACA AGGA CGAC GATGACAAGTAACATATGAATATCCTCCTTAG-3′), which is designed to have the FLAG sequence immediately before the stop codon of the *yqjA* gene, followed by priming site 2 sequence of pKD3 (Datsenko and Wanner, 2000), and 2051 (5′-AAATGAGTTGTCGGGCAGTTA TGCGTGGTT TAAGCAT AAGGTGTAGGCTGGAGCTGCTTC-3′), which harbours the sequence downstream of the *yqjA* gene followed by the priming site 1 of pKD3. The junction region of the *ugtL* and *yqjA* genes and the sequence encoding the FLAG epitope were amplified from the chromosome by PCR and confirmed to have the predicted sequence by nucleotide sequencing.

We generated a *lac* transcriptional fusion at the deleted *yqjA* gene locus as described previously (Ellermeier *et al*., 2002). The Cm^r cassette of EG13233 was removed using plasmid pCP20 (Datsenko and Wanner, 2000), and the *lac* transcriptional fusion plasmid pCE36 was integrated into the FLP recombination target sequence of the deleted *yqjA* locus.

Survival enrichment selection for peptide-resistant genes

Chromosomal DNA prepared from an overnight culture of the *phoP* strain MS7953s was digested with *Sau*3AI (1 U μl^{-1}) for 15, 20, 30 or 40 min. The digested DNA was separated on 0.8% agarose gel, and 2–5 kb fragments were collected and ligated to *Bam*HI-digested pUC19 plasmid DNA. The ligation mixture was transformed into *E. coli* DH5α selecting for ampicillin-resistant transformants. Plasmid DNA was prepared from a pool of 16 000 transformants and used to transform strain MS7953s. To select clones exhibiting magainin 2 resistance, 1–2 $\times$ 10^6 cells were grown in LB-ampicillin to mid-log phase and used to inoculate two microtitre plate wells each containing 50 μl of LB-ampicillin and magainin 2 (50 $\mu g\ ml^{-1}$; Bachem), and then incubated for 4 h at 37°C with aeration. The cultures from both wells were plated together on to LB-ampicillin plates after being diluted 10 times with fresh LB medium. Plasmids were isolated, reintroduced into MS7953s by electroporation, and transformants were selected on LB-ampicillin plates. These colonies were collected as a pool and used for a new round of selection by repeating the procedure just described twice more. We picked 211 random ampicillin-resistant transformants from eight LB-ampicillin plates, prepared plasmid DNA from each of these transformants and introduced each of these plasmids into strain MS7953s selecting for ampicillin-resistant transformants on LB-ampicillin plates. To examine the magainin 2 resistance of these clones, two random single colonies from each transformation were tested as follows: 1–2 $\times$ 10^6 cells were grown in LB-ampicillin to mid-log phase, used to inoculate LB medium containing magainin 2 (50 $\mu g\ ml^{-1}$) and incubated for 4 h at 37°C with aeration. Plasmids conferring significant resistance to magainin 2 upon strain MS7953s (i.e. >5% survival compared with <0.01% survival for strain MS7953s harbouring the pUC19 vector) were used to retransform MS7953s. To determine the identity of the inserted fragments, the plasmids were sequenced using primers 2391 (5′-GCGGATAA CAATTTCACACAGG-3′) and 2392 (5′-CGCCAGGGTTTTC CCAGTCACGAC-3′).

Peptide killing assays

Antimicrobial peptide susceptibility assays were conducted

by a modification of a previously described method (Groisman *et al*., 1992a). Bacteria were inoculated into N-minimal medium at pH 7.4, 10 mM $MgCl_2$ for overnight growth at 37°C with aeration. These cultures were harvested and washed three times in N-minimal medium, pH 7.4, 10 μM $MgCl_2$. Cells were then diluted 1:100 into N-minimal medium, pH 7.4, 10 μM $MgCl_2$. Bacteria were incubated for 4 h at 37°C with aeration to an OD_{600} of 0.3–0.4 and diluted to $1–2 \times 10^5$ ml^{-1} in N-minimal medium, pH 7.4, 10 μM $MgCl_2$. Peptides were dissolved and serially diluted with autoclaved distilled water. Samples of 5 μl of peptide solution (concentration 10 times higher than the final concentration) were placed in wells of a 96-well plate (Cell Culture Cluster, Costar®), and 45 μl of the bacterial culture was added. After a 1 h incubation at 37°C with aeration, 20 μl was mixed with 180 μl of LB broth, and 50 μl of this solution was plated on to LB agar plates. The number of colony-forming units (cfu) was counted after overnight aerobic incubation at 37°C. The percentage survival was calculated as follows: survival (%) = cfu of peptide-treated culture/cfu of no peptide culture × 100. Susceptibility to defensin HNP-1 was performed as described previously (Fields *et al*., 1989) with the following modifications. Bacterial strains were grown as described above, harvested, washed once with TSB medium–sodium phosphate buffer, pH 7.4, and diluted to $1–2 \times 10^5$ bacteria ml^{-1} in the same buffer. Defensin HNP-1 (Bachem) was dissolved in autoclaved distilled water and serially diluted in 0.01% acetic acid to 10 times higher than the final concentration. An aliquot of 5 μl of 10× defensin HNP-1 solution was placed in wells in a 96-well plate, and 45 μl of bacterial solution was added. After incubation for 90 min at 37°C with aeration, 20 μl was mixed with 180 μl of LB broth, and 50 μl was plated on an LB agar plate. The number of cfu was counted after 24 h aerobic incubation at 37°C. The calculation of percentage survival was performed as described above.

Localization of the UgtL protein

Strains harbouring a chromosomal *ugtL* gene encoding a protein with a C-terminal FLAG epitope were grown in 300 ml as described above to OD_{600} of 0.3–0.4, washed with PBS once, resuspended in 4 ml of PBS and opened by sonication. A whole-cell lysate was loaded on the top phase of sucrose gradient with 4 ml of 60% and 4 ml of 70% sucrose in a Beckman Ultra-Clear™ centrifuge tube. Sucrose gradient ultracentrifuge procedure (Yamato *et al*., 1975) with modifications (http://www.cmdr.ubc.ca/bobh/methodsall.html) was used to isolate inner and outer membranes of bacteria by centrifuging these tubes in a SW41 rotor at 38 000 r.p.m. for 18 h at 4°C. Upper and lower bands were collected and dialysed. Purity of the inner membrane fraction (upper brown band) was assayed by measuring the NADH oxidase activity (Orndorff and Dworkin, 1980): the enzymatic activity is defined as ΔOD_{340} β-NADH μg^{-1} protein min^{-1}. Inner and outer membrane preparations (10 μg of protein) were run on 12% SDS–PAGE gel, transferred to nitrocellulose membrane and analysed by Western blot using anti-FLAG M2 monoclonal antibody (Sigma). UgtL–FLAG bands were developed using anti-mouse IgG horseradish peroxidase-linked antibody and the ECL detection system (Amersham Biosciences).

Western blot analysis of FLAG-tagged proteins

Strains harbouring chromosomally encoded UgtL or YqjA proteins with a C-terminal FLAG epitope were grown in 50 ml as described above to an OD_{600} of 0.3–0.4, washed with PBS once, resuspended in 1 ml of PBS and opened by sonication. For cross-linking of the UgtL–FLAG protein, the cell culture was incubated with 1.36 ml of 37% formaldehyde and 0.5 ml of 1 M phosphate buffer, pH 7.6, for 15 min at room temperature; then, 2.6 ml of 2.5 M glycine solution was added to stop the reaction, which was washed once before sonication. These whole-cell lysates were used for SDS–PAGE (12% polyacrylamide) and Western blotting detection (ECL, Amersham Biosciences).

Mass spectrometry analysis of lipid A

The lipid A portion from the lipopolysaccharide was extracted by treating the outer membrane preparation described above with SDS in acidic conditions (Zhou *et al*., 1999). Outer membrane solution (0.8 ml) was dialysed against PBS, then mixed with a single-phase Bligh–Dyer mixture (Bligh and Dyer, 1959) made by the addition of 2 ml of methanol and 1 ml of chloroform. After 60 min at room temperature, the insoluble material was collected by centrifugation in a clinical centrifuge at top speed for 20 min. This pellet was washed once with 5.0 ml of a fresh single-phase Bligh–Dyer mixture, consisting of chloroform–methanol–water (1 : 2 : 0.8 v/v). The pellet was then dispersed in a 1.8 ml portion of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, with sonication in a bath apparatus. The mixture was incubated at 100°C for 30 min to cleave the glycosidic linkage between Kdo and lipid A (Rosner *et al*., 1979). To recover the lipid A, the hydrolysed material was converted to a two-phase Bligh–Dyer mixture by the addition of 2 ml of chloroform and 2 ml of methanol. After centrifugation at low speed, the lower phase was collected and washed twice with 4 ml of the upper phase derived from a fresh neutral two-phase Bligh–Dyer mixture, consisting of chloroform–methanol–water (2 : 2 : 1.8 v/v). MALDI-TOF mass spectrometry analyses of lipid A were performed in the negative ion mode on a Voyager DE STR mass spectrometer (PerSeptive Biosystems) equipped with a 337 nm nitrogen laser and delayed extraction. Analyses were carried out in the reflector mode at a mass range of *m/z* 1500–3000 with an accelerating voltage of 20 kV and a delay time of 300 ns. The instrument was externally calibrated. A low-mass gate value of *m/z* 500 was selected to avoid saturation of the detector. 2,5-Dihydroxybenzoic acid (DHB) at 10 μg μl^{-1} in 70% acetonitrile (ACN)–0.1% trifluoroacetic acid (TFA) was used as a matrix. The final mass spectra were from an average of 5–10 spectra, in which each spectrum is a collection from 200 laser shot.

β-Galactosidase assays

Assays were carried out in triplicate, and the activity was determined as described previously (Miller, 1972).

Acknowledgements

This work was supported, in part, by grant AI49561 from the NIH to E.A.G., who is an Investigator of the Howard Hughes Medical Institute.

References

Bechinger, B. (1997) Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J Membr Biol* **156:** 197–211.

Bishop, R.E., Gibbons, H.S., Guina, T., Trent, M.S., Miller, S.I., and Raetz, C.R. (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. *EMBO J* **19:** 5071–5080.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37:** 911–917.

Brodsky, I.E., Ernst, R.K., Miller, S.I., and Falkow, S. (2002) *mig-14* is a *Salmonella* gene that plays a role in bacterial resistance to antimicrobial peptides. *J Bacteriol* **184:** 3203–3213.

Chan, S., and Reinhold, V.N. (1994) Detailed structural characterization of lipid A: electrospray ionization coupled with tandem mass spectrometry. *Anal Biochem* **218:** 63–73.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158:** 9–14.

Dartigalongue, C., Missiakas, D., and Raina, S. (2001) Characterization of the *Escherichia coli* sigma E regulon. *J Biol Chem* **276:** 20866–20875.

Darveau, R.P., Blake, J., Seachord, C.L., Cosand, W.L., Cunningham, M.D., Cassiano-Clough, L., and Maloney, G. (1992) Peptides related to the carboxyl terminus of human platelet factor IV with antibacterial activity. *J Clin Invest* **90:** 447–455.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Davis, R.W., Bolstein, D., and Roth, J.R. (1980) *Advanced Bacterial Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Detweiler, C.S., Monack, D.M., Brodsky, I.E., Mathew, H., and Falkow, S. (2003) *virK*, *somA* and *rcsC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. *Mol Microbiol* **48:** 385–400.

Ellermeier, C.D., Janakiraman, A., and Slauch, J.M. (2002) Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* **290:** 153–161.

Ernst, R.K., Yi, E.C., Guo, L., Lim, K.B., Burns, J.L., Hackett, M., and Miller, S.I. (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **286:** 1561–1565.

Ernst, R.K., Guina, T., and Miller, S.I. (2001) *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect* **3:** 1327–1334.

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.

Garcia Vescovi, E., Soncini, F.C., and Groisman, E.A. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84:** 165–174.

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

Groisman, E.A., Heffron, F., and Solomon, F. (1992b) Molecular genetic analysis of the *Escherichia coli phoP* locus. *J Bacteriol* **174:** 486–491.

Groisman, E.A., Kayser, J., and Soncini, F.C. (1997) Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J Bacteriol* **179:** 7040–7045.

Guina, T., Yi, E.C., Wang, H., Hackett, M., and Miller, S.I. (2000) A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar Typhimurium promotes resistance to alpha-helical antimicrobial peptides. *J Bacteriol* **182:** 4077–4086.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S.I. (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27:** 1171–1182.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., and Miller, S.I. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95:** 189–198.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580.

Hilbert, F., del Portillo, F.G., and Groisman, E.A. (1999) A periplasmic D-alanyl-D-alanine dipeptidase in the gram-negative bacterium *Salmonella enterica*. *J Bacteriol* **181:** 2158–2165.

Johnson, R.S., Her, G.R., Grabarek, J., Hawiger, J., and Reinhold, V.N. (1990) Structural characterization of monophosphoryl lipid A homologs obtained from *Salmonella minnesota* Re595 lipopolysaccharide. *J Biol Chem* **265:** 8108–8116.

Karbarz, M.J., Kalb, S.R., Cotter, R.J., and Raetz, C.R. (2003) Expression cloning and biochemical characterization of a *Rhizobium leguminosarum* lipid A 1-phosphatase. *J Biol Chem* **278:** 39269–39279.

Kox, L.F., Wosten, M.M., and Groisman, E.A. (2000) A small protein that mediates the activation of a two-component system by another two-component system. *EMBO J* **19:** 1861–1872.

Llama-Palacios, A., Lopez-Solanilla, E., Poza-Carrion, C., Garcia-Olmedo, F., and Rodriguez-Palenzuela, P. (2003) The *Erwinia chrysanthemi phoP–phoQ* operon plays an important role in growth at low pH, virulence and bacterial survival in plant tissue. *Mol Microbiol* **49:** 347–357.

Ludtke, S.J., He, K., Heller, W.T., Harroun, T.A., Yang, L., and Huang, H.W. (1996) Membrane pores induced by magainin. *Biochemistry* **35:** 13723–13728.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., *et al.* (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413:** 852–856.

McLeod, G.I., and Spector, M.P. (1996) Starvation- and

stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in *Salmonella typhimurium* is RpoS (sigma S) independent and occurs through both PhoP-dependent and -independent pathways. *J Bacteriol* **178:** 3683–3688.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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.

Moss, J.E., Fisher, P.E., Vick, B., Groisman, E.A., and Zychlinsky, A. (2000) The regulatory protein PhoP controls susceptibility to the host inflammatory response in *Shigella flexneri*. *Cell Microbiol* **2:** 443–452.

Nicolas, P., and Mor, A. (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annu Rev Microbiol* **49:** 277–304.

Norrander, J., Kempe, T., and Messing, J. (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26:** 101–106.

Orndorff, P.E., and Dworkin, M. (1980) Separation and properties of the cytoplasmic and outer membranes of *Myxococcus xanthus*. *J Bacteriol* **141:** 914–927.

Peschel, A. (2002) How do bacteria resist human antimicrobial peptides? *Trends Microbiol* **10:** 179–186.

Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M.C., and Trieu-Cuot, P. (2003) Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. *Mol Microbiol* **49:** 1615–1625.

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.

Rosner, M.R., Tang, J., Barzilay, I., and Khorana, H.G. (1979) Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. *J Biol Chem* **254:** 5906–5917.

Sanderson, K.E., Hessel, A., and Rudd, K.E. (1995) Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol Rev* **59:** 241–303.

Sarvamangala Devi, J.N., and Shivananda, P.G. (1983) Frogs as natural reservoirs of *Salmonella*. *Ind J Med Res* **78:** 465–470.

Snavely, M.D., Miller, C.G., and Maguire, M.E. (1991) The *mgtB* Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. *J Biol Chem* **166:** 815–823.

Soncini, F.C., and Groisman, E.A. (1996) Two-component regulatory systems can interact to process multiple environmental signals. *J Bacteriol* **178:** 6796–6801.

Soncini, F.C., Vescovi, E.G., and Groisman, E.A. (1995) Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* **177:** 4364–4371.

Uzzau, S., Figueroa-Bossi, N., Rubino, S., and Bossi, L. (2001) Epitope tagging of chromosomal genes in *Salmonella*. *Proc Natl Acad Sci USA* **98:** 15264–15269.

Wosten, M.M., Kox, L.F., Chamnongpol, S., Soncini, F.C., and Groisman, E.A. (2000) A signal transduction system that responds to extracellular iron. *Cell* **103:** 113–125.

Yamato, I., Anraku, Y., and Hirosawa, K. (1975) Cytoplasmic membrane vesicles of *Escherichia coli*. A simple method for preparing the cytoplasmic and outer membranes. *J Biochem (Tokyo)* **77:** 705–718.

Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* **84:** 5449–5453.

Zasloff, M. (1992) Antibiotic peptides as mediators of innate immunity. *Curr Opin Immunol* **4:** 3–7.

Zasloff, M. (2001) The commercial development of the antimicrobial peptide pexiganan. In *Development of Novel Antimicrobial Agents: Emerging Strategies*. Lohner, K. (ed.). Wymondham, UK: Horizon Scientific Press, pp. 261–270.

Zhou, Z., Lin, S., Cotter, R.J., and Raetz, C.R. (1999) Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH_4VO_3 in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. *J Biol Chem* **274:** 18503–18514.