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Cloning of a Catabolite Repression Control (*crc*) Gene from *Pseudomonas aeruginosa*, Expression of the Gene in *Escherichia coli*, and Identification of the Gene Product in *Pseudomonas aeruginosa*

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Mutants which are defective in catabolite repression control (CRC) of multiple independently regulated catabolic pathways have been previously described. The mutations were mapped at 11 min on the *Pseudomonas aeruginosa* chromosome and designated *crc*. This report describes the cloning of a gene which restores normal CRC to these Crc$^-$ mutants in *trans*. The gene expressing this CRC activity was subcloned on a 2-kb piece of DNA. When this 2-kb fragment was placed in a plasmid behind a phage T7 promoter and transcribed by T7 RNA polymerase, a soluble protein with a molecular weight (MW) of about 30,000 was produced in *Escherichia coli*. A soluble protein of identical size was overproduced in a Crc$^-$ mutant when it contained the 2-kb fragment on a multicopy plasmid. This protein could not be detected in the mutant containing the vector without the 2-kb insert or with no plasmid. When a 0.3-kb *Acc*I fragment was removed from the *crc* gene and replaced with a kanamycin resistance cassette, the interrupted *crc* gene no longer restored CRC to the mutant, and the mutant containing the interrupted gene no longer overproduced the 30,000-MW protein. Pools of intracellular cyclic AMP and the activities of adenylate cyclase and phosphodiesterase were measured in mutant and wild-type strains with and without a plasmid containing the *crc* gene. No consistent differences between any strains were found in any case. These results provide original evidence for a 30,000-MW protein encoded by *crc*$^+$ that is required for wild-type CRC in *P. aeruginosa* and confirms earlier reports that the mode of CRC is cyclic AMP independent in this bacterium.

Pseudomonas aeruginosa, like *Escherichia coli* and other enteric bacteria, is able to preferentially metabolize one carbon source over another when both are present in the growth medium and can repress induction of multiple catabolic pathways by growth on a preferred carbon source (17). Included among the pathways found in a *Pseudomonas* spp. which are regulated by catabolite repression control (CRC) are the inducible enzymes and transport proteins of carbohydrate catabolism (42), histidase (28), amidase (36), protocatechuate dioxygenase (43), alkyl sulfatase (7), urocanase (30), and choline transport (32).

In the enteric bacteria, growth on glucose results in repression of other catabolic pathways, but in *P. aeruginosa* and other *Pseudomonas* species, intermediates of the tricarboxylic acid cycle cause repression. CRC has been extensively studied in *E. coli* (27, 35, 40, 41), and the mechanism of regulation is known to involve a protein (CAP) which, when bound to cyclic AMP (cAMP), interacts with promoter regions to facilitate the binding of RNA polymerase, thereby initiating transcription. In the presence of glucose, the cAMP concentration 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:sugar phosphotransferase system (PTS) (29, 31). Both genetic and biochemical evidence support the current theory that cAMP levels are regulated by the interaction of enzyme III^{Glc} (the PTS component which is responsible for the phosphorylation of glucose-specific enzyme II) with adenylate cyclase (31). Although most genes appear to be positively regulated by the CAP-cAMP complex, there are also examples of negative regulation by this complex (24, 25). In *P. aeruginosa* and *Pseudomonas putida*, the mechanism of CRC elicited by organic acids is not known, but several studies indicate that cAMP is not involved (28, 34). This is consistent with the differences between *P. aeruginosa* and *E. coli* both in mechanism of glucose transport and in structure of the PTS components (4, 5, 17, 37).

In this paper, we report the cloning of a gene involved in CRC in *P. aeruginosa* and its subcloning to a 2-kb piece of DNA. The product of this *crc* gene appears to be a soluble protein with a molecular weight (MW) of 30,000. We found no evidence of any involvement of cAMP in the regulation of the CRC phenomenon.

(A portion of these results are from a Ph.D. dissertation submitted by J. A. Wolff to the graduate faculty of Virginia Commonwealth University, Richmond, 1986.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, phage, and plasmids used in this study are listed in Table 1. Basal salts medium (BSM) contained 50 mM K_2PO_4 (pH 7), 15 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 2 μM $FeSO_4$. Carbon sources were used at a final concentration of 20 mM unless otherwise indicated. Amino acid supplements were used at 0.5 mM. For *pyrE* mutants, uracil was added to a final

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TABLE 1. Strains, plasmids, and phage used

Strain[a]	Relevant characteristics	Source or reference[b]
P. aeruginosa		
PAO1	Prototroph	13
PAO6093	*met-9011 amiE200 strA thr-6093*::Tn*5*-751 Km^r Tp^r	D. Haas
PAO8005	*crc-5 rec-102*	This study
PAO8007	*crc-20 rec-102*	This study
PAOA50N	*plcA*	9
PRP705	*crc-5*	42
PRP707	*argB18 rec-102*	This study
PRP708	*pyrE rec-102*	This study
PRP709	*crc-5 rec-102*	This study
PRP720	*crc-20*	42
PT066	*his-4 lys-12 ilv-1118 trp-6 pro-82 rec-102*	10
E. coli		
HB101	*recA13 hsdS20 ara-14 proA2 leuB6 thi-1 lacY1 mcrB mrr galK2 rpsL20 mtl-1 xyl-5 supE44*	20
DH1	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA*	20
DH5α	Φ80d*lacZ*ΔM15Δ(*lacZYA-araF*)U169 in DH1	BRL
Plasmids		
pTZ18R	α-Peptide *lacZ* Amp^r, pBR322 *ori*, f1 *ori*	Pharmacia
pGP1-2	T7 RNA polymerase	39
pT7-6	T7 promoter	39
pT7-5	T7 promoter	39
pHC79	Cb^r Tc^r *cos*	12
pRO1614	*oriV* in pBR322	26
pRK2013	Km^r *tra*$^+$	6
pPZ101	Cb^r Tc^r *cos oriV*	This study
pPZ102	*argB*$^+$ *pyrE*$^+$ *crc*$^+$ in pPZ101	This study
pPZ103	*argB*$^+$ *pyrE*$^+$ *crc*$^+$ in pPZ101	This study
pPZ104	*argB*$^+$ *pyrE*$^+$ *crc*$^+$ in pPZ101	This study
pPZ115	*argB*$^+$ *pyrE*$^+$ *crc*$^+$ *plcA*$^+$ in pPZ101	This study
pPZ343	*oriV* in pTZ18R	This study
pPZ353	*crc*$^+$ in pTZ18R	This study
pPZ354	*crc*$^+$ in pPZ343	This study
pPZ403	Δ*crc*::Km^r in pPZ343	This study
pPZ360	*crc*$^+$ in pT7-6	This study
pPZ362	*crc*$^+$ in pT7-5	This study
Phage E79*tv-2*		23, 38

[a] Strains with the PRP prefix were derived from PAO1. All *P. aeruginosa* chromosomal gene designations are according to Holloway and Zhang (14). All *E. coli* chromosomal gene designations are according to Bachmann (1).
[b] BRL, BRL Life Technologies Inc., Gaithersburg, Md.; Pharmacia, Pharmacia LKB Biotechnologies, Piscataway, N.J.

concentration of 1 mM. All other growth was on rich Luria-Bertani (LB) medium (22). Antibiotics were used at the following concentrations: tetracycline, 100 μg/ml for *P. aeruginosa* and 10 μg/ml for *E. coli*; carbenicillin, 500 μg/ml for *P. aeruginosa*; kanamycin, 500 μg/ml for *P. aeruginosa* and 25 to 75 μg/ml for *E. coli*; trimethoprim, 500 μg/ml for *P. aeruginosa*; ampicillin, 50 to 100 μg/ml for *E. coli*. When plasmid-containing cells were induced by growth for 3 to 4 h in liquid BSM prior to enzyme assays, carbenicillin was not added, since it slowed the growth rate significantly. Cells were grown at 37°C, and growth was measured in a Klett-Summerson colorimeter with a no. 66 filter.

Plasmid isolation, mapping, and subcloning. All procedures were essentially as described in Maniatis et al. (20). Plasmids derived from pTZ18R were propagated in *E. coli* DH5α (Table 1).

Construction of *rec-102* strains. The *rec-102* mutation of PT066 was introduced into other strains by cotransduction with *thr*$^+$ using phage E79*tv-2*. Phage propagated on PTO66 (*thr*$^+$ *rec-102*) were used to transduce *thr* mutant recipient strains; *thr*$^+$ transductants were selected and scored for coinheritance of the *rec-102* allele (unpublished data).

Isolation of PAO1 chromosomal DNA and construction of PAO1 clone bank. Chromosomal DNA was isolated by the procedure of Marmur (21). DNA fragments were generated by partial digestion with *Sau*3A, layered on a 10 to 45% sucrose gradient, and centrifuged for 24 h at 25,000 × *g*. Fractions were collected, and those containing 35- to 45-kb DNA fragments were pooled, dialyzed, and concentrated by using an Amicon Centricon-30. Cosmid vector pPZ101 (0.5 μg), which had been digested with *Bam*HI and treated with phosphatase, was ligated with 1 μg of chromosomal *Sau*3A restriction fragments in a total volume of 20 μl. The ligated DNA was packaged by using commercially obtained lambda packaging extracts according to the supplier (Amersham Corp., Arlington Heights, Ill.) and transduced into *E. coli* DH1.

Transfer of recombinant plasmids. A triparental mating system was used to mobilize the PAO1 library from *E. coli* to *P. aeruginosa*. The donor (*E. coli* DH1 containing the library) was grown to 50 Klett units, and the recipient (*P. aeruginosa* auxotroph) and helper (*E. coli* HB101 carrying the helper plasmid pRK2013) were grown to about 300 Klett units. These were mixed (1:1:4), and 0.6 ml of the mating mixture was filtered onto a 0.45-μm-pore-size filter (Millipore HAWP) and washed with 0.5 ml of LB medium. The filter was transferred to an LB plate and incubated overnight at 37°C. Cells were rinsed from the filter and suspended in BSM, and the entire suspension was spread onto selective BSM plates containing carbenicillin and incubated at 37°C.

Other plasmids were transformed into *E. coli* strains made competent by the $CaCl_2$ procedure (20). *P. aeruginosa* strains were made competent by a similar procedure but with $MgCl_2$ substituted for $CaCl_2$.

T7 expression. Expression of the *crc* gene in *E. coli* was performed by using the plasmids (pT7-5, pT7-6, and pGP1-2) and the methods of Tabor and Richardson (39).

Cell fractionation. Cell extracts for cAMP phosphodiesterase and adenylate cyclase assays were made by suspending washed cells in 0.1 M Tris (pH 8), disrupting the cells by sonic oscillation, and centrifuging them at 105,000 × *g* for 2 h at 4°C (15).

Extracts for glucose-6-phosphate dehydrogenase assays were made by suspending cells in 5 mM Tris (pH 8) containing 5 mM EDTA and 200 μg of lysozyme per ml. This cell suspension was incubated for 5 min at room temperature, $MgCl_2$ was added to a final concentration of 15 mM, DNase was added to 20 μg/ml, and the suspension was vortexed until it was no longer viscous. Supernatant fractions were collected after centrifugation at 35,000 × *g* for 1 h.

To isolate various cellular fractions prior to sodium dodecyl sulfate (SDS)-gel electrophoresis, whole cells were suspended in 100 mM Tris (pH 7.4) with 5 mM EDTA. This suspension was passed through a French pressure cell, $MgCl_2$ was added to 15 mM, DNase was added to 200 μg/ml, and the crude extract was centrifuged at 3,600 × *g* for 15 min to remove whole cells. The supernatant was centrifuged at 229,000 × *g* for 30 min, and the resulting supernatant was

referred to as the soluble fraction. The pellet (cell envelope) was washed in 25 mM Tris (pH 7.4) with 10 mM $MgCl_2$ and resuspended in the same buffer. To further fractionate the cell envelope, Triton X-100 was added to 2%, and the envelope suspension was incubated for 15 min at room temperature. After centrifugation at 229,000 × *g* for 30 min, Triton-soluble and Triton-insoluble fractions were obtained.

Enzyme assays. Adenylate cyclase (EC 4.6.1.1) was measured in cell extracts by the conversion of [^{3}H]ATP to [^{3}H]cAMP (34). After the purification of radiolabeled cAMP by paper chromatography, the cAMP was located by UV illumination, and spots were cut out and quantitated by counting in a liquid scintillation counter.

3′,5′-cAMP phosphodiesterase (EC 3.1.4.17) activity was determined from the conversion of 3′,5′-[^{3}H]cAMP to 5′-[^{3}H]AMP by cell extracts followed by the conversion of 5′-[^{3}H]AMP to [^{3}H]adenosine by snake venom (34). Any [^{3}H]cAMP not converted to [^{3}H]adenosine was bound to an anion-exchange resin and removed by centrifugation, and the radiolabeled adenosine remaining in the supernatant was quantitated by counting in a liquid scintillation counter.

Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically by monitoring the increase in NADPH concentration as previously described (15). The enzyme was induced by incubating cells in liquid BSM for 3 h in the presence of 20 mM mannitol. Activity is expressed as milli-international units per milligram of extract protein.

Amidase (EC 3.4.1.5) was measured colorimetrically in whole-cell suspensions by following the production of acetohydroxymate as previously described (3). The enzyme was induced by growth for 3 to 4 h in the presence of 20 mM lactamide or as described in the figure legends. Activity is expressed as milli-international units per milligram of total cell protein.

Other assays. To measure mannitol uptake and incorporation, cells growing on succinate BSM were inoculated into fresh medium containing 40 mM succinate plus 5 mM [^{14}C]mannitol (0.5 μCi/mmol) and incubated at 37°C for 3 h. Cell samples were collected on a 0.45-μm-pore-size HAWP filter, and the filters were washed and counted in a liquid scintillation counter. Activity is expressed as nanomoles of mannitol taken up per milligram of total cell protein. The intracellular cAMP concentration was quantitated by using the displacement of [^{3}H]cAMP from cAMP-binding protein as previously described (34). Protein was quantitated by the method of Lowry et al. (19).

Analysis of proteins by SDS-polyacrylamide gel electrophoresis. Samples to be analyzed on SDS-polyacrylamide gels were solubilized in sample buffer and heated to 100°C for 5 min prior to electrophoresis, as described by Laemmli (16). Gels were stained with Coomassie blue or dried and placed against Kodak XAR-5 film overnight for autoradiography.

RESULTS

Cosmid cloning of *crc*. In order to efficiently clone large DNA inserts, the cosmid vector pPZ101 was constructed. The broad-host-range replicator (*oriV*) was cut from pRO1614 on a 1.8-kb *Pst*I fragment and inserted into the *Pst*I site of the cosmid pHC79 (Fig. 1). The resulting cosmid, pPZ101, was 8.2 kb in size, retained the β-lactamase function, and allowed the efficient cloning of *P. aeruginosa* DNA up to 44 kb in size. A library of *P. aeruginosa* chromosomal DNA was constructed in pPZ101 as described in Materials and Methods. The cosmid clone bank was mobilized by triparental mating from the library in *E. coli* DH1 to *P.*

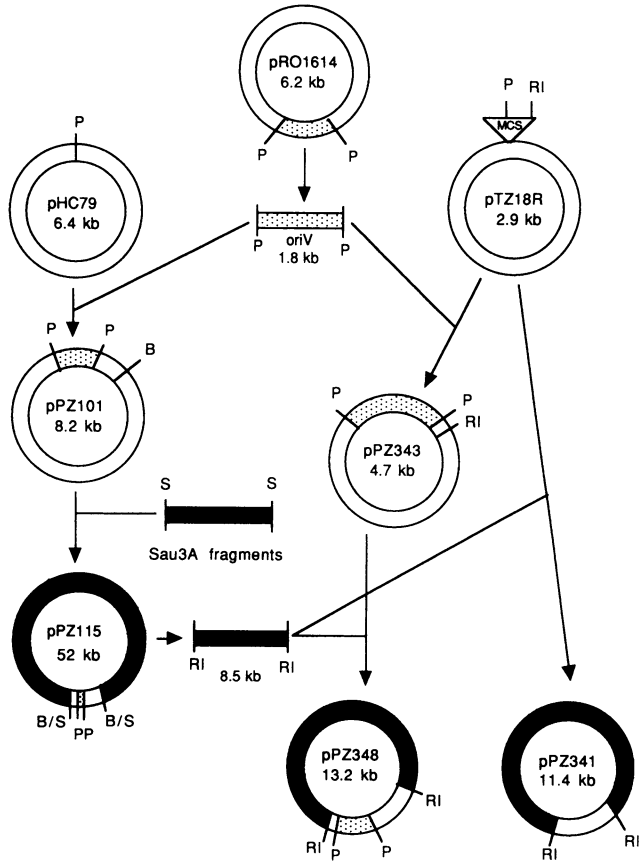

FIG. 1. Cloning of *crc* gene in pPZ101 and construction of subclones. The broad-host-range replicator (*oriV*) was cut from pRO1614 as a 1.8-kb *Pst*I fragment and inserted into the *Pst*I site of cosmid pHC79 to form pPZ101. *Sau*3A-digested *P. aeruginosa* DNA was cloned into pPZ101, resulting in pPZ115 which contains the *argB*$^+$, *pyrE*$^+$, *plcA*$^+$, and *crc*$^+$ genes. Vector pPZ343 was constructed from pTZ18R in the same manner as pPZ101. An 8.5-kb *Eco*RI fragment containing the *crc* gene was cut from pPZ115 and inserted into pTZ18R and pPZ343 to form pPZ341 and pPZ348, respectively. Further subcloning from pPZ341 was carried out with these two smaller vectors. Stippled areas represent the *oriV* regions, and shaded areas represent *P. aeruginosa* DNA. B, *Bam*HI; RI, *Eco*RI; P, *Pst*I; S, *Sau*3A; S/B, ligation of *Sau*3A fragments into a *Bam*HI site; MCS, multiple cloning site.

aeruginosa PRP707, a recombination-deficient strain derived from PAO303 (*argB18*). Since *crc* had previously been mapped close to *argB* (42), transconjugants were selected simultaneously for growth in the absence of arginine and for carbenicillin resistance. After purification of colonies on selective medium, an agar plate containing 50 independently isolated, purified colonies was washed with BSM, and the pooled cell suspension was grown under selective conditions in succinate-BSM with carbenicillin. Plasmid DNA was extracted from this pooled culture and purified on a CsCl gradient. The pool of presumptive *argB*$^+$ recombinant plasmids was used to transform *P. aeruginosa* strains carrying *argB* and other mutations in the 11-min region of the PAO chromosome (*pyrE* and *plcA*). Transformants were selected for inheritance of carbenicillin resistance and scored for the unselected alleles *argB*$^+$, *plcA*$^+$, *pyrE*$^+$, and *crc*$^+$ (Table 2). Uptake of ^{14}C-labeled mannitol on succinate-mannitol plates (42) was used to score the Crc$^+$ phenotype. Carbenicillin-

TABLE 2. Ordering of mutations by frequency of allelic coinheritance in carbenicillin-resistant transformants

Strain complemented[a]	Plasmid	Coinheritance	
		No. of unselected alleles/total no.	Frequency (%)
PRP707[b] (*argB18*)	pPZ101	0/5	0
	argB$^+$ pool	48/53	91
PRP708[b] (*pyrE*)	pPZ101	0/5	0
	argB$^+$ pool	58/108	54
PRP709[c] (*crc-5*)	pPZ101	0/12	0
	argB$^+$ pool	23/158	15
PAOA50N[d] (*plcA*)	pPZ101	0/5	0
	argB$^+$ pool	6/69	9

[a] Strains were transformed with vector pPZ101 or with plasmids from a pool of 50 transconjugants selected for complementation of *argB18* (see Materials and Methods).

[b] Scored by growth on unsupplemented BSM containing 20 mM succinate plus carbenicillin.

[c] Scored by autoradiography for uptake of [^{14}C]mannitol on BSM plates containing 50 mM succinate, 10 mM [^{14}C]mannitol, and carbenicillin as previously described (42).

[d] Scored by production of extracellular phospholipase C as previously described (2).

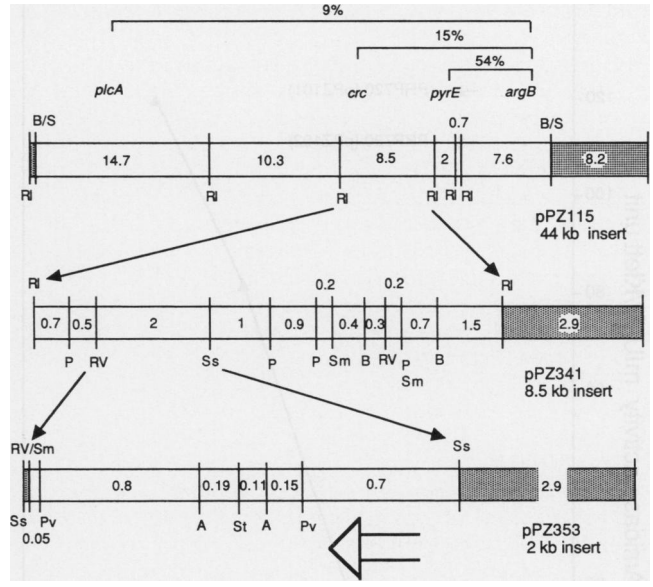

FIG. 2. Restriction map of pertinent Crc$^+$ clones. pPZ115 is the original cosmid clone containing the *argB*$^+$, *pyrE*$^+$, *plcA*$^+$, and *crc*$^+$ alleles. Their relative locations are indicated as cotransformation frequencies from the pooled *argB*$^+$ Cb^r cosmid clones (Table 2). pPZ341 contains an 8.5-kb *Eco*RI fragment from pPZ115 cloned into pTZ18R. This 8.5-kb fragment was reduced in size through several intermediate subclones to form pPZ353. The large arrow below pPZ353 indicates direction of transcription of the *crc* gene as determined from the T7 expression experiments. The shaded areas represent vector DNA, and the clear areas represent *P. aeruginosa* DNA. These maps are drawn approximately to scale except for the vector region of pPZ353 (note the break). B, *Bam*HI; S/B, ligation of *Sau*3A fragments into a *Bam*HI site; RI, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; RV, *Eco*RV; Sm, *Sma*I; RV/Sm, ligation of *Eco*RV end into a *Sma*I site; Ss, *Sst*I; A, *Acc*I.

resistant transformants exhibited a 91% coinheritance of the unselected *argB* allele (a portion of the original large cosmids apparently spontaneously deleted the *argB*$^+$ locus during selection on carbenicillin). The frequency of coinheritance of carbenicillin resistance with *pyrE* fell to 54%, with *crc* it fell to 15%, and with *plcA* it fell to 9% (Fig. 2). Four different *crc*$^+$ plasmids (pPZ102, pPZ103, pPZ104, and pPZ115) were purified and used to demonstrate linkage of the plasmid-encoded alleles (data not shown). Plasmids pPZ102, pPZ103, and pPZ104 complemented the *argB*, *pyrE*, and *crc* mutations but not the *plcA* mutation; however, pPZ115 complemented all four mutations (Fig. 2). Electrophoretic analysis of *Bam*HI-cut DNA prepared from these plasmids showed that each of these plasmids was different (data not shown). See Fig. 1 for a summary of all constructions.

Restoration of Crc$^+$ phenotype to PRP720 transformed with pPZ102. Since plasmids carrying the *crc* gene could be used to restore repression of mannitol uptake by succinate (i.e., Crc$^+$) in PRP705 (*crc-5*), these same plasmids should also restore repression of amidase activity. To test this, PRP720 (*crc-20*) was transformed with pPZ102 (*crc*$^+$) and pPZ101 (vector), and both transformants were induced for amidase by the addition of lactamide during exponential growth in succinate-BSM. Figure 3 shows that the presence of pPZ102 in PRP720 restored normal CRC. This is illustrated by an eightfold-higher expression of amidase activity in PRP720 with the vector than in PRP720 with pPZ102 (*crc*$^+$).

Subcloning the *crc* gene. Two pUC-derived vectors, pTZ18R and pPZ343 (pTZ18R containing the *oriV* from pRO1614 inserted into the multiple cloning site; Fig. 1), were used for subcloning the *crc* gene. pTZ18R will replicate only in *E. coli*, whereas pPZ343 will replicate in *E. coli* and *P. aeruginosa*. pPZ115 (*arg*$^+$ *pyr*$^+$ *plc*$^+$ *crc*$^+$) was digested with *Eco*RI (Fig. 2), and fragments were ligated into the *Eco*RI site of pPZ343. These subclones were transformed into the recombination-deficient Crc$^-$ mutants PAO8005 (*crc-5* derived from PRP705) and PAO8007 (*crc-20* derived from PRP720), and complementation of these *crc* mutations was measured as described in the following paragraph. The 8.5-kb *Eco*RI fragment from pPZ115 was found to complement both mutants. This 8.5-kb fragment was also inserted into pTZ18R to form pPZ341. Further subclones were derived from pPZ341. To allow for testing of these subclones, purified *P. aeruginosa* DNA fragments were also ligated into pPZ343, or, if the fragment lacked a *Pst*I site, the *oriV* was ligated directly into the *Pst*I site of the multiple cloning region. In the latter case, the new construct was selected in the *P. aeruginosa* mutant strain. Figure 1 and 2 illustrate some of these constructions. Currently, we are using a new vector which contains the *oriV* inserted into the *Aat*II site of pGEM-3zf. This permits the use of *Pst*I for cloning into this vector and retains the use of β-galactosidase for selection of vectors containing DNA inserts.

Effects of subcloned *crc* gene on expression of multiple regulatory units in Crc$^-$ mutants. To test for CRC activity, each of the subclones was used to transform the *rec-102* derivatives of *crc* mutants, PAO8005 and PAO8007. These mutants were chosen for complementation tests because they were isolated by using different chemical mutagens and different enrichment procedures prior to selection (42). To determine which subclones contained the *crc* gene, the plasmid-containing mutant strains were tested for mannitol uptake and incorporation. The smallest subclone which restored CRC of the mannitol pathway to these mutants was pPZ354 (the 2-kb fragment from pPZ353 plus *oriV*; Fig. 2). This subclone was tested further for its ability to restore CRC of glucose-6-phosphate dehydrogenase and amidase in these same mutants (Table 3). The levels of all three inde-

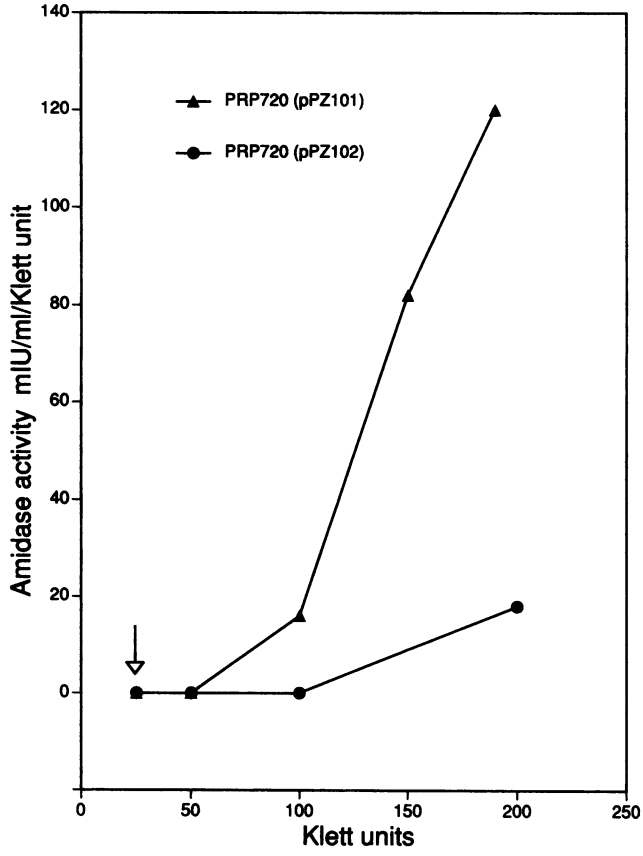

FIG. 3. Restoration of CRC of expression of amidase activity in Crc$^-$ mutant PRP720 transformed with pPZ102 containing the *crc*$^+$ gene. Cultures of PRP720 containing either the vector (pPZ101) or the cloned *crc*$^+$ gene (pPZ102) were grown in succinate-BSM and diluted into fresh medium, and 20 mM lactamide was added at the time indicated by the arrow. The cultures were sampled before and after the addition of inducer, and amidase activity was assayed at the times indicated, as described in Materials and Methods. Each value varied by less than 10% from the mean of five or more determinations.

pendently regulated enzymes were compared with enzyme levels found in the mutants containing vector pPZ343 or no plasmid. In cultures grown in succinate, where repression is maximal, all three activities were similar in the wild-type strain and the mutant strains which contained the 2-kb subclone of the *crc* gene (pPZ354) and were much lower than those values for the Crc$^-$ mutants with no plasmid or with the vector alone. In cultures grown in lactate, where repression is minimal or absent, the activities in the mutant containing the *crc* gene were again approximately equal to or lower than the wild-type activity.

Construction of defective *crc* gene. To locate the *crc* gene on the 2-kb fragment and to provide a defective *crc* gene for further experiments, a deletion was constructed in the center of this fragment. After removal of the *Acc*I site from the multiple cloning site of pPZ353, a 0.3-kb *Acc*I fragment was removed from the center of the 2-kb subclone, and a kanamycin resistance cassette (Pharmacia LKB Biotechnology, Piscataway, N.J.) was inserted in place of the *Acc*I fragment (Fig. 4). The *oriV* was inserted in the *Sst*I site, and this plasmid, pPZ403, was transformed into the Crc$^-$ mutant PAO8007. Table 3 shows that mannitol uptake and incorporation and amidase activity in PAO8007 containing pPZ403 remained the same as in PAO8007 with no plasmid or with only the vector and were much higher than activities in PAO8007 containing pPZ354 (*crc*$^+$).

Expression of *crc* gene in *E. coli*. The vectors pT7-7 and pT7-6 allow for the insertion of a gene in opposite orientations behind a phage T7 promoter. When used in conjunction with plasmid pGP1-2, which carries the T7 RNA polymerase, genes can be expressed in *E. coli*, often in very large quantities (39). The 2-kb fragment containing the *crc* gene was cut from pPZ353 with *Eco*RI and *Hin*dIII and inserted in the vectors pT7-5 and pT7-6 to form pPZ362 and pPZ360, respectively. Each plasmid was then transformed into *E. coli* DH5α along with a second plasmid, pGP1-2. Following the protocol of Tabor and Richardson (39), the host cell polymerase was inhibited by the addition of rifampin, the T7 polymerase was induced by raising the temperature to 42°C, and, after 20 min, [^{35}S]methionine was added to detect

TABLE 3. [^{14}C]mannitol uptake and amidase and glucose-6-phosphate dehydrogenase activities in Crc$^-$ mutants containing the *crc* gene, the defective (Δ*crc*::Km^r) *crc* gene, or the plasmid vector[a]

Strain/plasmid	[^{14}C]mannitol uptake and incorporation (nmol/mg of cell protein)		Amidase activity (mIU/mg of cell protein)		Glucose-6-phosphate dehydrogenase activity (mIU/mg of extract protein)	
	Succinate	Lactate	Succinate	Lactate	Succinate	Lactate
PAO1 (wild type)	115	1,063	0.11	1.51	3	121
PAO8007 (*crc-20*)	604	1,065	7.08	6.99	14	203
PAO8007/pPZ343 (*crc-20*/vector)	591	ND	6.19	ND	33	ND
PAO8007/pPZ354 (*crc-20*/*crc*$^+$)	32	363	0.29	1.66	6	66
PAO8007/pPZ403 (*crc-20*/Δ*crc*::Km^r)	681	ND	9.6	ND	ND	ND
PAO1 (wild type)	155	ND	0.23	ND	3	ND
PAO8005 (*crc-5*)	971	ND	0.88	ND	26	ND
PAO8005/pPZ343 (*crc-5*/vector)	1,063	ND	1.07	ND	17	ND
PAO8005/pPZ354 (*crc-5*/*crc*$^+$)	84	ND	0.34	ND	8	ND

[a] Each value is the average of two or more experiments. Activities were induced by transferring cells grown overnight on succinate-BSM plates or lactate-BSM plates (with carbenicillin for plasmid-containing strains) into liquid BSM containing 40 mM succinate or 40 mM lactate plus inducer. The liquid culture was incubated at 37°C for 3 to 4 h, and enzyme activity or uptake and incorporation were measured as described in Materials and Methods. ND, not done.

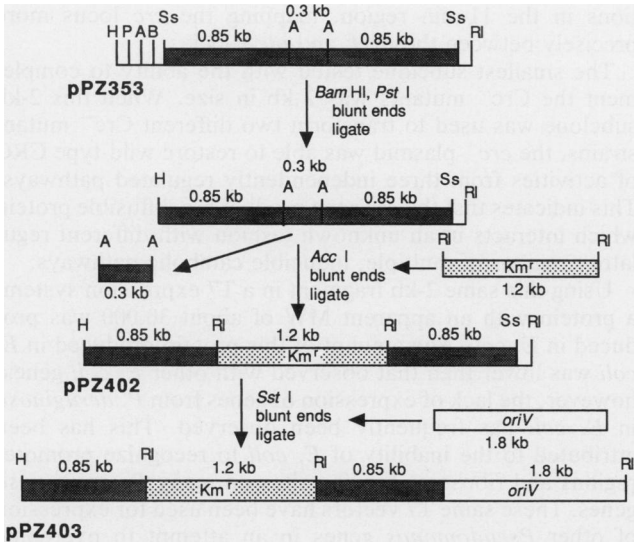

FIG. 4. Construction of defective *crc* gene. The *Acc*I site was removed from the multiple cloning site of pPZ353 by digestion with *Bam*HI and *Pst*I, removal of unpaired 3′ tails, and ligation of the blunt ends. During this process, the *Sst*I site was also lost. The resulting plasmid was cut with *Acc*I, precipitated to remove the 0.3-kb *Acc*I fragment, and mixed with a 1.2-kb kanamycin resistance cassette. Unpaired 5′ ends were filled, and the two pieces were ligated. The resulting plasmid, pPZ402, contained the kanamycin cassette inserted at the *Acc*I deletion and restored *Eco*RI sites at either end of the cassette. The *oriV* was inserted into pPZ402 at the blunted *Sst*I site to produce pPZ403. H, *Hin*dIII. Other abbreviations are as in Fig. 2. Dark filled area indicates *P. aeruginosa* DNA.

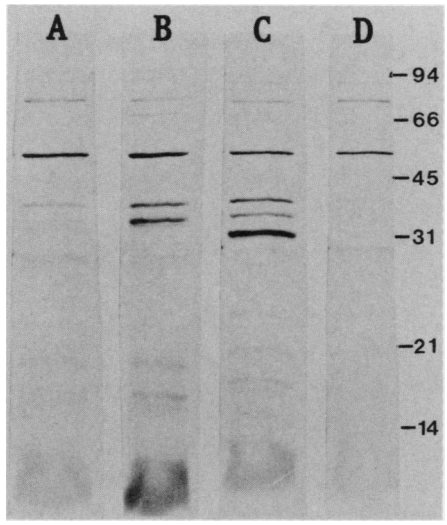

FIG. 5. Autoradiogram showing expression of the *crc*$^+$ gene product in *E. coli*. The 2-kb fragment of *P. aeruginosa* DNA containing the *crc* gene was cut from pPZ353 and inserted behind a T7 promoter in opposite orientations, producing pPZ360 and pPZ362. When these plasmids were used in *E. coli*, the protein product of T7-specific mRNA was determined as described in Results. After inhibition of the host RNA polymerase and induction of the T7 polymerase, the proteins produced from the T7-specific message were labeled with [^{35}S]methionine. These cells were solubilized and electrophoresed on SDS-polyacrylamide gels, and labeled proteins were identified by autoradiography. Lane B, labeled proteins produced from pPZ360; lane C, labeled proteins produced from pPZ362; lanes A and D, proteins produced from the vectors pT7-6 and pT7-5, respectively, containing no *P. aeruginosa* DNA. The locations of the MW standards are indicated on the right.

protein synthesized from T7-specific mRNA. The use of rifampin along with the production of large amounts of T7 RNA polymerase provides nearly exclusive expression of genes contained in pT7-5 and pT7-6, provided they are in the correct orientation with respect to the T7 promoter. The cells from four separate cultures containing pGP1-2 with pPZ360, pPZ362, pT7-5, or pT7-6 were solubilized, and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Autoradiograms showed that a unique protein with an apparent MW of about 30,000 was found only in cells containing pPZ362 (Fig. 5). When soluble and envelope fractions were analyzed separately, the 30,000-MW protein (30K protein) was found in the soluble fraction (not shown). The amount of this 30K protein which was produced by *E. coli*, however, appeared to be less than that observed by others using the same methods for different *E. coli* genes. To determine whether the relatively low level of protein detected was due to its instability in *E. coli*, pulse-chase experiments were performed. The amount of labeled protein detected after a 5-min incubation with [^{35}S]methionine remained constant for at least 1 h after continued incubation of the cells with a 1,000-fold excess of unlabeled methionine (data not shown).

In a similar experiment, unlabeled cells with pPZ362 or pPZ360 were incubated at 42°C in LB medium containing rifampin for a longer time (2 h). When the soluble proteins were analyzed on SDS gels by Coomassie blue staining, the 30K protein could be identified among the soluble proteins. As with the labeled protein, the stained protein was visible only in cells containing pPZ362, but once again, amounts of this protein were lower than expected (data not shown). The direction of transcription of the 30K protein from pPZ362 is indicated on the map of pPZ353 in Fig. 2.

Identification of *crc* gene product in *P. aeruginosa*. To determine whether this 30K protein produced in *E. coli* from pPZ362 could also be identified in *P. aeruginosa*, we compared the proteins produced in the wild type and in a Crc$^-$ mutant strain with and without pPZ354 (*crc*$^+$). Cells were grown in succinate-BSM for several generations, broken in a French pressure cell, and separated into soluble and envelope fractions as described in Materials and Methods. Cell fractions were analyzed on SDS-polyacrylamide gels stained with Coomassie blue. In the soluble fraction of PAO8007 (*crc-20*) containing pPZ354, the most intensely stained protein band had an apparent MW of about 30,000 (Fig. 6). This intense protein band did not appear in mutants containing the vector (pPZ343) or the defective *crc* gene (pPZ304), nor was it visible in the wild type (PAO1) (Fig. 6). It was not observed in the Triton-soluble or Triton-insoluble envelope fractions from any strain (not shown).

Roles of cAMP, adenylate cyclase, and 3′,5′-nucleotide phosphodiesterase activities in CRC. cAMP plays an important role in CRC in *E. coli*, with cAMP pools and adenylate cyclase levels varying with the concentration of glucose in the growth medium (27). To look for a similar regulatory role of cAMP in *P. aeruginosa*, the intracellular pools of cAMP and the activities of adenylate cyclase and cAMP phosphodiesterase were examined in mutant and wild-type cultures grown under repressing and nonrepressing conditions. The intracellular cAMP pools in the wild type (PAO1/pPZ101) were relatively unchanged regardless of the carbon source,

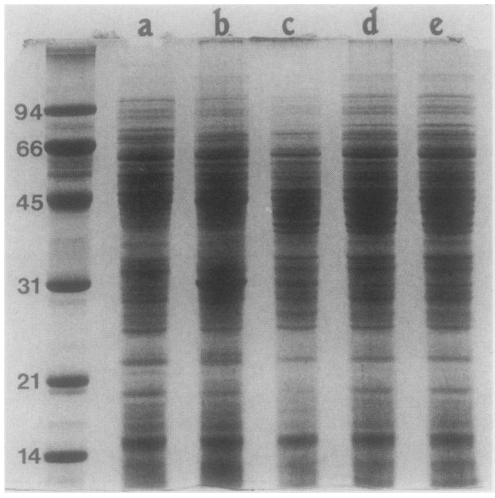

FIG. 6. SDS-polyacrylamide gel analysis of soluble proteins in *P. aeruginosa* Crc$^-$ and wild-type strains containing various plasmids. Cultures were grown for several generations in succinate-BSM and fractionated as described in Materials and Methods. Soluble proteins were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue. Proteins were from PAO1 (lane a), PAO8007 (*crc-20*) (lane e), and PAO8007 with plasmids pPZ354 (*crc*$^+$) (lane b), pPZ403 (Δ*crc*::Km^r) (lane c), and pPZ343 (vector) (lane d). The locations of the MW standards are indicated on the left.

and there was neither a loss nor a restoration of cAMP in the mutant strains regardless of the carbon source or plasmid present (Table 4). This was also the case for the activities of adenylate cyclase and cAMP phosphodiesterase (Table 4). All measurements were consistent with those previously reported (34) for PAO1.

DISCUSSION

In a previous report (42), we described the isolation and characterization of mutants which were defective in CRC of the mannitol and glucose active transport systems, glucose-6-phosphate dehydrogenase, Entner-Doudoroff dehydratase and aldolase, glucokinase, and amidase. All mutations mapped near *argB18* in the 11-min region of the *P. aeruginosa* chromosome, and the locus for this mutation was designated *crc*. The isolation of these mutants facilitated the cloning of the *crc* gene, allowing us to use complementation of these Crc$^-$ mutants as an assay for the *crc* gene. We also used a pool of Arg$^+$ subclones to complement other mutations in the 11-min region, mapping the *crc* locus more precisely between the *pyrE* and *plcA* loci.

The smallest subclone tested with the ability to complement the Crc$^-$ mutants was 2 kb in size. When this 2-kb subclone was used to transform two different Crc$^-$ mutant strains, the *crc*$^+$ plasmid was able to restore wild-type CRC of activities from three independently regulated pathways. This indicates that the *crc* gene product is a diffusible protein which interacts in an unknown fashion with different regulatory regions of multiple, inducible catabolic pathways.

Using this same 2-kb fragment in a T7 expression system, a protein with an apparent MW of about 30,000 was produced in *E. coli*. The amount of this protein produced in *E. coli* was lower than that observed with other *E. coli* genes; however, the lack of expression of genes from *P. aeruginosa* in *E. coli* has frequently been observed. This has been attributed to the inability of *E. coli* to recognize promoter regions and ribosome-binding sites on many *P. aeruginosa* genes. These same T7 vectors have been used for expression of other *Pseudomonas* genes in an attempt to overcome these promoter differences. Some genes have been expressed well in *E. coli* with only the substitution of the T7 promoter (8, 18, 33), while others are expressed poorly or not at all without the further addition of a translation initiation site recognized by *E. coli* (11). We have not yet tested the extent of *crc* gene expression in pT7-7, which contains a T7 promoter plus a T7 ribosome-binding site followed by an ATG (39).

When the *crc* gene was present in a *P. aeruginosa* Crc$^-$ mutant on a multicopy plasmid, overproduction of a protein with a MW of about 30,000 occurred. This protein appeared as the major soluble protein in this strain, but only when the plasmid was present. It could not be visualized on SDS-polyacrylamide gels of wild-type extracts without the plasmid. Thus, this protein appears normally to be present in very small amounts but is overproduced when carried on a multicopy plasmid. These data, together with the identification of an electrophoretically identical protein produced in *E. coli* from the same 2-kb DNA fragment, support our conclusion that this *crc* gene codes for a soluble protein with a monomer MW of 30,000. Although we have not yet ruled out a periplasmic location for this protein, its location in the cytoplasm would be consistent with that expected for a protein which must interact with the regulatory regions of multiple operons.

No consistent differences in the activities of adenylate cyclase or phosphodiesterase or in the intracellular pools of cAMP were observed in mutant or wild-type cultures regardless of the carbon source or the presence of any plasmid.

TABLE 4. Intracellular cAMP concentration and adenylate cyclase and cAMP-phosphodiesterase activities in cells from late-log phase cultures[a]

Strain/plasmid	cAMP concn (μM)[b]		Sp act (mIU/mg of protein)[c]			
			Adenylate cyclase		cAMP-phosphodiesterase	
	Lactate	Succinate	Lactate	Succinate	Lactate	Succinate
PAO1/pPZ101	36 ± 8	33 ± 3	2.2 ± 0.2	2.1 ± 0.2	51 ± 11	29 ± 4
PRP709/pPZ101	39 ± 12	43 ± 7	1.5 ± 0.2	1.5 ± 0.3	43 ± 4	118 ± 27
PRP720/pPZ101	24 ± 2	15 ± 3	2.7 ± 1.8	2.6 ± 0.9	116 ± 39	77 ± 22
PRP720/pPZ102	31 ± 7	37 ± 23	1.8 ± 0.4	1.3 ± 0.1	27 ± 7	45 ± 9

[a] Cells were grown in BSM with succinate or lactate as the carbon source.
[b] Quantified as described in Materials and Methods.
[c] Enzyme activities in cell extracts were measured as described in Materials and Methods.

This is consistent with previous reports (28, 34) that the molecular mechanism of CRC in *P. aeruginosa* is fundamentally different from that in *E. coli* and other enteric bacteria. In view of the observation that the *crc* gene product is overproduced when the *crc* gene is present on a plasmid, a dramatic change in the activities of adenylate cyclase or phosphodiesterase or in the intracellular pools of cAMP might be expected to occur in plasmid-bearing strains if regulation of CRC in *P. aeruginosa* were related to cAMP pools. This lack of regulation of CRC by cAMP and glucose is not surprising when one considers the differences in the mechanism of glucose transport and in the nature of the PTS components between *P. aeruginosa* and the enteric bacteria. In *P. aeruginosa*, only one sugar which is transported by a PTS, i.e., fructose, has been identified (5). The components of this PTS differ from those found in the enteric bacteria in two ways. (i) The only soluble component which could be identified was an enzyme I-like protein. No equivalent of the low-MW HPr could be found. (ii) In the enteric bacteria, some PTS sugars require a third soluble protein, enzyme III. It is this enzyme III^{Glc} which is thought to regulate adenylate cyclase and, thus, cAMP levels (31). No equivalent of enzyme III could be identified in the fructose PTS (5). Moreover, it is known that the transport of glucose in *P. aeruginosa* is not carried out by a PTS but by an inducible binding-protein-dependent active-transport system (4, 17, 37). Further investigations into the structure and function of this *crc* gene product are currently in progress.

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