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# The *pmrF* polymyxin-resistance operon of *Yersinia pseudotuberculosis* is upregulated by the PhoP–PhoQ two-component system but not by PmrA–PmrB, and is not required for virulence

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The *Yersinia pseudotuberculosis* chromosome contains a seven-gene polycistronic unit (the *pmrF* operon) whose products share extensive homologies with their *pmrF* counterparts in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), another Gram-negative bacterial enteropathogen. This gene cluster is essential for addition of 4-aminoarabinose to the lipid moiety of LPS, as demonstrated by MALDI-TOF mass spectrometry of lipid A from both wild-type and *pmrF*-mutated strains. As in *S. typhimurium*, 4-aminoarabinose substitution of lipid A contributes to *in vitro* resistance of *Y. pseudotuberculosis* to the antimicrobial peptide polymyxin B. Whereas *pmrF* expression in *S. typhimurium* is mediated by both the PhoP–PhoQ and PmrA–PmrB two-component regulatory systems, it appears to be PmrA–PmrB-independent in *Y. pseudotuberculosis*, with the response regulator PhoP interacting directly with the *pmrF* operon promoter region. This result reveals that the ubiquitous PmrA–PmrB regulatory system controls different regulons in distinct bacterial species. In addition, *pmrF* inactivation in *Y. pseudotuberculosis* has no effect on bacterial virulence in the mouse, again in contrast to the situation in *S. typhimurium*. The marked differences in *pmrF* operon regulation in these two phylogenetically close bacterial species may be related to their dissimilar lifestyles.

## INTRODUCTION

Cationic peptides (typically containing 30–40 amino acid residues) are important effectors of innate immunity of both plants and animals to microbes (Hancock & Scott, 2000). To date, several hundred antimicrobial peptides have been identified in nature and classified into different groups according to their structure. Two families have been extensively studied: the first (of which defensins are the prototype representatives) consists of molecules stabilized by one or several intramolecular disulphide bridges, whereas the second includes α-helix-folded compounds such as cecropins, magainins or melittin. Due to their common cationic and amphipathic properties, antimicrobial peptides are able to bind easily to negatively charged microbial membranes. However, their mode of action may dramatically differ from one class of compounds to another: some peptides may alter bacterial membrane integrity by solubilization or pore formation, whereas others are able to translocate across these membranes in order to access and inhibit intracellular targets (Hancock & Rozek, 2002; Wu *et al.*, 1999). Microbial pathogens must thus have evolved distinct mechanisms for resisting such a broad range of peptide effectors (Devine & Hancock, 2002; Wu *et al.*, 1999). Antimicrobial peptide resistance mechanisms in the Gram-negative facultative intracellular bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) have been extensively studied. In this species, a seven-gene polycistronic unit (*pmrHFIJKLM*, the *pmrF* operon) plays an essential role in resistance to polymyxin: at least six of the seven *pmrF* operon genes are necessary for the

Abbreviation: MALDI-TOF, matrix assisted laser desorption ionization-time of flight.

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The GenBank/EMBL/DDBJ accession numbers for the *Y. pseudotuberculosis pmrF*, *phoP–phoQ* and *pmrA–pmrB* operons are AF336802, AF333125 and AY259243 respectively.

biosynthesis and export of 4-deoxy-4-amino-L-arabinose (hereafter referred to as 4-aminoarabinose) and esterification of the 4′ phosphate group of lipid A with this amino sugar – a modification which contributes to a reduction in the net negative charge of lipid A (Groisman *et al.*, 1997; Gunn *et al.*, 1998, 2000). In *Salmonella*, transcriptional activation of this operon requires the PmrA–PmrB two-component regulatory system, where PmrB is the integral membrane sensor kinase that responds to high $Fe^{3+}$ levels and PmrA is the cognate regulatory protein that controls *pmrF* operon expression directly (Wosten & Groisman, 1999). The PmrA–PmrB system is encoded by the *pmrCAB* operon, where the *pmrC* gene has recently been shown to mediate substitution of lipid A with phosphoethanolamine (Lee *et al.*, 2004). A decrease in extracellular $Mg^{2+}$ concentration (i.e. from the usual millimolar range down to micromolar levels) also promotes PmrA-dependent up-regulation of the *pmrF* operon. This process additionally requires the PhoP (regulator)–PhoQ (sensor) two-component regulatory system (Soncini *et al.*, 1996). PhoP positively controls the *pmrF* operon at the transcriptional level by increasing production of PmrD (an 85 amino acid polypeptide), which then activates the PmrA protein (Kox *et al.*, 2000; Roland *et al.*, 1994).

It has been reported that the Gram-negative enteropathogen *Yersinia pseudotuberculosis* (a member of the *Enterobacteriaceae* related to *Salmonella*) is highly resistant to polymyxin and α-helical peptides (Bengoechea *et al.*, 1998a, b). In *Yersinia pestis* (a clone that recently emerged from *Y. pseudotuberculosis*: Achtman *et al.*, 1999), it has been shown that this resistance is controlled by PhoP–PhoQ (Oyston *et al.*, 2000) and probably depends on the production of 4-aminoarabinose (Rebeil *et al.*, 2004). In the present work, we confirm the crucial role of a *Salmonella*-like *pmrF* operon in the control of resistance of *Y. pseudotuberculosis* to certain classes of antimicrobial peptides. However, this operon may play a role in cellular processes other than those allowing survival in mammals, and its two-component-system-mediated expression differs from that of the corresponding *Salmonella* chromosomal locus.

## METHODS

**Bacterial strains and growth conditions.** The main characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used as a host for the cloning experiments. *E. coli* SY327λ*pir* and SM10λ*pir* were recipients for replication of suicide plasmids. *Y. pseudotuberculosis*, *S. typhimurium* and *E. coli* strains were grown at 28 °C, 37 °C and 37 °C, respectively, in Luria–Bertani (LB) broth or on agar plates. Mating experiments between *E. coli* and *Y. pseudotuberculosis* were plated on M9 minimum medium agar, as described previously (Carnoy *et al.*, 2000). Ampicillin (100 μg $ml^{-1}$), kanamycin (50 μg $ml^{-1}$), tetracycline (12·5 μg $ml^{-1}$), chloramphenicol (35 μg $ml^{-1}$) and sucrose (10 %) were added to media for bacterial selection when necessary. IPTG (1 mM) and X-Gal (200 μg $ml^{-1}$) were used for blue/white colony screening.

**Nucleic acid manipulations.** Standard procedures were used for genomic DNA extraction, small-scale plasmid preparation, endonuclease digestion, DNA ligation, agarose gel electrophoresis, elution of DNA fragments and *E. coli* transformation (Sambrook & Russell, 2001). Large-scale plasmid DNA preparations were purified on Qiagen columns. Recombinant plasmid DNA was introduced into *Y. pseudotuberculosis* by mating or electroporation (Conchas & Carniel, 1990). Southern and slot blots were performed according to standard procedures. RNA extraction was performed with the SV total RNA isolation kit (Promega). The DIG hybridization and detection kit (Roche Diagnostics) was used for nucleic acid hybridization. Slot blot densitometry analyses were performed using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**DNA sequencing and sequence analysis.** DNA was sequenced by the dideoxy chain-termination method using the ABI PRISM dichloRhodamine Dye Terminator Sequencing kit with AmpliTaq DNA polymerase FS (Perkin Elmer) according to the manufacturer's instructions. Extension products were analysed with the Applied Biosystems model ABI 377XL automated DNA sequencer (Perkin Elmer). The nucleotide sequences obtained were analysed with Perkin-Elmer software (Sequence Navigator). The nucleotide sequence data for the *Y. pseudotuberculosis pmrF*, *phoP–phoQ* and *pmrA–pmrB* operons have been deposited in the GenBank nucleotide sequence database under accession numbers AF336802, AF333125 and AY259243 respectively.

**Synthetic oligonucleotides and PCR.** Oligonucleotide primers (Table 2) were custom-synthesized (Sigma and Genset) for PCR generation of DNA fragments used for cloning or probing. PCR amplification was performed as described elsewhere (Sebbane *et al.*, 2001) with AmpliTaq Gold polymerase (Perkin Elmer Applied Biosystems). Digoxigenin-labelled PCR products were generated with the PCR DIG Labelling Mix (Roche Diagnostics) and purified on Dye-ex Spin columns or Qiaquick PCR purification kit (Qiagen).

**Mutagenesis.** Gene inactivation in *Y. pseudotuberculosis* was performed by exchange of the wild-type gene with its inactivated allele through homologous recombination using a suicide plasmid. Briefly, we engineered pUC18 plasmid derivatives containing a deleted copy of the gene or DNA region of interest. Each construct was obtained by fusing PCR fragments yielded by amplification of the target gene's upstream and downstream flanking regions with, respectively, the 5′ and 3′ ends of an antibiotic (Km or Tet) resistance gene. DNA inserts were then subcloned into the suicide vector pCVD442. Mutants were selected after mating the *Y. pseudotuberculosis* wild-type strain with *E. coli* λ*pir* harbouring recombinant suicide plasmids (see Table 1). Mutant genotypes were confirmed by PCR and Southern blot hybridization with appropriate DNA probes (data not shown). Random Tn*5* mutagenesis of *Y. pseudotuberculosis* cells was performed by mating the recipient strain with the donor strain *E. coli* SM10 λ*pir* harbouring the recombinant suicide vector pMS90 as described previously (Riot *et al.*, 1997).

**Lipid A extraction and MALDI-TOF mass spectrometry analysis.** Lipid A was isolated as previously described (Zhou *et al.*, 1999). Briefly, an overnight culture grown at 37 °C on LB was centrifuged and the cell pellet was then washed once with PBS. Cells were resuspended in 0·8 ml PBS, and a single-phase Bligh/Dyer mixture was made by addition of 2 ml methanol and 1 ml chloroform. After 60 min at room temperature, the insoluble material was collected by centrifugation for 20 min. The pellet was washed once with 5 ml of a fresh, single-phase Bligh/Dyer mixture consisting of chloroform/methanol/water (1 : 2 : 0·8, by vol.) and was then dispersed in 1·8 ml 12·5 mM sodium acetate (pH 4·5) containing 1 % SDS; the mixture was boiled for 30 min in order to cleave the glycosidic bond between lipid A and KDO. Lipid A was recovered by converting the hydrolysed material into a two-phase Bligh/Dyer mixture by addition of 2 ml

**Table 1.** Strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or origin |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
| DH5α | $F^-\phi80dlacZ\Delta M15\ \Delta(lacZYA–argF)U169\ recA1\ endA1\ hsdR17\ (r_K^-\ m_K^+)\ supE44\ \lambda^-$ *thi-1 gyrA relA1* | Invitrogen |
| SY327λ*pir* | Δ(*lac pro*) *argE* (Am) *recA rif nalA* λ*pir*; host for pCVD442 and derivatives | Miller & Mekalanos (1988) |
| SM10λ*pir* | *thi thr leu sup tonA lacY recA*::RP4-2Tc::MuKm λ*pir*; host for pCVD442 and derivatives | Simon *et al.* (1983) |
| SG13009(pREP4) | $F^-$ *his pyrD* Δ(*lon-100*) *rpsL*(pREP4) | Gottesman *et al.* (1981) |
| *S. enterica* | | |
| CIP 60.62 | Serovar Typhimurium wild-type strain LT2 (ATCC 43971); referred to as *S. typhimurium* | Collection de l'Institut Pasteur |
| *Y. pseudotuberculosis* | | |
| 32777 | Wild-type strain, harbouring the virulence plasmid pYV | Sebbane *et al.* (2001) |
| 27RB1 | 32777 $LacZ^-$ mutant; engineered with plasmid pFS41 | This work |
| 32777ΔPhoPA | 32777 $PhoP^-$ derivative ($\Delta_{283–583}$ *phoP* Ω*aphA-3*); engineered with plasmid pAP4A-1 | Marceau *et al.* (2004) |
| 32777β1ΔPhoPT | 27RB1 $PhoP^-$ derivative ($\Delta_{283–583}$ *phoP* Ω*tet*); engineered with plasmid pAP4T-1 | This work |
| 32777ΔPmrA | 32777 $PmrA^-$ derivative ($\Delta_{152–492}$ *pmrA* Ω*aphA-1a*); engineered with plasmid pMRA1 | This work |
| 32777ΔPmrF | 32777 $PmrF^-$ derivative ($\Delta_{15–833}$ *pmrF* Ω*aphA-3*); engineered with plasmid pMRF1 | This work |
| **Plasmids** | | |
| pUC18 | Cloning vector; Ap | Appligene |
| pACYC184 | Cloning vector; Tet Cm | New England Biolabs |
| pCVD442 | Suicide vector containing the counter-selectable marker *sacB*; Ap | Donnenberg & Kaper (1991) |
| pUC4K | Km; source of aminoglycoside phosphotransferase *aphA-1a* gene | Amersham Pharmacia |
| pUC1318-KmII | Km; source of aminoglycoside phosphotransferase *aphA-3* gene | Gift from P. Trieu-Cuot |
| pQE60 | Expression vector; Ap | Qiagen |
| pGP704 | Suicide vector, pBR322 derivative with *oriR6K mobRP4*; Ap | Miller & Mekalanos (1988) |
| pMS90 | Suicide vector derived from pJM703.1, containing transposon Tn*5*; Ap Km | Riot *et al.* (1997) |
| pAP4A-1 | pCVD442 Ω4·7 kbp (1516 bp *aphA-3* kanamycin-resistance cassette flanked by 1115 bp *Sac*I/*Xba*I PCR fragment generated with primers A1 and AP2 and 2029 bp *Xba*I/*Sph*I PCR fragment generated with primers AP3 and B2 generated from the *Y. pseudotuberculosis phoPQ* locus); carries the inactivated allele of the *Y. pseudotuberculosis phoP* gene | Marceau *et al.* (2004) |
| pAP4T-1 | pCVD442 Ω4·6 kbp (1409 bp tetracycline-resistance cassette from pACYC184 flanked by 1115 bp *Sac*I/*Xba*I PCR fragment with primers A1 and AP2 and 2029 bp *Xba*I/*Sph*I PCR fragment generated with primers AP3 and B2 generated from the *phoPQ* locus from *Y. pseudotuberculosis* 32777); carries an inactivated copy of the *Y. pseudotuberculosis phoP* gene | This work |
| pMRA1 | pCVD442 Ω2·3 kbp (1516 bp *aphA-3* kanamycin-resistance cassette flanked by 1303 bp *Sac*I/*Bam*HI PCR fragment with primers PBP41 and PMRA2 and 1185 bp *Bam*HI/*Sph*I PCR fragment generated with primers PMRA303 and PMRB2 from the *Y. pseudotuberculosis dacB–pmrAB* locus); carries an inactivated copy of the *Y. pseudotuberculosis pmrA* gene | This work |
| pMRF1 | pCVD442 Ω4·3 kbp (1516 bp *aphA-3* kanamycin-resistance cassette flanked by 1128 bp *Sac*I/*Bam*HI PCR fragment generated with primers UNK4 and UNK5 and 1732 bp *Bam*HI/*Sph*I PCR fragment generated with primers PMRF3 and UNK2 from the *Y. pseudotuberculosis pmrF* operon); carries an inactivated copy of the *Y. pseudotuberculosis pmrF* gene | This work |
| pFS41 | pCVD442 containing a copy of the *Y. pseudotuberculosis lacZ* region with a complete deletion (3181 bp) of the LacZ-coding sequence | This work |
| pMM501 | pACYC184 $\Delta_{1523–2149}$, $\Delta P_{lacZ}$, $\Omega P_{pmrF}$::*lacZ* | This work |
| pQE60PhoH6.2 | pQE60 Ω669 bp (*Nco*I/*Bam*HI PCR fragment generated from the *Y. pseudotuberculosis* 32777 *phoP* gene, with primer set 60f1Nco and 60f2Bam) | This work |

**Table 1.** cont.

| Strain or plasmid | Relevant characteristics | Reference or origin |
|---|---|---|
| pF12 | pUC18 Ω3·58 kb (*Nhe*I/*Sph*I fragment containing the first and second genes of the *Y. pseudotuberculosis* 32777 *pmrF* operon under control of their own promoter); used for complementation of *pmrF* mutants | This work |

chloroform and 2 ml methanol. After low-speed centrifugation, the lower phase was collected and washed twice with 4 ml of the upper phase derived from a fresh, neutral, two-phase chloroform/methanol/water (2 : 2 : 1·8, by vol.) Bligh/Dyer mixture. The washed lower phase was dried under nitrogen.

MALDI-TOF mass spectroscopy analyses of lipid A were performed with the Bruker-Reflex III two-stage reflection time-of-flight mass analyser (Bruker Daltonics) in a linear TOF configuration with an acceleration voltage of 20 kV. Details of the methods used are given by Lindner (2000). Mass scale calibration was performed externally with similar compounds of known chemical structure.

**Purification of His-tagged recombinant PhoP proteins under native conditions.** The recombinant *E. coli* strain harbouring pQE60PhoH6.2 was grown in 1 litre of LB medium supplemented with ampicillin (100 μg $ml^{-1}$) and kanamycin (25 μg $ml^{-1}$). When the $OD_{600}$ reached 0·8, expression of the gene encoding the recombinant protein was induced with 1 mM IPTG for 0·5 h. The cells were harvested by centrifugation and then resuspended in 5 ml of lysis buffer (300 mM NaCl, 50 mM $Na_2HPO_4$, 10 mM imidazole, pH 8) per g fresh weight. Cells were lysed using a French press with a pressure of 1000 p.s.i. (approx 7 MPa). The lysate was clarified by centrifugation at 10 000 ***g*** for 20 min. The supernatant was filtered on a 0·45 μm filter before being loaded onto a 1 ml HiTrap Affinity column (Amersham) pre-equilibrated with lysis buffer. The column was washed first with 10 ml lysis buffer and then with 5 ml lysis buffer containing 50 mM imidazole. Elution was performed with 10 ml lysis buffer containing 250 mM imidazole. The collected fractions were analysed by SDS-PAGE, using a 12·5 % polyacrylamide gel and Coomassie blue staining. Samples were dialysed overnight against PBS/10 % (v/v) glycerol, dispensed into aliquots and stored at −20 °C.

**Table 2.** Oligonucleotide primers

| Name | Sequence (5′→3′) |
|---|---|
| 60f1Nco | catgccatggatgcgggttctggttgtggaagataacgcg |
| 60f2Bam | cgcggatccgttgacgtcaaaacgatatccctgaccac |
| A1 | aaaagagctccgcgttgttgcgtcaccatctgacagtgcaaa |
| AP2 | ctagtctagaccggcttccagtacagcgact |
| AP3 | ctagtctagatgggtcgactacgtaaaaaac |
| B2 | acatctgcaggagctcaaacatcatattgatcaaaa |
| BTU449E | aataaaatagtgtcttcctaaggttc |
| PBP41 | aaaagagctccgattactgatggtgtgttgc |
| PMRA2 | ctagtctagatcaagaataatcatgctgtac |
| PMRA303 | gacggatccgctgggcaaacagttaaccgc |
| PMRB2 | acatgcatgctacgatcaaggcggttttccaacgt |
| PMRF3 | ctagtctagatcatttagtgacacggtgtga |
| UNK2 | acatctgcaggagctctcagtaaaacaacgtttttggg |
| UNK4 | ctagtctagatcatttagtgacacggtgtga |
| UNK5 | acatcccggggagctcctaggccagcaataggtagt |
| UNK70 | cgccagcaatgtaatg |
| YOPH1 | catcgtcaggtatctcga |
| YOPH2 | caatcagttgcgcagtac |

**Electrophoretic mobility shift assays.** The binding of purified $PhoP(His)_6$ protein to PCR products from gene promoter regions was assessed by electrophoretic mobility shift assays, as previously described (Himpens *et al.*, 2000).

**Antimicrobial peptide activity assays.** Polymyxin B and cecropin B were purchased from Sigma; defensin A was purified from *Phormia terranovae* (a kind gift from P. Bullet). The formulation of the incubation medium was identical to that of RPMI 1640 (Invitrogen, ref. 21870) except for $Ca(NO_3)_2$ and $MgSO_4$, which were added separately at appropriate concentrations. The bactericidal effects of antimicrobial peptides were determined using *Y. pseudotuberculosis* cells obtained from cultures in LB broth (which naturally contains iron) at 28 °C for 16–18 h ($OD_{620}$ 0·4) followed by an additional 3 h incubation in LB broth at the appropriate temperature and in the presence of 300 μM of the iron chelators deferoxamine mesylate or 2,2′-dipyridyl (Sigma) when necessary. Bactericidal assays were performed as follows. First, stock solutions of cecropin B, polymyxin B and defensin A (0·1 mg $ml^{-1}$ in sterile distilled water) were diluted in the incubation medium at the appropriate concentrations. Then 100 μl volumes of these working solutions of defined peptide concentration were placed in each well of a 96-well microtitre plate. Ten microlitres of *Y. pseudotuberculosis* cell suspension was added to each well (final bacterial concentration $5 \times 10^6$ c.f.u. $ml^{-1}$). As a control, bacterial suspensions were concomitantly added to wells containing 100 μl incubation medium lacking added antimicrobial peptides. Microtitre plates were shaken gently for 30 s and then incubated at 37 °C for 2 h. To assess viability of *Y. pseudotuberculosis*, 50 μl of mixture from each well was serially diluted in sterile distilled water. Diluates were plated on LB and incubated at 28 °C for 48–72 h. Bacterial survival was defined as the ratio of the number of viable bacteria after 2 h contact with peptides to the number of viable bacteria in the absence of peptides. *Micrococcus luteus* (highly sensitive to defensins) and *S. enterica* serovar Typhimurium strain LT2 were used as internal controls for defensin A and iron chelator (deferoxamine mesylate or 2,2′-dipyridyl) activity respectively.

**Experimental infection in a mouse model.** Six-week-old female outbred OF1 mice (Iffa Credo) were challenged either by the intravenous (i.v.) route (0·3 ml bacterial suspension in sterile PBS) or by the intragastric (i.g.) route (0·2 ml bacterial suspension in sterile distilled water, using a gastric tube). Mice were starved for 18 h prior to gastric inoculation. Bacterial inocula were prepared from overnight cultures in LB at 28 °C. The cultures were centrifuged and the bacterial pellets were washed once and resuspended in distilled water or PBS. Animals were kept in positive-pressure cabinets during experimentation, and mortality was monitored daily for 21 days after challenge. For each experimental infection, the presence of the virulence plasmid pYV was confirmed by PCR on bacterial thermolysates using primers YOPH1 and YOPH2, which are internal to *yopH*, a gene located on pYV (Carnoy *et al.*, 2000). Infected animals were monitored for 3 weeks, and the 50 % lethal dose ($LD_{50}$) was calculated from groups of 5 (i.v. model) or 10 (i.g. model) mice according to the method of Reed & Muench (1938).

## RESULTS

### *Y. pseudotuberculosis* harbours a *Salmonella pmrF*-like operon which contributes to lipid A substitution with 4-aminoarabinose and resistance to cecropin B and polymyxin B

We first performed *in silico* analysis of the genome of *Y. pestis* CO92 (Parkhill *et al.*, 2001), a strain which is genetically closely related to *Y. pseudotuberculosis* (Achtman *et al.*, 1999). This investigation revealed the presence of a chromosomal locus of seven genes, apparently organized into an operon and displaying significant similarities with the *Salmonella pmrF* operon, i.e. 49–78 % identity at the protein level for the *pmrH*, *pmrF*, *pmrI*, *pmrG*, *pmrK* and *pmrL* genes respectively, and 37 % for the last one, *pmrM*, which makes a less obvious contribution (if any) to 4-aminoarabinose biosynthesis and export in *Salmonella* (Gunn *et al.*, 2000). PCR analysis of *Y. pseudotuberculosis* 32777 DNA with primers designed from the *Y. pestis* CO92 *pmrF* sequence confirmed that a *pmrF*-like locus was indeed present in this species. As expected, its nucleotide sequence (GenBank accession no. AF336802) was more than 98 % identical to that of *Y. pestis*. By analogy with *Salmonella*, we hypothesized that this locus might also contribute to substitution of lipid A with 4-aminoarabinose in *Y. pseudotuberculosis* and might, as a consequence, play a role in bacterial resistance to α-helical antimicrobial peptides. To verify this hypothesis, we inactivated the second gene of the operon (*pmrF*, whose product is necessary for 4-aminoarabinose substitution on bactoprenol phosphate and its subsequent export through the inner membrane) in wild-type strain 32777 and then compared the mutant's lipid A composition and susceptibility to antimicrobial peptides with those of the parent. MALDI-TOF mass spectrometry analysis of lipid A extracted from the wild-type and mutant strains (Fig. 1) confirmed that the *pmrF* locus was involved in addition of 4-aminoarabinose to lipid A. Inactivation of *pmrF* was associated with a decrease in bacterial resistance to both cecropin B and polymyxin B, and the magnitude of the reduction varied almost linearly with the peptide dose (Fig. 2). This deficiency was fully restored after *trans*-complementation with the *pmrF* wild-type gene (Fig. 2). In contrast, susceptibility to the insect defensin A did not change when the *pmrF* gene was inactivated, whatever the antimicrobial peptide concentration used in the assay.

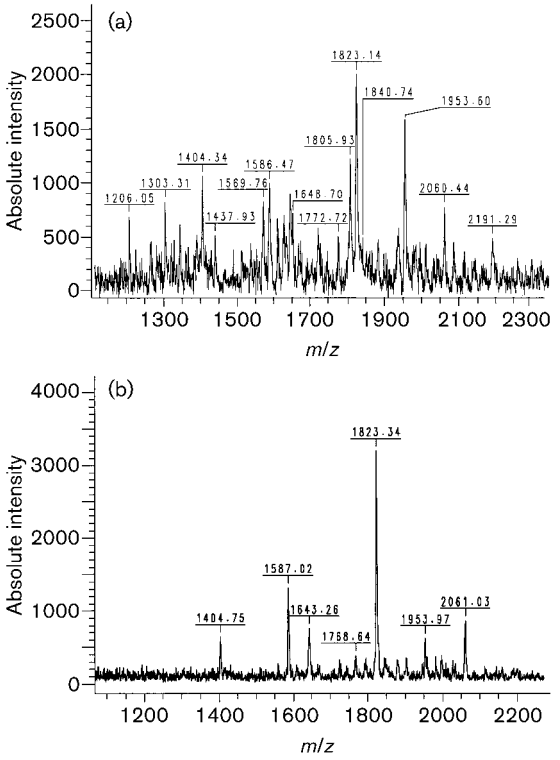


**Fig. 1.** Negative-ion MALDI-TOF mass spectrometry analysis of *Y. pseudotuberculosis* lipid A. The lipid A structure of the wild-type strain 32777 (a) was similar to that reported for *Y. pestis* (Aussel *et al.*, 2000). The prominent peak detected at *m/z* 1823 may correspond to a hexa-acylated molecular species containing two glucosamines, two phosphates, four OH–$C_{14}$, one $C_{12:0}$ and one $C_{16:1}$, whereas the one obtained at *m/z* 2060 is consistent with a hepta-acylated form following the addition of $C_{16:0}$ to the hexa-acyl molecule. Peaks at *m/z* 1953 and at *m/z* 2191 are compatible with 4-aminoarabinose substitution on hexa-acylated and hepta-acylated lipid A, respectively. In lipid A from the *pmrF* mutant strain 32777ΔPmrF (b), the peak at *m/z* 1953 is close to the background level whereas the peak at *m/z* 2191 was not detected. Similar peaks at *m/z* 2060 in both strains show that hepta-acylation is *pmrF*- independent.

### Unlike PhoP–PhoQ, the PmrA–PmrB two-component regulatory system is not essential for activation of the *Y. pseudotuberculosis pmrF* operon

$Mg^{2+}$/$Ca^{2+}$ and $Fe^{3+}$ cation limitations (as well as pH) influence the ability of *Salmonella* to resist polymyxin (Garcia Vescovi *et al.*, 1996; Groisman *et al.*, 1997; Wosten *et al.*, 2000). We tested whether these environmental cues could also have an impact on the transcription of the *pmrF* operon in *Y. pseudotuberculosis*. As in *Salmonella*, a low (1 μM) $Mg^{2+}$/$Ca^{2+}$ concentration and a pH decrease from 8 to 6 were both found to induce polymyxin resistance (Fig. 3a). Transcription of the *pmrF* operon in *Y. pseudotuberculosis* was found to increase accordingly, by a factor of 7 and 2·5 for the $Mg^{2+}$/$Ca^{2+}$ ion starvation and acidic pH conditions respectively (Fig. 3b), as determined by slot blot densitometry analysis. In contrast to *Salmonella*, no reduction in polymyxin resistance was observed when *Y. pseudotuberculosis* cells were incubated with the iron chelators deferoxamine mesylate (Fig. 3a) or 2,2′-dipyridyl (data not shown); nor did we see an increase in peptide resistance in the presence of an excess of ferric chloride. Similar results were noted with the α-helical peptide cecropin B at 3·25 μg $ml^{-1}$ (data not shown). Finally, as depicted

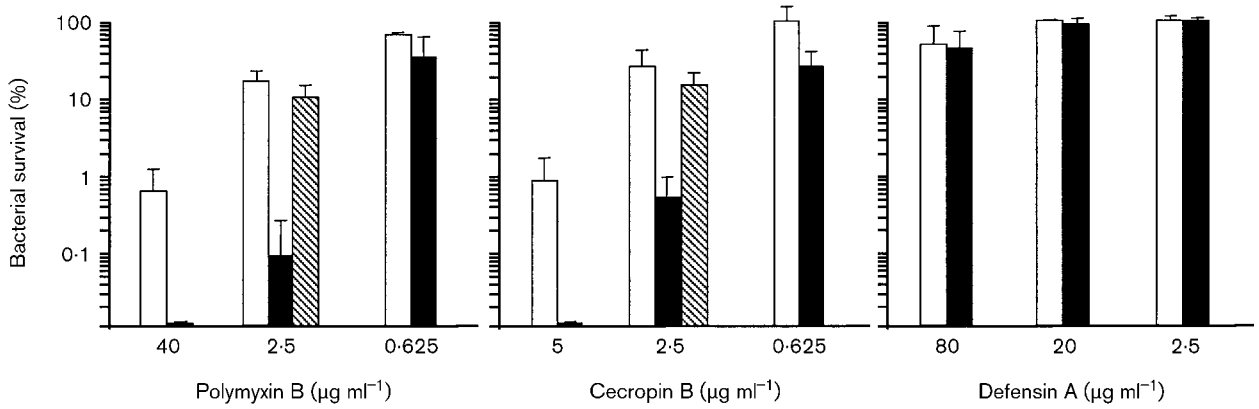


**Fig. 2.** Role of the *pmrF* locus in resistance of *Y. pseudotuberculosis* to various antimicrobial peptides. Wild-type strain 32777 and the isogenic *pmrF* (the second gene of the *pmrF* operon) mutant 32777ΔPmrF were incubated at 37 °C in RPMI with various amounts of the antimicrobial peptides polymyxin B, cecropin B and defensin A. Bacterial survival was assessed after a 2 h incubation period. Each bar (white for wild-type, black for *pmrF* mutant, hatched for *trans*-complemented mutant) represents the mean value (± SD) of five independent experiments. *pmrF* inactivation was associated with decreased bacterial survival versus 2·5 μg ml$^{-1}$ polymyxin B or cecropin B. Complementation of mutant 32777ΔPmrF with *pmrF in trans* (plasmid pF12) fully restored the resistance of *Y. pseudotuberculosis* to 2·5 μg ml$^{-1}$ concentrations of these two antimicrobial peptides.

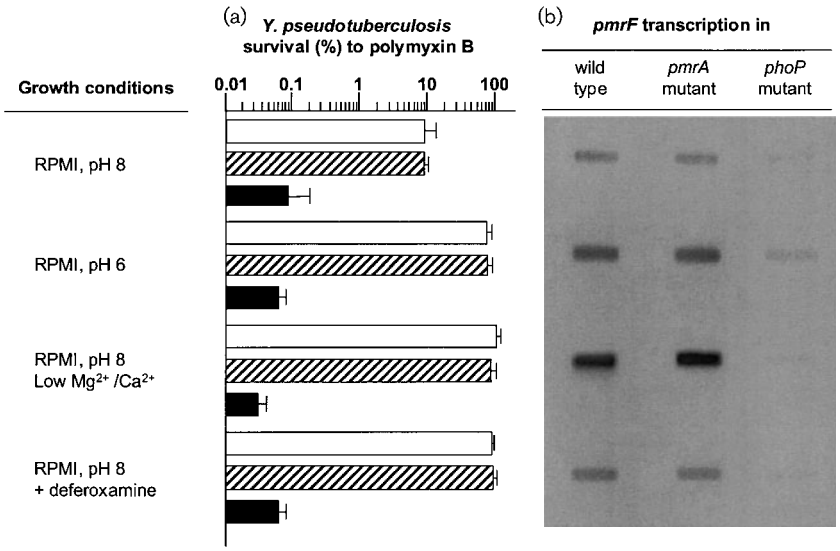


**Fig. 3.** *pmrF* transcription and resistance to polymyxin B in the wild-type strain and isogenic *pmrA* and *phoP* mutants of *Y. pseudotuberculosis* as a function of $Mg^{2+}/Ca^{2+}$ and $Fe^{3+}$ concentrations and environmental pH. (a) Strains 32777 (wild-type), 32777ΔPmrA (a *pmrA* mutant) and 32777ΔPhoPA (a *phoP* mutant) were incubated at 37 °C under different *in vitro* conditions and in the presence or absence of 2·5 μg polymyxin B ml$^{-1}$. Bacterial survival after 2 h contact with the antimicrobial peptide is depicted. Each bar (white for the wild-type strain, dashed for the *pmrA* mutant, grey for the *phoP* mutant) represents the mean value (± SD) of five independent assays. No resistance inductions were observed in either condition with the *pmrF* mutant. As a control assay, the resistance of *S. typhimurium* LT2 to polymyxin B was assessed under identical conditions: antimicrobial peptide resistance was found to increase sixfold and eightfold at low $Mg^{2+}/Ca^{2+}$ ion concentrations and at pH 6 respectively, and was decreased tenfold when the assay was performed using iron-depleted medium (data not shown). (b) Total RNAs (10 μg) extracted from bacteria incubated for 30 min in medium free of antimicrobial peptide were spotted onto nitrocellulose membranes and hybridized with a digoxigenin-labelled 600 bp *pmrF* probe. A 16S rRNA probe from *Y. pseudotuberculosis* was used as a control for constitutive gene expression (data not shown). No signal was detected when RNA samples were pre-treated with RNase before hybridization. Experiments were repeated three times and representative results are shown.

in Fig. 3(b), *pmrF* transcription was found not to require iron. Taken together, these results suggest that the *pmrF* locus is regulated differently in *Y. pseudotuberculosis* when compared to *Salmonella*. In this latter bacterium, iron-mediated regulation of the *pmrF* locus is controlled by the PmrA–PmrB two-component system (Wosten *et al.*, 2000). Two corresponding tandemly arranged open reading frames (YPO3507 and YPO3508, whose products exhibit 56·4 and 50·4 % identity with the *Salmonella* PmrA and PmrB proteins respectively) have been identified in the *Y. pestis* CO92 genome. As expected, homologue genes with 99·5 and 99·4 % identity at the nucleotide level were also found in *Y. pseudotuberculosis* 32777 (GenBank accession no. AY259243). In line with a possible contribution to iron sensing, *Y. pseudotuberculosis* PmrB contains two ExxE iron receptor motifs at positions 30–33 and 55–58 (in the periplasmic loop) – just like its *Salmonella* or *Erwinia carotovora* counterparts (which are able to sense iron: Hyytiäinen *et al.*, 2003; Wosten *et al.*, 2000) but unlike PmrB from *Pseudomonas aeruginosa* (which apparently lacks this tetrapeptide and does not exhibit iron-sensing activity: McPhee *et al.*, 2003). We engineered a non-polar mutation in the *Y. pseudotuberculosis pmrA* gene and assessed *pmrF* expression in the mutant relative to wild-type under two different *in vitro* conditions (iron starvation and pH 6). As shown in Fig. 3(b), transcription of this operon in the *pmrA* mutant did not differ from that of the wild-type strain in either case, and inactivation either of *pmrB* alone or of both genes encoding this two-component system yielded identical results. We thus conclude that the PmrA–PmrB two-component system is not essential for *pmrF* transcriptional activation in *Y. pseudotuberculosis* – at least under the *in vitro* conditions established for *Salmonella*. Whether or not the *Y. pseudotuberculosis* PmrA–PmrB two-component system responds to iron levels remains to be established experimentally.

However, transcription of the *Y. pseudotuberculosis pmrF* operon was also found to be induced upon $Mg^{2+}$ and $Ca^{2+}$ ion starvation (Fig. 3b) – consistent with the fact that lipid A substitution with 4-aminoarabinose is mediated by the PhoP–PhoQ two-component system (Rebeil *et al.*, 2004). Hence, it is possible that the *pmrF* operon was still under the control of PhoP–PhoQ but was independent of PmrA–PmrB. To verify this hypothesis, we assessed the impact of a *phoP* knock-out mutation on the transcription level of the *pmrF* operon when bacteria were in an acidic (pH 6) environment or exposed to low concentrations of $Mg^{2+}$/$Ca^{2+}$ or $Fe^{3+}$ ions. As depicted in Fig. 3(b), PhoP inactivation was associated with a reduction in *pmrF* transcript production by a factor of between 3 and 20, depending on the bacterial growth conditions. This result indicates that the PhoP–PhoQ two-component system is essential for efficient expression of the *pmrF* operon. DNA mobility shift assays with purified, His-tagged PhoP protein revealed that PhoP binds to the *pmrF* operon promoter region (Fig. 4), strongly suggesting that PhoP regulates *pmrF* operon transcription directly.

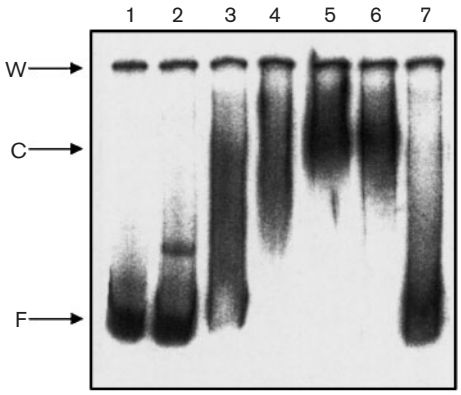


**Fig. 4.** Assay for PhoP binding to the promoter region of the *Y. pseudotuberculosis pmrF* operon. A 353 bp fragment encompassing the putative *pmrF* operon promoter region was PCR-generated with primers UNK70 and BTU449E. The figure shows electrophoretic mobilities of the $^{32}$P-labelled 353 bp fragment alone (lane 1), mixed with 0·1, 0·5, 1 and 2 μg purified PhoP(His)$_6$ (lanes 2, 3, 4 and 5, respectively) and mixed with 2 μg purified PhoP(His)$_6$ and a 100-fold excess of non-specific DNA (lane 6) or a 50-fold excess of the same 353 bp unlabelled *pmrF* operon promoter region (lane 7). As an internal control of the electrophoretic mobility shift assay, PhoP(His)$_6$ was found to bind to the $^{32}$P-labelled promoter region of the *Y. pseudotuberculosis phoP* gene; this binding was not observed after addition of a 50-fold excess of unlabelled *pmrF* promoter region (data not shown). The positions of the well (W), complexed DNA (C) and free DNA (F) are indicated on the left of the figure. As illustrated, PhoP binds specifically to the 353 bp DNA segment encompassing the putative promoter of the *pmrF* operon.

## PhoP is the only detectable transcriptional regulator controlling expression of the *Y. pseudotuberculosis pmrF* operon

To search for other transcriptional regulators involved in the regulation of the *pmrF* operon, we used wild-type strain 32777 to construct a *lacZ* null mutant (strain 27RB1), which was next *trans*-complemented with plasmid pMM501, a pACYC184 derivative carrying the *lacZ* gene under the control of the *pmrF* operon's promoter region. Screening for *pmrF* activators was performed by Tn*5* transposon insertional mutagenesis of *Y. pseudotuberculosis* strain 27RB1(pMM501) and isolation of mutants on X-Gal agar. Of the three million Tn*5* mutants generated over ten experiments, 65 simultaneously formed pale blue colonies on selective agar and exhibited decreased resistance (ranging from 50- to 200-fold) to polymyxin B. Sequencing of the 200 bp upstream and downstream of the Tn*5* insertion site revealed that the transposon had disrupted the *phoP* or *phoQ* gene in 60 mutants. In the remaining five, Tn*5* was found to be inserted within an ORF whose product was highly homologous (82·2 % amino acid identity) to that of the *E. coli miaA* gene. Rather than encoding a transcriptional regulator as such, *miaA* produces a protein that

acts at the post-transcriptional level by allowing tRNA maturation (Caillet & Droogmans, 1988). We also explored the possibility that the *pmrF* operon might be under the control of a repressor. The same experimental strategy was thus used to identify potential negative regulators, although here Tn*5* mutagenesis was carried out on a *phoP* mutant from strain 27RB1(pMM501). Tn*5* mutants were screened on X-Gal agar for their capacity to give blue colonies darker than those yielded by the parental $PhoP^-$ strain. Using this approach, we were not able to isolate any repressors other than one displaying 44 % amino acid identity with the *E. coli* LacI repressor. These white-to-blue reverting mutants displayed similar polymyxin resistance to that of the 27RB1(pMM501) *phoP* mutant, suggesting that mutation of this *lacI*-like gene caused derepression of a regulon required for sugar metabolism (possibly lactose) rather than derepression of the *pmrF* operon.

### The *pmrF* operon is not required for full virulence of *Y. pseudotuberculosis* in a mouse model

Antimicrobial peptides are major effectors of innate immunity in mammals, and so we investigated whether the *pmrF* operon contributes to the progression of host infection by *Y. pseudotuberculosis*. The virulence of the *pmrF* mutant was assessed in the OF-1 mouse. The *Y. pseudotuberculosis pmrF* mutant was found to be as virulent as the parental strain when administered intravenously ($LD_{50} < 10^2$). Its $LD_{50}$ also did not differ significantly from that of the wild-type in an oral model of infection, and the kinetics of animal death were similar, regardless of the infecting strain – again, in contrast to the situation in *Salmonella* (Gunn *et al.*, 2000).

## DISCUSSION

The absence of 4-aminoarabinose and the high susceptibility to antimicrobial peptides in *Y. pestis phoP* mutants (Rebeil *et al.*, 2004) had already suggested that of all the possible PhoP-regulated elements, it is this sugar moiety which is particularly involved in resistance of *Y. pseudotuberculosis* to antimicrobial peptides. In this work, we provide direct evidence that the *Y. pseudotuberculosis pmrF* operon plays a major role in bacterial resistance *in vitro* to killing by polymyxin B and at least some α-helical antimicrobial peptides (cecropin B), but is not needed for insect defensin tolerance. A contribution to polymyxin resistance was previously reported for the *Salmonella pmrF* gene cluster (Gunn *et al.*, 1998). In the pathogenic yersiniae, the *pmrF* operon is inserted between the *btuCDE* operon (required for the passage of vitamin $B_{12}$ through the outer and inner bacterial membranes (de Veaux *et al.*, 1986) and the *nplC* gene (encoding a putative lipoprotein of unknown function). An identical genetic organization was described very recently in the insect pathogen *Photorhabdus luminescens* (Derzelle *et al.*, 2004). In contrast, *btu* and *nplC* homologue genes are clustered on the *Salmonella* chromosome, at some distance (985 kb) from the *pmrF* operon. As in *Ps. aeruginosa* (Moskowitz *et al.*, 2004), the *pmrD* gene (which flanks the right extremity of the *Salmonella pmrF* operon and encodes a *pmrA* activator) was not found in the immediate vicinity of the *Y. pseudotuberculosis pmrF* operon, and subsequent whole-genome *in silico* analysis revealed that the gene was missing in the micro-organism studied here.

In *S. typhimurium*, *pmrF* transcription is mediated by both the PhoP–PhoQ and PmrA–PmrB two-component regulatory systems but is only under the control of PhoP–PhoQ in *Y. pseudotuberculosis*. In line with this result, PhoP was found to participate directly in the transcriptional activation of this operon in the latter species. This situation is similar to what was observed in *Ps. aeruginosa* (McPhee *et al.*, 2003), although both two-component systems are suspected to control *pmrF* expression in this latter organism. It is noteworthy that no putative *Salmonella*-, *Ps. aeruginosa*- or *E. coli*-like *phoP* promoter-binding direct repeats were found upstream of either the *pmrF* or *phoPQ* operons. A lack of conserved motifs in these promoting regions has also been observed in *Ph. luminescens* (Derzelle *et al.*, 2004).

*Y. pseudotuberculosis* PmrA and PmrB exhibit relatively low levels of identity (56 and 51 %, respectively) with the respective homologous proteins in *Salmonella*, thus raising the possibility that the two *Y. pseudotuberculosis* and *Salmonella* PmrA–PmrB systems may not in fact be homologues. Interestingly, this situation is very similar to that recently observed in *Er. carotovora*, where the PmrA and PmrB subunits (Hyytiainen *et al.*, 2003) share only 59 % and 56 % protein identity with their respective *Salmonella* counterparts and 65 % and 58 % with those of *Y. pseudotuberculosis*. Just as in *Salmonella*, the *Erwinia* PmrB protein is likely to be able to sense iron as an input signal directly, as illustrated by the presence of conserved iron-receptor-like EXXE motifs. However, in *Er. carotovora*, the PmrA–PmrB system functions differently and, moreover, has been shown to regulate virulence functions specific to plant pathogens, in addition to antimicrobial peptide resistance (Hyytiäinen *et al.*, 2003). These observations strongly suggest that this two-component system has diverged differently in these three species, contrasting with 'core' systems like OmpR–EnvZ, which remain highly conserved (i.e. > 90 % identical). The CtrA–CtrB two component system has recently been shown to regulate distinct pathways (and thus different genes) in *Brucella abortus* and *Caulobacter crescentus*, despite the high degree of homology (> 80 % identity at the amino acid level) for the corresponding regulator subunits in these two species (Bellefontaine *et al.*, 2002). PmrA–PmrB is thus a second example showing that a ubiquitous two-component regulatory system may control different regulons in different bacterial species.

What, then, might be the role of PmrA–PmrB? In *Yersinia*, this system is probably involved in regulation of genes other than those contributing to lipid A substitution with

4-aminoarabinose. In contrast to *Salmonella*, *E. coli* and *Er. carotovora*, the *Yersinia pmrA* and *pmrB* genes are not associated with *pmrC* to form the *pmrCAB* operon – in fact, *pmrC* is probably absent from the *Yersinia* genome (Chain *et al.*, 2004). Instead, as judged by the presence of a very short intergenic region (5 nucleotides), *pmrA* and *pmrB* are putatively co-transcribed with *dacB*, which encodes a product 79 % identical to an *E. coli* penicillin-binding DD-carboxypeptidase/DD-endopeptidase (Korat *et al.*, 1991). This latter finding suggests that PmrA–PmrB could be involved in peptidoglycan homeostasis in *Yersinia* cells. Fully annotated genome sequences of pathogenic yersiniae are either available now (for *Y. pestis* and *Y. pseudotuberculosis*) or will be released soon (for *Y. enterocolitica*): transcriptome analysis of *pmrA* and *pmrB* mutants and their parental counterparts should clarify this point.

As shown in Fig. 3(b), *pmrF* transcription responds to pH changes, and our results indicate that this regulation was PmrA–PmrB-independent. As already mentioned by other workers, the PhoP–PhoQ system is unlikely to participate in this process either (Garcia Vescovi *et al.*, 1996). Accordingly, a (twofold) pH-induced transcriptional activation was still observed in a *phoP*-null background (Fig. 3), suggesting that an additional regulator may control *pmrF* expression. To identify other potential activators or repressors, Tn*5* transposon mutagenesis was carried out in *Y. pseudotuberculosis*. The regulation mutants isolated (other than *phoP* and *phoQ* mutants) were all found to have a transposon copy inserted within the *miaA* gene. The *miaA* product is a tRNA $N^6$-isopentenyladenosine synthetase involved in the first step of the transformation of the adenosine located at position 37 ($ms^2i^6A37$) of some tRNAs into 2-methyl-$N^6$-isopentenyladenosine. Recently, this tRNA modification was found to be essential for optimal expression of virulence factors in the bacterial pathogens *Agrobacterium tumefaciens* and *Shigella flexneri* (Durand *et al.*, 1997; Gray *et al.*, 1992). In the latter organism, it has been reported that a *miaA* mutant displayed a tenfold reduction in transcription of the *virF* regulon due to a post-transcriptional downregulation of VirF activator expression (Durand *et al.*, 2000). *miaA* inactivation in *Y. pseudotuberculosis* was associated with a slight inhibition of bacterial growth (as in *Sh. flexneri*). Transcription of the *pmrF* operon in two independent mutants (which differed only in the orientation of Tn*5* within *miaA*) was significantly reduced, although it was not as low as that obtained in *Y. pseudotuberculosis phoP* mutants (data not shown). The exact mechanism of this unexpected additional control of *pmrF* operon transcription is unknown. However, the dramatic decrease in *phoP* mRNA levels in these insertion mutants (unpublished data) suggests that MiaA might modulate expression of the *pmrF* operon via regulation of PhoP – either by controlling the production of a regulator that mediates *phoPQ* transcription or by interfering with autoregulation of the PhoP–PhoQ system. These hypotheses are currently undergoing further investigation. However, to the best of our knowledge, MiaA activity has yet to be reported as being pH-dependent: therefore, we assume that MiaA per se is unlikely to account for the pH-dependence of *pmrF* transcription, and that other *pmrF*-regulating elements exist. Failure to detect putative regulatory elements by transposon mutagenesis could be due to the deleterious effects of inactivation on the bacterial cell or, alternatively, to a very low frequency of Tn*5* intragenic insertion.

*pmrF* inactivation attenuates *Salmonella* virulence when bacteria are administered by an oral route (Gunn *et al.*, 2000). In contrast, we found that arabinosylation of LPS had no detectable effect on the oral pathogenicity of *Y. pseudotuberculosis* in a mouse model. This discrepancy might be partly explained by the distinctly different lifestyles of the two enteropathogens in the host. Following oral ingestion, *Salmonella* cells enter the intestinal mucosa and then are taken up by (and survive within) resident phagocytes. Bacterial survival and replication in these cells depend, at least in part, on resistance (through lipid A modification of LPS) to killing by antimicrobial peptides (Gunn *et al.*, 2000). Like *Salmonella*, *Y. pseudotuberculosis* is an enteroinvasive pathogen but after having crossed the epithelial barrier, it resists attack by mucosal macrophages via a plasmid-encoded type III secretion process (Cornelis, 2002), thus blocking phagocytosis.

Growth of *Y. pseudotuberculosis* at temperatures below 37 °C results in increased resistance to polymyxin B, consistent with previous *Yersinia* studies showing that temperature downshifts are associated with higher levels of incorporation of 4-aminoarabinose into lipid A (Bhagya Lakshmi *et al.*, 1989; Kawahara *et al.*, 2002). Nevertheless, we found that transcription of the *Y. pseudotuberculosis pmrF* operon was abolished at low temperatures (M. Marceau and others, unpublished results). Upregulation of lipid A substitution must therefore occur at a post-transcriptional level by an as yet unknown, cold-induced regulatory mechanism. The existence of such a mechanism (designed to counterbalance reduced transcription levels of the *pmrF* operon) strongly suggests that lipid A modifications may also play a crucial role outside the mammalian host.

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