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

Tracey A. Hunt,† Wen-Tao Peng, Isabelle Loubens and Douglas G. Storey

Author for correspondence: Douglas G. Storey. Tel: +1 403 220 5274. Fax: +1 403 298 9311. e-mail: storey@ucalgary.ca

**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

---

† Present address: Dept of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada.

**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 PA103ΔregAB::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 toxA 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), Vir (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 pyoveridine synthesis (Cunliffe et al., 1995; Miyazaki et al., 1995). The PvdS (pyoveridine sigma factor) protein shows homology to Escherichia coli FedI, 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 Vasili & 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 FeCl\(_3\) (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 PstI-SmaI fragment from a genomic clone of regA was ligated into pUC12. A 3.2 kbp BamHI fragment from pZ1918 (Schweizer, 1993b) containing a promoterless lacZ gene was then ligated into the first BamHI site of the regA ORF. A sacRB gene from pMH1801 (Hynes et al., 1989) was digested with PstI and the fragment containing the sacRB gene was ligated into the PstI 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
can be monitored in this strain by performing β-galactosidase assays.

Plasmid pTHpvds 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: ACC AAT TCT ACC TGG ACG GT C CGG TGT). 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 pTHpvds 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 multicopy 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, knockout strains were constructed. In knock-outs of PA103 and IL-1, the pvds ORF was interrupted with an Ω cassette from pHP45 or a Gm cassette from pUCGM respectively, at the unique Stul site within the pvds ORF. The interrupted pvds gene was constructed on a suicide plasmid that contained a sacB cassette (pQJ200SK) to facilitate the double crossover onto the chromosome. The resulting plasmids, pJQpvds::Ω and pJQpvds::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 [γ-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 pTHpvds.
containing the \textit{pvdS} gene. Probes for \textit{toxA} and \textit{algD} were prepared as described by Storey \textit{et al.} (1997). Statistical analysis was as described by Storey \textit{et al.} (1998).

\textbf{RESULTS}

\textbf{Transcription of pvdS in the lungs of patients with CF}

We have previously shown that both \textit{regAB} transcripts are produced in the lungs of patients with CF (Raivio \textit{et al.}, 1994). We have also demonstrated that \textit{toxA} is transcribed in the CF lung (Storey \textit{et al.}, 1992). It was, therefore, of interest to determine if \textit{pvdS} is transcribed in the lungs of CF patients, and whether or not there was a correlation between the transcript accumulation of \textit{pvdS}, \textit{regAB} and \textit{toxA}. Fig. 1 shows that we were able to detect \textit{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 \textit{pvdS} is regulated in the lungs of patients with CF. We hypothesize that \textit{pvdS} may be regulating the \textit{regAB} operon and subsequently \textit{toxA}. We would have liked to correlate \textit{pvdS} expression to the \textit{regAB} operon since \textit{pvdS} directly regulates this operon. However, the half-life of the two \textit{regA} transcripts is very short and, therefore, they are difficult to detect in sputa (Raivio \textit{et al.}, 1994). Thus, we have compared the transcript accumulation of \textit{toxA} to \textit{pvdS} since it appears that \textit{pvdS} indirectly regulates \textit{toxA}. A Spearman rank correlation was performed on the 16 samples shown in Fig. 1. This analysis showed a statistically significant correlation between \textit{pvdS} and \textit{toxA} transcript accumulation \((r = 0.647, P < 0.005)\). Notably, we did not detect a statistically significant correlation between either \textit{algD} and \textit{pvdS} \((r = 0.430 P < 0.05)\) or \textit{algD} and \textit{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 \textit{et al.} (1996) showed that the pattern of \textit{regAB} transcript accumulation in the lungs of patients with CF was similar to the pattern found in the hypoxigenic 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.

\textbf{P. aeruginosa pvdS knock-out strains produce less ETA than the parental strain PA103}

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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{PvdS is transcribed in the lungs of patients with CF: population transcript accumulation of \textit{P. aeruginosa pvdS}, \textit{toxA} and \textit{algD} in RNA extracted from CF sputa. All samples contained equivalent numbers of \textit{P. aeruginosa} \((10^8 \text{ ml}^{-1})\) and \textit{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 \(\mu\text{g}\) 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.}
\end{figure}
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 µg 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 pvdS::Gm (●, V), PA103 pvdS::Gm with the vector control pUC181.1 (▲, △), and PA103 pvdS::Gm with the plasmid pTHpvdS (E, D). (b) PAO1 ∆pvdS (U, V), PAO1 ∆pvdS with the vector control pUC181.1 (▲, △), and PAO1 ∆pvdS::Gm with the plasmid pTHpvdS (E, D).

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
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 knockout. 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

<table>
<thead>
<tr>
<th>Iron-regulated promoter</th>
<th>Binding sequence†</th>
<th>Base-pair match</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa pvdA promoter</td>
<td>3CCTAAATTC&lt;sup&gt;−30&lt;/sup&gt;</td>
<td>8/9</td>
</tr>
<tr>
<td>P. aeruginosa pvdD promoter</td>
<td>3GCTAAATCC&lt;sup&gt;−29&lt;/sup&gt;</td>
<td>8/9</td>
</tr>
<tr>
<td>P. aeruginosa pvdE promoter</td>
<td>3GCTAAATAC&lt;sup&gt;−13&lt;/sup&gt;</td>
<td>9/9</td>
</tr>
<tr>
<td>P. aeruginosa pvdF promoter</td>
<td>3CTAAATGG&lt;sup&gt;−30&lt;/sup&gt;</td>
<td>9/9</td>
</tr>
<tr>
<td>P. aeruginosa toxA promoter</td>
<td>3ACTAAATCC&lt;sup&gt;−54&lt;/sup&gt;</td>
<td>7/9</td>
</tr>
<tr>
<td>P. putida siderophore promoter</td>
<td>3CCCTAAATCC&lt;sup&gt;−87&lt;/sup&gt;</td>
<td>8/9</td>
</tr>
<tr>
<td>P. sp. strain M114 iron-regulated promoter</td>
<td>3GCTAAAT</td>
<td>6/9</td>
</tr>
<tr>
<td>P. aeruginosa regAB promoter P2</td>
<td>3CTAGATAC&lt;sup&gt;−8&lt;/sup&gt;</td>
<td>8/9</td>
</tr>
<tr>
<td>Consensus PvdS binding sequence</td>
<td>(G/C) (G/C) TAAAT (T/A) (C/G)</td>
<td>9/9</td>
</tr>
</tbody>
</table>

* 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 (†), which is in relation to the translational start site. Nucleotides that differ from the consensus are shown in bold.

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

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 P. aeruginosa, therefore an indirect regulator of ETA production in P. aeruginosa.
A critical difference in 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 on pDF191.8-202 into strains IL-1 and IL-1 pvdS::Ω complemented with the regAB operon on pDF191.8-202.

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 on 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.

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...
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. aeruginosaa, 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 transcription 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 PA103ΔpvdS::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 PAO1ΔpvdS (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 into 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 hypoxotogenic 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 regA 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 Fur may 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.

ACKNOWLEDGEMENTS
We thank Drs Vasil, Schweizer and Hynes for the gifts of strains and plasmids, and Leila Donegan for plasmid construction. This research was supported by MRC grant MT15680 to D.G.S.

REFERENCES
Pseudomonas aeruginosa: PvdD has similarity to peptide synthetase.

on iron-regulated promoters.


