JOURNAL OF BACTERIOLOGY, May 1997, p. 2802–2809 Vol. 179, No. 9
0021-9193/97/$04.00+0

A Divergently Transcribed Open Reading Frame Is Located Upstream of the *Pseudomonas aeruginosa vfr* Gene, a Homolog of *Escherichia coli crp*

LAURA J. RUNYEN-JANECKY,† ALLEN K. SAMPLE,‡ TRICIA C. MALENIAK, AND SUSAN E. H. WEST*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 27 June 1996/Accepted 21 February 1997

The *Pseudomonas aeruginosa* homolog of the *Escherichia coli* global transcriptional regulator CRP (or CAP) was recently identified and designated Vfr (S. E. H. West, A. K. Sample, and L. J. Runyen-Janecky, J. Bacteriol. 176:7532–7542, 1994). Nucleotide sequence analysis of the region 5′ to *vfr* identified a 423-bp open reading frame (ORF), which was designated *orfX*. The deduced amino acid sequence of ORFX was 53% identical and 87% similar to a divergent ORF of unknown function located 5′ to the *E. coli crp* gene. When *orfX* was expressed from a phage T7 promoter in *E. coli*, a protein with an apparent molecular mass of approximately 18 kDa was produced. We constructed a chromosomal deletion of the region containing the 5′ end of *orfX* (*orfX′*), *vfr*, and the 3′ end of *trpC* (*trpC′*) in *P. aeruginosa* strains PAO1 and PA103. The cloned *vfr* gene restored Vfr-dependent production of exotoxin A and protease in the PA103 *orfX′-vfr-trpC′* deletion mutant, suggesting that ORFX is not required for Vfr production or activity. To determine whether transcription of *orfX* and *vfr* are controlled by the same mechanisms that control transcription of the region of the divergent ORF (d*orf*) and of *crp*, we compared the *vfr-orfX* and *crp*-d*orf* intergenic regions. Using S1 nuclease analysis, we determined that the distance between the *orfX* and *vfr* transcriptional start sites was 105 bp. Thus, the *P. aeruginosa orfX* and *vfr* promoters are arranged in a back-to-back orientation rather than the face-to-face orientation of the d*orf* and *crp* promoters. A CRP recognition site is associated with each promoter in the *crp*-d*orf* intergenic region; binding of the CRP-cyclic AMP complex to the stronger d*orf* CRP recognition site activates transcription from the d*orf* promoter and represses transcription from the *crp* promoter. The *vfr-orfX* intergenic region does not contain an obvious CRP recognition site. In addition, *vfr* was not required for transcription of *orfX*. Unlike the d*orf* and *crp* mRNAs, the 5′ ends of the *orfX* and *vfr* mRNAs were not complementary. Thus, the *orfX* mRNA cannot hybridize to the 5′ end of the *vfr* mRNA to inhibit *vfr* transcription, a mechanism that has been postulated to control *crp* transcription in *E. coli*.

In *Escherichia coli*, the cyclic AMP (cAMP) receptor protein (CRP or CAP) is a transcriptional regulator of numerous genes, most notably those required for the utilization of various carbon sources. Other genes in the CRP regulon include those involved in pH-regulated gene expression, flagellum synthesis, iron acquisition, enterotoxin production, transfer of F plasmids, and the heat shock response (7, 39). When complexed with cAMP, CRP binds a specific recognition sequence in the promoter of a gene that it activates and interacts with RNA polymerase to initiate transcription; at more complex promoters, the CRP-cAMP complex may also interact with a promoter-specific regulatory protein (14). The CRP-cAMP complex may also repress transcription of some genes (14). Recently, a gene encoding the *Pseudomonas aeruginosa* homolog of CRP was cloned and designated *vfr* (for virulence factor regulator) due to its effect on the production of several virulence factors or their regulators, including exotoxin A, RegA (a positive regulator of exotoxin A production), and LasR (a positive regulator of elastase and alkaline protease production) (19, 43). Vfr is 67% identical and 91% similar to the *E. coli* CRP protein. The amino acid residues in CRP, which constitute the structural features that are required for cAMP binding, DNA binding, and interaction with RNA polymerase, are identical to or conserved in the amino acid residues of Vfr (43). The conservation of these structural features between Vfr and CRP implied that Vfr can substitute for CRP in *E. coli*. The cloned *P. aeruginosa vfr* gene complemented the β-galactosidase and tryptophanase deficience phenotype of an *E. coli crp* deletion mutant (43). Additionally, in an *E. coli crp cya* deletion mutant, which does not produce cAMP, complementation of the β-galactosidase deficience phenotype by *vfr* required the addition of exogenous cAMP, indicating that Vfr requires cAMP for activity in *E. coli* (43).

The mechanisms that regulate *vfr* expression in *P. aeruginosa* have not been elucidated; however, *crp* expression in *E. coli* is known to be both negatively and positively autoregulated at the transcriptional level (1, 9, 12). The negative autoregulation of *crp* transcription is a direct result of CRP-dependent transcription of an open reading frame that diverges from the *crp* gene (which we designated d*orf*) (11, 23, 24). The promoter of d*orf* controls the synthesis of two mRNA species of approximately 300 and 550 nucleotides (6). The longer mRNA contains an ORF encoding a putative protein (dORF) of unknown function (6). The shorter mRNA results from transcriptional termination at a transcriptional terminator located within the d*orf* protein-coding sequence. Transcription from the d*orf* promoter initiates 2 bp 5′ to the *crp* transcription start site (11)

* Corresponding author. Mailing address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, 2015 Linden Dr. West, Madison, WI 53706. Phone: (608) 263-7884. Fax: (608) 263-0438. E-mail: wests@svm.vetmed.wisc.edu.

† Present address: Department of Microbiology, University of Texas, Austin, TX 78712-1095.

‡ Present address: IDEXX Laboratories, Westbrook, ME 04092.

and requires the binding of the CRP-cAMP complex to a CRP recognition site, designated CRP site I, that is centered 43 bp 5′ to the d*orf* transcriptional start site (1).

Two separate hypotheses that describe the molecular mechanism which governs the negative autoregulation of *crp* transcription by CRP-dependent transcription from the d*orf* promoter have been proposed (11, 23). Both of the proposed mechanisms are influenced by the structure of the *crp*-d*orf* intergenic region. Hanamura and Aiba (11) proposed that repression of *crp* transcription occurs because binding of the CRP-cAMP complex to CRP site I positions RNA polymerase so that transcription initiates primarily from the d*orf* promoter (11). Using DNase I footprinting analysis, they demonstrated that when the CRP-cAMP complex was present, RNA polymerase preferentially bound the d*orf* promoter (11). In an alternative model, Okamoto and Freundlich (23) proposed an antisense mRNA model in which the 5′ end of the d*orf* mRNA hybridizes to the 5′ end of the *crp* mRNA to cause premature termination of *crp* transcription. This model was based on the observation that the *E. coli crp*-d*orf* intergenic region contains an imperfect direct repeat that encompases the *crp* and d*orf* transcriptional start sites such that the 5′ ends of the mRNAs are complementary. The antisense mRNA model is supported by the observation that in a *crp* in vitro transcription assay, addition of the d*orf* mRNA decreased the amount of *crp* mRNA produced (23). However, using abortive initiation assays, Hanamura and Aiba (11) demonstrated that production of the d*orf* mRNA was not required for repression of *crp* transcription.

CRP also activates its own transcription. The *crp* promoter contains a weak CRP recognition site, designated CRP site II, which is centered 60 bp 5′ of the *crp* transcriptional start site. Under conditions in which the level of cAMP is extremely high, the CRP-cAMP complex binds CRP site II and positions RNA polymerase so that transcription of *crp* is initiated (12). Hanamura and Aiba (12) suggest that this positive autoregulation of *crp* transcription ensures maximal production of CRP during periods of carbon starvation.

We report here the identification and sequence of a divergent ORF, designated *orfX*, which is located upstream of the *P. aeruginosa vfr* gene and is similar to the divergent ORF located 5′ of the *E. coli crp* gene. We also evaluated whether ORFX and Vfr were required for expression of each other. Because of the high level of conservation between the protein products encoded by the *vfr* and *crp* genes and the *orfX* and d*orf* genes, we undertook an investigation to determine whether the intergenic regions were similar. We found significant differences between the two intergenic regions, suggesting that expression of *orfX* and *vfr* in *P. aeruginosa* occurs by a mechanism(s) distinct from those that mediate transcription of d*orf* and *crp* in *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. All strains were routinely maintained on LB agar (21) at 37°C. Antibiotics were used at the following concentrations (per milliliter): 100 μg of ampicillin and 25 μg of tetracycline for *E. coli* and 200 μg of carbenicillin, 100 μg of tetracycline, and 30 to 100 μg of gentamicin for *P. aeruginosa.*

TSBD broth, an iron-deficient medium used for optimal production of exotoxin A, was prepared as described by Ohman et al. (22). Briefly, tryptic soy broth (Difco Laboratories, Detroit, Mich.) was treated with Chelex-100 (minus 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) to remove iron and was then dialyzed. The dialysate was supplemented with 0.05 M monosodium glutamate and 1% glycerol. For iron-replete conditions, TSBD broth was supplemented with 37 μM $FeCl_3 \cdot 6H_2O$.

Recombinant DNA techniques. Standard recombinant DNA techniques were performed as described by Sambrook et al. (30) and Ausubel et al. (2). *E. coli* SURE (Stratagene Cloning Systems, La Jolla, Calif.), XL1-Blue (Stratagene Cloning Systems), and HB101 (30) were used as the host strains in the cloning experiments. Plasmid DNA was introduced into *P. aeruginosa* by electroporation as described by Smith and Iglewski (37).

To facilitate construction of the probes for mapping the *orfX* and *vfr* transcriptional start sites and construction of the *orfX-lacZ* and *vfr-lacZ* reporter plasmids, the *vfr-orfX* intergenic region was cloned into pUC18 and pGEM-7 (Promega Corp., Madison, Wis.) by the following strategy. pKF402, which contains the 5′ ends of *orfX* and *vfr*, was digested with *Bfa*I, treated with the Klenow fragment of DNA polymerase I (United States Biochemical Corp., Cleveland, Ohio), and digested with either *Eco*RI or *Hin*dIII to generate 0.55-kb fragments containing the *vfr-orfX* intergenic region. These fragments were ligated into either *Eco*RI- and *Sma*I-digested pUC18 or *Hin*dIII- and *Sma*I-digested pGEM-7 to construct pWNP10 and pTM10, respectively. pWNP11 was constructed as described above for pWNP10 except that pKF402 was digested with *Hph*I instead of *Bfa*I and treated with mung bean nuclease (New England Biolabs, Beverly, Mass.) instead of the Klenow fragment of DNA polymerase I.

DNA sequencing. Double-stranded DNA sequencing was accomplished by the dideoxy chain termination method (31) with a Sequenase 2.0 sequencing kit (United States Biochemical Corp.). A 0.6-kb *Nar*I fragment containing the *orfX* gene was subcloned into the vector pBluescript KS^+ (Stratagene Cloning Systems) to generate pKF416. Additional subclones were constructed on the basis of convenient restriction enzyme sites. Plasmid DNA was prepared by the acid-phenol miniprep procedure of Weickert and Chambliss (40); 7-deaza-dGTP was routinely used in place of dGTP to reduce the number of GC compressions (3). To minimize sequence artifacts caused by enzyme pausing, unlabeled dideoxy nucleotides and terminal transferase were added to the completed termination reaction mixture and the incubation was continued at 30°C for 30 min as described by Li and Schweizer (16). *orfX*-specific oligonucleotide primers were synthesized by Gibco Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Sequence data were analyzed with Genetics Computer Group, Inc. (Madison, Wis.), software (version 8). The TFASTA algorithm for protein homology (26) was used to compare the deduced amino acid sequence of *orfX* with sequences in the GenBank database (release 88.0, April 1995).

Expression of the ORFX protein in *E. coli*. We used the phage T7-based expression system described by Tabor and Richardson (38) to label selectively the ORFX protein. A 2.6-kb *Sal*I-*Pst*I fragment containing both the *orfX* and *vfr* genes was cloned into the phage T7 gene 10 promoter-containing vectors pT7-5 and pT7-6 to generate pT7-5-906 and pT7-6-906. A 0.9-kb *Nar*I fragment containing only the *vfr* gene was cloned into pT7-6 to generate pT7-6-917. These plasmids were transformed into *E. coli* KM381 pGP1-2. Expression of polypeptides encoded on the cloned fragments was analyzed by labeling the induced gene products with 20 μCi of ^{35}S-labeled methionine (NEN Research Products, Boston, Mass.) as described by Tabor and Richardson (38). The labeled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% [wt/vol] acrylamide) (15) and visualized by autoradiography.

Construction of a chromosomal deletion of the region containing the 5′ end of *orfX*, *vfr*, and the 3′ end of *trpC*. The mutants PA103-9001 and PAO9001, which have a 1.2-kb deletion of the chromosomal region containing the 5′ end of *orfX* (*orfX′*), *vfr*, and the 3′ end of *trpC* (*trpC′*), were constructed by allelic exchange. Briefly, a 2.6-kb *Sal*I-*Pst*I DNA fragment containing *orfX*, *vfr*, and *trpC′* was cloned into pNOT19 (33) to generate pNOT906. The 1.2-kb *Xho*I fragment containing the 5′ end of *orfX*, *vfr*, and the 3′ end of *trpC* was deleted from pNOT906 and was replaced with a 0.85-kb *Sal*I fragment containing the gentamicin resistance cassette from pUCGM (34) to generate pNOT906Δvfr:Gm. This construct was electroporated into PA103 and PAO1. ColE1-derived vectors are not maintained in *P. aeruginosa*; therefore, stable gentamicin-resistant colonies can be obtained only if the plasmid has integrated into the chromosome. Recombinants were selected on media containing 100 μg of gentamicin per ml for PA103 or 15 to 30 μg of gentamicin per ml for PAO1 and were screened for loss of vector sequences by selection on 200 μg of carbenicillin per ml. Southern hybridization analysis of chromosomal DNA from the gentamicin-resistant and carbenicillin-sensitive recombinants confirmed that the 1.2-kb *Xho*I fragment containing *orfX′*, *vfr*, and *trpC′* had been deleted.

Exotoxin A activity. For measurement of exotoxin A production, TSBD broth (10 ml) was inoculated with 100 μl of an overnight TSBD culture and was incubated at 32°C with shaking for 18 h. The cultures were centrifuged for 10 min at 12,000 × *g*, and the supernatant fractions were stored at −80°C until they were assayed for exotoxin A activity. Thawed supernatants (10 μl) were activated by treatment with urea and dithiothreitol and were assayed for ADP-ribosyl transferase activity as described by Ohman et al. (22) and Chung and Collier (8). Partially purified elongation factor 2 from wheat germ (8) and [^{14}C]NAD (NEN Research Products) were used as the substrates. Exotoxin A activity was expressed as counts per minute per 10 μl of culture supernatant.

Assay for proteolytic activity. Proteolytic activity was assayed by spotting 10 μl of an overnight culture on tryptic soy agar plates containing 1.5% (wt/vol) skim milk as described by Wretlind and Pavlovskis (45). The plates were incubated at room temperature for 48 h. Proteolytic activity was measured as zones of clearing around the colonies.

RNA extraction. A guanidine thiocyanate-hot phenol method was used to isolate total cellular RNA from PAO1 grown to stationary phase in 100 ml of TSBD medium. Cells were collected by centrifugation at 6,000 × *g* and resus-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
P. aeruginosa strains		
PA103	Prototroph; exotoxin A hyperproducer; elastase deficient	17
PA103-9001	Derivative of PA103 in which the 1.2-kb chromosomal region containing the 5′ end of *orfX*, *vfr*, and the 3′ end of *trpC* was replaced with an 0.85-kb *Sal*I fragment carrying the *aacC1* gene from pUCGM; Gm^r	This study
PAO1	Prototroph; Cm^r	13
PAO9001	Derivative of PAO1 in which the 1.2-kb chromosomal region containing the 5′ end of *orfX*, *vfr*, and the 3′ end of *trpC* was replaced with an 0.85-kb *Sal*I fragment carrying the *aacC1* gene from pUCGM; Cm^r Gm^r	This study
E. coli KM381	Host for the phage T7 expression system	38
Plasmids		
pKF416	pBluescript KS^- carrying *orfX* on a 0.6-kb *Nar*I fragment; Ap^r	This study
pUCP19	*E. coli-P. aeruginosa* shuttle vector; Ap^r Cb^r	32
pKF906	pUCP19 carrying *vfr* and *orfX* on a 2.6-kb *Pst*I-*Sal*I fragment; Ap^r Cb^r	43
pKF917	pUCP19 carrying *vfr* on a 0.9-kb *Nar*I fragment; Ap^r Cb^r	43
pT7-5	pBR322 carrying a multiple-cloning site downstream of the gene 10 promoter of phage T7; Ap^r	38
pT7-6	pT7-5 with a multiple-cloning site in the orientation opposite to that of pT7-5; Ap^r	38
pGP1-2	pACYC177 carrying the phage T7 RNA polymerase gene under p_L control and the *c*I857 repressor gene; Km^r	38
pT7-5-906	pT7-5 carrying the 2.6-kb *Sal*I-*Pst*I fragment containing *vfr* on an *Eco*RI-*Pst*I fragment from pKF906; Ap^r	43
pT7-6-906	pT7-6 carrying the 2.6-kb *Sal*I-*Pst*I fragment containing *vfr* on an *Eco*RI-*Pst*I fragment from pKF906; Ap^r	43
pT7-6-917	pT7-6 carrying the 0.9-kb *Nar*I fragment containing *vfr* on an *Eco*RI-*Pst*I fragment from pKF917; Ap^r	43
pNOT19	pUC19 containing a 10-bp *Nde*I-*Not*I adaptor in the *Nde*I site; Ap^r	33
pUCGM	pUC1918 carrying the *aacC1* gene; Gm^r	34
pNOT906	pNOT19 carrying a 2.6-kb *Sal*I-*Pst*I fragment containing *orfX*, *vfr*, and *trpC′*; Ap^r	This study
pNOT906Δvfr:Gm	pNOT906 in which the 1.2-kb *Xho*I fragment was replaced with the 0.85-kb *Sal*I fragment containing the *aacC1* gene from pUCGM; Ap^r Gm^r	This study
pKF402	pBluescript KS^- carrying the 5′ ends of *orfX* and *vfr* on a 1.2-kb *Xho*I fragment; Ap^r	This study
pWNP10	pUC18 carrying a 0.55-kb *Eco*RI-*Bfa*I fragment containing the *vfr-orfX* intergenic region from pKF402; Ap^r	This study
pWNP11	pUC18 carrying a 0.55-kb *Eco*RI-*Hph*I fragment containing the *vfr-orfX* intergenic region from pKF402; Ap^r	This study
pTM10	pGEM-7 carrying a 0.55-kb *Hin*dIII-*Bfa*I fragment containing the *vfr-orfX* intergenic region from pKF402; Ap^r	This study
pRS415	Promoterless *lacZYA* transcriptional fusion vector; Ap^r Cb^r	36
pMLB1034	Promoterless *lacZ* translational fusion vector; Ap^r Cb^r	35
pLTxn	Transcriptional fusion vector carrying the promoterless *E. coli lacZ* gene derived from pRS415 and pMLB1034; Ap^r	This study
pRO1614	Broad-host-range vector; Ap^r Cb^r	25 and 44
pLJR0	pLTxn carrying a 1.9-kb *Pst*I fragment from pRO1614; Ap^r Cb^r	This study
pWNP12	pLJR0 carrying a 0.55-kb *Eco*RI-*Bam*HI fragment from pWNP10; the *vfr* promoter directs *lacZ* expression; Ap^r Cb^r	This study
pTM100	pLJR0 carrying a 0.55-kb *Eco*RI-*Bam*HI fragment from pTM10; the *orfX* promoter directs *lacZ* expression; Ap^r Cb^r	This study

[a] Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance.

pended in 3 ml of 4 M guanidine thiocyanate (30). Three milliliters of phenol was added, and the samples were vortexed for 30 s and then incubated at 60°C for 1 min. One and one-half milliliters of 100 mM sodium acetate (pH 5.2)–10 mM Tris-HCl (pH 7.4)–1 mM sodium EDTA and 3 ml of chloroform-isoamyl alcohol (24:1) were added. The samples were vortexed for 30 s and incubated at 60°C for 1 min. The suspension was then centrifuged, and the aqueous phase was collected. Additional phenol-chloroform-isoamyl alcohol extractions (60°C) were done until no visible protein remained. The RNA was then ethanol precipitated. Residual DNA and protein were removed by treatment with DNase I (Pharmacia Biotech, Inc., Piscataway, N.J.) at 37°C for 1 h followed by digestion with proteinase K (Calbiochem, La Jolla, Calif.) at 37°C for 1 h. Residual DNase I and proteinase K were removed by phenol-chloroform-isoamyl alcohol extraction (25:24:1 [vol/vol]) at 60°C. The RNA was ethanol precipitated and redissolved in diethylpyrocarbonate-treated water.

S1 nuclease mapping. To identify the 5′ end of the *orfX* mRNA, two probes complementary to the *orfX* mRNA but differing at their labeled 5′ termini were used. To generate probe 1, pWNP11 was digested with *Nco*I, which cuts 94 bp 3′ of the *orfX* ATG, labeled at the 5′ ends with [γ-^{32}P]dATP (NEN Research Products) with T4 polynucleotide kinase (Gibco Bethesda Research Laboratories, Inc.), and then digested with *Bfa*I to liberate a 503-bp fragment labeled on the DNA strand complementary to the *orfX* mRNA sequence. Probe 2, which contains a 5′ terminus located 109 bases 3′ of the *orfX* ATG, was generated as described above except that pWNP10 was digested with *Ava*I and *Sac*I. Two probes were also generated to identify the 5′ end of the *vfr* mRNA. For probe 3, pWNP10 was digested with *Bsa*I, which cuts 68 bp 5′ to the *vfr* ATG, labeled at the 5′ end with [γ-^{32}P]dATP with T4 polynucleotide kinase, and digested with *Eco*RI, which liberated a 462-bp fragment labeled on the DNA strand complementary to the *vfr* mRNA sequence. Probe 4, which contains a 5′ terminus 46 nucleotides 3′ to the *vfr* ATG, was isolated as described above except that an *Eco*RI-*Bam*HI fragment from pWNP11 was digested with *Bfa*I and *Xho*I.

The S1 nuclease protection experiments were done by the method of Berk and Sharp (5). Total cellular RNA from PAO1 and the end-labeled probes were mixed in 80% formamide–40 mM PIPES (pH 6.7)–400 mM NaCl–1 mM EDTA, denatured for 10 min at 85°C, and then allowed to hybridize for 16 h at 55°C. Hybridization products were digested with 100 U of S1 nuclease (Gibco Bethesda Research Laboratories) for 45 min at 37°C in 8% formamide–4 mM

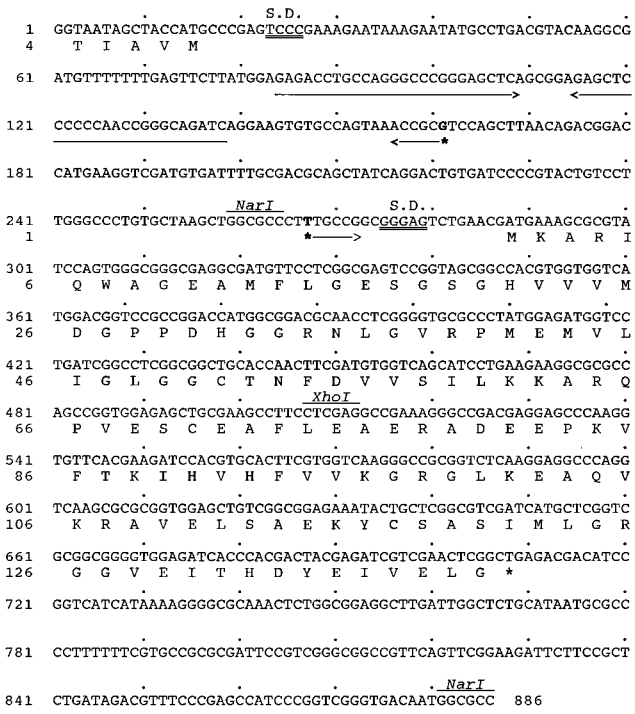

FIG. 1. Nucleotide sequence of the region located 5′ to the *P. aeruginosa vfr* gene. The derived amino acid sequences of *orfX* and *vfr* are shown below the coding sequence. The putative ribosome binding sites are double underlined, and the putative *orfX* stop codon is indicated with an asterisk. The start sites for *vfr* and *orfX* transcription (in boldface type) are indicated by boldface asterisks; the arrows indicate the directions of transcription. A 52-bp imperfect inverted repeat is designated by facing arrows. The *Xho*I and *Nar*I restriction sites are indicated. S.D., Shine-Dalgarno sequence.

PIPES (pH 6.7)–290 mM NaCl–1 mM EDTA–30 mM sodium acetate (pH 4.6)–1 mM zinc acetate–5% glycerol. A G+A sequencing ladder was generated according to the method of Maxam and Gilbert (20) with the end-labeled probes as templates. S1 nuclease-protected DNA fragments were analyzed on an 8 M urea–6% polyacrylamide sequencing gel.

β-Galactosidase assays. For measurement of the activities of the *vfr* and *orfX* promoters, we constructed transcriptional fusions of the *vfr-orfX* intergenic region, in both orientations, to the *E. coli* promoterless *lacZ* gene. The *lacZ* transcriptional fusion vector pLJR0 was constructed by the following strategy. The 2.4-kb *Sca*I-*Cla*I DNA fragment from pRS415 (45), which contains four transcriptional terminators from the *E. coli rrnB* operon, a multicloning site, translational stop codons, and the amino terminus of the promoterless *lacZ* gene, was ligated to the 4.8-kb *Cla*I-*Sca*I fragment from pMLB1034 (35), which contains the carboxyl terminus of the *lacZ* gene and the ColE1 origin of replication, to generate pLTxn. To enable pLTxn to replicate in *P. aeruginosa*, a 1.9-kb *Pst*I fragment from pRO1614 (25, 44) was inserted into the single *Pst*I site to generate pLJR0. An *Eco*RI-*Bam*HI fragment containing the *vfr-orfX* intergenic region from pWNP10 was cloned into pLJR0 to generate pWNP12. pWNP12 contains 487 bp 5′ and 46 bp 3′ to the *vfr* ATG; the *vfr* promoter directs *lacZ* expression. The same *Eco*RI-*Bam*HI fragment containing the *vfr-orfX* intergenic region from pTM10 was cloned into pLJR0 in the opposite orientation to generate pTM100 so that the *orfX* promoter directs *lacZ* expression. PAO1 or PA103 carrying either pWNP12, pTM100, or pLJR0 was grown overnight in TSBD broth at 32°C with shaking. Promoter activity was assessed by measuring β-galactosidase activities as described by Miller (21).

Nucleotide sequence accession number. The *orfX* nucleotide sequence has been submitted to GenBank and assigned accession no. U89892.

RESULTS

Nucleotide sequence analysis of the *P. aeruginosa orfX* gene. We determined the nucleotide sequence of an 886-bp fragment which contained the region upstream of the *P. aeruginosa vfr* gene and 15 bp of the *vfr* coding sequence (Fig. 1). This fragment contained a 423-bp ORF divergent from *vfr* beginning 273 bp 5′ to the *vfr* translational start codon. The ORF, designated *orfX*, encoded a putative protein of 140 amino acids (ORFX) which had a predicted molecular mass of 15,237 Da and a predicted pI of 6.36. The codon usage pattern of ORFX agreed with typical codon usage patterns for *P. aeruginosa* (41). A potential ribosome binding site, GGAG, was found 8 bp 5′ to a putative ATG translational start codon, and a stop codon was located at bp 708. However, a Rho-independent transcriptional terminator was not found 3′ to the stop codon. This observation suggests that termination of *orfX* transcription occurs by a Rho-dependent mechanism or that *orfX* is the first gene in an operon. A search of the 180-bp region 3′ to the *orfX* translational stop codon revealed several putative ATG and GTG start codons. However, because these putative start codons were not preceded by a recognizable ribosome binding site and the codon usage patterns of these ORFs were not typical of *P. aeruginosa*, it is unlikely that a functional ORF is present in the 180-bp region downstream of *orfX.*

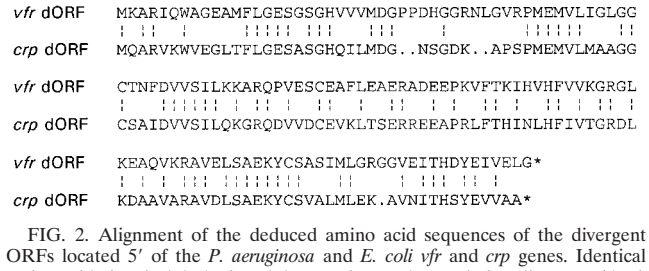

FIG. 2. Alignment of the deduced amino acid sequences of the divergent ORFs located 5′ of the *P. aeruginosa* and *E. coli vfr* and *crp* genes. Identical amino acids (vertical dashes) and the gaps inserted to optimize alignment (dots) are indicated. The stop codons are indicated by asterisks.

We conducted a TFASTA search of the GenBank database using the deduced amino acid sequence of *orfX* and found that ORFX was 53% identical and 87% similar to the deduced amino acid sequence of a divergent ORF located immediately upstream of the *E. coli crp* gene (Fig. 2). One significant difference was detected in the nucleotide sequence of these two ORFs: the factor-independent transcriptional terminator, which is found within the d*orf* coding sequence, is not present in the *P. aeruginosa orfX*-coding sequence. The carboxy termini of *E. coli* dORF and ORFX were also 52 and 65% identical, respectively, to the deduced amino acid sequence of an uncharacterized 195-bp region of DNA located 3′ to the *Alcaligenes eutrophus rnp*B gene, which encodes RNase P. Thus, this uncharacterized region may contain the *A. eutrophus orfX* homolog.

Transcription directed by the *P. aeruginosa orfX* promoter. To determine whether the *orfX* promoter functions in *P. aeruginosa*, we measured β-galactosidase activity directed by the *orfX* promoter and compared it to the level of β-galactosidase activity directed by the *vfr* promoter in *P. aeruginosa* PA103. For this analysis, we fused the *vfr-orfX* intergenic region, in both orientations, to a plasmid-borne promoterless *lacZ* gene. *lacZ* expression was directed by the *orfX* promoter on pTM100 and by the *vfr* promoter on pWNP12 (Fig. 3). The patterns of β-galactosidase production controlled by the *orfX* and *vfr* promoters were similar. During the logarithmic phase of growth, the level of β-galactosidase produced under the control of either the *orfX* or the *vfr* promoter remained constant and gradually increased twofold during stationary phase. Negligible amounts of β-galactosidase were produced in PA103 containing only the transcriptional fusion vector. Thus, these results indicate that the *orfX* promoter functions in *P. aeruginosa.*

Production of the *P. aeruginosa orfX* gene product in *E. coli.* To demonstrate that a translated protein is produced from the

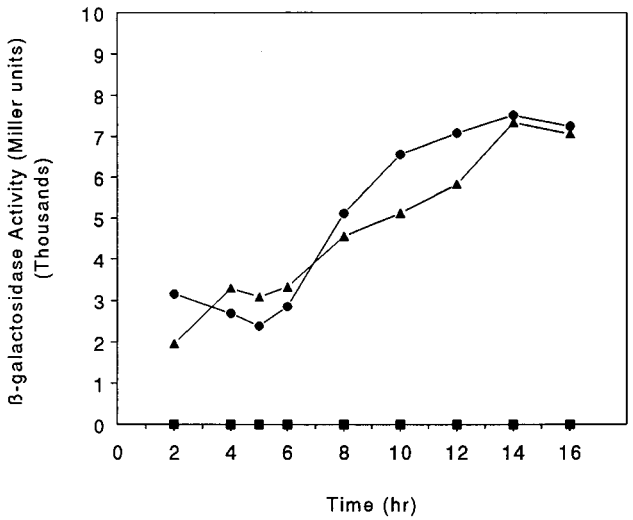

FIG. 3. *orfX* promoter functions in *P. aeruginosa*. PA103 containing an *orfX* promoter-*lacZ* fusion carried on pTM100 (triangles), a *vfr* promoter-*lacZ* fusion carried on pWNP12 (circles), or the vector plasmid pLJR0 (squares) was grown in TSBD broth at 32°C. β-Galactosidase activities are expressed in Miller units. No difference was detected in the rates of growth of the three strains.

423-bp *P. aeruginosa orfX* gene, we used the phage T7-based expression system to express and label selectively the *P. aeruginosa orfX* gene product in *E. coli* (38). We cloned the 2.6-kb *Sal*I-*Pst*I fragment containing the *orfX* gene in both orientations relative to the inducible phage T7 gene 10 promoter to generate pT7-5-906 and pT7-6-906. In addition to the *orfX* gene, the 2.6-kb *Sal*I-*Pst*I fragment carried the *vfr* gene and a truncated *trpC* gene that contained a deletion of its first 18 bp (Fig. 4A). A unique protein with an apparent molecular mass of approximately 18 kDa was produced in cells containing pT7-6-906 but not in cells containing pT7-5-906, which carries the *orfX* gene in the opposite orientation (Fig. 4B). This apparent molecular mass is slightly larger than the 15.2-kDa predicted molecular mass for ORFX and is probably due to aberrant migration of ORFX on the sodium dodecyl sulfate-polyacrylamide gel. The 27.5-kDa protein produced by cells carrying pT7-5-906 is Vfr (43). Termination of transcription at the transcriptional terminator located between *trpC* and *vfr* and also at a 52-bp stem-loop structure located in the *orfX-vfr* intergenic region may account for the low level of ORFX production. Cells containing pT7-6-906 also produced a polypeptide with an apparent molecular mass of less than 6.4 kDa. This polypeptide was not produced from the 0.9-kb *Nar*I fragment carried on pT7-6-917. This fragment is an internal

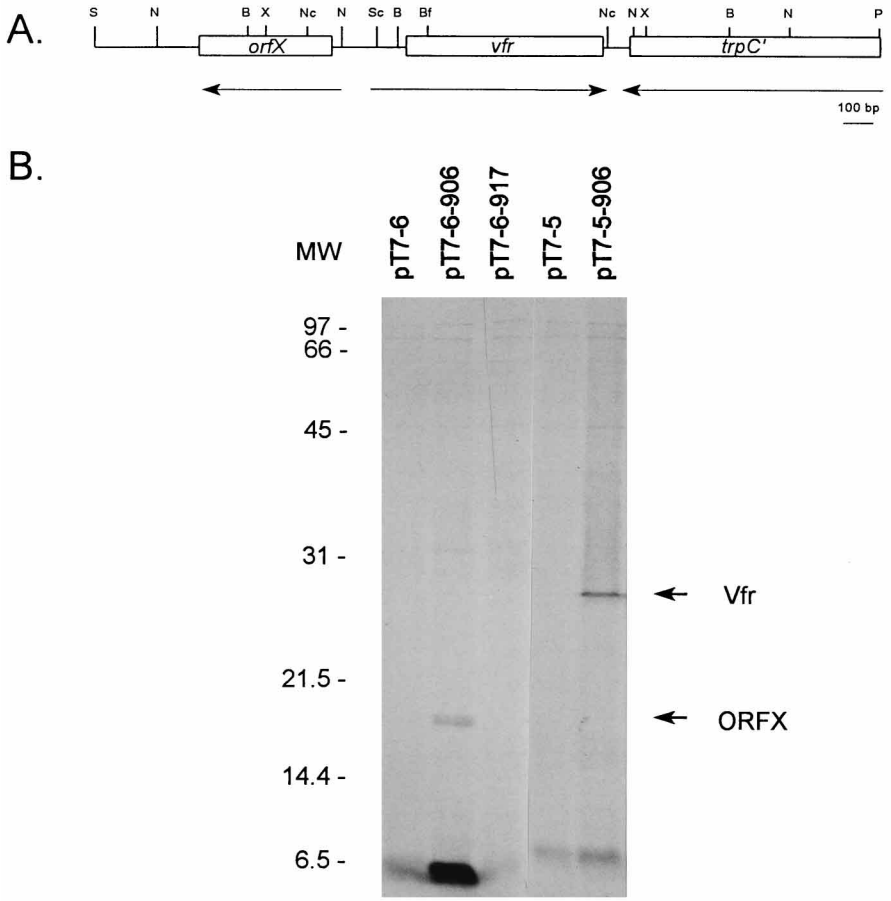

FIG. 4. Expression of the *P. aeruginosa orfX* gene product in *E. coli*. (A) Partial restriction map of the *orfX-vfr* locus. The direction of transcription of each gene is indicated by an arrow. Restriction endonucleases: S, *Sal*I; N, *Nar*I; X, *Xho*I; A, *Ava*I; Nc, *Nco*I; Sc, *Sac*I; B, *Bsa*I; Bf, *Bfa*I; H, *Hph*I; P, *Pst*I. (B) Autoradiogram showing expression of the *orfX* gene product in *E. coli*. Expression of *orfX* was induced from the inducible phage T7 promoter as described in Materials and Methods. Plasmid constructs are indicated above the lanes. pT7-6-906 and pT7-5-906 contain the entire 2.6-kb *Sal*I-*Pst*I fragment; pT7-6-917 contains the 0.9-kb *Nar*I fragment encoding only *vfr*. The locations of the molecular weight standards (MW) in thousands are indicated on the left. The locations of ORFX and Vfr are shown on the right.

TABLE 2. ORFX is not required for exotoxin A production in the *P. aeruginosa orfX′-vfr-trpC′* mutant PA103-9001

Strain	ADP-ribosyl transferase activity[a] (cpm/10 μl of supernatant) in indicated strain with:		
	No plasmid	pUCP19 (vector)	pKF917 (*vfr*)
PA103-9001 (*orfX′-vfr-trpC′*)	101 ± 14	51 ± 15	8,659 ± 377
PA103	11,941 ± 2,871	7,901 ± 3,525	17,685 ± 4,349

[a] Determined as described by Ohman et al. (22) and Chung and Collier (8). The data shown are the means ± standard deviations of three experiments.

fragment of the 2.6-kb *Sal*I-*Pst*I fragment in pT7-6-906, encodes *vfr*, and was present in the same orientation as that of the phage T7 gene 10 promoter in pT7-6-906 (Fig. 4A). Thus, this small polypeptide must be encoded at either the *trpC′* or the *orfX* end of the 2.6-kb *Sal*I-*Pst*I fragment from pT7-6-906. A 111-bp ORF which potentially encodes a 4-kDa polypeptide and is preceded by a potential ribosome binding site (AGGA GG) is located at the *trpC′* end of the 2.6-kb *Sal*I-*Pst*I fragment in pT7-6-906. The high level of expression of this polypeptide is consistent with its location immediately 3′ of the strong T7 gene 10 promoter.

Effect of ORFX on Vfr production or activity. We hypothesized that ORFX may regulate Vfr production or modulate its activity. To examine this possibility, we determined whether *vfr* carried on pKF917 could restore Vfr-dependent exotoxin A and protease production in the *orfX′-vfr-trpC′* deletion mutant PA103-9001. pKF917 restored exotoxin A production in PA103-9001 to levels that were comparable to those observed for the parental strain, PA103, carrying the vector plasmid pUCP19 and to 50% of the level of PA103 carrying pKF917 (Table 2). *vfr* carried on pKF917 also restored protease production in PA103-9001 (data not shown). The observation that Vfr-dependent production of these exoproteins did not require the cloned *orfX* gene suggests that ORFX is not required for Vfr production or activity. However, we cannot rule out the possibility that transcription of *orfX* may repress Vfr production as transcription of d*orf* represses *crp* transcription in *E. coli.*

Identification of the transcriptional start sites for *orfX* and *vfr*. Transcription from the *E. coli* d*orf* promoter, which initiates 2 bp from the *crp* transcriptional start site, represses transcription of *crp* (11, 23, 24). To determine if a similar mechanism might control transcription of *vfr*, we identified the apparent initiation sites for transcription of *orfX* and *vfr* in *P. aeruginosa* PAO1 using S1 nuclease mapping (Fig. 5). From this analysis, we determined that transcription of *orfX* begins at a thymidine residue located 21 bp 5′ to the *orfX* ATG and that transcription of *vfr* begins at a cytosine located 146 bp 5′ of the *vfr* ATG. The same transcriptional start sites were obtained with a second set of DNA probes with different labeled 5′ ends (data not shown). Thus, the distance between the *orfX* and *vfr* transcriptional start sites is 105 bp, which implies that the promoters of these two genes may function simultaneously.

Effect of Vfr on *orfX* promoter activity. Transcription from the *E. coli* d*orf* promoter requires the binding of a CRP-cAMP complex to a CRP recognition sequence centered 43 bp 5′ to the d*orf* transcriptional start site (23). To determine whether Vfr was required for *orfX* transcription, we measured the activity of the *orfX-lacZ* fusion carried on pTM100 in stationary-phase cells (23 h) of the *P. aeruginosa orfX′-vfr-trpC′* deletion mutants PAO9001 and PA103-9001. PAO9001 and PA103-9001 carrying pTM100 produced 7,026 and 5,302 Miller units

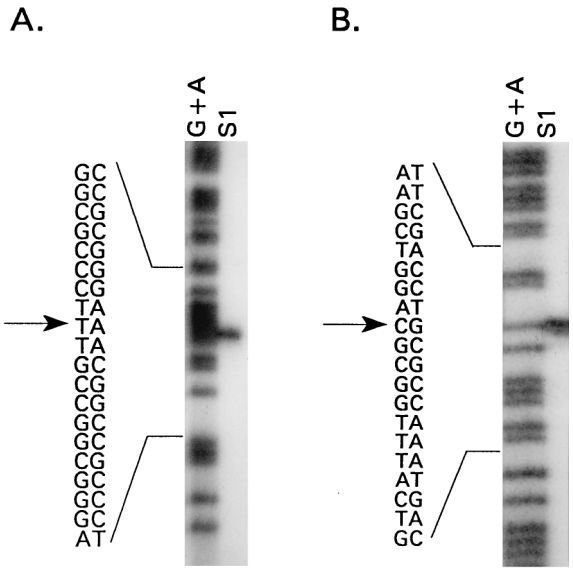

FIG. 5. Mapping of the *orfX* and *vfr* transcriptional start sites. S1 nuclease analysis was done as described in Materials and Methods. S1 nuclease (S1)-protected fragments and G+A Maxam and Gilbert sequencing ladders of the corresponding DNA fragments were electrophoresed on a 6% polyacrylamide–8 M urea gel. Arrowheads indicate S1 nuclease-resistant products. (A) Total cellular mRNA that was probed with a 503-bp *Nco*I-*Bfa*I DNA fragment labeled at the *Nco*I end on the strand complementary to *orfX* mRNA. (B) Total cellular mRNA that was probed with a 462-bp *Bsa*I-*Eco*RI DNA fragment labeled at the *Bsa*I end on the strand complementary to *vfr* mRNA.

of β-galactosidase, respectively. These levels were comparable to those of the parental strains PAO1 and PA103 carrying pTM100, which produced 6,907 and 7,953 Miller units of β-galactosidase activity, respectively. Negligible amounts of β-galactosidase were produced in strains containing only the transcriptional fusion vector (data not shown). These observations suggest that Vfr was not required for *orfX* transcription in *P. aeruginosa.*

Characterization of the *vfr-orfX* intergenic region. Even though the nucleotide sequences of the *vfr-crp* and *orfX*-d*orf* coding regions are 65 and 68% identical, respectively, over the entire lengths of the coding sequences, the intergenic regions associated with these genes appear to have diverged significantly. Figure 6 is a comparison of the *crp*-d*orf* and *vrf-orfX* intergenic regions. With the exception of two 20- to 22-bp sequences, no sequence similarity was detected between the *vfr-orfX* and *crp*-d*orf* intergenic regions. A 20-bp region that overlaps the *orfX* start of transcription and the putative −10 region and the *E. coli* CRP site I were 75% identical, and a 22-bp region centered 34 bp from the *vfr* translational start site and a region centered 31 bp from the *E. coli* d*orf* translational start site were 68% identical. Because the distance between the *vfr* and *orfX* transcriptional start sites is 105 bp, the arrangement of the *P. aeruginosa vfr* and *orfX* promoters is a back-to-back arrangement with intervening DNA between the two promoters. The *E. coli crp* and d*orf* promoters are arranged in a face-to-face configuration in which the transcriptional start sites are separated by 1 bp (4, 11). The *E. coli crp*-d*orf* intergenic region contains two CRP recognition sequences; CRP site I is centered 43 bp 5′ of the d*orf* transcriptional start site, which is required for transcription from d*orf*, and CRP site II is centered at 60 bp 5′ of the *crp* transcriptional start site (23). An obvious CRP recognition sequence was not present in the *P. aeruginosa vfr-orfX* intergenic region. The *E. coli crp*-d*orf*

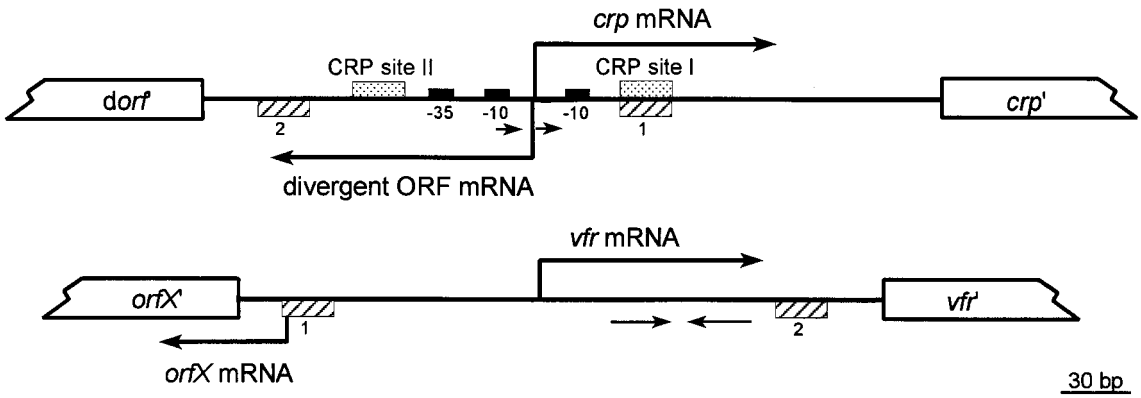

FIG. 6. Comparison of the *P. aeruginosa vfr-orfX* and *E. coli crp*-d*orf* intergenic regions. The protein-coding sequences are indicated by open boxes. Large arrows originating from the transcriptional start sites indicate the directions of transcription. The *E. coli* −10 and −35 sigma^{70} recognition sequences are represented by filled boxes. The CRP recognition sequences in the *crp*-d*orf* intergenic region are represented by stippled boxes. Small arrows indicate direct and indirect repeats. The two conserved regions within the *vfr-orfX* and *crp*-d*orf* intergenic regions are indicated by correspondingly numbered and cross-hatched boxes.

intergenic region also contains a pair of direct repeats located at the *crp* and d*orf* transcriptional start sites such that the 5′ ends of the mRNAs are complementary (23). The *P. aeruginosa vfr-orfX* intergenic region does not contain any direct repeats. The 5′ ends of the *vfr* and *orfX* mRNAs are also not complementary to each other. An imperfect 52-bp inverted repeat is located 22 bp 3′ to the *vfr* transcriptional start site and may be capable of forming a large hairpin structure with a 6-nucleotide loop. This structure is followed by an adenosine-rich stretch. Such a structure is not present in the *E. coli crp*-d*orf* intergenic region.

We also examined the *orfX-vfr* intergenic region for putative *P. aeruginosa* sigma factor recognition sequences (28). We did not find strong matches to the putative *P. aeruginosa rpoD* and *rpoN* consensus recognition sequences (YSTTGR-17 to 18 bp-YRTAAT and RR-TGGCAT–9 bp–YTGCTTR, respectively, where Y is C or T, R is A or G, and S is G or C) (28). The lack of an RpoD or RpoN recognition sequence suggests that transcription from the *orfX* and/or *vfr* promoters requires a different sigma factor and/or an accessory transcription-activating protein (10). In addition, like many other *P. aeruginosa* genes (28), the 5′ noncoding regions of *vfr* or *orfX* have little or no recognizable sequence similarity to promoter sequences in enteric bacteria.

DISCUSSION

In the facultative anaerobe *E. coli*, a divergent ORF (d*orf*) is located upstream of the *crp* gene (6). Whether a protein product is produced from this ORF has not been investigated. Therefore, it is not known if this putative protein has a function. We have found in the obligate respiring organism *P. aeruginosa* a d*orf* homolog, designated *orfX*, which is located upstream of the *crp* homolog *vfr* and is 53% identical and 87% similar at the amino acid level to the *E. coli* d*orf*. Additionally, a putative d*orf* homolog was found in the chemolithotrophic bacterium *A. eutrophus*. The conservation of this ORF among three metabolically distinct bacterial species suggests that it may have an important, and therefore conserved, cellular function. The deduced amino acid sequence of ORFX contained no obvious signal sequences for secretion or membrane-spanning domains (27); thus, ORFX may be a cytoplasmic protein. ORFX is not essential for cell viability, as we were able to construct viable *orfX′-vfr-trpC′* deletion mutants of *P. aeruginosa* PA103 and PAO1. The *orfX′-vfr-trpC′* deletion mutants grew on minimal media supplied only with tryptophan (data not shown) and possessed wild-type catabolite repression control (18). These observations suggest that ORFX is not involved in the synthesis of amino acids and vitamins and, like Vfr, is not required for catabolite repression control. Other than tryptophan auxotrophy due to the deletion of *trpC*, the phenotype of the *orfX′-vfr-trpC′* deletion mutants was unchanged from that of the *vfr* mutants PA103-15, PA103-16, and PA103-19, which we have previously described (42, 43). Thus, we cannot associate a distinct phenotype with an *orfX* mutation. We also considered the possibility that the function of ORFX may be related to the transcription of *vfr* or Vfr activity. Our observation that only *vfr* was required to restore Vfr-activated exotoxin A and protease production in the *orfX′-vfr-trpC′* deletion mutant suggests that ORFX is not required for production or activity of Vfr; however, we cannot rule out the possibility that ORFX may have a subtle effect on Vfr, perhaps by affecting the level of Vfr that is produced or by modulating Vfr activity.

Our comparison of the two intergenic regions revealed that many of the structural features associated with transcription of either *crp* or d*orf* were altered or missing in the *vfr-orfX* intergenic region. These observations imply that transcriptional regulation of these two highly conserved gene pairs occurs by different molecular mechanisms. For example, the *E. coli crp* and d*orf* promoters are classified as competing promoters arranged in divergent orientations, specifically in a face-to-face arrangement where the 5′ termini of the mRNA are only 1 bp apart (4, 11). Because of this arrangement, these two promoters compete for binding of RNA polymerase. The *P. aeruginosa vfr* and *orfX* promoters are arranged in a back-to-back configuration with 105 bp of intervening DNA between the two transcriptional start sites. Since the RNA polymerase binding site in many promoters extends only 45 bp 5′ of the transcriptional start site (13), two RNA polymerase molecules may bind the *orfX* and *vfr* promoters simultaneously to initiate transcription. However, we cannot eliminate the possibility that the RNA polymerase binding sites at the *orfX* and *vfr* promoters overlap since it has been shown that at some promoters, RNA polymerase can bind sequences 60 bp 5′ of the transcriptional start site (29). The binding of a transcriptional activator or repressor to either the *vfr* or the *orfX* promoter may block binding of RNA polymerase or a transcriptional regulator at the other promoter. Neither OrfX nor Vfr are required for the other's transcription. It is not known if accessory transcrip-

tional factors are involved in transcription of *vfr* and/or *orfX*. The lack of complementarity between the 5′ ends of the *vfr* and *orfX* mRNAs implies that the regulation of *vfr* transcription by formation of an *orfX-vfr* mRNA hybrid does not occur. The 52-bp imperfect inverted repeat which is followed by an adenosine-rich stretch is not present in the *E. coli crp*-d*orf* intergenic region. Although the significance of the potential hairpin structure is unclear, since transcription of *vfr* must proceed through this region, the hairpin may result in premature termination of the *vfr* mRNA. Alternatively, the hairpin may protect the *vfr* mRNA from degradation at the 5′ end.

The *P. aeruginosa vfr-orfX* and *E. coli crp*-d*orf* structural genes are highly conserved; however, the sequences and structures of the intergenic regions containing the promoters have diverged significantly. Differences between the *P. aeruginosa* and *E. coli* transcriptional machinery may have necessitated a change in the promoter structure for optimal gene expression in each of these organisms. This intergenic region appears to be an excellent model for the study of the evolution of bacterial promoters.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI31477 from the National Institute of Allergy and Infectious Diseases awarded to S.E.H.W. L.J.R.-J. was a trainee under Public Health Service grant T32 GM 07215.

REFERENCES

1. **Aiba, H.** 1983. Autoregulation of the *Escherichia coli crp* gene: CRP is a transcriptional repressor for its own gene. Cell **32:**141–149.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing and Wiley Interscience, New York N.Y.
3. **Barr, P. J., R. M. Thayer, P. Laybourn, R. C. Najarian, F. Seela, and D. R. Tolan.** 1986. 7-Deaza-2′-deoxyguanosine-5′-triphosphate: enhanced resolution in M13 dideoxy sequencing. BioTechniques **4:**428–432.
4. **Beck, C. F., and R. A. J. Warren.** 1988. Divergent promoters, a common form of gene organization. Microbiol. Rev. **52:**318–326.
5. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12:**721–732.
6. **Bhasin, R., and M. Freundlich.** 1991. The nucleotide sequence of the *Escherichia coli crp* divergent RNA and an overlapping ORF. Biochim. Biophys. Acta **1129:**109–111.
7. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100–122.
8. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16:**832–841.
9. **Cossart, P., and B. Gicquel-Sanzey.** 1985. Regulation of expression of the *crp* gene of *Escherichia coli* K-12: in vivo study. J. Bacteriol. **161:**454–457.
10. **Gralla, J. D.** 1990. Promoter recognition and mRNA initiation by *Escherichia coli* Eσ^{70}. Methods Enzymol. **185:**37–54.
11. **Hanamura, A., and H. Aiba.** 1991. Molecular mechanism of negative autoregulation of *Escherichia coli crp* gene. Nucleic Acids Res. **19:**4413–4419.
12. **Hanamura, A., and H. Aiba.** 1992. A new aspect of transcriptional control of the *Escherichia coli crp* gene: positive autoregulation. Mol. Microbiol. **6:**2489–2497.
13. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
14. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
16. **Li, M., and H. P. Schweizer.** 1993. Resolution of common DNA sequencing ambiguities of GC-rich DNA templates by terminal deoxynucleotide transferase without dGTP analogues. Focus **15:**19–20.
17. **Liu, P. V.** 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. **128:**506–513.
18. **MacGregor, C. H., L. J. Runyen-Janecky, N. A. Zielinski, P. V. Phibbs, Jr., and S. E. H. West.** 1995. The *vfr* gene, a member of the *crp* family, is not required for catabolite repression control in *Pseudomonas aeruginosa*, abstr. K-56, p. 546. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
19. **Maleniak, T. C., R. J. Callan, S.-J. Suh, and S. E. H. West.** 1996. *Pseudomonas aeruginosa* Vfr regulates expression of numerous proteins including elastase, alkaline protease, and phospholipase C, abstr. B-33, p. 160. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
20. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
21. **Miller, J. H.** 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28:**899–908.
23. **Okamoto, K., and M. Freundlich.** 1986. Mechanism for the autogenous control of the *crp* operon: transcriptional inhibition by a divergent RNA transcript. Proc. Natl. Acad. Sci. USA **83:**5000–5004.
24. **Okamoto, K., S. Hara, R. Bhasin, and M. Freundlich.** 1988. Evidence in vivo for autogenous control of the cyclic AMP receptor protein gene (*crp*) in *Escherichia coli* by divergent RNA. J. Bacteriol. **170:**5076–5079.
25. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150:**60–69.
26. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
27. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
28. **Ronald, S., M. A. Farinha, B. J. Allan, and A. M. Kropinski.** 1992. Cloning and physical mapping of transcriptional regulatory (sigma) factors from *Pseudomonas aeruginosa*, p. 249–257. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
29. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. Science **262:**1407–1413.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109–112.
33. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
34. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site specific insertion and deletion mutagenesis. BioTechniques **15:**831–834.
35. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
37. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
38. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
39. **Ullmann, A., and A. Danchin.** 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nucleotide Res. **15:**1–53.
40. **Weickert, M. J., and G. H. Chambliss.** 1989. Acid-phenol minipreps make excellent sequencing templates. U.S. Biochemical Corp. Editorial Comments **16:**5–6.
41. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16:**9323–9335.
42. **West, S. E. H., S. A. Kaye, A. N. Hamood, and B. H. Iglewski.** 1994. Characterization of *Pseudomonas aeruginosa* mutants that are deficient in exotoxin A synthesis and are altered in expression of *regA*, a positive regulator of exotoxin A. Infect. Immun. **62:**897–903.
43. **West, S. E. H., A. K. Sample, and L. J. Runyen-Janecky.** 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J. Bacteriol. **176:**7532–7542.
44. **West, S. E. H., H. P. Schweizer, C. Dahl, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and nucleotide sequence of the region required for their replication in *Pseudomonas*. Gene **148:**81–86.
45. **Wretlind, B., and O. R. Pavlovskis.** 1984. Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. J. Bacteriol. **158:**801–808.