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Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*

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Summary

The two-component regulatory system PhoP-PhoQ of *Pseudomonas aeruginosa* regulates resistance to cationic antimicrobial peptides, polymyxin B and aminoglycosides in response to low Mg^{2+} conditions. We have identified a second two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides. This system responds to limiting Mg^{2+}, and is affected by a *phoQ*, but not a *phoP* mutation. Inactivation of the *pmrB* sensor kinase and *pmrA* response regulator greatly decreased the expression of the operon encoding *pmrA-pmrB* while expression of the response regulator *pmrA in trans* resulted in increased activation suggesting that the *pmrA-pmrB* operon is autoregulated. Interposon mutants in *pmrB*, *pmrA*, or in an intergenic region upstream of *pmrA-pmrB* exhibited two to 16-fold increased susceptibility to polymyxin B and cationic antimicrobial peptides. The *pmrA-pmrB* operon was also found to be activated by a number of cationic peptides including polymyxins B and E, cattle indolicidin and synthetic variants as well as LL-37, a component of human innate immunity, whereas peptides with the lowest minimum inhibitory concentrations tended to be the weakest inducers. Additionally, we showed that the putative LPS modification operon, PA3552-PA3559, was also induced by cationic peptides, but its expression was only partially dependent on the PmrA-PmrB system. The discovery that the PmrA-PmrB two-component system regulates resistance to cationic peptides and that both it and the putative LPS modification system are induced by cationic antimicrobial peptides has major implications for the development of these antibiotics as a therapy for *P. aeruginosa* infections.

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Introduction

Infections with *Pseudomonas aeruginosa* are particularly difficult to cure through antimicrobial therapy because of the bacterium's intrinsically impermeable outer membrane and active efflux of toxic agents from the cytoplasm (Nikaido, 1996). Virtually no novel antibiotics are available for this organism although cationic antimicrobial peptides hold the promise of improving the success of anti-*Pseudomonas* therapy (Hancock, 1997).

Cationic antimicrobial peptides are a structurally diverse group of molecules that are found in virtually all eukaryotes examined to date (Hancock *et al*., 1995; Hancock and Chapple, 1999). In addition to their proven role in killing a wide variety of potential pathogens including Gram-positive and Gram-negative bacteria, fungi and viruses, they are also multifunctional modulators of innate immunity (Scott and Hancock, 2000). They are known to interact with the outer membrane via the self-promoted uptake pathway permitting good activity against Gram-negative bacteria (Hancock *et al*., 1995; Hancock and Chapple, 1999).

As a result of the wide variety of environments in which *Pseudomonas* exists, it has evolved mechanisms for responding to many different stimuli. This diversity of responsiveness is reflected in the genome sequence, in which 9.4% of ORFs encode regulatory proteins (Stover *et al*., 2000). One major class of regulatory systems found in *P. aeruginosa* is the two-component regulatory system family. These systems generally involve a cytoplasmic membrane-spanning histidine kinase sensor protein and a cytoplasmic response regulator. Generally, upon engagement of a periplasmic binding site on the sensor kinase by an effector molecule, this protein autophosphorylates at a histidine residue on the cytoplasmic domain and transfers this phosphate to an aspartate residue of the response regulator leading to increased transcription

of multiple genes, and/or repression of others (Rodrigue *et al*., 2000). The *Pseudomonas* genome contains 64 response regulators and 63 histidine kinases as well as 16 atypical kinases (Rodrigue *et al*., 2000). The function of most of these regulatory proteins is undetermined.

In *Salmonella*, the PhoP-PhoQ two-component regulatory system is a global regulatory system that responds to limiting concentrations of Mg^{2+} and other divalent cations to activate virulence, as well as polymyxin B and cationic antimicrobial peptide resistance, by affecting the transcription of more than 40 genes (Gunn *et al*., 1998; Heithoff *et al*., 1999; Soncini *et al*., 1996). In *P. aeruginosa*, the PhoP-PhoQ system has been shown to control resistance to aminoglycosides, polymyxin B, and cationic antimicrobial peptides (Macfarlane *et al*., 1999; Macfarlane *et al*., 2000; Groisman, 2001). Insertional inactivation of PhoQ, but not PhoP, also decreases the virulence of *P. aeruginosa* in a burned mouse model by 100-fold relative to a wild-type parent strain (Brinkman *et al*., 2001). Lipopolysaccharide isolated from the sputum isolates of patients with cystic fibrosis chronically infected with *P. aeruginosa* showed modifications that reflect those observed in *Salmonella* when grown in Mg^{2+} limiting conditions (Ernst *et al*., 1999).

In *Salmonella*, PhoP-PhoQ-mediated resistance to polymyxin B and cationic antimicrobial peptides largely occurs through changes in the structure of Lipid A, including the addition of N_4-aminoarabinose, ethanolamine, and palmitic acid (Zhou *et al*., 2001), decreasing self-promoted uptake across the outer membrane. The addition of aminoarabinose to the Lipid A-phosphates of LPS is catalysed by the seven-gene operon, *pmrHFIJKLM* (Trent *et al*., 2001a, b) which is controlled indirectly by PhoP-PhoQ via another two-component regulatory system, PmrA-PmrB (Soncini *et al*., 1996). In addition to this regulatory hierarchy between the two response regulators, *Salmonella* PmrB can be independently activated by high (100 μM) concentrations of Fe^{3+} or by reduced pH (possibly by influencing Fe^{3+} solubility) (Soncini *et al*., 1996; Wosten *et al*., 2000). Homologues of the *pmrHFIJKLM* LPS modification system (PA3552-PA3558) are present in the genome of *P. aeruginosa*. In *Pseudomonas*, the operon contains an eighth gene (PA3559) that is homologous to the *ugd* gene (UDP-glucose dehydrogenase) that in *Salmonella* is required for LPS modification and is regulated by the PhoP-PhoQ, PmrA-PmrB and the YojN-RcsA-RcsB systems (Mouslim and Groisman, 2003).

Pseudomonas aeruginosa possesses several closely related homologues of the response regulator PmrA, but not the signal sensor kinase, PmrB. The most closely related *Pseudomonas* homologues of the PmrB sensor kinase share similarity only in the C-terminal kinase domain and, to date, the identity of PmrA and PmrB in *P. aeruginosa* has not been determined.

We present here data that identifies the PmrA-PmrB encoding operon and shows that certain cationic antimicrobial peptides and the polymyxins are capable of inducing the *pmrA-pmrB* genes, and the putative LPS modification operon, thus increasing the resistance of *Pseudomonas* to these agents. This argues that the development of cationic peptide derivatives that do not induce these genes will represent an important improvement in therapeutic treatment of *Pseudomonas* infections of cystic fibrosis patients. We also show that Mg^{2+} regulates the LPS modification operon via a mechanism that is dependent upon both PmrA-PmrB and PhoP-PhoQ systems. These and other observations, demonstrate that the PmrA-PmrB and PhoP-PhoQ systems, although sharing several features, operate differently in *Pseudomonas* and *Salmonella*.

Results

Identification of pmrA-pmrB

To permit screening for Mg^{2+} responsive promoters, a library of mini-Tn*5 luxCDABE* mutants was constructed. Strain H974 contained a transposon insertion between ORFs PA4773 and PA4774 and luminescence was strongly activated under Mg^{2+}-limitation (Fig. 1A; Table 1). These open reading frames are situated in an operon upstream of a two-component regulatory system PA4776 (response regulator) and PA4777 (sensor kinase) and a MerR-type regulator, PA4778. The sensor kinase and response regulator pair exhibited significant similarity at the amino acid level to the PmrA-PmrB system of *Salmonella* (PmrA: 45% identical, 60% similarity; PmrB: 27% identical, 48% similarity), although the periplasmic sensing domain of the sensor protein shared very little homology with that of PmrB from *Salmonella* and this sensor protein was not the closest homologue of *Salmonella* PmrB in the *P. aeruginosa* genome (data not shown).

Regulation of pmrA-pmrB *by* Mg^{2+}

As shown in Table 1, the PA4773::*lux* fusion in strain H974 was strongly induced by low (20 μM) Mg^{2+} and repressed by high (2 mM) Mg^{2+}. To determine if the Mg^{2+}-regulation observed for this fusion was controlled by the PhoP-PhoQ system or other Mg^{2+} responsive elements, a series of double mutants were created. Because PhoQ-null mutants are known to express PhoP-activated genes under normally repressing conditions (Macfarlane *et al*., 1999), *phoQ* mutants were constructed in strain H974, producing strain H975 (*phoQ::xylE*-Gm^R; PA4773::*lux*). This strain exhibited increased activation of the *luxCDABE* cassette under high Mg^{2+} conditions, suggesting that the *luxCDABE* fusion in this strain was controlled in part by

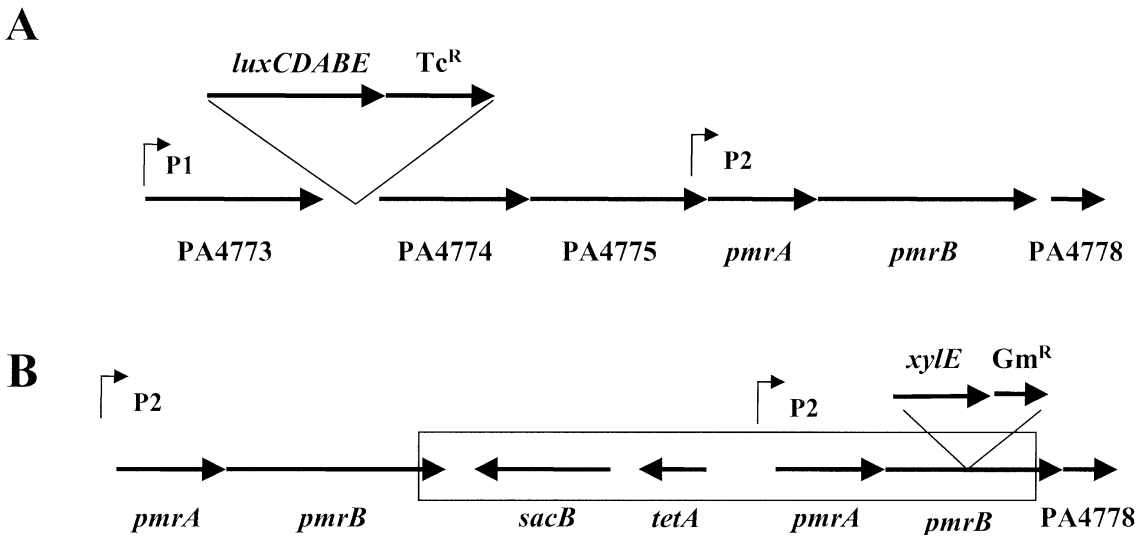

Fig. 1. A. Structure of the operon containing *pmrA-pmrB*. The site of transposon insertion in strain H974 is shown. P1 and P2 represent hypothetical PmrA-regulated and weakly constitutive promoters, respectively, positioned approximately based on the data presented in the text. B. Structure of the merodiploid fusion in strain H973. Plasmid encoded sequences are boxed or the site of insertion of the *xylE*-Gm^R cassette is indicated.

phoQ (Table 1, Fig. 2), although some level of Mg^{2+} regulation was still observed. A *phoP* mutant was also created in the H974 background. This strain (H980) showed wild-type response to Mg^{2+} indicating that the effect of *phoQ* deletion was not mediated via PhoP (Fig. 2).

An interposon mutant, H970, was created in the putative *pmrB* sensor kinase gene by cloning the *pmrA-pmrB* genes and interrupting the *pmrB* gene with a cassette containing the reporter gene *xylE* before recombining the *pmrB::xylE*-Gm^R gene back into *P. aeruginosa*. The presence of the *xylE* cassette allowed examination of the response of *pmrB* transcription to changing environmental conditions. Initial examination of this response showed very little activity regardless of Mg^{2+} concentration, with only 6.4 ± 0.4 pmols·$\mu g^{-1}min^{-1}$ of 2-hydroxymuconic semialdehyde produced under high Mg^{2+} conditions and 21.7 ± 5.0 pmols·$\mu g^{-1}min^{-1}$ produced under low Mg^{2+} conditions. We reasoned that this could be due to a strict requirement for the PmrA and PmrB proteins to activate the operon. To determine whether or not this was the case strain H973 was created (Fig. 1B), a co-integrate merodiploid mutant that contained both a wild-type *pmrB* gene in addition to a *pmrB::xylE* fusion. Strain H973 (merodiploid strain) produced 29.6 ± 1.3 pmols·$\mu g^{-1}min^{-1}$ 2-hydroxymuconic semialdehyde when grown in high Mg^{2+}, increasing 39-fold to 1150 ± 30 pmols·$\mu g^{-1}min^{-1}$ when grown in low Mg^{2+}. This demonstrates that a functional copy of *pmrB* is required for maximal expression of *pmrB*.

To further confirm the strict requirement for *pmrB,* strain H976 was constructed, in which *pmrB* was interrupted by the *xylE*::Gm^R cassette in a H974 (PA4773::*lux*) background. Strain H976 exhibited 10 to 16-fold lower expres-

Table 1. Effect of overexpression of *phoP* or *pmrA* on luminescence of the PA4773::*luxCDABE* fusion in strains H974 (PA4773::*lux*), H975 (PA4773::*lux*, *phoQ*) and H976 (PA4773::*lux*, *pmrB*) fusion under high and low Mg^{2+} conditions. Measurements are expressed in thousands of RLU/OD_{620}.

Conditions	Plasmid	H974	H975	Fold-change[a]	H976	Fold-change[b]
2 mM Mg^{2+} (high)	None	16.6 ± 3.1	78.8 ± 7.3	4.7	2.0 ± 0.8	0.12
	pUC*phoP*	8.8 ± 3.6	52.8 ± 4.2	6	1.3 ± 0.5	0.14
	pUC*pmrA*	20.2 ± 1.7	ND	ND	1.8 ± 0.8	0.09
2 μM Mg^{2+} (low)	None	876 ± 23	829 ± 14	0.94	91.8 ± 1.3[c]	0.10
	pUC*phoP*	951 ± 38	650 ± 18	0.68	40 ± 1.1[c]	0.04
	pUC*pmrA*	2630 ± 30	ND	ND	16.7 ± 0.6[c]	0.006

a. Indicates the fold-change of H975 compared to H974.
b. Indicates the fold-change of H976 compared to H974.
c. The modest 2.5–5.5 fold decrease in RLU in the *pmrA*-overexpressing strain H976 compared to the same strain with no plasmid or the *phoP*-overexpressing strain H976 may be due to the overexpression of PmrA in the absence of phosphorylation by PmrB, causing this PmrA to act as a repressor.

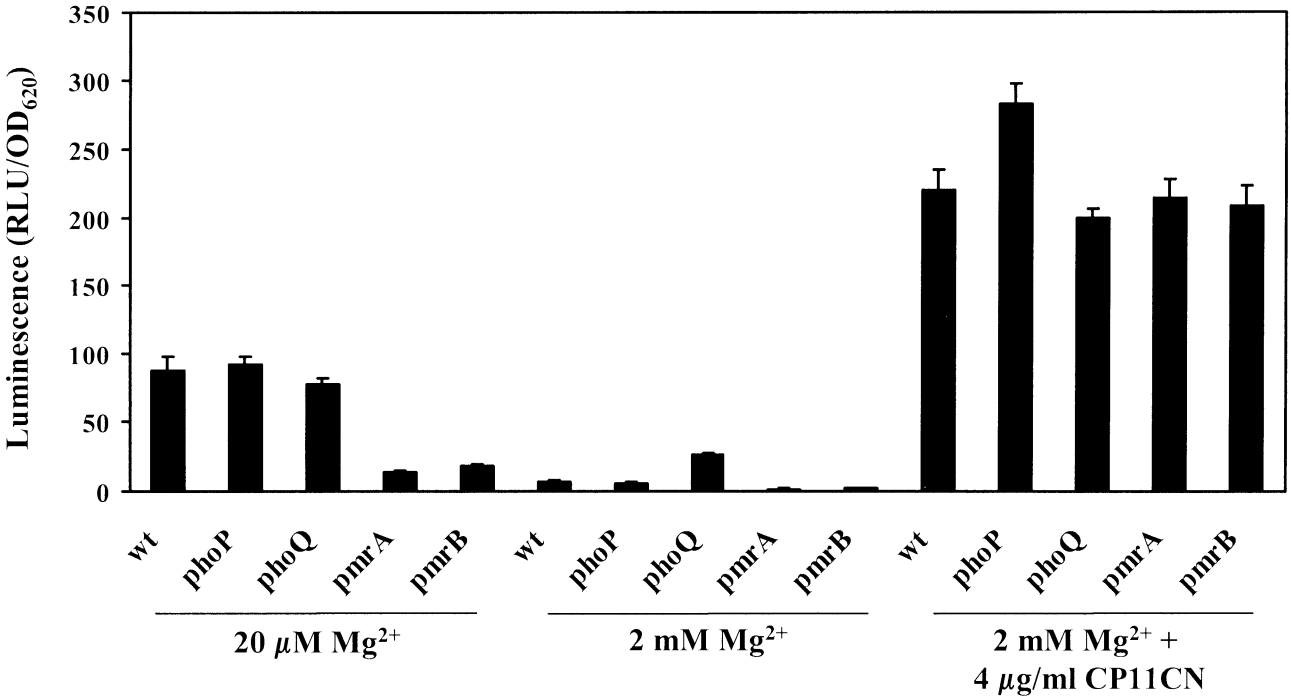

Fig. 2. Induction of PA4773::*lux* fusion in BM2-glucose minimal media supplemented with 20 μM Mg^{2+}, 2 mM Mg^{2+}, and 2 mM Mg^{2+} with 4 μg ml^{-1} CP11CN in H974 (PA4773::*lux*), H980 (PA4773::*lux*; *phoP*), H975 (PA4773::*lux*; *phoQ*), H981 (PA4773::*lux*; *pmrA*) and H976 (PA4773::*lux*; *pmrB*).

sion of the *luxCDABE* reporter (Table 1 and Fig. 2) compared to H974. However both H974 and H976 were inducible in low Mg^{2+} concentrations, which is probably a result of PhoQ-mediated regulation of PmrA phosphorylation as discussed below. Strain H981, a *pmrA* mutant also showed abrogation of the induction of the PA4773:*lux* fusion under low Mg^{2+} conditions (Fig. 2).

When *pmrA* was supplied on a plasmid (pUC*pmrA*) to strain H974 (PA4773::*lux*), luminescence was increased threefold in low Mg^{2+} media (Table 1). When pUC*pmrA* was added to strain H976, which lacked a functional copy of *pmrB*, very little expression was observed from the PA4773::*lux* fusion, even in low Mg^{2+} media. These results are consistent with the hypothesis that PmrB was required to activate PmrA, leading to increased expression of the PA4773::*lux* fusion. Addition of pUC*phoP* did not have any effect on expression of the PA4773::*lux* fusion (Table 1).

The pmrA-pmrB *genes regulate resistance to polymyxin B and cationic antimicrobial peptides*

Strains H103 (wt), H970 (*pmrB::xylE*-Gm^R), and H974 (PA4773::*lux*) were examined for sensitivity to killing by 2 μg ml^{-1} polymyxin B after growth in medium containing high (2 mM) or low (20 μM) Mg^{2+} (Fig. 3). Under high Mg^{2+} conditions, all strains exhibited complete susceptibility to polymyxin B, with 6 log orders of killing taking place within 2 min. In low Mg^{2+} medium, the parent strain was more resistant to polymyxin B, exhibiting only two-log orders of killing in 10 min, whereas the mutants, H970 and H974 were completely susceptible to killing (i.e.>6 log orders reduction in colonies within 5 min) upon exposure to this concentration of antibiotic.

Minimum inhibitory concentration (MIC) data also confirmed the increased susceptibility of strains H970 (*pmrB::xylE*), H974 (PA4773::*lux*), and H988 *(pmrA*::*xylE*) to polymyxin B, and certain cationic antimicrobial peptides (Table 2). The mutant strains showed eight to 32-

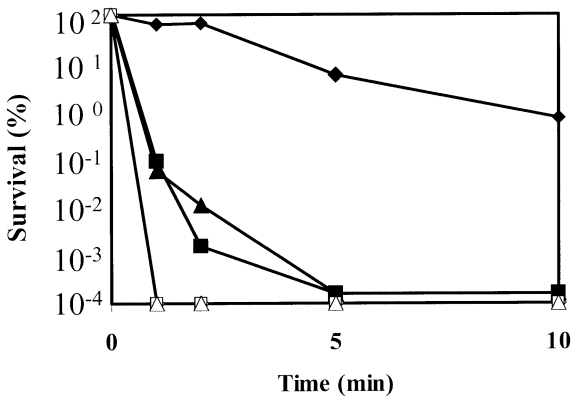

Fig. 3. Killing of *P. aeruginosa* by 2 μg ml^{-1} polymyxin B. Strains are ◇ H103 (WT) grown in 2 mM Mg^{2+}; △ H970 (*pmrB::xylE*), 2 mM Mg^{2+}; □ H974 (PA4773::*luxCDABE*), 2 mM Mg^{2+}; ◆ H103, 10 μM Mg^{2+}; ▲ H970, 10 μM Mg^{2+}; ■ H974, 10 μM Mg^{2+}.

Table 2. Minimal inhibitory concentrations (μg ml^{-1}) of peptides and aminoglycosides toward *P. aeruginosa* grown in low Mg^{2+} medium. MICs were determined by two-fold serial dilution in BM2-glucose minimal media with 20 μM Mg^{2+} added. Results shown are the mode of 4–8 independent experiments.

	MIC (μg ml^{-1})			
Antibiotic	H103 (wt)	H974 (PA4773::*lux*)	H970 (*pmrB::xylE*)	H988 (*pmrA::xylE*)
CP10A	32	2	2	2
indolicidin	32	4	16	16
CP11CN	>32	4	32	32
cycCP11	16	2	16	16
LL-37	16	8	8	16
polyphemusin	1	0.5	0.5	0.5
linear polyphemusin	>32	>32	>32	>32
CEMA	2	1	0.5	1
CP208	>32	>32	>32	>32
polymyxin B	8	0.25	1	0.25
PMBN[a]	>32	>32	>32	>32
colistin	32	16	4	8
tobramycin	2	1	2	2
amikacin	4	1	4	2

a. Polymyxin B nonapeptide.

fold lower MICs to polymyxin B. Strains H974 (PA4773::*lux*), H970 (*pmrB::xylE*), and H988 *(pmrA::xylE*) showed marginal MIC changes to peptides LL-37, polyphemusin, linear polyphemusin and polymyxin B nonapeptide. Linear polyphemusin and CP208 showed no antimicrobial activity toward any of the strains at the concentrations examined. Strain H974 showed eightfold decreases in MIC to indolicidin, CP11CN and cycCP11CN, whereas strains H970 and H988 showed no significant change in MIC to these compounds. CP10A exhibited a 16-fold reduced MIC to all mutant strains. There was little effect of these mutations on the susceptibility of the strains to amikacin and tobramycin. Thus, strain H974 was generally more sensitive than H970 (*pmrB::xylE*-Gm^R) or H988 (*pmrA::xylE*-Gm^R) to most of the antimicrobial agents examined. This may be a result of a polar effect of the transposon on the downstream genes PA4774 and PA4775. The functions of the PA4773, PA4774, and PA4775 genes are unknown, but they share some similarity with polyamine biosynthetic genes.

Induction of the pmrA-pmrB *containing operon by polymyxin B and cationic antimicrobial peptides*

Two types of antibiotic resistance that can influence clinical outcome are mutational resistance and adaptative resistance. Adaptive antibiotic resistance can involve multiple mechanisms, one of which could involve pre-exposure to antibiotic. In the case of polymyxin B resistance in *Salmonella* sp., detection of low Mg^{2+} by PhoQ or high Fe^{3+}/low pH by PmrB leads to induction of genes responsible for the LPS modifications that reduce self-promoted uptake across the outer membrane. We utilized the reporter gene fusions in strains H973 and H974 to determine whether or not these signals and/or polymyxin B and cationic antimicrobial peptides could induce expression of *pmrB::xylE* or PA4773::*luxCDABE*.

No induction was observed in response to increased Fe^{3+} concentrations or lowered pH, the signals responsible for activation of PmrB in *Salmonella* (Fig. 4A). However, a dose-dependent induction of *pmrB::xylE* in the merodiploid strain H973 was observed over a range of concentrations from 0 to 750 ng ml^{-1} of polymyxin B (Fig. 4B). Similarly, growth of the *pmrB::xylE* fusion in high (2 mM) Mg^{2+} with the addition of various subinhibitory concentrations of cationic antimicrobial peptides led to a very strong induction of the *pmrB::xylE* fusion in response to certain cationic peptides (Fig. 4A). Cattle indolicidin caused a 50-fold increase in expression whereas CP11CN, an improved indolicidin variant, increased expression of the fusion by 45-fold. Polyphemusin was considerably less effective with only a fourfold increase.

Utilization as a reporter of the *lux* fusion in H974 permitted rapid and sensitive analysis of the effects on the *pmrA-pmrB* operon of a variety of peptides at different concentrations (Table 3). The patterns observed using the *lux* reporter matched very closely to those observed using the *xylE* reporter although the fold-changes for induction appeared to be higher when measured by *lux* expression, probably because baseline expression could be more reliably determined by luminescence. Thus, both of these reporters appeared to be regulated in the same manner. The PA4773::*lux* fusion in H974 was strongly induced in high Mg^{2+} by indolicidin and the indolicidin variants CP10A, cycCP11 and especially CP11CN (Table 3). These structurally related peptides appeared to affect the expression to similar degrees and importantly, very low

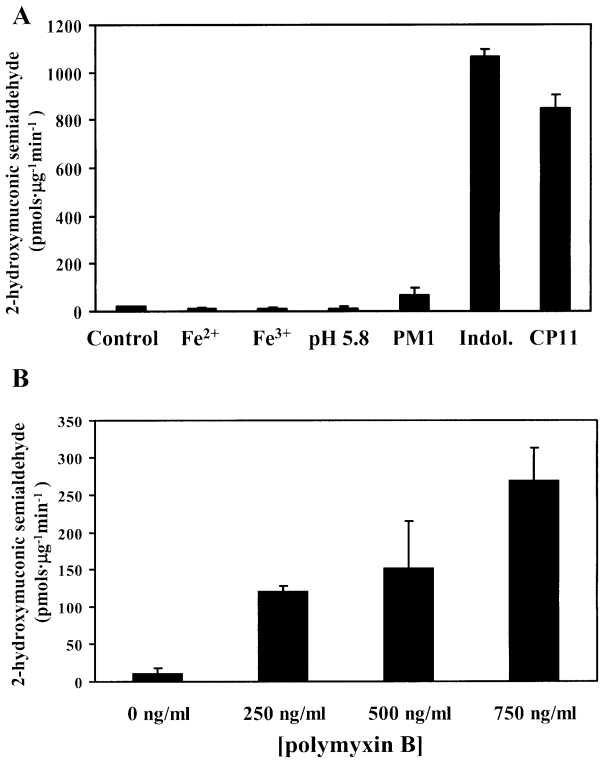

Fig. 4. A. Induction of *pmrB::xylE* fusion in strain H970 by various stimuli. Control – BM2 medium supplemented with 2 mM Mg^{2+} pH 7.0; Fe^{2+} – addition of 100 μM $FeSO_4$ + 300 μM desferroximine mesylate; Fe^{3+} – addition of 100 μM $FeCl_3$ + 300 μM 2, 2 dipyridyl; pH 5.8 – altering the pH of BM2 medium to pH 5.8; PM1 – addition of 62 ng ml^{-1} polyphemusin; Indol. – addition of 500 ng ml^{-1} indolicidin; CP11 – addition of 500 ng ml^{-1} CP11CN.
B. Dose-dependent response to increasing concentration of polymyxin B of the pmrB::xylE fusion in strain H970.

concentrations (31–250 ng ml^{-1}; <1% of MIC) still caused significant induction of this fusion.

The a-helical peptide CEMA was also capable of causing modest induction of luminescence in the PA4773::*lux* fusion, although the lowest concentration causing induction was significantly higher (0.5 mg ml^{-1}) than that observed for CP11CN (31 ng ml^{-1}), despite the fact that the MIC of CEMA toward *P. aeruginosa* is 16-fold lower than that for CP11CN (Table 2). Conversely, the inactive a-helical variant, CP208 still caused induction of the fusion at extremely high concentrations, but induction fell off rapidly as the concentration of CP208 fell below 8 mg ml^{-1}. The weakly antimicrobial human cathelicidin peptide LL-37 demonstrated similar effects to CP208. Nevertheless it is clear that induction of this operon was not related to the ability of the peptides to kill bacteria.

Horseshoe crab polyphemusin and related peptides were also examined for their ability to induce luminescence in the PA4773::*lux* fusion, since polyphemusin displays strong antibacterial activity toward *Pseudomonas*. Interestingly, the β-hairpin peptide polyphemusin induced only a 17-fold increase in luminescence in the PA4773::*lux* fusion in H974 (Table 3), despite the fact that *P. aeruginosa* is >32 fold more sensitive to killing by this peptide compared to indolicidin and its derivatives (Table 2). Interestingly, the inactive (with respect to killing) linear polyphemusin variant P1L caused very high induction of the fusion at concentrations from 0.5 to 4 μg ml^{-1}, equivalent to that observed with the indolicidin-like peptides.

The response to a number of aminoglycoside antibiotics and polymyxins was also examined. None of the aminoglycosides studied were able to induce luminescence in the PA4773::*lux* fusion strain. Five to 11-fold induction was observed in response to polymyxin B concentrations between 0.125 and 1 μg ml^{-1}, to colistin (polymyxin E) between 0.25 and 2 μg ml^{-1}, and polymyxin B nonapeptide between 0.5 and 2 μg ml^{-1}. Thus, induction by polymyxins was substantially lower that that observed with certain peptides.

Table 3. Induction of PA4773:*luxCDABE* fusion in strain H974 in response to cationic peptides.

Antibiotic	Fold-induction	Maximal luminescence (RLU/OD_{620})	Peptide concentration (μg ml^{-1}) leading to 10% luminescence	50% luminescence	100% luminescence
CP10A	82	81500	0.25	1	4
indolicidin	86	85600	0.25	2	8
CP11CN	130	130000	0.031	1	4
cycCP11	119	119000	0.031	1	4
LL-37	25	24500	2	4	16
polyphemusin	17	16800	0.015	0.062	0.5
linear polyphemusin	132	132000	0.5	2	4
CEMA	7	6800	0.5	0.5	2
CP208	16	16100	8	16	32
polymyxin B	5	5200	0.125	0.25	0.5
PMBN[a]	6	5600	0.5	2	4
colistin	11	11000	0.25	0.5	2
gentamicin	1	1260	–	–	–
tobramycin	1	1220	–	–	–

a. Polymyxin B nonapeptide.

A series of mutants were created in strain H974 to determine whether or not the induction, by peptides, of the PA4773:*lux* fusion depended upon the products of the *phoP*, *phoQ*, *pmrA*, or *pmrB* genes. Interestingly, the fusion showed strong induction by CP11CN in all of these strains (Fig. 2), implying that an unknown regulator may be playing a role in the peptide-mediated activation of this operon.

Induction of putative LPS modification operon (PA3552-PA3559) by cationic antimicrobial peptides

Lipopolysaccharide modifications in *Salmonella* are directly regulated by PmrA and PmrB. In order to determine how the eight-gene operon encoding the putative LPS modification system (PA3552-PA3559) of *Pseudomonas* is regulated, we created a plasmid-encoded promoter fusion to the entire intergenic region between PA3551 and PA3552. This *p*PA3552::*lux* fusion was then mobilized into strains H103 (WT), H851 (*phoP*), H854 *(phoQ*), H988 *(pmrA*), and H970 *(pmrB*) and the luminescence of these strains under varying Mg^{2+} and peptide conditions was examined. As shown in Fig. 5, the expression of this fusion was induced 86-fold in low Mg^{2+}. Expression was reduced 13-fold in strain H851 (*phoP*) whereas in strain H988 (*pmrA*) expression was reduced by twofold, indicating that the PhoP activator is the most important determinant of low Mg^{2+} induced activation of the fusion. In high Mg^{2+}, the *p*PA3552::*lux* fusion in the *phoQ* mutant was strongly derepressed, similar to the phenotype observed for the PA4773::*lux* fusion in strain H976 (Fig. 2) and for OprH expression (Macfarlane *et al*., 1999). In addition to this regulation by Mg^{2+}, the *p*PA3552*::lux* fusion was also induced 50 to 80-fold by 4 μg ml^{-1} CP11CN in all strains examined (Fig. 5)

The results obtained using plasmid encoded fusions were then confirmed by carrying out real-time PCR experiments using RNA isolated from H103, H851, and H988, grown in high Mg^{2+}, low Mg^{2+} and high Mg^{2+} + 4 μg ml^{-1} CP11CN. The results were consistent with the observations made for the plasmid encoded pPA3552*::lux* fusion (data not shown).

Discussion

Previous work has demonstrated that the PhoP-PhoQ system of *P. aeruginosa* plays a role in regulating resistance to polymyxin B, α-helical peptides, and aminoglycosides (Ernst *et al*., 1999; Macfarlane *et al*., 1999; 2000) and hinted at the possibility that other regulatory systems are likely involved in regulation of these phenotypes. We have shown here an additional role for PmrAB. This is conceptually similar to the situation for *Salmonella* but this study illustrates several key differences between the PhoP-PhoQ and PmrA-PmrB systems of *Pseudomonas* compared to those of *Salmonella* (Table 4). In *Salmonella*, the PhoP-PhoQ system responds to decreased Mg^{2+} concentrations that are found within the *Salmonella*-

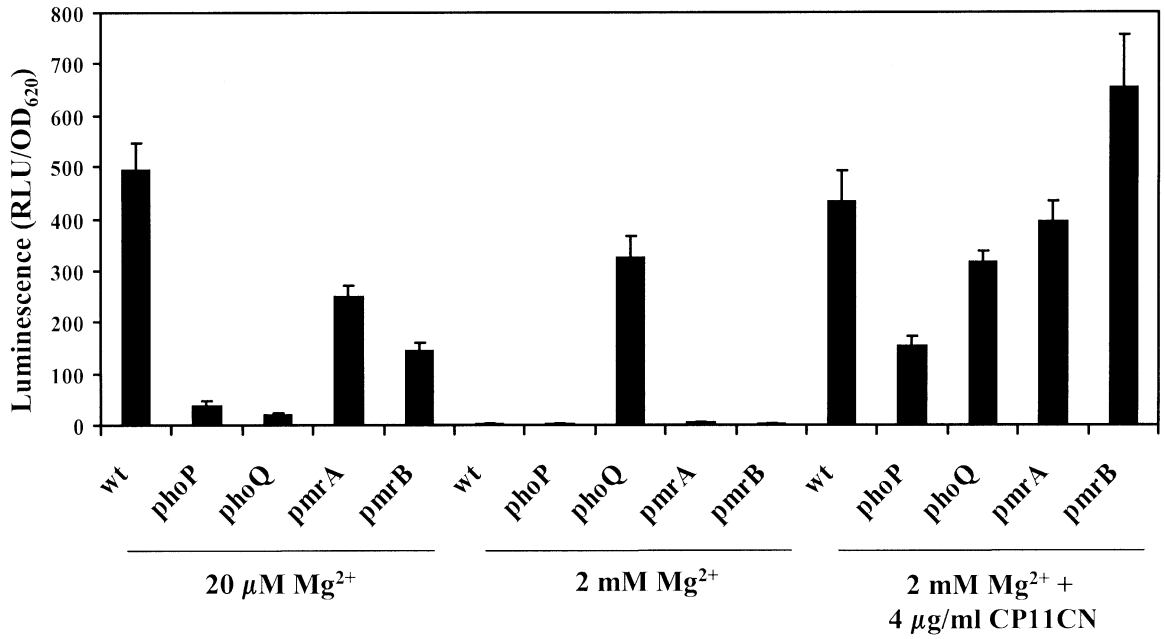

Fig. 5. Induction of *p*PA3552::*lux* fusion in BM2-glucose minimal media supplemented with 20 μM Mg^{2+}, 2 mM Mg^{2+}, or 2 mM Mg^{2+} with 4 μg ml^{-1} CP11CN in H103 (wt), H851 (*phoP*), H854 (*phoQ*), H988 (*pmrA*) and H970 (*pmrB*). The *p*PA3552::*lux* fusion contained a *lux* cassette fused to the entire intergenic region between PA3551 and PA3552 and reports on the transcription of the eight-gene operon encoding the putative LPS modification system (PA3552-PA3559) of *Pseudomonas*. In *Salmonella* this operon is PmrA-PmrB regulated.

Table 4. Differences between the PhoP-PhoQ and PmrA-PmrB systems of *Salmonella* and *Pseudomonas*.

Phenotype/property	*Salmonella*	*P. aeruginosa*
Lifestyle	Intracellular pathogen; SCV is an acidified, low Mg^{2+} environment	Extracellular pathogen associated with mucosal surfaces; commonly found in soil and water samples
Cationic peptide resistance	LPS modifications are important for resistance; PhoP mutants are susceptible	Unique three-gene operon (PA4773-PA4775) also contributes to resistance; PhoP mutants have no resistance phenotype
Virulence	PhoP and PhoQ mutants exhibit decreased virulence; PmrA mutants are also less virulent; constitutive PhoP expression attenuates virulence	PhoQ mutants show 100-fold less virulence than wild-type; PhoP mutants are four times more virulent than wild-type
PhoP	Indirectly regulates PmrA-PmrB via PmrD	Directly regulates OprH and LPS modification operon; 70% similar to Salmonella PhoP throughout entire sequence
PmrA	Directly regulates LPS modification operon	Directly regulates LPS modification operon and the *pmrA-pmrB* operon; *pmrA-pmrB* genes are induced by cationic peptides; 60% similar to *Salmonella* PmrA throughout entire sequence
PhoQ	Activated by limiting Mg^{2+}	Activated by limiting Mg^{2+}, 52% similar to *Salmonella* PhoQ throughout entire sequence, 23 residue insertion in signaling domain
PmrB	Activated by lowered pH and increased Fe^{3+}	Activated by limiting Mg^{2+}, 48% similar to *Salmonella* PmrB in the C-terminal sequence, no similarity in the N-terminal 170 residues

containing vacuole (SCV) and is essential for intramacrophage survival (Groisman, 2001). The PmrA-PmrB system of *Salmonella* is interconnected with PhoP-PhoQ, but also independently activated by increased concentrations of Fe^{3+} (Soncini and Groisman, 1996; Wosten *et al*., 2000). The concentration of iron in the mouse stomach and small intestine may approach the levels required for PmrB-signalling (Wosten *et al*., 2000). Thus, the *Salmonella* PhoP-PhoQ and PmrA-PmrB systems appear to be involved in adapting to the many environments encountered during the lifestyle of *Salmonella* species.

Pseudomonas aeruginosa however, is primarily a pathogen of mucosal surfaces. As such, it does not usually encounter decreases in pH, nor, generally speaking, are the Mg^{2+} concentrations within the airway surface fluid, which can be as high as 2.2 mM, limiting (Cowley *et al*., 1997; Baconnais *et al*., 1999) The airway surface fluid of CF patients can possess a very high concentration of cationic peptides and proteins (300 μg ml^{-1}) (Soong *et al*., 1997), yet the lungs of CF patients are often chronically infected with *P. aeruginosa*, which is capable of resisting these high concentrations of peptides. A previous report indicated that *P. aeruginosa* from the lungs of patients with cystic fibrosis had LPS modifications similar to those associated with the *Salmonella* PhoP-PhoQ-regulated changes and this was taken as evidence of activation of PhoP-PhoQ (Ernst *et al*., 1999). The data presented here provide an alternative explanation for these modifications as endogenous lung peptides and/or polymyxin E aerosol therapy could have resulted in direct activation of the LPS modification operon and the *pmrA-pmrB* operon. *In vitro* data demonstrates that LL-37, a peptide constitutively expressed in the human lung, is capable of inducing the *pmrA-pmrB* fusion at concentrations in the range of 2–16 μg ml^{-1}. This may be clinically relevant, as it is known that newborns with cystic fibrosis complicated by pulmonary infections have levels of approximately 6–8 μg ml^{-1} of LL-37 in their bronchoalveolar lavage fluid (Schaller-Bals *et al*., 2002), a level higher than that shown in non-CF individuals.

In this paper we have described the identification of a two-component regulatory system, PmrA-PmrB, in *P. aeruginosa* and shown that Mg^{2+} regulates this system. This Mg^{2+} regulation is largely independent of the PhoP-PhoQ system, which also responds to Mg^{2+}. PmrA and PmrB are required for high-level expression of this operon under Mg^{2+}-deficient conditions (Fig. 2). The promoter for the *pmrA-pmrB* operon contained an imperfect direct repeat that may form a binding site for PmrA (Fig. 6). Moreover, the fact that the PA4773::*lux* fusion is expressed despite a polar insertion indicates that there must be a second promoter in front of the downstream *pmrA-pmrB* genes.

Interestingly, overexpression of *phoP* in strain H974 (PA4773::*lux*), H975 (PA4773::*lux*; *phoQ::xylE*) and H976 (PA4773::*lux*; *phoP::xylE*) did not affect induction of the operon by low Mg^{2+} (Table 1). In contrast PhoQ is capable of affecting operon expression because a *phoQ* interposon mutant (H975) leads to fivefold increased expression of a PA4773::*luxCDABE* fusion (Table 1). This is consistent with the suggestion that in addition to the ability of PhoQ to act as both a kinase and phosphatase toward PhoP (Macfarlane *et al*., 1999), it may also be able to act as a phosphatase toward PmrA.

As mentioned above, certain classes of cationic antimicrobial peptides and polymyxin B were shown to activate the system in a Mg^{2+}-independent manner. The activation of the PA4773::*lux* fusion by peptides appears to take place in the absence of both the PhoP-PhoQ system and the PmrA-PmrB system. This suggests that another reg-

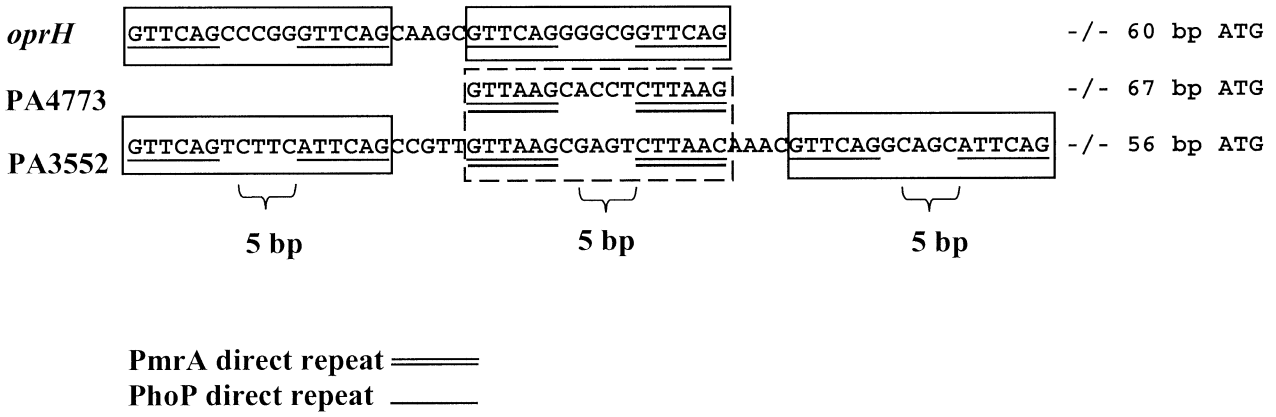

Fig. 6. Alignment of the promoter regions of *P. aeruginosa oprH*, PA4773 and PA3552 depicting putative PhoP and PmrA binding sites (boxed regions). The PhoP binding site (solid line) contains two 6 bp direct repeats separated by a 5-bp spacer region which resembles the known *E. coli* PhoP binding site (Yamamoto *et al*., 2002) and that identified in Macfarlane *et al*. (Macfarlane *et al*., 1999). The PmrA binding site (dashed line) contains similar but distinct, direct repeats with a 5-bp spacer region that resembles the *Salmonella* PmrA binding site (Aguirre *et al*., 2000). The distance between the predicted binding sites and the start codon (ATG) is indicated.

ulatory system that responds to peptides exists for this operon. The relationship between inducibility of the system and resistance to a given peptide was incomplete because it did not require peptide activity *per se* to induce the operon, and because the peptides used have different structures and mechanisms of action and are apparently differentially affected by the PmrAB-regulated resistance mechanism. Indeed, examination of the data in Tables 2 and 3 shows that when the antibacterial activity of an antibiotic was high (e.g. polyphemusin I, CEMA, polymyxin B) its ability to induce PmrA-PmrB was relatively weak. The converse did not hold true, indicating that there can be other reasons for weak antibacterial activity, but the generally poor activity of the indolicidins against *P. aeruginosa* might be explained in part by their strong ability to induce PmrA-PmrB. Another clear caveat is that the ability to induce the *pmrA-pmrB* operon is not related to the mechanism of action of specific peptides, as completely non-lethal peptides like CP208 and polymyxin B nonapeptide were able to induce this operon. Similarly there was no strong relationship between peptide structure and the ability to induce the *pmrA-pmrB*-containing operon as all cationic peptides, including lipopeptides, α-helical, cyclic, and β-hairpin molecules had some ability to induce.

Adaptive antibiotic resistance can involve multiple mechanisms one of which would involve pre-exposure to antibiotic. *P. aeruginosa* exhibits a phenotype known as adaptive polymyxin B resistance (Gilleland and Farley, 1982). This phenotype occurs when *P. aeruginosa* is passaged in medium containing serially increasing concentrations of polymyxin B, after which growth can be observed at concentrations 1000 times greater than the MIC. However, the resistance is not stable, so that when these cells are grown in medium containing no antibiotic, the cells revert to normal susceptibility. The mechanism by which this takes place is unknown, although the regulation of this type of resistance suggests that the cells may be responding directly to the presence of polymyxin B, thereby inducing resistance. The activation of the *pmrA-pmrB* and the PA3552-PA3559 operons by cationic peptides is consistent with the adaptive polymyxin B resistance phenotype.

In *Salmonella*, the PmrA-PmrB system directly controls the *pmrHFIJKLM* system, which is responsible for LPS modifications involved in resistance to cationic antimicrobial peptides. As *Pseudomonas* possesses orthologues of this system, we examined the regulation of these genes (PA3552-PA3559). The *p*PA3552*::lux* fusion was activated by growth on low (20 μM) Mg^{2+}. Deletion of either *phoP* or *phoQ* resulted in ~95% less expression under low Mg^{2+} conditions, whereas deletion of *pmrA* or *pmrB* resulted in ~50% less expression than wild-type cells, but still substantially more than in the *phoP* or *phoQ* mutants. Under high Mg^{2+}, this fusion was 700-fold derepressed in the *phoQ* mutant. This is consistent with the promoter structure of PA3552, which contains three binding motifs, two that resemble a PhoP-like binding sequence (Macfarlane *et al*., 1999), and one that resembles a putative PmrA-like binding sequence (Fig. 6).

Previous studies in *Pseudomonas* have shown that whereas deletion of *phoQ* results in resistance to cationic antimicrobial peptides under Mg^{2+}-replete conditions where the bacteria would normally be sensitive, *phoP* mutants had no susceptibility phenotype (Macfarlane *et al*., 1999; 2000). Data presented in this paper suggests that in addition to PhoQ acting as a kinase under low Mg^{2+} growth conditions, it also acts as a phosphatase under

Mg^{2+}-replete conditions. This difference is consistent with the observation that the PhoQ sensing domain of *P. aeruginosa* undergoes a large conformational shift when Mg^{2+} is non-limiting, while the sensing domain of *E. coli* PhoQ does not (Lesley and Waldburger, 2001). Intriguingly, the data also suggest that PhoQ exerts an effect on genes regulated by PmrA exclusively, since *phoQ* mutants exhibit derepression of a PA4773::*lux* fusion (Fig. 2). We have not eliminated the possibility that PhoQ acts via a PmrD-like intermediate, however, unlike the situation observed in *Salmonella* (Kox *et al*., 2000), the derepression/activation is not dependent upon PhoP (Fig. 2) and there is no gene homologous to *pmrD* in *P. aeruginosa*. Thus, although there are many similarities between the PhoP-PhoQ and PmrA-PmrB systems of *Pseudomonas* and *Salmonella*, there are many differences as well (Table 4).

Collectively, these data indicate that the regulation of cationic peptide resistance is very complex. The data indicate *P. aeruginosa* possesses at least two regulatory systems that respond independently to Mg^{2+}, and PmrB appears to also respond to signals other than limiting Mg^{2+} and different from those signals that *Salmonella* PmrB respond to. Clearly, more work is needed to elucidate the mechanism for cationic peptide-mediated gene induction. Although, the data presented here suggest a potential problem with induced resistance to cationic peptides, the results also point to one group of peptides, the polyphemusins, that are almost unaffected by PmrA-PmrB-mediated resistance and have a relatively poor ability to induce the *pmrA-pmrB* operon. Thus, this provides clear evidence that it is possible to design cationic antimicrobial peptides which are unaffected by the resistance mechanisms discussed here. Future peptide design efforts will be directed towards exploiting this observation.

Experimental procedures

Bacterial strains, primers and growth conditions

The bacterial strains and plasmids used in this study are described in Table 5. The sequences of DNA primers used in the study are available by request from the authors. Cultures were routinely grown in Luria–Bertani broth or BM2-glucose minimal medium containing low (20 μM) or high (2 mM) $MgSO_4$ concentrations. Antibiotics for selection were used at the following concentrations: tetracycline, 100 μg ml^{-1} for *P. aeruginosa* and 10 μg ml^{-1} for *E. coli*; ampicillin 100 μg ml^{-1} for *E. coli*; carbenicillin, 300–500 μg ml^{-1} for *P. aeruginosa*; gentamicin 50 μg ml^{-1} for *P. aeruginosa* and 15 μg ml^{-1} for *E. coli*. Routine genetic manipulations were carried out according to Maniatis *et al*. (1989).

DNA manipulations

A bank of *luxCDABE* fusion strains were made by mobilizing pUT mini-Tn5 *luxCDABE* (Winson *et al*., 1998) into H103 and selecting for resistance to 100 μg ml^{-1} tetracycline. Resulting mutants were screened for differential expression of the fusion in response to high (2 mM) or low (20 μM) $MgSO_4$ concentrations. Interrupted genes were identified by arbitrarily primed PCR using primers Tn*5*luxout (5′-GTCAT TCAATATTGGCAGGTAAACACTATTATCACC-3′) and ARB1 (5′-GGCCACGCGTCGACTAGTACNNNNNGATAT-3′) and the following cycling conditions: 95°C for 5 min followed by 5 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 1.5 min. Thirty cycles were then performed with cycling at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min followed by an extension step at 72°C for 5 min. The products from this PCR reaction were then used as the template in another PCR reaction using primers ARB2 (GGCCACGCGTCGACTAG TAC) and Tn*5*luxout. The cycling conditions for this second reaction were 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min followed by an extension step at 72°C for 5 min. All PCR reactions used 150 μM each dNTP, 5% DMSO, 1 × polymerase buffer and 0.5 μM each primer with 1.25 U of Vent polymerase (NEB, Mississauga, Canada). These amplicons were then sequenced using BigDye sequencing chemistry (PE Biosystems, Foster City, CA) on a BaseStation51 DNA fragment analyzer (MJ Research, Reno, NV). Strain H974 was identified as having an insertion in the intergenic region between PA4773 and PA4774 and is described hereafter as a PA4773::*lux* mutant.

For construction of a *pmrB* interposon mutant, a fragment containing the *pmrA* and *pmrB* genes was PCR amplified using primers designed from the genome sequence. These amplicons were then cloned into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. An *Eco*RI fragment containing the *pmrA-pmrB* genes was then ligated into *Eco*RI digested pEX18Tc (Hoang *et al*., 1998) creating pEX*pmrAB*. A *Bam*HI fragment from pX1918GT containing a *xylE*-Gm^R cassette was cloned into the unique *Bgl*II site in the *pmrB* gene of pEX*pmrAB* creating pEX*pmrB-xylE*. The inserted cassette was shown to be in the forward orientation by restriction analysis. For construction of strain H973, a *pmrB* merodiploid, pEX*pmrB-xylE* was mobilized into the chromosome without selection on 5% sucrose to maintain the plasmid vector sequence. To produce the *pmrA* interposon mutant, *pmrA* was PCR amplified and cloned into pEX100T (Schweizer and Hoang, 1995) producing pEX*pmrA*. A *xylE*-Gm^R cassette from plasmid pX1918GT was removed with *Pst*I and ligated into *Pst*I digested pEX*pmrA*. Gene-replacement constructs were mobilized into *P. aeruginosa* H103 using biparental mating and resolved by successive selection on 50 μg ml^{-1} gentamicin and 5% sucrose. The allelic exchanges were confirmed by PCR. The PCR amplified *pmrA* gene was cloned into pUCP23, producing pUC*pmrA*.

Plasmid pUCP*lux* was created by first replacing the MCS of pUCP23 (West *et al*., 1994) with a new linker (*Eco*RI-*Bam*HI-*Sma*I-*Xho*I-*Not*I-*Hin*dIII). The *luxCDABE* genes from plasmid pCS26 (M. Surette) were cloned as a *Not*I fragment into the new MCS. The orientation of the *luxCDABE* cassette was confirmed by restriction digestion. To determine how the eight-gene operon encoding the putative LPS modification system (PA3552-PA3559) of *Pseudomonas* is regulated, a

Table 5. *Pseudomonas aeruginosa* strains, plasmids, and peptides used in this study.

Strain	Genotype, characteristics, or sequence	Reference
H103	wild-type *P. aeruginosa* PAO1	
H851	*phoP::xylE*-Gm^R derivative of H103	Macfarlane *et al.* (1999)
H854	*phoQ::xylE*-Gm^R derivative of H103	Macfarlane *et al.* (1999)
H970	*pmrB*:: *xylE*-Gm^R derivative of H103	This study
H973	*pmrB*$^+$ *pmrB*::*xylE*-Gm^R merodiploid derivative of H103	This study
H974	PA4773::*luxCDABE* derivative of H103; Tc^R	This study
H975	PA4773::*luxCDABE*; *phoQ*::*xylE*-Gm^R derivative of H103	This study
H976	PA4773::*luxCDABE*; *pmrB*::*xylE*-Gm^R derivative of H103	This study
H980	PA4773::*luxCDABE; phoP::xylE*-Gm^R derivative of H103	This study
H981	PA4773::*luxCDABE*; *pmrA::xylE*-Gm^R derivative of H103	This study
H988	*pmrA::xylE*-Gm^R derivative of H103	This study
Plasmids		
pCR2.1-TOPO	PCR cloning vector, Ap^R	Invitrogen
pCS26	source of a 6-kb NotI *luxCDABE* cassette	M. Surette
pEX100T	suicide vector containing *sacB* gene,Ap^R	Schweizer and Hoang (1995)
pEX18Tc	suicide vector containing *sacB* gene, Tc^R	Hoang *et al.* (1998)
pX1918GT	source of *xylE*-Gm^R cassette; Gm^R, Ap^R	Schweizer and Hoang (1995)
pUC*phoP*	formerly pEMR3, *phoP* cloned into pUCP19	Macfarlane *et al.* (1999)
pEX*pmrA*	pEX100T containing *pmrA* gene	This study
pEX*pmrAB*	pEX100T containing *pmrA-pmrB* genes	This study
pUC*pmrA*	*pmrA* cloned into pUCP23	This study
pEX*pmrA::xylE*	suicide vector containing pmrA::xylE-Gm^R fusion	This study
pEX*pmrB::xylE*	suicide vector containing *pmrB::xylE*-Gm^R fusion	This study
pEXQ-*xylE*F1	suicide vector containing *phoQ::xylE*-Gm^R fusion	Macfarlane *et al.* (1999)
pUT mini-Tn*5 luxCDABE*	mini-Tn*5-luxCDABE* containing plasmid	Winson *et al.* (1998)
pUCP*lux-p*$_{PA3552}$	pUCP23 containing the entire intergenic region between PA3551 and PA3552 fused to *luxCDABE*	
Peptides		
CP10A	ILAWKWAWWAWRR-NH_2[a]	Friedrich *et al.* (2001)
CEMA	KWKLFKKIGIGAVLKVLTTGLPALKLTK[a]	Macfarlane *et al.* (2000)
CP208	KKKSFIKLLTSAKVSVLTTAKPLISS[a]	Friedrich *et al.* (1999)
indolicidin	ILPWKWPWWPWRR-NH_2[a]	Selsted *et al.* (1992)
CP11CN	ILKKWPWWPWRRK-NH_2[a]	Friedrich *et al.* (2000)
cycCP11	$IC_1LKKWPWWPWRRC_1K$[a]	A. Rozek and R. Hancock, unpublished
LL-37	LLGDFFRKSKEKIFKEFKRIVQRIKDFLRNLVPRTES[a]	Gudmundsson *et al.* (1996)
polyphemusin	$RRWC_1FRVC_2YRGFC_2YRKC_1R$[a]	Zhang *et al.* (2000)
linear polyphemusin	RRWAFRVAYRGFAYRKAR[a]	Zhang *et al.* (2000)
polymyxin B	fa-B^L-T^L-B(B^L-B^L-FL^L-B^L-B^L-T^L-)[b]	Windholz *et al.* (1976)
PMBN	B^L-T^L-B(B^L-B^L-FL^L-B^L-B^L-T^L-)[b]	Windholz *et al.* (1976)
colistin	fa-B^L-T^L-B(B^L-B^L-LL^L-B^L-B^L-T^L-)[b]	Windholz *et al.* (1976)

a. Sequence in the one letter amino acid code; -NH_2 indicates amidation of the carboxyl terminus; numbered cysteines represent residues joined by disulphide bonds.
b. Sequence in the one letter amino acid code; B indicates α, γ diamino butyrate; a superscripted L indicates that amino acid is the L-enantiomer; fa indicates a 6-methyloctanoyl or 6-methylheptanoyl fatty acid chain.

plasmid-encoded promoter fusion to the entire intergenic region between PA3551 and PA3552 was created by PCR amplifying the promoter for PA3552 and cloning it in front of the *luxCDABE* cassette, to produce pUCP*lux-p*$_{PA3552}$.

Real-time PCR assays

Total RNA was isolated from mid-log *P. aeruginosa* grown in BM2-glucose minimal media with 20 μM Mg^{2+}, 2 mM Mg^{2+}, or 2 mM Mg^{2+} and 4 μg ml^{-1} CP11CN using RNeasy mini columns (Qiagen). Four micrograms of total RNA was combined with 0.5μM dNTPs, 500 U ml^{-1} SuperaseIN (Ambion), 10 μM DTT, in 1× reaction buffer and reverse transcribed for 1 h at 37°C and 2 h at 42°C with 10 000 U ml^{-1} Superscript II reverse transcriptase (Invitrogen). The RNA was destroyed by the addition of 170 mM NaOH and incubation at 65°C for 10 min. The reaction was neutralized by addition of HCl. The cDNA was then used as template for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The real-time PCR was carried out in 1 × SYBR Green Master Mix, with 200 nM forward and reverse primers designed to the PA3552 ORF, and 1 μl cDNA. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

Gene reporter assays

XylE assays were performed as described previously (Macfarlane *et al.*, 1999). Briefly, a 25 ml *P. aeruginosa* culture was grown in 125 ml Erlenmeyer flasks to an OD_{600} of 0.3–0.6. The cells were pelleted by centrifugation, resuspended in 750 μl of 50 mM potassium phosphate buffer pH 7.5 containing 10% v/v acetone, and broken by sonication. Unbroken

cells and debris were removed by centrifugation. The protein content of the crude extracts was determined by the modified Lowry assay. Aliquots of the cell extracts were then added to 1 ml of 50 mM potassium phosphate buffer, pH 7.5 containing 0.3 mM catechol. The absorbance change of the solution was monitored at 375 nm and the rate of change was used to determine the enzyme activity in the sample using an E375 for 2-hydroxymuconic semialdehyde of 44 000.

Induction of the *luxCDABE* fusion in liquid media was measured using a SPECTRAFluorPlus luminometer (Tecan, San Jose, CA). Luminescence was corrected for growth by simultaneously monitoring the absorbance at 620 nm. Image analysis of colonies grown on agar plates was performed with a Luminograph LB980 photon-imaging video system (EG and G Berthold, Bundoora, Australia).

Killing curves

Pseudomonas aeruginosa cultures were grown to OD_{600} of 0.3–0.6 in BM2-glucose minimal media containing 20 μM Mg^{2+}. These cultures were then diluted 1:100 into prewarmed sodium phosphate buffer, pH 7.5 containing 2 μg ml^{-1} polymyxin B sulphate (Sigma). Samples were shaken at 37°C and aliquots were withdrawn at specified times, and then assayed for survivors by plating onto LB agar.

Minimal inhibitory concentrations (MICs)

Minimal inhibitory concentrations were assessed using standard broth microdilution procedures in BM2 glucose minimal medium containing 10 μM or 2 mM Mg^{2+} (Macfarlane *et al*., 1999). Growth was scored following 24 h incubation at 37°C. For measuring MICs against cationic antimicrobial peptides, a modified assay was used to prevent artificially high MICs due to aggregation of peptides and binding to polystyrene (Zhang *et al*., 2000).

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