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Identification of the lipopolysaccharide modifications controlled by the *Salmonella* PmrA/PmrB system mediating resistance to Fe(III) and Al(III)

Kunihiko Nishino,[1†] Fong-Fu Hsu,[2] John Turk,[2] Michael J. Cromie,[1] Marc M. S. M. Wösten[1‡] and Eduardo A. Groisman[1*]

[1]*Department of Molecular Microbiology, Howard Hughes Medical Institute, and* [2]*Mass Spectrometry Resource, Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA.*

Summary

Iron is an essential metal but can be toxic in excess. While several homeostatic mechanisms prevent oxygen-dependent killing promoted by Fe(II), little is known about how cells cope with Fe(III), which kills by oxygen-independent means. Several Gram-negative bacterial species harbour a regulatory system – termed PmrA/PmrB – that is activated by and required for resistance to Fe(III). We now report the identification of the PmrA-regulated determinants mediating resistance to Fe(III) and Al(III) in *Salmonella enterica* serovar Typhimurium. We establish that these determinants remodel two regions of the lipopolysaccharide, decreasing the negative charge of this major constituent of the outer membrane. Remodelling entails the covalent modification of the two phosphates in the lipid A region with phosphoethanolamine and 4-aminoarabinose, which has been previously implicated in resistance to polymyxin B, as well as dephosphorylation of the Hep(II) phosphate in the core region by the PmrG protein. A mutant lacking the PmrA-regulated Fe(III) resistance genes bound more Fe(III) than the wild-type strain and was defective for survival in soil, suggesting that these PmrA-regulated lipopolysaccharide modifications aid *Salmonella*'s survival and spread in non-host environments.

Accepted 6 June, 2006. *For correspondence. E-mail groisman@borcim.wustl.edu; Tel. (+1) 314 362 3692; Fax (+1) 314 747 8228. Present addresses: †Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, 8–1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan; ‡Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, the Netherlands.

Introduction

Two major forms of iron are found on our planet: Fe(II) in reducing intracellular environments, and Fe(III) in aerobic atmosphere. While iron is essential for several biological processes, Fe(II) overload leads to cellular malfunctions as a result of oxygen-dependent iron-stimulated free radical reactions (Halliwell and Gutteridge, 1992; Touati, 2000). Thus, organisms control the Fe(II) concentration in the various cellular compartments by tightly regulating uptake, storage and secretion (Nelson, 1999). Although once considered non-cytotoxic (Braun, 1997; Bruins *et al*., 2000), Fe(III) has bactericidal activity and this activity is different from that promoted by Fe(II) as Fe(III) exerts its bactericidal effect in an oxygen-independent fashion (Chamnongpol *et al*., 2002).

The PmrA/PmrB two-component regulatory system is activated specifically by extracytoplasmic Fe(III) and Al(III) (Wosten *et al*., 2000) and is required for resistance to Fe(III) in several enteric species (Chamnongpol *et al*., 2002). The PmrA/PmrB system governs the expression of proteins that confer resistance to the cationic antibiotic polymyxin B by modifying the lipid A phosphates in the lipopolysaccharide (LPS) with 4-aminoarabinose and phosphoethanolamine (Groisman *et al*., 1997; Gunn *et al*., 1998; Lee *et al*., 2004). The connection that exists between resistance to polymyxin B and to Fe(III), however, is presently unclear. Although a non-cytotoxic form of polymyxin B – termed polymyxin B nonapeptide (Vaara and Vaara, 1983) – could partially protect a *Salmonella pmrA* mutant from Fe(III)-mediated killing (Chamnongpol *et al*., 2002) inactivation of the PmrA-activated loci responsible for the lipid A modification with 4-aminoarabinose (i.e. *pbgP*) or phosphoethanolamine (i.e. *pmrC*) did not render the organism susceptible to Fe(III) (Lee *et al*., 2004).

The PmrA-activated *pbgPE* operon [also referred to as *arn* (Breazeale *et al*., 2003) and *pmrF* (Gunn *et al*., 1998)] consists of seven genes. The first five genes encode proteins that participate in the biosynthesis and incorporation of 4-aminoarabinose into lipid A (Raetz and Whitfield, 2002), but are not required for resistance to Fe(III) (Wosten *et al*., 2000). On the other hand, strains with in-frame deletions in either of the last two genes of the *pbgPE* operon (designated *pbgE2* and *pbgE3*) could

not grow in the presence of 100 μM Fe(III) (Wosten *et al*., 2000). They differed instead in their resistance to polymyxin B: a *pbgE2* mutant was hypersensitive to polymyxin B whereas a *pbgE3* mutant was not (Gunn *et al*., 2000). The biochemical function of the PbgE2 and PbgE3 proteins remains unknown. The PmrA-activated *yibD* and *dgoA* genes are dispensable for resistance to polymyxin B and Fe(III) (Tamayo *et al*., 2002).

Here we report the identification of the PmrA-regulated cell envelope modifications mediating resistance to Fe(III) and Al(III). We establish that Fe(III) resistance entails both modifications of the lipid A region of the LPS previously implicated in resistance to polymyxin B as well as dephosphorylation of one of the phosphates in the core region of the LPS by the PmrA-activated PmrG protein. We demonstrate that these modifications lower the overall negative charge in the bacterial cell surface, which reduces binding of Fe(III) and helps *Salmonella* survive in soil.

Results

The pbgE2 *and* pbgE3 *genes are not directly involved in Fe(III) resistance*

We determined that the minimal inhibitory concentration (MIC) for Fe(III) of strains deleted for the *pbgE2* or *pbgE3* genes is 50 μM, which is similar to the MIC of the Δ*pmrAB* mutant (i.e. 100 μM) and much lower than the MIC of the wild-type strain or mutants defective in either the *pbgP* or *ugd* genes (i.e. 3.2 mM) (Table 1). Plasmids expressing the *pbgE2* or *pbgE3* genes from a derivative of the *tet* promoter restored wild-type levels of resistance to the *pbgE2* and *pbgE3* mutants respectively (Table 1). Interestingly, wild-type levels of Fe(III) resistance could also be restored to the *pbgE2* or *pbgE3* mutants upon inactivation of the *pbgP* or *ugd* genes (Table 1), suggesting that the *pbgE2* and *pbgE3* gene products are not directly involved in Fe(III) resistance. Consistent with this notion, the plasmids expressing the *pbgE2* or *pbgE3* genes or one that expressed both *pbgE2* and *pbgE3* from the *tet* promoter derivative failed to confer Fe(III) resistance upon the Δ*pmrAB* mutant (Table 1). Thus, the connection between the *pbgE2* or *pbgE3* genes and Fe(III) resistance was not investigated further.

Table 1. MIC of Fe^{3+} against mutants deleted in PmrA-regulated genes and in mutants expressing identified iron-resistance genes.

Strain	$FeCl_3$ MIC (μM)
Wild-type	3200
Δ*pmrAB*	100
Δ*pbgE2*	50
Δ*pbgE3*	50
pbgP	3200
Ugd	3200
Δ*pbgE2*/vector	50
Δ*pbgE2*/p*pbgE2*	3200
Δ*pbgE3*/vector	50
Δ*pbgE3*/p*pbgE3*	3200
*pbgP*Δ*pbgE2*	3200
*pbgP*Δ*pbgE3*	3200
*ugd*Δ*pbgE2*	3200
*ugd*Δ*pbgE3*	3200
Δ*pmrAB*/vector	100
Δ*pmrAB*/p*pmrAB*	3200
Δ*pmrAB*/vector	100
Δ*pmrAB*/p*pbgE2*	100
Δ*pmrAB*/p*pbgE3*	100
Δ*pmrAB*/p*pbgE2E3*	100
Δ*pmrG*	3200
Δ*pmrC*	3200
Δ*pbgPE*	3200
Δ*ugd*	3200
Δ*yibD*	3200
Δ*pbgPE pmrC ugd pmrG yibD*	100
Δ*pmrC ugd pmrG yibD*	100
Δ*pmrC ugd*	1600
Δ*pmrC pmrG*	3200
Δ*pmrC yibD*	3200
Δ*yibD ugd*	3200
Δ*yibD pmrG*	3200
Δ*ugd pmrG*	3200
Δ*pmrC ugd yibD*	1600
Δ*pmrC pmrG yibD*	3200
Δ*ugd pmrG yibD*	3200
Δ*pmrC ugd pmrG*	100
*pmrA505*Δ*pmrC ugd pmrG*	100
Δ*pmrC ugd pmrG*/vector	100
Δ*pmrC ugd pmrG*/p*pmrC*	3200
Δ*pmrC ugd pmrG*/p*ugd*	1600
Δ*pmrC ugd pmrG*/p*pmrG*	1600
Δ*pmrG pbgPE*	3200
Δ*pmrC pbgPE*	1600
Δ*pmrC pbgPE pmrG*	100

Identification of Fe(III) resistance genes

To uncover the PmrA-regulated genes mediating Fe(III) resistance, we prepared a genomic library from the Fe(III)-susceptible Δ*pmrAB* mutant in the multicopy number plasmid pBR322 (Bolivar *et al*., 1977). Plasmid DNA was isolated from a pool of 26 000 transformants, introduced into the same Δ*pmrAB* mutant and transformants that could grow on agar plates containing 200 μM Fe(III) and 50 μg ml^{-1} ampicillin were recovered. The rationale behind this strategy was that a PmrA-regulated Fe(III) resistance gene(s) might be expressed from the *tet* promoter in pBR322 and phenotypically rescue the Δ*pmrAB* mutant. Then, we isolated plasmid DNA from each of 52 purified transformants and used it to retransform the Δ*pmrAB* mutant. All 52 plasmids conferred Fe(III) resistance, indicative that the plasmids harboured Fe(III) resistance genes.

Sequence analysis of the inserts in the 52 plasmid clones revealed that DNA originating from seven different regions of the *Salmonella* chromosome could confer Fe(III) resistance upon the Δ*pmrAB* mutant (Fig. S1). These inserts encompassed a total of 16 open reading frames (ORFs), which were individually subcloned into

pBR322. Eight of the resulting subclones could still confer Fe(III) resistance upon the Δ*pmrAB* mutant but to different degrees (Fig. S2). We focused our attention on the *pmrC* and *pmrG* genes because they bestowed the highest levels of Fe(III) resistance upon the Δ*pmrAB* mutant (Fig. S2), and because they were known to be directly regulated by the PmrA protein (Gunn *et al*., 1998; Wosten and Groisman, 1999); this is in contrast to the six other genes, whose expression was not PmrA-dependent (data not shown).

The PmrA-activated ugd, pbgP, pmrC *and* pmrG *genes are required for Fe(III) resistance*

To examine whether the *pmrG* and *pmrC* genes are necessary for Fe(III) resistance, we constructed strains deleted for the chromosomal copies of these genes (see *Experimental procedures*). Both Δ*pmrG* and Δ*pmrC* single mutants and a Δ*pmrG pmrC* double mutant behaved like the wild-type parent (Table 1). We also made double mutants deleted in additional PmrA-regulated genes or operons (i.e. *ugd*, *yibD*, and the *pbgPE* operon). These mutants also resembled the wild-type parent in terms of MIC for Fe(III) (Table 1). Thus, we constructed strains deleted for multiple PmrA-regulated genes with the hope of creating a strain that recapitulated the Fe(III) hypersensitivity of the Δ*pmrAB* mutant.

A strain deleted for the *pbgPE* operon and the *pmrC*, *ugd*, *pmrG* and *yibD* genes exhibited the same hypersensitivity to Fe(III) as the Δ*pmrAB* mutant (Table 1). This was also true for a strain deleted for the *pmrC*, *ugd*, *pmrG* and *yibD* genes (Table 1), which is consistent with the fact that the proteins encoded by the *ugd* gene and *pbgP* operon participate in the same pathway of synthesis and incorporation of 4-aminoarabinose into lipid A (Gunn *et al*., 1998; Zhou *et al*., 2001; Breazeale *et al*., 2003). We investigated all possible combinations of double and triple mutants with deletions in the *pmrC*, *ugd*, *pmrG* and *yibD* genes and determined that only the Δ*pmrC ugd pmrG* triple mutant was as hypersensitive to Fe(III) as the Δ*pmrAB* mutant (Table 1). Furthermore, a strain deleted for of all three *pmrC*, *ugd* and *pmrG* genes and harbouring the *pmrA505* allele, which encodes a PmrA protein that promotes transcription of PmrA-activated genes even under non-inducing conditions (Kox *et al*., 2000), was as susceptible to Fe(III) as the Δ*pmrAB* mutant (Table 1). Collectively, these results strongly suggest that the *pmrC*, *ugd* and *pmrG* genes and the *pbgPE* operon are the only PmrA-regulated determinants required for Fe(III) resistance.

The Fe(III)-susceptible mutants display increased Fe(III) binding

The LPS is one of the major components and the most accessible surface molecule in the outer leaflet of the bacterial outer membrane. It consists of three structurally distinct regions: the outermost O-antigen, a central core, and the innermost lipid A (Raetz and Whitfield, 2002). The *pmrC* gene product and the proteins encoded in the *ugd* gene and *pbgPE* operon mediate the modification of the two lipid A phosphates with phosphoethanolamine (Lee *et al*., 2004) and 4-aminoarabinose (Gunn *et al*., 1998) respectively. This suggested that Fe(III) resistance may result from hindering electrostatic interactions between the positively charged Fe(III) and the negatively charged phosphates in the lipid A; and it predicted that the Fe(III)-susceptible mutants would bind more iron than the wild-type strain. Consistent with this notion, there were 3–3.5 times more ^{59}Fe associated with the Δ*pmrAB* mutant and the Δ*pmrC ugd pmrG* triple mutant than with the wild-type strain (Fig. 1A). On the other hand, ^{59}Fe association with the *pmrC*, *ugd* and *pmrG* single mutants was similar to that exhibited by the wild-type parent (Fig. 1A). These results demonstrated that those mutants that bind more Fe(III) are more readily killed by this metal.

Polymyxin B nonapeptide rescues the ΔpmrC ugd pmrG *triple mutant from Fe(III)-mediated killing*

Modification of the lipid A phosphates with phosphoethanolamine and 4-aminoarabinose is required for resistance to both polymxyin B (Gunn *et al*., 1998; Lee *et al*., 2004) and Fe(III) (Table 1), raising the possibility that these bactericidal agents bind to similar or adjacent sites in lipid A. Consistent with this notion, the non-toxic polymyxin B nonapeptide, which binds to lipid A like the bactericidal polymyxin B (Yin *et al*., 2003), could partially rescue the Δ*pmrC ugd pmrG* triple mutant from Fe(III)-mediated killing (Fig. 1B). These results reinforce the notion that Fe(III) resistance is associated with modification or occlusion of the lipid A phosphates in the LPS.

PmrG is a phosphatase that targets the Hep(II) phosphate in the core region of the LPS

We then turned our attention to the *pmrG* mutant and determined that it exhibited a wild-type lipid A profile and resistance to polymyxin B (Fig. S3 and data not shown), which was in contrast to the phenotype of strains defective in the *pmrC*, *pbgP* or *ugd* genes (Gunn *et al*., 1998; Lee *et al*., 2004). As overexpression of the *pmrG* gene did not alter the lipid A profile of the Δ*pmrC ugd pmrG* triple mutant (Fig. S3), our findings implied that the PmrG protein was not likely to target the lipid A.

A hint to the potential function of the PmrG protein came from an independent genetic screening in which we

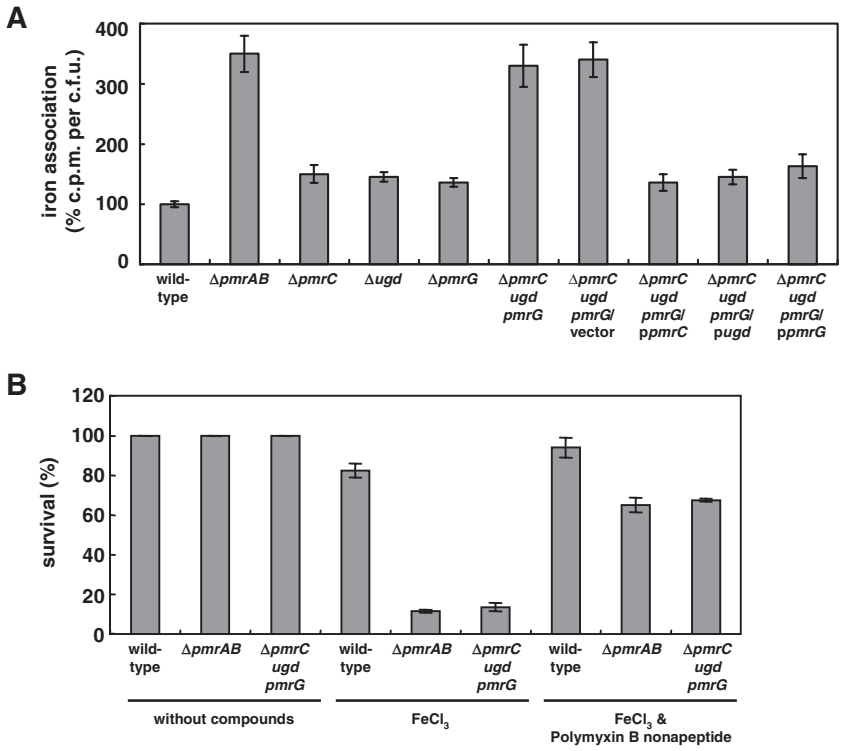

Fig. 1. Increased Fe(III) binding to the bacterial cell surface of Fe(III) hypersensitive mutants and rescue by polymyxin B nonapeptide.
A. Association of ^{59}Fe with wild-type (14028s), Δ*pmrAB* (EG13937), Δ*pmrC* (EG16626), Δ*ugd* (EG16627), Δ*pmrG* (EG16628) and Δ*pmrC ugd pmrG* (EG16639) strains, and with the Δ*pmrC ugd pmrG* mutant expressing the *pmrC* (EG15771), *ugd* (EG15753), or *pmrG* (EG15751) genes.
B. Survival of wild-type(14028s), Δ*pmrAB* (EG13937) and Δ*pmrC ugd pmrG* (EG16639) strains after incubation in the presence of $FeCl_3$ in the presence or absence of the non-toxic polymyxin B nonapeptide or with no compounds added.

mutagenized the Δ*pmrAB* strain and isolated pseudorevertants that could grow on agar plates containing 400 μM Fe(III). One of the pseudorevertants harboured a EZ-Tn*5* (http://www.epibio.com/category.asp?id=284) transposon insertion in the *rfaY* gene (also known as *waaY*), which is necessary for phosphorylating the Hep(II) heptose in the core region of the LPS (Yethon *et al*., 1998) (Fig. 2A). Reconstruction experiments demonstrated that deletion of the *rfaY* gene suppressed Fe(III)-mediated killing not only in the Δ*pmrAB* strain but also in the Δ*pmrC pmrG ugd* triple mutant (Table 2), restoring Fe(III) resistance to the levels exhibited by the Δ*pmrAB* strain harbouring the plasmid expressing the wild-type *pmrG* gene (Table 2).

Table 2. MIC of Fe^{3+} and iron survival of wild-type (14028s), Δ*pmrAB* (EG13937) and Δ*pmrC ugd pmrG* (EG16639) strains with additional mutation of the *rfaY* gene (EG14913 and EG16919) or overexpressing the *pmrG* gene (EG16765).

Strain	$FeCl_3$ MIC (μM)
Wild-type	3200
Δ*pmrAB*/vector	100
Δ*pmrAB*/p*pmrG*	1600
Δ*pmrAB*	100
Δ*pmrAB rfaY*	1600
Δ*pmrC ugd pmrG*	100
Δ*pmrC ugd pmrG rfaY*	1600

We hypothesized that the PmrG protein might counteract the action of the RfaY protein, removing the phosphate from the Hep(II) heptose. Thus, we purified a C-terminally His-tagged PmrG protein (Fig. 2B) and investigated its ability to dephosphorylate the core region of the LPS. The PmrG-His protein exhibited phosphatase activity towards core oligosaccharide (OS) prepared from the wild-type strain (Fig. 2C), but not against OS originating from the Δ*rfaY* mutant (Fig. 2C), which lacks the phosphate on Hep(II) (Yethon *et al*., 1998). The data presented above indicate that the PmrG protein encodes a phosphatase that targets the Hep(II) phosphate in the core region of the LPS.

PmrG protein is a periplasmic protein

To examine the subcellular location of the PmrG protein, we used a *Salmonella* strain that expressed a PmrG-FLAG protein from the normal chromosomal *pmrG* promoter, and also the cytoplasmic enzyme β-galactosidase, which served as a marker for the purity of the extracytoplasmic fractions. Western blot analysis with anti-FLAG antibodies demonstrated that the PmrG-FLAG protein localizes to the periplasmic region (Fig. 3D), as predicted by the PSORT program (http://www.psort.org/). We also noticed a slightly larger band in the cytoplasmic fraction (Fig. 3D), which could correspond to the full-length (i.e. unprocessed) form of the PmrG protein.

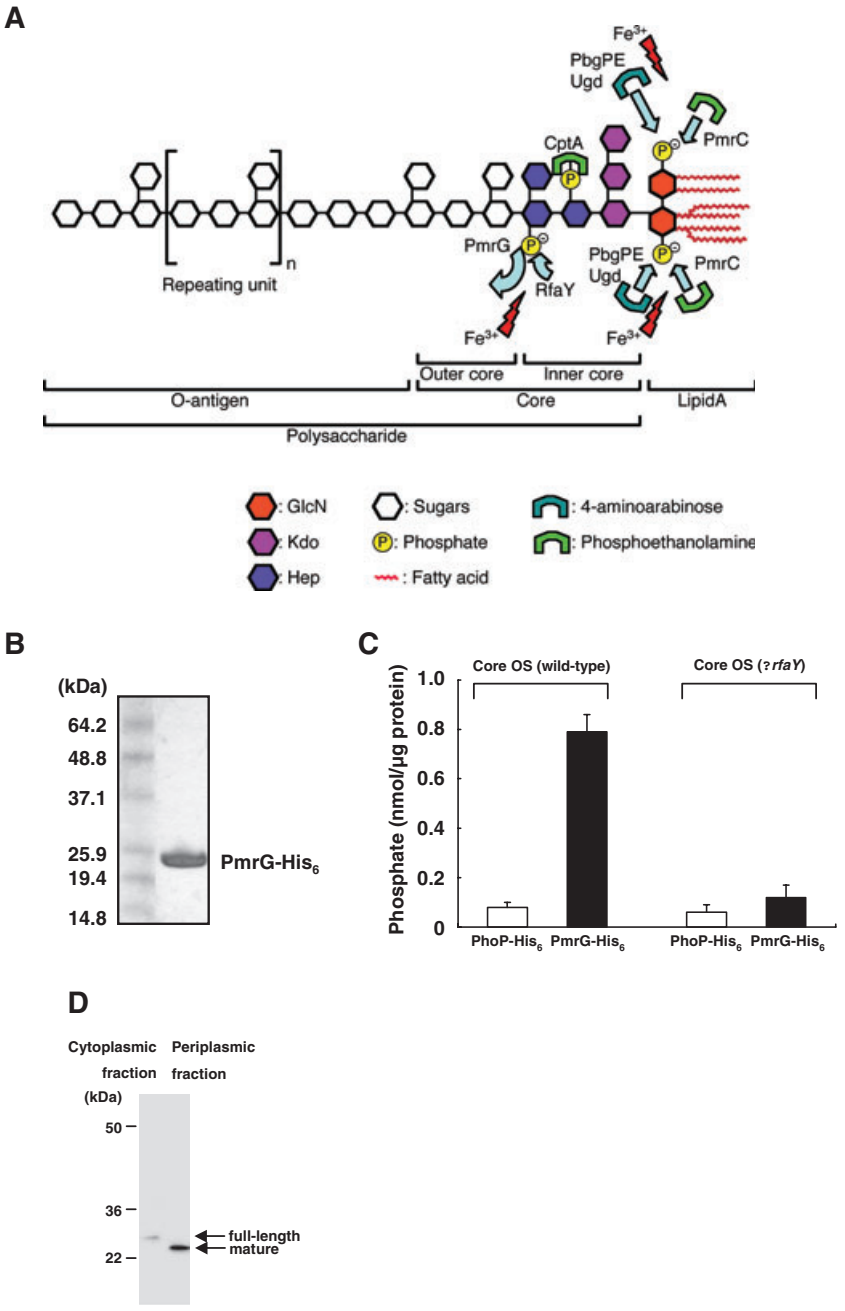

Fig. 2. PmrG is a phosphatase that targets the Hep(II) phosphate and localizes to the periplasm.
A. Schematic representation of the LPS structure indicating the phosphates that are targeted by the Fe(III) resistance determinants. The PmrC protein mediates the modification of the lipid A phosphates with phosphoehanolamine whereas the Ugd and PbgPE proteins modifies the lipid A phosphates with 4-aminoarabinose. The PmrG protein removes the phosphate from Hep(II) in the inner core region of the LPS, which is normally introduced by the RfaY protein.
B. SDS-PAGE gel of the purified PmrG-His_6 protein prepared as described in *Experimental procedures*.
C. Phosphatase activity of the purified PmrG-His protein was determined on core OS prepared from wild-type (14028s) or the phosphate-deficient Hep(II) Δ*rfaY* mutant (EG16916). The PhoP-His_6 protein was used as negative control.
D. Subcellular localization of chromosomally encoded PmrG-FLAG protein was determined by Western blotting of cytoplasmic and periplasmic extracts from strain EG16142. Purity of the preparations was verified by measuring the β-galactosidase activity of the cytoplasmic and periplasmic fractions.

Modification of the Hep(I) phosphate with phosphoethanolamine is not required for Fe(III) resistance

The core region of the LPS has two phosphates: one at Hep(II) that is targeted by PmrG (Fig. 2C), and one at Hep(I) that can be modified with phosphoethanolamine by the PmrA-activated *cptA* gene product (Tamayo *et al.*, 2005). We determined that the latter modification is not required for Fe(III) resistance because deletion of the *cptA* gene did not alter the susceptibility of the wild-type strain, the Δ*pmrAB* mutant, the Δ*pmrC ugd*, Δ*pmrC pmrG* and Δ*ugd pmrG* double mutants, or the Δ*pmrC ugd pmrG* triple mutant (Table S3).

Metals that induce the PmrA/PmrB system promote killing of the ΔpmrC ugd pmrG *mutant*

Al(III), which had been previously shown to be an inducer of the PmrA/PmrB system (Wosten *et al.*, 2000), also displayed bactericidal activity against the Δ*pmrC ugd pmrG* triple mutant (Table 3). On the other hand, neither Ca^{2+} nor Co^{2+}, K^{+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ga^{3+} or Ru^{3+} (at

Table 3. MIC of Al(III) for wild-type (14028s) and mutants (EG13937 and EG16639) exhibiting increased susceptibility to Fe(III).

Strain	$Al_2(SO_4)_3$ MIC (μM)
Wild-type	3200
Δ*pmrAB*	100
Δ*pmrC ugd pmrG*	100
Δ*pmrC ugd pmrG*/vector	100
Δ*pmrC ugd pmrG*/p*pmrC*	3200
Δ*pmrC ugd pmrG*/p*ugd*	1600
Δ*pmrC ugd pmrG*/p*pmrG*	1600

concentrations of up to 1 mM) could induce PmrA-activated genes (Wosten *et al*., 2000) or kill the Δ*pmrC ugd pmrG* triple mutant (data not shown). This suggests that there is correlation between the ability of a cation to activate the PmrA/PmrB system and its ability to kill the Δ*pmrC ugd pmrG* triple mutant.

The LPS modifications mediating Fe(III) resistance enhance survival in soil

Salmonella can survive in non-host environments such as soil and water for extended periods of time (Winfield and Groisman, 2003). Because Fe(III) and Al(III) are the most abundant metals in soil (Pina and Cervantes, 1996; Giesler *et al*., 2005) and soil is an environment that activates the PmrA/PmrB system (Chamnongpol *et al*., 2002), we hypothesized that the PmrA-regulated genes implicated in resistance to Fe(III) and Al(III) would be required for survival in soil. Indeed, the Δ*pmrC ugd pmrG* triple mutant was recovered 100-fold less than the wild-type strain following incubation in soil (Fig. 3).

Discussion

The LPS is the major constituent of the outer layer of the bacterial outer membrane. This makes the LPS a critical determinant of the permeability barrier that renders Gram-negative bacteria resistant to a variety of noxious compounds (Raetz and Whitfield, 2002). The phosphates in the lipid A and core regions of the LPS are responsible for the negative charge that characterizes the bacterial cell surface. This negative charge mediates the electrostatic interaction with cationic bactericidal compounds such as certain antimicrobial peptides and the metals Fe(III) and Al(III). Consistent with this notion, covalent modification and/or removal of the LPS phosphates can prevent binding and killing by these bactericidal agents.

Fe(III) and Al(III) are the specific signals that activate the *Salmonella* PmrA/PmrB regulatory system (Wosten *et al*., 2000), and exhibit bactericidal activity towards a Δ*pmrAB* mutant (Tables 1 and 3). The PmrA/PmrB system has been previously shown to control the expression of proteins mediating the modification of the two lipid A phosphates with 4-aminoarabinose and phosphoethanolamine (Groisman *et al*., 1997; Gunn *et al*., 1998; Lee *et al*., 2004), which results in resistance to the cationic peptide antibiotic polymyxin B. Likewise, the PmrA-activated *cptA* gene is responsible for the phosphoethanolamine modification of the Hep(I) phosphate in the core region (Tamayo *et al*., 2005). We have now determined that the PmrA-activated PmrG protein is a phosphatase that removes the phosphate from the Hep(II) phosphate. Together, these data suggest that a major role of the PmrA/PmrB system is to govern the removal of negative charges conferred by the phosphates in the LPS.

Inactivation of the PmrA-activated genes mediating the modification of the two lipid A phosphates and the Hep(II) phosphate in the core region resulted in a strain (i.e. the Δ*pmrC ugd pmrG* triple mutant) that was as susceptible to Fe(III) as the Δ*pmrAB* mutant (Table 1) and that bound more iron than the wild-type strain (Fig. 1B). This is in contrast to *Salmonella* strains defective in only one or two PmrA-regulated Fe(III) resistance genes, which retained wild-type levels of resistance (Table 1) and binding (Fig. 1B) to Fe(III). That the latter mutants did not exhibit intermediate susceptibility phenotypes between those displayed by the wild-type strain and the triple mutant implies that the bactericidal activity of Fe(III) and Al(III) may require concurrent binding of these trivalent metals to the three LPS phosphates discussed above. This binding may be responsible for the destabilization of the outer membrane that renders *Salmonella* susceptible to lysis by the

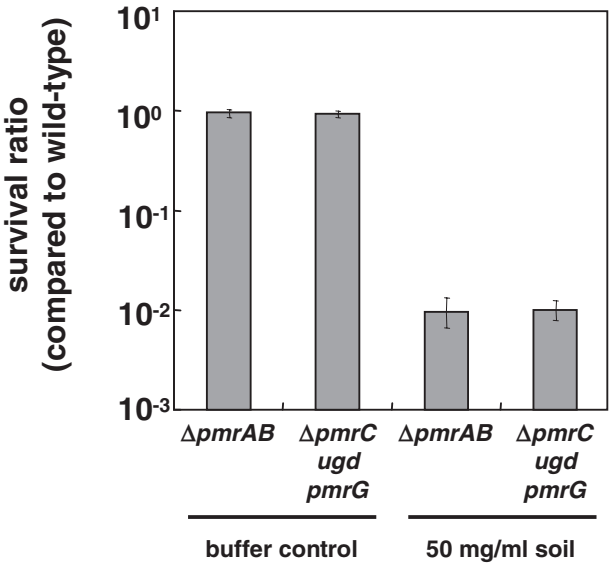

Fig. 3. Fe(III) resistance determinants are necessary for survival in soil. Fe(III) sensitive mutants are impaired for growth in soil (EG13937 and EG16639). Survival ratio is the ratio of mutants to wild-type *Salmonella* (14028s).

detergent deoxycholate and to killing by vancomycin (Chamnongpol *et al*., 2002), agents that cannot normally traverse the outer membrane of Gram-negative bacteria (Nikaido, 2003). This effect could be due to the displacement of divalent cations such as Mg^{2+} that stabilize the outer membrane by cross-linking adjacent LPS molecules (Raetz and Whitfield, 2002). Indeed, excess Mg^{2+} prevented Fe(III)-mediated killing (Chamnongpol *et al*., 2002), suggesting that Mg^{2+} and Fe(III) share binding sites in the LPS.

There appears to be an overlap between the sites targeted by Fe(III) and Al(III) and by polymyxin B because modification of the lipid A with phosphoethanolamine and 4-aminoarabinose is required for resistance to both the trivalent metals (this work) and the antibiotic (Groisman *et al*., 1997; Gunn *et al*., 1998; Lee *et al*., 2004). Moreover, the non-toxic polymyxin B nonapeptide could rescue the Δ*pmrC ugd pmrG* triple mutant from Fe(III)-mediated killing (Fig. 1C). Yet, this overlap is only partial because the PmrG-mediated dephosphorylation of the Hep(II) phosphate is necessary for resistance to Fe(III) but not to polymyxin B, and because an *rfaY* mutation restored resistance to Fe(III) but not to polymyxin B upon the Δ*pmrC ugd pmrG* mutant.

There is a correlation between the ability of a metal to activate the *Salmonella* PmrA/PmrB system and its ability to kill the Δ*pmrC ugd pmrG* triple mutant. This is most striking in the inability of Ga^{3+} to exert either activity (this work) (Wosten *et al*., 2000) despite having similar solution and co-ordination chemistries as Fe(III), and to a lesser extent Al(III). The reasons for these findings are presently unclear but do not appear to be related to the contrasting capacities of Fe(III) and Ga^{3+} to be reduced (which are high and low respectively) because aluminium's only valency is three, yet it behaves like Fe(III) with respect to activation of the PmrA/PmrB system and bacterial killing.

Whereas the PmrA/PmrB system responds to Fe(III) and is required for resistance to Fe(III) in both *Salmonella* and *Escherichia coli* (Wosten *et al*., 2000; Chamnongpol *et al*., 2002; Winfield and Groisman, 2004), it appears that its role in sensing and responding to Zn^{2+} is different in these two enteric species. In *Salmonella*, the PmrA/PmrB system neither responds to Zn^{2+} (Wosten *et al*., 2000) nor is required for resistance to Zn^{2+} (this work). By contrast, Zn^{2+} has been shown to promote transcription of the *pmrA* and *pmrB* genes as well as that of several PmrA-activated genes in *E. coli* (Lee *et al*., 2005). Zn^{2+} also inhibited the growth of *pmrA* and *pmrB* mutants of *E. coli*, which displayed a longer lag phase in the presence of Zn^{2+} than the wild-type strain (Lee *et al*., 2005).

Finally, the Δ*pmrC ugd pmrG* triple mutant could not survive in soil (Fig. 3), which is an environment that induces transcription of PmrA-activated genes (Chamnongpol *et al*., 2002), possibly because Fe(III) and Al(III) are the most abundant metals in soil (Pina and Cervantes, 1996; Giesler *et al*., 2005). This defect may be exacerbated in mild-acid conditions, such as those created by acid rain, which accelerate the elution of Fe(III) and Al(III) from soil (Taborsky, 1991; Pina and Cervantes, 1996; Morrill *et al*., 2004). This implicates the PmrA/PmrB system in microbial survival in non-host environments, which may aid *Salmonella* transmission to new hosts.

Experimental procedures

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table S1. For plasmid constructions, genes were amplified from the wild-type strain 14028s genomic DNA by PCR with primers listed in Table S2. The PCR fragments were cloned between the restriction enzyme sites of vector pBR322 as indicated in Table S1, which resulted in these genes being present in the same orientation and downstream of the *tet* promoter of the pBR322 vector (Bolivar *et al*., 1977). The construction of strains with deletions in particular genes was performed as described (Datsenko and Wanner, 2000) with the primers listed in Table S2. The drug-resistance genes were eliminated by using plasmid pCP20 as described (Datsenko and Wanner, 2000).

A strain expressing a chromosomally encoded PmrG-FLAG protein was constructed as described (Datsenko and Wanner, 2000) with primers 5′-AAACGGAAAACTCTTTTTAGATGGTGAATTTGTTCCCGGC**GACTACAAGGACGACGATGACAAG**TAGGTGTAGGCTGGAGCTGCTTC-3′ encoding the FLAG sequence immediately upstream of the stop codon of the *pmrG* gene following the priming site 1 sequence (Datsenko and Wanner, 2000) (sequence in bold face corresponds to the FLAG coding sequence) and 5′-GACAGCCGCTTCAGGCTGTCTTTACGCTTTTAACATGCGGCATATGAATATCCTCCTTAG-3′ harbouring the sequence immediately downstream of the stop codon of *pmrG* attached to priming site 2 (Datsenko and Wanner, 2000).

Selecting for Fe(III) resistance genes

A genomic library from the Δ*pmrAB* strain EG13937 was made in pBR322 as follows: chromosomal DNA prepared from an overnight culture of the Δ*pmrAB* strain was digested with Sau3AI *(*1 U μl^{-1}) for 15, 20, 30 or 40 min. The digested DNA was separated on 0.8% agarose gel, and fragments of approximately 2–10 kb in size were purified and ligated into the BamHI site of vector pBR322. The ligation products were transformed into *E. coli* DH5α selecting for ampicillin-resistant transformants. Plasmid DNA was prepared from a pool of 26 000 transformants and used to transform the Δ*pmrAB* deletion strain EG13937. Cells were plated on N-minimal pH 5.8 10 μM $MgCl_2$ agarose plates containing 50 μg of ampicillin/ml and 200 μM $FeCl_3$.

Determination of the MIC for Fe(III) and Al(III)

The MIC was determined on N-minimal pH 5.8 10 μM $MgCl_2$ agarose plates containing twofold dilutions of $FeCl_3$ or $Al_2(SO4)_3$. To determine the MIC, bacteria were grown overnight in N-minimal media with 10 mM $MgCl_2$, pH 7.7 at 37°C, then diluted into N-minimal media with 10 μM $MgCl_2$, pH 5.8, and organisms were tested at a final inoculum size of 10^4 colony-forming units (cfu) per μl with the use of a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan), following a 20 h incubation at 37°C. The MIC was the lowest concentration of compound that inhibited cell growth. MIC determinations were repeated at least three times.

Iron and soil killing assays, and polymyxin B nonapeptide competition assays

Overnight cultures grown in N-minimal media 10 mM $MgCl_2$ pH 7.7 at 37°C were washed and used to inoculate N-minimal media 10 μM $MgCl_2$ pH 5.8. After a 4 h incubation at 37°C, cells were diluted 1:100 in the same media. Fifty microlitres of diluted cells was mixed with 50 μl of $FeCl_3$ dissolved in the same media to a final concentration of 100 μM $FeCl_3$. For the competition assay, polymyxin B nonapeptide (Sigma) was added to this mixture at a final concentration of 1.25 mM. For polymyxin B killing assay, 50 μl of diluted cells were mixed with 50 μl of polymyxin B (Sigma) solution dissolved in the same media at a final concentration of 2.5 μg ml^{-1}. The mixtures in U-bottom 96-well microtiter plate were incubated at 37°C with shaking for 120 min. At this time, cells were put on ice, serial dilutions were conducted, 50 μl were plated onto LB agar plates and the number of colonies was recorded following overnight incubation at 37°C.

To investigate killing in soil, bacterial cultures were mixed with an equal volume of soil solution (dissolved in N-minimal media 10 μM $MgCl_2$ pH 5.8). The mixture was incubated for 5 h at 37°C before bacterial viability was assessed by dilution and plating as described above for the iron killing assay.

^{59}Fe association assay

^{59}Fe associated with cells was determined using a Packard Cobra II γ counter as described (Chamnongpol *et al*., 2002). The percentage of ^{59}Fe association was calculated as the amount of ^{59}Fe per colony-forming unit (cpm cfu^{-1}).

Mass spectrometry analysis of lipid A

Lipid A samples were prepared as described previously (Yi and Hackett, 2000), with a slight modification. Bacteria were grown overnight in N-minimal media with 10 mM $MgCl_2$, pH 7.7 at 37°C. Then, the cells were washed and diluted into N-minimal media with 10 μM $MgCl_2$, pH 5.8 to be a final inoculum size of 10^4 cfu μl^{-1}. Sixty microlitres (60 spots of 1 μl diluted cells) of each strain was spotted onto an N-minimal pH 5.8 10 μM $MgCl_2$ agarose plate containing 50 μM $FeCl_3$ with the use of a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan), followed by incubation at 37°C for 20 h. The cells were then harvested, washed, and suspended in 0.8 ml PBS, pH 7.4. Preparation of lipid A samples and MALDI-TOF mass spectrometry analyses of lipid A were performed in the negative ion mode on a Voyager DE STR mass spectrometer (PerSptive Biosystems) as described (Lee *et al*., 2004).

Phosphatase activity assay

Core OS was prepared as described (Yethon *et al*., 1998). The PhoP-His and PmrG-His proteins were purified using the Ni-NTA Spin kit (Qiagen) as described by the manufacturer. Production of free phosphate was assayed as described (Harder *et al*., 1994). Purified protein (1 μg) was incubated with core OS substrate for 30 min at 37°C. Free Pi generated in the assay was measured by malachite green assay using BIOMOL GREEN™ reagent (BIOMOL Res Laboratories) and a microplate reader at 620 nm. Data were corrected for background activity by subtracting the absorbance of the samples lacking core OS from those incubated in the presence of core OS.

β-Galactosidase assays

β-Galactosidase activity was determined as described (Miller, 1972).

Localization of the PmrG protein

Periplasmic and cytoplasmic fractions were prepared by the osmotic shock procedure as described (Neu and Heppel, 1965).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Genomic regions of the inserts present in the pBR322 plasmid derivatives conferring Fe(III) resistance upon the Δ*pmrAB* mutant.

Fig. S2. Overexpression of the *pmrG* and *pmrC* genes confers Fe(III) resistance to the Δ*pmrAB* strain.
Fig. S3. Negative-ion-mode MALDI-TOF mass spectrometry of lipid A from *Salmonella* strains exhibiting differential susceptibility to Fe(III).
Table S1. Bacterial strains and plasmids.
Table S2. Primers used in this study.
Table S3. MIC of Fe^{3+} against mutants deleted in the PmrA-regulated *cptA* and other genes implicated in iron resistance.

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