

Analysis of Promoters Recognized by PvdS, an Extracytoplasmic-Function Sigma Factor Protein from *Pseudomonas aeruginosa*

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The alternative sigma factor PvdS is required by *Pseudomonas aeruginosa* for initiation of transcription from pyoverdine (*pvd*) promoters. Two divergent PvdS-dependent promoters (*pvdE* and *pvdF*) were characterized by deletion analysis, and the minimal promoter region for each included a sequence element, the iron starvation (IS) box, that is present in other *pvd* promoters. Site-directed mutagenesis showed that the IS box elements were essential for promoter activity in vivo. Band shift assays and in vitro transcription experiments showed that a complex of PvdS and core RNA polymerase required the presence of an IS box in order to bind to and initiate transcription from *pvd* promoters. These results indicate that IS box elements participate in sequence-specific recognition by PvdS to enable initiation of transcription from *pvd* promoters and are likely to represent a –35 sequence element for this sigma factor.

Fluorescent pseudomonads are characterized by the production of yellow-green siderophores termed pyoverdines or pseudobactins that enable iron uptake under conditions where little free iron is available. These molecules are thought to be associated with biocontrol of fungal pathogens in the biosphere (7), and pyoverdine is required for virulence of the opportunist mammalian pathogen *Pseudomonas aeruginosa* (14). Production of pyoverdine by *P. aeruginosa* strain PAO is dependent on a protein, PvdS, that has been shown to be an alternative sigma factor protein (9, 27). The presence of PvdS is essential for the expression of all other pyoverdine synthesis genes that have been examined, including *pvdA*, *pvdD*, *pvdE*, *pvcA* to *D*, and *ptxR* (4, 8, 23, 24). These genes encode a variety of proteins that all contribute to the biosynthesis of pyoverdine. PvdS is also required for the synthesis of exotoxin A (18) and an extracellular proteinase, PrpL (P. J. Wilderman and M. L. Vasil, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. B-331, 2000), two enzymes that are secreted by *P. aeruginosa*. Expression of *pvdS* is regulated by the iron-responsive Fur repressor protein such that there is no transcription of *pvdS* and no production of pyoverdine or exotoxin A by cells grown in iron-rich media (reviewed in reference 25).

The DNA sequences that are recognized by PvdS to enable transcription from cognate promoters have not yet been identified. However, a DNA sequence element named the iron starvation (IS) box has been identified in the promoters of iron-regulated genes from *Pseudomonas* and been shown to be required for promoter function (20). The purpose of the experiments described here was to test the hypothesis that this sequence element is a DNA recognition site for PvdS. This was done through a detailed characterization of two divergent PvdS-dependent *pvd* promoters, the promoters of the *pvdE* and *pvdF* genes. Both of these genes are essential for pyover-

dine production by *P. aeruginosa* PAO, with *pvdE* encoding an ATP binding cassette-2 type transporter that is likely to be involved in secretion of pyoverdine or a precursor (11) and *pvdF* encoding an enzyme required for synthesis of formyl-hydroxyornithine residues that are present in the pyoverdine produced by this strain (B. McMorran et al., unpublished data).

Identification of the *pvdF* gene transcriptional start sites.

The strains and plasmids used in this study are described in Table 1, and the intergenic sequence between the *pvdE* and *pvdF* genes is shown in Fig. 1. As a prelude to promoter characterization, it was necessary to determine the transcriptional start site of the *pvdF* gene; the *pvdE* transcriptional start site has been mapped previously (20). The initiation site of the *pvdF* transcript was determined by primer extension analysis (Fig. 2) using an avian myeloblastosis virus reverse transcriptase primer extension kit (Promega). Primer extension analysis was carried out using 5'-end-labeled oligonucleotides, primer 1 (CCGCGGGGTTTCTCAGGGACCAGATATAGGCCAG),

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>P. aeruginosa</i> strains		
OT11	Pvd ⁺	19
OT11 <i>pvdS</i>	<i>pvdS</i> ::Kan Pvd ⁻	4
Plasmids		
pMP190	Broad-host-range promoter probe vector; Cm ^r	22
pUC19	Cloning vector; Ap ^r	28
pUC:: <i>pvdE/F</i>	<i>pvdE-pvdF</i> promoter region (–91 to +34 of <i>pvdF</i>) cloned into pUC19	27
pUC::mut1	<i>pvdE-pvdF</i> region with mutation in <i>pvdE</i> -proximal IS box	This study
pUC::mut2	<i>pvdE-pvdF</i> region with mutation in <i>pvdF</i> -proximal IS box	This study
pUC:: <i>pvdD</i>	<i>pvdD</i> promoter (–96 to +39) cloned into pUC19	27
pUC:: <i>pvdD</i> mut	<i>pvdD</i> promoter (–96 to +39) with mutation in IS box	This study

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A. *pvdE* promoter fragments

β-Gal (U)		
-Fe	+Fe	
24(10)	21(4)	vector control
17(11)	21(2)	← ⁻¹⁷
367(65)	19(1)	← ⁻³⁸
358(49)	19(1)	← ⁻⁵⁵
560(37)	8(2)	← ⁻⁷⁶
535(64)	19(3)	← ⁻⁹⁵
1226(140)	11(3)	← ⁻¹²¹
1075(203)	29(5)	← ⁺⁴ → ⁺³⁶¹
1222(92)	26(5)	← ⁺⁹⁵ → ⁺¹²¹

B. *pvdF* promoter fragments

β-Gal (U)		
-Fe	+Fe	
77(18)	57(10)	→ ⁻²⁸ → ⁺¹
98(4)	69(9)	→ ⁻³⁴ → ⁺¹
211(20)	30(7)	→ ⁻⁵¹ → ⁺¹
744(41)	84(0)	→ ⁻⁷² → ⁺¹
770(177)	32(3)	→ ⁻⁹¹ → ⁺¹
34(4)	38(3) (OT11 <i>pvdS</i>)	→ ⁻⁹¹ → ⁺¹
2240(248)	48(2)	→ ⁻²⁸² → ⁺¹
3138(505)	119(0)	→ ⁻⁹¹ → ⁺³⁴
3193(365)	92(8)	→ ⁻⁹¹ → ⁺²³⁷

C. IS box mutations: *pvdE* promoter activity

β-Gal (U)		
-Fe	+Fe	
136 (32)	16 (4)	← ⁻¹²¹ mutE
85 (8)	55 (3)	← ⁻¹²¹ mutF

D. IS box mutations: *pvdF* promoter activity

β-Gal (U)		
-Fe	+Fe	
3729 (365)	175 (36)	← ⁻⁹¹ mutE → ⁺³⁴
129 (14)	91 (12)	← ⁻⁹¹ mutF → ⁺³⁴

FIG. 3. Promoter activities of the *pvdE* and *pvdF* promoter deletion fragments in *P. aeruginosa*. The positions of the fragments are shown relative to a map of the *pvdE-pvdF* promoter region, with the nucleotides present in each fragment given relative to the transcript start site of the relevant gene. The transcript start sites (+1) and positions of IS box elements are also shown. The amounts of β-Gal produced from each construct in *P. aeruginosa* during growth in iron-deficient (-Fe) and iron-replete (+Fe) media, with standard deviations from three independent assays in parentheses, were determined using the method of Miller (15) as described previously (4, 20). Promoter fragments cloned into pMP190 were oriented such that *lacZ* expression was dependent upon *pvdE* promoter activity (A and C) or *pvdF* promoter activity (B and D) as shown. Fragments with the wild-type promoter sequence (A and B) or with mutations in the IS box sequence elements (C and D) were used. Vector control, *P. aeruginosa* OT11 containing pMP190 vector DNA. OT11*pvdS*, the pMP190 promoter construct was transformed into OT11*pvdS* (3) and assays were carried out.

ment (Fig. 1) (20). Further increases in promoter activity were observed with larger fragments, and the data indicate that sequences in the -72 to -51 and -282 to -91 regions contribute to promoter activity. The -72 to -51 region contains almost all of the IS box sequence (nucleotides -65 to -74) shown to be important for activity of the divergent *pvdE* promoter (Fig. 3) (20). Thus, this element may contribute to the activities of both promoters. Expression from the *pvdF* promoter was further increased by the presence of DNA downstream from the transcriptional start site (nucleotides +1 to +34), indicating that a sequence element in this region is required for maximal expression from this promoter. A study of the PvdS-dependent *pvdA* promoter also found that DNA downstream from the +1 site is required for promoter activity in *P. aeruginosa* (8). However, DNA downstream from the *pvdE* transcription start site, to nucleotide +195, did not increase *pvdE* promoter activity (Fig. 3A); it remains to be seen whether other *pvd* promoters need DNA elements downstream from the transcription start site for maximal activity. It should be noted that DNA downstream from nucleotide +1 is likely to be involved in promoter recognition by other extracytoplasmic-function (ECF) sigma factor proteins. DNA from nucleotide +50 to +120 of the *ctrl* promoter (a CarQ-dependent promoter) is required for promoter activity (10). PCR mutagenesis of an ECF-dependent promoter from *Escherichia coli*, *pfcA*, revealed that residues clustered around the +13 site were required for promoter function (1).

Mutations were introduced into the IS box elements by PCR mutagenesis using a protocol developed by Datta (5) (Fig. 1) in order to assess their involvement in promoter activity more directly. The mutated fragments were cloned into pMP190 in both orientations, the resulting constructs were transformed into *P. aeruginosa*, and the production of β-Gal was assayed (Fig. 3C and D). Mutation of the IS box nearest to *pvdE* reduced *pvdE* promoter activity, consistent with a previous study (20), but had no effect on *pvdF* promoter activity (Fig. 3C and D, mutE). The mutation to the IS box nearest to *pvdF* abolished expression from the *pvdF* promoter and also greatly reduced expression from the *pvdE* promoter (Fig. 3C and D, mutF). This indicates that this IS box is important for expression from both promoters.

In vitro analysis of PvdS-IS box interactions. The above data, in conjunction with earlier studies (8, 20), indicate the critical role of IS box sequence elements in *pvd* promoter function in *P. aeruginosa*. These promoters are PvdS dependent and therefore must contain a PvdS recognition sequence. Furthermore, we (4) and others (8, 17) have previously shown that *pvd* promoters are active in *E. coli*, although only if the *pvdS* gene is present; promoter constructs carrying the site-directed IS box mutations were inactive in *E. coli* expressing *pvdS* (data not shown). Collectively, these data lead to the hypothesis that the IS box forms part of the DNA recognition sequence of the PvdS sigma factor. To test this hypothesis, promoter fragments containing wild-type and mutated IS box

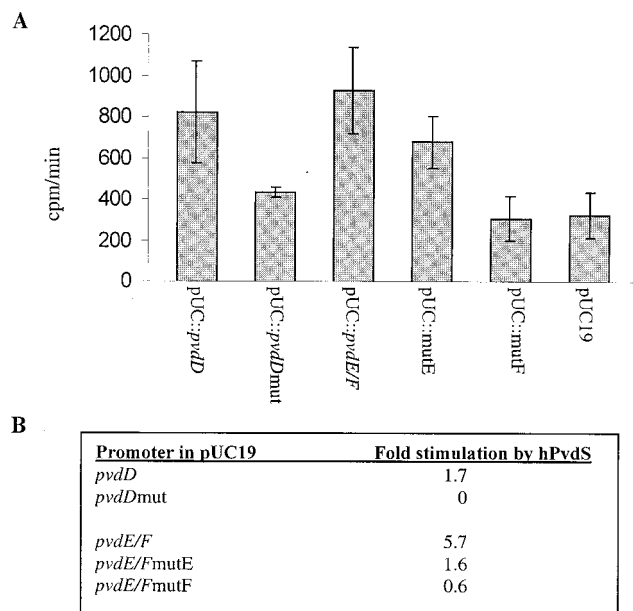


FIG. 4. Activities of mutant promoter fragments in vitro with purified PvdS protein. (A) Purified hPvdS was incubated with core RNA polymerase and different plasmid templates as shown. Templates carried wild-type promoter fragments (*pvdD* [nucleotides -96 to $+39$] or *pvdE-pvdF* [-121 to $+4$ of *pvdE* and -92 to $+34$ of *pvdF*]) or mutations in the IS box of the *pvdD* promoter (*pvdDmut*), the *pvdE*-proximal IS box (*mutE*), or the *pvdF*-proximal IS box (*mutF*) (Fig. 1; Table 1); the mutation in the *pvdD* IS box is the same as that described previously (20). The rate of RNA production from each plasmid template was measured as described previously (27). Error bars indicate standard deviations. (B) Fold stimulation represents the ability of hPvdS to stimulate activity above that of core enzyme with a *pvd* promoter template minus the corresponding value obtained with a vector template, calculated using the following equation (2, 27): fold stimulation = $[(\text{hPvdS-core} - \text{hPvdS only})/\text{core only}]_{\text{pUC::pvd template}} - [(\text{hPvdS-core} - \text{hPvdS only})/\text{core only}]_{\text{pUC}_{\text{vector template}}}$.

elements were used in in vitro transcription assays. PvdS was purified as a His-tagged protein (hPvdS) and incubated with core RNA polymerase and plasmid templates in an in vitro transcription assay as described previously (27). Experiments were carried out with the *pvdD* promoter, in which a mutation in the IS box abolishes promoter activity (20), as well as the *pvdE* and *pvdF* promoters. PvdS stimulated transcription from the *pvdD* promoter but failed to stimulate transcription from the *pvdD* promoter if the IS box had been mutated (Fig. 4). Mutation of the IS box nearest to *pvdE* (*mutE*) (Fig. 4) resulted in a reduction in PvdS-mediated transcription from the *pvdE-pvdF* promoter fragment, and mutating the other IS box in this fragment (*mutF*) (Fig. 4) virtually eliminated PvdS-mediated transcription (Fig. 4). These data are consistent with the hypothesis that the IS box forms part of the DNA recognition sequence of the PvdS sigma factor. They also suggest that the IS box nearest *pvdF* is required for PvdS-mediated expression from both of the divergent promoters, whereas the *pvdE*-proximal IS box is required only for transcription from one promoter. This is the same pattern as that found in vivo (Fig. 3C and D).

Interactions between promoter fragments and the core-PvdS complex were also analyzed using band shift assays. Core-PvdS

bound the *pvdD* wild-type promoter fragment but failed to cause any shifting of the corresponding fragment containing a mutated IS box (Fig. 5). For the *pvdE-pvdF* promoter fragment, a mutation in the *pvdE*-proximal IS box (*mutE*) prevented binding of core-PvdS, indicating that PvdS requires this sequence to bind to the *pvdE-pvdF* promoter fragment. Core-PvdS still retarded a *pvdE-pvdF* promoter fragment containing the *pvdF* IS box mutation (*mutF*), although slightly less effectively than the wild-type promoter fragment, suggesting that this sequence is not essential for binding of the protein to the promoter fragments used in these experiments. Consistent with these results, a fragment containing only the *pvdF* IS box (nucleotides -72 to $+34$ of *pvdF*) failed to bind core-PvdS, whereas a fragment containing only the *pvdE* IS box (nucleotides -38 to $+195$ of *pvdE*) still showed binding by core-PvdS in gel shift assays (data not shown). Collectively these results indicate that PvdS requires the presence of an IS box to enable binding of RNA polymerase at both the *pvdE-pvdF* and *pvdD* promoter fragments.

It is surprising that the mutation in the *pvdE*-proximal IS box essentially abolished binding of core-PvdS to the promoter fragment, as this mutation had no effect on expression from the *pvdF* promoter (Fig. 3D). Similarly, the mutation in the *pvdF*-proximal IS box essentially abolished activity from both promoters (Fig. 3C and D and 4), whereas it reduced but did not prevent binding of core-PvdS to the promoter fragment (Fig. 5). These apparently conflicting data may reflect differences in the use of linearized DNA fragments in band shift assays, instead of the supercoiled plasmid templates of the in vitro transcription and in vivo studies, or may be a consequence of complex interactions between the two divergent promoters. It

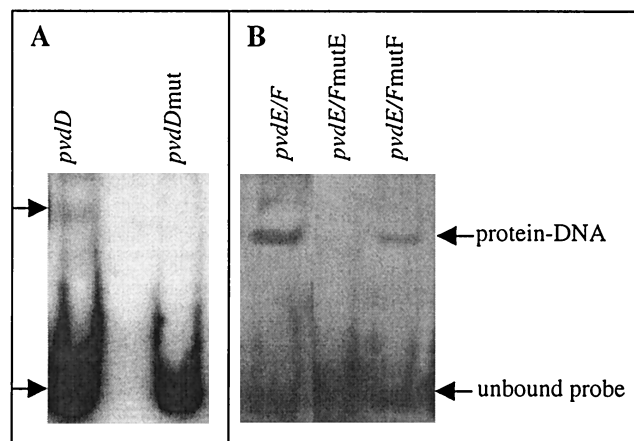


FIG. 5. Band shift assays with hPvdS and mutant promoters. (A) Band shift assays were carried out as described previously (27). Core enzyme (3.4 pmol) was incubated with hPvdS (13 pmol) and digoxigenin-labeled DNA fragments containing either the *pvdD* wild-type promoter fragment (nucleotides -96 to $+39$) or a *pvdD* promoter fragment containing a mutation in the IS box. Following electrophoresis and transfer to a nylon membrane, the DNA-protein complexes were detected by immunoblotting with antibodies against digoxigenin. (B) Core and hPvdS were incubated with the wild-type *pvdE-pvdF* promoter fragment ($-121/+4$ with respect to the *pvdE* transcription start site) or with promoter fragments containing a mutation in either the *pvdE* IS box (*mutE*) or the *pvdF*-proximal IS box (*mutF*), as shown. The positions of DNA-protein complexes are indicated by arrows.

TABLE 2. IS boxes in PvdS-dependent promoters^a

Promoter	IS box										
<i>pvdA</i>	-38	C	T	T	A	A	A	T	T	C	-30
<i>pvdD</i>	-39	G	C	T	A	A	A	T	C	C	-29
<i>pvdE</i>	-23	G	C	T	A	A	A	T	A	C	-15
<i>pvdF</i>	-38	C	G	T	A	A	A	T	T	G	-30
<i>ptxR^b</i>	?	A	G	T	A	A	A	T	A	G	?
<i>pvdY</i>	?	G	G	T	A	A	A	T	T	C	?
Consensus		(G/C)	(G/C)	T	A	A	A	T	(T/A)	(C/G)	

^a The *pvdA*, *-D*, *-E*, and *-F* genes have been characterized and are required for pyoverdine synthesis (11, 12, 13, 26). The IS boxes of these genes have been mutated and shown to be required for activity (8, 20; this study). Expression of *ptxR*, which encodes a positive regulator of the *pvc* genes, and the *pvdY* gene, which encodes a 17-kDa protein of unknown function, has been shown to be PvdS dependent (24, 25). ?, the transcriptional start site is not known.

^b The transcriptional start site can be estimated on the basis of RNase protection data (24) as being between nucleotide -28 and -20 downstream from the IS box.

may also be possible that a complex of core-PvdS bound at the *pvdE* IS box is more stable in the gel shift assay than the complex formed at the *pvdF* IS box, reflecting a more stringent requirement of protein-DNA binding in this assay than in transcription assays.

Investigation of two other promoters, *pvdY* and *ptxR*, that have been shown to require PvdS for activity (24, 25) reveals that they also contain IS box-like sequences (Table 2). Thus, all promoters that have been shown to be PvdS dependent contain IS box sequences, further supporting the proposition that this sequence motif is recognized by PvdS during initiation of transcription from *pvd* genes. For those promoters whose transcription sites have been mapped, the IS box is at the -35 region of the promoter (with the exception of the *pvdE* promoter), consistent with other ECF family members, which tend to have a recognition motif centered at about -35 (10, 16). The position of the *pvdE* IS box, centered at -19, is likely to be atypical and a consequence of the divergent and interacting natures of the *pvdE* and *pvdF* promoters.

In summary, characterization of the divergently transcribed *pvdE* and *pvdF* promoters has allowed the minimal promoter regions to be determined and emphasizes the involvement of the IS box sequence. Gel shift and in vitro transcription data very strongly suggest that PvdS recognizes the IS box in pyoverdine promoters. Further studies will be required to unravel the complex protein-DNA interactions that take place at these promoters as well as to identify and characterize the other sequence elements that contribute to the activity of these promoters.

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