The *Pseudomonas aeruginosa* alternative sigma factor PvdS controls exotoxin A expression and is expressed in lung infections associated with cystic fibrosis

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PvdS is an alternative sigma factor regulated by the global iron regulator Fur. It has been demonstrated that PvdS plays a role in the iron-dependent regulation of exotoxin A (ETA) in *Pseudomonas aeruginosa* strain PAO1. The goals of this research were to determine if *pvdS* was transcribed by the bacteria in the chronic lung infections associated with cystic fibrosis (CF) and to determine how PvdS interacts with the *regAB* promoters of the hyper-toxigenic strain PA103. It was found that *pvdS* is transcribed in the lungs of patients with CF and that it appears to be involved with the regulation of *toxA* in this environment. This correlated with the finding that in strain PA103, a mutation in *pvdS* reduced ETA activity while the same mutation in strain PAO1 abrogated ETA production. It was also shown that in strain PA103, *pvdS* was absolutely required for activation of the *regAB* P2 promoter. The effect of PvdS on the P2 promoter may be direct or indirect; however, in support of a direct role, an eight-out-of-nine base-pair match to the consensus sequence for PvdS binding was identified at the transcriptional start site for the P2 promoter. The effect of PvdS on the PA103 *regAB* P1 promoter under aerobic growth conditions was also examined. The results show that PvdS does modulate the expression from this promoter but that both the *regAB* operon and PvdS are required for optimal P1 promoter activity. These studies demonstrate that the alternative sigma factor PvdS acts as a regulator of ETA expression in *P. aeruginosa* strain PA103 through the *regAB* operon and that PvdS is expressed in lung infections associated with CF.

Keywords: RegA, *pvdS*, *toxA*, iron regulation, strain PA103

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes extensive morbidity and mortality in individuals who are immunocompromised or have underlying medical conditions such as cystic fibrosis (CF) (Bodey *et al.*, 1983). The virulence of *P. aeruginosa* during an infection is multi-factorial and includes the production of many virulence factors, one of the most toxic of which is exotoxin A (ETA) (Liu, 1974). ETA is

an ADP-ribosylating toxin that acts on eukaryotic elongation factor II to inhibit protein synthesis within the target host cell (Iglewski & Kabat, 1975). The regulation of ETA expression at the level of transcription is complex and involves a cascade of regulators that result in the maximal production of ETA in iron-limiting environments (Grant & Vasil, 1986; reviewed by Vasil & Ochsner, 1999). In high-iron conditions (100 µM), *toxA* transcripts cannot be detected, while in low-iron conditions, *toxA* mRNA can be identified during the late-exponential and stationary phases of growth (Lory, 1986).

Many regulators have been speculated to act on the *toxA* gene, but RegA is believed to act directly on the *toxA* promoter. RegA is a positive regulatory protein

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Abbreviations: CF, cystic fibrosis; ETA, exotoxin A.

that is required for the specific enhancement of ETA synthesis (Hedström *et al.*, 1986). ETA expression was demonstrated to be completely abrogated in the *regAB*-knockout strain PA103 Δ *regAB*::Gm, confirming that the *regAB* locus is required for ETA production (Raivio *et al.*, 1996). Because RegA is required for ETA production, the environmental regulation of *tox*A expression is believed to be mediated through the *regAB* operon.

The regulation of the *regAB* operon is strain dependent. Strain PAO1, the prototypical strain, when grown under aerobic conditions, transcribes only *regA*, and primary transcription occurs from an iron-regulated promoter designated P2 (Wick *et al.*, 1990). PAO1 is missing the start codon of the *regB* ORF (Wick *et al.*, 1990). In the hypertoxigenic strain PA103, the *regAB* operon is preceded by two independent promoters that regulate the transcription of separate transcripts (Frank *et al.*, 1989; Storey *et al.*, 1990). The P1 promoter is located 164 bp upstream from the translational start site of *regA* and is not tightly regulated by iron. The P2 promoter is active only in low-iron conditions and is located 75 nucleotides upstream of the *regA* start codon. In the hypertoxigenic strain PA103, when grown under aerobic conditions, it has been demonstrated that a functional *regB* gene is required in order for transcription from the P1 promoter to occur but activity from the P2 promoter is independent of *regB* (Wick *et al.*, 1990). Interestingly, it has been shown that when strain PAO1 is grown under microaerobic conditions, both P1 and P2 promoters are active (Ochsner *et al.*, 1996; Barton *et al.*, 1996). These observations further demonstrate the complexity of the regulation and the importance of environmental effects.

To date, at least seven regulators have been implicated in regulation of the *regAB* promoters: *regB* (Wick *et al.*, 1990; Storey *et al.*, 1991), PtxR (Hamood *et al.*, 1996), PtxS (Swanson *et al.*, 1999), Vfr (West *et al.*, 1994; Albus *et al.*, 1997), LasR (Gambello *et al.*, 1993), Fur (Prince *et al.*, 1991) and PvdS (Ochsner *et al.*, 1996). Of these regulatory elements, only PvdS appears to be involved in the regulation of both promoters in strain PAO1 (Ochsner *et al.*, 1996). *pvdS* was originally identified as a regulatory gene involved in activating pyoverdine synthesis (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). The PvdS (pyoverdine sigma factor) protein shows homology to *Escherichia coli* FeC1, an alternative sigma factor. Based on this strong homology, and the presence of a putative DNA-binding motif at the C-terminal end, PvdS was postulated to act as an alternative sigma factor for RNA polymerase (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). The PvdS protein from *P. aeruginosa* has been overexpressed and purified (Leoni *et al.*, 2000). The protein binds to the promoter regions of *pvdA*, *pvdE* and *pvdF*, and acts as an alternative sigma factor with *E. coli* RNA polymerase to initiate transcription of *pvdA* (Wilson & Lamont, 2000; Wilson *et al.*, 2001). PvdS expression is repressed by iron and this regulation is mediated through the global iron regulator Fur (Ochsner *et al.*, 1995). PvdS plays a key role in iron regulation in

P. aeruginosa and has been demonstrated to be involved in the regulation of pyoverdine biosynthesis genes and ETA production (Miyazaki *et al.*, 1995; Leoni *et al.*, 1996; Ochsner *et al.*, 1996). Recently, Lamont *et al.* (2002) have shown that PvdS may also be controlled by an anti-sigma factor FpvR. Ochsner *et al.* (1996) have also demonstrated that in the prototypical strain PAO1, PvdS was necessary for *regA* expression. Their data suggested that PvdS interacts either directly or indirectly with the P2 promoter of *regA* (Ochsner *et al.*, 1996; reviewed by Vasil & Ochsner, 1999). PvdS may modulate expression from the *regA* P1 promoter but is not required for activity in strain PAO1. On a functional basis, *pvdS* has been recently demonstrated to be required for optimal virulence in an infective endocarditis model (Xiong *et al.*, 2000). The role of PvdS in the pathogenesis of *P. aeruginosa* may, therefore, be more important than previously considered.

In this study, we wanted to establish whether *pvdS* is expressed and involved in the regulation of ETA during the chronic lung infections associated with the genetic disease CF. Our second objective was to determine if PvdS plays a role in the regulation of the *regAB* operon in strain PA103, since in PA103 the operon appears to be regulated differently from the *regA* gene in strain PAO1.

METHODS

Media. *E. coli* cultures were grown in Luria-Bertani broth (LB; Sambrook *et al.*, 1989) and *P. aeruginosa* cultures were grown in trypticase soy broth dialysate chelexed (TSBDC; Liu, 1973) as previously described. For maintenance of plasmids in *E. coli*, antibiotics were added to a final concentration of 100 µg ampicillin ml⁻¹, 15 µg gentamicin ml⁻¹ or 50 µg streptomycin ml⁻¹. For growth of *P. aeruginosa* strains containing plasmids or chromosomal insertions, media contained antibiotics at final concentrations of 400 µg carbenicillin ml⁻¹, 300 µg gentamicin ml⁻¹ or 500 µg streptomycin ml⁻¹. All antibiotics were purchased from Sigma. In liquid TSBDC media, high-iron conditions were achieved by adding FeCl₃ (Sigma) to a final concentration of 10 µg ml⁻¹. Low-iron conditions were achieved by acid washing all glassware with 20% HCl before use. Wherever possible, sterile iron-free plastic equipment was used in place of glassware.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. The strain *P. aeruginosa* IL-1 was constructed by the gene replacement systems reported by Quandt & Hynes (1993) and Schweizer (1992). To construct the gene replacement plasmid, a 6 kb *Pst*I-*Sma*I fragment from a genomic clone of *regA* was ligated into pUC12. A 3.2 kbp *Bam*HI fragment from pZ1918 (Schweizer, 1993b) containing a promoterless *lacZ* gene was then ligated into the first *Bam*HI site of the *regA* ORF. A *sacRB* gene from pMH1801 (Hynes *et al.*, 1989) was digested with *Pst*I and the fragment containing the *sacRB* gene was ligated into the *Pst*I site. The resulting plasmid was electroporated into *P. aeruginosa* PA103 and recombinants were selected for as previously described (Quandt & Hynes, 1993; Schweizer, 1992). The recombinant clone IL-1 was used in this study. The reporter construct prevents transcription from the P1 promoter because a functional *regB* is required for P1 activity in strain PA103 and *regAB* is not transcribed from IL-1. Transcription unique to the P2 promoter of the *regAB* operon

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
<i>P. aeruginosa</i>		
PA103	<i>regA</i> ⁺ <i>regB</i> ⁺ hypertoxigenic lab. strain	Liu (1966)
PAO1	<i>regA</i> ⁺ <i>regB</i> prototrophic lab. strain	Holloway <i>et al.</i> (1979)
IL-1	PA103 <i>regA::lacZ</i> ; P2- <i>lacZ</i> P1 ⁻ phenotype	This study
PA103 <i>pvdS::Gm</i>	Gm cassette at the <i>StuI</i> site of the <i>pvdS</i> gene in strain PA103; Gm ^R	This study
PAO1Δ <i>pvdS</i>	PAO1 with a 460 bp deletion in the <i>pvdS</i> gene; Gm cassette at the <i>pvdS</i> locus; Gm ^R	Ochsner <i>et al.</i> (1996)
IL-1 <i>pvdS::Ω</i>	Ω cassette at the <i>StuI</i> site of the <i>pvdS</i> gene in strain IL-1; Sm ^R /Spec ^R	This study
Plasmids		
pUC181.8	pUC18 containing the 1.8 kb <i>PstI</i> stabilizing fragment (SF) for <i>Pseudomonas</i> replication; Cb ^R	Frank & Iglewski (1988)
pTH <i>pvdS</i>	1005 bp <i>pvdS</i> PCR product ligated in the <i>EcoRI</i> site of pUC181.8; Cb ^R (<i>pvdS</i> is under the control of its native promoter)	This study
pDF191.8-202	pUC19 containing the 1.8 <i>PstI</i> fragment from pRO1614 and the <i>regAB</i> operon from strain PA103 (the <i>regAB</i> operon is under the control of its native promoters)	Frank <i>et al.</i> (1989)
pUCGm	Plasmid containing the Tn1696-derived Gm ^R cassette flanked by the pUC19 MCS; Gm ^R	Schweizer (1993a)
pHP45Ω	1.9 kb Sm ^R /Spec ^R interposon cassette in pHP45; Ap ^R Sm ^R /Spec ^R	Prentki & Krisch (1984)
pJQ200SK	Suicide vector containing the <i>sacB</i> cassette; pBluescript II SK MCS; Gm ^R	Quandt & Hynes (1993)
pJQ <i>pvdS::Gm</i>	Gm ligated into the <i>StuI</i> site of <i>pvdS</i> ; <i>pvdS::Gm</i> ligated into the <i>XhoI</i> and <i>XbaI</i> sites of pJQ200SK; Gm ^R	This study
pJQ <i>pvdS::Ω</i>	Ω ligated into the <i>StuI</i> site of <i>pvdS</i> ; <i>pvdS::Ω</i> ligated into the <i>XhoI</i> and <i>XbaI</i> sites of pJQ200SK; Sm ^R /Spec ^R	This study

can be monitored in this strain by performing β-galactosidase assays.

Plasmid pTH*pvdS* was constructed from the 1005 bp PCR product of the *pvdS* locus from *P. aeruginosa* strain PA103 (forward primer AAG AAT TCC TCC CCT CCA TCA TTC GCA G; reverse primer: ACG AAT TCT ACC TGG ACG GTC CCG TTG). The PCR product was flanked by *EcoRI* sites to aid in cloning. Sequence analysis confirmed that this PCR product contained the complete 563 bp *pvdS* gene, the 148 bp promoter region, and 294 bp of flanking chromosomal DNA. Plasmid pTH*pvdS* contains the entire *pvdS* ORF and expression is mediated from its native promoter. This plasmid was used to study the effect of *pvdS* expression from a multi-copy plasmid, but under the regulatory control of its native iron-regulated promoter.

In order to investigate the effects of interrupting the *pvdS* gene on the chromosome of strains IL-1 and PA103, knock-out strains were constructed. In knock-outs of PA103 and IL-1, the *pvdS* ORF was interrupted with an Ω cassette from pHP45Ω or a Gm cassette from pUCGm respectively, at the unique *StuI* site within the *pvdS* ORF. The interrupted *pvdS* gene was constructed on a suicide plasmid that contained a *sacB* cassette (pJQ200SK) to facilitate the double crossover onto the chromosome. The resulting plasmids, pJQ*pvdS::Ω* and pJQ*pvdS::Gm*, were electroporated into *P. aeruginosa* strains IL-1 and PA103 to produce strains IL-1 *pvdS::Ω* and PA103 *pvdS::Gm* respectively.

DNA manipulations. Plasmid DNA was isolated based on the alkaline lysis method developed by Birnboim & Doly (1979). Chromosomal DNA was isolated from *P. aeruginosa* using a modified version of the method described by Ausubel *et al.*

(1991). All restriction enzymes and buffers used were purchased from Gibco-BRL or Pharmacia. Electroporations were performed on *P. aeruginosa* following the method described by Smith & Iglewski (1989).

Southern hybridization. Southern blotting was performed as described by Ausubel *et al.* (1991) with some modifications. DNA fragments to be used as probes were labelled with [³²P]dCTP (Dupont NEN Products) using an Oligolabelling Kit (Pharmacia).

Growth curve methodology. ADP-ribosyltransferase and β-galactosidase assays were performed on culture samples collected over the course of a growth curve. Growth curves were performed over a 24–30 h time period and samples were collected at the time points indicated. All growth curves were repeated at least twice. Samples for β-galactosidase assays were collected and stored at –80 °C until β-galactosidase assays were performed on each sample in duplicate using a modification of the method of Miller (1972). Samples of cell-free supernatant were collected and stored at –80 °C until extracellular ADP-ribosyltransferase activity was assayed in triplicate for each sample as described by Chung & Collier (1977).

Analysis of RNA from bacterial populations found in sputa. The patient population analysed was as previously described by Storey *et al.* (1998). All patients and their guardians gave their consent to participate in this study. The study design was ethically reviewed and approved by the Conjoint Research Ethics Board at the University of Calgary. All techniques used in the collection, handling and extraction of the samples were as previously described by Storey *et al.* (1992). The DNA probe for *pvdS* was a 1 kb *EcoRI* fragment from pTH*pvdS*

containing the *pvdS* gene. Probes for *toxA* and *algD* were prepared as described by Storey *et al.* (1997). Statistical analysis was as described by Storey *et al.* (1998).

RESULTS

Transcription of *pvdS* in the lungs of patients with CF

We have previously shown that both *regAB* transcripts are produced in the lungs of patients with CF (Raivio *et al.*, 1994). We have also demonstrated that *toxA* is transcribed in the CF lung (Storey *et al.*, 1992). It was, therefore, of interest to determine if *pvdS* is transcribed in the lungs of CF patients, and whether or not there was a correlation between the transcript accumulation of *pvdS*, *regAB* and *toxA*. Fig. 1 shows that we were able to detect *pvdS* transcripts at levels above background in 80% of our CF samples. There is a range of transcript accumulation from background levels to greater than 20 times the optimal transcription when cells are grown in the laboratory (see sample 24 in Fig. 1b). These results represent 16 samples in which the RNA was extracted from equivalent numbers of bacteria and equivalent amounts of RNA were loaded on the slot blot. The variation in expression levels also suggested that *pvdS* is regulated in the lungs of patients with CF. We hypothesize that *pvdS* may be regulating the *regAB* operon and subsequently *toxA*. We would have liked to correlate *pvdS* expression to the *regAB* operon since *pvdS* directly regulates this operon. However, the half-life of the two *regA* transcripts is very short and, therefore, they are difficult to detect in sputa (Raivio *et*

al., 1994). Thus, we have compared the transcript accumulation of *toxA* to *pvdS* since it appears that *pvdS* indirectly regulates *toxA*. A Spearman rank correlation was performed on the 16 samples shown in Fig. 1. This analysis showed a statistically significant correlation between *pvdS* and *toxA* transcript accumulation ($r=0.647$, $P<0.005$). Notably, we did not detect a statistically significant correlation between either *algD* and *pvdS* ($r=0.430$ $P<0.05$) or *algD* and *toxA* ($r=0.385$ $P<0.1$) transcript accumulation in these samples, as expected. Overall this analysis suggests that PvdS is an important regulator of ETA in the lungs of patients with CF. Raivio *et al.* (1996) showed that the pattern of *regAB* transcript accumulation in the lungs of patients with CF was similar to the pattern found in the hypertoxigenic strain PA103 when growing under aerobic conditions. This led us to question if the role of PvdS in strain PA103 is identical to or different from its role in the regulation of the prototypical strain PAO1.

P. aeruginosa *pvdS* knock-out strains produce less ETA than the parental strain PA103

Initially we wanted to determine if a mutation in *pvdS* affected ETA production in the hypertoxigenic strain PA103 in the same way as in the prototypical strain PAO1. Fig. 2 shows the ETA production for the *pvdS* mutants and their complemented controls over 28 h of growth. Strain PA103 *pvdS*::Gm produced approximately 18–25% of the maximum ETA produced by the complemented strain (Fig. 2a). Interestingly, the ETA produced by PA103 *pvdS*::Gm remains tightly iron

Fig. 1. PvdS is transcribed in the lungs of patients with CF: population transcript accumulation of *P. aeruginosa* *pvdS*, *toxA* and *algD* in RNA extracted from CF sputa. All samples contained equivalent numbers of *P. aeruginosa* (10^8 ml $^{-1}$) and in all samples *P. aeruginosa* was the only pathogenic bacterium isolated. A soft laser scanning densitometer was utilized to determine the relative intensity from an autoradiograph of the blot. The results represent the signal from 10 μ g RNA extracted from an equivalent number of bacteria. Panels (a) and (b) represent the results from one experiment set. Panel (b) was separated to reflect the much higher values for sample no. 24. Control hybridizations (cont.) are identical for both panels.

Fig. 2. PvdS is required for optimal ETA production in *pvdS* knockouts of PA103 and PAO1. Cultures were grown for 28 h under high-iron conditions ($10 \mu\text{g ml}^{-1}$; black symbols) or iron-limiting conditions (open symbols) and aliquots were removed at the indicated time points. Extracellular ADP-ribosyltransferase activity was assayed in triplicate at each time point. (a) ETA production in a *pvdS* knockout of the hypertoxigenic strain PA103. PA103 $\Delta pvdS$::Gm ($\blacklozenge$, $\blacktriangle$), PA103 $\Delta pvdS$::Gm with the vector control pUC181.1 ($\blacktriangle$, $\triangle$), and PA103 $\Delta pvdS$::Gm with the plasmid pTHpvdS ($\bullet$, $\circ$). (b) PAO1 $\Delta pvdS$ ($\blacklozenge$, $\blacktriangle$), PAO1 $\Delta pvdS$ with the vector control pUC181.1 ($\blacktriangle$, $\triangle$), and PAO1 $\Delta pvdS$::Gm with the plasmid pTHpvdS ($\bullet$, $\circ$).

regulated (Fig. 2a). These results suggest that *pvdS* is required for maximal ETA production in PA103; however a mutation in *pvdS* does not abrogate the iron regulation of the residual ETA activity. In contrast, in strain PAO1 the *pvdS* mutation completely abrogates ETA activity, as previously shown by Ochsner *et al.* (1996) (Fig. 2b). In this experiment the residual ETA activity in PAO1 $\Delta pvdS$ (1% of maximal ETA activity in the complemented strain) was not affected by the iron concentration of the medium. Thus, it appears that there are significant differences in the effect that a mutation in

Fig. 3. ETA activity is increased under iron-limiting conditions in the presence of multi-copy *pvdS* in strain PA103 and strain PAO1. Cultures grown under high-iron conditions ($10 \mu\text{g ml}^{-1}$) are indicated by black symbols; cultures grown under iron-limiting conditions are indicated by open symbols. (a) PA103 ($\blacklozenge$, $\blacktriangle$), PA103 with the vector control pUC181.1 ($\blacktriangle$, $\triangle$), and PA103 with the plasmid pTHpvdS, expressing *pvdS* under the control of the native promoter ($\bullet$, $\circ$). (b) PAO1 ($\blacklozenge$, $\blacktriangle$), PAO1 with the vector control pUC181.1 ($\blacktriangle$, $\triangle$), and PAO1 with the plasmid pTHpvdS, expressing *pvdS* under the control of the native promoter ($\bullet$, $\circ$).

pvdS has on the ETA regulation of PA103 as compared to the prototypical PAO1.

ETA production is increased when PvdS is expressed on a multi-copy plasmid in low-iron conditions

pvdS expression is controlled by the iron-regulated repressor Fur (Barton *et al.*, 1996; Ochsner *et al.*, 1996). It therefore was of interest to determine if multiple copies of *pvdS* would enhance the expression of ETA in either high- or low-iron media. Plasmids pUC181.8 and pTHpvdS were electroporated into the hypertoxigenic strain PA103. Growth of the strains was observed over 28 h. In all cases no growth differences were observed between the strains; however growth was slower in low-iron conditions than in high-iron conditions (data not shown). The results in Fig. 3(a) demonstrate that when

Fig. 4. The reduced P2 activity from strain IL-1 *pvdS*:: Ω is restored in low-iron conditions when the mutation is complemented *in trans* with *pvdS*. Strain IL-1 contains a chromosomal P2-*lacZ* fusion. The six cultures were grown for 28 h and aliquots were assayed for β -galactosidase activity in duplicate at each indicated time point. Cultures grown under high-iron conditions ($10 \mu\text{g ml}^{-1}$) are indicated by black symbols; cultures grown under iron-limiting conditions are indicated by open symbols. $\blacklozenge$, $\blacktriangle$, IL-1 *pvdS*:: Ω ; $\blacklozenge$, $\blacktriangle$, IL-1 *pvdS*:: Ω with the vector control pUC181.1; $\bullet$, $\circ$, IL-1 *pvdS*:: Ω with the plasmid pTH*pvdS*.

pvdS is provided *in trans*, ETA production of strain PA103 is increased by approximately 16–27% in low-iron conditions. Enhanced ETA production solely in low-iron conditions demonstrates that multiple copies of the *pvdS* promoter are not sufficient to dilute out the iron-bound Fur at high iron concentrations.

In strain PAO1, multiple copies of *pvdS* also did not affect ETA production in high-iron conditions (Fig. 3b). However, in low-iron conditions we observed a more dramatic effect in this strain, with multiple copies of *pvdS* giving a 35–70% increase in maximal ETA production (Fig. 3b) as compared to strain PA103, which showed only a 16–27% increase. Thus, it appears that multiple copies of *pvdS* have different effects on ETA regulation in PAO1 as compared to PA103.

Influence of PvdS on *regA* P2 activity when *P. aeruginosa* strain PA103 is grown in low-iron conditions

Previous research in strain PAO1 (Ochsner *et al.*, 1996), and the effect on ETA production that we observed in PA103*pvdS*::Gm and in PA103 with multiple copies of *pvdS* (Fig. 2a and 3a) suggested that PvdS may be interacting differently with the PA103 *regA* promoters than with the *regA* promoters of PAO1. In order to monitor activity from the P2 promoter of the *regAB*

operon, strain IL-1 was constructed from the parental strain PA103. *P. aeruginosa* strain IL-1 contains a *lacZ* reporter gene insertion within the *regAB* ORF integrated into the chromosome. The reporter construct in IL-1 lacks transcription from the P1 promoter because a functional *regB* is required for P1 activity (Wick *et al.*, 1990) and *regAB* is not transcribed in this strain. Therefore, activity from the P2 promoter of the *regAB* operon can be examined in this strain by monitoring the expression from the chromosomal *lacZ* reporter gene. To examine the effect of *pvdS* on the P2 promoter, we generated a mutant of IL-1 containing a *pvdS* knock-out. No P2 activity was detected in either high- or low-iron conditions from strain IL-1 *pvdS*:: Ω (Fig. 4). However, P2 activity from this knock-out strain was restored to parental levels when complemented with *pvdS* and grown under iron-limiting conditions. Notably, the expression of the P2 promoter remains highly regulated in the complemented strain, as P2 activity is evident only during the late-exponential and early stationary phases of growth under iron-limiting conditions. These results suggest that PvdS is required for the iron-regulated activity from the P2 promoter of the *regAB* operon in PA103. A similar finding was shown for the P2 promoter in PAO1 (Ochsner *et al.*, 1996). Together these findings support the hypothesis that the alternative sigma factor PvdS acts as a regulator of the P2 promoter of the *regAB* operon, and that PvdS may directly bind the *regAB* P2 promoter.

The P2 promoter of the *regAB* operon may contain a consensus sequence for PvdS binding

The sequence upstream of the *regAB* operon was analysed for homologies to known iron-regulatory consensus sequences. In the *regAB* promoter region we identified an eight-out-of-nine base-pair match (Table 2) to the published PvdS consensus sequence (Rombel *et al.*, 1995; Wilson *et al.*, 2001). This partial consensus sequence is located between –77 and –68 from the translational start site for the RegA protein and between –1 and +8 of the transcriptional start site for the T2 transcript. The one mismatched base was in the centre of the sequence with a G instead of an A. The sequence in the P2 promoter region was compared to known and putative PvdS binding sequences (Table 2). The central adenine was conserved in all the other binding sites (Table 2). Another difference between the RegA binding site and the others is that in the RegA regulatory sequence the putative PvdS binding site sits directly over the transcriptional start site. All the other binding sites appear to be located upstream of the respective transcriptional start sites. It should be noted that *pvdA*, *E* and *F* have been demonstrated to be regulated by PvdS (Leoni *et al.*, 1996, 2000; Wilson & Lamont, 2000). The other promoters in Table 2, including the *regA* promoter, have not been examined with respect to *pvdS* regulation. The identification of this partial PvdS consensus binding sequence in the P2 promoter region suggests that PvdS may be a regulator of the iron-regulated P2 promoter of the *regAB* operon, and

Table 2. Promoter region of PvdS-regulated genes

Iron-regulated promoter*	Binding sequence†	Base-pair match
<i>P. aeruginosa pvdA</i> promoter ^{1,2}	-38 CT TAAATTC-30	8/9
<i>P. aeruginosa pvdD</i> promoter ^{2,3}	-39GCTAAAT CC -29	8/9
<i>P. aeruginosa pvdE</i> promoter ^{2,3}	-23GCTAAATAC-15	9/9
<i>P. aeruginosa pvdF</i> promoter ²	-38CGTAAATTG-30	9/9
<i>P. aeruginosa toxA</i> promoter ⁴	-62 CA TAAAT CC -54	7/9
<i>P. putida</i> siderophore promoter ⁴	-75CCTAAAT CC -67	8/9
<i>P. sp.</i> strain M114 iron-regulated promoter ⁴	-91 ACTAAT TCC-83 ^x	6/9
<i>P. aeruginosa regAB</i> promoter P2 ⁵	-1GCTAGATAC ⁺ 8	8/9
Consensus PvdS binding sequence	(G/C) (G/C) TAAAT (T/A) (C/G)	9/9

* The consensus sequence similarity was identified by: 1, Miyazaki *et al.* (1995); 2, Wilson *et al.* (2001); 3, Merriman *et al.* (1995); 4, Rombel *et al.* (1995); 5, this work.

† Nucleotide positions are indicated with respect to the predicted transcriptional start site except (^x), which is in relation to the translational start site. Nucleotides that differ from the consensus are shown in bold.

Fig. 5. Activity from the P2 promoter is increased in the presence of multiple copies of *pvdS* under iron-limiting conditions. Cultures grown under high-iron conditions (10 µg ml⁻¹) are indicated by black symbols; cultures grown under iron-limiting conditions are indicated by open symbols. ◆, ◇, IL-1; ▲, △, IL-1 with the vector control pUC181.1; ●, ○, IL-1 with the plasmid pTHpvdS, overexpressing *pvdS*.

therefore an indirect regulator of ETA production in *P. aeruginosa*.

Activity from the P2 promoter of *regAB* is increased when PvdS is expressed from a multi-copy plasmid with the native promoter

The results in Fig. 3(a) revealed that multiple copies of *pvdS* enhance ETA production but that this production remained iron regulated. Our hypothesis is that in strain

PA103, PvdS may be acting on the P2 promoter to regulate expression of the *regAB* operon. To examine the effect of multiple copies of *pvdS* on the PA103 *regAB* P2 promoter, plasmids pUC181.8 and pTHpvdS were electroporated into strain IL-1 and P2 promoter activity was monitored over 26 h of growth. The results (Fig. 5) demonstrate that after growth for 14–24 h with *pvdS* expressed from a multi-copy plasmid, P2 activity is increased by a factor of 93–111%. Furthermore, this enhancement in P2 activity is dependent on iron concentration, indicating that iron regulation is maintained. Thus it appears that the effect of multiple copies of *pvdS* on the *regAB* P2 promoter is very similar to the effect on production of ETA, supporting a regulatory link between PvdS and *toxA* occurring through RegA.

The *toxA* promoter is not regulated by PvdS independently of RegA

An eight-out-of-nine base-pair match to the PvdS consensus binding sequence has previously been identified in the *toxA* promoter region (Table 2) (Rombel *et al.*, 1995; Wilson *et al.*, 2001). Because of the existence of this potential binding sequence, we examined whether PvdS could regulate the production of ETA directly at the *toxA* promoter in addition to regulating ETA through the P2 promoter of *regAB*. In order to examine the possible role of PvdS regulation at the *toxA* promoter, extracellular ETA production was assayed from *P. aeruginosa* strain IL-1. Strain IL-1 possesses a functional *toxA* locus but an interrupted *regAB*, so any ETA produced from this strain is independent of the transcriptional activator RegA. ETA production was assayed from strains IL-1, IL-1(pUC181.8) and IL-1(pTHpvdS) in both high- and low-iron TSBDC and these assays confirmed that only negligible amounts of ADP-ribosyltransferase activity were observed from all three strains in both high- and low-iron conditions (data not shown). This indicates that PvdS cannot activate the

Fig. 6. Effect of PvdS on the *regAB* promoters. The cultures were grown in low-iron medium (a) or high-iron medium (b) for 24 h and aliquots were assayed for β -galactosidase activity in duplicate at each time point. $\triangle$, IL-1 with the vector control pUC181.8; $\blacktriangle$, IL-1 *pvdS::Ω* with the vector control pUC181.8; $\diamond$, IL-1 complemented with the *regAB* operon on pDF191.8-202; $\circ$, IL-1 *pvdS::Ω* complemented with the *regAB* operon on pDF191.8-202.

toxA promoter independently of the transcriptional activator RegA and that the regulation of ETA production by this alternative sigma factor may be indirect through the *regAB* operon.

Interaction of PvdS with the P1 promoter of *regAB* in strain PA103

A critical difference in *regA* regulation between strains PA103 and PAO1 is that when cells are grown under aerobic conditions, the P1 promoter of *regA* is active in PA103 whereas in PAO1 it is inactive. In strain PA103, a requirement for P1 activity is the presence of a functional *regAB* operon. To examine the effect of PvdS on the P1 promoter, we introduced the *regAB* operon *in trans* on plasmid pDF191.8-202 into strains IL-1 and IL-1 *pvdS::Ω* because the chromosomal copy of the *regAB* operon in these strains is inactivated by an insertion of *lacZ*. The results in Fig. 6(a) demonstrate that expression of *regAB* from a multi-copy plasmid dramatically increases the early activity from the chromosomal *regAB* operon in both IL-1 and IL-1 *pvdS::Ω*, suggestive of P1 activity. During stationary phase, the

levels of β -galactosidase activity in IL-1 *pvdS::Ω* (pDF191.8-202) drop to below those of IL-1 (pDF191.8-202), suggesting that the P2 promoter is not active in strain IL-1 *pvdS::Ω* (pDF191.8-202) when the *regAB* operon is expressed *in trans*. Fig. 6(a) also shows that in the *regAB*-complemented strains, the P1 activity is greater in strain IL-1 than in strain IL-1 *pvdS::Ω* at both early and late stages of growth. This suggests that activity from the P1 promoter may be modulated by PvdS but that it is not required. To confirm these results, we repeated these experiments in high-iron conditions, where the *regAB* P1 promoter is active but the P2 promoter is repressed. The results in Fig. 6(b) show that when the strains are grown in high-iron conditions, early expression is observed in the strains complemented by the *regAB* operon *in trans*. Furthermore, levels were lower in the *pvdS* knock-out strain, further supporting the hypothesis that PvdS is not required for P1 activity but may modulate it. As expected, in both complemented strains, there is a decrease in activity in stationary phase, indicating that the P2 promoter is not active when the strains are grown under high-iron conditions. Northern blot analysis of the parental strains PA103 and PA103 *pvdS::Ω* also demonstrates that PvdS may modulate transcription from the P1 promoter and it is essential for transcription from the P2 promoter (data not shown). This is a clear difference from the previously described strain PAO1, where the P1 promoter is not active under these conditions.

It is interesting that the early P1 activity in a strain with an active *pvdS* is unaffected by the iron concentration of the medium. In the *pvdS* knock-out strain, there is a slight iron effect, with the culture growing in high iron having about 20 % less activity than the culture growing in low iron. Overall, our data suggest that PvdS has a general modulatory effect on the P1 promoter. Interestingly, we could not detect a putative binding site in the area of the *regAB* P1 promoter, suggesting that PvdS may act indirectly on this promoter, probably through an additional regulatory element.

DISCUSSION

PvdS is an alternative sigma factor for RNA polymerase. This classification was originally based on protein homologies to known alternative sigma factors and the presence of a putative DNA-binding motif at the C-terminal end of the protein (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). *P. aeruginosa* PvdS binds to *E. coli* core RNA polymerase and to *pvdA* promoter DNA (Leoni *et al.*, 2000; Wilson & Lamont, 2000). The PvdS-core RNA polymerase complex also directs transcription of the pyoverdine biosynthesis genes *pvdE* and *pvdF* (Wilson *et al.*, 2001). Furthermore, it has been demonstrated that PvdS expression is regulated by iron through the global iron repressor Fur (Ochsner *et al.*, 1996). PvdS is transcribed only in low-iron conditions; in high-iron conditions transcription is repressed by Fur. In this study, we examined the expression of *pvdS* by *P. aeruginosa* during the chronic lung infections associated

with CF, and the role of this alternative sigma factor in the regulation of *toxA* in both the hypertoxigenic strain PA103 and the prototypical strain PAO1.

Recently, the oxygen- and iron-dependent sigma factor PvdS has been shown to be important in an infective endocarditis model (Xiong *et al.*, 2000). What is less clear is the role that PvdS may play in human infections. In some infections, such as the chronic lung infections associated with the genetic disease CF, expression of PvdS could be used as a marker for specific environmental conditions. In these infections, it appears that iron is limiting, as siderophores have been detected in the sputa of these patients (Haas *et al.*, 1991). However, the oxygen concentration that bacteria are exposed to in the lungs of patients with CF has not been determined. It is likely that over the course of these chronic infections the bacterial populations are exposed to varying oxygen levels. Also, it is likely that within the environment of the lung, different bacterial populations would be exposed to varying levels of oxygenation. With these possibilities in mind, we have extracted RNA from the total bacterial populations found in sputa from CF patients and analysed the sputa for transcription of *pvdS*. These samples were solely from paediatric patients, where it might be expected to see considerable variation in their lung disease and, hence, variation in the oxygen levels. We demonstrated that in samples with equivalent numbers of bacteria and containing only *P. aeruginosa*, there were varying levels of *pvdS* transcript accumulation (from background levels to 20 times the control levels of transcript accumulation; Fig. 1). This variability suggests that the bacteria are exposed to different concentrations of iron and/or oxygen within these samples. The results also suggest that the levels of *pvdS* transcription in the lungs of CF patients is being regulated. We then analysed the transcript accumulation patterns of *pvdS*, *toxA* and *algD*. Even though we analysed a relatively small sample size ($n = 16$), we were able to detect a significant correlation between *pvdS* transcript accumulation and *toxA* transcript accumulation. In contrast, we were not able to show a significant correlation between *pvdS* transcript accumulation and *algD* transcript accumulation. Taken together, our data suggest that *pvdS* is transcribed in the lungs of patients with CF and that PvdS may at least be partially responsible for the regulation of *toxA* within the lungs of CF patients. Since *pvdS* is repressed by anoxic conditions, our data also suggest that the bacterial population is exposed to a reasonable level of oxygen in some samples.

We also examined the functional role of PvdS in strain PA103. This strain was chosen because its ETA regulatory pattern seems to match that of *P. aeruginosa* in the lungs of individuals with CF (Raivio *et al.*, 1994). Further, PA103 differs from the prototypical strain PAO1 in the regulation of ETA production. The *pvdS* knockout strain PA103*pvdS*::Gm produced only 20% of the ETA produced by the complemented strain (Fig. 2a). This result was different from the findings in strain PAO1, where ETA production was totally abrogated in

strain PAO1Δ*pvdS* (Fig. 2b) (Ochsner *et al.*, 1996). The tighter control by PvdS in strain PAO1 was also observed in experiments where *pvdS* was introduced into PAO1 and PA103 on a multi-copy vector. These experiments revealed a greater percentage increase in ETA activity in PAO1 than that in PA103 (Fig. 3). This difference is probably due to some residual RegA production from the *regAB* promoters in strain PA103.

Ochsner *et al.* (1996) showed that PvdS was essential for the iron regulation of the *regA* P2 promoter in strain PAO1. When the *pvdS* gene of strain PA103 was disrupted, activity from the P2 promoter was not detected, irrespective of the iron conditions (Fig. 4). P2 promoter activity was only observed from this knock-out strain when *pvdS* was expressed *in trans* under iron-limiting conditions. Likewise, when *pvdS* was introduced on a multi-copy vector in to the parental PA103 strain, *regA* P2 activity was enhanced (Fig. 5). These studies suggest that in strain PA103, as in strain PAO1, PvdS is essential for *regA* P2 promoter activity. Interestingly, these functional studies are supported by the discovery of a putative PvdS binding site in the P2 promoter region of the *regAB* operon (Table 2).

The regulation of the *regA* P1 promoter was also analysed in strain PA103 to determine if it might account for the differences in ETA activity between strains PA103 and PAO1. In strain PAO1, the P1 promoter is not active under aerobic conditions (Ochsner *et al.*, 1996). Here we show that in strain PA103 growing under aerobic conditions, P1 promoter activity is modulated by the presence of *pvdS* but that PvdS is not required for activity (Fig. 6). Our results also suggest that both the *regAB* operon and PvdS are required for optimal aerobic activity of the *regAB* P1 promoter.

Recent evidence has suggested that a regulatory effect of PvdS on *regAB* may be mediated through PtxR (Hamood *et al.*, 1996). Vasil *et al.* (1998) also demonstrated that PvdS regulates *ptxR*, and the regulatory cascade postulated by this group suggests that Fur regulates the expression of *pvdS* in response to iron conditions. PvdS then activates the expression of *ptxR* and PtxR regulates ETA production either by activation of the P1 promoter of *regAB*, or indirectly by enhanced siderophore production. This model suggests that the regulation of ETA production involves a complex regulatory cascade. We support this hypothesis and believe that the proposed regulation by PtxR acts through the P1 promoter and not the iron-regulated P2, at least in strain PA103 growing under aerobic conditions. The evidence for this hypothesis is fourfold. (1) A putative PtxR binding site is located upstream of the transcriptional start of the P1 promoter (Hamood *et al.*, 1996). (2) A *regA*–*lacZ* fusion which contains only the P2 promoter remains tightly iron regulated (Storey *et al.*, 1990), suggesting that PvdS-mediated iron regulation is mediated through the P2 promoter. (3) A deletion of the *ptxR* gene results in decreased expression of siderophore biosynthesis genes and probably culminates in a reduction in intracellular iron levels. This would increase expression of PvdS, as

Fur would become active under these conditions (Stintzi *et al.*, 1999). (4) Overexpression of PtxR increases ETA synthesis but this production declines over time, reminiscent of P1 promoter activity, suggesting that overexpression of PtxR enhances P1 promoter activity (Colmer & Hamood, 1999). An alternative possibility is that *P. aeruginosa* Fur may directly act on the *regA* P1 promoter.

In summary, our studies demonstrate that in the chronic lung infections associated with the disease CF, the colonizing *P. aeruginosa* populations transcribe *pvdS*. Furthermore, *pvdS* transcript accumulation correlates with *toxA* transcript accumulation, further suggesting that *pvdS* is involved in the regulatory cascade controlling transcription of the virulence factor ETA. We also show that in the hypertoxigenic *P. aeruginosa* strain PA103, PvdS, a member of the ECF family of alternative sigma factors, is required for approximately 80% of the ETA production. Moreover, PvdS is essential for the activity of the P2 promoter of the *regAB* operon. The regulation of the *regAB* P2 promoter by PvdS may be direct or indirect. Finally we have demonstrated that in strain PA103 grown in an aerobic environment, PvdS along with the *regAB* operon is required for optimal activity from the *regAB* P1 promoter. Growth of strain PAO1 in similar conditions does not lead to detectable activity from the *regA* P1 promoter. This difference could account for the residual ETA activity (20% of wild-type) found in a knock-out strain of PA103. Taken together our results indicate that different isolates of *P. aeruginosa* vary in their response to iron concentration; however, on a population basis, PvdS is an important iron-regulated sigma factor that is expressed in the lungs of patients with CF.

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