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Iron-Regulated Transcription of the *pvdA* Gene in *Pseudomonas aeruginosa*: Effect of Fur and PvdS on Promoter Activity†

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The *pvdA* gene, encoding the enzyme L-ornithine N^5-oxygenase, catalyzes a key step of the pyoverdin biosynthetic pathway in *Pseudomonas aeruginosa*. Expression studies with a promoter probe vector made it possible to identify three tightly iron-regulated promoter regions in the 5.9-kb DNA fragment upstream of *pvdA*. The promoter governing *pvdA* expression was located within the 154-bp sequence upstream of the *pvdA* translation start site. RNA analysis showed that expression of PvdA is iron regulated at the transcriptional level. Primer extension and S1 mapping experiments revealed two 5′ termini of the *pvdA* transcript, 68 bp (T1) and 43 bp (T2) 5′ of the PvdA initiation. The *pvdA* transcripts were monocystronic, with T1 accounting for 90% of the *pvdA* mRNA. Fur box-like sequences were apparently absent in the regions 5′ of *pvdA* transcription start sites. A sequence motif resembling the −10 hexamer of AlgU-dependent promoters and the iron starvation box of pyoverdin genes controlled by the σ^E-like factor PvdS were identified 5′ of the T1 start site. The minimum DNA region required for iron-regulated promoter activity was mapped from bp −41 to −154 relative to the ATG translation start site of *pvdA*. We used *pvdA′::lacZ* transcriptional fusions and Northern (RNA) analyses to study the involvement of Fur and PvdS in the iron-regulated expression of *pvdA*. Two *fur* mutants of *P. aeruginosa* were much less responsive than wild-type PAO1 to the iron-dependent regulation of *pvdA* expression. Transcription from the *pvdA* promoter did not occur in a heterologous host unless in the presence of the *pvdS* gene in *trans* and was abrogated in a *pvdS* mutant of *P. aeruginosa*. Interaction of the Fur repressor with a 150-bp fragment encompassing the *pvdS* promoter was demonstrated in vivo by the Fur titration assay and confirmed in vitro by gel retardation experiments with a partially purified Fur preparation. Conversely, the promoter region of *pvdA* did not interact with Fur. Our results support the hypothesis that the *P. aeruginosa* Fur repressor indirectly controls *pvdA* transcription through the intermediary sigma factor PvdS; in the presence of sufficient iron, Fur blocks the *pvdS* promoter, thus preventing PvdS expression and consequently transcription of *pvdA* and other pyoverdin biosynthesis genes.

Iron is an essential element for almost all bacteria but is not readily available under aerobic conditions at neutral pH (38). Free iron is extremely limited also in the tissues and fluids of mammalian hosts because of the presence of transferrin in serum and lactoferrin in mucosal secretions (10). To acquire iron from the environment, most microorganism have evolved high-affinity uptake systems based on the synthesis of low-molecular-weight chelators, termed siderophores, and of their cognate receptors (reviewed in reference 64). *Pseudomonas aeruginosa* is an opportunistic human pathogen which produces in response to iron-limited growth a water-soluble, yellow-green fluorescent siderophore termed pyoverdin. In *P. aeruginosa* PAO1, this molecule is composed of a 6,7-dihydroxyquinoline-containing fluorescent chromophore joined to the N terminus of a partly cyclic octapeptide (D-Ser-L-Arg-D-Ser-L-N^5-OH-Orn-L-Lys-L-N^5-OH-Orn-L-Thr-L-Thr [7]). Pyoverdin has a very high affinity for Fe(III) (K_f about 10^{32} at neutral pH) and stimulates *P. aeruginosa* growth in the presence of human transferrin or serum (3, 54), and thus it probably plays a predominant role in iron mobilization in vivo (25, 27).

Over the last years, molecular studies have focused on the biochemical and regulatory aspects of pyoverdin synthesis in *P. aeruginosa* PAO1. The enzyme L-ornithine N^5-oxygenase, encoded by the *pvdA* gene, catalyzes the formation of the hydroxamate ligands (L-N^5-OH-Orn residues) of pyoverdin (60), and the product of the *pvdD* gene has been proposed to be a synthase involved in the assembly of the peptidic moiety of the siderophore by a nonribosomal mechanism (35). The products of the *envCD* gene cluster are implicated in pyoverdin secretion (43), while the outer membrane protein encoded by the *fpvA* gene (44) functions in ferripyoverdin uptake. The genetic loci involved in pyoverdin synthesis and uptake have been mapped in *P. aeruginosa* and found to be located within a 103-kb DNA region at about 47 min of the PAO1 chromosome map (1, 57, 62). Pyoverdin synthesis is negatively regulated by the level of available iron, and this probably derives from reduced transcription of corresponding biosynthetic genes (47). A *fur* homolog has recently been identified in *P. aeruginosa*, and *fur* mutants which constitutively produce pyoverdin have been isolated (5, 39, 45, 46). In the presence of sufficient iron, the Fur repressor is known to bind to a highly conserved region, called the Fur box, upstream of iron-regulated genes, thus blocking their transcription. Under conditions of iron deficiency, repression by Fur is relieved and the genes are transcribed (17, 28, 40, 45, 59). Interestingly, recognition sequences for the Fur repressor were not identified in the control regions of genes for pyoverdin biosynthesis and uptake (35, 44, 47, 60), whereas Fur box-like motifs were recognized in the putative promoters of transcriptional units involved in pyo-

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chelin- and enterochelin-mediated iron transport systems in *P. aeruginosa* (2, 16, 26). The consensus sequence (G/T)CTA AATCCC, which likely acts as a binding site for a transcriptional activator protein, was identified in the iron-regulated promoters of some pyoverdin biosynthetic genes and in the *P. aeruginosa* exotoxin A promoter (47). A new model for the iron-dependent regulation of pyoverdin synthesis in *P. aeruginosa* was proposed after the discovery of the novel transcriptional factor PvdS, a protein belonging to the extracytoplasmic function (ECF) subfamily of RNA polymerase sigma factors (σ^E [34]), which is required for transcription of pyoverdin biosynthetic genes (15, 37). According to this model, expression of the *pvdS* gene is negatively regulated by Fur. It has been hypothesized that under conditions of iron sufficiency, the Fur repressor blocks transcription from the *pvdS* promoter, thus indirectly preventing the expression of pyoverdin biosynthetic genes. During iron starvation, repression is relieved and cells are reprogrammed through the expression of the alternative sigma factor PvdS, which confers to RNA polymerase specificity for pyoverdin promoters. Whether this regulatory model can be extended to additional pyoverdin biosynthetic genes has yet to be established.

We previously showed that expression of the enzyme L-ornithine N^5-oxygenase in *P. aeruginosa* PALS124 (*pvdA*) harboring the complementing plasmid pPV225 was negatively regulated by iron (60). However, sequence analysis did not allow the detection of transcription start signals in the 154-bp DNA sequence preceding the translation start codon of *pvdA*, nor was it possible to demonstrate significant homology with Fur box-like consensus sequences or iron-regulated promoters known to exist in *P. aeruginosa* and other *Pseudomonas* spp. (60). In this report, we demonstrate that *pvdA* is finely regulated by iron at the transcriptional level. We identified three tightly iron-regulated promoter regions in the 5.9-kb DNA sequence upstream of *pvdA*. We localized two transcription start points for *pvdA* and analyzed the DNA sequence upstream of the start sites for putative promoter elements. We also found experimental evidence that *fur* indirectly regulates *pvdA* expression, through the transcriptional control of the *pvdS* gene, and demonstrated in vivo and in vitro interaction between the Fur repressor protein and the putative *pvdS* promoter. Our results suggest that *pvdS* plays a master regulatory role in the activation of pyoverdin biosynthetic genes.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown in LB medium or in M9 minimal medium (49). *P. aeruginosa* was grown in NYB (62), in SM9 (62), or on nutrient agar (Difco). DCAA (61) was used as the low-iron medium for *P. aeruginosa*. Media were solidified with 1.2% agar N.1 (Unipath). To reduce iron availability, the iron chelator 2,2′-dipyridyl was added to M9 minimal medium at 150 μM. Antibiotics were used in selective media at the following concentrations: tetracycline (Tc), 12.5 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin (Km), 25 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*; ampicillin (Ap), 100 μg/ml for *E. coli*; carbenicillin (Cb), 500 μg/ml for *P. aeruginosa*; and nalidixic acid (Nal) and streptomycin (Str), 25 μg/ml for *E. coli.*

Enzymatic assays and chemical determinations. The broad-host-range plasmid pMP220 (53), carrying a promoterless *lacZ* gene downstream of multiple cloning sites, was used to subclone internal fragments of pPV5 (60). The presence of two *Hin*dIII sites flanking the linker of pMP220 made possible the cloning of fragments in opposite orientations. For measurements of *lacZ* reporter gene activity, *P. aeruginosa* strains harboring pMP220-derived plasmids were grown for 12 to 18 h at 37°C in DCAA containing 100 μg of tetracycline per ml; they were then diluted 1:1,000 in the same medium with or without the addition of 100 μM $FeCl_3$ and subcultured for additional 8 to 10 h with shaking until the A_{620} reached approximately 0.4. *E. coli* MC4100 (11) carrying different transcriptional fusion constructs was grown in M9 minimal medium supplemented with the appropriate antibiotics and inoculated to an A_{620} of ≅0.01 in the same medium containing 100 μM $FeCl_3$ or 200 μM 2,2′-dipyridyl for additional 8 h of growth at 37°C (final A_{620}s of ≅0.8 in low-iron medium and ≅1.2 in high-iron medium). The cells were lysed with toluol, and β-galactosidase activity was determined spectrophotometrically, using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Activity was normalized to the A_{600} of the bacterial suspension and expressed in Miller units (36). Strains were assayed at least three different times, with duplicate assays each time.

The concentration of hydroxamate nitrogen in *P. aeruginosa* culture supernatant was determined by iodine oxidation as described by Gillam et al. (24). Levels of pyoverdin in culture supernatants of *P. aeruginosa* strains were estimated by means of UV-visible and fluorescence spectroscopy, using a Beckman 25 spectrophotometer and a Jasco FP770 spectrofluorometer as reported elsewhere (60, 62).

DNA manipulations and genetic techniques. Genomic DNA from *P. aeruginosa* PAO1 was prepared as previously described (60). For analytical purposes, plasmid DNA from *E. coli* and *P. aeruginosa* was prepared by the alkaline lysis method (49). Large-scale preparations of plasmids from *E. coli* were performed with Qiagen Tip-500 kits (Diagen) as specified by the manufacturer. The DNA was analyzed by agarose gel electrophoresis in Tris-borate buffer (49), and DNA bands were extracted from the gels with the Qiaex resin (Diagen) or by digestion of low-melting-point agarose (Sigma) with agarase (Boehringer) for fragments greater than 4 kb. *E. coli* was transformed with plasmid DNA by the standard $CaCl_2$ method (49). *P. aeruginosa* cells were made competent by using a minor modification of the $CaCl_2$ procedure (60). Plasmids were transferred from *E. coli* to *P. aeruginosa* by triparental matings with the helper plasmid pRK2013 (20).

Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were used under the conditions recommended by the manufacturer (Boehringer).

DNA sequencing and analysis. DNA fragments generated by digestion of pPV5 (60) were cloned at compatible sites of the phagemids pTZ18R and pTZ19R (Pharmacia). The inserts of single-stranded forms of the phagemid constructions were sequenced by the dideoxy-chain termination method (49) with T7 DNA polymerase (Pharmacia) and [α-^{35}S]dATP for labeling. To resolve band compressions due to high G+C content of *P. aeruginosa* DNA, sequencing reactions were performed with both dGTP and 7-deaza-dGTP. Multiple sequence alignments were made by using the programs FASTA and PILEUP of the Genetics Computer Group (University of Wisconsin) package (version 8.0).

RNA purification, Northern (RNA) blot analysis, S1 nuclease mapping, and primer extension. RNA was isolated from cells grown under different culture conditions at specified optical densities, using a modification of the hot-phenol extraction method (8). Cells from 1.5-ml cultures were collected by centrifugation (8,000 × *g*, 2 min, 4°C), suspended in 200 μl of lysis buffer (20 mM sodium acetate [pH 5.5], 0.5% sodium dodecyl sulfate [SDS], 1 mM EDTA), and extracted for 5 min at 65°C with 200 μl of prewarmed phenol saturated with 20 mM sodium acetate (pH 5.5). After centrifugation, the aqueous phase was extracted with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]), and the nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 6) and 3 volumes of absolute ethanol. The precipitated material was treated for 15 min at 37°C with 20 U of DNase I (RNase free; Boehringer) and 25 U of RNasin (Boehringer) in a total volume of 50 μl. The RNA was subsequently phenol extracted twice, ethanol precipitated, and redissolved in diethylpyrocarbonate-treated water. The RNA concentration was estimated by measuring the optical density of the solution at 260 and 280 nm.

Aliquots of 10 μg of total RNA were denatured at 65°C for 15 min in the presence of 2 M formaldehyde and 50% formamide and electrophoresed for 4 h at 4 V/cm on a 1% agarose gel containing 2 M formaldehyde in morpholine propanesulfonic acid (MOPS) buffer (49). The RNA was transferred onto nylon filters (Hybond-N; Amersham Corp.) as described by Sambrook et al. (49) and heat fixed. DNA probes were generated either by random primer labeling with the DIG-High Prime kit (Boehringer) or by PCR with the PCR DIG Probe Synthesis kit (Boehringer), using digoxigenin (DIG)-11-dUTP as directed by the manufacturer. Hybridization was performed for 12 h at 50°C in 7% SDS–50 mM Na_3PO_4 (pH 7.0)–50% formamide–5× SSC (0.75 M NaCl, 0.075 M sodium citrate [pH 8.0])–0.1% *N*-lauroylsarcosine–2% blocking reagent (Boehringer). Filters were washed twice for 5 min in 2× SSC–0.1% SDS at room temperature and twice for 15 min in 0.1× SSC–0.1% SDS at 65°C. DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase antibody conjugate and the chemiluminescent substrate Lumigen PPD (Boehringer) as specified by the manufacturer. For the detection of the chemiluminescent signal, the membranes were exposed to Kodak XAR film. For quantitative transcript estimation, DNA probes were labeled with [α-^{32}P]dCTP (3.0 Ci/nmol; Amersham Corp.) with a primer extension kit purchased from Pharmacia, and then unincorporated nucleotides were removed by passage through a Sephadex G-50 spin column. Filters were hybridized and washed as reported elsewhere (49) and analyzed with a Betascope model 603 blot analyzer (Betagen). Results were normalized by reprobing of the filter with an *arcA* probe from pME3702 (23).

A single-stranded, uniformly labeled DNA probe for S1 nuclease protection assays was generated by using a modification of the procedure described by Espelund et al. (19). A 1,134-bp *Bgl*II fragment encompassing the 5′ end of *pvdA* was ligated to the *Bam*HI site of pUCP18 in the transcriptional orientation opposite that of the *lacZ*′ gene of the vector, yielding pPV226. The cloned fragment was amplified by using a biotinylated M13/pUC universal primer (5′-biotin-CGTTGTAAAACGACGGCCAG-3′, annealing to the noncoding strand downstream of the *Hin*dIII site of the vector) and a reverse M13/pUC primer

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or relevant characteristics	Reference or source
E. coli		
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 [ϕ80d*lacZ*Δ*M15*] Nal^r	51
H1717	*aroB fhuF*::λp*lac*Mu	55
JM109	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*] Nal^r	66
MC4100	*araD139 rpsL150 relA1 flbB5301 deoC1 pstF25 rbsR* Δ(*lacZYA-argF*)U169 Str^r	11
P. aeruginosa		
PAO1 (ATCC 15692)	Prototroph	American Type Culture Collection
PAO6261	*anr*^{0}	67
PAO OT11	*leu-1 pro-1*	15
PAO1 A4	*fur-4* (His-86→Tyr substitution at the Fur protein)	5
PAO6261 C6	*anr*^{0} *fur-6* (Ala-10→Gly substitution at the Fur protein)	5
PAO OT11*pvdS*	*leu-1 pro-1 pvdS*::*kan* Kan^r	15
PAK	Prototroph	29
PAK-N1	PAK mutant with Tn*5G* insertion in the *rpoN* locus, Tc^r	29
Plasmids		
pTZ18R and -19R	Phagemid vectors for DNA cloning and sequencing; ColE1 replicons, *lacZ*α Ap^r	Pharmacia
pBR322	ColE1 replicon, Ap^r Tc^r	49
pQE60	Expression vector; ColE1 replicon, P_{T5} *lacO* RBSII Ap^r	Diagen
pDMI,1	Repressor plasmid; p15A replicon, *lacI*^{q} Km^r	12
pRK2013	Helper plasmid; ColE1 replicon, Km^r Mob$^+$ Tra$^+$	20
pUCP18 and -19	*E. coli-Pseudomonas* shuttle vectors derived from pUC18 and -19; ColE1 pRO1600 replicons, *lacZ*α Ap^r Cb^r	51
pMP220	Broad-host-range, low-copy-number promoter probe vector; IncP replicon, *lacZ* Tc^r Tra$^-$. In pMP220R, the multiple cloning site is in inverted orientation.	53
pME3536	Broad-host-range, low-copy-number promoter probe vector derived from pKT240; IncQ replicon, *lacZ* Ap^r Cb^r Tra$^-$	4, 65
pPV5	8.5-kb *Xho*I fragment of pPV4 ligated to pUCP19	60
pBRXB	1.8-kb *Xho*I-*Bam*HI fragment containing *pvdS* ligated to pBR322	15
pPV226	1.1-kb *Bgl*II fragment of pPV5 ligated to pUCP18	This study
pPV51/52	0.3-kb *Sph*I-*Bgl*II fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV53/54	0.5-kb *Pst*I-*Bgl*II fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV55/56	1.1-kb *Bgl*II fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV57/58	3.6-kb *Pst*I fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV59/510	1.3-kb *Pst*I-*Sph*I fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV511/512	1.2-kb *Sph*I fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV513/514	1.9-kb *Pst*I fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV515/516	5.0-kb *Bam*HI-*Bgl*II fragment of pPV5 ligated to pMP220 in both orientations	This study (Fig. 1B)
pPV51Δ1-19	5′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ1-38	5′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ1-54	5′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ1-81	5′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ1-101	5′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ87-334	3′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ115-334	3′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pPV51Δ137-334	3′-deletion derivative of the *pvdA* promoter in pPV51	This study (Fig. 7)
pBL2	1.1-kb *Bgl*II fragment of pPV5 ligated to pME3536	This study
pBRP_{pvdA}	0.3-kb *Sph*I-*Bgl*II fragment of pPV5 ligated to *Sph*I-*Bam*HI sites of pBR322	This study
pBRP_{pvdS}	150-bp sequence encompassing the putative *pvdS* promoter blunt-end ligated to the *Eco*RV site of pBR322	This study
pQE*pvdS*	593-bp fragment encompassing the entire *pvdS* gene ligated to the *Eco*RI-*Hin*dIII sites of pQE60 under P_{T5} *lacO* control	This study
pQE*fur*	402-bp *fur* coding sequence ligated to the *Nco*I-*Hin*dIII sites of pQE60 under P_{T5} *lacO* RBSII control	This study

(5′-TCACACAGGAAACAGCTATGAC-3′, annealing to the coding strand 9 nucleotides [nt] downstream of the *Eco*RI site). Amplification reactions were carried out by using 1 ng of circular template in a 100-μl reaction mixture containing 1× PCR buffer (Perkin-Elmer), 1.5 μM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1 μM each primer, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). Thirty cycles were performed in a Perkin-Elmer 480 thermal cycler, each cycle comprising 30 s at 95°C (denaturation), 1 min at 60°C (annealing), and 45 s at 74°C (extension). The biotinylated PCR product (500 ng) was bound to 25 μl of magnetic streptavidin beads (Dynabeads M-280 Streptavidin; Dynal) by incubation in BW buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 20 mM NaCl) for 15 min at 20°C. Beads were washed once with BW buffer, and the nonbiotinylated strand was removed by two cycles of denaturation with 100 μl of 125 mM NaOH in 100 mM NaCl. After two washes with BW buffer and two washes with water, the single-stranded DNA captured onto the streptavidin beads was used as the template for antisense DNA synthesis. Five picomoles of an oligonucleotide designated RV*P*pa (5′-GGCGGTTGCAGTTGC CTGAGTCAT-3′; M-Medical) complementary to the coding strand of the *pvdA* gene from the eighth codon to the ATG translation start site was annealed with the biotinylated DNA in a 25-μl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 4 mM dithiothreitol (DTT), 20 μM dGTP, 20 μM dTTP, 6.5 U of T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemical), and 2 μl each of [α-^{32}P]dATP and [α-^{32}P]dCTP (10 mCi/ml). The mixture was incubated 20 min at 37°C and chased with the four deoxynucleoside triphosphates at a final concentration of 150 μM. After further 10 min at 37°C, the reaction was stopped by the addition of 1 μl of 500 mM EDTA. Unincorporated nucleotides and primer were removed by two washes with BW buffer, and the ^{32}P-labeled DNA strand was eluted from the immobilized template by NaOH-NaCl denaturation as described above. An aliquot of 10 ng (corresponding to 2×10^6 cpm) of the single-stranded DNA probe was mixed with 100 μg of total RNA extracted from *P. aeruginosa* PAO1 grown in DCAA to an A_{620} of

≅0.6 and coprecipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol. Nuclease S1 mapping of the *pvdA* messenger with the single-stranded DNA probe was performed as described by Sambrook et al. (49) at a hybridization temperature of 50°C. Controls without RNA or S1 nuclease were included in the experiment.

Primer extension reactions were carried out essentially as reported elsewhere (22). One picomole of the oligonucleotide (24-mer) used to generate the S1 probe was end labeled with 10 U of T4 polynucleotide kinase (New England Biolabs) and 100 μCi of [γ-^{32}P]ATP (Amersham Corp.) for 1 h at 37°C. The labeling mixture was heated at 65°C for 20 min, and the primer was coprecipitated with 100 μg of total RNA by the addition of 0.5 volume of 6 M ammonium acetate and 3 volumes of absolute ethanol. The pellet was washed three times with 70% ethanol, dried under vacuum, and resuspended in 50 μl of a mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT. The sample was incubated 30 min at 65°C, supplemented with 10 U of RNasin (Boehringer), and transferred at 45°C for 1 h. Reverse transcription was started by adding the four nucleotides (dATP, dCTP, dGTP, and dTTP) at a final concentration of 2.5 mM each and 60 U of reverse transcriptase (SuperScript II; Gibco BRL). Incubation was carried out for 1 h at 37°C and 1 h at 45°C. The sample was ethanol precipitated after the addition of 0.5 volume of 6 M ammonium acetate. The pellet was resuspended in 6 μl of water containing 1 μg of DNase-free RNase A (Boehringer) per ml, supplemented with 4 μl of formamide dye (49), and denatured at 90°C for 5 min.

The unlabeled primer was used to sequence the DNA region upstream of the *pvdA* gene from plasmid pPV226, using a T7 sequencing kit (Pharmacia) and [α-^{32}P]dATP. S1 nuclease-protected fragments and primer extension products were run in parallel to the sequencing reactions to map the start sites of the transcript. For quantitative estimation of S1-protected fragments and primer extension products, dried gels were analyzed in a Betascope model 603 blot analyzer (Betagen).

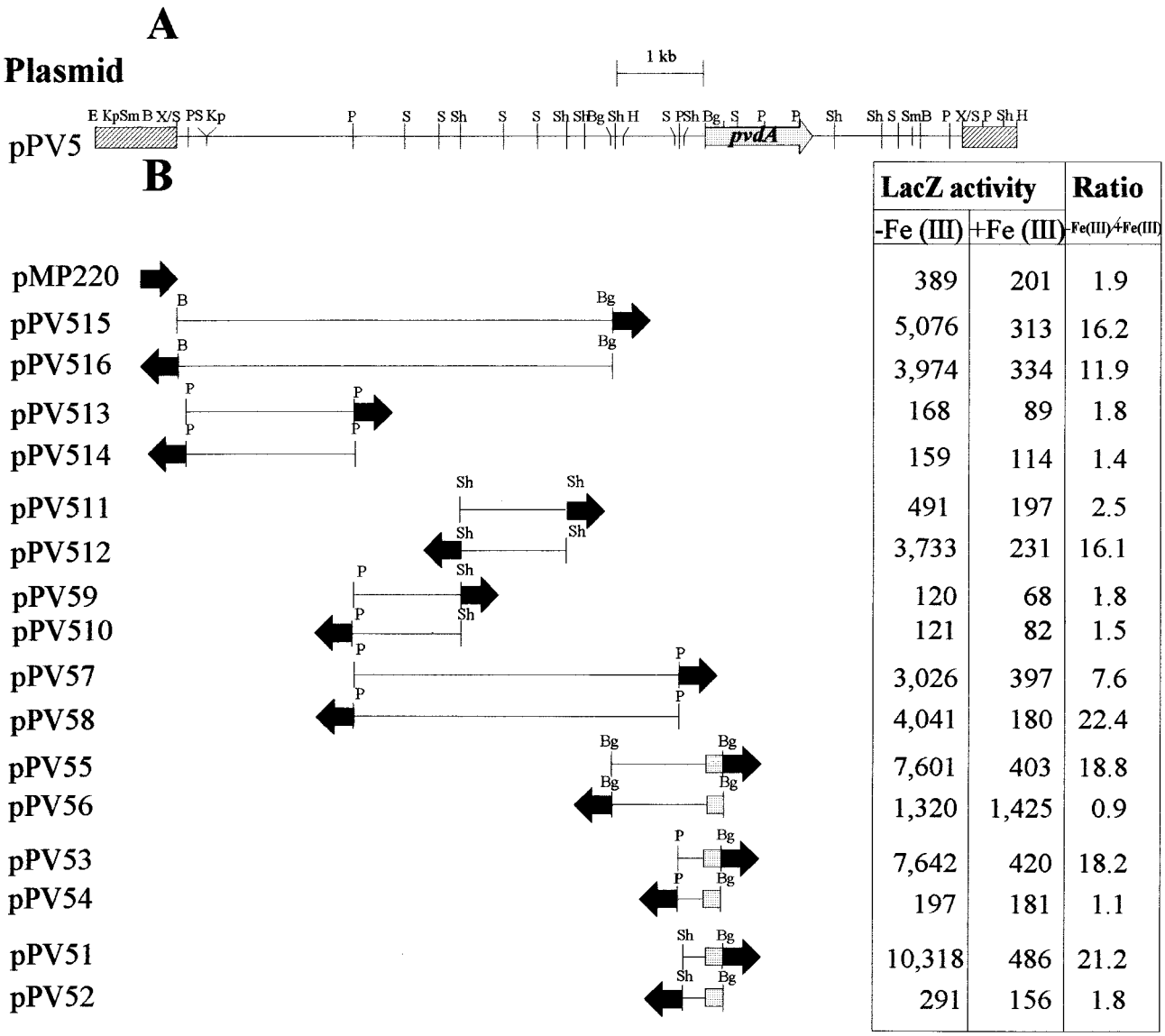
PCR-mediated deletions of the *pvdA* promoter. For 5′-deletion analysis of the *pvdA* promoter, five forward oligonucleotides (28-mers) were synthesized, the sequences of which were chosen to be conducive to priming of the coding strand from positions −135, −116, −100, −75, and −53 relative to the ATG translation start codon of the *pvdA* gene. All five primers contained an *Eco*RI site downstream of the triplet CCC at their 5′ termini. A reverse primer (28-mer) annealing to the coding strand from nt +498 to +525 relative to the ATG translation start codon of the *pvdA* gene was used for PCRs in combination with each of the forward primers. Amplification reactions were performed as described above, using pPV5 as the template at temperatures of 95°C (denaturation), 55°C (annealing), and 74°C (extension) for a total of 30 cycles. The PCR products were digested with *Eco*RI and *Bgl*II and ligated to compatible sites of pMP220R. The partial sequences of cloned fragments were checked by double-stranded sequencing using RV*P*pa as the primer. Deletions of DNA regions downstream of the major transcription initiation site of the *pvdA* gene were obtained by using pPV225HB (60) as the template for PCRs with primers 5′-GGGGGATCCG TTGACTAGAACCGA-3′, 5′-CCGGGATCCAAGTCGAGAGCAGCCCTGG -3′, and 5′-CCCGGATCCGGCCCCTTTCAAGCGGGAAC-3′ (annealing to the coding strand from nt −84 to −69, from nt −60 to −41, and from nt −38 to −19, respectively, relative to the ATG translation start codon of *pvdA*) in combination with the commercial M13/pUC reverse primer (20-mer; U.S. Biochemical). The amplification products were digested with *Sph*I and *Bam*HI and ligated to compatible sites of pMP220R. The sequences of the cloned fragments were checked by using the synthetic primers in double-stranded DNA sequencing.

Cloning and expression of *pvdS* under an exogenous promoter. The *pvdS* gene, including the complete coding sequence and the 20-bp region upstream of the ATG start codon, was cloned under phage T5 promoter (P_{T5}) and *lac* operator (*lacO*) control in the expression vector pQE60. Two oligonucleotides, designed on the basis of the published *P. aeruginosa pvdS* sequence (15) and identified as FW*pvdS* (5′-GCAGAATTCTCCGCAGCAAGGTGATTTCCATG-3′; the underlined region corresponds to nt 124 to 146) and RV*pvdS* (5′-CGCCAAGCT TAGCGGCGGGCGCTGAGATGGGT-3′; the underlined region corresponds to nt 705 to 684), were used to amplify the *pvdS* gene by PCR from *P. aeruginosa* PAO1 genomic DNA. Amplification reactions were performed as described above at temperatures of 95°C (denaturation), 60°C (annealing), and 74°C (extension) for a total of 30 cycles. The PCR product was cloned under control of an exogenous promoter in the expression vector pQE60. The pQE plasmids (Diagen) are pDS-derived vectors (9) containing a regulatory element consisting of P_{T5} and two modified *lacO* sequences upstream of a multiple cloning site, followed by translation stop codons in all reading frames and by lambda phage transcriptional terminators. The vector was digested with *Eco*RI and *Hin*dIII in order to remove the 21-bp sequence upstream of the ATG translation start site and then ligated to the *Eco*RI-*Hin*dIII-digested amplification product. The resulting construct, pQE*pvdS*, contained the *pvdS* open reading frame and the 21-bp upstream sequence under control of the promoter-operator element of pQE60. The DNA insert of pQE*pvdS* was checked by double-stranded sequencing with forward and reverse primers (Diagen) annealing to the DNA regions flanking the multiple cloning site of the pQE vector and with an internal primer corresponding to nt 320 to 355 of the *pvdS* sequence (15). Plasmid pQE*pvdS* was used to transform *E. coli* MC4100(pDMI,1) competent cells. Plasmid pDMI,1 (12) is a pACYC-derived replicon (13) containing the *lacI*^{q} gene and is used to repress the expression directed by the P_{T5}-*lacO* regulatory element of pQE60. Different transcriptional fusions in the vector pMP220 were conjugated in *E. coli* MC4100 harboring both plasmids pQE60*pvdS* and pDMI,1. Exconjugants were grown at 37°C in LB supplemented with ampicillin, kanamycin, and tetracycline. Expression of *pvdS* under P_{T5}-*lacO* control was obtained by the induction of cultures (A_{620} ≅ 0.4) with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by 3 h of incubation at 37°C prior to testing for β-galactosidase activity.

Fur titration assay. Plasmid pBRP_{pvdA} was obtained by ligation of the 0.3-kb *Sph*I-*Bgl*II fragment of pPV5 to *Sph*I-*Bam*HI sites of pBR322. A 150-bp DNA fragment encompassing the putative *pvdS* promoter was obtained by PCR with primers FW*P*ps (5′-CCATTCACGAATAAAGGTGAGATTGGT-3′) and RV*P*ps (5′-TTGTTCCGACATGGAAATCACCTTGCT-3′), corresponding to nt 6 to 32 and 155 to 129, respectively, of the sequence published by Cunliffe et al. (15). The PCR-generated fragment was blunt-end cloned at the *Eco*RV site of pBR322, generating pBRP_{pvdS}. The pBR322-derived constructs carrying putative Fur-binding sequences were introduced in *E. coli* H1717, and transformants were screened on MacConkey agar (Merck) supplemented with ampicillin (100 μg/ml) and increasing concentrations of $FeCl_3$ (20, 40, 60, 80, 100, and 120 μM) as reported elsewhere (55).

Expression and partial purification of the *P. aeruginosa* Fur protein. Two primers, designed on the basis of the published *P. aeruginosa fur* sequence (45) and designated FW*fur* (5′-CCCCCATGGTTGAAAATAGCGAACTTCG-3′; the underlined region corresponds to nt 26 to 49) and RV*fur* (5′-CCCAAGCTTA CTACTTCTTCTTGCGCACGTAG-3′; the underlined region corresponds to nt 432 to 410), were used to amplify the *fur* gene from *P. aeruginosa* PAO1 genomic DNA. Amplification reactions were performed as described above at temperatures of 95°C (denaturation), 60°C (annealing), and 74°C (extension) for a total of 30 cycles. The PCR product was digested with *Nco*I and *Hin*dIII and ligated to compatible sites in pQE60 to allow the inserted DNA to start with its authentic ATG codon (located within *Nco*I site) and to stop with a TAA codon (located within the *Hin*dIII site). The DNA insert of the resulting construct, termed pQE*fur*, was sequenced by using forward and reverse primers (Diagen) annealing to the DNA regions flanking the multiple cloning site of the pQE vector and with an internal primer annealing to nt 160 to 182 of the *fur* coding strand (45). Cells of *E. coli* JM109 (66) harboring pQE*fur* were grown overnight at 37°C in LB supplemented with ampicillin and diluted 1:1,000 in 500 ml of the same medium which had been prewarmed at 37°C. After approximately 5 h of growth (A_{620} ≅ 0.6), the cultures were induced with 1 mM IPTG and incubated at 37°C for an additional 4 h to allow expression of the cloned *fur* gene. Uninduced cultures were used as negative controls. Cells were harvested by centrifugation (8,000 × *g*, 10 min, 4°C), and the Fur protein was extracted by three cycles of freezing and thawing as described elsewhere (30). The released material, suspended in 20 ml of 1 mM DTT, was supplemented with streptomycin sulfate to give a concentration of 1%, and the nucleic acids were removed by centrifugation (20,000 × *g*, 10 min, 4°C). The clarified supernatant was brought to 60% saturation with ammonium sulfate, and after standing for 12 h at 4°C, the precipitate was collected and dissolved in 20 ml of 1 mM DTT. Salts and low-molecular-weight contaminants were removed by passage through a Centricon-10 ultrafiltration apparatus (Amicon) followed by three serial washes with 5 ml of 1 mM DTT in 10 mM BisTris-borate pH 7.5 (17). The protein content was determined by the method of Bradford (6), and the preparation was adjusted to a Fur concentration of 10 mg/ml and stored frozen at −80°C. Protein extracts were suspended in gel loading buffer (0.25 M Tris-HCl, 2% SDS, 10% 2-mercaptoethanol, 20% glycerol), heated at 100°C for 5 min, and analyzed in a 0.1% SDS–12.5% polyacrylamide gel as described by Laemmli (32). Polyacrylamide gel electrophoresis (PAGE) was carried out at 10 V/cm in Tris-glycine buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 1% SDS). After electrophoresis, gels were stained with Coomassie brilliant blue, destained, and photographed. The partial amino acid sequence of the purified protein was determined by automated Edman degradation. The protein sample (20 μg) was subjected to SDS-PAGE, electrotransferred onto a ProBlott membrane (Applied Biosystem), and sequenced on a Perkin Elmer-Applied Biosystem 476A sequencer equipped with a Blott cartridge, using an optimized liquid phase fast program.

Gel retardation assays. Promoter regions of the *pvdS* and *pvdA* genes were labeled with [α-^{32}P]dCTP (10 mCi/ml) by PCR as described by Peterson et al. (42). A 150-bp DNA probe encompassing the putative *pvdS* promoter was generated with primers FW*P*ps and RV*P*ps. A 177-bp *pvdA* probe was obtained by amplification of pPV225HB DNA (60) with primer RV*P*pa and the commercial M13/pUC reverse primer (20-mer; U.S. Biochemical); the amplification product was subjected to digestion with *Sph*I and Klenow fill-in. Unincorporated nucleotides and primers were removed by passage through a spin column (QIAquick; Diagen), and the PCR products were adjusted to a concentration of 100 nM in water. Gel retardation assays were performed as described by de Lorenzo et al. (17), using either $MnCl_2$ or freshly prepared $FeCl_2$ as the binding cofactor. The binding buffer consisted of 10 mM BisTris-boric acid buffer (pH 7.5), 100 μM $MnCl_2$ or $FeCl_2$, 1 mM $MgCl_2$, 40 mM KCl, 5 μg of denatured salmon sperm DNA per ml, 100 μg of bovine serum albumin per ml, 5% (vol/vol) glycerol, and variable amounts of the Fur proteins in a volume of 10 μl. The binding mixtures were equilibrated for 30 min at 37°C before loading onto a 5% nondenaturing polyacrylamide gel polymerized in 20 mM BisTris-boric acid buffer (pH 7.5) supplemented with 100 μM $MnCl_2$ or $FeCl_2$, without addition of dye. A control track with bromophenol blue and xylene cyanol was used to determine the extent of migration. Gels were run at 5 V/cm in 20 mM BisTris-boric acid (pH 7.5)–100



LacZ activity		Ratio
-Fe (III)	+Fe (III)	-Fe(III)/+Fe(III)
389	201	1.9
5,076	313	16.2
3,974	334	11.9
168	89	1.8
159	114	1.4
491	197	2.5
3,733	231	16.1
120	68	1.8
121	82	1.5
3,026	397	7.6
4,041	180	22.4
7,601	403	18.8
1,320	1,425	0.9
7,642	420	18.2
197	181	1.1
10,318	486	21.2
291	156	1.8

FIG. 1. (A) Restriction endonuclease map of the 8.5-kb *Xho*I insert of pPV5. The stippled arrow shows the location and direction of transcription of *pvdA*. Striped bars indicate the multiple cloning sites of pUCP19 (not to scale). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Kp, *Kpn*I; P, *Pst*I; S, *Sal*I; Sh, *Sph*I; Sm, *Sma*I; X, *Xho*I; X/S, hybrid *Xho*I-*Sal*I site. (B) Analysis of promoter activities of restriction fragments derived from pPV5. Plasmid designations are given on the left, and DNA inserts are aligned with the physical map in panel A. Arrows show the direction of transcription of the reporter *lacZ* gene from the promoter probe vector pMP220 (53); stippled bars indicate a portion of the *pvdA* gene. The expression of β-galactosidase (LacZ) from each of the constructs in *P. aeruginosa* PAO1 during growth under both low- and high-iron conditions is given on the right. LacZ activity values, in Miller units (36), are the means of at least six determinations. The standard deviation is <19% of each value. −Fe(III), growth under conditions of iron deficiency (DCAA); +Fe(III), growth under conditions of iron sufficiency (DCAA supplemented with 100 μM $FeCl_3$).

μM $MnCl_2$ or $FeCl_2$ until the xylene cyanol had approximatively reached the middle of the gel and were exposed without drying to Kodak XAR film at room temperature.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been included in the GenBank-EMBL database (release 46, updated version of December 11, 1995) as an extension of the formerly published *pvdA* sequence under accession number Z25465.

RESULTS

Localization of iron-regulated promoter regions in plasmid pPV5. The recombinant plasmid pPV5 contains an 8.5-kb *Xho*I fragment encompassing the *pvdA* gene ligated to pUCP19 (60). To identify promoter regions within the 5.9-kb DNA preceding *pvdA*, a high-resolution restriction map of pPV5 was generated (Fig. 1A), and various restriction fragments originating from pPV5 by single or double restriction enzyme digestions were subcloned in both orientations upstream of the reporter *lacZ* gene of the promoter probe vector pMP220. The resulting constructs were used to measure the β-galactosidase activity in *P. aeruginosa* PAO1. Figure 1B shows the different transcriptional fusions obtained and the β-galactosidase levels in lysates of cells grown under low-iron (DCAA) and high-iron (DCAA supplemented with 100 μM $FeCl_3$) conditions. Plasmids pPV51, pPV53, pPV55, pPV58, pPV512, pPV515, and pPV516 exhibited strong iron-regulated promoter activity, with 11.9- to 22.4-fold increases of β-galactosidase levels under conditions of limiting iron. Plasmid pPV57 also showed iron-regulated *lacZ*

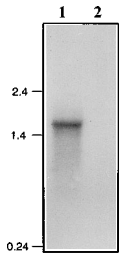


FIG. 2. Northern analysis and transcriptional regulation of *pvdA*. Total RNA was isolated from *P. aeruginosa* cells grown under conditions of iron deficiency (DCAA; lane 1) and iron sufficiency (DCAA supplemented with 100 μM $FeCl_3$; lane 2). The A_{620} of the bacterial cultures was approximately 0.6 for both conditions. Total RNA samples (10 μg in each lane) were hybridized with a 0.5-kb *Pst*I probe internal to the open reading frame of *pvdA* (60) which was labeled with DIG-11-dUTP as described in Materials and Methods. Sizes of RNA standards (in kilobases) are shown on the left.

gene expression, but β-galactosidase activity was increased by a factor of <8 in response to iron deficiency. The other constructs lacked significant promoter activity or directed the expression of the reporter gene in an iron-independent way, as in the case of pPV56. Comparative analysis of β-galactosidase activities expressed by different fusion constructs suggests that at least three main iron-regulated promoters can be located within the 5.9-kb DNA region upstream of *pvdA*. The very high expression levels of the reporter gene in iron-deficient cells and the poor expression in iron-replete cells imply a very stringent control of the transcription directed by these promoters. The *pvdA* promoter, designated P_{pvdA}, is contained within the 154-bp sequence preceding the translation start site of *pvdA*, as revealed by comparing the β-galactosidase activities expressed by deletion constructs originated from pPV55 (i.e., pPV53 and pPV51). A second iron-regulated promoter is located within the 1.3-kb *Sph*I fragment of pPV512 and directs transcription in opposite orientation with respect to P_{pvdA}. The presence of this element might also account for the iron-dependent promoter activity observed for plasmids pPV58 and pPV516. A third strong promoter is located in pPV515, likely within the 0.5-kb DNA region flanking the right *Sph*I site of pPV511, approximately 1.1 kb upstream of the *pvdA* gene (Fig. 1A). This promoter region, which activates transcription in the same orientation as P_{pvdA}, is also located in the proper orientation within plasmid pPV57, although β-galactosidase expression with this construct is lower than that with pPV515. This discrepancy is not understood but might reflect differences in transcript stability due to the presence of additional DNA sequences which negatively affect the expression of the reporter gene in pPV57.

The effect of iron concentration on the expression of the reporter *lacZ* gene under control of P_{pvdA} was evaluated by growing *P. aeruginosa* PAO1(pPV51) and PAO1(pPV55) to mid-logarithmic phase in low-iron medium supplemented with increasing concentrations of $FeCl_3$. Addition of iron at up to 10 μM had a negligible effect on expression of the reporter gene, while promoter activity was completely repressed after addition of 25 μM $FeCl_3$ (data not shown).

Northern analysis and transcriptional regulation of *pvdA*. To investigate whether iron regulates the expression of *pvdA* at the transcriptional level and to estimate the size of the RNA transcript encoding PvdA, Northern blot analyses were performed with total cellular RNA from *P. aeruginosa* PAO1 following logarithmic growth in low- and high-iron medium. The DNA probe was a 0.5-kb *Pst*I fragment internal to the open reading frame of the *pvdA* gene (60). A single hybridization band of approximately 1.6 kb was observed with the RNA extracted from iron-deficient cells, while no bands were detectable in RNA from cells grown in the high-iron medium (Fig. 2), indicating a stringent negative control of *pvdA* transcription by iron. Reprobing of the filter with an *arcA* probe (23) demonstrated the presence of approximately equivalent amounts of transcript, independently of the iron concentration of the medium (data not shown). The size of the transcript was estimated to be approximately 1.6 kb. This value, compared with the length of the *pvdA* open reading frame and with the location of the putative promoter region, suggests that the *pvdA* transcript is monocystronic and that a transcriptional stop signal might be located approximately 300 bp downstream of the translation stop codon of the gene.

To correlate *pvdA* expression with pyoverdin synthesis over the growth cycle, a time course experiment was performed. Synthesis of pyoverdin and transcription of *pvdA* were monitored after subculturing in low-iron medium (DCAA) at the early logarithmic (5 h), late logarithmic (7.5 h), and stationary (10 h) phases. The results reported in Fig. 3 show that the increase in cell density over time is paralleled by the release of pyoverdin in the medium, as determined by both spectrofluorometric and chemical analyses of culture supernatants. Conversely, Northern analysis with the *pvdA* probe clearly shows that maximum level of *pvdA* expression is attained during the early logarithmic phase and that the amount of *pvdA* mRNA is dramatically reduced (by a factor 8.3) when the culture reaches the stationary phase.

Sequencing of the DNA regions flanking *pvdA* and identification of *pvdA* transcriptional start sites. To localize possible transcriptional initiation and termination signals, the DNA regions flanking the *pvdA* open reading frame were sequenced (Fig. 4). Analysis of the 3′ region revealed the presence of an extended inverted repeat (bp 1661 to 1717) generating a potential stem-loop-like element downstream of the *pvdA* open reading frame. This secondary structure may be predicted to have a high thermodynamic stability (−58.3 kcal [ca. −244 kJ]/mol, as deduced by computer analysis), and it might be involved in the generation of the 3′ terminus of the *pvdA*

	Time (h)		
	5	7.5	10
Growth (A_{600} of the culture)	0.37	0.72	0.90
Relative fluorescence emission	0.62	4.47	10.42
Hydroxamate concn (μM)	13	98	203
Northern analysis of the *pvdA* transcript			
Specific β-emission (cpm/μg RNA)	6.7	5.4	0.8

FIG. 3. Correlation of pyoverdin synthesis with *pvdA* transcription over the growth cycle of *P. aeruginosa* PAO1 in low-iron medium (DCAA). Cells and culture supernatants were collected from early exponential (5 h, A_{600} = 0.37), late exponential (7.5 h, A_{600} = 0.72), and stationary (10 h, A_{600} = 0.90) cultures. Pyoverdin production was detected by fluorometric determinations (emission at 455 nm after excitation at 405 nm) of supernatants diluted 1:10 in 200 mM Tris-HCl (pH 11) as reported previously (60, 62). Hydroxamate levels were determined in culture supernatants as described elsewhere (24), using a standard curve obtained with known quantities of hydroxylamine hydrochloride in DCAA. Total RNA (10 μg of each sample) extracted from cells at the different growth stages was hybridized with a 0.5-kb *Pst*I probe internal to the open reading frame of *pvdA* (60) which was labeled with [α-^{32}P]dCTP as described in Materials and Methods. Filters were analyzed with a Betascope model 603 blot analyzer (Betagen). The specific β-emission value of each band was normalized by subtracting the background level of the filter.

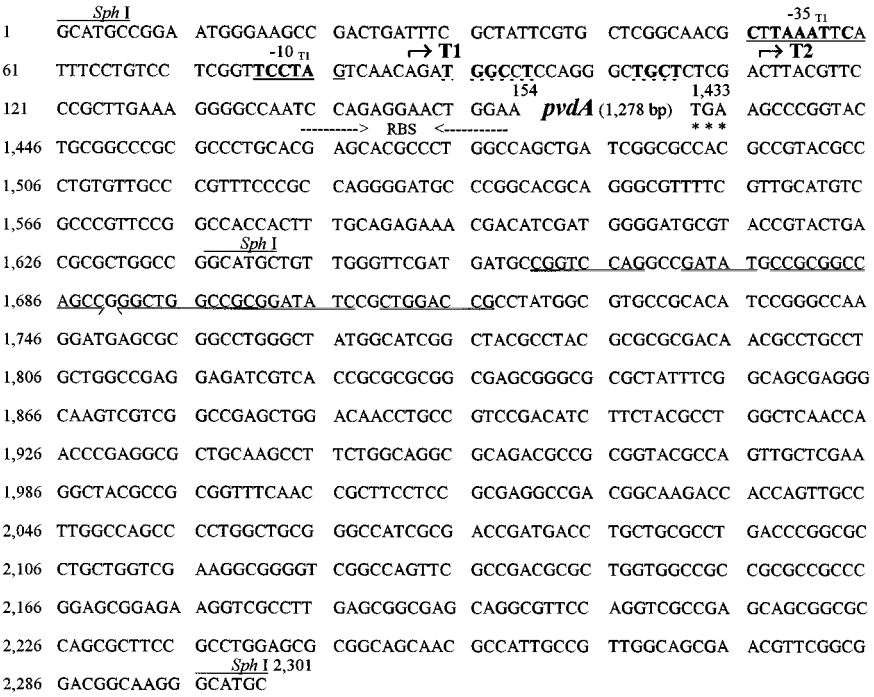


FIG. 4. Nucleotide sequence of the DNA regions flanking the *pvdA* gene. The location of *Sph*I sites is indicated, as is the location of the *pvdA* coding sequence (60; GenBank-EMBL accession number Z25465) from the ATG translation start site (not shown) to the termination codon TGA (nt 1433 to 1435; marked by asterisks). The positions of the 5′ ends of transcripts T1 and T2, as determined by S1 nuclease mapping and primer extension analysis, are indicated by bent arrows above the nucleotides at position +1 (see Fig. 5 for details). -10_{T1} and -35_{T1} indicate homology regions between the −10 and −35 motifs of T1 and the consensus sequences TCCTAT (34, 50, 68) and (G/C)CTAAATCCC (47), respectively. Dotted lines underscore the sequence elements upstream of the T2 start site with sequence similarity to σ^{54}-dependent promoters (18, 48). Conserved nucleotides are in boldface. A stem-loop structure encompassing the potential ribosome-binding site (RBS) of *pvdA* is underscored by dashed arrows. An inverted repeat probably involved in the generation of the 3′ terminus of the *pvdA* mRNA is double underlined.

mRNA, in agreement with the transcript length estimate as deduced from Northern analyses.

The initiation sites of *pvdA* transcription were determined by both S1 mapping and primer extension analyses. A 1,134-bp single-stranded antisense DNA probe spanning from the eighth codon of *pvdA* to the upstream *Bgl*II site (Fig. 1A) was hybridized with total RNA extracted from iron-deficient *P. aeruginosa* cultures. Comparison of the S1 mapping products with the sequence obtained by using the primer used for pPV226 DNA sequencing indicated the presence of two protected fragments (Fig. 5, lane 2) with 5′ ends corresponding to the A and C residues located at positions 87 and 112 of the sequence shown in Fig. 4. To substantiate this pattern of initiation, primer extension analysis was carried out on a different RNA preparation from iron-deficient *P. aeruginosa* PAO1 cultures, with the oligonucleotide, RV*P*pa, used to prime antisense DNA synthesis for S1 mapping. The results (Fig. 5, lane 3) indicated that the cDNA transcripts comigrated with G and T residues of the reference sequence, thus confirming the two transcriptional start sites deduced from the S1 mapping experiment. Quantitative estimation of S1-protected fragments and primer extension products showed that the level of the main transcript, originating from the A residue at position 87 (T1), was nearly 10-fold higher than that of the transcript arising from the C residue at positions 112 (T2).

The untranslated leaders of the *pvdA* transcripts contained poor secondary structure except for the presence of a 5-bp inverted repeat encompassing the ribosome-binding site of the *pvdA* gene. This sequence element could generate a stem-loop secondary structure which could affect mRNA stability or translation efficiency (28).

No sequence similarity was detected when the −10 and −35 sequences 5′ of T1 were aligned with the corresponding regions of T2. Thus, DNA sequences directly upstream of the start sites of T1 and T2 were compared with a number of well-characterized *P. aeruginosa* promoter sequences. Interestingly, the −10 region upstream of the T1 (TCCTAG) was found to be fairly similar to the corresponding regions of the *algD* promoter (TCCTAT) and of the *algU* P_1 and P_3 promoters (TCTACT and TCCAAA, respectively). The *algD* gene, encoding the enzyme GDP-mannose dehydrogenase involved in alginate biosynthesis, is positively regulated by the *algU* gene product (50, 68). AlgU belongs to the ECF family of sigma factors (34, 68) and is known to control its own expression by activating transcription directed by the *algU* P_1 and P_3 promoters (50). Homology (66% conservation) was also detected between sequences spanning from positions −32 to −24 of the *algD* promoter and from positions −33 to −25 of the T1 start site. Within this sequence range, 66% similarity was also observed between the *regAB* P2 promoter (63) and the T1 control region. However, the proposed −35 consensus of σ^E-dependent promoters ($^{-36}$CCGGAACTT^{-28} [34]), which is highly conserved in the AlgU-dependent promoters, appeared to be poorly represented (55% similarity) in both the *regAB* P2 promoter and the putative T1 control region (data not shown).

Because of the peculiar structural and regulatory features of the putative *pvdA* promoter, we aligned the sequence 5′ of the T1 initiation site with promoter sequences of iron-regulated genes involved in the biosynthesis of fluorescent siderophores in *Pseudomonas* spp. Promoters were selected among those known to be activated by σ^E-like transcription factors, i.e., the PvdS protein of *P. aeruginosa* (15, 37) and PbrA protein of

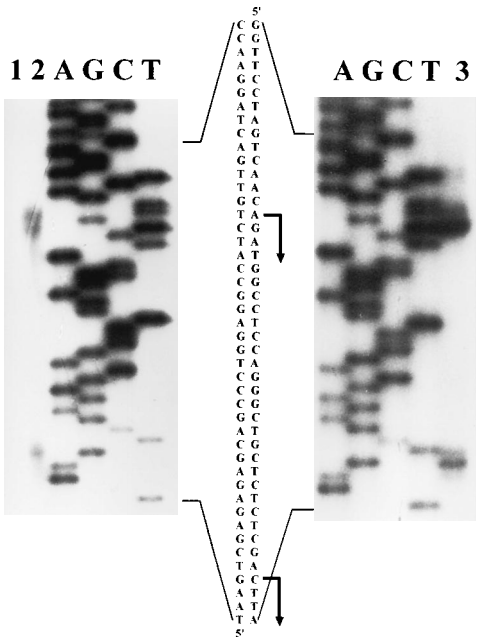


FIG. 5. Localization of the transcriptional start sites of *pvdA*. S1 nuclease mapping of the *pvdA* transcript was performed with an antisense single-stranded DNA probe spanning from the eighth codon of *pvdA* to the upstream *Bgl*II site (Fig. 1A). Primer extension was carried out with a 5′-end-labeled 24-mer, designated RV*P*pa, complementary to the initial eight codons of *pvdA*. Experimental details regarding S1 mapping and primer extension reactions are given in Materials and Methods. Lane 1, S1 protection assay without RNA (negative control); lane 2, S1 protection assay with RNA; lane 3, primer extension analysis of the *pvdA* mRNA. Lanes A, G, C, and T represent sequencing ladders of pPV226 with the oligonucleotide RV*P*pa. These were run in parallel with S1-protected fragments and primer extension products to determine exactly the 5′ ends of the transcripts. The double-stranded DNA sequence of the region is shown between the two autoradiograms, with transcriptional start sites and direction of transcription indicated by arrows.

Pseudomonas fluorescens (52). Multiple alignments evidenced poor homology among sequences 5′ of the start sites of T1, *pvdD*, and *pvdE* transcripts of *P. aeruginosa* (37, 47) and of the SP1 promoter transcript of *P. fluorescens* M114 (41). Nevertheless, the sequence $^{-36}$CT TAAATTCA^{-27}, resembling the consensus (G/C)CTAAATCCC (47), was found to be located in the −35 region upstream of the T1 start site. This motif, termed the iron starvation box, was found in the promoter regions of some iron-regulated transcriptional units of fluorescent pseudomonads and has been proposed to act as a recognition site for a transcriptional activator of pyoverdin genes (47). Of note, sequence motifs with different degrees of similarity to the iron starvation box were found to be centered close to the −35 region of both *pvdD* ($^{-37}$GCTAAATCCC^{-27}) and SP1 ($^{-42}$GGTAAACGC^{-33}) promoters. Additional regions of elevated homology with the iron starvation box were identified in the SP1 promoter from bp −92 to −83 and in the *pvdE* promoter from bp −23 to −14 relative to the transcription start site (data not shown).

Analysis of −10 and −35 regions upstream of the minor transcript T2 revealed the presence of the motif TGGCCT-7 bp-TGCT, from positions −22 to −6, resembling the consensus sequence of activated promoters transcribed by σ^{54}-like RNA polymerase holoenzyme of *P. aeruginosa* (18, 48). This apparent similarity prompted us to evaluate the role of RpoN in the transcriptional control of *pvdA* promoter. A transcriptional *pvdA*::*lacZ* fusion was generated in the promoter probe vector pME3536 (65), and β-galactosidase levels specified by the resulting construct, pBL2, were measured in *P. aeruginosa* PAO1, PAK (*rpoN*$^+$), and PAK-N1 (*rpoN*) (29) grown under low-iron (DCAA) and high-iron (DCAA supplemented with 100 μM $FeCl_3$) conditions. In *rpoN*$^+$ and *rpoN* backgrounds of *P. aeruginosa* PAK, the *pvdA*::*lacZ* fusion was expressed at nearly identical levels under low-iron conditions (12,983 ± 814 and 12,188 ± 971 Miller units, respectively), and similar regulation by iron was observed (approximately 30-fold reduction of β-galactosidase activity in iron-rich cultures). Moreover, the specific β-galactosidase activities were not significantly different in the two *rpoN*$^+$ strains PAK and PAO1 grown under low- and high-iron conditions (for the latter strain, 12,623 ± 1,022 and 439 ± 56 Miller units, respectively).

Primer extension experiments performed on total RNA extracted from iron-deficient cells of *P. aeruginosa* PAK and PAK-N1 showed no difference in the transcription initiation pattern between *rpoN*$^+$ and *rpoN* strains. As in the case of PAO1, the most abundant transcript (T1) originated from bp 87 of the sequence reported in Fig. 4, while the minor transcript (T2), accounting for approximately 1/10 of the total *pvdA* mRNA, started 25 bp downstream (Fig. 6). These results indicate that transcription directed by the *pvdA* promoter does not require the *P. aeruginosa* σ^{54}-like RNA polymerase holoenzyme.

Minimum sequence required for iron-regulated expression of the *pvdA* promoter. To define precisely the minimum sequence required for iron-regulated expression of the *pvdA* promoter, deletion analysis of the DNA region encompassing the *pvdA* transcription start points was carried out (Fig. 7). Derivatives of pPV51 carrying deletions extending from the *Sph*I site downstream to bp 38 (pPV51Δ1-19 and pPV51Δ1-38) exhibited severe reduction of β-galactosidase activity measured under low-iron conditions compared with the full-length construct. Deletion of more than 38 bp, relative to the *Sph*I site of pPV51, completely abrogated promoter activity, as revealed by the basal levels of β-galactosidase expressed under low-iron conditions by plasmids pPV51Δ1-54, pPV51Δ1-81, and pPV51Δ1-101. It is interesting that in these three constructs, the putative iron starvation box (47) was either mutagenized (CTCGAAT TCA in pPV51Δ1-54) or completely deleted (in pPV51Δ1-81

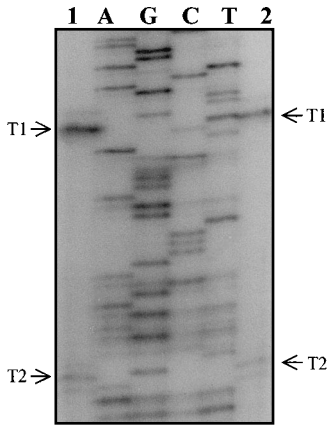
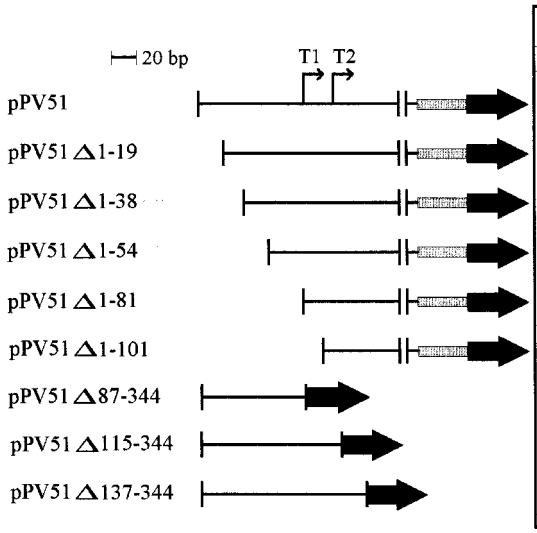


FIG. 6. Initiation patterns of the *pvdA* transcript in *rpoN*$^+$ and *rpoN* backgrounds of *P. aeruginosa* PAK (29). Primer extension was carried out with a 5′-end-labeled 24-mer, designated RV*P*pa, complementary to the initial eight codons of *pvdA*. Experimental details regarding primer extension reactions are given in Materials and Methods. Lanes 1 and 2, primer extension analysis of the *pvdA* mRNA from strains PAK (*rpoN*$^+$) and PAK-N1 (*rpoN*), respectively. Lanes A, G, C, and T represent sequencing ladders of pPV226 with the oligonucleotide RV*P*pa. These were run in parallel with primer extension products to determine exactly the 5′ ends of the transcripts.



	LacZ activity		Ratio	Promoter activity (%)
	-Fe (III)	+Fe (III)		
pPV51	10,318	486	21.2	100.0
pPV51 Δ1-19	1,643	265	6.2	15.9
pPV51 Δ1-38	1,655	302	5.5	16.0
pPV51 Δ1-54	395	288	1.4	3.8
pPV51 Δ1-81	278	280	1.0	2.7
pPV51 Δ1-101	292	312	0.9	2.8
pPV51 Δ87-344	314	321	1.0	3.0
pPV51 Δ115-344	10,621	495	21.5	102.9
pPV51 Δ137-344	10,412	472	22.1	100.9

FIG. 7. PCR-mediated deletion analysis of the *pvdA* promoter. Primers used for the amplification are described in Materials and Methods. Plasmid designations are given on the left, and DNA inserts are aligned with the pPV51 map. Bent arrows denote the locations of transcription initiation sites of T1 and T2. Large arrows show the direction of transcription of the reporter *lacZ* gene from the promoter probe vector pMP220 (53); stippled bars indicate a portion of the *pvdA* gene. Note that the reference bar is relative only to bp 1 to 160, while the rest of the figure is not to scale. The expression of β-galactosidase (LacZ) from each of the constructs in *P. aeruginosa* PAO1 during growth under low- and high-iron conditions is given on the right. LacZ activity values, in Miller units (36), are the means of six determinations. The standard deviation is <18% of each value. −Fe(III), growth under conditions of iron deficiency (DCAA); +Fe(III), growth under conditions of iron sufficiency (DCAA supplemented with 100 μM $FeCl_3$). Promoter activity was estimated for iron-deficient cultures and expressed as percent of β-galactosidase activity relative to the level of pPV51.

and pPV51Δ1-101). Deletions extending from the minor transcription start point downstream to the *Bgl*II site at bp 344 in the *pvdA* coding region (pPV51Δ115-344 and pPV51Δ137-344) did not affect β-galactosidase expression directed by the *pvdA* promoter, while a deletion extending upstream of the minor transcription start point (pPV51Δ87-344) completely abrogated promoter activity. Taken together, these results suggest that the DNA region encompassing bp 1 to 114 (Fig. 4) contains the minimum sequence required for full iron-regulated promoter activity.

Regulation of *pvdA* expression by iron in *P. aeruginosa* Fur$^+$ and Fur$^-$ backgrounds. To evaluate the role of the Fur protein in the regulation of iron-dependent *P. aeruginosa* promoters, plasmids pPV51, pPV55, and pPV512 were transferred to a set of *P. aeruginosa fur* mutants, including PAO1 A4 and PAO6261 C6. The *fur-4* and *fur-6* mutations in PAO1 A4 and PAO6261 C6, respectively, lead to deregulation of siderophore synthesis (5) and to constitutive production of pyoverdin in DCAA supplemented with 100 μM $FeCl_3$ (data not shown). Moreover, the *anr*^{0} mutation in PAO6261 has no appreciable effect on siderophore synthesis (5). Strain PAO6261 showed iron-dependent expression of β-galactosidase activity with all four constructs, comparable to that of wild-type PAO1. Conversely, expression of the reporter gene in the *fur* mutants was significantly increased under high-iron conditions (Table 2). However, the ratios of reduction of β-galactosidase activities by addition of $FeCl_3$ were ≥1.8, and the β-galactosidase activities expressed in the *fur* mutants grown under iron-rich conditions did not attain the levels of the parental strain grown in the low-iron medium. Thus, promoters cloned in pPV51, pPV55, and pPV512, although active under high-iron conditions in a *fur* mutant background, display residual iron-dependent regulation. This finding might indicate that Fur proteins expressed by the three mutants retain partial repressor activity, although the possible involvement of factors other than Fur in the transcriptional control of P_{pvdA} and of other iron-regulated pyoverdin promoters cannot be ruled out.

To confirm that transcription of *pvdA* is regulated by Fur, Northern blot analyses on total RNA extracted from PAO6261, PAO6261 C6, and PAO1 A4 grown under low- and high-iron conditions were conducted. Figure 8 shows that levels of *pvdA* transcript are slightly higher in iron-deficient cells of the *fur* mutants (lanes 3 and 5) than in the *fur*$^+$ strain (lane 1). As expected, addition of 100 μM $FeCl_3$ to the culture medium totally repressed *pvdA* expression in PAO6261 (lane 2), while transcription of *pvdA* in iron-rich cultures of both *fur* mutants was not less than in iron-deficient medium (lanes 4 and 6). These results clearly indicate that *fur* is involved in transcriptional control of *pvdA*.

Regulation of *pvdA* expression by iron in *P. aeruginosa* PvdS$^+$ and PvdS$^-$ backgrounds and PvdS-dependent transactivation of *pvdA* expression in *E. coli*. Previous results (15, 57) have shown that the ECF-like sigma factor PvdS is required for expression of promoters of pyoverdin synthesis genes in *E. coli*, in which they are otherwise inactive. The *pvdS* gene itself is iron regulated, and at least in *E. coli*, *pvdS* transcription has been proposed to be under the control of the Fur repressor protein (15, 39).

To assess the role of PvdS in transcriptional control of additional iron-regulated promoters of pyoverdin genes, the β-galactosidase levels expressed under low- and high-iron conditions by pPV51, pPV55, and pPV512 were compared in *P. aeruginosa* PAO OT11 and PAO OT11*pvdS*, which are isogenic except for a *pvdS*::*kan* mutation in the latter strain. Table 2 shows that for all constructs, the expression of the reporter gene was normally regulated by iron in the *pvdS*$^+$ background but was dramatically reduced when pPV51, pPV55, and pPV512 were tested in the *pvdS* mutant. These results indicate that expression of the reporter gene directed by P_{pvdA} and by the promoter cloned in pPV512 is dependent on the *pvdS* gene. In line with these observations, both Northern blot and S1 mapping experiments did not enable detection of *pvdA* mRNA in iron-deficient cultures of PAO OT11*pvdS* (data not shown).

To confirm the involvement of PvdS in the activation of iron-

TABLE 2. Effects of *fur* and *pvdS* mutations on promoter activity of iron-regulated transcriptional fusions in *P. aeruginosa* PAO

Strain	Genomic mutation	Plasmid	LacZ activity[a]		Ratio	
			−Fe(III)	+Fe(III)	−Fe(III)/+Fe(III)	Parental/mutant[b]
PAO6261	*anr*^{0}	pMP220	266	148	1.8	
	anr^0	pPV51	9,871	423	23.3	
	anr^0	pPV55	8,205	387	21.2	
	anr^0	pPV512	3,454	312	11.1	
PAO OT11	*leu-1 pro-1*	pMP220	310	163	1.9	
	leu-1 pro-1	pPV51	11,437	502	22.8	
	leu-1 pro-1	pPV55	6,284	322	19.5	
	leu-1 pro-1	pPV512	3,146	323	9.7	
PAO1 A4	*fur-4*	pMP220	212	144	1.5	1.2
	fur-4	pPV51	10,622	5,923	1.8	11.8
	fur-4	pPV55	7,290	2,551	2.8	6.7
	fur-4	pPV512	4,208	1,718	2.4	6.6
PAO6261 C6	*anr*^{0} *fur-6*	pMP220	251	138	1.8	1.0
	anr^0 *fur-6*	pPV51	10,297	4,102	2.5	9.4
	anr^0 *fur-6*	pPV55	11,542	3,205	3.6	5.9
	anr^0 *fur-6*	pPV512	3,693	1,921	1.9	5.8
PAO OT11*pvdS*	*leu-1 pro-1 pvdS*::*kan*	pMP220	297	179	1.7	0.9
	leu-1 pro-1 pvdS::*kan*	pPV51	545	488	1.1	<0.1
	leu-1 pro-1 pvdS::*kan*	pPV55	386	411	0.9	<0.1
	leu-1 pro-1 pvdS::*kan*	pPV512	606	372	1.6	0.2

[a] β-Galactosidase (LacZ) activity was determined in culture lysates of *P. aeruginosa* grown in DCAA [−Fe(III)] and DCAA supplemented with 100 μM $FeCl_3$ [+Fe(III)]. Units of β-galactosidase are as defined by Miller (36) and correspond to means of six determinations. The standard deviation is <18% of each value.
[b] Obtained dividing the ratio −Fe(III)/+Fe(III) of the parental strain by the corresponding value of the mutant strain. The values for *P. aeruginosa* PAO1 were derived from Fig. 1B.

dependent *P. aeruginosa* promoters, plasmids pPV51, pPV55, and pPV512 were introduced into *E. coli* MC4100 carrying plasmid pBRXB, which contains a DNA insert encompassing the entire *pvdS* gene with its putative control region (15). Promoter activity was monitored by measuring β-galactosidase levels under conditions of iron deficiency (M9 supplemented with 150 μM 2,2′-dipyridyl) and iron sufficiency (M9 supplemented with 100 μM $FeCl_3$). Table 3 shows that P_{pvdA} and the iron-regulated promoter cloned in pPV512 were silent in *E. coli* MC4100 unless in the presence of the *pvdS* gene. There were differences in the extent of transactivation between P_{pvdA} (plasmids pPV51 and pPV55) and the promoter region cloned in pPV512. Levels of β-galactosidase expressed in *E. coli* MC4100 carrying pBRXB and pPV51 or pPV55 were greatly increased under conditions of limiting iron, although reporter gene expression could also be demonstrated in the presence of sufficient iron. Conversely, promoter activity expressed by pPV512 was much lower than that expressed by the other constructs and was undetectable in iron-sufficient cultures. These results may indicate that the dosage of PvdS, which has been proposed to be regulated at the transcriptional level by

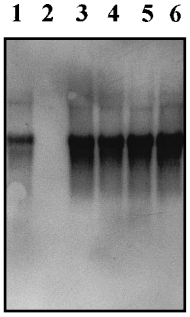


FIG. 8. Effect of the *fur* mutation on the transcription of *pvdA*. Northern hybridization analysis was carried out with 10 μg of total RNA extracted from *P. aruginosa* PAO6261 (*anr*^{0}; lanes 1 and 2), PAO6261 C6 (*anr*^{0} *fur-6*; lanes 3 and 4), and PAO1 A4 (*fur-4*; lanes 5 and 6). Transcript levels were compared in cells grown at an A_{600} of ≅0.6 in iron-poor medium (DCAA; lanes 1, 3, and 5) and iron-rich medium (DCAA supplemented with 100 μM $FeCl_3$; lanes 2, 4, and 6). Filters were hybridized with the 0.5-kb *Pst*I probe internal to the open reading frame of *pvdA* which was labeled by random priming with DIG-11-dUTP as outlined in Materials and Methods.

TABLE 3. PvdS-dependent transactivation of iron-regulated transcriptional fusions in *E. coli* MC4100

Plasmids	LacZ activity[a]			
	M9 + 2,2′-dipyridyl[b]	M9 + $FeCl_3$[c]	LB	LB + IPTG[d]
pMP220 + pBR322	47	44		
pPV51 + pBR322	41	41		
pPV55 + pBR322	46	42		
pPV512 + pBR322	40	42		
pMP220 + pBRXB	48	51		
pPV51 + pBRXB	299	142		
pPV55 + pBRXB	347	145		
pPV512 + pBRXB	81	49		
pMP220 + pQE60			47	51
pPV51 + pQE60			45	47
pPV55 + pQE60			49	49
pPV512 + pQE60			48	45
pMP220 + pQE*pvdS*			49	44
pPV51 + pQE*pvdS*			63	1,926
pPV55 + pQE*pvdS*			64	2,103
pPV512 + pQE*pvdS*			65	148

[a] Units of β-galactosidase (LacZ) are as defined by Miller (36) and correspond to means of six determinations. The standard deviation is <14% of each value.
[b] M9 minimal medium was supplemented with 150 μM 2,2′-dipyridyl to reduce iron availability.
[c] M9 minimal medium was supplemented with 100 μM $FeCl_3$ to increase iron availability.
[d] Cultures (A_{620} ≅ 0.4) were induced with 1 mM IPTG and then incubated for 3 h at 37°C prior to testing for β-galactosidase activity. Uninduced cultures were used as controls.

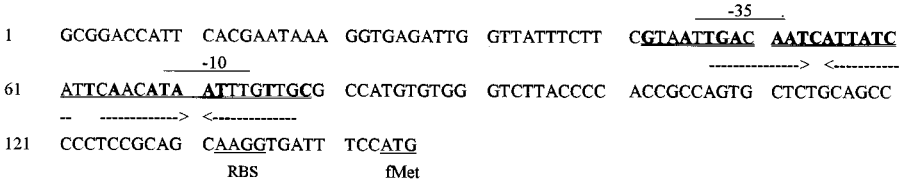


FIG. 9. Organization of the putative promoter region of the *pvdS* gene (15). The proposed −10 and −35 recognition hexamers for vegetative (σ^{70}) RNA polymerase are overlined. The potential ribosome-binding site (RBS) and the ATG translation start codon (fMet) are underlined. Regions of homology to the Fur consensus are doubly underlined, with conserved nucleotides in boldface. Two imperfect inverted repeats overlapping the putative Fur-binding motifs in the −10 and −35 regions are shown by dotted arrows below the sequence.

the Fur repressor protein (15, 39), modulates the activities of promoters cloned in pPV51, pPV55, and pPV512. To assess the role of PvdS in the transcriptional activation of each of the pyoverdin promoters and to rule out the possibility that the observed effects were due, at least in part, to a direct activity of Fur on the *pvdA* promoter, we removed the indigenous iron-regulated promoter with its putative Fur box from the *pvdS* gene and cloned the *pvdS* open reading frame under the control of the hybrid P_{T5}-*lacO* promoter-operator in the expression vector pQE60. The resulting construct, pQE*pvdS*, was introduced into *E. coli* MC4100 carrying the repressor plasmid pDMI,1, and β-galactosidase activities expressed by pPV51, pPV55, and pPV512 were measured in LB (an iron-rich medium) with or without IPTG induction. Results shown in Table 3 demonstrate that β-galactosidase is expressed after IPTG induction from plasmids pPV51, pPV55, and pPV512. The extent of PvdS-dependent activation of transcriptional fusions was greater in induced cultures carrying pQE*pvdS* than in iron-poor cultures carrying pBRXB, probably because of the higher expression of the PvdS protein in the pQE system. Also in this case, however, pPV51 and pPV55 exhibited stronger promoter activities (approximately 30-fold increase after IPTG induction) than pPV512 (nearly 2-fold increase after IPTG induction), suggesting that additional factors may be required for complete activation of the iron-regulated *P. aeruginosa* promoter cloned in pPV512. These results also indicate that at least in *E. coli*, the sigma factor PvdS is sufficient to activate transcription from P_{pvdA}. Repression of *pvdA* expression by iron could therefore be attributed to a direct activity of the Fur repressor protein on the *pvdS* promoter-operator region.

In vitro and in vivo interaction of Fur with the *pvdS* putative promoter region. Analysis of the putative promoter region of the *pvdS* gene (15) revealed the presence of two imperfect inverted repeats overlapping the −10 and −35 recognition sequences for vegetative RNA polymerase. These structures are shifted 3 and 5 bp downstream of sequence motifs exhibiting 48 and 79% homology with the *E. coli* Fur consensus, respectively (Fig. 9). To determine whether these sequences can function as Fur-binding sites, plasmids pBRXB and pBRP_{pvdS} were introduced into *E. coli* H1717 and assayed in the Fur titration assay (55). *E. coli* H1717 is an enterochelin-defective strain containing a *fhuF*::*lacZ* fusion which is exceptionally sensitive to changes of iron concentration because of the weak affinity of the Fur-Fe(II) repressor complex for the *fhuF* promoter (55). Introduction of a high-copy-number plasmid carrying a Fur-binding sequence in *E. coli* H1717 will therefore titrate out the available Fur pool, thus allowing transcription of the *fhuF*::*lacZ* fusion. Introduction of pBRXB and pBRP_{pvdS} into *E. coli* H1717 produced red (Lac^+) colonies at iron concentrations of ≥120 μM, while both pBRP_{pvdA} and the control vector pBR322 gave white (Lac^-) colonies at ≥40 μM $FeCl_3$. This result indicates that the *E. coli* Fur repressor protein recognizes sequence motifs in the putative *pvdS* promoter region, suggesting that in *P. aeruginosa*, iron-regulated expression of PvdS is controlled by the Fur homolog. Conversely, the negative response of pBRP_{pvdA} in the Fur titration assay provides further evidence of the absence of Fur-binding motifs within the *pvdA* promoter.

To demonstrate in vitro binding of the Fur repressor to the putative promoter region of *pvdS*, gel retardation assays were performed. The *P. aeruginosa* PAO1 *fur* gene was amplified by PCR and cloned in the expression vector pQE60 under P_{T5}-*lacO* control. The resulting construct, designated pQE*fur*, was introduced into *E. coli* JM109 and used for the production of the Fur protein. *E. coli* JM109 (pQE*fur*) was grown to the early logarithmic phase and induced with 1 mM IPTG to allow expression of the cloned *fur* gene. Comparative analysis of SDS-PAGE profiles of uninduced and IPTG-induced crude cell lysates indicated that Fur was overexpressed after induction, accounting for approximately 50% of total cellular proteins (data not shown). To obtain partial purification of Fur, induced cells were permeabilized by repeated cycles of freezing and thawing. This procedure liberated a substantial fraction of the overexpressed Fur, with poor contamination by the bulk of endogenous cellular protein. Partially purified Fur was analyzed on SDS-PAGE and found to be 72% pure after densitometric scanning of the gel (data not shown). Sequencing of the whole DNA insert of pQE*fur* indicated that mispriming did not occur during amplification. Automated Edman degradation of the protein sample electroblotted after SDS-PAGE gave the sequence MVENSELRKAGLKVTLPRVKILQMLD SAEQ, corresponding to the expected N-terminal sequence of the *P. aeruginosa* Fur protein (45).

A 150-bp DNA probe encompassing the putative *pvdS* promoter and containing the proposed consensus sequence for binding of the Fur repressor protein (15, 39) was used in protein binding assays with increasing amounts of Fur in the presence of $MnCl_2$, (Fig. 10). Addition of 50 to 60 pmol of Fur to the *pvdS* probe (form I) produced one poorly represented transitional species with lower mobility (form II) that gives way to a maximally shifted fragment (form III) at higher concentrations of Fur (from 7 up to 30 μM). Under the same experimental conditions, the mobility of the *pvdA* probe was not affected by the addition of Fur in the gel retardation assay (Fig. 10). Remarkably, no shift of the *pvdS* probe was observed when the Fur repressor was replaced by an equivalent amount of protein extracted from uninduced cells of JM109(pQE*fur*) or in the absence of the metal cofactor $MnCl_2$, while replacement of $MnCl_2$ with freshly prepared $FeCl_2$ allowed Fur binding, although at higher protein concentrations (data not shown). These observations indicate that the *P. aeruginosa* Fur repressor protein interacts with the putative promoter-operator region of the *pvdS* gene only as a complex with a metal cofactor [Mn(II) or Fe(II)], but it does not recognize sequence elements located within the *pvdA* promoter. The identification of two transitional forms of the Fur-DNA complex suggests the

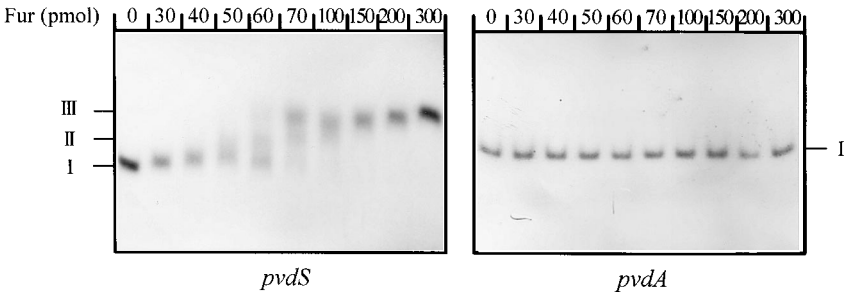


FIG. 10. Gel retardation assays of in vitro binding of the *P. aeruginosa* Fur repressor to the promoter regions of *pvdS* (left) and *pvdA* (right). The DNA probes were labeled with [α-^{32}P]dCTP by PCR and incubated with increasing amounts of the Fur protein under the experimental conditions described in Materials and Methods. Fur-bound fragments (forms II and III) were separated from the unbound form (I) on a 5% polyacrylamide gel run in BisTris-boric acid buffer (pH 7.5)–100 μM $MnCl_2$. In each case, 0.1 pmol of DNA probe was used in a 10-μl reaction volume with various amounts of the Fur protein as indicated at the top.

presence in the *pvdS* promoter of primary and secondary binding sites for the Fur repressor, as demonstrated by previous studies of Fur-regulated promoters (5, 17, 28, 39, 40).

DISCUSSION

Iron deficiency is an important environmental stimulus to bacteria for coordinate regulation of gene expression. A number of pathogenic bacteria, including *P. aeruginosa*, respond to the sensing of low iron levels in the environment by the coordinate activation of siderophore and virulence genes (33). Iron-dependent expression of *P. aeruginosa* virulence-associated factors is primarily controlled at the transcriptional level and involves multiple regulatory components (21, 45–47, 56, 63).

The overall aim of this investigation was to gain insight into the molecular mechanisms controlling the expression of the *pvdA* gene, encoding the enzyme L-ornithine N^5-oxygenase in *P. aeruginosa* PAO1. Preliminary observations indicated that the 154-bp sequence upstream of the *pvdA* open reading frame contained sufficient information to govern the iron-repressible expression of PvdA from a multicopy plasmid in *P. aeruginosa* (60) but lacked homology with known promoter-like motifs of *P. aeruginosa* (18, 48) and with the Fur consensus sequence (16, 33). It was also noticed that the presence of the multicopy *pvdA* gene in *trans* did not cause expression of the L-Orn hydroxylating activity in iron-rich *P. aeruginosa* cells (60). These results suggested that iron-dependent repression of *pvdA* expression by binding of the Fur repressor protein to the putative promoter-operator regions of *pvdA* was unlikely.

In this study, we localized three tightly iron-regulated promoters within the 5.4-kb DNA region upstream of *pvdA*. A strong promoter was contained within the 154-bp fragment 5′ of the translation start site of the *pvdA* gene, while the remaining two promoters were located more than 1 kb upstream of *pvdA* and appeared to be divergently oriented. Recently, three insertions of Tn*1737*KH, a type I transcription probe transposon containing a promoterless *lacZ* gene, were mapped in the DNA region encompassing the *pvdA* gene, all of which abolished pyoverdin synthesis (57). Two insertional mutations, *pvd-30* and *pvd-45*, gave rise to elevated iron-regulated β-galactosidase activity and, by comparison with the pPV5 physical map reported in this paper, can be located within and downstream of the *pvdA* gene. The third Tn*1737*KH insertion, *pvd-38*, was mapped close to the *Hin*dIII site upstream of *pvdA* and exhibited a low level of iron-regulated transcription. According to these data, transcriptional directions of the reporter *lacZ* gene in pPV51 and pPV55 are the same as in *pvd-30* and *pvd-45*, while the promoter cloned in pPV56, although oriented as the reporter gene in *pvd-38*, did not appear to be regulated by iron in *P. aeruginosa*. Cumulatively, these results also suggest that additional iron-regulated transcriptional units of pyoverdin genes could be located upstream of *pvdA*, within the so-called pyoverdin region A (57).

Northern blot analyses provided direct evidence that *pvdA* expression is negatively regulated at the transcriptional level by the iron concentration in the culture medium. Quantitative estimation of the *pvdA* mRNA during a growth cycle in low-iron medium showed that the highest transcript levels were reached during the early logarithmic phase. Conversely, the maximum pyoverdin concentration was attained at the stationary phase. These results agree with a previous report indicating that aerobic *P. aeruginosa* cultures produce the highest siderophore levels during the late portion of the logarithmic phase, probably as a consequence of the increased iron demand for heme synthesis (14). Therefore, it can be speculated that an initial response of *P. aeruginosa* to iron-deficient growth is the activation of transcription of pyoverdin biosynthesis genes, which are switched off when the amount of released siderophore is sufficient to support its strictly aerobic metabolism.

S1 mapping and primer extension analyses of the *pvdA* mRNA demonstrated that transcription arises from two distinct start sites. This is not unusual for *P. aeruginosa*, as multiple promoters and/or multiple transcription initiation sites have been identified upstream of iron-regulated genes, including *pvdD*, *regAB*, and *toxA* (47, 56, 63). The most abundant transcript, T1, originated 68 bp upstream of the translation start site of *pvdA*, while the second transcript, T2, originated 25 bp downstream of the T1 initiation site and accounted for approximately 1/10 of the total *pvdA* mRNA. The *pvdA* transcripts were monocistronic, with a length of approximately 1.6 kb, as estimated from Northern analysis. In line with this observation, sequencing of the DNA region 3′ of the *pvdA* gene revealed the presence of a 57-bp potential stem-loop-like structure centered 255 bp downstream of the PvdA coding sequence, probably involved in the generation of the 3′ terminus of the *pvdA* mRNA either by protection from 3′ exonucleolytic digestion or by 3′ processing.

Results of homology studies suggest that the *pvdA* promoter has unique structural features. The −10 region of T1 exhibited a fair degree of similarity with −10 regions of AlgU-dependent promoters (18, 50, 68) and showed partial similarity to the −10 consensus sequence identified by Lonetto and colleagues in a group of promoters dependent on the activation by ECF-like sigma factors (34). It was also interesting that the DNA region extending from bp −33 to −25 relative to the start site of the T1 transcript matched 6 of 9 nt with the corresponding region

of the *regAB* P2 promoter, which has been reported to be tightly regulated by iron (21, 63). Furthermore, the −35 region of T1 showed a high degree of conservation when compared with the consensus (G/C)CTAAATCCC found in the promoter regions of *P. aeruginosa* iron-regulated genes, including *pvdD*, *pvdE*, and *toxA*, as well as in iron-regulated promoters of fluorescent *Pseudomonas* spp. (47). The importance of this sequence element for the iron-regulated gene expression has previously been demonstrated by the observation that mutations in the conserved CTAAAT motif strongly reduced promoter activity (47, 56). Our analysis also showed that 5′ deletions extending within or downstream of the iron starvation box completely abrogated the *pvdA* promoter activity, although the 50-bp sequence upstream of this motif also appeared to be essential for full iron-regulated promoter activity. An alignment of promoter sequences of iron-regulated pyoverdin genes dependent on the activation by PvdS-like sigma factors revealed that sequence motifs resembling the iron starvation box were shared by *pvdA*, *pvdD*, and *pvdE* promoters of *P. aeruginosa* (47) and by the SP1 promoter of *P. fluorescens* M114 (41), being centered from positions −32 to −37 in all but one of the four promoters studied. Except for the −10 sequence of the T1 transcript of *pvdA*, it was not possible to recognize any similarity between the canonical −10 and −35 motifs recognized by σ^E-dependent RNA polymerases (34) and the corresponding regions of pyoverdin promoters. This is difficult to explain, since the ECF-like sigma factors PvdS and PbrA, which activate transcription of pyoverdin genes in *P. aeruginosa* PAO1 and pseudobactin genes in *P. fluorescens* M114, show extensive homology with other ECF members in structural domains 2.4 and 4, responsible for −10 and −35 recognition, respectively (15, 34, 52). Moreover, the distance between the iron starvation box and transcription start sites was variable in the four promoters examined (from 14 bp in the *pvdE* promoter to 33 bp in the SP1 promoter), consistent with the hypothesis that this sequence element is recognized by a transcriptional activator rather than being involved in RNA polymerase binding (47).

Despite some resemblance between the DNA region directly upstream of the T2 transcription start site and the proposed consensus of σ^{54}-dependent promoters (18, 48), our conclusion is that T2 is not transcribed by the σ^{54}-like RNA polymerase since both the activity and the transcription initiation pattern of the *pvdA* promoter did not differ in *rpoN*$^+$ and *rpoN* backgrounds of *P. aeruginosa*. However, it is of interest that the DNA region located between the T1 and T2 initiation sites is essential to P_{pvdA}, since a deletion extending upstream of the T2 start point completely abrogated promoter activity. It remains to be determined whether T1 and T2 arise from separate, tandemly arranged promoters or whether T2 represents a processing product of the major *pvdA* transcript.

Regulation studies demonstrated that *pvdA* expression is under the control of the Fur repressor protein of *P. aeruginosa*. Introducing *pvdA′*::*lacZ* transcriptional fusions into *P. aeruginosa* Fur$^-$ mutants partially abrogated iron regulation of *lacZ* expression. Loss of iron-dependent repression of *pvdA* transcription in a *fur*-defective background was confirmed by Northern analyses of the *pvdA* transcripts in *fur* mutants grown in iron-rich medium. Because of the lethal effect of a *fur* null mutation in *P. aeruginosa* (5, 45), we used for our study two *fur* mutants producing Fur repressor proteins with single amino acid substitutions at critical positions of the peptide sequence (5). It is therefore possible that the mutated Fur proteins retain a partial regulatory function. Consistent with this hypothesis, Barton and colleagues (5) demonstrated that mutated Fur repressors carrying the A4 and C6 substitutions retained partial DNA-binding activity. This might account for the residual iron-dependent regulation observed in the different *fur* mutant backgrounds with both P_{pvdA} and the promoter cloned in pPV512.

In the light of the complex regulatory network governing the expression of siderophore genes in fluorescent pseudomonads, we assumed that *pvdA* transcription was dependent on the activity of the ECF-like sigma factor PvdS. In this case, Fur would indirectly modulate *pvdA* transcription by controlling the expression of the *pvdS* gene. In line with this hypothesis, introduction of *pvdA′*::*lacZ* fusions (plasmids pPV51 and pPV55) and of plasmid pPV512 in a *pvdS* mutant resulted in a dramatic reduction of β-galactosidase levels in iron-starved cells compared with the parental strain. Moreover, both *pvdA* mRNAs were undetectable in the *pvdS* mutant grown under conditions of limiting iron, indicating an absolute requirement of PvdS for transcription directed by P_{pvdA}. We also observed that P_{pvdA} and the promoter cloned in pPV512 were silent in *E. coli*, probably because of the lack of appropriate transcriptional activator protein(s). This behavior seems to be a general feature of promoters of pyoverdin synthesis genes (15, 37, 52). Introduction of the *pvdS* gene with its authentic promoter in an *E. coli* strain carrying plasmid pPV51, pPV55, or pPV512 caused iron-controlled expression of the reporter gene from the three fusion constructs. It was also noticed that an excess of iron was not sufficient to entirely repress transcription from P_{pvdA}. Similar results have been observed with the *pvdD* and *pvdE* promoters (15) and might be due to the excessive levels of Fur-binding sequences on the plasmid-located *pvdS* promoter which titrate out the *E. coli* Fur repressor pool. Thus, we replaced the indigenous *pvdS* control region with an inducible P_{T5}-*lacO* hybrid promoter and monitored transactivation of pyoverdin promoters under iron-rich conditions. Overproduction of PvdS upon IPTG induction resulted in a dramatic increase of β-galactosidase activities expressed by the different fusion constructs, even in the presence of sufficient iron. It can therefore be deduced that PvdS confers to the *E. coli* RNA polymerase specificity for the pyoverdin promoters. The extent of transactivation of the *P. aeruginosa* promoters in *E. coli* appeared to be related to the level of expression of this alternative sigma factor, which, in turn, was controlled by the Fur repressor. Interestingly, this effect was more evident for P_{pvdA} than for the promoter cloned in pPV512 and, for both promoters, the activity was significantly lower in *E. coli* carrying the multicopy *pvdS* gene than in *P. aeruginosa*. A similar effect was observed by Cunliffe et al. (15) for the promoters of different pyoverdin genes, thus raising the possibility that activating factors other than PvdS could play a role in the expression of pyoverdin promoters in *P. aeruginosa*. Consistent with this hypothesis, multiple regulatory components have recently been shown to control transcription of pseudobactin genes in *Pseudomonas putida* WCS358 (31, 58, 59).

As the last step of our study, we showed that Fur is not involved in direct control of the *pvdA* promoter and provided evidence of in vivo and in vitro binding of Fur to the *pvdS* promoter. Introduction of pBR322-derived constructs carrying the *pvdS* control region in *E. coli* H1717 (55) led to a positive result in the Fur titration assay, while the construct carrying the entire *pvdA* promoter did not. These results were substantiated by gel retardation assays showing metal-dependent binding of the Fur repressor protein to the *pvdS* promoter but not to the *pvdA* promoter. The band shift patterns observed with the *pvdS* promoter were suggestive of the presence of two recognition sites with different affinities for the Fur repressor. In line with this observation, tandemly arranged Fur-binding sites have recently been identified by an independent research group in the

pvdS promoter (39) and mapped by DNase I footprinting at bp 13 to 37 (low-affinity site) and 38 to 77 (high-affinity site) of the sequence reported in Fig. 9. Thus, P_{pvdA} appears to differ significantly from the iron-regulated SP1 promoter of *P. fluorescens* M114 (41). Both promoters are activated by iron-regulated σ^E homologs (52), but unlike the SP1 promoter, P_{pvdA} lacks a Fur-binding motif and is not directly controlled by Fur (40).

In conclusion, we have shown that iron-regulated expression of *pvdA* is indirectly controlled at the transcriptional level by *fur* and requires the alternative sigma factor PvdS. In the presence of sufficient iron, Fur binds to the *pvdS* promoter, thus preventing transcription of this gene. Under conditions of iron deficiency, repression by Fur is relieved and *pvdS* is expressed, acting as a master regulatory switch of pyoverdin biosynthesis genes. Such a hierarchical organization of negative and positive transcription factors might provide a finer model for global control of iron-regulated gene expression.

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