Genetic analysis of genes involved in dipeptide metabolism and cytotoxicity in *Pseudomonas aeruginosa* PAO1

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Received 13 November 2007
Revised 2 April 2008
Accepted 11 April 2008

The dipeptide transport operon in bacteria comprises genes for the transport and metabolism of amino acids and dipeptides, as well as haem and haem precursors such as aminolaevulinic acid. Such nutrient and mineral sources are vital for bacteria to survive in and colonize a range of niches. *In silico* analysis of the dipeptide transport systems in sequenced *Pseudomonas* species identified the presence of two genes in *P. aeruginosa* strains that were absent in other sequenced pseudomonads. These genes encode a putative metallopeptidase, PA4498, and a putative transcriptional regulator, PA4499. Proteomic profiling of wild-type PAO1 and a PA4499 mutant strain indicated that PA4499 negatively regulated the putative peptidase, PA4498. Transcriptional fusion analysis verified that expression of PA4498 (*mdpA*, metallo-dipeptidase *aeruginosa*) was negatively regulated by the downstream putative transcriptional regulator PA4499 (*psdR*, *Pseudomonas* dipeptide regulator). Transcriptional fusion analysis also showed that the *dppABCD* operon was under the negative control of *psdR*. Functional genomic analysis of *mdpA* indicated that it is required for the metabolism of a range of dipeptides and that it contributes to the cytotoxicity of PAO1 on an epithelial cell line.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium commonly isolated from soil and water and is renowned for its nutritional and ecological versatility. The ability to transport and metabolize an array of nutrients enables this bacterium to colonize a wide variety of niches. As an opportunistic human pathogen, *P. aeruginosa* is a common cause of nosocomial infections. It is responsible for persistent infection of immunocompromised individuals and can cause chronic lung infections in patients with cystic fibrosis (Govan & Deretic, 1996). Given that epithelial cells act as the initial barrier to infection, an understanding of the interactions between these cells and the bacterium is critical. *P. aeruginosa* has also been shown to cause infections in non-mammalian hosts such as insects (Jander *et al.*, 2000) and plants (Rahme *et al.*, 1995; Silo-Suh *et al.*, 2002; Walker *et al.*, 2004). The metabolic versatility of this bacterium is reflected in the fact that 10% of its genome encodes membrane-associated transporters, with a further 8% involved in the metabolism of carbon-containing compounds (Stover *et al.*, 2000). One transport system of particular interest is the dipeptide (*dpp*) transport operon because of its involvement in the transport of not only dipeptide containing compounds

but also aminolaevulinic acid (Carter *et al.*, 2002; King & O'Brian, 1997), haem (Letoffe *et al.*, 2006) and single amino acids (Tamber & Hancock, 2006).

The *dpp* transport operon has been well characterized and shows conservation in structure across both Gram-negative (Abouhamad & Manson, 1994) and Gram-positive (Guedon *et al.*, 2001; Slack *et al.*, 1995) bacterial species. The *dpp* operon has been shown to be maximally expressed in the stationary phase of growth, with expression driven from the promoter region upstream of the first gene, *dppA* (Abouhamad & Manson, 1994). In *Salmonella typhimurium* and *Escherichia coli*, *dppA* has been shown to play a role in chemotaxis (Abouhamad *et al.*, 1991). The role of the *dpp* transport machinery in utilizing haem iron has also been shown in *E. coli*. Binding of haem by the DppA protein allows transport of this molecule into the cell via the *dppBCDF* ATP-binding cassette transporter (Letoffe *et al.*, 2006).

To date relatively little information is available regarding the regulation of this transport machinery. Regulation of the *dpp* transport operon in *Bacillus subtilis* has been shown to be exerted by the pleiotropic negative regulators CodY and AbrB (Slack *et al.*, 1995). In *Lactococcus lactis*, a homologue of CodY was shown to respond to the levels of branched-chain amino acids, thereby sensing aspects of the nutritional supply for the cell. Additionally an *lrp* (encoding a leucine-responsive regulatory protein) gene

Abbreviations: HTH, helix–turn–helix; LES, Liverpool epidemic strain; XRE, xenobiotic response element.

from *Bradyrhizobium japonicum* has been shown to complement an *E. coli* *dpp* mutant strain for the uptake of dipeptides (King & O'Brian, 1997). In *E. coli* the *gcvB* gene, which encodes a small untranslated RNA, has been shown to regulate expression of both the Dpp and oligopeptide (Opp) transport systems. This small untranslated RNA appears to regulate genes associated with oligopeptide transport at the translational level whereas it appears to regulate *dppA* expression at the mRNA level (Urbanowski *et al.*, 2000).

P. aeruginosa PAO1 contains a gene cluster which shows a high level of homology to previously characterized *dpp* transport operons (<http://www.pseudomonas.com>). Interestingly, transcriptome analysis has shown that *dppA3* in *P. aeruginosa* PAO1 is induced under low-iron conditions (Ochsner *et al.*, 2002). The porin OpdP, which is associated with this putative dipeptide transport machinery in *P. aeruginosa* PAO1, is required for the transport of the dipeptide glycyl-glutamic acid (Tamber & Hancock, 2006). The *opdP* gene is induced to a high level when bacteria are grown on the amino acid L-arginine, suggesting involvement of OpdP in the uptake of single amino acids as well as dipeptides (Tamber & Hancock, 2006).

Previous transcriptome analysis of *P. aeruginosa*, carried out in the presence of a human airway epithelial cell line,

showed that expression of the gene *PA4498*, encoding a putative metallopeptidase, was upregulated 4.8-fold (Frisk *et al.*, 2004). Variations in gene expression have been shown to influence the virulence of the Liverpool epidemic strain (LES) of *P. aeruginosa*. The highly virulent, epidemic strain LES431 has shown increased resistance to antimicrobials and has caused mortality in both immunocompromised and non-immunocompromised patients. Transcriptome analysis of this strain has shown that it also overexpresses the *mdpA* gene, encoding a putative metallopeptidase (Salunkhe *et al.*, 2005).

This paper identifies the *psdR* gene as a transcriptional repressor of the *mdpA* and *dppA3* genes. It also demonstrates that the *mdpA* gene is essential for the growth of *P. aeruginosa* on dipeptides as sole carbon source and suggests an involvement of this gene in *P. aeruginosa* cytotoxicity.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. All cultures of *P. aeruginosa* PAO1 and associated mutants were routinely grown on Casamino Acid (CAA) medium supplemented with 100 µM FeCl₃, LB or M9 minimal medium (Sambrook *et al.*, 1989) at 37 °C. The mPA4498 (mPA*mdpA*) mutant strain was obtained from the University of Washington Mutant Library (Jacobs *et al.*, 2003).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild-type	Holloway & Morgan (1986)
PA <i>psdR</i>	Δ <i>psdR</i> ::Gm ^r	This study
PAO1/pMP220::P <i>psdR</i>	P <i>psdR</i> ; <i>lacZ</i> ; Tc ^r Km ^r	This study
PAO1/pMP220::P <i>dppA</i>	P <i>dpp</i> ; <i>lacZ</i> ; Tc ^r Km ^r	This study
PA <i>psdR</i> /pMP220::P <i>dppA</i>	Δ <i>psdR</i> ::Gm ^r ; P <i>dppA</i> ; <i>lacZ</i> ; Tc ^r Km ^r	This study
PA <i>psdR</i> /pMP220::P <i>dppA</i> pBBR <i>psdR</i>	Δ <i>psdR</i> ::Gm ^r ; P <i>dppA</i> ; <i>lacZ</i> ; Tc ^r Km ^r ; <i>psdR</i> ; Cm ^r	This study
PAO1/pMP220::P <i>mdpA</i>	P <i>mdpA</i> ; <i>lacZ</i> ; Tc ^r Km ^r	This study
PA <i>psdR</i> /pMP220::P <i>mdpA</i>	Δ <i>psdR</i> ::Gm ^r ; P <i>mdpA</i> ; <i>lacZ</i> ; Tc ^r Km ^r	This study
PA <i>psdR</i> /pMP220::P <i>mdpA</i> pBBR <i>psdR</i>	Δ <i>psdR</i> ::Gm ^r ; P <i>dppA</i> ; <i>lacZ</i> ; Tc ^r Km ^r ; <i>psdR</i> ; Cm ^r	This study
mPAO1	Wild-type	Jacobs <i>et al.</i> (2003)
mPA <i>mdpA</i>	<i>mdpA</i> ::Tn5 Tc ^r	Jacobs <i>et al.</i> (2003)
<i>Escherichia coli</i>		
TOP 10 F' DH5α	TOPO chemically competent cells	Invitrogen
XL1 Blue	Chemically competent cells	NEBioLabs
Plasmids		
pBBR1MCS	Cloning vector, BHR, Cm ^r	Kovach <i>et al.</i> (1994)
TOPO Z	TOPO cloning vector	Invitrogen
pMP220	Transcriptional reporter	Spaink <i>et al.</i> (1987)
PΔ <i>psdR</i> 1	TOPO-Frg1-Gen-Frg2, <i>psdR</i> ::Gen	This study
pK19Δ <i>psdR</i>	pK19-Frg1-Gen-Frg2, <i>psdR</i> ::Gen	This study
pTF99	P <i>psdR</i> : <i>lacZ</i> pmp220	This study
pTF98	P <i>mdpA</i> : <i>lacZ</i> pmp220	This study
pTF00	P <i>dppA</i> : <i>lacZ</i> pmp220	This study
pRK2013	Helper plasmid, Tra ⁺ Mob ⁺ ColE1 Km ^r	Figurski & Helinski (1979)
pK19 <i>mobsac</i>	Suicide vector	Schafer <i>et al.</i> (1994)

E. coli strains were routinely grown in LB medium at 37 °C. Where appropriate, antibiotics were added to growth media at the following concentrations: for *P. aeruginosa*, 50 µg kanamycin ml⁻¹, 200 µg chloramphenicol ml⁻¹ and 50 µg gentamicin ml⁻¹; for *E. coli*, 25 µg gentamicin ml⁻¹, 50 µg ampicillin ml⁻¹ and 12.5 µg tetracycline ml⁻¹.

Mammalian cell culture. Immortalized human epithelial cell line 16HBE140 S-1 was obtained from P. B. Davis (Case Western Reserve University) (Kube *et al.*, 2001; Rajan *et al.*, 2000). Cells were cultured on BSA-collagen-fibronectin-coated plastic, using minimum essential medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 µg penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 400 µg neomycin G-418 ml⁻¹. Cells were incubated at 37 °C in a humidified 5 % CO₂ atmosphere. All cell culture reagents were supplied by Sigma, unless stated otherwise.

DNA and RNA manipulations. Restriction digests, ligations, transformations and agarose gel electrophoresis were performed as described by Sambrook *et al.* (1989). All restriction enzymes were obtained from Roche Pharmaceuticals. Small-scale plasmid DNA isolation was performed using the Qiagen Plasmid Mini-kit according to the manufacturer's instructions. Prior to cloning, PCR products were purified either from agarose gels using the Qiagen Gel Extraction kit or directly from solution using the Qiagen PCR Purification kit and cloned into the pCR2.1TOPO plasmid using the TA cloning kit according to the manufacturer's specifications (Invitrogen). Plasmids were mobilized into *Pseudomonas* strains by triparental mating using the helper plasmid pRK2013 (Figurski & Helinski, 1979).

Determination of nucleotide sequence and sequence analysis. All transcriptional fusions and *psdR* gene disruption were confirmed by nucleotide sequencing performed at Lark Technologies. The sequence data were assembled using the DNASTAR software package and analysed using the University of Wisconsin genetic computer group (GCG) program FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1990) at the National Centre for Biotechnology Information (NCBI). Multiple sequence alignments were performed using the CLUSTAL W program (Chenna *et al.*, 2003).

Construction of the *psdR* mutant. To construct a PA4499 (*psdR*)-negative mutant we first amplified two genomic fragments. Fragment 1 (Frg1) covers the region extending from +1435 to -130 relative to the PA4498 (*mpdA*) ATG start codon, and fragment 2 (Frg2) covers the region extending from +270 relative to the *psdR* ATG start codon to +507 relative to the PA4500 (*dppA3*) ATG start codon. These two fragments are located 348 bp apart. The joining of Frg1 and Frg2 would delete 348 bp of the *psdR* gene including 58 bp of the untranslated region upstream to the ATG and the first 96 codons of the *psdR* ORF. We engineered *PstI* and *NotI* restriction sites at the 5' and the 3' ends of Frg1 and *NotI* and *XbaI* restriction sites at the 5' and the 3' ends of Frg2 respectively. These fragments were separately cloned into the TOPO cloning vector. Frg1 and Frg2 were then joined at the *NotI* site. The vector containing Frg1 and Frg2 was linearized at the *NotI* site and ligated to the gentamicin-resistance cassette digested with the same restriction enzyme to produce the recombinant plasmid PΔ*psdR1* containing a disrupted *psdR* gene. The disrupted gene was transferred to into the allelic-exchange vector pK19*mobsac* using *PstI* and *XbaI* restriction sites to create pK19Δ*psdR*. The *sacB* gene contained on pK19*mobsac* codes for the enzyme levansucrase, which confers sucrose susceptibility to cells expressing the intact gene. Triparental mating was carried out to transfer this construct from *E. coli* XL1 to *P. aeruginosa* PAO1 wild-type strain. The positive clones, which had integrated the plasmid into the chromosome, were selected for on 50 µg gentamicin ml⁻¹, 50 µg ampicillin ml⁻¹. The loss of the *sacB* gene by a second crossover allows the host to grow on medium supplemented with 5 % sucrose. Thus to select for a double crossover

event we plated all PAO1 transconjugants that were gentamicin resistant on LB supplemented with 5 % sucrose. All gentamicin- and sucrose-resistant colonies were considered as candidates to possibly contain the double crossover. Cells were subsequently screened by PCR using primers PK4499CKF and PK4499CKR (Table 2). The confirmed disruptants with the double crossover were named PΔ*psdR*.

Complementation of the *psdR* mutant strain. For the complementation of the strain carrying the mutation in *psdR*, primers PKCOMP99F and PKCOMP99R were designed, which would amplify the entire *psdR* gene and its promoter region. This fragment was cloned into the TOPO cloning vector. This was then digested back out from the TOPO vector using the restriction enzymes *HindIII* and *XbaI*. Plasmid pBBR1MCS was also digested using *HindIII* and *XbaI*. Both insert and linearized pBBR1MCS backbone were ligated at 16 °C. The ligated construct, pBBR*psdR*, was electroporated initially into *E. coli* and following this into the *psdR* mutant strain of *P. aeruginosa*. The PΔ*psdR*/pBBR*psdR* strain was selected for on 50 µg gentamicin ml⁻¹, 200 µg chloramphenicol ml⁻¹.

Construction of the transcriptional fusions. To characterize the transcriptional activity of the *psdR*, *mdpA* and *dppA3* genes, we used the pMP220 vector bearing a promoterless *lacZ* reporter gene. Two pairs of primers, PKTF4499F/PKTF4499R and PKTF4498F/PKTF4498R (Table 2), were used to amplify a 592 bp fragment containing the *psdR*-*mdpA* intergenic region with the *psdR* and *mdpA* predicted promoters and part of the ORF of both genes. To facilitate directional cloning into pMP220 and a correct fusion of *psdR* and *mdpA* promoters with the *lacZ* reporter gene we introduced a *PstI* site downstream and an *EcoRI* site upstream of each promoter. Therefore, both transcriptional fusions, pTF99 (*psdR*) and pTF98 (*mdpA*), contain the full-length *mdpA*-*psdR* intergenic region but in different orientation. The two constructs were transferred into *P. aeruginosa* PAO1 and the PΔ*psdR* mutant strain by electroporation to produce pMP220::P*psdR* and pMP220::P*mdpA* respectively. To characterize the transcriptional activity of *dppA* the primers PKTF4500F and PKTF4500R were used to amplify a 414 bp region. This consisted of the entire *psdR*-*dppA* intergenic region and the first 62 bp of the *dppA* gene. To facilitate directional cloning into pMP220 and correct fusion of the *dppA* promoter with the *lacZ* reporter gene, a downstream *PstI* and upstream *EcoRI* site were introduced. This construct was then transferred into *P. aeruginosa* PAO1 and the PΔ*psdR* mutant strain by

Table 2. Primers used in this study

Name	Sequence (5'-3')
FRG1F	CTGCAGCGCAGATCATCGGTTCTGTTGC
FRG1R	GCGGCCGCGCGGGCGTCAATAAAGTCTG
FRG2F	GCGGCCGCGCCTACGAACGCCTGTCCAG
FRG2RT	CTAGAGAGATCTTCACGTCTTCGGGTTTCC
PK4499CKF	GTCACATTAAAATAGGGGTTTCG
PK4499CKR	GGTCAGGACTTCCGCCACAT
PKCOMP99F	TCCATCATGTTCCGCAGAC
PKCOMP99R	TCAACGAAATTAAGCACGACT
PKTF4499F	GCCGCCGAATTCTACTCCCGCCAACAC
PKTF4499R	GCCGCCCTGCAGGCTGCAGGCTCTTCT
PKTF4498F	GCCGCCCTGCAGTACTCCCGCCAACAC
PKTF4498R	GCCGCCGAATTCTCGGTGCAGGCTCTTCT
PKTF4500F	GGCAGCGATGTGGCGGAAGTC
PKTF4500R	TTGCTGGCGCGTGGGAGAAC

electroporation to produce PAO1/pMP220::P*ddpA* and P*ApsdR*/pMP220::P*ddpA* respectively.

To verify the transcriptional fusion analysis the strains P*ApsdR*/pMP220::P*ddpA* and P*ApsdR*/pMP220::P*mdpA* were complemented using the vector pBBR*psdR*. The construct was transferred into P*ApsdR*/pMP220::P*ddpA* and P*ApsdR*/pMP220::P*mdpA* by electroporation to produce P*ApsdR*/pMP220::P*ddpA* pBBR*psdR* and P*ApsdR*/pMP220::P*mdpA* pBBR*psdR* respectively.

Proteomic analysis

Protein isolation. Cells were inoculated at an OD₆₀₀ of 0.01 and grown to an OD₆₀₀ of 0.6 in 200 ml CAA medium. Total protein from the bacterial suspension was isolated by removing whole cells from the culture by centrifugation (7000 r.p.m., 10 min at 4 °C) using a Sorvall SS-34 rotor in a Sorvall RC-5B centrifuge. The pellet was resuspended in 6 ml HEPES buffer (0.1 M, pH 7.4), protease inhibitor cocktail, DNase (50 U), RNase (10 mg) and 200 µl MgCl₂ (100 mM). Resuspended cells were lysed by sonication (5 × 20 s). Cellular debris was removed by centrifugation (6000 r.p.m., 10 min at 4 °C). Aliquots of 1–2 ml of extracts were extracted twice with 1 ml phenol heated for 10 min at 70 °C. The proteins (solid phase) were precipitated by adding 2 ml acetone.

Two-dimensional gel electrophoresis. Pellets were resolubilized in solution A [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 50 mM DTT] and diluted to a concentration of 10 mg protein ml⁻¹. Then 100 µl solution B [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, carrier ampholytes, Triton X-100 and bromophenol blue (0.005 g ml⁻¹)] were added to 3 mg protein. Proteins were loaded by in-gel rehydration on 24 cm pH 4–7 immobilized pH gradient (IPG) strips (Amersham Biosciences). Isoelectric focusing was conducted for a total of 89 kV h. IPG strips were then reduced, alkylated and loaded for second-dimension SDS-PAGE as described previously (Nouwens *et al.*, 2000). Coomassie-blue-stained gels were imaged using a Fluor-S Multimager. To reduce the likelihood of false variations in spot intensity, protein mix from each sample was separated in triplicate gels. The 2D gel electrophoresis was repeated twice in two different experiments. To reduce gel-to-gel variation within the three repeated gels for each strain, one average gel for the proteome of each strain was generated using Photoretix PG200 software (Nonlinear Dynamics).

Peptide mass mapping. Protein spots were excised from the gel, washed three times with 120 µl ammonium bicarbonate (pH 7.8, 25 mM)/acetonitrile 50% (v/v) and dried using a SpeedVac (Savant Instruments). Gel pieces were rehydrated with 8 µl ammonium bicarbonate (25 mM, pH 7.8) containing 10 ng porcine modified trypsin µl⁻¹ (Promega) for 10 min at room temperature. Proteins were then digested at 37 °C for 16 h. Peptides were extracted from the gel in 8 µl 50% acetonitrile/1% trifluoroacetic acid (TFA). A 1 µl aliquot of each extract was spotted onto a target plate, covered with 1 µl 8 mg α-cyano-4-hydroxycinnamic acid ml⁻¹ in 50% acetonitrile/1% TFA and allowed to dry. Peptide masses were generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Maldi-TOF Voyager DE-Pro. All spectra were obtained in reflectron mode using an accelerating voltage of 20 kV. Mass calibration was performed using Calibration Mix 2 (PE Biosystems) as an external standard. The peptide mass profile was analysed using Data Explore (PerSeptive Biosystems) software and tested against the Mascot Peptide Mass Fingerprint database (<http://www.matrixscience.com>). The parameters for a successful identification included a mass tolerance of 150 p.p.m. per peptide and total sequence coverage greater than 30%. Identified proteins were reconfirmed by checking their predicted mass and pI values against their location in the gels.

Epithelial cell infection conditions. For infection studies, cells were washed twice with phosphate-buffered saline, detached and resuspended in the described cell culture medium and seeded at the appropriate cell number 5 days prior to infection to obtain confluent monolayers on the day of infection. Bacterial strains were cultured overnight aerobically for 16–18 h in cell culture medium without antibiotics (infection medium) at 37 °C. Following PBS washing to remove extracellular components, bacterial densities (in infection medium) were adjusted so as to infect PBS-washed, fully confluent cell monolayers at an m.o.i. of approximately 50:1. Serial dilutions were plated onto LB agar to confirm both the m.o.i. used and the bacterial numbers after 9 h of infection. C.f.u. ml⁻¹ values for mutant and wild-type strains showed no significant difference. Quantification of cytotoxicity was carried out for mPAO1 and the *mdpA* mutant strain as previously described (O'Grady *et al.*, 2006).

Growth curve analysis. Mutants and parent strains were grown overnight in M9 minimal medium (Sambrook *et al.*, 1989) supplemented with glucose. Cells were then washed in quarter-strength Ringer's solution and resuspended at a final OD₆₀₀ of 0.01 in M9 minimal medium supplemented with different dipeptides (Gly-Asp, Gly-Gln, Gly-Gly, Gly-Phe, Gly-Ala, Ala-Val, Val-Val, Phe-Pro, Gly-Pro) at a concentration of 10 mM. OD₆₀₀ readings were taken over a 4 day period (30 s every 15 min) with cultures grown with shaking at 37 °C. Analysis of growth was carried out using the Bioscreen C (Oy Growth Curves, Helsinki, Finland) 200-well culture growth monitoring instrument.

RESULTS

Bioinformatic analysis identifies novel genes associated with the *ddp* operon

Bioinformatic analysis of the dipeptide transport genes in all sequenced *Pseudomonas* strains has highlighted different levels of complexity in the operon structure (Fig. 1). In particular the cluster of genes associated with *ddp* transport in *P. aeruginosa* strains PAO1 and PA14 contains an extra four genes which encode a putative transcriptional regulator (PsdR), a putative metalloproteinase (MdpA) and two predicted dipeptide-binding proteins (DppA1, DppA2). The *mdpA* and *psdR* genes are not found in the genomes of *Pseudomonas entomophila* L48 (Vodovar *et al.*, 2006), *P. putida* KT2440 (Nelson *et al.*, 2002), *P. syringae* pv. tomato DC3000 (Buell *et al.*, 2003), *P. syringae* pv. *syringae* B728a (Feil *et al.*, 2005) and *P. syringae* pv. *phaseolicola* 1448A (Joardar *et al.*, 2005). Bioinformatic analysis of *mdpA*, located upstream of the putative transcriptional regulator *psdR*, has identified the protein as a putative metalloproteinase. BLASTP pairwise comparisons showed that MdpA has substantial amino sequence similarity to and domain conservation with Xaa-Pro aminopeptidases. Domain analysis showed that PsdR is a MerR family-like regulator with a helix–turn–helix (HTH) XRE (xenobiotic response element) domain and a cupin sensor domain. In *P. fluorescens* Pf-5 bioinformatic analysis identified a region with DNA sequence homology to *psdR*. However, this DNA sequence was found to contain an internal stop codon.

Fig. 1. Genomic organization of genes associated with *dpp* transport in sequenced *Pseudomonas* species and *E. coli* K-12. The *dppA4BCDF* gene cluster encodes proteins involved in single and dipeptide amino acid transport (in *E. coli*: Abouhamad & Manson, 1994); *opdP* has been shown to play a role in the transport of the dipeptide glycyl-L-glutamic acid (Tamber *et al.*, 2006). In *P. aeruginosa* PAO1 and PA-14 the presence of genes encoding two extra putative binding proteins, *dppA1*, and *dppA2*, and the genes *mdpA* and *psdR* are found. The gene *psdR* encodes a putative transcriptional regulator, and domain analysis has shown that PsdR belongs to the MerR family of transcriptional regulators containing a HTH XRE domain and cupin sensor residue. The gene *mdpA* encodes a putative metallo-dipeptidase. In *P. fluorescens* Pf-5 a region directly upstream of the *dpp* operon is homologous to the probable transcriptional regulator, *psdR*. This, however, is predicted to be a non-coding region because it contains an internal stop codon. The *dppA1,2* and *mdpA* genes are not present in this strain. The transport operon identified in *P. entomophila* L48 is similar to that of *P. fluorescens* and contains two additional genes encoding dipeptide-binding proteins. Other fully sequenced *Pseudomonas* species, including *P. putida* KT2440, *P. syringae* DC3000 and *P. syringae* b728a, contain the more conventional, condensed dipeptide transport operon and lack the upstream *dppA1,2*, *mdpA* and *psdR* genes. The dipeptide transport operon in *E. coli* K-12 lacks a copy of the *opdP* gene, and contains only a single *dppA* gene.

Proteomic analysis identifies targets of *psdR*

In order to investigate the effect of the *psdR* gene product on the genes located immediately downstream and upstream of it, we constructed a *psdR* mutant (PA*psdR*) with a deletion covering most of the predicted promoter and half of the ORF which includes the HTH domain. Proteomic analysis was performed to examine the global impact of *psdR* deletion. Total proteins were extracted from PAO1 and the PA*psdR* mutant strains grown to an OD₆₀₀ of 0.6 in CAA medium. Up to 250 protein spots were visible on gels of both mutant and wild-type strains, representing approximately 5% of the total proteome (Fig. 2). In order to reduce gel-to-gel variation within the three repeated gels for each strain, one average gel was generated for each set using Photoretix PG200 software. The use of this approach to compare proteome profiles from wild-type and the *psdR* mutant strain identified one genuine difference (Fig. 2a, b). The protein spot that was present in the *psdR* mutant and undetectable in the parent strain was characterized using MALDI-TOF MS and identified as the putative metallopeptidase, MdpA. These results suggest that the putative transcriptional regulator, PsdR, negatively regulates expression of the metallopeptidase, MdpA.

PsdR negatively regulates transcription of *mdpA* and *dppA3*

In silico analysis of the promoter region of the *mdpA* gene was carried out to predict whether PsdR plays a direct role in regulating its expression. The promoter region identified contained a 21 bp spacer region between the –10 and –35 consensus sites. This elongated spacer region is associated with promoters that could be under the control of MerR-type regulators (Brown *et al.*, 2003), to which PsdR shows homology. This observation suggests that the expression of *mdpA* could be under the control of PsdR. Inspection of the promoter region of *dppA3* identified a promoter region homologous to that of *mdpA* (Table 3). This suggests that PsdR may also function in regulating expression of the *dpp* transport machinery.

Expression of *mdpA* in a *psdR* mutant strain was much higher than in the wild-type based on β -galactosidase transcriptional fusion analysis. Expression was also growth phase dependent, with maximum levels seen at mid-exponential phase (Fig. 3a). This result indicates that expression of *mdpA* is negatively regulated by PsdR. Expression of *dppA3* was significantly higher in the mutant background, and levels of expression increased throughout

Fig. 2. Two-dimensional gel electrophoresis proteome profiles of the *psdR* mutant (a) and wild-type PAO1 (b) strains. The 2D analysis was carried out in two independent experiments with triplicate gels for the mutant and the wild-type. The arrows indicate the protein spot corresponding to MdpA present in the mutant and undetectable in the wild-type. Triplicate gels were analysed using Photoretix PG200 software. The protein spot was identified by MALDI-TOF.

growth. This result suggested that *dppA3* expression is also negatively regulated by *psdR*. When the *psdR* mutant was complemented with the plasmid pBBR*psdR*, the level of expression of *mdpA* was similar to wild-type and remained below 400 Miller units over the 24 h period. A similar pattern emerged when expression of *dppA* was analysed in a *psdR* complemented strain, with levels of expression similar to those of the wild-type (data not shown). This verifies that a mutation in *psdR* results in increased expression of both *mdpA* and *psdR*.

MdpA and PsdR are involved in the metabolism of a range of dipeptides

The characterization of the porin OpdP and its specificity for the transport of the dipeptide glycyl-L-glutamic acid (Tamber *et al.*, 2006) in *P. aeruginosa* PAO1 prompted us to hypothesize that MdpA may be involved in the metabolism of this dipeptide. This was examined by assessing the wild-type and an *mdpA* mutant strain for growth in the presence of different dipeptide carbon sources including glycyl-L-glutamic acid. Wild-type and *mdpA* mutant strains showed indistinguishable growth in M9 minimal medium supplemented with glucose as a

carbon source (data not shown). Unlike the wild-type the *mdpA* mutant strain was unable to utilize a range of dipeptides as a sole carbon source (Table 4). A *psdR* mutant strain was also compared to wild-type for growth when these dipeptides were used as sole carbon sources. Disruption of *psdR* resulted in a shorter lag phase of growth when these dipeptides, including Gly-Glu (Fig. 4b), were used. When X-Pro dipeptides, including Phe-Pro and Gly-Pro (Fig. 4c), were used as carbon sources, the growth kinetics of wild-type and mutant strains were more similar.

The *mdpA* gene is induced in the presence of X-Pro dipeptides

In an attempt to determine whether *mdpA* is induced in the presence of dipeptides, its expression was analysed using a reporter strain with an *mdpA-lacZ* transcriptional fusion grown in M9 minimal medium supplemented with different dipeptides: Gly-Asp, Gly-Gln, Gly-Gly, Gly-Phe, Gly-Ala, Ala-Val, Val-Val, Phe-Pro, Gly-Pro. Cells were harvested at an OD₆₀₀ of 0.7, a value at which cultures had previously been shown to have the highest level of expression of *mdpA* in M9 minimal medium (data not shown).

Table 3. Alignment of putative promoter regions of *mdpA* and *dppA* genes of *P. aeruginosa*

Promoter	Putative regulator	Sequence*	Spacer
<i>mdpA</i>	<i>psdR</i>	CATGGAAGGCGAGAGTTGCCATGGCCCATAAAGGC	21 bp
<i>dppA</i>	<i>psdR</i>	GACTGAAAATT-TGTTTTGACCTGAAT-TTAAGCT	19 bp
		* * * * * * * * * * * *	

*The -35 (left) and -10 (right) sequences are underlined.

Fig. 3. Role of PsdR in regulating expression of *mdpA* and *dppA3*: kinetics of expression of (a) *mdpA* and (b) *dppA3* transcriptional reporter *lacZ* fusions in *P. aeruginosa* PAO1 and the *psdR* mutant. Cultures were grown in M9 minimal medium supplemented with glucose. Experiments were carried out in triplicate; means $\pm$ SE are presented. (a) *lacZ* expression of *mdpA* is significantly higher in the mutant strain disrupted in *psdR* compared to wild-type (LSD, $P=0.05$), and this difference is growth phase dependent. (b) Expression of *dppA* is also significantly higher in the mutant strain disrupted in *psdR* (LSD, $P=0.05$) but the pattern of expression as a function of growth phase is different. Complementation of *psdR* restores expression to wild-type levels (data not shown).

Expression of *mdpA* was significantly induced, about fourfold, only in the presence of the X-Pro dipeptides (Phe-Pro, Gly-Pro) (Fig. 5). This result indicated that the *mdpA* gene promoter is directly or indirectly induced by X-Pro dipeptides.

Dipeptidase MdpA plays a role in cytotoxicity of *P. aeruginosa* PAO1

Transcriptomic evidence shows induction of *mdpA* in the presence of epithelial cells in the highly virulent LES isolates of *P. aeruginosa* (Frisk *et al.*, 2004; Salunkhe *et al.*, 2005). This prompted us to test the role of *mdpA* in virulence using a cytotoxicity assay on human epithelial cells. Cytotoxicity of airway epithelial cells was measured by quantifying the release of lactate dehydrogenase into culture supernatants. At an m.o.i. of 50:1, PAO1 was about four times more cytotoxic to airway epithelial cells than the *mdpA* mutant at 6 h post-infection (Fig. 6). Thus *mdpA* appears to contribute to cytotoxicity in PAO1.

DISCUSSION

We have identified a transcriptional regulator, PsdR, and characterized its role in the regulation of the *dpp* transport operon and dipeptide metabolism in *P. aeruginosa* PAO1. PsdR has a HTH domain at its N-terminal region. The HTH domain is a common DNA-binding motif found in several bacterial and eukaryotic repressors as well as activators. Domain analysis showed that PsdR is a MerR-like regulator. Deletion of most of the predicted promoter and the HTH region of PsdR led to significant increase in transcription of *mdpA* and *dppA3*. It is noteworthy that the *psdR* mutation affects the levels and not the pattern of expression of both genes. The structure of PsdR and its effect on *mdpA* and *dppA3* expression strongly suggest that it is a repressor of the expression of these two genes and may directly interact with their promoters. At this stage it is not possible to exclude an indirect control of *mdpA* and *dppA3* transcription by PsdR. The *psdR* mutant strain shows a shorter lag phase of growth than the wild-type when different dipeptides are supplied as the sole carbon source. This is consistent with the observation that the mutant would have increased expression of genes responsible for both dipeptide transport and metabolism. Interestingly, the dipeptide carbon sources on which the wild-type and the *psdR* mutant strain have similar growth rates are the X-Pro dipeptides. These dipeptides have been

Table 4. Growth of mPAO1 and an *mdpA* mutant strain on different dipeptides as the sole carbon source

–, No growth; +, OD₆₀₀ 0.1–0.3; ++, OD₆₀₀ >0.3.

	Gly-Glu	Gly-Phe	Phe-Pro	Gly-Pro	Ala-Val	Gly-Ala	Gly-Asp
mPAO1	+	+	++	++	+	+	–
mPA Δ mdpA	–	–	–	–	–	–	–

Fig. 4. Growth of PAO1 (plain line) and a *psdR* mutant strain (squared line) in M9 minimal medium supplemented with different dipeptide carbon sources. (a) Growth in M9 supplemented with glucose is the same for both strains. (b) The *psdR* mutant has a shorter lag phase compared to wild-type in M9 supplemented with Gly-Glu. Similar growth kinetics were seen for Gly-Phe, Ala-Val and Gly-Ala. (c) Growth of mutant and wild-type strains was more similar when X-Pro dipeptides are used as the sole carbon source (shown for Gly-Pro). Complementation of *psdR* restored growth to wild-type levels (data not shown). Growth was assayed over 52 h; means $\pm$ SE are presented (error bars not shown where smaller than symbols).

shown to specifically induce *mdpA* expression, which could explain the similar pattern of growth kinetics between the *psdR* mutant and wild-type strains. The induction of *mdpA* expression in the presence of these specific dipeptides presumably allows for faster metabolism of these carbon sources and therefore faster growth.

The results reported here show that expression of *psdR* in the wild-type strain does not fully repress *mdpA* expression and that the transcription of this gene is growth phase

Fig. 5. Specific dipeptides induce expression of the *mdpA* β -galactosidase transcriptional fusion in *P. aeruginosa* PAO1. Cultures were grown in M9 minimal medium supplemented with different dipeptides and grown to an OD₆₀₀ of 0.7. Experiments were carried out in triplicate; means $\pm$ SE are presented. Expression of *mdpA* is higher in the presence of the X-Pro (Phe-Pro, Gly-Pro) dipeptides.

dependent. This suggests that *mdpA* is under control of other regulators which are not affected by *PsdR*. Previous experimental evidence suggests that the regulation of expression of *mdpA* is complex and is controlled by many regulatory inputs. Transcriptome analysis suggests that *mdpA* is regulated by quorum-sensing-dependent regulators LasR and RhlR in *P. aeruginosa* PAO1. Expression was shown to be ninefold higher when the PAO1 and *lasR/rhlR* double mutant strains were compared (Schuster *et al.*, 2003). A similar phenotype is seen in the post-transcriptional regulator *rsmA* mutant background (Burrowes *et al.*, 2006).

Transcriptome-profiling experiments had previously shown that *mdpA* is induced both in the presence of an epithelial cell line (Frisk *et al.*, 2004) and in a highly virulent LES isolate of *P. aeruginosa* PAO1 (Salunkhe *et al.*, 2005). This result suggested to us that this gene could play a specific role in interactions with epithelial cells. Interestingly, a mutation in *mdpA* results in reduced cytotoxicity of the bacterium to an epithelial cell line (Fig. 6). *P. aeruginosa* produces several proteases that are implicated in the process of infections caused by this organism (Doring *et al.*, 1985). Most clinical strains of *P. aeruginosa* produce elastase, a zinc metallo-protease that is implicated in the pathogenesis of infections related to these organisms. Indeed, numerous other examples of proteases and aminopeptidases that are linked to virulence have been highlighted (Coffey *et al.*, 2000; Corbett *et al.*, 2003; Feil *et al.*, 2005; Hidalgo-Grass *et al.*, 2006; Kooi *et al.*, 2006; Song *et al.*, 2004; Spratt *et al.*, 1995). Dipeptidyl aminopeptidase activity has previously been implicated in the virulence of *Porphyromonas gingivalis* (Kumagai *et al.*, 2000). This aminopeptidase has been shown to function extracellularly. However, sequence analysis of MdpA does not show the presence of a signal peptide involved in triggering protein translocation; it rather suggests a cytoplasmic location for this protein. The exact mechanism of how the dipeptidase MdpA is involved in virulence is unknown.

Fig. 6. Analysis of cytotoxicity of *P. aeruginosa* strains mPAO1 (black bars) and mPAmdpA (white bars). Cytotoxicity was determined in triplicate experiments by quantifying the release of lactate dehydrogenase (LDH) into culture supernatants. Means $\pm$ SE are presented. Strain mPAmdpA had reduced cytotoxicity compared to the wild-type.

Analysis of *Pseudomonas* genomes has shown the presence of the *mdpA* dipeptidase gene specifically in *P. aeruginosa* strains and not in other sequenced *Pseudomonas* species. Questions remain regarding what biological roles both the regulator and peptidase play in *Pseudomonas*–host interactions. The contribution of MdpA is potentially explained by its role in cytotoxicity, presumably because of the advantages in nutrient metabolism it affords. The physiological role of PsdR, which functions as a repressor of both dipeptide transport and metabolism, is less clear. Perhaps a tighter regulation of genes involved in the uptake and metabolism of small molecules into the cell is required for the successful colonization of environments in which *P. aeruginosa* is prominent.

It is assumed that the genetic complexity of *P. aeruginosa* allows for its ecological and metabolic versatility. Analysis of the *dpp* gene cluster in *P. aeruginosa*, the characterization of novel genes associated with both nutrient transport and metabolism and the role of *mdpA* in cytotoxicity would support this view.

ACKNOWLEDGEMENTS

We acknowledge Joanne Cummins, Simon Miller, Claire Adams and Pat Higgins for useful advice and discussions. This research was supported in part by grants awarded by the European Commission (MTKD-CT-2006-042062; O36314), the Science Foundation of Ireland (SFI 02/IN.1/B1261; 04/BR/B0597), the Department of Agriculture and Food (DAF RSF 06 321; DAF RSF 06 377), Irish Research Council for Science, Engineering and Technology (IRCSET) (05/EDIV/FP107;), the Health Research Board (RP/2004/145; RP/2006/271; RP/2007/290), the Environmental Protection Agency (EPA 2006-PhD-S-21) and the Higher Education Authority of Ireland (PRTL13).

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Edited by: M. A. Curtis