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The Nucleotide Sequence of the *Pseudomonas aeruginosa pyrE-crc-rph* Region and the Purification of the *crc* Gene Product

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The gene (*crc*) responsible for catabolite repression control in *Pseudomonas aeruginosa* has been cloned and sequenced. Flanking the *crc* gene are genes encoding orotate phosphoribosyl transferase (*pyrE*) and RNase PH (*rph*). New *crc* mutants were constructed by disruption of the wild-type *crc* gene. The *crc* gene encodes an open reading frame of 259 amino acids with homology to the apurinic/apyrimidinic endonuclease family of DNA repair enzymes. However, *crc* mutants do not have a DNA repair phenotype, nor can the *crc* gene complement *Escherichia coli* DNA repair-deficient strains. The *crc* gene product was overexpressed in both *P. aeruginosa* and in *E. coli*, and the Crc protein was purified from both. The purified Crc proteins show neither apurinic/apyrimidinic endonuclease nor exonuclease activity. Antibody to the purified Crc protein reacted with proteins of similar size in crude extracts from *Pseudomonas putida* and *Pseudomonas fluorescens*, suggesting a common mechanism of catabolite repression in these three species.

The genus *Pseudomonas* is noteworthy for its diversity in habitat and physiology. Some *Pseudomonas* strains are able to use over 100 organic compounds as the sole or principal source of carbon. *Pseudomonas aeruginosa* can utilize at least 80 different organic compounds (41). A mechanism of catabolite repression control exists in these organisms (18) which prevents them from wasting energy maintaining the enzymes for all these catabolic pathways and ensures the preferential utilization of the most efficient source of carbon and energy. Such a regulatory mechanism has also been identified in the enteric bacteria (20) and in *Bacillus* spp. (6).

In the enteric bacteria, the molecular mechanism of catabolite repression control involves a catabolite activator protein (Cap) which, when bound to cyclic AMP (cAMP), interacts with promoter regions of regulated genes to facilitate the binding of RNA polymerase, thereby initiating transcription (20). In the presence of glucose, the cAMP pool is lowered, Cap is not bound, and the regulated genes are not transcribed (i.e., they are repressed). The effect of glucose on cAMP pools is mediated by components of the phosphoenolpyruvate phosphotransferase system which also serve to activate adenylate cyclase (33). Since the initial identification of these regulatory components, catabolite repression has proven to be a global mechanism in the enteric bacteria, affecting at least 28 separate promoters which regulate biosynthetic as well as catabolic operons (7). It also has been found to act as a negative regulator as well as a positive regulator (1, 7, 23, 24).

In *Bacillus* spp. catabolite repression is not mediated by glucose, nor is cAMP involved (6, 37, 40). Genetic evidence has implicated the catabolite control protein (CcpA), a member of the GalR family of repressor proteins (6), Hpr, a component of the phosphoenolpyruvate phosphotransferase system (9), and a *cis*-acting DNA sequence (CRE) (15, 48). Recent work has shown that HPr specifically phosphorylated at Ser-46 forms a complex with CcpA capable of protecting the CRE from DNase digestion (11), suggesting a global regulatory model for catabolite repression in Bacillus spp. (11, 14).

In *P. aeruginosa* and *Pseudomonas putida*, acetate and intermediates of the tricarboxylic acid cycle are the strongest repressing substrates, although glucose can cause repression of some catabolic pathways, such as that of mannitol utilization (38). Further, neither cAMP nor adenylate cyclase appears to play a role in catabolite repression control (29, 38). To date, the molecular mechanism of catabolite repression control in these organisms is completely unknown. Recently we described the isolation of the first mutants in catabolite repression control (*crc*) in *P. aeruginosa* (47). Their phenotype is the failure to repress multiple independently regulated pathways when grown in the presence of tricarboxylic acid cycle intermediates (47). We subsequently cloned a gene which restores wild-type regulation to these mutants (19). Here we report the nucleotide sequences for the *crc* region, the comparison of the derived protein sequences to other known proteins, and the significance of this comparison to the function of the Crc protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* was grown at 37°C in basal salts medium (BSM) (16) or in Luria-Bertani (LB) medium (22). *Pseudomonas fluorescens* and *P. putida* were grown at 30°C in LB medium. Antibiotics were used at the following concentrations: tetracycline (TET), 100 μg/ml for *P. aeruginosa* and 20 μg/ml for *Escherichia coli*; carbenicillin (CB), 500 μg/ml for *P. aeruginosa*; ampicillin (AMP), 100 μg/ml for *E. coli.*

Mutant construction and transfer of plasmids. *Pseudomonas* strain PAO8020, carrying the *crc* mutation, was constructed as follows: from the 2-kb insert of plasmid pPZ353, a 0.3-kb *Acc*I fragment was replaced with a Tc^r cassette (see Fig. 1 for diagram; pPZ353 is identical to pPZ354 except that it lacks an *oriV*). The truncated *crc* gene containing the Tc^r cassette was cut from this plasmid and inserted into pUC19*mob* to produce pPZ407. pUC19*mob* carries the *mob* region of pRP4 (26) which acts in *trans* with the *tra* genes carried on the chromosome of *E. coli* S17-1 to mobilize the conjugal transfer of this plasmid into a broad range of gram-negative bacteria (39). Donor cells (S17-1 containing pPZ407) and recipient cells (*P. aeruginosa* PAO1) were grown to log phase in LB medium. These cells were mixed in a 1:1 ratio, and 2 ml of this mix was filtered onto a sterile 0.45-μm-pore-size filter. Filters were incubated at 37°C on LB plates overnight, and then removed from the plates and washed with 50 mM potassium phosphate, pH 7. Washed cells were concentrated by centrifugation and suspended in 1 ml of the same buffer, and 100 μl was plated on selective medium. Plates were incubated at 37°C for up to 60 h. Transconjugants which were Tc^r

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
P. aeruginosa		
PAO1	Prototroph	Holloway,[a] 13
PAO8005	*crc-5 rec-102*	18
PAO8007	*crc-20 rec-102*	18
PAO8020	Δ*crc*::Tc^r	This study
PAO8023	*crc*::Cb^r	This study
PRP701	*crc-1*	47
ADD1976	T7 polymerase, *lac* promoter	Darzins,[b] 5
P. fluorescens	ATCC 33512, prototroph	ATCC[c]
P. putida	PPN1225, Rif^r	Holloway
E. coli		
DH5α	ϕ80d/*lac*ZΔM15 Δ(*lacZYA-araF*)*recA*	GIBCO/BRL[d]
M15	*lac ara gal mtl*	Qiagen[e]
BW287	*dut-1 xth* A3(Ts)	Weiss,[f] 8
BW549	*dut-1 xth* A3(Ts) *nfo*	Weiss, 8
S17-1	RP4-2 Tc::Mu Km::Tn*7* Tp^r Sm^r *pro*	39
Plasmids		
pTZ18R	*lac*Z, α-peptide, Amp^r ϕ f1*ori*	Pharmacia[g]
pUC19*mob*	*mob* from pRP4 in pUC19	Lory,[h] 26
pEB16	T7 promoter	Darzins, 5
pQE32	T5 promoter, *lacO*, RBS,[i] 6 His	Qiagen
pUCP18	multicopy shuttle vector Amp^r	West,[j] 46
pKF812	*vfr* gene in pUCP18	West, 46
pPZ343	*oriV* in pTZ18R	19
pPZ352	*Pst*I-*Eco*RV, *crc*$^+$ in pPZ343	This study
pPZ353	*Sst*I-*Eco*RV, *crc*$^+$ in pTZ18R	19
pPZ354	*Sst*I-*Eco*RV, *crc*$^+$ in pPZ343	19
pPZ407	Δ*crc*::Tc^r in pUC19*mob*	This study
pPZ441	Δ*crc* in pPZ343	This study
pPZ445	*Sst*I-*Apa*I, *crc*$^+$ in pPZ343	This study
pPZ448	*Sst*I-*Stu*I, *crc*$^+$ in pPZ343	This study
pPZ456	*Sst*I-*Stu*I, *crc*$^+$ in pEB16	This study
pPZ461	*Kpn*I-*Stu*I, *crc*$^+$ in pQE32	This study
pPZ471	5′, 3′ Δ*crc* in pUC19*mob*	This study
pKK223/HAP1	*ref-1*$^+$ in pKK223	Hickson,[k] 31

[a] B. Holloway, Monash University, Melbourne, Australia.
[b] A. Darzins, Ohio State University, Columbus.
[c] American Type Culture Collection, Rockville, Md.
[d] GIBCO/BRL Life Technologies, Gaithersburg, Md.
[e] Qiagen Inc., Chatsworth, Calif.
[f] B. Weiss, The Johns Hopkins University, Baltimore, Md.
[g] Pharmacia LKB Technologies, Piscataway, N.J.
[h] S. Lory, University of Washington, Seattle.
[i] rbs, ribosome binding site.
[j] S. West, University of Wisconsin, Madison.
[k] I. Hickson, University of Oxford, Oxford, England.

and Cb^s were assumed to be double crossovers in which the chromosomal wild-type *crc* gene was replaced by the mutant *crc* gene encoding a C-terminal deletion of the Crc coding sequence. *Pseudomonas* strain 8023, carrying the *crc* mutation, was constructed as follows: an *Apa*I restriction site was introduced at codon 35 of the Crc coding sequence (see Fig. 1) by using an in vitro oligonucleotide mutagenesis kit (Amersham Corporation, Arlington Heights, Ill.). Then the 0.5-kb *Apa*I-*Pvu*II fragment with 5′ and 3′ deletions of the *crc* gene was cloned into pUC19mob to create pPZ471. pPZ471 was introduced into *P. aeruginosa* PAO1 as described above, and Cb^r transconjugants were selected and assumed to be single crossovers in which the chromosomal wild-type *crc* gene was replaced by tandem *crc* alleles, one containing a C-terminal and one containing an N-terminal deletion of the Crc coding sequence.

Transformation was employed to move plasmids into *E. coli* and *P. aeruginosa*. *E. coli* was made competent by the $CaCl_2$ procedure (21). *P. aeruginosa* strains were made competent by a similar procedure but with $MgCl_2$ substituted for $CaCl_2$ (19).

Plasmid isolation, mapping, and subcloning. All procedures were essentially as described in Maniatis et al. (21). Plasmids were propagated in *E. coli* DH5α.

DNA sequencing. DNA sequencing was carried out by the dideoxy chain termination method by using *Taq* polymerase as described by the manufacturer (Promega Corporation, Madison, Wis.). The reaction mixtures included 7-Deaza dGTP and were run on polyacrylamide gels containing 20% dimethylformamide. Single-stranded DNA was used as the template for sequencing reactions. It was derived from subclones in pTZ18R, produced in *E. coli* DH5αF′ with the helper phage M13K07, and prepared according to the supplier of the helper phage (Promega Corporation). Oligonucleotides prepared by the East Carolina University School of Medicine Biotechnology Program DNA Synthesis Core Laboratory were used to sequence both DNA strands. Additional DNA sequencing was done by the automated DNA sequencing facility at the University of North Carolina, Chapel Hill.

Sequence analyses. Nucleotide and protein sequence analyses were carried out using the University of Wisconsin Genetics Computer Group software package and the Basic Local Alignment Search Tool (BLAST) Network Service at the National Center for Biotechnology Information, National Institutes of Health (2).

Enzyme assays. Measurements of [^{14}C]mannitol uptake and incorporation and glucose-6-phosphate dehydrogenase and amidase activities were described previously (19). Glucose-6-phosphate dehydrogenase was measured by using the soluble fraction from crude cell extracts. Extract preparation is described below. Mannitol uptake and amidase activities were measured with washed whole cells. Apurinic/apyrimidinic (AP) endonuclease assays were done with partially depurinated plasmid DNA and analyzed by gel electrophoresis (3). Purified human Ref-1 used as a control was the generous gift of Steven Xanthodakis (Roche Biomedical, Nutley, N.J.). Additional AP endonuclease assays were performed in the laboratory of Miriam Sander (NIEHS, Research Triangle Park, N.C.) by using a synthetic oligonucleotide substrate (25). Exonuclease assays were performed on linearized plasmid DNA with *E. coli* ExoIII (Promega Corporation) as a positive control (3).

Expression of the *crc* gene product in *P. aeruginosa* and *E. coli*. To express the *crc* gene product in *P. aeruginosa*, the 922-bp fragment (pPZ448) (see Fig. 1) was cloned into pEB16, placing the Crc coding sequence downstream of a T7 promoter (5). The resulting plasmid, pPZ456, was transformed into *P. aeruginosa* ADD1976, which contains the T7 polymerase under the control of the *lac* promoter on the chromosome (4). To achieve maximum expression of the *crc* gene product, ADD1976 containing pPZ456 was grown for several generations in LB medium and, at an optical density at 600 nm of 0.8, 2 mM isopropylthio-β-D-galactopyranoside (IPTG) was added. After continued growth for 4 h at 37°C, rifampin (200 μg/ml) was added, and the cells were incubated for another 4 h. The cells were harvested by centrifugation, suspended in 20 mM Tris, pH 6.8, and disrupted in a French pressure cell at 16,000 psi. The cell envelope was removed by centrifugation at 200,000 × *g*, and the resulting supernatant was loaded onto a column containing Macro-Prep High Q anion exchange resin (Bio-Rad Laboratories, Hercules, Calif.). The column was washed with 2 column volumes of 20 mM Tris, pH 6.8; this was followed by 1 column volume of 20 mM Tris, pH 6.8, containing 100 mM NaCl. Subsequently, the Crc protein was eluted with 20 mM Tris, pH 6.8, containing 160 mM NaCl.

For expression of the *crc* gene product in *E. coli*, the *crc* gene was cloned into the vector pQE32 (Qiagen Inc., Chatsworth, Calif.). This vector contains a T5 promoter, two *lac* operators, and a ribosome binding site at the appropriate distance from an ATG codon. It also places six histidine residues at the N terminus of the gene product, allowing the specific binding of the expressed protein to a nickel-nitrilo-tri-acetic acid (Ni-NTA) resin. In order to insert the *crc* gene in frame with the ATG, a *Kpn*I site was introduced 6 bp upstream of the *crc* initiation codon by using an oligonucleotide-directed in vitro mutagenesis system (see above). The *crc* gene was then excised as an 883-bp *Kpn*I-*Stu*I fragment which was inserted into the *Kpn*I-*Sma*I site in the vector pQE32. The resulting Crc fusion protein produced from this plasmid contains an additional 20 amino acids (including the six histidines added by the vector) at the N terminus. pQE32 containing the *crc* gene, pPZ461, was transformed into *E. coli* M15 which also contained a second plasmid with the *lacI*^{q} gene, permitting tight control of *crc* gene expression in the absence of IPTG. These cells were grown to an optical density at 600 nm of approximately 0.8 in LB medium, at which time 2 mM IPTG was added and growth was continued for an additional 4 h. The culture was harvested by centrifugation, suspended in 100 mM Tris, pH 7.4, and soluble cell extracts were prepared as above. The Crc fusion protein was purified on a Ni-NTA column used as recommended by the supplier (Qiagen).

Analysis of cell extracts by SDS-polyacrylamide gel electrophoresis. To isolate soluble protein fractions, cells were disrupted in a French pressure cell at 16,000 psi and centrifuged at 200,000 × *g* for 30 min. The resulting supernatants were solubilized in an equal volume of sample buffer, heated to 100°C for 5 min, and run on sodium dodecyl sulfate (SDS) polyacrylamide gels (19).

Protein sequencing. Sequencing was performed in the East Carolina University School of Medicine Biotechnology Program Protein Sequencing Core Laboratory on a Milligen-Biosearch Model 6625 Prosequencer (Millipore Corp., Marlborough, Mass.). For each run, 10 μg of protein was coupled to a diisothiocyanate disk.

Antibody production. Crc fusion protein (500 μg) was combined with 2 volumes of complete Freund's adjuvant and injected intramuscularly into a New Zealand White rabbit. The rabbit was boosted in a similar fashion with 500 μg of the same protein mixed with incomplete Freund's adjuvant and, finally, boosted twice more with 50 μg of the protein alone, injected subcutaneously.

Western blots (immunoblots). Purified Crc protein or cell extracts from the various *Pseudomonas* or *E. coli* strains were run on SDS polyacrylamide gels, and proteins were transferred to Immobilon-P membranes (Millipore Corp., Bed-

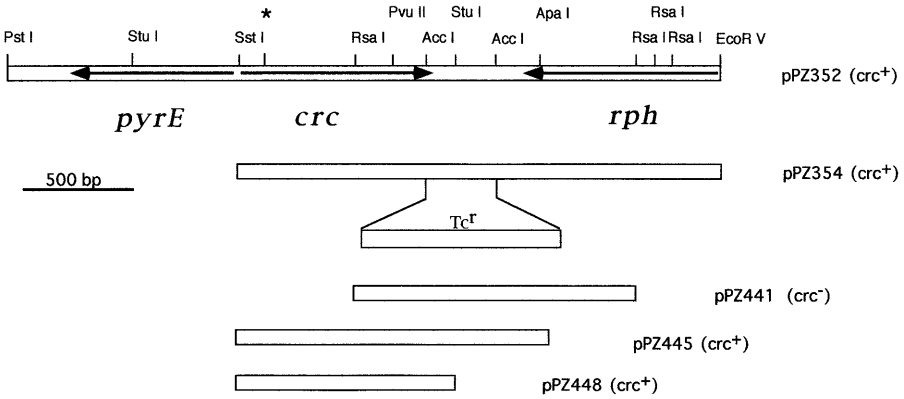

FIG. 1. A summary of the 3.1-kb *crc* region. Identified genes and the orientation of their coding sequences are indicated. Subclones of this region which complement *crc* are indicated (*crc*$^+$ summarized from Fig. 2). pPZ352 was constructed by subcloning a 3.1-kb *Pst*I-*Eco*RV fragment from pPZ341 into pPZ343 (19). Vector pPZ343 is pTZ18R containing a broad-host-range origin (*oriV*) derived from pRO1614 (28) to allow replication in *Pseudomonas* spp. The construction of pPZ354 was described previously (19) (plasmid pPZ353 is identical except that it lacks an *oriV*). pPZ441 was constructed by isolating a 1.2-kb *Rsa*I fragment from pPZ354 and inserting this into the *Sma*I site of pPZ343. pPZ445 was created by deleting a 0.7-kb *Apa*I-*Sma*I fragment from pPZ354. pPZ448 was created by deleting a 1-kb *Stu*I-*Sma*I fragment from pPZ354. In all plasmids, except pPZ352, the *lac* promoter of the vector is in the same orientation as *crc*. The location of the tetracycline resistance cassette used to disrupt the *crc* gene is indicated. *, location of the engineered *Apa*I site subsequently used in the construction of the *crc* deletion strain PAO8023.

ford, Mass.). Visualization of the transferred antigens was carried out as described by Ey and Ashman (10) with the following minor modifications. After blocking, the membranes were incubated with (i) anti-Crc rabbit serum (or nonimmune serum), (ii) goat anti-rabbit immunoglobulin G biotin conjugate (Sigma Chemical Co., St. Louis, Mo.), and (iii) alkaline phosphatase-streptavidin (Zymed Labs, San Francisco, Calif.).

Nucleotide sequence accession numbers. The 3.1-kb DNA sequence of the *crc* region has been assigned GenBank accession number U38241. The sequence between the *Sst*I and *Stu*I sites (55 to 976 in Fig. 4) containing the Crc coding sequence was assigned GenBank accession number L12038.

RESULTS

Nucleotide sequence of the *crc* region. In previous cloning experiments (19), we identified a 2-kb piece of *P. aeruginosa* chromosomal DNA that was able to restore a wild-type phenotype to *crc* mutants (i.e., plasmid pPZ354). This DNA codes for a protein with a molecular mass of approximately 30 kDa, assumed to be the *crc* gene product (19). We sequenced 3 kb of the *crc* region and found three genes, *crc*, *pyrE*, and a novel *P. aeruginosa* gene which we have designated *rph* (Fig. 1). Since the *crc* gene was known to be linked to *pyrE* (19), it was no surprise to find the coding sequence for orotate phosphoribosyl transferase, the gene product of *pyrE*. On the other side of *crc* the DNA sequence encodes a protein with 68% identity to the RNase PH of *E. coli*. Given that the *rph* gene of *E. coli* (encoding RNase PH) is adjacent to *pyrE*, we have named this the *rph* gene for *P. aeruginosa*. Figure 2 compares the deduced amino acid sequences of orotate phosphoribosyl transferase and RNase PH with their *E. coli* homologs.

The nucleotide sequence of the *crc* gene and the predicted amino acid sequence of the Crc protein are shown in Fig. 3. Amino acid analysis of the purified *crc* gene product (see below) demonstrated that protein synthesis initiates at the ATG located at position 97. All of these genes show a strong bias for G/C residues in the third base of their codons (Crc, 81%; PyrE, 86%; and Rph, 91%) consistent with other genes expressed in *P. aeruginosa*. A potential ribosome binding site (GGGGG) was identified 7 to 11 bp upstream of the ATG for Crc. This is an atypical ribosome binding site for *P. aeruginosa* and might explain the lower expression of the *crc* gene in *E. coli* (see below). A stop codon (TGA) at position 874 produced an open reading frame (ORF) of 777 bp. A protein produced from this ORF would have a molecular mass of 28.5 kDa, in close agreement with the 30-kDa protein identified on SDS polyacrylamide gels (19). At positions 894 to 919, 18 bp after the stop codon, a strong factor-independent transcriptional terminator 26 bp in length was identified by using the TERMINATOR program. The PEPTIDESTRUCTURE program predicts that the Crc protein is very hydrophilic, which is consistent with the identification of this gene product in the soluble fraction of *P. aeruginosa* (19).

A

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PAO1    MQAYQRDFIRFAIERGVLRFGEFTLKSGRTSPYFFNAGLFDSGLALARLGR 51
E. coli KPYQRQFIEFALSKQVLKFGEFTLKSGRKSPYFFNAGLFNTGRDLALLGR 50

PAO1    FYAEAVIDSGIDFDVLFGPAYKGIPLAATTAVALAEQHQRDLPWCFNRKE 101
E. coli FYAEALVDSGIEFDLLFGPAYKGIPIATTTAVALAEHHDLDLPYCFNRKE 100

PAO1    AKEHGEGGTLVGAPLSGRVLIIDDVITAGTAIREVMQIIDAQGARAAGVL 151
E. coli AKDHGEGGNLVGSALQGRVMLVDDVITAGTAIRESMEIIQANGATLAGVL 150

PAO1    IALNRQERGKGELSAIQEVERDFGMPVVSIVSLEQVLEYLAEDAELKKHL 201
E. coli ISLDRQERGRGEISAIQEVERDYNCKVISIITLKDLIAYLEEKPEMAEHL 200

PAO1    PAVEAYRAQYGI 213
E. coli AAVKAYREEFGV 212
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B

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PAO1    MNRPSGRAADQLRPIRITRHYTKHAEGSVLVEFGDTKVICTVSAESGVPRFL 52
E.coli  MRPAGRSNNQVRPVTLTRNYTKHAEGSVLVEFGDTKVLCTASIEEGVPRFL 51

PAO1    KGQGQGWLTAEYGMLRRSTGERNQREASRGKQGGRTLEIQRLIDRSLRAA 102
E.coli  KGQGQGWITAEYGMLPRSTHTRNAREAAKGKQGGRTMEIQRLIARALRAA 101

PAO1    LDLSKLGENTLYIDCDVIQTDGGTRTASITGATVSLIDALAVLKKRVALK 152
E.coli  VDLKALGEFTITLDCDVLQADGGTRTASITGACVALVDALQKLVENGKLK 151

PAO1    GNPLKQIVAAVSVGIYQGVPVLDLDYLEDSAAESDLNVVVMTDCRRFIEVQ 202
E.coli  TNPMKGMVAAVSVGIVNGEAVCDLEYVEDSAAETDMNVVMTEDGRIIEVQ 201

PAO1    GTAEGAPFRPAELNAMLELAQQGMQELVRTQRAALAE 239
E.coli  GTAEGEPFTHEELLILLALARGGIESIVATQKAALAN 238
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FIG. 2. Homology between *E. coli* and *P. aeruginosa pyrE* and *rph* gene products. (A) Alignment of orotate phosphoribosyl transferase sequences (*pyrE*). (B) Alignment of RNase PH sequences (*rph*).

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    R  Q  Y  A  Q  M   ←  pyrE start
 1  GctGatAcgCctGcaTgaatgacaccaagccgcatgaatttagctaaaccgtttGAGCTC 60
           .        .        .        .        .        .

 61 gggtatcatacacgcacgtgattttgggggccattt ATG CGG ATC ATC AGT GTG 114
  1                    crc start  →     M  R  I  I  S  V   6

115 AAC GTG AAT GGT ATT CAG GCT GCG GCC GAG CGC GGT TTG CTC AGT  159
  7 N  V  N  G  I  Q  A  A  A  E  R  G  L  L  S   21

160 TGG CTG CAA GCA CAG AAT GCC GAC GTG ATC TGC TTG CAG GAC ACC  204
 22 W  L  Q  A  Q  N  A  D  V  I  C  L  Q  D  T   36

205 CGA GCC TCC GCC TTC GAT CTG GAT GAC CCG TCC TTC CAA CTG GAC  249
 37 R  A  S  A  F  D  L  D  D  P  S  F  Q  L  D   51

250 GGC TAC TTC CTT TAT GCC TGC GAT GCC GAG CTA CCC GAA CAG GGC  294
 52 G  Y  F  L  Y  A  C  D  A  E  L  P  E  Q  G   66

295 GGT GTC GCA CTC TAC AGC CGT TTG CAG CCC AAA GCT GTG ATC AGC  339
 67 G  V  A  L  Y  S  R  L  Q  P  K  A  V  I  S   81

340 GGC TTA GGT TTC GAA ACG GCC GAT CGT TAC GGG CGC TAC CTG CAA  384
 82 G  L  G  F  E  T  A  D  R  Y  G  R  Y  L  Q   96

385 GCC GAC TTC GAC AAG GTG AGT ATC GCC ACC CTG CTG CTG CCT TCC  429
 97 A  D  F  D  K  V  S  I  A  T  L  L  L  P  S  111

430 GGG CAG AGC GGA GAC GAG AGC TTG AAC CAG AAA TTC AAG TTC ATG  474
112 G  Q  S  G  D  E  S  L  N  Q  K  F  K  F  M  126

475 GAC GAC TTC ACC CAT TAC TTG AGC AAG CAG CGT CGC AAG CGC CGC  519
127 D  D  F  T  H  Y  L  S  K  Q  R  R  K  R  R  141

520 GAA TAC ATC TAC TGC GGC TCG CTG TAC GTC GCC CAT CAG AAG ATG  564
142 E  Y  I  Y  C  G  S  L  Y  V  A  H  Q  K  M  156

565 GAT GTG AAG AAC TGG CGC GAA TGT CAG CAG ATG CCG GGC TTC CTC  609
157 D  V  K  N  W  R  E  C  Q  Q  M  P  G  F  L  171

610 GCG CCC GAA CGG GCC TGG CTG GAC GAA GTG TTC GGC AAC CTC GGC  654
172 A  P  E  R  A  W  L  D  E  V  F  G  N  L  G  186

655 TAT GCC GAC GCC CTG CGC GAA GTC AGC CGC GAA GGC GAC CAG TTC  699
187 Y  A  D  A  L  R  E  V  S  R  E  G  D  Q  F  201

700 AGC TGG TGG CCG GAC AGC GAA CAG GCC GAG ATG CTC AAC CTC GGC  744
202 S  W  W  P  D  S  E  Q  A  E  M  L  N  L  G  216

745 TGG CGC TTC GAC TAC CAG GTG CTG ACC CCC GGC CTA CGC CGC TTC  789
217 W  R  F  D  Y  Q  V  L  T  P  G  L  R  R  F  231

790 GTG CGC AAC GCC AAG CTG CCG CGC CAG CCG CGC TTC TCC CAG CAT  834
232 V  R  N  A  K  L  P  R  Q  P  R  F  S  Q  H  246

835 GCG CCG CTG ATC GTC GAC TAC GAC TGG CAG TTG AGC ATC TGA gccc 880
247 A  P  L  I  V  D  Y  D  W  Q  L  S  I  *      260

881 tgtcagacaccgaaaaagccggcgcatgcgccggctttttctatttcccgtgcggcctact 940

941 tgatcagccgcaggcagaacggatagcgatAGGCCT                        976
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FIG. 3. Nucleotide sequence of the *P. aeruginosa* DNA fragment contained in pPZ352, extending from 16 bp within the *pyrE* coding sequence to the *Stu*I site (976). The region underlined (894 to 919) is a strong stem-loop structure with the properties of a factor-independent terminator. The *crc* structural gene starts at the ATG at position 97. A potential Shine-Dalgarno sequence, GGGGG, is located at positions 86 to 90.

Genetic identification of the *crc* structural gene. In order to confirm our identification of the *crc* gene, we subcloned the *crc* region and tested these clones for complementation of the *crc* mutation. In addition we have constructed new defined mutations (knockouts) of the chromosomal copy of *crc.*

PAO1 (wild type) shows repression of the mannitol pathway and amidase when grown in the presence of succinate plus the appropriate inducer. However, these activities are not repressed in *crc* mutants (46). When a plasmid carrying the *crc* gene is introduced into *crc* mutants, these activities become sensitive to repression by succinate and are lowered to the levels seen in the wild type (19). Figure 1 summarizes the complementation results that are presented in Fig. 4. The smallest plasmid able to restore a wild-type phenotype to the

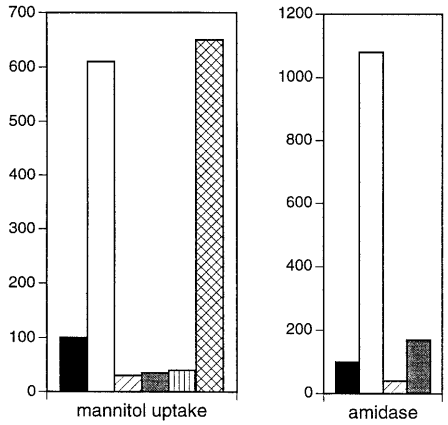

FIG. 4. Complementation of *crc* mutant PAO8007 with plasmids shown in Fig. 1. Each plasmid was transformed into PAO8007, and all strains were grown for two generations in BSM containing 40 mM succinate plus 5 mM [^{14}C]mannitol (for uptake and incorporation) or 40 mM succinate plus 20 mM lactamide (for amidase). Assays were performed as previously described (19). Enzyme activity in PAO1 is expressed as 100% of each specific activity (1.2 μmol/mg of protein for amidase and 115 nmol/mg of protein for mannitol uptake). All others are expressed as a percentage of that found in PAO1. Symbols: ■, PAO1; □, PAO8007; ▨, PAO8007/pPZ354; ▩, PAO8007/pPZ448; ▥, PAO8007/pPZ445; ▩, PAO8007/pPZ441.

crc mutants was pPZ448. This plasmid contained an *Sst*I-*Stu*I fragment 922 bp in length. When soluble proteins from PAO8007 containing pPZ448 or the original 2-kb subclone, pPZ354, were analyzed on SDS polyacrylamide gels stained with Coomassie blue, a major band in both of these extracts had an approximate molecular mass of 30 kDa (data not shown) (19). This band was not present in PAO8007 containing only the vector. This confirmed our previous conclusion (19) that the *crc* gene product has a molecular mass of approximately 30 kDa.

To create new *crc* mutants, the *crc* gene was disrupted by replacing the 0.3-kb *Acc*I fragment from the insert in pPZ354 (see Fig. 1) with a tetracycline resistance cassette and inserting this defective *crc* gene into the chromosome of PAO1 by homologous recombination. This construction deleted the last 24 bp from the end of the gene as well as the terminator and some downstream sequence. This construct was derived from pPZ353, which is identical to pPZ354 except that it cannot replicate in *Pseudomonas* spp. Figure 5 shows that this deletion mutant, PAO8020, has a phenotype identical to other *crc* mutants which either arose spontaneously or were produced by chemical mutagenesis (46). Southern analysis of PAO8020 showed that it contained only a single copy of the *crc* gene and that the Tc^r cassette was located within this gene (3). The wild-type phenotype could be restored to PAO8020 by transformation with both pPZ354 and pPZ448 (Fig. 3). Thus the DNA contained within the 922-bp *Sst*I-*Stu*I fragment is sufficient to complement this mutation.

We subsequently constructed a second strain, PAO8023, by recombination of a 5′- and 3′-deleted copy of the *crc* gene (containing the coding sequence for amino acids 36 to 201 of the Crc sequence) into PAO1. PAO8023 is also phenotypically a *crc* mutant (data not shown).

Homology of the *crc* gene product to AP endonucleases. We compared the deduced amino acid sequence of the *crc* gene product to that of other proteins by using the FASTA, TFASTA, and BLAST programs. The only strong candidates for related proteins belonged to a family of DNA repair enzymes found in both prokaryotes and eukaryotes. The repair

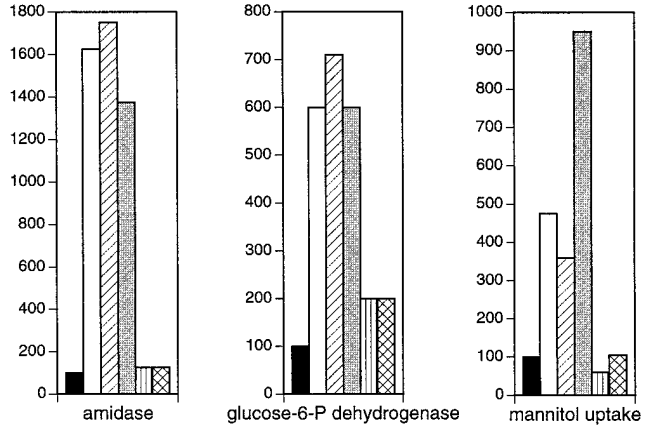

FIG. 5. Complementation of *crc* mutant PAO8020 with *crc*$^+$ plasmids shown in Fig. 1. Plasmids pPZ448 and pPZ354 and the vector, pPZ343, were transformed into PAO8020, and all strains were grown for two generations in BSM containing 40 mM succinate plus 5 mM [^{14}C]mannitol (for uptake and incorporation), 40 mM succinate plus 20 mM mannitol (for glucose-6-phosphate dehydrogenase), or 40 mM succinate plus 20 mM lactamide (for amidase). Assays were performed as previously described (19). Enzyme activity in PAO1 is expressed as 100% of specific activity (0.83 μmol/mg of protein for amidase, 10.4 nmol/mg of protein for glucose-6-phosphate dehydrogenase, and 215 nmol/mg of protein for mannitol uptake). All others are percentages of that found in PAO1. Symbols: ■, PAO1; □, PAO8007; ▨, PAO8020; ▩, PAO8020/pPZ343; ▥, PAO8020/pPZ448; ▩, PAO8020/pPZ354.

enzymes in this family include human Ref-1 (redox factor) (45, 49), bovine BAP1 (bovine AP endonuclease) (32), *Drosophila* Rrp1 (recombination repair protein) (34, 35), *Arabidopsis thaliana* Arp (apurinic endonuclease-redox protein) (4), *E. coli* ExoIII (exonuclease) (36), *Streptococcus pneumoniae* ExoA (exonuclease) (30), and *Bacillus subtilis* ExoA (exonuclease) (27). They range in identity to Crc from 25 to 32% and in similarity to Crc from 51 to 57%. Homologies in these same ranges are found when the prokaryotic enzymes are compared with the eukaryotic enzymes or when the prokaryotic enzymes are compared with each other. Areas of identity are not located at a particular region of each protein but exist throughout all these proteins, including Crc. These homologies are illustrated in Fig. 6. All these proteins (with the exception of *B. subtilis* ExoA which has yet to be isolated) possess AP endonuclease activity and are theorized to be involved in some way in DNA repair. Recently, an ORF was identified in *Coxiella burnetii* (44) which codes for a putative protein with a 44% identity to *crc*. This putative protein is also shown in Fig. 6. In *C. burnetii* this ORF is adjacent to a homolog of orotate phosphoribosyl transferase. It is intriguing to note that this ORF and *crc* are adjacent to and divergently expressed from the orotate phosphoribosyl transferases. In *P. aeruginosa* there are 80 bp between the ATG start codons for *pyr*E and *crc*, while in *C. burnetii* there are 50 bp between ATGs.

As an initial test for a DNA repair activity in the Crc protein, we compared the sensitivities of *P. aeruginosa* PAO1 (*crc*$^+$), PAO8007 (*crc-20*), and PAO8020 (Δ*crc*) to DNA damaging agents. *E. coli xth* (ExoIII) mutants are extremely sensitive to alkylating agents such as methyl methanesulfonate and mitomycin C and oxidizing agents such as H_2O_2 (8). At concentrations of these agents which caused a decrease in survival of PAO1 of less than 1 log, no greater sensitivity was observed in either PAO8007 or PAO8020 (data not shown).

Since there may be redundant recombination repair activities in *Pseudomonas* spp. that would mask the phenotype of a single mutation, we also tested the Crc protein for AP endo-

```
human Ref-1          LKIcSWNVdG LRAwik.... ..Kkg...Ld WvkeEapDIl CLQETKcse.
bovine BAP1          LKIcSWNVdG LRAwik.... ..Kkg...Ld WvkeEapDIl CLQETKcse.
Drosophila Rrp1      LKIcSWNVaG LRAwlk.... ..Kdg...Lq lidlEepDIf CLQETKcan.
Arabidopsis Arp      vKVmtWNVNG LRgllk.... ..fesfsaLq laqrEnfDIl CLQETKlqv.
B. subtilis ExoA     MKlISWNVNG LRAvmr.... ..Kmdf..Ls YlkeEdaDII CLQETKiqd.
S. pneumoniae ExoA   MKlISWNIds LnAaltsdsa raKlsqevLq tlvaEnaDII aiQETKlsak
E. coli ExoIII       MKfVSFNING LRAr...... ....phqlea ivekhqpDVI gLQETKvhdd
C. burnetii          MrIItLNlNG iRAaarr... ......gfFd WlkrqkaDIV CLQETKacl.
P. aeruginosa CRC    MrIISvNVNG iqAaaer... ......glLs WlqaqnaDVI CLQDTrasa.
          Consensus  MKIISWNVNG LRA------- --K-----L- W---E--DII CLQETK----

human Ref-1          ...nklpael qEL.pGlshq YWsap.sdKe GYSGVgLLSR qc...PlkVs
bovine BAP1          ...nklpvel qEL.sGlshq YWsap.sdKe GYSGVgLLSR qc...PlkVs
Drosophila Rrp1      ...dqlpeev trL.pGyhpy Wlcmp.g... GYaGVAiYSk im...PihVe
Arabidopsis Arp      ...kdveeik ktLidGydhs FWscs.vsKl GYSGtAiiSR ik...PlsVr
B. subtilis ExoA     ...gqv.... .DL.qpedyh vYwny.avKk GYSGtAvFSk qe...PlqVi
S. pneumoniae ExoA   gptkkhveil eELfpGyent WRssqepark GYaGtmFLyk keltptisfp
E. coli ExoIII       mfpleevanv g......... .Ynvfyhgqk GhyGVALLtk et...PiaVr
C. burnetii          .......eit ngdqfhpkgy hcyyhdaeKs GYSGVgiYcR ek...PdrVt
P. aeruginosa CRC    .......fdl dDpsfqldgy Flyacdaelp eqgGVALYSR lq...PkaVi
          Consensus  ---------- -EL--G---- -W------K- GYSGVALLSR -----P--V-

human Ref-1          YGiGdEEhDq EGRVIvAEFD sFv....lVt aYvPNaGrGL vRLEY..R.q
bovine BAP1          YGiGeEEhDq EGRVIvAEYD aFv....lVt aYvPNaGrGL vRLEY..R.q
Drosophila Rrp1      YGiGnEEfDd vGRmItAEYE kFY....lIn vYvPNsGrkL vnLEp..R.m
Arabidopsis Arp      YGtGlsghDt EGRIVtAEFD sFY....lIn tYvPNsGdGL kRLsY..Rie
B. subtilis ExoA     YGiGvEEhDq EGRVItlEFE nvF....Vmt vYtPNsrrGL eRiDY..R.m
S. pneumoniae ExoA   eigapstmDl EGRIItlEFD aFF....Vtq vYtPNaGdGL kRLEe..R.q
E. coli ExoIII       rGfpgDDeEa qrRIImAEip sLLgnvtVIn gYfPqgesrd hpikFpakaq
C. burnetii          trlGwEhaDk EGRyIqADFg sLs....Vas lYmPsgttGe hRqki..kfd
P. aeruginosa CRC    sGlGfEtaDr yGRylqADFD kvs....Iat lllPsgqsGd esLnq..kfk
          Consensus  YG-G-EE-D- EGR-I-AEFD -F-----VI- -Y-PN-G-GL -RLEY--R--

human Ref-1          rWDeaFrkFl KgLasrKPlV lCGDLNVAHe EIDLrNPKgN kK......nA
bovine BAP1          rWDeaFrkFl KgLasrKPlV lCGDLNVAHe EIDLrNPKgN kK......nA
Drosophila Rrp1      rWEklFqaYv KkLdalKPVV iCGDMNVsHm pIDLeNPKnN tK......nA
Arabidopsis Arp      eWDrtLsnhi KeLeksKPVV ltGDLNcAHe EIDifNPagN kr......sA
B. subtilis ExoA     qWEeaLlsYi leLdqkKPVI lCGDLNVAHq EIDLkNPKaN rn......nA
S. pneumoniae ExoA   vWDakYaeYl aeLdkeKPVl atGDyNVAHn EIDLaNPasN rr......sp
E. coli ExoIII       fYqnlqnyLe teLkrdnPVl imGDMNIspt DlDigigeeN rKrwlrtgkc
C. burnetii          fmDrymkrLk nivhskrsfI iCGDwNIvHk EIDikNfKsN qK......ys
P. aeruginosa CRC    fmDdfthyLs KqrrkrreyI yCGsLyVAHq kmDvkNwrec qq......mp
          Consensus  -WD--F---- K-L---KPVV -CGDLNVAH- EIDL-NPK-N -K-------A

human Ref-1          GFTpqERqgF gELLqavpLa DsFRHlyPnt pyaYTFWtYm .mnARskNvG
bovine BAP1          GFTpqERqgF gELLqavpLt DsFRHlyPnt ayaYTFWtYm .mnARskNvG
Drosophila Rrp1      GFTqEERdkM tELL.glGFV DtFRHlyPdr kgaYTFWtYm .anARarNvG
Arabidopsis Arp      GFTiEERqsF ganLldkGFV DtFRkqhPgv .vgYTYWgYR .hggRktNkG
B. subtilis ExoA     GFsdqEReaF trFLea.GFV DsFRHvyPdl egaYsWWsYR .agARdrNiG
S. pneumoniae ExoA   GFTdEERagF tnLL.atGFt DtFRHvhgdv perYTWWaqR sktskinNtG
E. coli ExoIII       sFlpEERewM drLM.swGLV DtFRHanPqt adrFsWFdYR sk.gfddNrG
C. burnetii          GclpEERawL dEvFtkvGLV DaFRvvnqk. pdqYTWWssR gr........
P. aeruginosa CRC    GFlapERawL dEvFgnlGYa DaLRevsre. gdqFsWWpds eq.AemlNlG
          Consensus  GFT-EER--F -ELL---G-V D-FRH--P-- ---YTWW-YR ---AR--N-G

human Ref-1          WRLDYFLlSh sL....lpal cDskIrskal aSDHCPItLy Lal....
bovine BAP1          WRLDYFLlSq sv....lpal cDskIrskal gSDHCPItLy Lal....
Drosophila Rrp1      WRLDYcLvSe rF....vpkV vEheIrsqcl gSDHCPItif Fni....
Arabidopsis Arp      WRLDYFLvSq si....aanV hDsyIlpdin gSDHCPIgLi Lkl....
B. subtilis ExoA     WRiDYFvvSe sL....keqI eDasIsadvm gSDHCPVeLi ini....
S. pneumoniae ExoA   WRiDYWLtSn ri....adkV tksdmidsga rqDHtPIvLe idl....
E. coli ExoIII       lRiDlLLaSq pLaeccvetg iDyeIrsmek pSDHaPVwat Frr....
C. burnetii          .......... .......... .......... .......... .......
P. aeruginosa CRC    WRFDYqvltp gL....rrfV rnaklprqpr fSqHaPlivd Ydwqlsi
          Consensus  WRLDYFL-S- -L-------V -D--I----- -SDHCPI-L- L------
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FIG. 6. Comparison of Crc to AP endonucleases. Alignment was made using the PILEUP program. The consensus sequence contains those amino acids present in five of the nine sequences compared. Capitalized amino acids are similar or identical to the consensus. The alignments begin at amino acids 62 for Ref-1, 61 for BAP1, 427 for Rrp1, and 267 for Arp. All the remaining (prokaryotic) sequences begin at the amino terminus.

TABLE 2. Complementation analysis of *E. coli* ExoIII mutants with cloned *P. aeruginosa crc* and *crc-f*[a] genes and the human *ref-1* gene

Strain (plasmid, gene)	Relative plating efficiency[b]
BW287	1.2×10^{-3}
BW287 (pKK223-HAP1, *ref-1*)	0.93
BW287 (pPZ343, vector)	2.5×10^{-3}
BW287 (pPZ353, *crc*)	5.0×10^{-3}
BW287 (pQE32, vector)	1.5×10^{-3}
BW287 (pPZ461, *crc-f*)	3.2×10^{-3}
BW549	3.0×10^{-5}
BW549 (pKK223-HAP1, *ref-1*)	0.82
BW549 (pPZ343, vector)	4.0×10^{-5}
BW549 (pPZ353, *crc*)	5.0×10^{-5}
BW549 (pQE32, vector)	3.5×10^{-5}
BW549 (pPZ461, *crc-f*)	3.8×10^{-5}

[a] *crc* fusion gene.

[b] Cells were grown to late log phase in 2× YT (yeast extract-tryptone) medium (21). They were concentrated 25× by centrifugation and suspended in 50 mM potassium phosphate, pH 7.5. Samples of serial 10× dilutions were plated on two identical 2× YT plates. (Plates for strains carrying plasmids contained ampicillin.) One plate of each pair was incubated at 30°C, the other was incubated at 42°C. The numbers of colonies appearing on the plates were counted after 24 h. The relative plating efficiency is expressed as the ratio of the number of colonies growing at 42°C and 30°C.

nuclease activity in vivo by using two *E. coli* mutants. *E. coli* strains BW287 and BW549 contain a temperature-sensitive mutation in the *xth* gene (ExoIII), giving them lower survival rates at increased growth temperatures (8, 44). Both strains contain a *dut-1* mutation to generate AP sites in the DNA. BW549 also contains a mutation in the *nfo* gene which codes for endonuclease IV, giving it an even lower survival rate than BW287 (8). Previous studies indicated that other genes in this AP endonuclease family (*ref-1* and *rrp1*) can replace the *xth* gene in these mutants and restore wild-type survival rates at elevated temperature (12, 31). We performed a similar experiment by comparing the plating efficiency of BW287 and BW549 carrying plasmids containing the *ref-1* gene, the *crc* gene, and the *crc* fusion gene (*crc-f*) with the plating efficiency of each of these strains carrying no plasmid or vector only. Table 2 shows that neither form of the *crc* gene could replace the temperature-sensitive ExoIII protein at 42°C, while the Ref-1 protein could. To confirm that the *crc* gene was actually being expressed in these *E. coli* strains, Western blots were performed (as described below). In both strains containing the *crc* or *crc-f* genes, a band was recognized by the anti-Crc antisera at the correct location. No band was observed in the controls (3).

Expression of the purified *crc* gene product. We purified two *crc* gene products, a native Crc expressed in *P. aeruginosa* and a fusion protein expressed in *E. coli*. After placement of the *crc* gene behind a T7 promoter in pEB16 (creating pPZ456), the Crc protein became the major protein in soluble extracts of *P. aeruginosa* ADD1976 following induction of the T7 polymerase with IPTG (Fig. 7B). This protein had a MW of approximately 30 kDa, which was identical to that identified as the *crc* gene product produced in *P. aeruginosa* from pPZ354 (reference 19 and above). The native protein was purified to approximately 60% purity, allowing for the identification of the following 10 amino acids from the N terminus: M-R-I-I-S-V-N-V-N-G. These 10 amino acids are in exact agreement with the nucleotide sequence presented in Fig. 3. This purified protein was also recognized by antibodies prepared against the Crc fusion protein purified from *E. coli* (see below).

In *E. coli* M15, the *crc* gene product was produced from pPZ461 (Fig. 7A). Although the amount of Crc fusion protein produced in *E. coli* from this plasmid was increased over our original attempt (19), it was less than that produced in *P. aeruginosa* (compare lane 4 with lane 10 in Fig. 7). The vector, pQE32, contains *lac* and T5 promoters and a ribosome binding site which is followed by a multiple cloning site which allows for in-frame cloning of a desired gene after six histidine codons. The fusion protein produced from this vector containing the *crc* gene was slightly larger than 30 kDa because of the added six histidines and the amino acids added at the N terminus to allow placement of the gene in frame with the histidine codons. Purification of the fusion protein over a Ni-NTA column resulted in a preparation which was greater than 95% pure. When the complete amino acid composition of this fu-

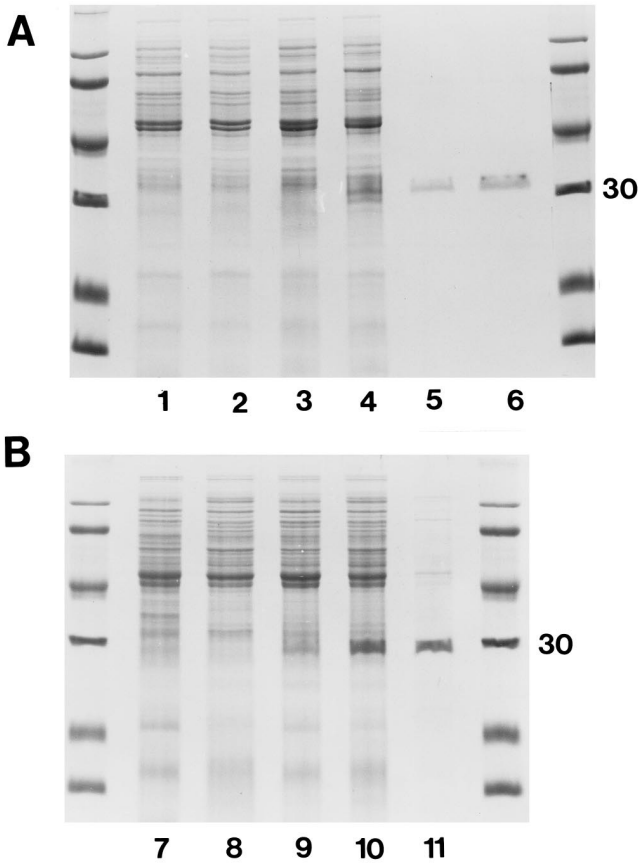

FIG. 7. SDS polyacrylamide gels showing expression and purification of the *crc* gene product from pPZ461 in *E. coli* M15 and from pPZ456 in *P. aeruginosa* ADD1976. Cells were grown as described in Materials and Methods either with (+) or without (−) IPTG. The crude soluble protein fractions and purified fractions (see Materials and Methods) were solubilized in sample buffer and run on SDS polyacrylamide gels (19) which were stained with Coomassie blue. Molecular mass markers contain proteins of 97, 66, 45, 30, 21, and 14 kDa. The 30-kDa band is labeled. (A) Crc fusion protein purified from *E. coli* M15. Lanes on either end contain molecular mass markers. Lane 1, crude soluble fraction from M15 (+); lane 2, crude soluble fraction from M15 containing vector pQE32 (+); lane 3, crude soluble fraction from M15 containing pPZ461 (−); lane 4, crude soluble fraction from M15 containing pPZ461 (+); lanes 5 and 6 contain 244 ng and 540 ng, respectively, of Crc fusion protein purified by elution from the Ni-NTA column. Samples of crude extracts contained 4 to 6 μg of protein. (B) Native Crc purified from *P. aeruginosa*. Lanes on either end contain molecular mass markers. Lane 7, crude soluble fraction from ADD1976 (+); lane 8, crude soluble fraction from ADD1976 containing vector pEB16 (+); lane 9, crude soluble fraction from ADD1976 containing pPZ456 (−); lane 10, crude soluble fraction from ADD1976 containing pPZ456 (+); lane 11 contains 260 ng of partially purified Crc protein. Samples of crude extracts contained 6 to 7 μg of protein.

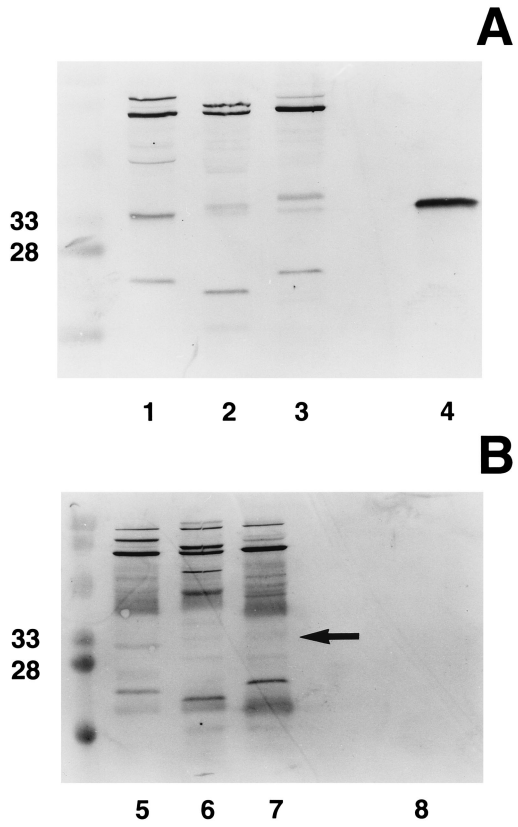

FIG. 8. Identification of a protein antigenically similar to Crc in crude extracts from *P. putida* and *P. fluorescens*. Crude extracts were prepared from log-phase cultures of *Pseudomonas* spp. grown on LB medium. Samples of each extract containing 8 to 9 μg of protein were run on SDS polyacrylamide gels (see Materials and Methods). Western blot analysis was performed as described in Materials and Methods. Bands shown in panel A were visualized with anti-Crc antiserum and those shown in panel B were visualized with nonimmune serum. Prestained molecular mass markers of 19, 28, 33, 50, 80, and 106 kDa are shown in the left lane. Because of the bound stain, these markers migrate more rapidly than the unstained markers. Lanes 1 and 5 contain extracts from *P. aeruginosa*, lanes 2 and 6 contain extracts from *P. putida*, lanes 3 and 7 contain extracts from *P. fluorescens*, and lanes 4 and 8 contain 50 ng of partially purified native Crc protein from *P. aeruginosa*. The arrow in panel B indicates the position of the Crc protein, just above the 33-kDa marker.

sion protein was determined, it was found to be in close agreement with the amino acid composition predicted from the nucleotide sequence (3).

We assayed both the purified native Crc protein and the fusion protein for both AP endonuclease and for exonuclease activities. Neither activity could be identified in either Crc protein, although we were able to detect AP endonuclease activity in purified human Ref-1 and both activities in purified *E. coli* ExoIII (3).

Detection of Crc in *Pseudomonas* species. Purified Crc fusion protein from *E. coli* was used to produce polyclonal antibody in a rabbit. Western blots were performed with this antiserum to look for antigenically similar proteins in crude soluble cell extracts from *P. putida* and *P. fluorescens*. Sera from nonimmune rabbits served as a control for specificity of the anti-Crc antibody. Figure 8 shows that crude extracts from the *Pseudomonas* strains each have a protein that is recognized only by anti-Crc antibody. These proteins from both strains have molecular weights similar to Crc. No protein which reacted specifically with anti-Crc antibody could be found in crude extracts from *E. coli* (3).

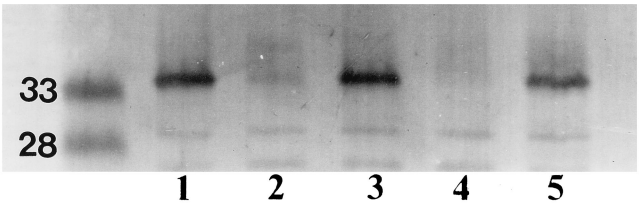

FIG. 9. Identification of Crc protein in PAO1 and various *crc* mutant strains. Crude cell extracts from wild-type and mutant cultures were prepared from LB-grown cultures, run on SDS polyacrylamide gels, and analyzed by Western blotting as shown in Fig. 7. The region of the gel containing proteins which reacted only with anti-Crc antiserum (not with preimmune serum) are shown. The left lane shows the 28- and 33-kDa markers. Lane 1, extract from PAO1; lane 2, extract from PAO8005 (*crc-5*); lane 3, extract from PAO8007 (*crc-20*); lane 4, extract from PAO8020 (Δ*crc*); lane 5, extract from PRP701 (*crc-1*).

This same anti-Crc antiserum was used to look for the presence of Crc protein in crude extracts of *crc* mutants. Figure 9 shows that the knockout mutant, PAO8020, described above lacks a cross-reacting 30-kDa protein. Our second knockout, PAO8023, also lacks a cross-reacting 30-kDa protein (data not shown). Of two other mutants produced by chemical mutagenesis (47), PAO8005 contains a very small amount of the 30-kDa protein, while PAO8007 produces a 30-kDa protein which we assume to be nonfunctional. Both of these mutants are complemented by a 2-kb DNA fragment containing the crc^+ gene and have a phenotype identical to PAO8020 (19). PRP701 is a spontaneous *crc* mutant which also has a phenotype identical to PAO8005, PAO8007, and PAO8020 (48). This mutant also presumably contains a defective Crc protein.

Crc is distinct from *E. coli* Cap. Recently, West et al. identified a gene from *P. aeruginosa*, *vfr*, which has 67% identity and 91% similarity to the *E. coli crp* gene and which complements the *E. coli crp* mutation (46). To confirm that the Crc protein does not function in the same manner as the Cap protein in *E. coli* (also known as Crp), we attempted to complement the *crc* mutation with the *vfr* gene. The *crc* mutants, PAO8007 and PAO8020, were transformed with the vector pUCP18 and with the same vector carrying the *vfr* gene, pKF812. Each of these was grown in BSM-succinate plus mannitol or BSM-succinate plus lactamide, and the activities of mannitol dehydrogenase, glucose-6-phosphate dehydrogenase, and amidase were measured. In contrast to the complementation data shown in Fig. 4 and 5, all of these activities were identical and independent of the plasmid present (17). The wild-type phenotype was not restored by the vfr^+ plasmid as it was by the crc^+ plasmids shown in Fig. 2 and 3. Thus, the function of the Crc protein could not be replaced by the *vfr* gene product.

Since the phenotype of the *crc* mutants suggests the loss of a regulatory protein, we compared the sequence of Crc with that of other regulatory proteins. No significant homology was found to Cap from *E. coli* or to any other DNA-binding protein. The MOTIFS program was also used to look for helix-loop-helix regions, common to DNA-binding proteins. No such regions were identified. No regions of the protein similar to any other sequence pattern in the Prosite database were found (at the level of zero mismatches). Nor were any matches found by using the PROFILESCAN program, which looks for structural and sequence motifs within proteins. For all searches, the entire amino acid sequence of the *crc* gene product was used as well as smaller portions of this sequence.

DISCUSSION

We have isolated and sequenced 3.1 kb of *P. aeruginosa* chromosomal DNA which contains three genes, *pyrE*, *rph*, and *crc*. We have shown that a 922-bp fragment complements previously described *crc* mutants (19). These mutants lack a gene essential for catabolite repression control. When the 922-bp sequence was interrupted with a tetracycline resistance gene and the defective *crc* gene was inserted into the chromosome of wild-type strain PAO1, a mutant with a phenotype identical to the original mutants was generated. Western analysis of crude extracts of this mutant showed that it lacked the 30-kDa protein. The construction of this knockout should produce an almost full-length Crc protein (lacking eight amino acids), suggesting that the truncated protein is unstable in *P. aeruginosa.*

The 922-bp fragment contains an ORF encoding a protein of 259 amino acids with a relative molecular mass of approximately 28.5 kDa. Eighteen base pairs following the TGA stop codon is a strong factor-independent transcriptional terminator which is 26 bp in length. A potential ribosome binding site was identified upstream of the initiating ATG; however, this site was unique in that it contained only G residues. This lack of a typical *E. coli* ribosome binding site may provide a partial explanation for the low level of expression of the *crc* gene in *E. coli*, even from a strong promoter (19). However, even when an appropriate ribosome binding site was provided in pPZ461, Crc represented a smaller percentage of the total protein in *E. coli* than in *P. aeruginosa*. Crc protein was purified from *P. aeruginosa*, and analysis of the first 10 amino acids are in exact agreement with those predicted from the DNA sequence.

A comparison of the predicted *crc* gene product to other known protein sequences led to the surprising finding that this protein shows homology to a group of DNA repair enzymes; however, all attempts to show that the Crc protein functions as an endonuclease proved negative. *crc* mutants show no increased sensitivity to DNA damaging agents, and the *crc* gene cannot complement *E. coli xth* or *nfo* mutants. The purified Crc proteins lack both endonuclease and exonuclease activity. Exonuclease activity is a feature of the bacterial enzymes belonging to this group. This suggests that, although the Crc protein may be related to this family of endonucleases, it does not have the same activity. Crc appears to be most closely related to the *C. burnetii* putative protein, with more similarity in the C-terminal region where Crc is less similar to the endonucleases (see Fig. 6). This suggests that these two proteins may have the same function. Perhaps, like the Ref-1 protein, the Crc protein has a regulatory function and a second function involved in DNA modification. Ref-1 stimulates the DNA-binding activity of the Fos and Jun transcription factors by an unknown mechanism and has endonuclease activity (45, 49).

In order to identify a regulatory mechanism for the Crc protein, we have also tested both native and fusion Crc proteins for their ability to bind to small DNA fragments containing upstream regions of several Crc-regulated genes involved in carbohydrate catabolism. To date, gel shift assays indicate that the Crc protein has no specific DNA-binding activity. The possibility exists that this lack of binding is due to the absence of a cofactor required for binding of the Crc protein to the DNA. Therefore, experiments were carried out to look for DNA binding in the presence of various metabolites. Included in the binding mixtures were the following: acetyl coenzyme A, coenzyme A, AMP, ADP, ATP, cAMP, GTP, NAD-NADH, NADP-NADPH, and glutathione. Each was added to a separate Crc-DNA mixture, and binding was assayed by gel shift. In no case were we able to find any DNA-binding activity with native or fusion Crc protein (3). Positive controls included a binding activity found in crude extracts of wild-type and *crc* mutant strains which does bind to these same regulatory regions of *P. aeruginosa* DNA (3).

To demonstrate that the Crc protein functions in a different manner from *E. coli* Cap, we attempted to replace the defective Crc protein in *crc* mutants with the *P. aeruginosa* homolog of *E. coli* Cap. As expected, the *vfr* gene, which does complement *E. coli crp* mutants (46), did not complement *crc* mutants.

Thus, the Crc protein has been examined for modes of action suggested by its sequence and by its function. It has been exhaustively tested for endonuclease, exonuclease, and DNA-binding activities and was found to possess none of these. Moreover, the predicted amino acid sequence for the Crc protein showed no significant homology to any other protein(s) other than this family of eukaryotic and prokaryotic DNA repair enzymes, nor did it contain a structural motif common to any group of proteins in the Prosite bank. The actual mechanism of action of the Crc protein in catabolite repression control is likely to be quite different from that of any previously described prokaryotic regulatory protein. Perhaps, like Ref-1, instead of binding directly to DNA itself, it regulates gene expression by activating a DNA-binding protein. This could explain its ability to regulate multiple independent catabolic systems. Whatever its mechanism, it is likely that the same catabolite repression control system exists in the fluorescent pseudomonads, since we were able to identify proteins which were antigenically similar to Crc in *P. putida* and *P. fluorescens* but not in *E. coli*. The existence of a common mechanism of catabolite repression control in these closely related species is not surprising, since repression by organic acids has been observed in all three species (16, 42).

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