Regulation of the *Pseudomonas aeruginosa* *toxA*, *regA* and *ptxR* genes by the iron-starvation sigma factor PvdS under reduced levels of oxygen

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The level of environmental oxygen (EO) within various *Pseudomonas aeruginosa* infection sites is low (microaerobic), and this can affect the production of different virulence factors. Expression of the *toxA* gene, encoding exotoxin A (ETA), is regulated by *regA*, *ptxR* and *pvdS*. Moreover, the iron-starvation sigma factor PvdS directs the transcription of pyoverdine siderophore genes (e.g. *pvdD*). DNA–protein binding analysis using recombinant PvdS showed that the PvdS–RNA polymerase holoenzyme complex specifically bound the *toxA*, *regA* and *ptxR* promoter regions. All three promoters contain a PvdS-binding site, the iron-starvation box. To determine the relationship between these different genes and PvdS, we conducted a comparative analysis of *toxA*, *regA*, *ptxR* and *pvdD* transcription throughout the growth cycle of wild-type *P. aeruginosa* and its *pvdS* mutant in iron-deficient medium under aerobic-shaking (A-sh) and microaerobic-static (M-st) conditions. Under both EO conditions, optimal *toxA*, *regA* and *pvdD* expression and pyoverdine production required PvdS, while *ptxR* expression was moderately dependent on PvdS only under A-sh conditions. Expression of *regA*, *pvdD* and pyoverdine production in wild-type *P. aeruginosa* was significantly lower under M-st in comparison with A-sh conditions, while the opposite was observed for *toxA* and *ptxR*. Although low, the level of *toxA* expression and ETA production in the *pvdS* mutant were higher under M-st than under A-sh conditions. Transcription of *pvdS* and PvdS expression were also reduced by low EO. We propose that the regulation of *toxA* expression under aerobic conditions primarily involves PvdS, while an additional EO-responsive regulator(s) besides PvdS is required under low EO levels. Thus, PvdS may control the transcription of the *ptxR*, *regA* and *toxA* genes, and respond to EO by acting at different levels of the *toxA* regulatory cascade.

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INTRODUCTION

Cystic fibrosis (CF) is a genetic disease characterized by airway obstruction, chronic bacterial infection, and excessive host inflammatory response (Hassett *et al.*, 2002; Sadikot *et al.*, 2005; Yoon *et al.*, 2002). *Pseudomonas aeruginosa* is the predominant opportunistic pathogen associated within the thickened mucus layer of the CF lung (Baltch, 1994; Hassett *et al.*, 2002). As disease progresses, *P. aeruginosa* accelerates lung deterioration and establishes a

chronic infection with frequent exacerbations (Baltch, 1994; Davis *et al.*, 1996). *P. aeruginosa* produces an arsenal of virulence factors that facilitate its survival within the CF lung and are important for pathogenesis (van Delden, 2004).

Exotoxin A (ETA) is considered one of the most powerful extracellular virulence factors produced by *P. aeruginosa* (Iglewski & Kabat, 1975). The 68 kDa ETA protein, encoded by *toxA*, is an ADP-ribosyl transferase that irreversibly inhibits protein synthesis in eukaryotic cells causing cell death (Hamood *et al.*, 2004; Iglewski & Kabat, 1975). The regulation of ETA production is an intricate process that involves environmental factors and several regulators (Hamood *et al.*, 2004). Environmental factors include cation concentration, temperature, oxygen and iron levels (Hamood *et al.*, 2004; Liu, 1973). The most

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Abbreviations: A-sh, aerobic shaking conditions (~21% O₂); M-st, microaerobic static conditions (~10% O₂); EMSA, electrophoretic mobility shift assay(s); EO, environmental oxygen; ETA, exotoxin A; IS box, iron-starvation box.

extensively analysed of these factors is iron which represses ETA production at the transcriptional level (Frank & Iglewski, 1988; Grant & Vasil, 1986; Hamood *et al.*, 2004; Lory, 1986). Therefore, to achieve maximum ETA levels *in vitro*, *P. aeruginosa* is usually grown in iron-deficient medium at 32 °C with good aeration (Frank & Iglewski, 1988; Grant & Vasil, 1986; Hamood *et al.*, 2004; Lory, 1986).

The regulation of *toxA* expression involves at least three positive regulators, including RegA, PtxR and the iron-starvation (alternative) sigma factor PvdS (Frank & Iglewski, 1988; Hamood *et al.*, 2004; Wick *et al.*, 1990). The 29 kDa protein RegA is essential for *toxA* expression (Frank & Iglewski, 1988; Hamood *et al.*, 2004; Wick *et al.*, 1990). Neither ETA nor *toxA* mRNA were detected in a *regA*-deficient mutant of the *P. aeruginosa* strain PA103 (Wick *et al.*, 1990). At this time, the mechanism through which RegA regulates *toxA* expression has not been completely determined (Frank & Iglewski, 1988; Hamood & Iglewski, 1990; Hamood *et al.*, 2004; Raivio *et al.*, 1996; Wick *et al.*, 1990). The LysR-type transcriptional activator PtxR is not essential, but does increase *toxA* expression by four- to fivefold, although the exact mechanism of this regulation is unknown (Hamood *et al.*, 1996, 2004). PtxR also co-regulates the expression of several genes controlled by quorum sensing, and of the *pvc* operon (Carty *et al.*, 2006; Stintzi *et al.*, 1999). PvdS enhances *toxA* expression and directs the transcription of genes required for production of the siderophore pyoverdine (e.g. *pvdD*, *pvdE*, *pvdF*) and two extracellular proteases (Cunliffe *et al.*, 1995; Ochsner *et al.*, 1996). The iron-starvation (IS) box (consensus TAAAT, followed by the CGT triplet 16–17 nt downstream) is the DNA signature recognized by the PvdS sigma factor (Rombel *et al.*, 1995; Visca *et al.*, 2002; Wilson *et al.*, 2001). Confirmed or potential IS boxes have been detected in the promoter region of PvdS-regulated genes of *P. aeruginosa* PAO, including *toxA*, *regA*, *ptxR*, *pvdD*, *pvdEF* and several other genes (Hunt *et al.*, 2002; Ochsner *et al.*, 2002; Wilson *et al.*, 2001). Evidence suggests PvdS regulates *toxA* expression through *regA* (Hamood *et al.*, 2004; Ochsner *et al.*, 1996). Repression of *toxA* and the pyoverdine genes occurs through the negative regulation of *pvdS* expression by iron and the Fur repressor protein (Hamood *et al.*, 2004; Visca, 2004). Moreover, PvdS activity is controlled at the post-translational level by the FpvR anti-sigma factor through a surface signalling mechanism which is triggered by pyoverdine binding to the outer-membrane receptor FpvA (Beare *et al.*, 2003; Lamont *et al.*, 2002; Visca *et al.*, 2002).

Within the thickened CF mucus layer in the lung alveoli, iron availability is limited and oxygen is reduced, forming a hypoxic gradient that ranges from microaerobic [10% environmental oxygen (EO)] to anaerobic conditions (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002). Traditionally described as an obligate aerobe, *P. aeruginosa* survives the reduced oxygen conditions within the CF mucus by utilizing nitrate within the airway surface fluid as

an alternative electron acceptor (Hassett *et al.*, 2002; Yoon *et al.*, 2002). Sufficient levels of nitrate within the mucus layer allow for favourable growth of *P. aeruginosa*, which leads to chronic colonization (Hassett *et al.*, 2002; Yoon *et al.*, 2002). Such a unique microniche within the CF mucus is likely to affect the production of virulence factors by *P. aeruginosa*. Notably, local production of ETA and pyoverdine, and expression of corresponding genes has been documented in sputa of CF patients chronically infected with *P. aeruginosa* (Haas *et al.*, 1991; Hunt *et al.*, 2002; Jaffar-Bandjee *et al.*, 1995).

We have shown that reduced oxygen levels obtained under static culture conditions considerably increase *toxA* transcription and ETA production in iron-deficient medium (Gaines *et al.*, 2005). Preliminary analysis suggested that the influence of reduced oxygen on ETA production does not occur directly through *regA* or *pvdS* (Gaines *et al.*, 2005). Therefore, the influence of reduced oxygen on ETA synthesis in *P. aeruginosa* may not occur through *pvdS* or *regA*. In this study, we examined the effect of reduced oxygen on the *toxA* regulatory circuit. We also examined how PvdS would affect the regulation of *regA*, *toxA*, and *ptxR* under different conditions of EO availability.

METHODS

Bacterial strains, plasmids and growth media. All strains and plasmids used in this study are listed in Table 1. For general growth experiments, including preparation of overnight cultures, plasmid DNA extraction and electroporation, *P. aeruginosa* or *Escherichia coli* strains were grown in Luria–Bertani (LB) broth (Miller, 1972). To examine gene expression, ETA synthesis and pyoverdine production, *P. aeruginosa* strains were grown in iron-depleted medium (TSB-DC). TSB-DC is a Chelex 100-treated trypticase soy broth dialysate containing 1% (v/v) glycerol, 500 mM monosodium glutamate, 2.5 mM MgSO₄ and 0.5 mM CaCl₂ (Ohman *et al.*, 1980). Iron-sufficient conditions were obtained by adding 25 µg ml⁻¹ FeCl₃ to TSB-DC. Antibiotics were added to the growth medium at the following concentrations: for *P. aeruginosa* – carbenicillin 300 µg ml⁻¹, gentamicin 60 µg ml⁻¹, streptomycin 300 µg ml⁻¹, tetracycline 50 µg ml⁻¹; and for *E. coli* – carbenicillin 100 µg ml⁻¹, kanamycin 50 µg ml⁻¹.

Growth conditions. *P. aeruginosa* containing different plasmids was routinely grown overnight in LB broth with aeration at 37 °C. Cells were pelleted, washed, resuspended in fresh TSB-DC, and inoculated into TSB-DC as previously described (Gaines *et al.*, 2005). Individual 125 ml flasks were incubated at 32 °C under aerobic conditions with shaking at 250 r.p.m. (referred to as A-sh, ~21% O₂ atmosphere) or, alternatively, under microaerobic static conditions (referred to as M-st, ~10% O₂ atmosphere) as previously described (Gaines *et al.*, 2005). M-st conditions were achieved using GasPak Jars with Campy Pak Plus envelopes (Becton Dickinson). Throughout the 24 h growth cycle, individual flasks for each time point were removed from their incubation condition, and samples of the cultures were obtained for analysis. Each growth curve experiment was repeated at least three times.

DNA manipulations. Routine genetic manipulations were conducted following standard protocols as described by Sambrook & Russell (2001). Plasmid DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega). Plasmid DNA was introduced into PAO or PAO:: *pvdS* by electroporation (Smith & Iglewski, 1989). To construct the *ptxR*-P2 expression plasmid, a 430 bp fragment of

Table 1. Strains and plasmids used in this study

^r, resistant; Gm, gentamicin; Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

Strains and Plasmids	Description	Reference
Strains		
PAO	<i>P. aeruginosa</i> PAO1 prototypic laboratory strain	Cunliffe <i>et al.</i> (1995)
PAO::pvdS	PAOIL with 460 bp <i>pvdS</i> internal deletion; Gm ^r	Cunliffe <i>et al.</i> (1995)
6424::regA-lacZ	In-frame <i>lacZ</i> fusion in <i>regA</i> (PA0707) in MPAO1; Tet ^r	Jacobs <i>et al.</i> (2003)
M15	<i>E. coli</i> lacI ^q ara-14 galK2 mtl-1 F ⁻ ; expression host for pPvdSF	Stuber <i>et al.</i> (1990)
Plasmids		
pPvdSF	564 bp fragment containing the <i>pvdS</i> coding sequence cloned in-frame with the FLAG coding sequence in pQE60; Cb ^r , Km ^r	Leoni <i>et al.</i> (2000)
pSW200	pUC18 carrying the 1.8 kb stability fragment from pRO1614; Cb ^r	Gambello <i>et al.</i> (1993)
pSW205	<i>lacZ</i> translational fusion vector carrying the <i>P. aeruginosa</i> 1.8 kb stability fragment; Cb ^r	West <i>et al.</i> (1994)
pMP190	Broad-host-range, low-copy-number promoter-probe vector; IncP replicon, <i>lacZ</i> Cm ^r Sm ^r Tra ⁻	Spaink <i>et al.</i> (1987)
pMP220	Broad-host-range, low-copy-number promoter-probe vector; IncP replicon, <i>lacZ</i> Tc ^r Tra ⁻	Spaink <i>et al.</i> (1987)
pSW228	760 bp of the <i>tox</i> A upstream region plus the region encoding the first 7 amino acids fused in-frame with the <i>lacZ</i> gene in pSW205; Cb ^r	West <i>et al.</i> (1994)
pRL88	598 bp of the <i>regA</i> upstream region plus the first 90 bp of the open reading frame fused in-frame with the <i>lacZ</i> gene in pSW205; Cb ^r	Storey <i>et al.</i> (1990)
pIN10	<i>regA</i> constitutively expressed from the <i>lac</i> promoter in pSW200; Cb ^r	This study
pPVD31	pVLT31 (broad-host-range expression vector) containing <i>pvdS</i> under the <i>tac</i> promoter; Tc ^r	Ochsner <i>et al.</i> (1996)
ppvdD	550 bp of the <i>pvdD</i> upstream region cloned in pMP190 with the promoter oriented toward the <i>lacZ</i> gene; Sm ^r	Rombel <i>et al.</i> (1995)
pJAC24 (<i>ptxR</i> -P1/2)	555 bp of the <i>ptxR</i> upstream region plus the region encoding the first 55 amino acids fused in-frame with the <i>lacZ</i> gene in pSW205; Cb ^r	Colmer & Hamood (1999)
pJAC73	430 bp of the <i>ptxR</i> upstream region plus the region encoding the first 55 amino acids cloned in pUC18; Cb ^r	This study
pJH2 (<i>ptxR</i> -P2)	The 430 bp <i>ptxR</i> upstream region from pJAC73 fused in-frame with the <i>lacZ</i> gene in pSW205; Cb ^r	This study
pMP190::PpvdE	Promoter of <i>pvdE</i> pyoverdine biosynthesis gene directionally cloned into pMP190; Sm ^r	Rombel <i>et al.</i> (1995)
pMP220::PpvdS	587 bp of the <i>pvdS</i> upstream region cloned in pMP220 with the promoter oriented toward the <i>lacZ</i> gene; Tc ^r	Ambrosi <i>et al.</i> (2002)

the *ptxR*-*ptxS* intergenic region containing the putative *ptxR* P2 promoter (Vasil *et al.*, 1998) together with the region encoding the first 55 aa of PtxR was obtained from pJAC24 by PCR. The blunt-ended PCR product was cloned into the *Sma*I site of pUC18, producing pJAC73. The orientation of the *ptxR* promoter in pJAC73 was determined by restriction digestion with *Bam*HI. The 430 bp fragment was then obtained from pJAC73 by *Eco*RI/*Bam*HI digestion and cloned in-frame with the *lacZ* gene in the previously described translational vector pSW205 (West *et al.*, 1994). Construction of this recombinant plasmid (pJH2) was confirmed by sequencing. Plasmid pIN10 was constructed by cloning the 1.5 kb *Ava*I-*Pst*I fragment from pDF18-202 (Frank *et al.*, 1989) into the *Sal*I-*Pst*I sites of pUC18. The fragment carries intact *regA* from the *P. aeruginosa* strain PA103 and approximately 500 bp of the *regA* upstream region. In the resulting recombinant plasmid (pIN9), *regA* is constitutively expressed from the *lac* promoter. The 1.8 kb stability fragment that allows ColEI plasmids to replicate stably in *P. aeruginosa* was cloned into the *Pst*I site of pIN9 generating pIN10.

Purification of PvdS. PvdS was purified by affinity chromatography from *E. coli* M15 carrying the expression plasmid pPvdSF, in which the *pvdS* coding sequence is cloned in-frame with the C-terminus FLAG peptide (Leoni *et al.*, 2000). *E. coli* M15/pPvdSF was grown overnight in LB broth supplemented with 0.4% glucose (v/v). A 5 ml aliquot of the overnight culture was subcultured into 500 ml LB broth supplemented

with 0.4% glucose and grown at 37 °C for 2.5 h to approximately 0.5 OD₆₀₀. Cultures were then induced with 500 μM IPTG for 3 h at 37 °C. Cells were centrifuged and pellets stored at -80 °C.

Analysis of the different cell fractions indicated that the recombinant PvdS (PvdSF) was localized to inclusion bodies (data not shown). The insoluble fraction was lysed and purified using CellLytic B and the inclusion bodies solubilized using CellLytic IB according to the manufacturer's recommendations (Sigma-Aldrich). Proteins were refolded by dialysis in 6 M urea for 24 h at 4 °C with an additional 250 ml of 25 mM Tris/HCl (pH 7.4) added at 6, 12 and 18 h. PvdSF was purified using a 1 ml anti-FLAG M2 affinity gel chromatography column as instructed by the manufacturer (Sigma-Aldrich). Proteins in the eluates were estimated by the Bradford protein assay kit with BSA as the standard (Pierce Biotechnology). Proteins (10 μg) from the eluates were separated by SDS-PAGE, transferred to membrane and probed for PvdSF using anti-FLAG M2 antibody (Sigma-Aldrich). Eluates containing PvdSF were combined, concentrated and exchanged into DNA binding buffer for a final PvdSF concentration of 0.582 μg μl⁻¹.

SDS-PAGE and immunoblotting. Samples from A-sh and M-st PAO cultures were collected at 4 h intervals, and total bacterial protein concentration was determined using the DC protein assay kit (Bio-Rad) with BSA as the standard. Comparable protein samples (40 μg of total bacterial proteins for PvdS detection or 10 μg for

RpoD and RpoA detection) were separated in duplicate on SDS-PAGE gels prepared according to Laemmli (1970). After electrophoresis, gels were either stained with Coomassie brilliant blue to assess protein resolution, or electrotransferred to nitrocellulose membrane (Hybond C extra; Amersham) and probed for PvdS, RpoD and RpoA quantification using a mouse polyclonal anti-PvdS antiserum (Ambrosi *et al.*, 2005) and commercial monoclonal anti-RpoD or anti-RpoA antibodies (Neoclone). Immune complexes were detected using secondary anti-mouse antibodies conjugated with either alkaline phosphatase (Promega) or horseradish peroxidase (Calbiochem). Membranes were developed with the 5-bromo-4-chloro-3-indoyl-phosphate or nitro blue tetrazolium chloride reagents for colorimetric determinations (Promega), or with the Amersham ECL chemiluminescent reagents (Amersham Biosciences), followed by exposure to X-ray film (Kodak).

Electrophoretic mobility shift assays (EMSA). DNA fragments of 211, 251, 354 and 129 bp carrying the PvdS-specific IS box were obtained by PCR from the promoter regions of *tox*A, *reg*A, *ptx*R and *pvd*EF, respectively. Oligonucleotide primers were designed using Primer Express 1.0 software (Applied Biosystems) (Table 2). PCR products were purified from agarose gels using the Qiaex II Gel Extraction Kit (Qiagen). Purified DNA fragments were end-labelled with [γ - 32 P]ATP using T4 polynucleotide kinase (Sambrook & Russell, 2001). To determine optimum concentrations required to produce a specific PvdS–core RNA polymerase (RNAP)–DNA complex, several preliminary EMSA were performed using different concentrations of PvdS and *E. coli* RNAP with the *pvd*EF control probe. Where indicated, purified PvdSF (2.91 μ g, equivalent to 130 pmol, in 5 μ l) and core RNAP (0.70 μ g, equivalent to 1.8 pmol, in 1 μ l) (Epicentre) were preincubated for 30 min at 37 °C in DNA-binding buffer [10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM KCl, 1 mM DTT/5 % glycerol with 50 μ g ml $^{-1}$ BSA and 5 μ g ml $^{-1}$ poly(dI–C)] for a total reaction volume of 20 μ l to reconstitute the holoenzyme (Dupuy & Matamouros, 2006; Sambrook & Russell, 2001). Radiolabelled probe (0.1 pmol, corresponding to 10 5 –10 7 c.p.m., depending on the probe) was added to the mixture and incubated for 1 h at room temperature. Reactions were stopped with the addition of 5 μ l of 0.8 μ g ml $^{-1}$ heparin-loading dye (1 % bromophenol blue, 50 %

glucose) and allowed to incubate for 5 min at room temperature. Reactions were separated by 5 % PAGE in 0.5 $\times$ Tris-borate-EDTA buffer for 16 h at 4 °C. Gels were then dried and exposed to X-ray film.

β -Galactosidase and pyoverdine assays and sandwich ELISA.

Assays for β -galactosidase were performed as previously described (Miller, 1972), and activity was expressed in units (U). Triplicate samples were obtained for each growth condition and time point throughout the growth cycle. The OD $_{600}$ of the culture was used to compensate for growth differences.

Pyoverdine levels were determined throughout the growth cycle for PAO grown in TSB-DC at 32 °C. Supernatant fractions collected every 4 h were appropriately diluted in TSB-DC at pH 7.3. Pyoverdine levels were quantified spectrophotometrically at A $_{405}$ according to Stintzi *et al.* (1996). Normalization of pyoverdine levels for growth was obtained by dividing A $_{405}$ by OD $_{600}$.

Sandwich ELISA for ETA quantification was done as previously described by Gaines *et al.* (2005). Values were standardized by dividing the amount of ETA (pg μ l $^{-1}$) from each supernatant fraction by the OD $_{600}$ of the culture from which that fraction was obtained.

Reverse transcriptase PCR (RT-PCR). Oligonucleotide primers for RT-PCR are listed in Table 2. PAO cultures were grown until early stationary phase in TSB-DC under A-sh and M-st conditions. Total RNA was extracted by the hot phenol/chloroform method as previously described (Carty *et al.*, 2003). Residual DNA was removed from the RNA with RQI DNase I (1 U μ l $^{-1}$) for 1.5 h at 37 °C in the presence of RNase inhibitor (RNasin; Promega) and the RNA was purified using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using 1 μ g of RNA, 250 ng of random hexamers (Promega), 10 mM dNTPs, and 6 U of StrataScript reverse transcriptase (Stratagene). After a 2 h incubation at 42 °C, reactions were stopped by heating at 94 °C for 5 min. PCR was performed on 250 ng of cDNA using 3 μ M concentration of each of the appropriate primers, 10 mM dNTPs and 1.25 U of GoTaq DNA Polymerase (Promega) per reaction. PCR to detect *rps*L or *pvd*S messages was conducted as described by Sobel *et al.* (2003); an initiation cycle of 94 °C for 5 min, 20 cycles (*rps*L) or 29 cycles (*pvd*S) including 30 s at

Table 2. Primer sets utilized in this study and location of IS boxes in PvdS-regulated promoters

Gene	Primer sets for EMSA	IS box*
<i>ptx</i> R	Forward: 5'-AATGAATGTGCCATATCTCCATAAATAA-3' Reverse: 5'-ACAGGGCGGCGCTCA-3'	²² TAAAT-N $_{16}$ -Caa
<i>pvd</i> EF	Forward: 5'-GTCGACCTGCCACGGGCGG-3' Reverse: 5'-GACGAAGCAGGATGCCCGGT-3'	¹⁰⁰ TAAAT-N $_{16}$ -CGT (<i>pvd</i> E)† ⁵⁷ TAAAT-N $_{16}$ -CGT (<i>pvd</i> F)†
<i>reg</i> A	Forward: 5'-CGGAAAAATCGGTAACAGCG-3' Reverse: 5'-AGTGATGGCTCTATGGGCAAA-3'	¹⁴⁷ TtgAT N $_{16}$ -CGT
<i>tox</i> A	Forward: 5'-CCCTGCATGTATCCTCCGA-3' Reverse: 5'-GATGGCTCCTTTGATGGGTG-3'	⁶⁶ TAAAT-N $_{16}$ -Tcc
Gene	Primer sets for RT-PCR	
<i>pvd</i> S	Forward: 5'-ACCGTAGATCCTGGTGAAGA-3' Reverse: 5'-CGAGTATTTCTGTTCGAGCGC-3'	
<i>rps</i> L‡	Forward: 5'-GCAACTATCAACCAGCTG-3' Reverse: 5'-GCTGTGCTCTTGCAGGTTGTG-3'	

*Putative IS boxes were identified using the consensus sequence described by Ochsner *et al.* (2002). Start positions (apex) of the IS boxes are given relative to the 5'-end of forward primer (reverse primer in case of *pvd*F).

†Confirmed by Wilson *et al.* (2001).

‡Described by Sobel *et al.* (2003).

95 °C, 1 min at 60 °C, 1 min at 72 °C, and a final elongation cycle at 72 °C for 7 min. The amount of product was assessed on 1.5% agarose gels and visualized with GelStar stain (Cambrex).

Statistical analysis. Statistics were calculated using InStat (Graph Pad Software). ANOVA and the Student-*t* test were used to determine significant differences among the various conditions.

RESULTS

Purified PvdS binds to the promoter regions of *toxA*, *regA*, and *ptxR* genes of *P. aeruginosa* PAO

Previous studies have shown that the iron-starvation sigma factor PvdS complexed with core RNAP binds the IS box within the promoters of different pyoverdine genes (Leoni *et al.*, 2000; Wilson *et al.*, 2001). Potential IS boxes were also identified in the upstream regions of *toxA*, *regA* and *ptxR* (Table 2) (Hunt *et al.*, 2002; Ochsner *et al.*, 2002; Wilson *et al.*, 2001). However, specific binding of the PvdS-RNAP holoenzyme to these upstream regions has not yet been demonstrated. It is possible that PvdS regulates the expression of these three genes at different levels, either indirectly (through a regulatory cascade) or directly (by binding to their upstream regions and promoting transcription initiation). Therefore, we examined the binding of the PvdS-RNAP to the *toxA*, *regA* and *ptxR* promoters. PvdSF was overexpressed in *E. coli* M15(pPvdSF) and purified by immunoaffinity chromatography using the FLAG-M2 antibody as previously described (Leoni *et al.*, 2000). The specific interaction of the PvdSF-RNAP holoenzyme with the promoter regions of the PvdS regulated genes was examined by electrophoretic mobility shift assays (EMSA) using radiolabelled DNA fragments encompassing the promoter regions of *toxA*, *regA* and *ptxR*. The *pvdEF* promoter was used as the positive control for DNA binding by PvdSF-RNAP, as previously demonstrated by Wilson *et al.* (2001). The specificity of the holoenzyme binding to the DNA was enhanced by the addition of heparin, a polyanion that prevents non-specific binding of RNAP to DNA (Leoni *et al.*, 2000; Pfeffer *et al.*, 1977). Binding reactions were conducted as described in Methods and primarily consisted of *E. coli* core RNAP, purified PvdSF, probe and heparin. Titration of core RNAP with purified PvdSF was done to determine optimal binding conditions (data not shown). As expected, the PvdSF-RNAP holoenzyme produced two gel shift bands with the *pvdEF* probe, likely due to the presence of two divergently oriented IS boxes which could generate 1:1 and 1:2 DNA-protein complexes (Fig. 1a). DNA-protein complexes were detected with the PvdSF-RNAP and the *toxA*, *regA* and *ptxR* probes (Fig. 1b, c, d). Despite repeated experiments, the core RNAP also produced smaller sized complexes with the *regA* and *ptxR* probes (Fig. 1c, d), and minor bands were also observed with the *pvdEF* and *toxA* probes (Fig. 1a, b). However, additional unique gel shift bands were detected when any of the probes was incubated with the PvdSF-RNAP complex (Fig. 1). Leoni *et al.* (2000) previously indicated that the PvdS-RNAP

Fig. 1. Binding of PvdSF complexed with *E. coli* core RNAP to DNA fragments that carry IS boxes (Table 2). The PvdSF-RNAP complex was incubated with the DNA fragments (18 : 1 molar ratio) that carry (a) the *pvdEF* intergenic region (positive control), (b) the *toxA* upstream region, (c) the *regA* upstream region and (d) the *ptxR* upstream region. EMSA were conducted in the presence of heparin ($0.8 \mu\text{g } \mu\text{l}^{-1}$). Arrows indicate specific PvdS-RNAP-DNA complexes.

holoenzyme bound the *pvdA* promoter with low efficiency. As shown in Fig. 1, we detected the same phenomenon in so far as most of the probe was detected in the free, unbound form. However, purified PvdSF alone did not specifically bind any of the probes, which indicates the requirement of PvdSF-RNAP holoenzyme formation for DNA recognition (Fig. 1). These results suggest that PvdS regulates, at least in part, the expression of *toxA*, and possibly *regA* and *ptxR*. However, additional experiments, including *in vitro* transcription of *toxA*, *regA*, and *ptxR* in the presence and absence of PvdS, will be required to confirm that the formation of these complexes enhances the transcription of these genes.

The increase in *toxA* expression by *P. aeruginosa* PAO under reduced EO conditions requires PvdS

Evidence based on the close correlation between *pvdS*, *regA*, *ptxR* and *toxA* expression indicates that PvdS

regulates *toxA* through *regA* and *ptxR* (Ochsner *et al.*, 1996; Vasil *et al.*, 1998). Moreover, binding of the PvdS–RNAP complex to both the *toxA* and *regA* upstream regions (Fig. 1) suggests that PvdS regulates *toxA* expression both directly and indirectly, through *regA* and *ptxR*. However, we recently showed that under reduced EO, *toxA* expression differs from that of *regA* and *pvdS* (Gaines *et al.*, 2005). Under microaerobic and anaerobic conditions, *toxA* expression in PAO increased while *regA* and *pvdS* expression decreased (Gaines *et al.*, 2005). Thus, we addressed the following questions: (i) does *toxA* expression differ from *regA* and *pvdS* expression microaerobically, and (ii) if so, does it occur throughout the growth cycle? (iii) Does the increase in *toxA* expression under microaerobic conditions still depend on PvdS? (iv) If PvdS regulates *toxA* expression microaerobically, does this regulation occur through *regA*? (v) How does PvdS regulate *ptxR* expression throughout the growth cycle aerobically and microaerobically? To answer these questions, we examined the role of *pvdS* on the expression of *toxA*, *regA*, *ptxR* and the pyoverdine genes throughout the growth cycle and under two different levels of EO, A-sh and M-st, using a *pvdS* deletion mutant of PAO.

We examined *toxA* expression using the *toxA*–*lacZ* fusion plasmid pSW228 in *P. aeruginosa* PAO and its *pvdS* deletion mutant PAO::*pvdS* (Table 1). In agreement with our previous report (Gaines *et al.*, 2005), we confirmed that the level of *toxA* expression in PAO under M-st conditions was significantly higher ($P<0.001$) compared with A-sh (data not shown). Comparison of *toxA* expression between PAO and PAO::*pvdS* showed that under A-sh and M-st conditions and between the 12- and 24 h time points, the level of *toxA* expression in PAO::*pvdS* was significantly lower ($P<0.001$) than that in PAO (Fig. 2a, b). This indicates that, under A-sh and M-st conditions, maximal *toxA* expression in PAO requires functional PvdS. Despite the loss of PvdS, *toxA* expression in PAO::*pvdS* during the 10–24 h time frame was slightly higher under M-st conditions in comparison with A-sh (Fig. 2c), suggesting that reduced EO causes a limited increase in *toxA* expression even in PAO::*pvdS*. To further confirm these results, we measured the amount of ETA secreted by PAO and PAO::*pvdS* under A-sh and M-st conditions. Cells were grown to mid-stationary phase, and the amount of ETA within the supernatant fraction was determined by sandwich ELISA as previously described (Gaines *et al.*, 2005). Both PAO and PAO::*pvdS* produced more ETA under M-st conditions than under A-sh conditions (data not shown). In addition, under both conditions, PAO::*pvdS* produced significantly less ETA ($P<0.001$, M-st; $P<0.01$, A-sh) than the wild-type PAO (data not shown).

Under microaerobic conditions, *regA* expression in *P. aeruginosa* PAO is PvdS-dependent

The transcriptional activator RegA is considered essential for *toxA* expression in *P. aeruginosa* (Storey *et al.*, 1991;

Fig. 2. Effect of reduced EO levels on *toxA* expression throughout the growth cycle of PAO and PAO::*pvdS*. PAO and PAO::*pvdS* carrying the *toxA*–*lacZ* fusion plasmid pSW228 were grown in TSB-DC medium under A-sh or M-st conditions as described in Methods. Six hours after inoculation, triplicate samples were obtained every 2 h, and the level of β -galactosidase activity was determined. (a) *toxA* expression in PAO and PAO::*pvdS* under A-sh conditions; (b) *toxA* expression in PAO and PAO::*pvdS* under M-st conditions; (c) *toxA* expression in PAO::*pvdS* under both conditions. Filled symbols, PAO; open symbols, PAO::*pvdS*; ● and ○, A-sh conditions; ■ and □, M-st conditions. Values represent the means of three independent experiments ($\pm$ SEM). *, $P < 0.001$.

Vasil *et al.*, 1989). Under A-sh conditions, PvdS regulates the expression of both *toxA* and *regA* (Ochsner *et al.*, 1996). We compared the role of PvdS in *regA* expression under A-sh and M-st conditions using plasmid pRL88, which

contains a *regA-lacZ* translational fusion (Storey *et al.*, 1990). Opposite to *toxA* expression, between 8 and 20 h, *regA* expression in wild-type PAO under A-sh conditions was significantly higher ($P < 0.001$) than that under M-st conditions (Fig. 3). Moreover, *regA* expression in PAO::*pvdS* was abrogated under both A-sh and M-st conditions (Fig. 3). These results suggest that reduced EO negatively influences *regA* expression in PAO, and that PvdS is essential for *regA* expression regardless of the level of EO.

We also explored the role of *regA* in the regulation of ETA production by PvdS. We examined the effect of plasmid pIN10, in which *regA* is constitutively expressed from the *lac* promoter, on ETA production by PAO::*pvdS* grown in iron-deficient (TSB-DC) and -sufficient (TSB-DC with $25 \mu\text{g ml}^{-1} \text{FeCl}_3$) media under A-sh and M-st conditions. Under A-sh conditions, pIN10 significantly enhanced ETA production in PAO::*pvdS* and deregulated it to a great extent with respect to iron (data not shown). Under A-sh conditions, the amount of ETA produced by PAO::*pvdS*(pIN10) was about $25 \text{ pg } \mu\text{l}^{-1} \text{OD}_{600}^{-1}$ in iron-deficient medium but $20 \text{ pg } \mu\text{l}^{-1} \text{OD}_{600}^{-1}$ in iron-sufficient medium (data not shown). Detection of the limited repressive effect of iron in the absence of functional PvdS, and upon *regA* overexpression, suggests that this effect of iron is independent of PvdS and RegA. We obtained similar results under M-st conditions (data not shown). These results suggest that PvdS regulates *toxA* expression through *regA* both aerobically and microaerobically. To confirm these results further, we used sandwich ELISA to examine the effect of pPVD31 (in which *pvdS* is expressed from the *tac* promoter) (Ochsner *et al.*, 1996) on ETA production in the PAO *regA* mutant

6424::*regA-lacZ* (Bailey & Manoil, 2002; Jacobs *et al.*, 2003) that was obtained from the University of Washington Genome Center, <http://www.genome.washington.edu/UWGC/pseudomonas/index.cfm>. In iron-deficient medium, strain 6424::*regA-lacZ*(pPVD31) produced no detectable ETA under A-sh and M-st conditions (data not shown). Ochsner *et al.* (1996) previously described similar results using semiquantitative immunoblot analysis of ETA production. Despite constitutive expression of *regA*, ETA is still slightly repressed by iron.

The growth of *P. aeruginosa* PAO under M-st conditions reduces pyoverdine production and the expression of pyoverdine genes

Previous studies showed that, under A-sh conditions, pyoverdine production by *P. aeruginosa* is stringently controlled by PvdS (Cunliffe *et al.*, 1995; Hunt *et al.*, 2002; Ochsner *et al.*, 1995). Therefore, we examined whether reduced EO alters pyoverdine production and if PvdS stringently controls pyoverdine production under these conditions. *P. aeruginosa* PAO was grown in TSB-DC under A-sh and M-st conditions, and samples were taken every 4 h to determine the level of pyoverdine in the supernatant fractions as previously described (Stintzi *et al.*, 1996). Between the 8 and 24 h time points, wild-type PAO produced significantly ($P < 0.001$) lower levels of pyoverdine under M-st conditions than under A-sh conditions (Fig. 4a). As previously demonstrated (Leoni *et al.*, 1996; Ochsner *et al.*, 1995; Visca *et al.*, 1992), PAO::*pvdS* produced no pyoverdine under either condition, and pyoverdine production by wild-type PAO was fully repressed by iron (data not shown).

To examine whether the reduction in pyoverdine production is the result of a decrease in the expression of the pyoverdine synthesis genes, we analysed in detail the expression of one of these genes, namely *pvdD*, using the *pvdD-lacZ* transcriptional fusion plasmid ppvdD (Rombel *et al.*, 1995). Between the 8 and 20 h time points, *pvdD* expression in PAO under M-st conditions was significantly ($P < 0.001$) reduced in comparison with A-sh conditions (Fig. 4b). Expression of *pvdD* was fully repressed in PAO::*pvdS*, irrespective of EO (Fig. 4b). Using a *pvdE-lacZ* fusion plasmid, we also observed that *pvdE* expression in PAO was significantly lower under M-st conditions than under A-sh conditions (data not shown). These results suggest that reduced EO affects the expression of *regA*, *pvdD* and *pvdE* by a mechanism(s) different from the one governing *toxA* expression.

PvdS influences the expression from the *ptxR*-P2 promoter under A-sh but not M-st conditions

The LysR transcriptional activator PtxR modulates ETA production in *P. aeruginosa* by increasing *toxA* expression by four- to fivefold (Hamood *et al.*, 1996). We have previously demonstrated that, similar to *toxA* expression,

Fig. 3. Effect of reduced EO on *regA* expression in PAO and PAO::*pvdS*. PAO and PAO::*pvdS* carrying the *regA-lacZ* fusion plasmid pRL88 were grown in TSB-DC medium under A-sh or M-st conditions as described in Methods. Beginning 8 h post-inoculation, triplicate samples were obtained every 4 h, and the level of β -galactosidase activity was determined. Filled symbols, PAO; open symbols, PAO::*pvdS*; ● and ○, A-sh conditions; ■ and □, M-st conditions. Values represent the means of three independent experiments ($\pm$ SEM).

Fig. 4. (a) Effect of reduced EO on pyoverdine production by PAO in iron-deficient medium. Beginning 4 h post-inoculation, samples were obtained every 4 h, and the supernatant fractions were separated. Pyoverdine levels were determined by measuring the A_{405} of the supernatant fraction. Values were standardized by dividing the amount of pyoverdine in each fraction by the OD_{600} of the culture from which the fraction was obtained. *, $P < 0.001$. (b) Effect of reduced EO on *pvdD* expression throughout the growth cycle of PAO and PAO::pvdS. PAO or PAO::pvdS carrying the *pvdD-lacZ* fusion plasmid *ppvdD* were grown in TSB-DC medium at 32 °C under A-sh or M-st conditions as described in Methods. Triplicate samples were obtained every 4 h beginning 8 h post-inoculation, and the level of β-galactosidase activity was determined. Filled symbols, PAO; open symbols, PAO::pvdS; ● and ○, A-sh conditions; ■ and □, M-st conditions. Values represent the means of three independent experiments (± SEM).

ptxR expression increases under reduced EO (Gaines *et al.*, 2005). Thus, we examined the role of PvdS in *ptxR* expression under A-sh and M-st conditions using the previously described *ptxR-lacZ* fusion plasmid pJAC24, which carries both P1 and P2 *ptxR* promoters (Colmer & Hamood, 1999). Under A-sh conditions, the pattern of *ptxR* expression in PAO::pvdS was essentially similar to that in wild-type PAO, reaching a peak at 6 h and gradually declining thereafter (data not shown). There were no differences in *ptxR* expression between wild-type and the PAO::pvdS mutant under M-st conditions (data not shown). However, under M-st conditions, *ptxR* expression in PAO::pvdS was significantly higher than that under A-sh conditions at 10 to 24 h time points (data not shown).

Available evidence suggests that *ptxR* is expressed from two separate promoters, P1 and P2 (Vasil *et al.*, 1998). Based on the results of RNase protection experiments, we previously suggested that *ptxR* expression from P2 is regulated by PvdS under microaerobic conditions (Vasil *et al.*, 1998). Therefore, we determined the effect of both reduced EO and PvdS on the expression of *ptxR*-P2 throughout the growth cycle of PAO. These experiments were conducted using the *ptxR*-P2-*lacZ* fusion plasmid pJH2, which carries only the P2 promoter (Table 1). Under M-st conditions and in both strains, *ptxR*-P2 expression was significantly higher than that under A-sh conditions at several time points (Fig. 5a, b). Throughout the growth cycle and under A-sh and M-st conditions, differences in *ptxR*-P2 expression between PAO and PAO::pvdS were limited and inconsistent (Fig. 5c, d). These results suggest that unlike its effect on *tox*A and *reg*A expression, PvdS slightly affects *ptxR* expression. In addition, the enhancement in *ptxR* expression by reduced EO is unlikely to occur through PvdS.

Reduced EO represses PvdS expression throughout the growth cycle of *P. aeruginosa* PAO

The above results suggest that PvdS regulates the expression of *tox*A, *reg*A and the pyoverdine genes under both A-sh and M-st conditions, and *ptxR* expression under A-sh conditions only (Figs 2–5). We have previously shown that anaerobic conditions reduce *pvdS* expression in PAO (Gaines *et al.*, 2005). Thus, to gain further insight into the role of PvdS in such regulation, the expression profile of *pvdS* was examined throughout the growth cycle of *P. aeruginosa* PAO under A-sh and M-st conditions. Gene fusions and RT-PCR assay were used to monitor *pvdS* transcription. As shown in Fig. 6(a), and similar to *reg*A, *pvdD* and *pvdE* expression, the level of *pvdS* expression in PAO under A-sh conditions was four- to sixfold higher at the 10 to 16 h time points than that under M-st conditions. Decreased *pvdS* transcription under M-st conditions was further confirmed by RT-PCR. The RT-PCR analysis was carried out on total RNA extracted from early stationary phase (10 h) *P. aeruginosa* PAO cultures. As a control, the level of the housekeeping gene *rpsL* that encodes the ribosomal subunit S12 was determined (Sobel *et al.*, 2003). As shown in Fig. 6(b), the amount of *pvdS* transcript produced under A-sh conditions was markedly (~ sixfold) higher than that produced under M-st conditions, in substantial agreement with the results of the *pvdS-lacZ* transcriptional fusion analysis. We also monitored the intracellular levels of PvdS throughout the growth cycle of PAO by Western blot analysis under both A-sh and M-st conditions. As controls, the levels of the vegetative σ^{70} factor RpoD and the α subunit (RpoA) of the RNAP were also determined (Fujita *et al.*, 1993). As shown in Fig. 6(c), PvdS production correlates well with the transcriptional profile. The level of PvdS under M-st conditions was markedly lower than that under A-sh conditions (Fig. 6c).

Fig. 5. Effect of reduced EO on *ptxR*-P2 expression throughout the growth cycle of PAO and PAO::pvdS. PAO and PAO::pvdS carrying the *ptxR*-P2-*lacZ* fusion plasmid pJH2, which contains only the putative P2 promoter, were grown in TSB-DC under A-sh or M-st conditions as described in Methods. Starting 4 h post-inoculation triplicate samples were obtained every 2 h, and the level of β -galactosidase activity was determined. (a) *ptxR*-P2 expression in PAO under A-sh and M-st conditions; (b) *ptxR*-P2 expression in PAO::pvdS under both conditions; (c) *ptxR*-P2 expression in both strains under A-sh conditions; (d) *ptxR*-P2 expression in both strains under M-st conditions. Filled symbols, PAO; open symbols, PAO::pvdS; ● and ○, A-sh conditions; ■ and □, M-st conditions. Values represent the means of three independent experiments ($\pm$ SEM). *, $P < 0.001$; ♦, $P < 0.05$

Levels of RpoD and RpoA were essentially the same under both conditions and throughout the growth cycle (Fig. 6c). These results suggest that lower EO reduces *pvdS* transcription and the intracellular levels of PvdS in PAO. Consequently, the expression of *toxA*, *regA* and the pyoverdine genes (that are controlled by the PvdS-dependent RNAP) is reduced. The failure of reduced EO to affect the levels of the two main components of the vegetative transcriptional machinery, RpoA and RpoD, indicates that low EO specifically reduces PvdS expression and hence the level of PvdS-dependent RNAP.

DISCUSSION

In certain *P. aeruginosa* infection sites, the environmental conditions are likely to be microaerobic (Allen *et al.*, 1997; Worlitzsch *et al.*, 2002; Xiong *et al.*, 2000). In particular,

within the mucus layer of the CF lung, EO forms a hypoxic gradient (microaerobic to anaerobic) (Hassett *et al.*, 2002), which could influence the expression of extracellular virulence factors by *P. aeruginosa*. We have previously reported that reduced EO levels significantly increase *toxA* expression in PAO, while reduced EO decreased rather than increased *pvdS* expression (Gaines *et al.*, 2005; this study). Therefore, in this study we have addressed the impact of PvdS and other *toxA* regulators on the expression of *toxA* under microaerobic (M-st) conditions. We selected aerobic (A-sh) conditions as a control since most laboratory studies have examined the interaction between PvdS and PvdS-regulated genes under aerobic conditions (Cunliffe *et al.*, 1995; Hunt *et al.*, 2002; Leoni *et al.*, 2000; Ochsner *et al.*, 1996; Wilderman *et al.*, 2001; Wilson *et al.*, 2001). Based on the results of our analyses, we propose the following models to explain the regulation of different genes under A-sh and M-st conditions.

Fig. 6. Effect of reduced EO on PvdS expression. (a) PAO carrying the *pvdS-lacZ* fusion plasmid, pMP220::PpvdS, was grown under A-sh (solid line) or M-st (broken line) conditions as described in Methods. Triplicate samples were obtained at 6, 10, 12 and 16 h, and the level of β -galactosidase activity was determined. Values represent the mean of three independent experiments ($\pm$ SEM). *, $P < 0.001$. (b) Total RNA extracted from early stationary phase PAO cultures under A-sh and M-st conditions was subjected to RT-PCR analysis. The PCR products were separated on a 1.5% agarose gel and stained with GelStar Stain (Cambrex) for visualization. Transcription of the constitutively expressed *rpsL* encoding ribosomal subunit S12 was used as a control. (c) Whole-cell lysates (40 μ g for PvdS or 10 μ g for both RpoA and RpoD) obtained at indicated time points from A-sh (A) and M-st (M) cultures were probed with anti-PvdS polyclonal mouse antiserum or with monoclonal anti-RpoD and anti-RpoA antibodies. Whole-cell extract (40 or 10 μ g) from PAO::pvdS under A-sh condition was used as a negative control. Purified PvdS (10 ng) was used as a positive control. Immunoblots are representative of one of three independent experiments.

Regulation of *toxA*, *regA*, *ptxR* and the pyoverdine genes by PvdS under A-sh conditions

Under aerobic conditions and in iron-deficient medium, *toxA*, *regA*, *pvdS* and the pyoverdine genes are highly expressed (Figs 2–4, 6). In addition, the expression of *regA*, *toxA* and the pyoverdine genes requires functional PvdS (Figs 2–4, 7a). With respect to the pyoverdine genes, previous studies showed that PvdS directly regulates the expression of at least *pvdD* and *pvdF* (Ochsner *et al.*, 2002; Visca, 2004; Wilson *et al.*, 2001). With respect to *toxA*, our results strongly suggest that PvdS regulates its expression through *regA* (data not shown; Fig. 7a). The expression of *regA* from the exogenous *lac* promoter complemented the defect of PAO::pvdS in ETA production (data not shown). Similar to its effect on the pyoverdine genes, PvdS regulates *regA* expression by binding directly to the *regA* upstream region (Fig. 1). More specifically, PvdS may regulate the expression from the *regA* P2 promoter. Computer analysis showed that the potential PvdS binding site within the *regA* upstream region is located -1 bp 5' to $+8$ bp 3' of the T2 transcription start site (Hunt *et al.*, 2002). Whether PvdS binding at this site affects *regA* transcription is not known. However, if further analysis confirms that PvdS binds to this sequence, it would be consistent with the notion that *regA* expression at mid log and early stationary phases of growth occurs through the iron-responsive *regA* P2

promoter (Frank & Iglewski, 1988; Storey *et al.*, 1990). The iron-insensitive *regA* P1 promoter influences the expression at earlier stages of growth (Frank & Iglewski, 1988; Storey *et al.*, 1990). Previous studies indicated that iron-Fur represses *regA* and *toxA* expression through PvdS (Ochsner *et al.*, 1996; Visca, 2004). Thus, it would be logical if PvdS regulates the iron-responsive *regA* P2 promoter (Fig. 7a). What is not clear at this time is the relevance of the PvdS–RNAP binding to the *toxA* upstream region (Fig. 1). Besides the physical interaction of the PvdS–RNAP with the *toxA* upstream region, the *toxA* upstream region carries a potential PvdS binding site that contains more conserved nucleotides than the PvdS binding site within the *regA* upstream region (Hunt *et al.*, 2002). However, we have excluded the possibility that an increased level of PvdS bypasses *regA* and enhances *toxA* expression directly by binding to the *toxA* upstream region. As Ochsner *et al.* (1996) previously reported in PAO Δ *regA*, and we could confirm in this study for the 6424::*regA-lacZ* mutant, constitutive *pvdS* expression from the *tac* promoter does not complement the defect in *toxA* expression in a *regA*-defective background (data not shown).

With respect to *ptxR*, under A-sh conditions PvdS has a limited effect on *ptxR* expression (data not shown; Figs 5, 7a). Under A-sh conditions and in comparison with PAO,

Fig. 7. Schematic diagram depicting the regulation of *toxA*, *regA*, *ptxR*, and the pyoverdine genes by PvdS in low iron medium under A-sh (a) and M-st (b) conditions. (a) Heavy solid lines indicate strong activation by increased levels of PvdS; thin solid line indicates mild activation by increased levels of PvdS. (b) Broken lines indicate limited activation by reduced levels of PvdS. '?' indicates a potential regulator that is induced by reduced EO and enhances *toxA* and *ptxR* expression independently of PvdS.

the level of *ptxR* expression from the P1/P2 promoter (pJAC24) in PAO::*pvdS* was significantly lower at the 8 h time point only (data not shown). In addition, throughout the growth cycle, and under A-sh conditions differences in *ptxR* expression from the P2 promoter (pJH2) between the two strains were inconsistent (Fig. 5c). Although statistically insignificant, *ptxR* expression from either P1/P2 or P2 was lower in PAO::*pvdS* than in PAO between the 16 and 24 h time points (Fig. 5c). Whether this limited effect is due to PvdS binding is not known at this time. The *ptxR* upstream region contains a well conserved IS box located -153 to -130 bp 5' of the T2 transcription start site (Table 2), and a second putative box located -25 to -2 bp 5' of it (Colmer-Hamood *et al.*, 2006). Both sites are included within the 354 bp fragment to which PvdS-RNAP binds (Table 2; Fig. 1d). We ruled out the possibility that PvdS regulates the expression of the *ptxR* P1 promoter. Using a *ptxR*-P1-*lacZ* fusion plasmid, we could confirm no difference in *ptxR* expression between wild-type PAO and PAO::*pvdS* (data not shown).

Regulation of *toxA*, *regA*, *ptxR* and the pyoverdine genes by PvdS under M-st conditions

Under these conditions, the expression of *regA*, *pvdD*, *pvdE* and *pvdS* was significantly reduced (Figs 3, 4, 6a; data not shown). The abolition of *regA*, *pvdD* and *pvdE* expression indicates that these genes are strictly controlled by PvdS under M-st as well as A-sh condition (Figs 3, 4, 7b). As discussed above and similar to the A-sh conditions, this control is likely to be accomplished through direct binding of the PvdS-RNAP complex to the upstream region of each gene (Fig. 1). Previous studies suggested that microaerobic conditions reduce PvdS production in *P. aeruginosa* PAO (Ochsner *et al.*, 1996). Using RNase protection experiments, Ochsner *et al.* (1996) previously showed that, under microaerobic conditions, *pvdS* transcription in PAO is reduced by about 10-fold (in comparison with aerobic conditions). We confirmed that the effect of reduced EO occurs at the level of *pvdS* transcription. Compared to A-sh conditions, *pvdS* expression under M-st conditions was reduced by about sixfold throughout the growth cycle of PAO (Fig. 6a). Also, PvdS expression from the exogenous *tac* promoter eliminated the effect of reduced EO on pyoverdine production. In fact, PAO(pPVD31) produced comparable levels of pyoverdine under A-sh and M-st conditions in the presence and absence of iron (data not shown). We also showed that the effect is specific and independent of the vegetative transcriptional machinery. As shown in Fig. 6(c), while the relative concentration of PvdS was reduced by low EO levels, the relative concentration of RpoD and RpoA was not affected. However, the mechanism(s) by which low EO reduces the expression of *pvdS* and other genes under M-st conditions is still not known. Certain factors that regulate PvdS production in *P. aeruginosa* including iron-Fur, the anti sigma factor FpvR and the quorum sensing regulator VqsR have been identified (Beare *et al.*, 2003; Juhas *et al.*, 2004; Lamont *et al.*, 2002; Visca, 2004). Since we examined *pvdS* expression in iron-deficient medium only, iron-Fur is less likely to be involved in the reduced *pvdS* expression under M-st conditions. Available evidence suggests that FpvR regulates *pvdS* expression post-transcriptionally (Beare *et al.*, 2003; Visca, 2004). Our analysis suggests that reduced EO affects primarily *pvdS* transcription, and consequently PvdS synthesis (Fig. 6). However, a role for FpvR in PvdS synthesis cannot be dismissed. Reduced EO may regulate PvdS expression through *vqsR*. Juhas *et al.* (2004) previously showed that a *vqsR* deletion in PAO reduced the transcription of more than 20 iron-regulated genes including *pvdS*, as well as the pyoverdine and pyochelin synthesis genes. We do not know at this time if the growth of PAO under M-st conditions reduces *vqsR* transcription.

Unlike *regA*, *pvdD*, *pvdE* and *pvdS*, the expression of *toxA* under M-st conditions is increased (Fig. 2). This increase is less likely to occur through *pvdS* whose expression is significantly reduced under M-st conditions (Figs 6a, 7b). Indeed, the increase in *toxA* expression under M-st

conditions was detected even in the absence of functional PvdS. Between the 10 and 22 h time points, *toxA* expression in PAO::pvdS under M-st conditions ranged from 1.5 to 3.7-fold higher than that under A-sh conditions (Fig. 2c), a difference comparable to that seen between the 16 and 24 h time points in wild-type PAO (direct comparison not shown). In comparison with PAO, *toxA* expression in PAO::pvdS was significantly reduced under both A-sh and M-st conditions (Fig. 2a, b). Again, whether PvdS binding to the *toxA* upstream region regulates *toxA* expression under these conditions is still unknown. Similar to the observation under A-sh conditions, PvdS still regulates *toxA* expression through *regA* under M-st conditions. As for A-sh conditions, constitutive PvdS expression from the exogenous *tac* promoter (in pPVD31) failed to complement the defect of the 6424::*regA-lacZ* mutant in ETA production under M-st conditions (data not shown). Thus, under M-st conditions, *toxA* expression in *P. aeruginosa* may be increased by a *regA*- and *pvdS*-independent mechanism (Fig. 7b). However, both *regA* and *pvdS* are required for maximum levels of ETA production by PAO (Fig. 7b). Although we did not examine the effect of iron on *toxA* expression in the present study, results of our previous study suggest that iron does not influence the increase in *toxA* expression under M-st conditions (Gaines *et al.*, 2005). In iron-sufficient medium, *toxA* expression in PAO was significantly reduced under both A-sh and M-st conditions (Gaines *et al.*, 2005). Despite this reduction, however, the level of *toxA* expression under M-st conditions was relatively higher than that under A-sh conditions (Gaines *et al.*, 2005).

Besides RegA and PvdS, other *P. aeruginosa* regulators that control *toxA* expression (directly or indirectly) are Vfr and LasR (Gambello *et al.*, 1993; Hamood *et al.*, 1996; Suh *et al.*, 2002; West *et al.*, 1994). In comparison with PAO, the level of *toxA* expression in PAOΔvfr was significantly reduced (West *et al.*, 1994). In addition, Vfr specifically binds to a Vfr consensus sequence within the *toxA* upstream region, suggesting that Vfr may regulate *toxA* expression directly (Kanack *et al.*, 2006). However, under M-st condition, the level of *toxA* expression in PAOΔvfr was higher than that under A-sh conditions (i.e. *vfr* deletion did not interfere with the increase in *toxA* expression). Under A-sh conditions and at the 16 h time point, the level of β -galactosidase activity produced by PAO/pSW228 was 1377.5 ± 88.68 , while that of PAOΔvfr/pSW228 was 352.5 ± 30.17 ($P=0.0023$). Under M-st conditions, PAO/pSW228 produced 3014.0 ± 79.30 units of β -galactosidase activity with PAOΔvfr/pSW228 producing 729.0 ± 23.24 ($P=0.0002$). Thus, similar to PvdS, Vfr is essential for efficient *toxA* expression, but is not the factor through which the increase in *toxA* expression under M-st conditions occurs. Using PAO and its *lasR* deletion mutant, we also ruled out a role for LasR in the observed increase in *toxA* expression under microaerobic conditions. Under A-sh conditions, and in comparison with PAO/pSW228, the level of β -galactosidase activity produced by

PAOΔ*lasR*/pSW228 was about 2.6-fold less (1060.5 ± 60.80 vs 411.5 ± 12.20 ; $P=0.0025$). Under M-st conditions, the level of β -galactosidase activity produced by both strains was increased by two- to threefold compared to A-sh conditions (2295.5 ± 107.16 for PAO/pSW228 and 1315.8 ± 66.78 for PAOΔ*lasR*/pSW228).

It is possible that *toxA* and *ptxR* expression is induced through the denitrification (anaerobic respiration) process in which nitrate functions as the terminal electron acceptor. We previously demonstrated that the most significant increase in *toxA* and *ptxR* expression occurs when PAO is grown anaerobically in the presence of nitrate (Gaines *et al.*, 2005). Schreiber *et al.* (2007) recently showed that the denitrification process in *P. aeruginosa* involves a regulatory network that includes the oxygen regulator Anr, the nitric oxide regulator Dnr and the nitrate responsive two-component regulatory systems NarXL. Whether any of these regulators enhance *toxA* and/or *ptxR* expression anaerobically or microaerobically in the presence of nitrate is yet to be determined.

Similar to *toxA* expression, *ptxR* expression in PAO was increased under M-st conditions. This increase occurred whether *ptxR* was expressed from the P1/P2 promoters or the P2 promoter alone (data not shown; Fig. 5d) (Gaines *et al.*, 2005). In addition, the increase was detected in both PAO and PAO::pvdS, thus appearing independent of PvdS (data not shown; Figs 5d, 7b). We hypothesize that reduced EO increases *toxA* and *ptxR* expression through a single mechanism or as a general effect on the expression of several genes (Fig. 7b). Due to the nature of the PAOΔ*ptxR* mutation, we cannot confirm or deny a role for *ptxR* in regulating ETA production under reduced EO. We previously reported that upon its initial characterization PAOΔ*ptxR* produced a significantly lower level of ETA than PAO (Hamood *et al.*, 1996). However, upon continuous subculturing, PAOΔ*ptxR* produced levels of ETA comparable to that produced by PAO (Hamood *et al.*, 1996). This phenomenon could be due to either a mutation in another gene that suppresses the *ptxR* mutation or to a compensation of PtxR function by another LysR protein. This effect is specific to ETA as it differs from the effect of *ptxR* on the production of other virulence factors such as pyocyanin (Carty *et al.*, 2006). Besides the genes described in this study, other *P. aeruginosa* genes whose expression varies under reduced EO in iron-deficient medium are not known. Previous studies that described the effect of reduced EO on the expression of *P. aeruginosa* genes were conducted in iron-sufficient media (Bragonzi *et al.*, 2005; Eschbach *et al.*, 2004). In addition, Ochsner *et al.* (2002) described a transcriptome analysis of PAO and PAO::pvdS in iron-deficient medium under A-sh conditions. Furthermore, Palma *et al.* (2003) compared PAO global transcription in iron-deficient and iron-sufficient media under A-sh conditions. Therefore, a comprehensive transcriptome analysis of PAO and PAO::pvdS in iron deficient medium under M-st conditions is essential to understand the relationship between *pvdS*, *regA*, *toxA* and *ptxR*.

In conclusion, our results show that reduced levels of EO enhance *tox*A, and to a lesser extent *ptx*R expression, but reduce the expression of *reg*A, *pvd*D, *pvd*E and *pvd*S (Fig. 7). The enhancement in *tox*A and *ptx*R expression does not appear to occur through PvdS. In addition, our results show that the PvdS–RNAP complex binds to the *ptx*R, *reg*A and *tox*A upstream regions. While the present evidence supports a direct interaction between PvdS and the promoters of *reg*A, *ptx*R and the pyoverdine genes, the effect of this interaction on the expression of these genes is yet to be determined. In addition, the environmental conditions under which PvdS interacts directly with the *tox*A promoter are still unknown.

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REFERENCES

- Allen, D. B., Maguire, J. J., Mahdavian, M., Wicke, C., Marcocci, L., Scheuenstuhl, H., Chang, M., Le, A. X., Hopf, H. W. & Hunt, T. K. (1997). Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg* **132**, 991–996.
- Ambrosi, C., Leoni, L. & Visca, P. (2002). Different responses of pyoverdine genes to autoinduction in *Pseudomonas aeruginosa* and the group *Pseudomonas fluorescens*–*Pseudomonas putida*. *Appl Environ Microbiol* **68**, 4122–4126.
- Ambrosi, C., Tiburzi, F., Imperi, F., Putignani, L. & Visca, P. (2005). Involvement of AlgQ in transcriptional regulation of pyoverdine genes in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **187**, 5097–5107.
- Bailey, J. & Manoel, C. (2002). Genome-wide internal tagging of bacterial exported proteins. *Nat Biotechnol* **20**, 839–842.
- Baltch, A. L. (1994). *Pseudomonas* bacteremia. In *Pseudomonas aeruginosa Infections and Treatments*, pp. 73–128. Edited by R. P. Smith & A. L. Baltch. New York: Marcel Dekker.
- Beare, P. A., For, R. J., Martin, L. W. & Lamont, I. L. (2003). Siderophore-mediated cell signalling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. *Mol Microbiol* **47**, 195–207.
- Bragonzi, A., Worlitzsch, D., Pier, G. B., Timpert, P., Ulrich, M., Hentzer, M., Andersen, J. B., Givskov, M., Conese, M. & Döring, G. (2005). Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. *J Infect Dis* **192**, 410–419.
- Carty, N. L., Rumbaugh, K. P. & Hamood, A. N. (2003). Regulation of *tox*A by PtxR in *Pseudomonas aeruginosa* PA103. *Can J Microbiol* **49**, 450–464.
- Carty, N. L., Layland, N., Colmer-Hamood, J. A., Calfee, M. W., Pesci, E. C. & Hamood, A. N. (2006). PtxR modulates the expression of QS-controlled virulence factors in the *Pseudomonas aeruginosa* strain PAO1. *Mol Microbiol* **61**, 782–794.
- Colmer, J. A. & Hamood, A. N. (1999). Expression of *ptx*R and its effect on *tox*A and *reg*A expression during the growth cycle of *Pseudomonas aeruginosa* strain PAO1. *Can J Microbiol* **45**, 1008–1016.
- Colmer-Hamood, J. A., Aramaki, H., Gaines, J. M. & Hamood, A. N. (2006). Transcriptional analysis of the *Pseudomonas aeruginosa* *tox*A regulatory gene *ptx*R. *Can J Microbiol* **52**, 343–356.
- Cunliffe, H. E., Merriman, T. R. & Lamont, I. L. (1995). Cloning and characterization of *pvd*S, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. *J Bacteriol* **177**, 2744–2750.
- Davis, P. B., Drumm, M. & Konstan, M. W. (1996). Cystic fibrosis. *Am J Respir Crit Care Med* **154**, 1229–1256.
- Dupuy, B. & Matamouros, S. (2006). Regulation of toxin and bacteriocin synthesis in *Clostridium* species by a new subgroup of RNA polymerase sigma-factors. *Res Microbiol* **157**, 201–205.
- Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D. & Schobert, M. (2004). Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J Bacteriol* **186**, 4596–4604.
- Frank, D. W. & Iglewski, B. H. (1988). Kinetics of *tox*A and *reg*A mRNA accumulation in *Pseudomonas aeruginosa*. *J Bacteriol* **170**, 4477–4483.
- Frank, D. W., Storey, D. G., Hindahl, M. S. & Iglewski, B. H. (1989). Differential regulation by iron of *reg*A and *tox*A transcript accumulation in *Pseudomonas aeruginosa*. *J Bacteriol* **171**, 5304–5313.
- Fujita, M., Tanaka, K., Takahashi, H. & Amemura, A. (1993). Organization and transcription of the principal sigma gene (*rpo*DA) of *Pseudomonas aeruginosa* PAO1: involvement of a sigma 32-like RNA polymerase in *rpo*DA gene expression. *J Bacteriol* **175**, 1069–1074.
- Gaines, J. M., Carty, N. L., Colmer-Hamood, J. A. & Hamood, A. N. (2005). Effect of static growth and different levels of environmental oxygen on *tox*A and *ptx*R expression in the *Pseudomonas aeruginosa* strain PAO1. *Microbiology* **151**, 2263–2275.
- Gambello, M. J., Kaye, S. & Iglewski, B. H. (1993). LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun* **61**, 1180–1184.
- Grant, C. C. & Vasil, M. L. (1986). Analysis of transcription of the exotoxin A gene of *Pseudomonas aeruginosa*. *J Bacteriol* **168**, 1112–1119.
- Haas, B., Kraut, J., Marks, J., Zanker, S. C. & Castignetti, D. (1991). Siderophore presence in sputa of cystic fibrosis patients. *Infect Immun* **59**, 3997–4000.
- Hamood, A. N. & Iglewski, B. H. (1990). Expression of the *Pseudomonas aeruginosa* *tox*A positive regulatory gene (*reg*A) in *Escherichia coli*. *J Bacteriol* **172**, 589–594.
- Hamood, A. N., Colmer, J. A., Ochsner, U. A. & Vasil, M. L. (1996). Isolation and characterization of a *Pseudomonas aeruginosa* gene, *ptx*R, which positively regulates exotoxin A production. *Mol Microbiol* **21**, 97–110.
- Hamood, A. N., Colmer-Hamood, J. A. & Carty, N. L. (2004). Regulation of *Pseudomonas aeruginosa* exotoxin A synthesis. In *Pseudomonas: Virulence and Gene Regulation*, pp. 389–423. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.
- Hassett, D. J., Cuppoletti, J., Trapnell, B., Lyman, S. V., Rowe, J. J., Yoon, S. S., Hilliard, G. M., Parvatiyar, K., Kamani, M. C. & other authors (2002). Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* **54**, 1425–1443.
- Hunt, T. A., Peng, W. T., Loubens, I. & Storey, D. G. (2002). The *Pseudomonas aeruginosa* alternative sigma factor PvdS controls

- exotoxin A expression and is expressed in lung infections associated with cystic fibrosis. *Microbiology* **148**, 3183–3193.
- Iglewski, B. H. & Kabat, D. (1975). NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc Natl Acad Sci U S A* **72**, 2284–2288.
- Jacobs, M. A., Alwood, A., Thaisittikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **100**, 14339–14344.
- Jaffar-Bandjee, M. C., Lazdunski, A., Bally, M., Carrere, J., Chazallete, J. P. & Galabert, C. (1995). Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. *J Clin Microbiol* **33**, 924–929.
- Juhas, M., Wiehlmann, L., Huber, B., Jordan, D., Lauber, J., Salunkhe, P., Limpert, A. S., von Götze, F., Steinmetz, I. & other authors (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* **150**, 831–841.
- Kanack, K. J., Runyen-Janecky, L. J., Ferrell, E. P., Suh, S. J. & West, S. E. (2006). Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. *Microbiology* **152**, 3485–3496.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I. & Vasil, M. L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **99**, 7072–7077.
- Leoni, L., Ciervo, A., Orsi, N. & Visca, P. (1996). Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. *J Bacteriol* **178**, 2299–2313.
- Leoni, L., Orsi, N., de Lorenzo, V. & Visca, P. (2000). Functional analysis of PvdS, an iron starvation sigma factor of *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 1481–1491.
- Liu, P. V. (1973). Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. *J Infect Dis* **128**, 506–513.
- Lory, S. (1986). Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa*. *J Bacteriol* **168**, 1451–1456.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Ochsner, U. A., Vasil, A. I. & Vasil, M. L. (1995). Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. *J Bacteriol* **177**, 7194–7201.
- Ochsner, U. A., Johnson, Z., Lamont, I. L., Cunliffe, H. E. & Vasil, M. L. (1996). Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. *Mol Microbiol* **21**, 1019–1028.
- Ochsner, U. A., Wilderman, P. J., Vasil, A. I. & Vasil, M. L. (2002). GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* **45**, 1277–1287.
- Ohman, D. E., Sadoff, J. C. & Iglewski, B. H. (1980). Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. *Infect Immun* **28**, 899–908.
- Palma, M., Worgall, S. & Quadri, L. E. (2003). Transcriptome analysis of the *Pseudomonas aeruginosa* response to iron. *Arch Microbiol* **180**, 374–379.
- Pfeffer, S. R., Stahl, S. J. & Chamberlin, M. J. (1977). Binding of *Escherichia coli* RNA polymerase to T7 DNA. Displacement of holoenzyme from promoter complexes by heparin. *J Biol Chem* **252**, 5403–5407.
- Raivio, T. L., Hoffer, D., Prince, R. W., Vasil, M. L. & Storey, D. G. (1996). Linker insertion scanning of *regA*, an activator of exotoxin A production in *Pseudomonas aeruginosa*. *Mol Microbiol* **22**, 239–254.
- Rombel, I. T., McMorran, B. J. & Lamont, I. L. (1995). Identification of a DNA sequence motif required for expression of iron-regulated genes in pseudomonads. *Mol Gen Genet* **246**, 519–528.
- Sadikot, R. T., Blackwell, T. S., Christman, J. W. & Prince, A. S. (2005). Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* **171**, 1209–1223.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning, a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M. & Jahn, D. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* **189**, 4310–4314.
- Smith, A. W. & Iglewski, B. H. (1989). Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res* **17**, 10509.
- Sobel, M. L., McKay, G. A. & Poole, K. (2003). Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother* **47**, 3202–3207.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.
- Stintzi, A., Cornelis, P., Hohnadel, D., Meyer, J. M., Dean, C., Poole, K., Kourambas, S. & Krishnapillai, V. (1996). Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa* PAO. *Microbiology* **142**, 1181–1190.
- Stintzi, A., Johnson, Z., Stonehouse, M., Ochsner, U., Meyer, J. M., Vasil, M. L. & Poole, K. (1999). The *pvc* gene cluster of *Pseudomonas aeruginosa*: role in synthesis of the pyoverdine chromophore and regulation by PtxR and PvdS. *J Bacteriol* **181**, 4118–4124.
- Storey, D. G., Frank, D. W., Farinha, M. A., Kropinski, A. M. & Iglewski, B. H. (1990). Multiple promoters control the regulation of the *Pseudomonas aeruginosa regA* gene. *Mol Microbiol* **4**, 499–503.
- Storey, D. G., Raivio, T. L., Frank, D. W., Wick, M. J., Kaye, S. & Iglewski, B. H. (1991). Effect of *regB* on expression from the P1 and P2 promoters of the *Pseudomonas aeruginosa regAB* operon. *J Bacteriol* **173**, 6088–6094.
- Stuber, D., Matile, H. & Garotta, G. (1990). System for high level production in *E. coli* and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure/function analysis. In *Immunological Methods*, pp. 121–152. Edited by I. Lefkowitz & I. Pernis. Orlando: Academic Press.
- Suh, S. J., Runyen-Janecky, L. J., Maleniak, T. C., Hager, P., MacGregor, C. H., Zielinski-Mozny, N. A., Phibbs, P. V., Jr & West, S. E. (2002). Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. *Microbiology* **148**, 1561–1569.
- van Delden, C. (2004). Virulence factors in *Pseudomonas aeruginosa*. In *Pseudomonas: Virulence and Gene Regulation*, pp. 3–45. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.
- Vasil, M. L., Grant, C. C. & Prince, R. W. (1989). Regulation of exotoxin A synthesis in *Pseudomonas aeruginosa*: characterization of *toxA-lacZ* fusions in wild-type and mutant strains. *Mol Microbiol* **3**, 371–381.
- Vasil, M. L., Ochsner, U. A., Johnson, Z., Colmer, J. A. & Hamood, A. N. (1998). The Fur-regulated gene encoding the alternative sigma

factor PvdS is required for iron-dependent expression of the LysR-type regulator *ptxR* in *Pseudomonas aeruginosa*. *J Bacteriol* **180**, 6784–6788.

Visca, P. (2004). Iron regulation and siderophore signalling in virulence by *Pseudomonas aeruginosa*. In *Pseudomonas: Virulence and Gene Regulation*, pp. 69–123. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.

Visca, P., Colotti, G., Serino, L., Verzili, D., Orsi, N. & Chiancone, E. (1992). Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. *Appl Environ Microbiol* **58**, 2886–2893.

Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45**, 1177–1190.

West, S. E., Sample, A. K. & Runyen-Janecky, L. J. (1994). The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol* **176**, 7532–7542.

Wick, M. J., Frank, D. W., Storey, D. G. & Iglewski, B. H. (1990). Structure, function, and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annu Rev Microbiol* **44**, 335–363.

Wilderman, P. J., Vasil, A. I., Johnson, Z., Wilson, M. J., Cunliffe, H. E., Lamont, I. L. & Vasil, M. L. (2001). Characterization of an

endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. *Infect Immun* **69**, 5385–5394.

Wilson, M. J., McMorran, B. J. & Lamont, I. L. (2001). Analysis of promoters recognized by PvdS, an extracytoplasmic-function sigma factor protein from *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 2151–2155.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J. & other authors (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* **109**, 317–325.

Xiong, Y. Q., Vasil, M. L., Johnson, Z., Ochsner, U. A. & Bayer, A. S. (2000). The oxygen- and iron-dependent sigma factor PvdS of *Pseudomonas aeruginosa* is an important virulence factor in experimental infective endocarditis. *J Infect Dis* **181**, 1020–1026.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Oschsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. & other authors (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593–603.

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