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The *pvc* Gene Cluster of *Pseudomonas aeruginosa*: Role in Synthesis of the Pyoverdine Chromophore and Regulation by PtxR and PvdS

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A putative operon of four genes implicated in the synthesis of the chromophore moiety of the *Pseudomonas aeruginosa* siderophore pyoverdine, dubbed *pvcABCD* (where *pvc* stands for pyoverdine chromophore), was cloned and sequenced. Mutational inactivation of the *pvc* genes abrogated pyoverdine biosynthesis, consistent with their involvement in the biosynthesis of this siderophore. *pvcABCD* expression was negatively regulated by iron and positively regulated by both PvdS, the alternate sigma factor required for pyoverdine biosynthesis, and PtxR, a LysR family activator previously implicated in exotoxin A regulation.

Although iron is an essential nutrient for most bacteria, the low solubility and, thus, bioavailability of this element in nature complicates bacterial iron acquisition (32). Many bacteria deal with this problem by synthesizing high-affinity iron-chelating molecules, termed siderophores (31), which function coordinately with cell surface receptors specific for the iron-siderophore complexes (30, 31) to transport iron into the cell. Pathogenic organisms also encounter an iron-limited environment in the host (41), and siderophore-mediated mechanisms of iron acquisition are important contributors to in vivo growth and, thus, pathogenesis of many disease-causing bacteria (e.g., see references 7 and 16).

Pseudomonas aeruginosa is an opportunistic human pathogen which produces two known siderophores, pyoverdine (9) and pyochelin (8). Production of pyoverdine in vivo has been documented (17), consistent with a demonstrated role for this siderophore in promoting in vivo growth and pathogenesis (27). This mixed hydroxymate-catecholate siderophore is characterized by a conserved hydroxyquinoline chromophore bound to an amino acid tail of variable length and composition (6). Synthesis of the chromophore is hypothesized to involve a condensation of D-tyrosine and L-2,4-diaminobutyric acid (DAB) (6), while synthesis of the peptide moiety apparently involves a nonribosomal mechanism (23).

Genes for the synthesis of pyoverdine have been mapped to three regions of the *P. aeruginosa* PAO chromosome, at 23, 47 (20), and 66 to 70 (44) min on the recalibrated PAO map. A 103-kb fragment of chromosomal DNA originating from the 47-min region has been cloned. Referred to as the *pvd* region (48), this DNA carries several genes shown to be involved in pyoverdine biosynthesis. DNA originating from the 66- to 70-min region of the PAO chromosome has also been cloned. Responsible for the synthesis of a chromophore-like molecule dubbed pseudoverdine, a gene(s) in this region is required for pyoverdine biosynthesis (44), presumably for the chromophore moiety.

Pyoverdine synthesis is dependent upon an alternate sigma factor, PvdS, required for gene expression from a variety of *pvd* promoters (10, 28). PvdS is negatively regulated by Fur (10, 28, 34), a repressor protein which mediates the iron-regulated expression of a number of genes, providing a likely explanation for the iron-regulated production of pyoverdine in *P. aeruginosa*.

In the present report we describe the sequencing of the pseudoverdine gene cluster and the identification and regulation of an operon of four genes (*pvcABCD*) required for pyoverdine (chromophore) production.

Methods. Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) (Difco), brain heart infusion (BHI) (BDH) and the iron-deficient King's B (KB) (22) or succinate minimal (24) media have been described previously. Strains cultivated for the purpose of extracting RNA for use in RNase protection assays were grown in low-iron Trypticase soy broth dialysate with (iron replete) or without (iron deficient) $FeCl_3$ supplementation (39 μM) (3). Minimal medium was supplemented with amino acids (1 mM) and adenosine (2 mM) as required. The following antibiotics were included in the growth media as required at the indicated concentrations: ampicillin, 100 μg/ml; kanamycin, 100 μg/ml; carbenicillin, 400 μg/ml; streptomycin, 500 μg/ml; chloramphenicol, 50 μg/ml (for *Escherichia coli* and *P. aeruginosa* K1081), 200 μg/ml (for *P. aeruginosa* ML5087), or 600 μg/ml (for *P. aeruginosa* PAO1); gentamicin, 75 μg/ml; Irgasan DP-300 (Ciba-Geigy), 50 μg/ml; and $HgCl_2$, 50 μg/ml.

Chromosomal DNA was prepared by using a modification of the Blin and Stafford procedure as described in reference 40. Large-scale plasmid DNA was prepared with a Plasmid Midi kit (Qiagen, Inc., Chatsworth, Calif.). Standard methods were used for the preparation of small-scale plasmid DNA, enzyme digestions, ligations, agarose gel electrophoresis (40), and transformation of *E. coli* (40) and *P. aeruginosa* (4). DNA fragments were purified from agarose gels with a Prep-a-gene kit (Bio-Rad Labs, Mississauga, Canada). Southern hybridizations were carried out as described previously (40), with a

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
E. coli		
GM2163	*hsdR2 mcrB1 dam-13*::Tn*9 dcm-6*	New England Biolabs
S17-1	*thi pro hsdR recA* Tra^+	43
P. aeruginosa		
PAO1	Prototroph	
PAO1 Δ*ptxR*		This work
PAO1 Δ*pvdS*		34
ML5087	*ilv-220 thr-9001 leu-9001 met-9001 pur-67 aphA*	36
K1081	ML5087Δ*ptxR*	This work
Plasmids		
pAS16	pK18*mobsacB* plasmid containing a 1-kb *Bam*HI-*Hin*dIII fragment from pAS20 encompassing the mutated *ptxR* gene	This work
pHP45ΩHg	pUC18 derivative carrying an Hg^r cassette	12
pK18*mobsacB*	Gene replacement vector derived from plasmid pK18; Mob^+ Km^r *sacB*	42
pKT240	Broad-host-range cloning vector; IncP-4 Mob^+ Km^r Cb^r	2
pMAL-c2	MalE fusion vector; Ap^r	Pharmacia
pPYP177	pKT240 derivative carrying a 7.8-kb *Sac*I-*Hin*dIII DNA fragment of the pPYP180 insert	44
pPYP180	pKT240 derivative carrying a 10.8-kb *Sac*I-*Cla*I DNA fragment from PAO1 which directs expression of pseudoverdine	44
pVLT31	Broad-host-range expression vector; *lacI* p*lac* Tc^r	11
pPTX990	pVLT31::*ptxR*	This work
pRK2013	Broad-host-range helper vector; Tra^+ Km^r	14
pSUP203	Gene replacement vector; Mob^+ Tc^r	43
pUC18	Cloning and sequencing vector; Ap^r	40
pUC19	Cloning and sequencing vector; Ap^r	40
pUC-Gm	pUC18::Gm; Ap^r Gm^r	33

[a] Gm^r, gentamicin resistant; Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant; Mob^+, mobilizable.

digoxigenin labelling kit (Boehringer, Mannheim, Germany) to process the hybridizations.

A region of pPYP180 responsible for pseudoverdine and necessary for pyoverdine synthesis was sequenced following the generation of a number of subclones (in pUC19) from which nested deletions were constructed by using a double-stranded nested deletion kit (Pharmacia Biotech). Plasmid DNA for sequencing was purified as described previously (1) and sequenced by Cortec DNA Services Laboratory Inc., using the M13 universal forward primer. The sequence overlapping the boundaries of the various subclones was obtained by using defined oligonucleotide primers and plasmid pPYP177 DNA as the template. Sequence analysis was carried out with the PCGENE software package (Intelligentics Inc., Mountain View, Calif.). The *ptxR* gene was cloned as a 990-bp promoterless gene which was generated by PCR using Deep Vent DNA polymerase (New England Biolabs) and the primers 5′-TCTAGACCCGTCCGGACCCACTTC-3′ (*Xba*I site underlined) and 5′-AAGCTTGCCCAGCCTCATTCGCTCTG-3′ (*Hin*dIII site underlined). Following cloning into pCR-blunt (Invitrogen Corporation, Carlsbad, Calif.), the fragment was directionally cloned as an *Xba*I-*Hin*dIII fragment into pVLT31 to yield pPTX990.

Two approaches were taken to generate Δ*ptxR* derivatives of *P. aeruginosa*. Initially, the 1.4-kb *Bam*HI-*Bgl*II DNA fragment of pPYP177 was cloned into the *Bam*HI site of pMAL-c2 (Pharmacia) in the same orientation as the *lac* promoter of this vector. The recombinant vector was introduced into and subsequently prepared from *E. coli* GM2163 before being digested with *Cla*I and *Nru*I to release a 0.4-kb fragment from the *ptxR* coding region. Following purification of the vector free of this 0.4-kb fragment, the *Cla*I 5′ end was blunt ended with Klenow fragment and the plasmid was recircularized with T4 DNA ligase. The *ptxR* coding region with the deletion was excised from the plasmid on a 1-kb *Bam*HI-*Hin*dIII DNA fragment and cloned into plasmid pK18*mobsacB*. The resultant vector, pAS16, was then introduced into *E. coli* S17-1 and mobilized into *P. aeruginosa* ML5087 via conjugation as described previously (37). Recipients carrying pAS16 in the chromosome were selected on LB agar containing kanamycin (100 μg/ml) and tetracycline (10 μg/ml). Kanamycin-resistant colonies appearing after 24 h of growth at 37°C were streaked onto LB agar containing sucrose (10%, wt/vol) (42). Sucrose-resistant colonies carrying the *ptxR* region with the deletion (e.g., K1081) were identified following amplification of the *ptxR* gene by

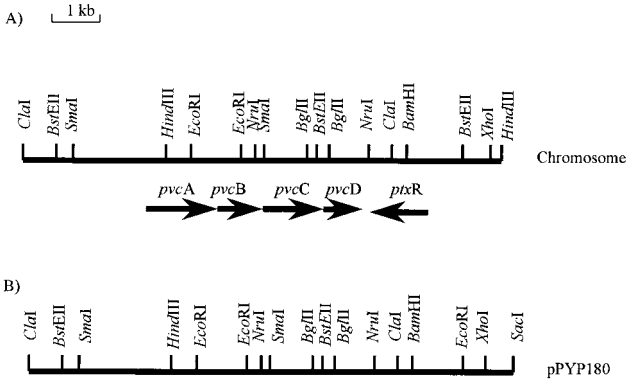

FIG. 1. Physical map of the *pvcABCD-ptxR* region of the *P. aeruginosa* PAO1 chromosome (A) and plasmid pPYP180 (B). Restriction mapping to the right of *ptxR* revealed differences between pPYP180 and the chromosome, indicating that some DNA rearrangement had occurred during the cloning of the *pvc* locus.

TABLE 2. Identification of proteins exhibiting similarity to deduced PvcABCD proteins[a]

Pvc protein (size [amino acids])	Similar protein	Strain	Size (amino acids)	Alignment score[b]	Function
PvcA (327)	Dit1	*S. cerevisiae*	536	14.693	Spore wall maturation
PvcB (289)	TfdA	*A. eutrophus* (*R. eutropha*)	297	6.294	2,4-Dichlorophenoxyacetate α-ketoglutarate dioxygenase
PvcC (500)	HpaA	*K. pneumoniae*	520	40.258	4-Hydroxyphenylacetate 3-hydroxylase
	HpaB	*E. coli*	520	39.115	4-Hydroxyphenylacetic hydroxylase
PvcD (215)	CC4	*Pseudomonas stutzeri*	210	13.977	Cytochrome c_4
	Cyc1	*Thiobacillus ferrooxidans*	213	13.437	Cytochrome *c*-552
	CycA	*A. vinelandii*	210	10.997	Cytochrome c_4
	CycC4	*P. aeruginosa*	181	10.544	Cytochrome c_4

[a] Proteins exhibiting sequence similarity to the deduced PvcABCD polypeptides were identified by using the network services offered by the National Center for Biotechnology Information (Bethesda, Md.) and the Swiss-Protein sequence database.

[b] Protein sequences exhibiting similarity to PvcA, PvcB, PvcC, or PvcD were individually aligned to the corresponding Pvc protein by using the PCOMPARE program available with the PCGENE software package. The alignment score was obtained by the method of Needleman and Wunsch (29) as implemented by Feng et al. (13), using the structure-genetic matrix with a gap penalty of 6 and a bias parameter of 0. A score above 3.0 is indicative of significant similarity.

using *Taq* polymerase and primers ptxR3 (5′-CAGGACTTC GTCAAGTGGCA-3′) and ptxR4 (5′-AGCTCTTCGAGAA C-GGCCTG-3′). Reaction mixtures were formulated as described previously (38) and subjected to 1 min at 94°C followed by 30 cycles of 40 s at 94°C, 50 s at 50°C, and 3 min at 72°C before finishing with 10 min at 72°C. A *ptxR* deletion mutant of PAO1 was subsequently generated following the tagging of a 0.8-kb deletion in this gene with a gentamicin resistance cartridge. Briefly, the 5′ and 3′ flanking regions of *ptxR* were amplified with the primer pair ptx247H (5′-AGGAAGCTTG TCCAATACTTGAG-3′, harboring a *Hin*dIII site) and ptx594X (5′-AGGTCTAGATGATTCAATCGCTCC-3′, harboring an *Xba*I site) or ptx1389K (5′-CCCGGTACCCCTCGGCGCGC TAC-3′, harboring a *Kpn*I site) and ptx1866E (5′-GCGGAA TTCCTGGCAACCCAGTTGC-3′, harboring an *Eco*RI site), respectively. PCR was performed on chromosomal PAO1 DNA with *Taq* polymerase and 30 cycles of 1 min at 94°C, 1 min at 55°C, and 40 s at 72°C. The PCR fragments were cloned into pCRII-2.1 (Invitrogen) and sequenced by using M13 primers and Sequenase (Amersham Life Science). The flanking regions were directionally transferred as 348-bp *Hin*dIII-*Xba*I and 478-bp *Kpn*I-*Eco*RI fragments into pUC-Gm which had been previously obtained by placing a 1.7-kb Gm^r cartridge (34) in the *Sma*I site of the pUC18 polylinker (33). The resulting plasmid, pUCΔ*ptxR*::Gm, was linearized with *Eco*RI and ligated to *Eco*RI-cut pSUP203 (43), yielding pSUPΔ*ptxR*::Gm. This vector was then mobilized into *P. aeruginosa* PAO1 via triparental mating (50), with *E. coli* HB101(pRK2013) as the helper strain. Gm^r transconjugants were isolated on BHI agar containing gentamicin and Irgasan DP-300 (for counterselection). Individual colonies were patched onto BHI agar containing tetracycline to screen for loss of the pSUP203 plasmid-borne tetracycline resistance gene, and candidate PAO1Δ*ptxR*::Gm mutants (Gm^r Tc^s) were screened for the deletion by Southern blot analysis (data not shown).

The riboprobes used for the RNase protection assays were

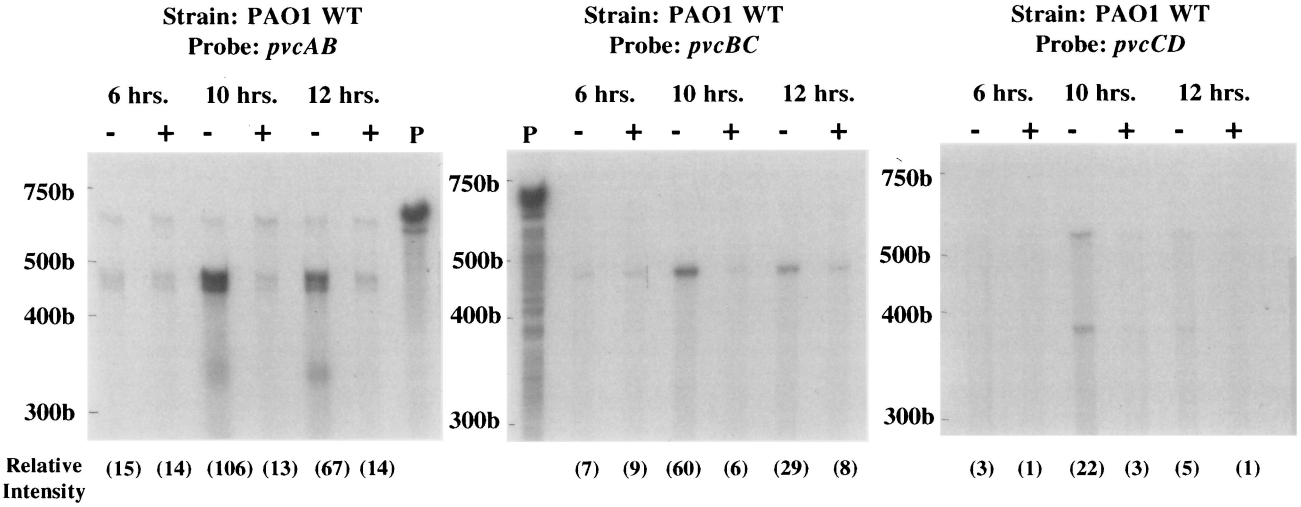

FIG. 2. RNase protection analysis of *pvcABCD* expression in wild-type strain PAO1. RNA samples were extracted from cells grown continuously for 6, 10, or 12 h in medium that was either iron deficient (−) or iron replete (+), and protection against RNase digestion was afforded by the *pvcAB*, *pvcBC*, or *pvcCD* probes assessed as described in Materials and Methods. Undigested ^{32}P-labelled probes (P) are also shown. ^{32}P-labelled RNA standards are shown to the left of each gel. The gels shown in this figure were exposed for 16 h. The relative intensities (in parentheses) of the major band in each lane were determined by using NIH Image software (version 1.55). Riboprobing with *omlA* (not iron regulated) yielded protected fragments with relative intensities of 155 (6 h, iron deficient), 155 (6 h, iron replete), 175 (10 h, iron deficient), 173 (10 h, iron replete), 175 (12 h, iron deficient), and 190 (12 h, iron replete).

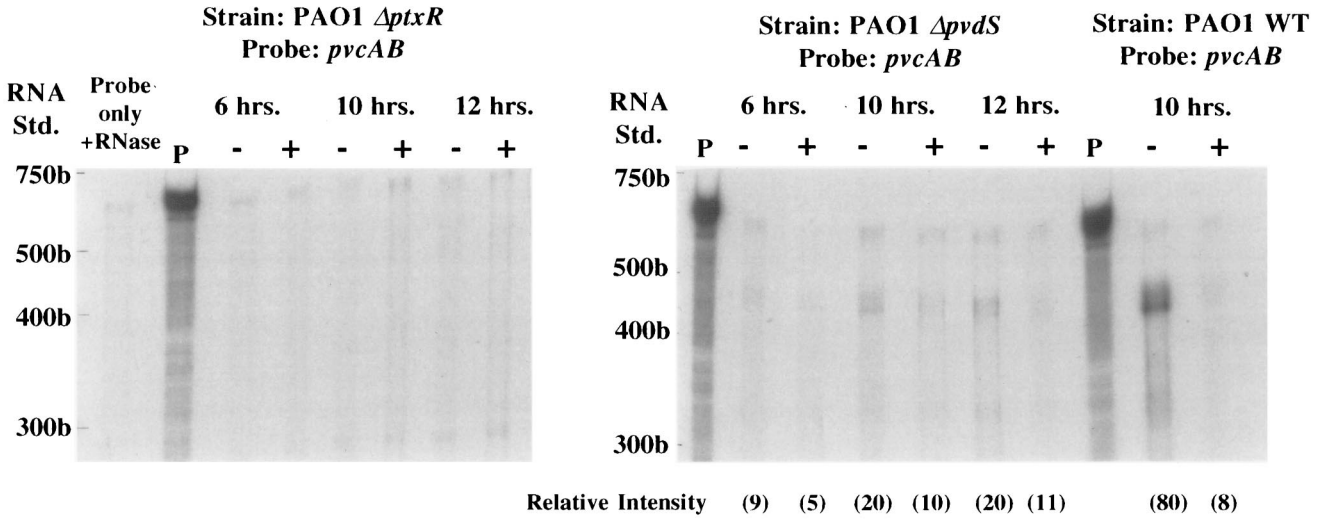

FIG. 3. RNase protection analysis of *pvcABCD* gene expression in the PAO1 parental strain (PAO1 WT) and PAO1 strains which carry deletion mutations in the *ptxR* (PAO1 Δ*ptxR*) or *pvdS* (PAO1 Δ*pvdS*) genes. RNA samples were extracted from cells grown continuously for 6, 10, or 12 h in medium that was either iron deficient (−) or iron replete (+), and protection against RNase digestion was afforded by the *pvcAB* probe assessed as described in Materials and Methods. Undigested (P) and RNase treated (Probe only + RNase) ^{32}P-labelled probes are also shown. The positions of ^{32}P-labelled RNA standards (Std.) are shown to the left of each gel. The gels shown in this figure were exposed for 16 h. The relative intensity of the major band in each lane (in parentheses) was determined by using NIH Image software (version 1.55). As no *pvcAB*-protected fragment (400 to 500 bp range) was observable in the PAO Δ*ptxR* lanes, relative intensities were not assessed. Riboprobing of total RNA from PAO1 Δ*ptxR* with *omlA* (not iron regulated) yielded protected fragments with relative intensities of 155 (6 h, iron deficient), 158 (6 h, iron replete), 170 (10 h, iron deficient), 136 (10 h, iron replete), 176 (12 h, iron deficient), and 184 (12 h, iron replete). Riboprobing of total RNA from PAO1 Δ*pvdS* with *omlA* yielded protected fragments with relative intensities of 189 (6 h, iron deficient), 192 (6 h, iron replete), 174 (10 h, iron deficient), 143 (10 h, iron replete), 175 (12 h, iron deficient), and 176 (12 h, iron replete).

generated following PCR amplification of selected regions of the genes of interest and cloning of the PCR fragments into the pCRII vector (Invitrogen). RNA probes were then generated from these cloned fragments by runoff transcription from the T7 promoter by using a Riboprobe kit (Promega), and the RNase protection assay was carried out as described previously (3). Autoradiographs of the dried gels were scanned and imported into Adobe Photoshop (version 4.0), and quantitative analysis was performed by using NIH Image software (version 1.55). The *pvcAB* probe (bp 1567 to 2015), covers 350 bp of *pvcA*, the *pvcA-pvcB* intergenic region, and 30 bp of *pvcB*. The *pvcBC* probe (bp 2640 to 3104) covers 180 bp of *pvcB*, the *pvcB-pvcC* intergenic region, and 132 bp of *pvcC*. Finally, the *pvcCD* probe (bp 3991 to 4367) covers 379 bp of *pvcC* and 125 bp of *pvcD*.

Identification and nucleotide sequence of the *pvc* pyoverdine biosynthetic gene cluster. The cloning of a 10.8-kb *Cla*I-*Sac*I DNA fragment of the 66- to 70-min region of the *P. aeruginosa* PAO1 chromosome (in pPYP180), which carries a gene(s) involved in pyoverdine biosynthesis, was previously described (44). This DNA fragment promoted the production of a pyoverdine chromophore-related fluorescent compound, termed pseudoverdine, in pyoverdine-deficient strains of *P. aeruginosa*, suggesting a role in the biosynthesis of the chromophore portion of the pyoverdine molecule (44). Almost 6 kb of the insert DNA present in pPYP180 was sequenced (deposited with the GenBank databases under accession no. AF002222), revealing a set of four open reading frames, designated *pvcA*, *pvcB*, *pvcC*, and *pvcD* (where *pvc* stands for pyoverdine chromophore), which comprise a putative operon. A fifth open reading frame was identified downstream of and in the opposite orientation to the *pvcABCD* genes (Fig. 1) and was subsequently identified as the *ptxR* gene described by Hamood et al. (18). This LysR family regulator is implicated in exotoxin A production (18). Deletion of a 500-bp *Bgl*II fragment, now known to encompass the 3′ end of *pvcC* and the 5′ end of *pvcD* (Fig. 1), completely abrogated pyoverdine production (44), confirming the involvement of the *pvcABCD* operon in pyoverdine biosynthesis.

The *pvcA* gene encodes a putative protein (37,019 Da) similar to the Dit1 protein of *Saccharomyces cerevisiae* (Table 2) (5). Dit1 catalyzes the formation of an uncharacterized tyrosine-containing precursor for a spore wall-specific dityrosine-containing macromolecule (5). As such, PvcD may function in the condensation of tyrosine and DAB, a proposed step in pyoverdine biosynthesis (6, 21). The second gene, *pvcB* (876 bp), encodes a putative protein (33,165 Da) exhibiting the greatest similarity to the TfdA proteins (oxygenases) of *Alcaligenes eutrophus* (*Ralstonia eutropha*) JMP134 (46) and *Burkholderia* sp. strain RASC (47) (Table 2). The third gene, *pvcC* (1,500 bp) encodes a predicted product (55,812 Da) which shows substantial similarity to the *Klebsiella pneumoniae* HpaA and the *E. coli* HpaB proteins (hydroxylases) (Table 2). It is tempting to suggest, then, that PvcB and PvcC play roles in the two proposed hydroxylation steps of pyoverdine chromophore biosynthesis (6). Finally, the *pvcD* gene (644 bp) encodes a putative product (23,076 Da) which exhibits similarity to proteins of the cytochrome *c* family (Table 2). The implied involvement of a *c* cytochrome in pyoverdine production in *P. aeruginosa* is reminiscent of an earlier observation that a cytochrome c_4 mutant of *Azotobacter vinelandii* lost its capacity to produce azotobactin (45), a pyoverdine-like siderophore. The recently described cytochrome *c* biogenesis protein CytA of *Pseudomonas fluorescens* ATCC 17400 also plays a role, hitherto unknown, in pyoverdine production in this organism (15). Intriguingly, a number of multicomponent aromatic amino acid hydroxylases of mammalian origin utilize components of electron transport to carry out the hydroxylation reaction (19), and PvcD may, therefore, assist PvcBC-mediated hydroxylation.

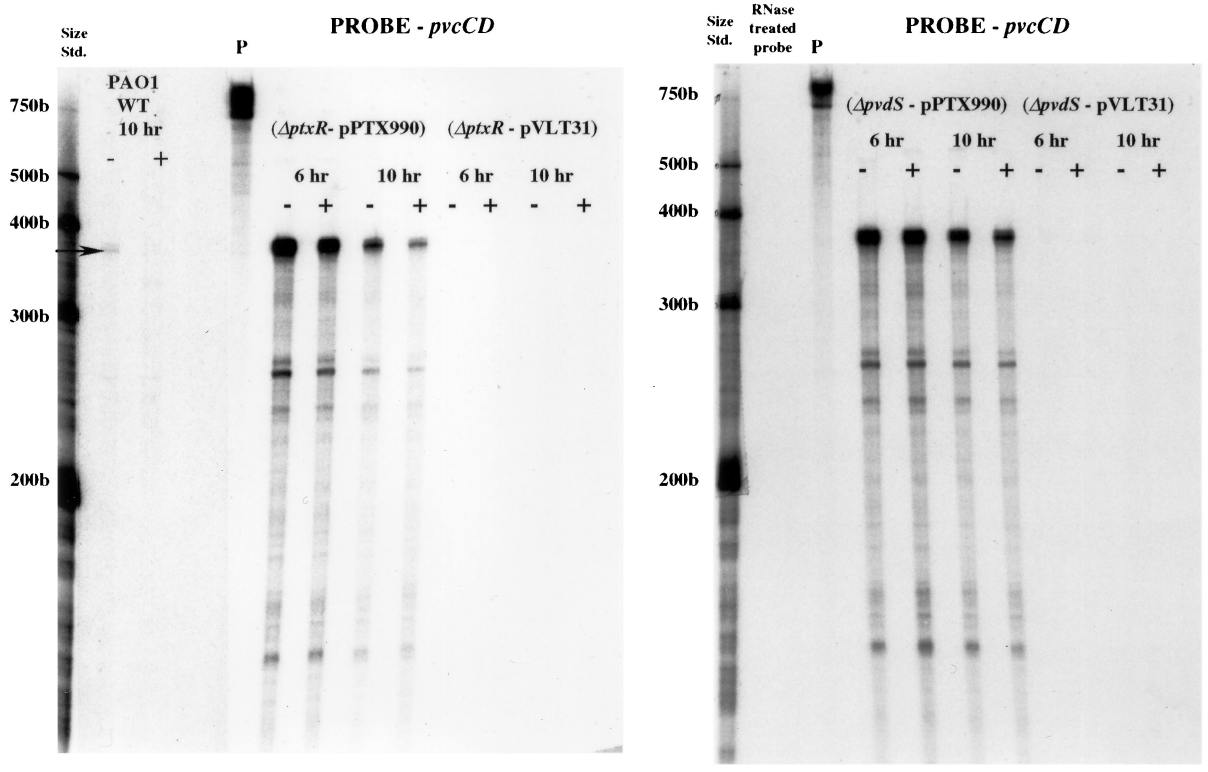

FIG. 4. RNase protection analysis of *pvcABCD* gene expression in the PAO1 Δ*ptxR* and PAO1 Δ*pvdS* strains harboring vectors pVLT31 and pVLT31::*ptxR* (pPTX990). RNA samples were extracted from cells grown continuously for 6 or 10 h in medium that was either iron deficient (−) or iron replete (+), and protection against RNase digestion was afforded by the *pvcCD* probe assessed as described in Materials and Methods. Results for the plasmid-free PAO1 wild-type strain are also shown. Undigested (P) ^{32}P-labelled probe and the positions of ^{32}P-labelled RNA standards (Std.) are shown to the left of each gel. The gels shown in this figure were exposed for 16 h.

Regulation of *pvcABCD* expression. Pyoverdine production is iron regulated, increasing inversely with the concentration of external iron (25). To determine whether *pvcABCD* expression was iron regulated, RNase protection assays using riboprobes derived from the *pvc* genes were performed. By using a *pvcAB* riboprobe an mRNA fragment of the expected size was protected in cells cultured under iron-limiting conditions (Fig. 2). Expression of *pvc* increased with time of growth in iron-limited medium, showing a maximum at 10 h, at which time it was eightfold higher in iron-limited cells than in iron-replete cells (Fig. 2). Similarly, RNase protection assays with the *pvcBC* and *pvcCD* probes demonstrated that iron-limited cells expressed ca. 8- to 10-fold-higher levels of *pvc* mRNA than did their iron-replete counterparts after 10 h of growth (Fig. 2). Assays carried out with a riboprobe for a gene whose expression is known not to be iron regulated, *omlA* (35) (see the legend to Fig. 2) confirmed that differences seen with or without iron were not attributable to variations in total RNA used in the assays above.

Although all probes used provided evidence of iron regulation of *pvcABCD* expression, the levels of *pvc* mRNA protected declined as the riboprobes used moved from the 5′ end to the 3′ end of the operon. Indeed, levels protected by the *pvcBC* and *pvcCD* probes were only 60 and 20%, respectively, of that protected by the *pvcAB* probe (Fig. 2), indicating that the *pvcCD* genes were underrepresented in the *pvc* mRNA population. Intriguingly, a sequence capable of forming a stem-loop structure was identified (CGCCGGCCGGTGCGC GCCACGGCCGGCG; $\Delta G = -30.4$ kcal) within the 51-bp intergenic region between *pvcB* and *pvcC*. Given the absence of an obvious promoter sequence in this region, this likely has an attenuating effect on expression of the *pvcC* and *pvcD* genes from a promoter upstream of *pvcA*. Consistent with this, the insertion of suicide vector pSUP202 sequences between wild-type copies of *pvcB* and *pvcC* in the chromosome of *P. aeruginosa* ML5087 (during an unsuccessful attempt at constructing a *pvcB* mutant) abrogated pyoverdine biosynthesis, despite the fact that the complete *pvcABCD* genes were present in this strain (data not shown). The separation of *pvcCD* from *pvcAB* by pSUP202 sequences likely leads to a lack of *pvcCD* expression, owing to the uncoupling of the latter from the promoter upstream of *pvcA*. Finally, the riboprobes used in this study invariably spanned portions of two adjacent genes, and the fact that fragments of the expected size were protected in each case supports the contention that these genes are encoded on the same (i.e., polycistronic) message.

Given the proximity of the *ptxR* gene to the *pvcABCD* operon it seemed possible that PtxR plays a role in the expression of *pvcABCD* and, thus, pyoverdine. Consistent with this, a mutant carrying a *ptxR* deletion (K1081) was examined for pyoverdine production. K1081 lacked visible pyoverdine (based on the pigmentation and fluorescence of spent culture supernatants). The involvement of *ptxR* in *pvc* expression was confirmed by the RNase protection assay with the *pvcAB* probe. As seen in Fig. 3, the *ptxR* deletion in strain PAO1 completely abrogated *pvc* expression, with no *pvc* mRNA detected under iron-limited or iron-replete conditions. Similar results were observed when the *pvcCD* probe was used (data not shown). Again, control experiments using *omlA* as the riboprobe confirmed that the decline in *pvc* expression was not

due to a decrease in RNA (see the legend to Fig. 3). Finally, constitutive *ptxR* expression from a multicopy plasmid (pPTX990) promoted high-level *pvc* expression, irrespective of iron availability in the growth medium (Fig. 4). Thus, PtxR activates *pvc* gene expression, and iron regulation of this putative operon is not mediated at the level of the *pvcABCD* genes.

The PvdS alternative sigma factor is required for expression of pyoverdine biosynthetic genes of the *pvd* locus (10, 28). To determine if PvdS was similarly required for *pvcABCD* expression, RNase protections assays were also carried out with the *pvcAB* probe and a *pvdS* deletion derivative of PAO1. As seen in Fig. 3, elimination of *pvdS* reduced *pvc* mRNA levels substantially (with no effect on *omlA* mRNA [see the legend to Fig. 3]), although, in contrast to the *ptxR* mutant, some *pvc* expression was still detectable in the *pvdS* mutant and it was iron-regulated (twofold increase in iron-limited cells). Again, control assays run using the *omlA* riboprobe did not show any variation related to iron levels in the growth medium (see the legend to Fig. 3), confirming that this modest effect of iron on *pvc* expression was real. The constitutively expressed cloned *ptxR* gene restored expression of *pvcABCD* to high levels in the *pvdS* deletion strain, indicating that PvdS does not act directly on *pvcABCD* expression. This is consistent with the absence of a putative PvdS binding site (39) upstream of the *pvc* genes and with the recent demonstration that PvdS acts on *ptxR* (49). Thus, PtxR mediates the PvdS effect on *pvcABCD* expression. This involvement of PvdS in *pvcABCD* expression also likely explains the iron-regulation of *pvc* expression, as *pvdS* is iron-regulated by the Fur repressor (26). Still, the observation that a *pvdS* deletion yields detectable, iron-regulated *pvc* gene expression while a *ptxR* deletion completely abrogates *pvc* expression suggests that some PtxR-mediated *pvc* expression can occur in the absence of *pvdS* and that it is still iron regulated.

The *ptxR* gene shown here to be linked to and involved in the expression of the *pvcABCD* operon and, hence, pyoverdine production was described previously (18) and was shown to play a positive regulatory role in the expression of exotoxin A. Hamood et al. (18) also indicated that the gene influenced siderophore production, an observation we have confirmed here. This connection between exotoxin A production and pyoverdine biosynthesis, first suggested by observations that PvdS plays a positive role in expression of exotoxin A (34), is intriguing, although its significance is unclear.

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