Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*

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Vfr of *Pseudomonas aeruginosa* is 91% similar to the cAMP receptor protein (CRP) of *Escherichia coli*. Based on the high degree of sequence homology between the two proteins, the question arose whether Vfr had a global regulatory effect on gene expression for *P. aeruginosa* as CRP did for *E. coli*. This report provides two-dimensional polyacrylamide gel electrophoretic evidence that Vfr is a global regulator of gene expression in *P. aeruginosa*. In a *vfr101::aacC1* null mutant, at least 43 protein spots were absent or decreased when compared to the proteome pattern of the parent strain. In contrast, 17 protein spots were absent or decreased in the parent strain when compared to the *vfr101::aacC1* mutant. Thus, a mutation in *vfr* affected production of at least 60 proteins in *P. aeruginosa*. In addition, the question whether Vfr and CRP shared similar mechanistic characteristics was addressed. To ascertain whether Vfr, like CRP, can bind cAMP, Vfr and CRP were purified to homogeneity and their apparent dissociation constants (K_d) for binding to cAMP were determined. The K_d values were 1.6 μ M for Vfr and 0.4 μ M for CRP, suggesting that these proteins have a similar affinity for cAMP. Previously the authors had demonstrated that Vfr could complement a *crp* mutation and modulate catabolite repression in *E. coli*. This study presents evidence that Vfr binds to the *E. coli lac* promoter and that this binding requires the presence of cAMP. Finally, the possible involvement of Vfr in catabolite repression control in *P. aeruginosa* was investigated. It was found that succinate repressed production of mannitol dehydrogenase, glucose-6-phosphate dehydrogenase, amidase and urocanase both in the parent and in two *vfr* null mutants. This implied that catabolite repression control was not affected by the *vfr* null mutation. In support of this, the cloned *vfr* gene failed to complement a mutation in the *P. aeruginosa crc* gene. Thus, although Vfr is structurally similar to CRP, and is a global regulator of gene expression in *P. aeruginosa*, Vfr is not required for catabolite repression control in this bacterium.

Keywords: Vfr, catabolite repression control, global regulator, cAMP binding, cAMP receptor protein

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterial pathogen that primarily affects cystic

fibrosis patients and immunocompromised patients. *P. aeruginosa* is found ubiquitously in nature and the recently completed genome sequencing demonstrated that it has the largest bacterial genome among the

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Abbreviation: CRP, cAMP receptor protein.

organisms that have been sequenced to date. One of the hypotheses for explaining the large genome size as well as the ubiquitous distribution of *P. aeruginosa* is based on the fact that this bacterium is able to metabolize various compounds as nutrients (Stover *et al.*, 2000). Catabolism of nutrients involves a large number of genes for the uptake and catalysis of the particular nutrient. In the presence of a preferred carbon source, genes that are necessary for catabolism of less preferred carbon sources are repressed. This control mechanism has been designated catabolite repression control (for a review see Ullmann, 1996).

A homologue of the *Escherichia coli* global transcriptional regulator CRP (cAMP receptor protein) was identified in *Pseudomonas aeruginosa* and it was designated Vfr for virulence factor regulator due to its effect on the production of several virulence factors (West *et al.*, 1994). Regulation of virulence factor production by Vfr is partially due to regulation of the *las* quorum-sensing system by Vfr (Albus *et al.*, 1997). The *las* quorum-sensing system in turn induces the *rhl* quorum-sensing system, and together the two quorum-sensing systems control expression of several genes for virulence factor production in *P. aeruginosa* (Pesci *et al.*, 1997). However, Vfr also regulates expression of genes independent of the *las* and *rhl* quorum-sensing systems. Vfr binds to the promoter of *tox*A and *reg*A to regulate the production of exotoxin A, thus demonstrating its direct role in the synthesis of this toxin (Runyen-Janecky *et al.*, 1996).

P. aeruginosa vfr encodes a 24225 Da polypeptide which is 67% identical and 91% similar to *E. coli* CRP (West *et al.*, 1994). CRP regulates the expression of over 100 genes in *E. coli*, most notably the genes involved in carbon source utilization whose expression is subject to catabolite repression by glucose (Botsford & Harman, 1992; Kolb *et al.*, 1993; Ullmann & Danchin, 1983). CRP functions by binding cAMP to form an active CRP-cAMP complex, which in turn binds to a specific sequence in the promoter of CRP-regulated genes.

Analysis of predicted amino acid sequences between the two proteins demonstrated that the majority of amino acid residues which constitute the structural features of CRP that are associated with cAMP binding, DNA binding and interaction with RNA polymerase are either identical or conserved in Vfr (West *et al.*, 1994). The conservation of major functional domains between Vfr and CRP implies that these two proteins have similar functions in their respective organisms and may regulate similar pathways, including catabolite repression control. Previously, we found that the cloned *P. aeruginosa* vfr gene could partially restore β -galactosidase activity in an *E. coli* *crp* mutant (West *et al.*, 1994). In an *E. coli* *crp cya* mutant, which does not produce cAMP, exogenous cAMP was required for Vfr to restore β -galactosidase activity. Thus, these observations suggest that Vfr is mechanistically similar to CRP in regard to cAMP binding, recognition of CRP-binding sites and interaction with *E. coli* RNA polymerase.

In *P. aeruginosa*, the enzymes of several carbohydrate-transport systems and catabolic pathways are subject to strong repression when acetate or tricarboxylic acid cycle intermediates are present in the growth medium along with the carbohydrate (for a review, see Collier *et al.*, 1996). The repressible enzymes include enzymes belonging to the *hex* regulon (the central pathway of catabolism), the glucose transporter regulon, the mannitol utilization regulon and the fructose regulon. Thus for *P. aeruginosa*, preferred carbon sources are the tricarboxylic acid cycle intermediates, and non-preferred substrates include glucose, gluconate, glycerate, glycerol, fructose and mannitol. Additionally, intracellular cAMP levels in *P. aeruginosa* do not fluctuate in response to growth phase or to various carbon sources, including glucose, succinate, acetamide, histidine, lactate, acetate, gluconate and glycerol (Phillips & Mulfinger, 1981; Siegel *et al.*, 1977). This observation suggests that cAMP does not play a role in catabolite repression.

A global regulator of catabolite repression control (CRC) in *P. aeruginosa* has been cloned, designated as *crc*, and characterized (MacGregor *et al.*, 1991). When *Crc*⁻ mutants were grown in the presence of succinate, production of glucose-6-phosphate dehydrogenase, glucokinase, Entner-Doudoroff dehydratase, aldolase, amidase, and the enzymes involved in glucose and mannitol transport were not repressed (Wolff *et al.*, 1991). The deduced amino acid sequence of the *Crc* protein showed that it was similar to a family of DNA repair enzymes composed of apurinic/apyrimidinic endonucleases (MacGregor *et al.*, 1996). However, MacGregor *et al.* (1996) were not able to demonstrate *in vitro* nuclease or DNA-binding activity with purified *Crc* protein. Thus, the mechanism by which *Crc* controls catabolite repression in *P. aeruginosa* is still unclear.

As a part of our continuing effort to characterize the role of Vfr in *P. aeruginosa* physiology, in this study we addressed whether Vfr is a global regulator of gene expression and whether it has a role in catabolite repression control in this bacterium. We demonstrate that although Vfr is mechanistically similar to CRP of *E. coli*, its role in *P. aeruginosa* physiology differs from the role of CRP in *E. coli*.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely maintained on LB agar at 37 °C. For proteomic analysis, cells were grown to stationary phase in trypticase soy broth dialysate (TSBD) (Ohman *et al.*, 1980) with aeration at 32 °C for 10 h. For analysis of catabolite repression control, cells were grown in basal salts minimal (BSM) medium (Hylemon & Phibbs, 1972) containing the appropriate carbon source (40 mM succinate or lactate) at 37 °C. Inducers were added as follows: 20 mM mannitol for mannitol dehydrogenase and glucose-6-phosphate dehydrogenase, 20 mM lactamide for amidase, and 0.2% (w/v) L-histidine for urocanase. Cells were inoculated to Klett readings

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype*	Reference
<i>E. coli</i>		
RZ1330	MG1655 <i>rpsL Δcrp lacP1⁺ ilv::Tn10 Δcya</i>	W. S. Reznikoff, Dept of Biochemistry, University of Wisconsin-Madison
<i>P. aeruginosa</i>		
PAO1	Prototroph	Holloway <i>et al.</i> (1979)
PAO8020	PAO1 <i>Δcrc</i> (3'-end); Tc ^r	MacGregor <i>et al.</i> (1996)
PAO9001	PAO1 <i>Δ(orfX, vfr, trpC')</i> <i>aacC1</i> ; Gm ^r	This study
PAO9002	PAO1 <i>vfr101::aacC1</i> ; Gm ^r	This study
Plasmids		
pMMB66HE (EH)	<i>tac</i> promoter expression vector, <i>lacI^a</i> ; Ap ^r	Fürste <i>et al.</i> (1986)
pRZ1306	pMMB66HE carrying promoterless <i>crp</i> under the control of the <i>tac</i> promoter; Ap ^r	W. S. Reznikoff
ptac917	pMMB66EH carrying <i>vfr</i> ; Ap ^r /Cb ^r	West <i>et al.</i> (1994)
pWNP28	<i>EcoRI</i> – <i>SmaI</i> deletion of ptac917 (<i>vfr</i> promoter deleted so <i>vfr</i> is under the control of the <i>tac</i> promoter); Ap ^r /Cb ^r	This study
pUCGM1	pUC1918 carrying the <i>aacC1</i> gene; Gm ^r Ap ^r	Schweizer (1993)
pNOT19	pUC19 carrying a 10 bp <i>NdeI</i> / <i>NotI</i> adaptor in the <i>NdeI</i> site; Ap ^r	Schweizer (1992)
pWNP128	pNOT19 carrying a 2.6 kb <i>Sall</i> – <i>PstI</i> fragment containing <i>orfX</i> , <i>vfr</i> and <i>trpC</i> ; Ap ^r	This study
pWNP129	pWNP128 with the 1.2 kb <i>XhoI</i> fragment carrying <i>vfr</i> replaced with the 0.85 kb <i>Sall</i> fragment carrying the <i>aacC1</i> gene from pUCGM1; Gm ^r Ap ^r	This study
pKF812	pUCP18 carrying <i>vfr</i> as a 1.2 kb <i>XhoI</i> fragment; Ap ^r /Cb ^r	West <i>et al.</i> (1994)
pWNP108	pKF812 carrying the <i>vfr101::aacC1</i> allele	This study
pWNP109	pUC18 carrying the <i>vfr101::aacC1</i> allele	This study
pUCP18	Multi-copy <i>E. coli</i> – <i>P. aeruginosa</i> shuttle vector; Ap ^r /Cb ^r	Schweizer (1991)

* Gm^r, gentamicin resistant; Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Tc^r, tetracycline resistant.

of 20–40 (no. 66 red filter) from plates containing BSM agar with 40 mM succinate and the appropriate antibiotics and grown to Klett readings of 150–210 (mid- to late-exponential). For PAO9001, tryptophan was included at 2 mM final concentration. Antibiotics were used at the following concentrations per ml: for *P. aeruginosa*, 250 µg carbenicillin, 50 µg tetracycline and 50 µg gentamicin; for *E. coli*, 100 µg ampicillin and 15 µg gentamicin.

Construction of *vfr* null mutants. The *vfr* deletion mutant PAO9001 and the *vfr* insertion mutant PAO9002 were constructed by allelic exchange. For construction of PAO9001, a 2.6 kb *Sall*–*PstI* DNA fragment containing *vfr* and flanking DNA was cloned into pNOT19 to generate pWNP128. A 1.2 kb *XhoI* fragment containing *vfr* was deleted from pWNP128 and was replaced with a 0.85 kb *Sall* fragment containing the gentamicin-resistance cassette from pUCGM1 to generate pWNP129. This cassette contains the *aacC1* gene encoding gentamicin acetyltransferase-3-1 (Gm^r). For PAO9002, the 0.85 kb *SmaI* fragment containing the gentamicin-resistance cassette from pUCGM1 was inserted into the unique *SphI* site (blunt-ended with mung bean nuclease) of *vfr* on pKF812 to generate pWNP108. The *vfr101::aacC1* allele with the gentamicin-resistance cassette was excised from pWNP108 as an *EcoRI*–*HindIII* fragment and ligated into pUC18 to generate pWNP109. pWNP129 and pWNP109 were introduced into PAO1 by electroporation. Since the ColE1-

derived vectors are not maintained in *P. aeruginosa*, stable gentamicin-resistant colonies can only be obtained if the plasmid has integrated into the chromosome. Recombinants were selected on media containing gentamicin (50 µg ml⁻¹) and were screened for loss of vector sequences by sensitivity to carbenicillin. Southern analysis of chromosomal DNA from the gentamicin-resistant, carbenicillin-sensitive recombinants confirmed that the 1.2 kb *XhoI* fragment carrying *vfr* was deleted from the chromosome and replaced with the *aacC1* gene for PAO9001 and that the *vfr101::aacC1* mutant allele replaced the *vfr* gene for PAO9002 (data not shown).

Two-dimensional electrophoretic analysis. Cells were grown in TSBD for 10 h (2 h into stationary phase) at 37 °C with aeration, harvested, and resuspended in 10 mM HEPES, pH 7.2, 5 mM EDTA and 1 mM PMSF. Cells were again harvested by centrifugation (15 000 g for 5 min), resuspended in SDS boiling buffer (5% SDS, 10%, v/v, glycerol and 60 mM Tris/HCl, pH 6.8), and boiled for 5 min. The insoluble particulate material was removed by centrifugation for 5 min at 15 000 g. Protein concentration was determined via the BCA method (Smith *et al.*, 1985), and β-mercaptoethanol was added to the samples at a final concentration of 5%. Approx. 30 µg protein was used to analyse the protein profiles between pl 4–8.5 and molecular mass 14–220 kDa via two-dimensional gel electrophoresis. The electrophoresis was performed by the Kendrick Laboratories, Inc. (Madison, WI), and the protein spots were visualized by silver staining.

Purification of CRP and Vfr. CRP and Vfr were purified from crude cell lysates of *E. coli* RZ1330/pRZ1306 or *P. aeruginosa* PA103/pWNP28, respectively, by affinity chromatography on cAMP-agarose as described by Ghosaini *et al.* (1988) with the following modifications. To overexpress CRP or Vfr, IPTG (1 mM final concentration) was added to 5-litre mid-exponential-phase cultures of RZ1330/pRZ1306 or PA103/pWNP28 respectively, grown in LB. *E. coli* RZ1330/pRZ1306 was harvested 15 h after induction and *P. aeruginosa* PA103/pWNP28 was harvested 6 h after induction by centrifugation for 15 min at 5000 g. To prepare crude cell lysates, the cell pellets were resuspended in 100 mM Tris/HCl, pH 8.0, 50 mM KCl, 2 mM EDTA, 5 mM β -mercaptoethanol, 1 mM sodium azide and 200 μ g lysozyme ml⁻¹ and incubated at 25 °C for 20 min. The solution was adjusted to 25 mM Tris/HCl, pH 8.0, 25 mM MgSO₄ and 1% BRIJ 35, and the lysates were incubated at 4 °C for 10 min. To decrease the viscosity due to nucleic acids, the lysate was sonicated at approx. 100 W for three 10 s intervals on ice using a Vibra Cell sonicator (Sonics and Materials). The cell-free extract was obtained by centrifugation at 16000 g for 30 min at 4 °C and was applied to a 5 ml cAMP-agarose column (Sigma) as described by Ghosaini *et al.* (1988).

cAMP-binding assay. This assay was performed by the ammonium sulfate precipitation method developed by Emmer *et al.* (1970) with slight modifications. Briefly, the assay was performed in 10 mM potassium phosphate, pH 7.4, with the protein, [8-³H]adenosine 3',5'-cyclic phosphate (cAMP, 25 Ci mmol⁻¹, 925 GBq mmol⁻¹; Amersham), and a carrier protein, casein (200 μ g), in a total volume of 100 μ l. After a 5 min incubation at 0–1 °C, 400 μ l ice-cold saturated (NH₄)₂SO₄ was added and the protein–cAMP complex was allowed to precipitate for 5 min on ice. The precipitate was collected by centrifugation in a microcentrifuge and the pellet was resuspended in 500 μ l double-distilled H₂O. The resuspended pellet was transferred to a scintillation vial containing 10 ml Opti-fluor (Packard) and the radioactivity was quantified in a Packard Tri-Carb 4530 scintillation counter. The control reaction contained all of the assay components except for the protein; therefore, the amount of [³H]cAMP bound specifically to the enzyme was determined by subtracting the level of nonspecific binding in the control reaction from the total level of radioactivity in the assay reaction. The saturation in cAMP binding was achieved by increasing the quantity of [³H]cAMP added to the assay. Approx. 0.3 μ g CRP and 1.1 μ g Vfr was used in the assays to determine the apparent dissociation constants for cAMP.

Gel retardation assays. A DNA fragment containing the *lacZ* promoter was isolated from pUCP19 as a 231 bp *Pvu*II–*Hind*III DNA fragment and was end-labelled with [α -³²P]dNTP using DNA polymerase Klenow fragment (United States Biochemical). The binding reactions, containing approximately 40 pM DNA and various concentrations of either purified Vfr or CRP, were carried out at room temperature for 20 min in 10 mM Tris/HCl pH 7.4, 100 mM KCl, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 50 μ g BSA ml⁻¹ and 20 μ M cAMP as described by DeVault *et al.* (1991), and were then electrophoresed on a 6% native polyacrylamide gel in 10 mM Tris/HCl pH 7.4, 1 mM EDTA and 20 μ M cAMP for 1.5 h at 10 V cm⁻¹. The gel was dried and exposed to Kodak X-OMAT AR5 film at –70 °C overnight with an intensifying screen.

Enzyme assays. Cells were disrupted by resuspending frozen cell pellets (from 30 ml cultures) in 2.5 ml 50 mM Tris/HCl pH 8.0 and pressing through a French pressure cell at

16000 p.s.i. (110 MPa). S-180 extracts were obtained after centrifugation at 180000 g for 30 min. The following enzyme assays were done as previously described: glucose-6-phosphate dehydrogenase (Hylemon & Phibbs, 1972), mannitol dehydrogenase (Siegel *et al.*, 1977), urocanase (Lessie & Neidhardt, 1967), except that urocanate was used at 50 μ M, and amidase (Brammar & Clarke, 1964). Glucose-6-phosphate dehydrogenase and mannitol dehydrogenase were assayed at 25 °C, while urocanase and amidase assays were at 37 °C. Amidase assays were done on cell suspensions; other enzyme assays were done on S-180 extracts. For the determination of specific activities, units refer to μ mol product min⁻¹. Protein concentrations were assayed relative to BSA standards by the Lowry method. For cell suspensions, total protein was first precipitated with trichloroacetic acid prior to the protein determination.

RESULTS

Construction of *vfr* null mutants

To assess the effect of a *vfr* mutation on *P. aeruginosa* physiology, we constructed two *vfr* null mutants: a complete *vfr* deletion mutant and a *vfr* insertion mutant. The deletion mutant (PAO9001) was constructed by replacing a 1.2 kb deleted DNA fragment that carried *vfr* as well as portions of an ORF upstream of *vfr* (*orfX*) (Runyen-Janecky *et al.*, 1997) and a portion of *trpC* gene that is located directly downstream of *vfr*, with the *aacC1* gene that encodes gentamicin resistance. The insertion mutant (PAO9002) was constructed by inserting the *aacC1* gene into the unique *Sph*I site of *vfr*. The mutant alleles were introduced into the *P. aeruginosa* wild-type strain PAO1 and the inheritance of *vfr* mutant alleles via allelic exchange was selected and screened as described in Methods. Within the scope of this study, both the deletion mutant and the insertion mutant behaved similarly, and thus indicated that deletion of *orfX* or *trpC* did not have a demonstrable effect on Vfr function.

Proteome analysis of a *vfr101::aacC1* mutant

A proteome analysis was performed to assess whether Vfr is a global regulator of gene expression in *P. aeruginosa*. As shown in Fig. 1, comparison of the silver-stained two-dimensional polyacrylamide gel electrophoresis patterns between the wild-type strain (PAO1) and the isogenic *vfr101::aacC1* null mutant (PAO9002) revealed that a null mutation in *vfr* resulted in altered production of numerous proteins. We found that at least 43 protein spots were either absent or significantly decreased in the proteome pattern of the *vfr* mutant when compared to that of the parent strain (Fig. 1, upper panel). This confirmed our hypothesis that Vfr acts as a positive regulator of gene expression in *P. aeruginosa* and that Vfr induces the expression of many genes. In contrast, we also found that production of 17 protein spots appeared to be repressed by the presence of Vfr (Fig. 1, lower panel). Thus, Vfr is a global regulator of gene expression in *P. aeruginosa* that has either a positive or negative effect on expression of at least 60 genes.

Fig. 1. Two-dimensional electrophoretic analysis showing the effect of *vfr101* mutation on overall protein synthesis in *P. aeruginosa*. PAO1 and its *vfr* mutant (PAO9002) were grown in TSBD into the late stationary phase of growth (10 h). Cells were harvested and total cellular proteins were separated by two-dimensional gel electrophoresis. Proteins were visualized by silver staining. The arrow indicates the single isoelectric focusing standard tropomyosin, which has a pI of 5.2 and a molecular mass of 33 kDa. Upper panel: PAO1. Circles indicate protein spots that are missing or reduced in the *vfr* mutant. Lower panel: PAO9002. Squares indicate protein spots that are missing or reduced in the parent strain.

Vfr binding to the *E. coli lacZ* promoter requires cAMP

In *E. coli*, expression of the *lacZ* gene, encoding β -galactosidase, requires the binding of the CRP–cAMP complex to the palindromic CRP-binding sequence in the *lac* promoter. The observation that *P. aeruginosa* Vfr can replace CRP to activate β -galactosidase production in *E. coli* suggested that, like CRP, Vfr binds the *lac* promoter (West *et al.*, 1994). To further test this hypothesis, we used gel retardation assays to examine

Fig. 2. Binding of Vfr and CRP to the *lacZ* promoter. A radiolabelled *PvuII*–*HindIII* DNA fragment containing the *lacZ* promoter from pUCP19 was incubated with purified Vfr or CRP as described in Methods. The first lane contained *lacZ* promoter DNA without any protein. Subsequent lanes contained increasing concentrations of either Vfr or CRP as indicated.

the ability of Vfr to bind the *lac* promoter. Both *P. aeruginosa* Vfr and *E. coli* CRP retarded the mobility of a DNA fragment that contains the *lac* promoter (Fig. 2). The addition of excess unlabelled *lac* promoter DNA, but not unlabelled nonspecific DNA, prevented Vfr from binding to the labelled *lac* promoter DNA fragment (data not shown). Additionally, when cAMP was not present in the reaction buffer, neither Vfr nor CRP bound the *lac* promoter DNA fragment (data not shown). These results confirm the previously demonstrated genetic evidence that Vfr complements an *E. coli crp* mutant for β -galactosidase production and that this complementation requires cAMP. Thus, the formation of a Vfr–cAMP complex is required for specific binding of the protein to the *lac* promoter, and therefore, the Vfr–cAMP complex should function in a mechanistically similar way to the CRP–cAMP complex. However, we note that a much higher concentration of Vfr than CRP was needed to bind and shift the *lacZ* promoter (Fig. 2). This result supports the earlier observation that Vfr partially complements the *E. coli crp* mutant, and it may indicate a difference in DNA sequence specificity of binding sites for the two proteins.

cAMP-binding properties of Vfr and CRP

To evaluate the specificity of the Vfr–cAMP interaction, we determined the apparent dissociation constants (K_d) of Vfr and CRP for this nucleotide. The data from the cAMP-binding experiments were analysed with the nonlinear regression data analysis program Enzfitter (Elsevier-Biosoft). The apparent K_d values were 0.4 μ M for CRP and 1.6 μ M for Vfr binding to cAMP (data not shown). Similar apparent K_d values were obtained by the competitive binding assay using non-radioactive cAMP as the competitor substrate (data not shown). The apparent K_d obtained for CRP agrees with the values of 1 μ M and 3–4 μ M reported for CRP isolated from *E. coli* and *Vibrio harveyi*, respectively (Chen *et al.*, 1985; Emmer *et al.*, 1970). The observation that the apparent K_d of Vfr for cAMP falls within the same range as K_d values reported for CRP from *E. coli* and *V.*

Table 2. Catabolite repression control is not affected by *vfr* null mutations

Strains were grown in basal salts medium supplemented with either succinate, a strong repressor of inducible catabolite operons, or lactate, a weak repressor of inducible catabolite operons. Metabolic enzyme synthesis was induced by addition of lactamide for amidase, mannitol for mannitol dehydrogenase and glucose-6-phosphate dehydrogenase, and histidine for urocanase. Amidase assays were done in triplicate, and all other assays in duplicate. Standard deviations are shown in parentheses.

Strain	Amidase (IU mg ⁻¹)		Mannitol dehydrogenase (mIU mg ⁻¹)		Glucose-6-phosphate dehydrogenase (mIU mg ⁻¹)		Urocanase (mIU mg ⁻¹)	
	Succinate	Lactate	Succinate	Lactate	Succinate	Lactate	Succinate	Lactate
PAO1 (Vfr ⁺)	0.5 (0.2)	14.8 (0.3)	2.4 (0.2)	36 (0.2)	8 (1.3)	137 (7.0)	3.7 (4.0)	40 (5.4)
PAO9001 (Vfr ⁻)	0.4 (0.1)	9.5 (0.6)	1.2 (0.4)	29 (0.8)	5 (0.6)	108 (15)	1.0 (1.3)	25 (0.4)
PAO9002 (Vfr ⁻)	0.6 (0.1)	10.6 (0.8)	1.4 (0.2)	40 (0.5)	8 (1.0)	140 (3.3)	2.1 (0.8)	42 (1.1)

Table 3. *vfr* does not complement a *crc* mutant for succinate mediated catabolite repression

Strains were grown in basal salts medium supplemented with succinate, and metabolic enzyme synthesis was induced by the addition of lactamide for amidase or mannitol for mannitol dehydrogenase and glucose-6-phosphate dehydrogenase. All assays were done in duplicate. Standard deviations are shown in parentheses.

Strain	Amidase (IU mg ⁻¹)	Mannitol dehydrogenase (mIU mg ⁻¹)	Glucose-6-phosphate dehydrogenase (mIU mg ⁻¹)
PAO1	0.3 (0.1)	2.5 (1.6)	2.7 (0.8)
PAO1/pUCP18	0.3 (0.05)	3.5 (0.4)	3.2 (0.2)
PAO1/pKF812	0.2 (0.02)	2.0 (0.5)	3.7 (0.1)
PAO8020	1.6 (0.5)	22 (0.8)	21 (4.0)
PAO8020/pUCP18	1.3 (0.1)	26 (0.1)	18 (3.5)
PAO8020/pKF812	2.3 (0.5)	23 (1.8)	15 (0.5)

harveyi suggests that these two proteins bind cAMP with a similar affinity.

Vfr is not required for catabolite repression control in *P. aeruginosa*

Since Vfr can function as a positive regulator of catabolite repression by substituting for the cAMP receptor in *E. coli*, Vfr may fulfil a similar role in catabolite repression control in *P. aeruginosa*. To examine this possibility, we determined whether catabolite repression control occurred in two *vfr* null mutants of PAO1. The parental strain PAO1 and the *vfr* null mutants, PAO9001 and PAO9002, were grown in basal salts medium that was supplemented with either succinate, a strong repressor of inducible catabolic operons, or lactate, a weak repressor of inducible catabolic operons, as sole carbon sources. Metabolic enzyme synthesis was induced by the addition of lactamide for amidase, mannitol for mannitol dehydrogenase and glucose-6-phosphate dehydrogenase, and histidine for urocanase. In both PAO1 and the *vfr* null mutants PAO9001 and PAO9002, the presence of succinate in the

growth medium repressed the synthesis of amidase, mannitol dehydrogenase, glucose-6-phosphate dehydrogenase and urocanase (Table 2). Based on these results, Vfr does not appear to be required for catabolite repression control in *P. aeruginosa*.

In *P. aeruginosa* *crc* mutants, catabolite-repressible genes are no longer repressed in the presence of preferred carbon sources such as succinate (Wolff *et al.*, 1991). To confirm that Vfr has no role in catabolite repression, we assayed whether Vfr could complement a *P. aeruginosa* *crc* mutant. The bacteria were grown in basal salts medium containing succinate to elicit catabolite repression. Metabolic enzyme synthesis was induced by the addition of lactamide for amidase or mannitol for mannitol dehydrogenase and glucose-6-phosphate dehydrogenase. The *crc* mutant PAO8020 carrying either the vector control (pUCP18) or the cloned *vfr* gene (pKF812) failed to repress the synthesis of amidase, mannitol dehydrogenase or glucose-6-phosphate dehydrogenase in the presence of succinate (Table 3). Thus, the function of Crc could not be replaced by multiple copies of Vfr and the results confirmed that Vfr is not

required for the catabolite repression response in *P. aeruginosa*.

DISCUSSION

The results of this study support our hypothesis that Vfr is a global regulator of gene expression in *P. aeruginosa*. Based on our limited proteome analysis, it appears that Vfr both activates and represses the production of numerous proteins in *P. aeruginosa*. Since Vfr regulates the quorum-sensing systems *las* and *rhl* (Albus *et al.*, 1997), it is possible that Vfr indirectly regulates production of many of the proteins via the quorum-sensing systems. Studies to identify genes that are directly regulated by Vfr are currently in progress.

We are also interested in elucidating the mechanism of Vfr function in *P. aeruginosa*. The requirement of cAMP for Vfr binding to the *E. coli lac* promoter further enhances our hypothesis that Vfr uses a similar molecular mechanism as CRP of *E. coli* (i.e. formation of a complex with cAMP and binding to a specific CRP-binding sequence) to activate transcription in *E. coli*. We found that Vfr bound cAMP with an apparent dissociation constant (K_d) of 1.6 μ M, which was similar to the apparent K_d of CRP. The similar binding constant implies that, like CRP, Vfr activity should be sensitive to fluctuations in the cAMP levels. We previously demonstrated that Vfr-dependent activation of β -galactosidase production in *E. coli* could be repressed in the presence of glucose (West *et al.*, 1994). Based on our data showing that Vfr binds cAMP with a similar dissociation constant as CRP, we propose that, as with CRP, glucose repression of Vfr-dependent β -galactosidase production in *E. coli* is a direct result of decreased cAMP levels. Thus, our data suggest that in *E. coli* Vfr functions in a manner mechanistically similar to CRP. However, in *P. aeruginosa*, it is not clear that cAMP is the only ligand which binds to and activates Vfr. cAMP levels remain constant and do not change in response to various carbon sources (Phillips & Mulfinger, 1981; Siegel *et al.*, 1977), suggesting that it may not function as a modulator of Vfr activity. A role for cAMP has not been established in *P. aeruginosa*. Additionally, even though there is a high degree of similarity between Vfr and CRP, the greatest variability between these two proteins occurs in the cAMP-binding domain (West *et al.*, 1994). Additional work is needed to determine if cAMP is the only ligand required for Vfr activity.

In contrast to the ability of Vfr to mediate catabolite repression control in *E. coli*, Vfr does not function in catabolite repression control in *P. aeruginosa*. Based on the differences in metabolic capabilities of *P. aeruginosa* and *E. coli*, it was not surprising that the *P. aeruginosa* catabolite repression control system did not require Vfr. First, the carbon source preference is very different between the two bacteria: while *E. coli* prefers a carbohydrate such as glucose *P. aeruginosa* prefers an organic acid such as succinate. Second, many of the genes that are catabolite repressed in *P. aeruginosa*, such as those encoding amidase and mannitol dehydrogenase,

are not found in *E. coli*, and therefore it is reasonable to suppose that a catabolite repression system other than a CRP-cAMP-like mechanism may control their expression. In addition, Vfr does not appear to regulate genes in *P. aeruginosa* that are regulated by CRP in enterobacteria. For example, CRP is required for the production of succinate dehydrogenase in *E. coli* (Wood *et al.*, 1984) and urocanase in *Klebsiella aerogenes* (*K. pneumoniae*) (Nieuwkoop *et al.*, 1984). These enzymes permit growth of these bacteria on succinate and histidine, respectively, as sole carbon sources. However, the *P. aeruginosa* PAO1 *vfr* insertion mutant PAO9002 was able to grow with either succinate or histidine as a sole carbon source (L. J. Runyen-Janecky, unpublished results). Furthermore, the *vfr* null mutants PAO9001 and PAO9002 produced a similar amount of urocanase as the parent strain. Finally, a Vfr-cAMP complex would be a poor candidate for mediating catabolite repression in response to various carbon sources, since cAMP levels are constant in *P. aeruginosa* (Phillips & Mulfinger, 1981; Siegel *et al.*, 1977).

In addition to *P. aeruginosa* Vfr, homologues of the *E. coli* CRP protein have been identified in numerous physiologically and ecologically distinct members of the gamma subdivision of the purple eubacteria including *Salmonella enterica* Serovar Typhimurium, *Vibrio fischeri*, *V. harveyi* and *Shigella flexneri*, members of the *Enterobacteriaceae* family; *Haemophilus influenzae*, a member of the *Pasturellaceae* family; and *Xanthomonas campestris*, a member of the *Pseudomonadaceae* family (Chandler, 1992; Cossart *et al.*, 1986; DeCrecy-Lagard *et al.*, 1990). Among these species, the members of the *Enterobacteriaceae* family (*E. coli*, *Salmonella enterica* and *Shigella flexneri*) are the most closely related, while *X. campestris* and *P. aeruginosa* are more distantly related to the *Enterobacteriaceae* family and also only distantly related to each other (Woese *et al.*, 1985). The presence of CRP in such a diverse group of bacteria suggests that CRP was present in the common ancestor of these species. Although the structure of a CRP homologue remained conserved as members of the gamma subdivision of the purple eubacteria evolved from this common ancestor, the cellular role of this protein did not. For instance, Vfr does not appear to play a role in catabolite repression in *P. aeruginosa*, and the genes identified thus far that are members of the Vfr regulon are secreted virulence determinants or regulators of secreted virulence determinants (Albus *et al.*, 1997; Maleniak *et al.*, 1996; West *et al.*, 1994). Likewise, the *X. campestris* CRP homologue, CLP, does not regulate metabolism but instead regulates phytopathogenicity (DeCrecy-Lagard *et al.*, 1990). In *H. influenzae*, CRP regulates development of competence for transformation (Chandler, 1992). The evolution of diverse roles of the CRP homologues in various species may reflect the different physiological needs of each species.

In conclusion, this report demonstrates that although Vfr, the *P. aeruginosa* CRP homologue, can function as a component of catabolite repression control in *E. coli*, Vfr is not required for catabolite repression in *P.*

aeruginosa. Although Vfr and CRP are both global transcriptional activators, their specific roles appear to have evolved to suit the particular needs of each species that are influenced by their natural niches and physiologies. Thus, it is not surprising that different sets of genes have evolved as members of the CRP/Vfr regulon in *E. coli* and *P. aeruginosa*. The corollary to this is that functions that are under the control of the CRP/Vfr regulon in one species may be part of a different regulon in another species, as seems to be the case for catabolite repression. Future study of identification of Vfr-regulated genes will help elucidate the function of this regulator on the physiology of *P. aeruginosa*.

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