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Cloning and Characterization of *pvdS*, a Gene Required for Pyoverdine Synthesis in *Pseudomonas aeruginosa*: PvdS Is Probably an Alternative Sigma Factor

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Cells of *Pseudomonas aeruginosa* secrete a fluorescent yellow-green siderophore, pyoverdine, when grown under iron-deficient conditions. We describe here the cloning and characterization of a gene, *pvdS*, which is required for this process. The *pvdS* gene is required for expression from promoters of at least two pyoverdine synthesis genes and can cause expression from these promoters in *Escherichia coli*, where they are otherwise inactive. Sequencing of *pvdS* revealed that it is a member of a subfamily of RNA polymerase sigma factors which direct the synthesis of extracellular products by bacteria. The *pvdS* gene is expressed only in iron-starved bacteria, and in *E. coli* cells at least, expression is regulated by the Fur repressor protein. We propose that in iron-rich cells of *P. aeruginosa*, Fur binds to the *pvdS* promoter and prevents expression of the gene; under conditions of iron starvation, repression is relieved and PvdS is made, reprogramming the cells for pyoverdine synthesis.

Fluorescent pseudomonads are characterized by the production of yellow-green fluorescent pigments. These act as siderophores and are variously termed pyoverdines or pseudobactins. The fluorescence and color are due to the presence of a dihydroxyquinoline group; this is attached to a peptide of 6 to 12 amino acids, and the exact nature of the peptide is species or strain specific (5). A pyoverdine-deficient mutant of the mammalian pathogen *Pseudomonas aeruginosa* showed greatly reduced virulence in a mouse model of infection (14), so that pyoverdine is a virulence factor for this organism. Synthesis of pyoverdines and pseudobactins is repressed by the presence of excess iron in the growth medium and this is due, at least in part, to reduced transcription of the corresponding biosynthetic genes (18, 31). However, the molecular mechanisms which control expression of these genes are not well understood. Regulation of expression of siderophore synthesis genes is best characterized in *Escherichia coli*, in which expression of the relevant genes is controlled by the Fur repressor protein. In the presence of iron (Fe^{2+}), Fur binds to the promoters of siderophore-related genes and represses transcription. A Fur-like protein is also present in *P. aeruginosa*, and Fur^- mutants of this species are derepressed for pyoverdine synthesis, implicating Fur in the regulation of expression of pyoverdine biosynthetic genes (29). However, there is evidence that transcriptional activators are also required for expression of siderophore genes in pseudomonads (12, 19, 27, 31, 33, 38, 39). A protein from *P. aeruginosa* which is likely to be an activator of gene expression is required for synthesis of pyochelin, a nonfluorescent siderophore (12). The *pfrA* gene is required for expression of pseudobactin synthesis genes in *P. putida* WCS358 (39), and a second gene required for expression of these genes has also been identified (38). A gene required for expression of siderophore genes in *P. fluorescens* M114 has been described very recently and is likely to encode an alternative sigma factor for RNA polymerase (33).

We describe here the cloning and characterization of a gene, *pvdS*, which is required for expression of pyoverdine synthesis genes in *P. aeruginosa*. The sequence of the gene and studies of its regulation provide a model for the mechanism whereby iron controls expression of pyoverdine synthesis genes in this species.

MATERIALS AND METHODS

Microbiological methods. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *E. coli* and *P. aeruginosa* were grown in Luria (L) broth (32) and brain heart infusion broth (GIBCO-BRL), respectively, solidified by addition of agar and supplemented as necessary with tetracycline and chloramphenicol as described previously (30). The concentrations of kanamycin used were routinely 50 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*; this was increased to 600 μg/ml for construction of a *pvdS* mutant, when the concentrations of tetracycline and chloramphenicol were 80 and 400 μg/ml, respectively. Media were also supplemented with $FeCl_3$ (60 μg/ml), ethylenediamine(*o*-hydroxy)phenylacetic acid (EDDA) (400 μg/ml), and bromochloroindolylgalactoside (120 μg/ml) as required.

Measurement of pyoverdine and β-galactosidase. King's B medium (15) was used to examine pyoverdine production; this medium promotes pyoverdine synthesis by *P. aeruginosa* while permitting the growth of pyoverdine-deficient mutants. The absorbances of culture supernatants were measured between 190 and 500 nm. Pyoverdine has a characteristic absorbance spectrum in this range, with a peak at 403 nm (13). β-Galactosidase was assayed by the method of Miller (25).

Nucleic acid methodology. Enzymes were purchased from Boehringer Mannheim, except for the Klenow fragment of DNA polymerase I (Amersham) and T4 polynucleotide kinase (New England Biolabs). Chromosomal DNA to be used for cloning was prepared and manipulated as described previously (22). Preparation of plasmid DNA, treatment of DNA with enzymes, subcloning of DNA, and transformation of plasmid constructs into *E. coli* were carried out by using standard methods (32) and using enzymes under the conditions recommended by the manufacturers. DNA molecules were end polished by using T4 DNA polymerase and the Klenow fragment. To subclone the *pvdS* promoter fragment into pMP190, PCR was used to amplify a 463-bp DNA fragment extending from upstream of the *pvdS* gene to nucleotide 381 (see Fig. 2). The DNA was end polished and then phosphorylated by using T4 polynucleotide kinase. This DNA was ligated with pMP190 DNA which had been treated with *Sal*I, end polished, and treated with calf intestinal phosphatase. The ligated DNA was then transformed into *E. coli* MC1061.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
MC1061	Δ*lacX74* Hsr$^-$ Hsm$^+$ *str*	6
S17-1	*hsdR* HsdM$^+$ *recA* chromosomally integrated RP4-2 Tc^r::Mu Km^r::Tn*7*	35
GC4468	Δ*lacU169*	8
QC1732	Δ*lacU169* Δ*fur*::*kan*	8
P. aeruginosa PAO		
OT11	Pvd$^+$	30
OT11 *pvdS*	*pvdS*::*kan*	This work
OT684	*res-4* Pvd$^+$	20
OT2102	OT684::Tn*5*-OT182 Pvd$^-$	This work
Plasmids		
pBR322	ColE1 cloning vector; Ap^r Tc^r	3
pNRE1	ColE1 plasmid containing *kan* gene cartridge	1
pSUP202	Mobilizable ColE1 plasmid; Ap^r Tc^r Cm^r	34
pOT182	Tn*5*-based DNA cloning vector	21
pOT306	pOT182-derived clone of pyoverdine synthesis genes	This work
pOT306 LacZ$^-$	LacZ$^-$ mutant of pOT306	This work
pBRXB	*Xho*I-*Bam*HI fragment containing *pvdS* gene from pOT306 cloned into pBR322	This work
pBRXBKm	*kan* gene cartridge from pNRE1 cloned into *pvdS* gene in pBRXB	This work
pMP190	Broad-host-range promoter probe vector	36
pMP190::P*pvdD*	Promoter of *pvdD* pyoverdine synthesis gene (previously termed fragment C1) cloned into pMP190	31
pMP190::P*pvdE*	Promoter of *pvdE* pyoverdine synthesis gene (previously termed fragment J) cloned into pMP190	31
pMP190::P*pvdS*	Promoter of *pvdS* gene cloned into pMP190	This work

For DNA sequencing, plasmid DNA was prepared with a Magic maxiprep (Promega). The DNA was then sequenced by using chemically synthesized oligonucleotides in conjunction with an automated sequencer, and the sequences were analyzed by standard methods with version 8.0-UNIX of the Genetics Computer Group package (11) in conjunction with other programs, as described previously (23). Southern analysis was done by standard methods (32) with chromosomal DNA prepared by the method of Chen and Kuo (7). Northern (RNA) analysis was done as described previously (31), with two adjacent *Sau*3A fragments internal to the *pvdS* gene (nucleotides 224 to 375) as the probe.

Creating a mutation in *pvdS*. Plasmid pBRXB DNA was treated with *Dra*III, which has a unique site within *pvdS*, and end polished. It was then ligated with the kanamycin resistance cassette (*kan*), which had been purified from agarose following treatment of pNRE1 (1) with *Hin*dII, and the DNA was transformed into *E. coli* MC1061 with selection for Ap^r Km^r bacteria. Plasmid DNA was prepared from one transformant, and restriction analysis confirmed that the *kan* gene had been inserted correctly into *pvdS*; the resulting plasmid was named pBRXBKm. To mutate *pvdS* in *P. aeruginosa*, pBRXBKm was treated with *Nde*I and *Bam*HI to release a fragment which contains the *kan* gene inserted into *pvdS*. This fragment was end polished and subcloned into pSUP202 (34), which had been treated with *Eco*RI and end polished. The DNA was transformed into *E. coli* S17-1 (35) and then transferred by conjugation into *P. aeruginosa* OT11 as described previously (22), with selection for Tc^r Km^r bacteria, which arose following recombination of the plasmid into the chromosome. A second recombination event was required for excision of the plasmid and loss of the wild-type *pvdS* gene, and bacteria in which this had occurred were identified as being Tc^s Km^r. Southern analysis (data not shown) confirmed that the expected recombination events had taken place.

Nucleotide sequence accession number. The DNA sequence presented here has been assigned accession number U12891 in the GenBank and EMBL databases.

RESULTS

Isolation of plasmid clone pOT306. We have previously described a transposon, Tn*5*-OT182, which facilitates the cloning of DNA adjacent to a transposon insertion site (22). This transposon was used to obtain mutants of *P. aeruginosa* which were deficient in pyoverdine synthesis. Tn*5*-OT182 was introduced into *P. aeruginosa* PAO strain OT684 (21) by conjugation from S17-1(pOT182), and tetracycline-resistant transconjugants were selected; 4,650 transconjugants were obtained from several conjugations. These were then screened for the ability to grow on King's B agar containing the iron-chelating agent EDDA, which prevents growth of Pvd$^-$ bacteria. Thirty-two pyoverdine-deficient mutants were obtained. One of these, mutant OT2102, and DNA cloned from it are the subjects of this report.

In contrast to parental strain OT684, mutant OT2102 made no detectable amounts of pyoverdine during growth in King's B broth (data not shown), and this is consistent with its inability to grow in the presence of EDDA. Tn*5*-OT182 contains a promoterless *lacZ* gene (Fig. 1) which is expressed only if the transposon is inserted into the chromosome downstream from an active promoter. There was low but significant expression of *lacZ* when the bacteria were grown in liquid culture and this was not iron regulated. In two different experiments, the bacteria gave an average of 13.5 U of β-galactosidase in King's B medium and 15.5 U in the same medium supplemented with $FeCl_3$.

Chromosomal DNA was prepared from OT2102, treated with restriction enzymes *Hin*dIII and *Xho*I, and then subjected to Southern analysis by using as the probe a 2.5-kb *Cla*I fragment from pOT182 (22) which contains 0.8 kb of the left part of Tn*5*-OT182. The probe hybridized to a *Hin*dIII band of about 20 kb and an *Xho*I band of about 17 kb (data not shown). DNA upstream of the insertion site in mutant OT2102 was then cloned as shown in Fig. 1, and the resulting plasmid clone was named pOT306. The size of pOT306 was about 18 kb, consistent with the Southern analysis of genomic DNA from mutant OT2102. A restriction map of pOT306 was constructed (Fig. 1), and as expected from the Southern analysis of chromosomal DNA, the cloned DNA included an *Xho*I site.

pOT306 contains an activator of pyoverdine gene promoters. *E. coli* MC1061 containing pOT306 was grown on nutrient agar containing bromochloroindolylgalactoside to test whether the *lacZ* gene in pOT306 was being expressed. The colonies turned blue, indicating that this was the case. This result was confirmed by carrying out liquid assays (Table 2) to measure the amount of β-galactosidase being produced. This result was

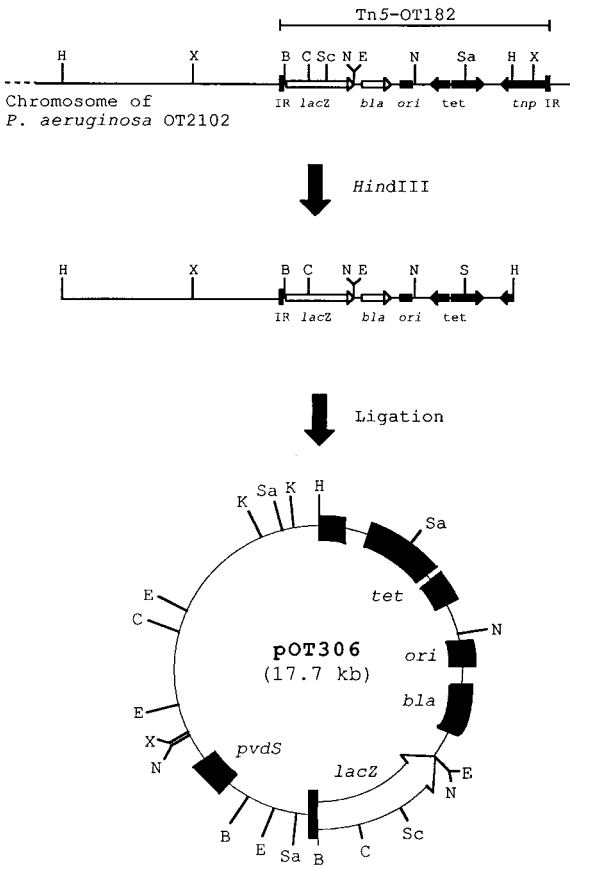

FIG. 1. Isolation of clone pOT306. *P. aeruginosa* mutant OT2102 contains transposon Tn*5*-OT182 (22) inserted into a gene required for synthesis of pyoverdine. Chromosomal DNA from this mutant was treated with *Hin*dIII, ligated under conditions favoring self-ligation (circularization) of the DNA molecules, and transformed into *E. coli* MC1061. Five clones were obtained from 100 ng of chromosomal DNA. Plasmid DNA was prepared from one of these and gave rise to a single *Hin*dIII fragment of approximately 18 kb; this plasmid was named pOT306. Restriction enzyme sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; Sa, *Sal*I; Sc, *Sac*I; X, *Xho*I. Genetic elements: *bla*, β-lactamase gene (conferring resistance to ampicillin and carbenicillin); IR, inverted repeat; *lacZ*, β-galactosidase gene; *ori*, ColE1 origin of DNA replication; *pvdS*, gene for pyoverdine synthesis; *tet*, tetracycline resistance gene. Genes shown in dark are expressed from their own promoters; those shown in white require an external promoter for expression.

unexpected, as promoters of other siderophore synthesis genes from pseudomonads are inactive in *E. coli* (19, 26), presumably because of the absence of relevant transcription factors. One possibility was that the promoter which caused expression of *lacZ* was recognized by *E. coli* RNA polymerase in the absence of transcription factors from *P. aeruginosa* so that *lacZ* expression occurred. A second possibility was that pOT306 contained a gene encoding a transcription factor which allowed pyoverdine gene promoters to be active in *E. coli*.

The second possibility was examined by testing pOT306 for the ability to cause expression from other pyoverdine gene promoters, fused to *lacZ*, in *E. coli*. We have characterized two such promoters, previously named C1 and J (31); these are from pyoverdine synthetase genes *pvdD* (23) and *pvdE* (20), respectively. The *lacZ* gene in pOT306 was first inactivated by treating pOT306 DNA with *Sac*I, which has a unique restriction site within *lacZ*, removing the protruding 3′ overhangs by treatment with T4 DNA polymerase, and then religating the DNA to create a frameshift mutation within the *lacZ* gene. The resulting plasmid, pOT306LacZ$^-$, had negligible *lacZ* activity (Table 2). The *pvdD* and *pvdE* promoter constructs, fused to *lacZ* in promoter probe vector pMP190 (36), were then transformed into *E. coli* containing pOT306LacZ$^-$, and the amounts of β-galactosidase made by the resulting bacteria were assayed. The results (Table 2) show that clone pOT306 LacZ$^-$ was able to cause expression from both of the pyoverdine gene promoters in iron-deficient medium, indicating that the plasmid encodes a protein capable of activating expression from these promoters in *E. coli*. Addition of $FeCl_3$ to the growth medium resulted in reduced expression of *lacZ*.

TABLE 2. Expression of *lacZ* from plasmid constructs in *E. coli*[a]

Plasmid(s)	Avg β-galactosidase activity[b] (SD)	
	L broth + $FeCl_3$	L broth + EDDA
pOT306	69.5 (6.2)	141 (25.2)
pOT306LacZ$^-$	<0.1	<0.1
pOT306LacZ$^-$ + pMP190	3.47 (0.84)	3.53 (0.97)
pMP190::P*pvdD*	1.23 (0.81)	2.43 (0.51)
pMP190::P*pvdE*	1.25 (0.54)	2.05 (0.48)
pOT306LacZ$^-$ + pMP190::P*pvdD*	44.9 (4.9)	136 (16.3)
pOT306LacZ$^-$ + pMP190::P*pvdE*	47.0 (4.01)	119 (19)
pBRXB + pMP190::P*pvdD*	21.5 (2.4)	61.7 (10.1)
pBRXB + pMP190::P*pvdE*	8.9 (1.65)	36.6 (3.64)
pBRXBKm + pMP190::P*pvdD*	1.17 (0.61)	3.87 (1.05)
pBRXBKm + pMP190::P*pvdD*	2.84 (0.71)	4.1 (0.99)

[a] Plasmid-containing strains of *E. coli* MC1061 were grown in L broth supplemented with $FeCl_3$ or the iron-chelating compound EDDA, as shown.
[b] β-Galactosidase activity is given in Miller units (25). The values shown are averages of at least three experiments.

The gene causing activation was then localized within the cloned DNA in pOT306. Preliminary experiments involving deletion and subcloning of restriction fragments from pOT306 LacZ$^-$ showed that the *trans*-activating activity was located within a 2.3-kb *Eco*RI-*Bam*HI fragment (data not shown). An *Xho*I-*Bam*HI fragment which is part of this fragment was subcloned into pBR322 to give plasmid pBRXB, and this construct was tested for transactivation of the *pvdD* and *pvdE* promoters. It was able to cause expression from both promoters (Table 2), indicating that the *Xho*I-*Bam*HI fragment contains an activating gene, although activation was less than that observed with pOT306LacZ$^-$.

Sequence analysis of the activating gene. The sequence of the 1.8-kb *Xho*I-*Bam*HI fragment was determined and found to contain a single extended open reading frame, which is shown in Fig. 2. The locus has a GC content of 60.6%, which is at the lower end of the range of genes from *P. aeruginosa* (40), but the open reading frame showed a pattern of codon usage similar to that of other *P. aeruginosa* genes (40; data not shown), indicating that it was likely to encode a protein. The translated sequence of this putative gene was compared to the translated sequences of genes in the GenBank (version 85.0) database by using FASTA (28). The most similar translation products were PupI (28.8% identity over 156 amino acids), which is required for expression of a ferric-siderophore uptake gene in *P. putida* (16), and FecI (31.5% over 130 amino acids), which is required for expression of genes required for iron citrate uptake in *E. coli* (37).

FecI is a member of a family of alternative sigma factors, the extracytoplasmic function (ECF) family, present in a range of bacteria (17). This prompted us to compare the translated sequences of the gene described here and that of PupI with those of the other members of the ECF family. There was significant similarity between the sequences (Fig. 3). The sim-

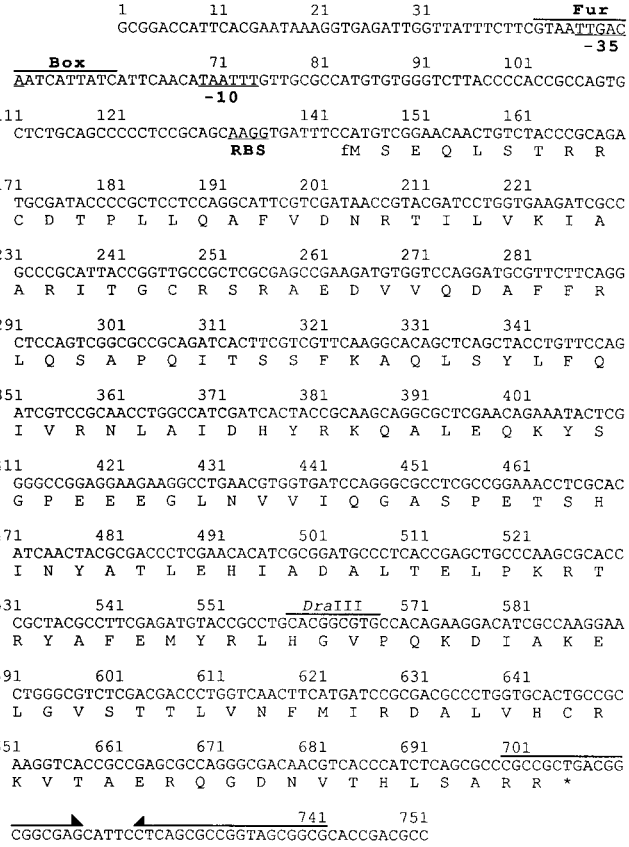

FIG. 2. Sequence of the *pvdS* gene. The open reading frame of the *pvdS* gene, along with the corresponding amino acid sequence of the protein, is shown. Other features which are marked are the likely binding site for the Fur repressor protein (Fur box), potential −35 and −10 recognition hexamers for RNA polymerase, a potential ribosome-binding site (RBS), a recognition site for restriction enzyme *Dra*III (used in construction of a *pvdS* mutant; see text), and a potential transcriptional termination region (inverted arrows).

ilarity of the product of the gene identified here to members of the ECF family makes it likely that it is also a sigma factor, and the gene was therefore named *pvdS*, for pyoverdine sigma factor. PupI also falls into this family and so is also likely to be a sigma factor; its sequence was unavailable when the ECF family was first identified.

The *pvdS* gene is 564 bp long and is preceded by a likely promoter for the vegetative (σ^{70}) RNA polymerase of *P. aeruginosa* (Fig. 2). The FoldRNA program (11) was used to identify stem-loop structures near the end of the gene which may act as transcriptional terminators. A predicted stem-loop structure with a stability of −24.2 kJ/mol was identified. This stem-loop includes the predicted termination codon of *pvdS*. Although it is not followed by several T residues, a feature common to rho-dependent terminators, the high stability of the stem-loop indicates that it may well act as the transcription terminator for *pvdS*.

Role of *pvdS* in pyoverdine synthesis. A DNA fragment containing the *pvdS* gene was able to cause expression from pyoverdine synthesis promoters in *E. coli* (Table 2), and sequence analysis indicated that PvdS is likely to be a sigma factor (Fig. 3). This led to the hypothesis that *pvdS* encodes an alternative sigma factor which directs RNA polymerase to the promoters of pyoverdine biosynthesis genes. This was tested by inserting a kanamycin resistance gene into the *pvdS* gene in plasmid pBRXB and then examining the effects of the mutation on expression from pyoverdine promoters in *E. coli* (Table 2). Plasmid pBRXBKm was unable to cause expression from either of the promoters, in contrast to pBRXB, which contains the intact *pvdS* gene. This showed that *pvdS* is indeed required for expression from pyoverdine promoters in *E. coli*.

To study the involvement of *pvdS* in pyoverdine synthesis in *P. aeruginosa*, the wild-type chromosomal gene was replaced with the mutated *pvdS* gene from pBRXBKm as outlined in Materials and Methods. The resulting mutant strain (OT11 *pvdS*) failed to make detectable pyoverdine when grown on King's B agar or in liquid medium. It was also unable to grow in the presence of EDDA, consistent with a failure to synthesize pyoverdine. The ability to make pyoverdine and grow in the presence of EDDA was restored when a *Sal*I fragment that includes *pvdS* was subcloned from pOT306 into pMP190 and then introduced into the OT11 *pvdS* mutant. These results show that *pvdS* is required for pyoverdine synthesis.

The effect of the mutation on expression from pyoverdine promoters was tested by introducing the *lacZ* fusion constructs into the mutant bacteria. As expected, expression from the promoters was very greatly reduced in the *pvdS* mutant relative to the wild-type strain (Table 3). This indicates that PvdS is required for iron-regulated expression from promoters of at least two genes encoding pyoverdine biosynthetic enzymes and explains the failure of the *pvdS* mutant to synthesize pyoverdine. The *pvdD* promoter retained low-level activity even in the absence of *pvdS*, although this was not iron regulated. This is consistent with the low-level transcription of *pvdD* observed in cells grown in iron-rich medium (31).

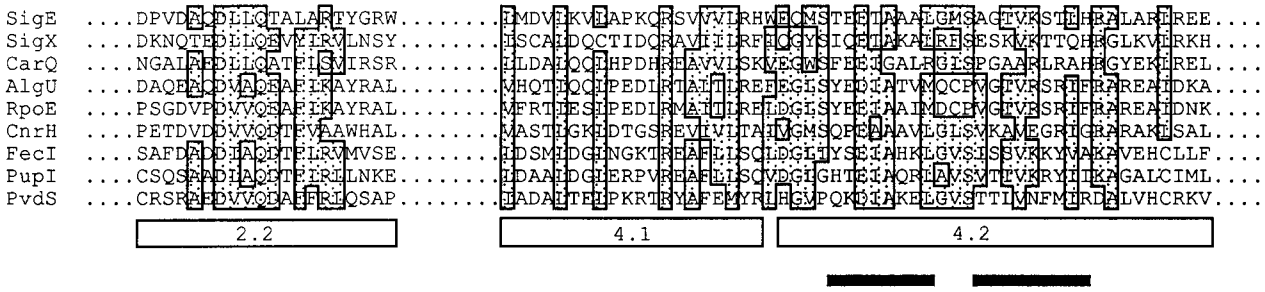

FIG. 3. Partial alignment of PvdS with members of the ECF family of sigma factors. An alignment of PvdS with regions 2.2, 4.1, and 4.2 of the ECF sigma family, the parts of the proteins which are most similar throughout the family (17), is shown. Residues which are identical or match in five or more of the nine sequences are marked. Residues which were considered to match were the same as described previously (17), i.e., D and E; N and Q; R and K; S and T; F, Y, and W; and I, L, V, and M. An alignment of the complete sequences is available on request. A helix-turn-helix DNA-binding motif predicted to be present in the C-terminal regions of the ECF proteins (17) is indicated by the black bars.

TABLE 3. Effect of the *P. aeruginosa pvdS* mutation on expression from the *pvdD* and *pvdE* promoters[a]

Strain (plasmid)	Avg β-galactosidase activity[b] (SD)	
	King's B broth + $FeCl_3$	King's B broth
OT11(pMP190)	21.8 (2.64)	20.3 (2.64)
OT11(pMP190::P*pvdD*)	262 (27.9)	1,272 (113)[c]
OT11(pMP190::P*pvdE*)	236 (37.0)	4,056 (346)[c]
OT11 *pvdS*(pMP190)	18.2 (2.55)	20.4 (3.16)
OT11 *pvdS*(pMP190::P*pvdD*)	74.4 (7.48)	90.3 (13.5)
OT11 *pvdS*(pMP190::P*pvdE*)	32.3 (2.21)	30.0 (4.86)

[a] Plasmid-containing strains of *P. aeruginosa* were grown in King's B broth with or without addition of $FeCl_3$, as shown.
[b] Units of β-galactosidase activity were defined as described in Table 2, footnote *b*. Unless noted otherwise, all values are averages of at least three experiments.
[c] Average of two experiments and very similar to value obtained previously with the same construct (31).

Regulation of expression of *pvdS* by iron. Synthesis of pyoverdine is repressed by the presence of iron in the growth medium, with regulation being at least in part at the transcriptional level (31). To test the possibility that expression of *pvdS* is also iron regulated, Northern analysis was done with RNA from bacteria grown in iron-rich and iron-deficient media (Fig. 4). A *pvdS* transcript of about 630 bases was present in cells grown under iron-deficient conditions, and this size is consistent with that of the gene (564 bp). This transcript was undetectable when the bacteria were grown in iron-replete media. The predicted −35 box of the *pvdS* promoter (Fig. 2) is spanned by a sequence which has a 15 of 19 match with the consensus sequence for binding of the Fur repressor protein (GATAATGATAATCATTATC; 10), indicating that the effect of iron on expression of *pvdS* may be due to binding of Fur at the *pvdS* promoter in iron-replete cells. To test the possibility that Fur regulates expression of *pvdS*, a DNA fragment containing the *pvdS* promoter was amplified by PCR and cloned into pMP190. The resulting construct was then transformed into a Fur^- mutant of *E. coli*, as well as the isogenic Fur^+ strain, and the effect of the *fur* mutation on expression from *pvdS* was examined (Table 4). Expression from the promoter was affected by the amount of iron in the growth medium in the Fur^+ strain but not in the Fur^- strain, indicating that the Fur protein does indeed repress transcription from the *pvdS* promoter in iron-replete cells. Levels of β-galactosidase were significantly higher in the *fur* mutant than in the Fur^+ strain, even under conditions of iron starvation, but the reason for this is unknown. Taken together, these data indicate that

FIG. 4. Northern analysis of expression of *pvdS*. RNA was prepared from *P. aeruginosa* OT11 which had been grown in broth supplemented with iron (+) or made iron deficient by addition of EDDA (−). Northern analysis was done by using as the probe an internal fragment of the *pvdS* gene. The hybridizing RNA is approximately 630 bases.

TABLE 4. Effect of a *fur* mutation on expression from the *pvdS* promoter[a]

E. coli strain (plasmid)	Avg β-galactosidase activity[b] (SD)	
	L broth + $FeCl_3$	L broth + EDDA
GC4468 Fur^+(pMP190::P*pvdS*)	34.3 (2.68)	124 (2.71)
QC1732 *fur*(pMP190::P*pvdS*)	223 (1.93)	206 (4.16)

[a] Bacteria were grown in broth supplemented with $FeCl_3$ or the iron-chelating compound EDDA, as shown.
[b] Units of β-galactosidase were defined as described in Table 2, footnote *b*. The values shown are averages of at least three experiments.

expression of *pvdS* is regulated by the presence of iron in the growth medium and that, in *E. coli* at least, regulation is mediated by the Fur protein.

DISCUSSION

The results presented here show that synthesis of pyoverdine requires a protein, PvdS, which is likely to be a sigma factor as it has sequence similarities to members of the ECF family of alternative sigma factors. PvdS is required for expression from the promoters of at least two pyoverdine genes, *pvdD* and *pvdE*. Promoters of other pyoverdine genes have not been characterized, but it is quite possible that PvdS is required for expression of many of these genes. Failure to express genes required for pyoverdine synthesis explains the pyoverdine-deficient phenotype of the *pvdS* mutant.

Expression of *pvdS* is normally repressed by the presence of iron in the growth medium (Fig. 4). The presence of a Fur box over the likely promoter of *pvdS* (Fig. 2) and the derepression of the promoter which occurs in a Fur^- mutant of *E. coli* (Table 4) indicate that the Fur protein regulates expression of *pvdS* in response to levels of iron by binding to the promoter in iron-replete cells. This leads to a simple model to explain the regulation of pyoverdine synthesis in response to levels of iron. In this model, Fur represses expression of *pvdS* when the bacteria are grown in iron-rich medium. The consequent absence of PvdS prevents the expression of pyoverdine synthesis genes. When the bacteria are grown in the absence of iron, Fur is inactive as a repressor and does not prevent synthesis of PvdS. This allows expression of pyoverdine synthesis genes, and consequent synthesis of pyoverdine, to occur. This model is consistent with the finding that Fur^- mutants of *P. aeruginosa* constitutively synthesize pyoverdine (29).

In this model, Fur controls expression of pyoverdine synthesis genes through an intermediary protein, PvdS. In *E. coli*, the Fur repressor binds to the promoters of genes required for siderophore biosynthesis and directly prevents their expression (4), rather than regulating expression through an intermediary protein such as PvdS. Why is an intermediary protein involved in expression of pyoverdine synthesis genes? One possible explanation is that this allows involvement of other elements in the regulatory process. The genes which are most similar to *pvdS*, *fecI* and *pupI*, are cotranscribed with genes which negatively regulate their activity, and many other members of the ECF sigma family are also negatively regulated by genes within the same operon (17). However, there is no evidence that *pvdS* is cotranscribed with another gene which regulates its activity. Northern analysis showed that the *pvdS* transcript (approximately 630 bases) is similar in size to the *pvdS* gene (564 bp) so that it is likely that the transcript is monocistronic. In addition, the DNA sequence shows that the gene is preceded by a likely promoter and followed by a possible transcription termination sequence. It remains to be seen whether, like that of FecI and

PupI, the activity of the PvdS protein is affected by other proteins in the cell or whether regulation is entirely at the level of transcription of the *pvdS* gene.

Other *Pseudomonas* species synthesize siderophores which are chemically similar to the pyoverdine made by *P. aeruginosa* PAO (5), and similarity extends to the genetic level, with hybridization occurring at high stringency between DNAs containing siderophore synthesis genes from different species (30). It therefore seems likely that, to some extent at least, similar regulatory mechanisms control expression of siderophore synthesis genes in different species of *Pseudomonas*. Consistent with this, genes have recently been identified in *P. fluorescens* M114 (*pbrA*; 33) and *P. putida* (*pfrI*; 39) which have sequences and functions similar to those of *pvdS* and are also likely to encode sigma factor proteins.

In addition to sigma factors, transcriptional activators may also be involved in regulating expression of siderophore synthesis genes in pseudomonads. In addition to *pfrI*, a second gene (*pfrA*) is required for expression from the promoter of at least one gene involved in biosynthesis of pseudobactin by *P. putida* WCS358 (39). This may allow regulation of siderophore synthesis to be influenced by other signals in addition to the levels of iron in the medium. The involvement of another protein(s) in expression from siderophore promoters in pseudomonads would explain why the pyoverdine promoters showed about 10-fold lower activity in *E. coli* containing the *pvdS* gene than in *P. aeruginosa* (Tables 2 and 3), although this would also be explained if PvdS were less effective in interacting with *E. coli* RNA polymerase or if the *pvdS* gene were inefficiently expressed in *E. coli*.

In addition to pyoverdine, cells of *P. aeruginosa* synthesize many other extracellular products in response to iron starvation, and many of these act as virulence factors during infection. These include exotoxin A, proteases, hemagglutinin, and the siderophore pyochelin (2, 9), although in some cases the effect of iron is strain dependent (2). Synthesis of a number of outer membrane proteins is also induced in response to iron starvation (24). These products are made under conditions (iron starvation) in which PvdS is present in the bacteria so that PvdS may be required for synthesis of some of these products as well as pyoverdine synthesis.

In conclusion, we have shown that synthesis of pyoverdine in *P. aeruginosa* requires the presence of a probable alternative sigma factor, PvdS. Expression of the *pvdS* gene is likely to be regulated by the Fur repressor in response to the levels of iron available to the bacteria. This provides a model which explains, at least in part, the molecular mechanisms which control pyoverdine synthesis in *P. aeruginosa*.

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ADDENDUM IN PROOF

The *pvdS* gene has been identified and characterized independently by M. Tsuda and co-workers (H. Miyazaki, H. Kato, T. Nakazawa, and M. Tsuda, Mol. Gen. Genet., in press).

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