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Role of *Acinetobacter baylyi* Crc in Catabolite Repression of Enzymes for Aromatic Compound Catabolism▽

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Here, we describe for the first time the Crc (*c*atabolite *r*epression *c*ontrol) protein from the soil bacterium *Acinetobacter baylyi*. Expression of *A. baylyi crc* varied according to the growth conditions. A strain with a disrupted *crc* gene showed the same growth as the wild type on a number of carbon sources. Carbon catabolite repression by acetate and succinate of protocatechuate 3,4-dioxygenase, the key enzyme of protocatechuate breakdown, was strongly reduced in the *crc* strain, whereas in the wild-type strain it underwent strong catabolite repression. This strong effect was not based on transcriptional regulation because the transcription pattern of the *pca-qui* operon (encoding protocatechuate 3,4-dioxygenase) did not reflect the derepression in the absence of Crc. *pca-qui* transcript abundance was slightly increased in the *crc* strain. Lack of Crc dramatically increased the mRNA stability of the *pca-qui* transcript (up to 14-fold), whereas two other transcripts (*pobA* and *catA*) remained unaffected. *p*-Hydroxybenzoate hydroxylase activity, encoded by *pobA*, was not significantly different in the absence of Crc, as protocatechuate 3,4-dioxygenase was. It is proposed that *A. baylyi* Crc is involved in the determination of the transcript stability of the *pca-qui* operon and thereby effects catabolite repression.

The introduction of aromatic compounds into the central energy conservation pathways is accomplished in *Acinetobacter baylyi* via the β-ketoadipate pathway (25). In *Acinetobacter*, this pathway contains two parallel branches separated in terms of enzymes, as well as regulation of expression, converting the two starting compounds protocatechuate and catechol into succinyl coenzyme A and acetyl coenzyme A (21). Protocatechuate breakdown requires six catalytic steps; the respective genes (*pca* genes), together with genes for one of several funneling pathways (*qui* genes), form a large operon (the *pca-qui* operon, about 14 kbp) (10). Regulation of the expression of this operon is directed from the intergenic region located upstream of this large gene cluster. Several levels of transcriptional regulation of *pca-qui* gene expression have been described, most of which have a negative effect and therefore serve to prevent gene expression. Only one mechanism causes induction at the otherwise weak promoter upstream of *pcaI* (*pcaIp*), namely, the activity of the regulator PcaU (22). In its absence, the *pca-qui* genes are expressed at a fairly high basal level. PcaU decreases this basal expression level. In the presence of the inducer protocatechuate, PcaU brings about high induction and is thus both a repressor and an activator (47, 53). PcaU is an IclR family member and binds to a site between the *pca-qui* genes and its own gene containing three repetitions of a 10-bp DNA sequence, which are all necessary for induction (29, 36). Additional regulatory levels of higher priority can prevent induction despite the continued presence of the inducer (Fig. 1). One is a mechanism that seems to organize gene expression priorities between the two branches of the pathway (cross-regulation) (5). A second repressing mechanism, carbon catabolite repression, is observed when other, nonaromatic carbon sources are present in addition to the aromatic carbon source. The *A. baylyi pca-qui* operon is downregulated during growth by a number of organic acids; acetate and succinate in combination have the strongest effect, reducing the activity of one encoded enzyme, protocatechuate 3,4-dioxygenase, by 95% (9). This repression has also been observed for other operons involved in aromatic compound degradation in *A. baylyi* (*pob* and *van*) and has been shown to be a gene-regulatory effect (47).

Carbon catabolite repression by organic acids, as well as by carbohydrates, has been described in numerous reports for other gammaproteobacteria (8, 19, 44). Despite this, the understanding of the mechanism(s) responsible for this layer of global regulation is still limited. Several lines of evidence indicate that the mechanism is different from that of enterobacteria, as well as gram-positive bacteria, whose mechanisms are quite well understood (4, 46, 52). For example, the *Pseudomonas* homologue of the *Escherichia coli* Crp protein, Vfr, was shown not to be involved in catabolite repression in *Pseudomonas aeruginosa* (31, 50). The alarmone (p)ppGpp was shown to be involved in the exponential silencing on complex medium of the *dmp* promoter (51). Several potential regulator proteins have been related to catabolite repression in *Pseudomonas*, CyoB (13, 42), PtsN and PtsO (2, 7), and the Crc (*c*atabolite *r*epression *c*ontrol) protein.

Crc was originally discovered in *P. aeruginosa* after random mutagenesis and a search for relief of catabolite repression of

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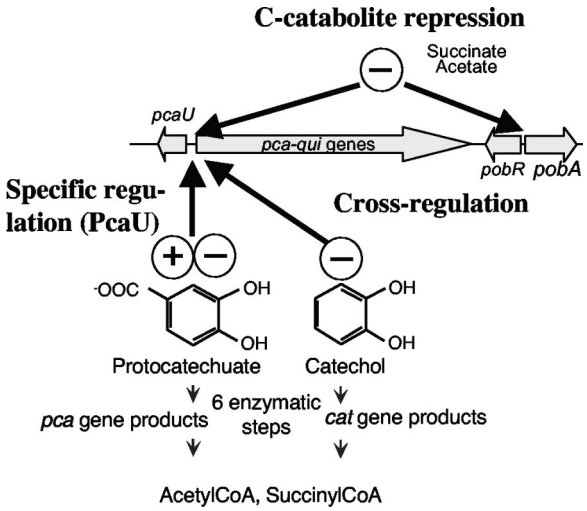

FIG. 1. Schematic overview of regulatory levels acting upon expression of the *pca-qui* operon encoding protocatechuate degradation in *A. baylyi*. CoA, coenzyme A.

amidase expression (34, 55). The *crc* mutant found displayed a pleiotropic loss of catabolite repression by succinate of multiple activities, such as mannitol and glucose transport, enzymes of sugar catabolism, and amidase. The purified Crc protein did not show any nuclease or DNA binding activity (33). Subsequently, Crc has been described in several *Pseudomonas* species as being involved in catabolite repression of the branched-chain keto acid dehydrogenase (26) and of alkane degradation (56), as well as of a number of enzymes involved in aromatic compound degradation (37). It was also related to the regulation of biofilm formation in *P. aeruginosa* (40). In-depth studies revealed the involvement of Crc, together with PtsN, in catabolite repression of toluene degradation (3) and, furthermore, in regulated Crc expression according to growth conditions (45). Two recent studies identified *Pseudomonas putida* Crc as an RNA-binding protein targeting the transcripts of the regulators AlkS and BenR. It is proposed that Crc represses translation of these RNAs, and thus, effective levels of BenR or AlkS cannot be reached (38, 39).

Despite the observation of carbon catabolite repression in many more gammaproteobacteria, attempts to unravel the molecular components and the mechanism in bacteria other than members of the *Pseudomonas* group are scarce. Here, we present the first investigation of the Crc protein of *A. baylyi*. It is shown that this Crc, like the *Pseudomonas* protein, is involved in the catabolite repression phenotype. It is also active posttranscriptionally. In contrast to its relative, *A. baylyi* Crc strongly influences transcript stability and is thus proposed to modify transcript levels.

MATERIALS AND METHODS

Growth conditions. Strains of *A. baylyi* (Table 1) were grown on mineral medium at 30°C as described previously (53). The carbon sources were used at the following final concentrations unless otherwise indicated: succinate, 10 mM; pyruvate, 20 mM; lactate, 60 mM; acetate, 15 mM; succinate plus acetate, 15 mM each; quinate, 5 mM; *p*-hydroxybenzoate, 5 mM; benzoate, 2 mM. Cultures (5 ml or 60 ml) were grown at 30°C aerobically on a rotary shaker. Cells used to inoculate growth experiments were grown under the same conditions as the main culture. For enzyme assays, 10-liter batch fermentation was used to produce sufficient biomass. The optical density was monitored at 546 nm. The mid-logarithmic growth phase was defined as the point where 50 to 80% of the maximal optical density was reached. Nutrient broth was 3 g liter beef extract^{-1}

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Acinetobacter		
ADP1	Wild type (strain BD413; ATCC 33305)	30
ADPU44	Δ*crc*-Ω; 50-bp BsgI fragment deleted from *crc* and replaced by Ω cassette from pHP45	28
ADPU47	*pcaI-luc* fusion inserted into *pcaI* (253 bp downstrem of start codon)	47
ADPU49	*pcaI-luc* fusion inserted into *pcaI*, Δ*crc*-Ω, 50-bp BsgI fragment deleted from *crc* and replaced by Ω cassette from pHP45	This work
ADPU53	*pobA-luc* transcriptional fusion inserted into *pobA*	47
ADPU54	*pobA-luc* transcriptional fusion inserted into *pobA*; Δ*crc*-Ω	This work
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	23
Plasmids		
pBluescript II SK(+)	Ap^r *lacZ*	Stratagene Europe, Amsterdam, The Netherlands; 1
pHP45Ω	Omega fragment for in vitro insertional mutagenesis; *aadA*$^+$ (Sm^r Spc^r)	43
pAC56	747-bp internal fragment of *A. baylyi crc* cloned into pBluescript II SK(+)	This work
pAC57	Δ*crc*-Ω; 50-bp BsgI fragment deleted from pAC56 and replaced by Ω cassette from pHP45Ω	This work
pZR2	2,392-bp HindIII fragment (*A. baylyi pcaK′CHG*) in pUC18	24
pZR9	5,407-bp Sau3A fragment (*A. baylyi pcaU′-pcaIJFBD′*) in pUC19	22
pZR430B	3.3-kbp SacI-HindIII fragment (*A. baylyi pobARS′*) in pUC18; multiple cloning site from pSL1180 in XhoI site of *pobA*	12
pZR504	9,964-bp BamHI-XhoI fragment (*A. baylyi pcaH′G-quiBCXA-pobSRA′*) in pUC18	16

and 5 g liter peptone^{-1}. Antibiotics for *A. baylyi* strains were used in the following concentrations: spectinomycin, 100 μg/ml; ampicillin, 150 μg/ml; streptomycin, 100 μg/ml; rifampin (rifampicin), 200 μg/ml. In the case of chromosomal integration of an antibiotic-encoding cassette, growth experiments were performed in the absence of antibiotics to ensure optimal growth conditions; after the experiment, several hundred cells were checked for the presence of the cassette by replica plating them on media containing the antibiotic. We never observed instabilities of chromosomally integrated resistance cassettes. *E. coli* strains were grown in LB medium at 37°C supplemented when appropriate with spectinomycin (100 μg/ml), ampicillin (100 μg/ml), and streptomycin (100 μg/ml).

Strain construction. For the construction of the *crc* strain *A. baylyi* ADPU44, a 747-bp *crc* internal fragment was amplified from chromosomal DNA of *A. baylyi* ADP1 using the oligonucleotides crc-ADP1-1 (5′-TCAACGTAAATGGC TTACG-3′) and crc-ADP1-2 (5′-TAATGACAGGCGCATGG-3′) (28). Blunt ends were produced by treatment with Vent DNA polymerase. The fragment was phosphorylated by applying T4 polynucleotide kinase and was ligated with the vector pBluescript II SK(+). The vector had been prepared by cleavage with SmaI and HincII and dephosphorylation using calf intestinal phosphatase. The resulting plasmid was named pAC56. The restriction endonuclease BsgI was used to delete a 50-bp fragment from the *crc* gene. Vent DNA polymerase was applied to produce blunt ends. The gap was closed by introducing the insert of plasmid pHP45Ω (containing transcriptional and translational terminators in both directions) after cleavage with SmaI. After ligation and transformation into *E. coli* DH5α, recombinant clones were selected by plating them on LB plates containing ampicillin, streptomycin, and spectinomycin. The construction was verified by restriction endonuclease analysis, and the plasmid was named pAC57. The complete insert of pAC57 was cut out of the plasmid using XbaI and PstI and used to transform *A. baylyi* ADP1. Transformants were selected for by growth on plates containing mineral medium with succinate, streptomycin, and spectinomycin. The successful integration of the modified *crc* gene into the correct chromosomal location was verified by PCR using primers hybridizing outside of the transforming DNA (crc3, 5′-ATGATACCAAAGGATAGC-3′, and crc4, 5′-TTGTGTAAGAAATTGGCG-3′) (see Fig. 3). A strain containing a *pcaIp-luc* fusion and the *crc* deletion described above was constructed and verified by introducing the insert from plasmid pAC57 into strain ADPU47 as described above. The *aad9* gene contained on the *luc* cassette in strain ADPU47 encodes resistance against spectinomycin (1 to 500 μg/ml tested), but not against streptomycin (1 to 500 μg/ml tested), whereas the *aadA*+ gene, which is part of the Ω cassette, encodes resistance against both antibiotics (up to 100 μg/ml in the case of streptomycin). Based on this difference, selection for strain ADPU49 could be done on streptomycin. Strain ADPU54 (*pobA-luc* Δ*crc*) was made from strain ADPU53 (*pobA-luc*) following the same procedure.

Transformation of *A. baylyi* with linear DNA. For transformation of *A. baylyi*, a 200-μl cell suspension of an overnight culture was transferred into 5 ml of fresh mineral medium supplied with 10 mM succinate and incubated for 2 h at 30°C to allow the cells to start growing again. To 500 μl of these cells, 0.1 to 1 μg linear DNA was added and incubated at 30°C for 3 h to overnight. One hundred microliters of the cells was spread on selective plates and incubated at 30°C. Colonies were transferred on fresh selective plates several times to ensure a pure culture.

Northern blot hybridization. For investigation of the *crc* transcript, cells were cultured on different media (nutrient broth and mineral medium with succinate, *p*-hydroxybenzoate, or pyruvate), and cells were harvested in the exponential growth phase. RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), and equal amounts (10 μg per lane) were loaded on a denaturing formaldehyde gel. Processing of the gel, blotting, probe preparation (a 461-bp BstNI-NheI fragment from plasmid pAC56), labeling with [^{32}P]dATP, hybridization, and detection were performed as described elsewhere (20). For the investigation of *pca-qui* transcript abundance, a 397-bp NsiI fragment cleaved out of plasmid pZR2 and containing parts of *pcaC* and *pcaH* was used. Analysis of transcript abundance was done by quantifying the specific signal in each lane after signal detection using a phosphorimager as described previously (9). For the half-life determinations, the following probes were applied: a 640-bp Eco47III fragment from plasmid pZR9 (*pcaI*), a 442-bp HpaI fragment from plasmid pZR504 (*quiA*), a 662-bp AflIII-PmlI fragment from plasmid pZR430B (*pobA*), and an 874-bp fragment generated by PCR applying primers catA1 (5′-GCGGACTTG AGCAAGAAGGTG-3′) and catA2 (5′-CGCTAGACGTGGACGATCAAC-3′) and chromosomal DNA from *A. baylyi* (*catA*).

Determination of enzyme activities. Previously described procedures were used for assay of protocatechuate 3,4-dioxygenase (53) and *p*-hydroxybenzoate hydroxylase (17). Each extract was assayed 5 to 10 times, and the standard deviation between individual assays was no more than 4%. The standard deviations for these enzymes between different cultures grown under the same conditions were 10 to 15%. For luciferase measurements, cells were pregrown in the same medium as in the experiment. Luciferase activity was determined throughout growth as outlined earlier (47). Data were taken from the mid-exponential growth phase. The standard deviation for data from independently grown cultures was 30%. For all enzyme assays, measurements were taken for a minimum of three individual cultures.

RESULTS

Identification of the *A. baylyi crc* gene. The deduced amino acid sequence of the *crc* gene from *P. aeruginosa* was used to identify the *A. baylyi* homologue. An open reading frame was found which displayed 40% identity in a pairwise sequence alignment with the *P. aeruginosa* Crc sequence. In the publicly accessible annotated genome sequence (http://www.genoscope.fr), this open reading frame is ACIAD3526 and was named *crc*. *A. baylyi* does not contain any other open reading frame with significant similarity to ACIAD3526. The chromosomal neighborhoods of the *crc* genes in *A. baylyi* and numerous other species (*Acinetobacter baumannii*, *P. aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas syringae*) are very similar, supporting the hypothesis that these genes are truly related (6, 41, 48, 49). They include a putative arsenate reductase downstream of *crc* and an orotate phosphoribosyltransferase (*pyrE*; pyrimidine biosynthesis) encoded upstream and transcribed in the opposite direction (Fig. 2A). A BLAST search revealed large numbers of genes encoding putative proteins with strong similarity to *A. baylyi crc*. Besides the *A. baumannii* homologue (70% identity), there are sequences from marine gamma- and betaproteobacteria with coding capacities for proteins about 45% identical to that of *A. baylyi crc* (Fig. 2B). Surprisingly, there is also a sequence from a eukaryote, the rodent malaria parasite *Plasmodium yoelii yoelii* (accession number XP_728665.1; 42% identical amino acids). Like most other sequences, this sequence was generated by genome sequencing and annotated as an exodeoxyribonuclease III. All of these related proteins are members of the large endonuclease/exonuclease/phosphatase family (Pfam entry PF03372) (14, 18).

Generation of a *crc* mutant strain of *A. baylyi*. To evaluate the function of Crc in *A. baylyi*, a *crc*-negative derivative of the wild-type organism was constructed (28). Therefore, an internal fragment (747 bp) of the *crc* gene was cloned into the vector pBSK II. A 50-bp fragment was deleted from the gene and replaced by the Ω cassette from plasmid pHP45Ω designed to interrupt transcription, as well as translation, in either direction (Fig. 3). The insert was cleaved out of the vector and used to transform *A. baylyi*. The resulting strain was verified by PCR using primers hybridizing to DNA external to the fragment that had been used for transformation; it was named ADPU44. Strain ADPU44 showed growth behavior (with respect to the doubling time, as well as the maximal optical density) on mineral medium with the carbon source succinate, quinate, or *p*-hydroxybenzoate comparable to that of the wild type. Northern blot hybridization was applied to verify that there was no RNA detectable in ADPU44 downstream of the Ω cassette by hybridization of total RNA with a *crc* probe downstream of the cassette (Fig. 4). Under the same culture conditions (mineral medium with the carbon sources succinate, pyruvate, and *p*-hydroxybenzoate), there was a clear band detected in total RNA of the wild type. A much weaker signal was

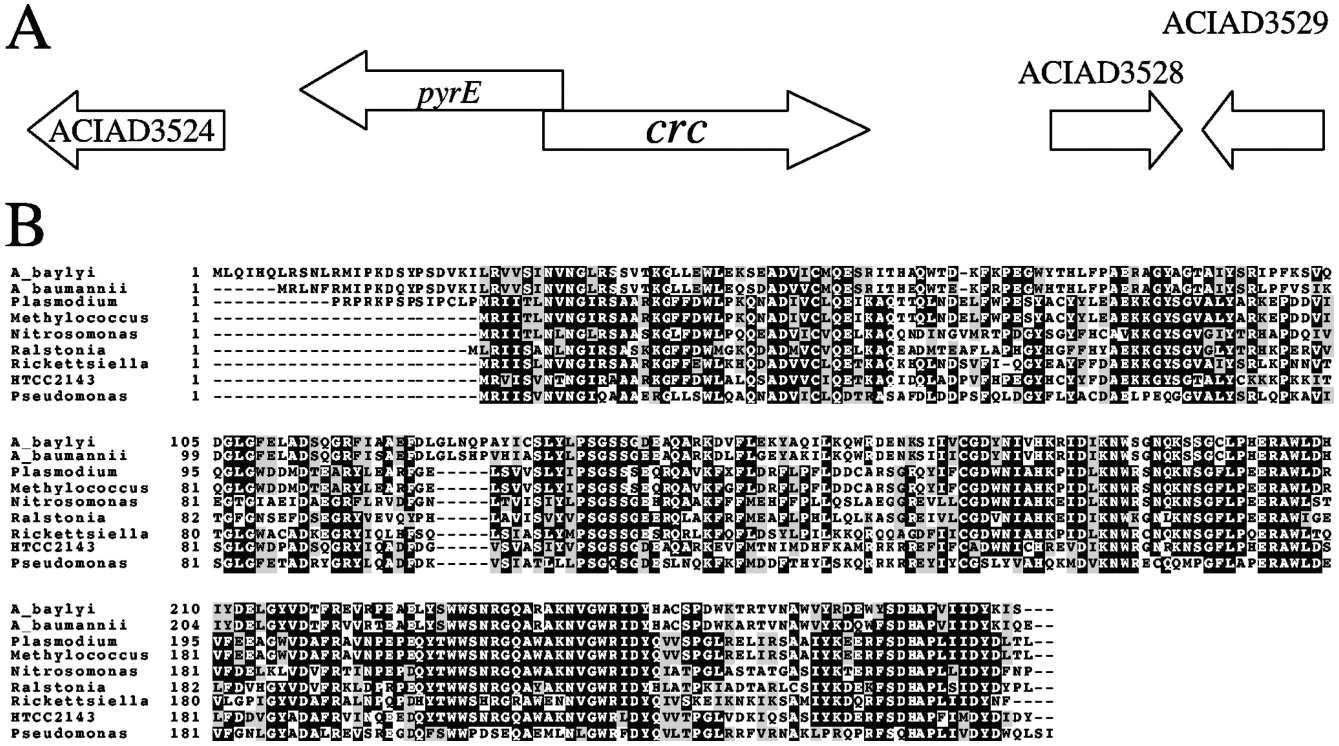

FIG. 2. (A) Location of the *crc* gene on the chromosome of *A. baylyi*. The gene products are ACIAD3524, a conserved hypothetical protein; *pyrE*, orotate phosphoribosyltransferase; ACIAD3528, putative arsenate reductase; and ACIAD3529, a conserved hypothetical protein. (B) *A. baylyi* Crc in a multiple-sequence alignment with related protein coding sequences using ClustalW2 (32). Residues identical with *A. baylyi* Crc in at least five sequences are indicated by black shading; gray shading indicates similar residues (done using BOXSHADE). The sequences are *A. baylyi* Crc (YP_047992.1), *A. baumannii* AYE (YP_001712136), marine gammaproteobacterium HTCC2143 (ZP_01617072.1), *P. yoelii yoelii* 17XNL1 exodeoxyribonuclease III (XP_728665.1), *Methylococcus capsulatus* Bath exodeoxyribonuclease III (YP_112853.1), *Nitrosomonas eutropha* C91 exodeoxyribonuclease III (YP_746928.1), *Ralstonia eutropha* H16 exodeoxyribonuclease III (YP_724743.1), a *Rickettsiella grylli* hypothetical protein (ZP_01301171.1), and *P. aeruginosa* Crc (NP_254019).

present after growth on nutrient broth, indicating regulated expression of *A. baylyi crc*. No signal was detected for the Δ*crc* strain ADPU44, confirming the successful inactivation of *crc*.

Absence of Crc strongly reduces carbon catabolite repression of protocatechuate 3,4-dioxygenase (PcaH and -G), but not of *p*-hydroxybenzoate hydroxylase (PobA). Protocatechuate 3,4-dioxygenase is known to undergo strong catabolite repression. Addition of acetate and succinate to medium containing a carbon source, which is metabolized via the protocatechuate branch of the β-ketoadipate pathway, leads to repression by 95%, whereas other organic acids, like pyruvate, do not have a repressing effect (9, 47). To evaluate the relevance of the Crc protein in this repression, we compared protocatechuate 3,4-dioxygenase activities in the wild type and the *crc* strain on different carbon sources. Enzyme activity was determined throughout growth; the levels in mid-log phase are sum-

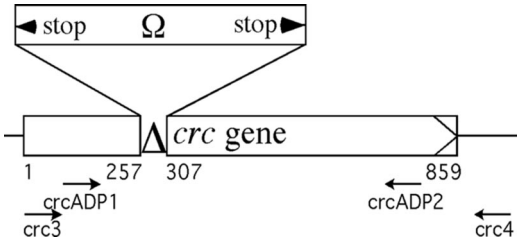

FIG. 3. Construction of the *crc* strain *A. baylyi* ADPU44. Shown is a schematic presentation of the *crc* gene with the deletion and the insertion of the Ω cassette. The numbers are related to residues in the gene, and the locations of oligonucleotides for cloning and verification of strain ADPU44 are indicated by arrows below the gene scheme.

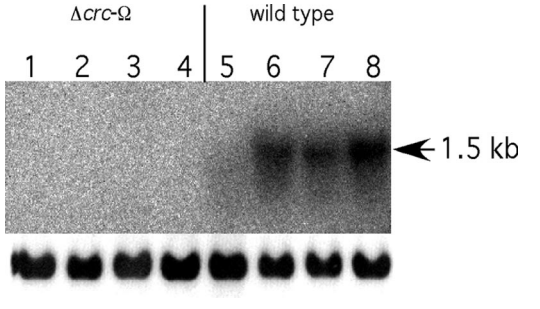

FIG. 4. Detection of the *crc* transcript in the *crc* strain (ADPU44) and the wild type (ADP1). The probe was a 461-bp fragment of DNA downstream of the locus of the insertion of the cassette. Each lane contained 10 μg of total RNA extracted from cells grown on the following media: nutrient broth (lanes 1 and 5), mineral medium plus pyruvate (lanes 2 and 6), mineral medium plus *p*-hydroxybenzoate (lanes 3 and 7), and mineral medium plus succinate (lanes 4 and 8). Below is shown the ethidium bromide-stained 16S rRNA from the same gel before blotting.

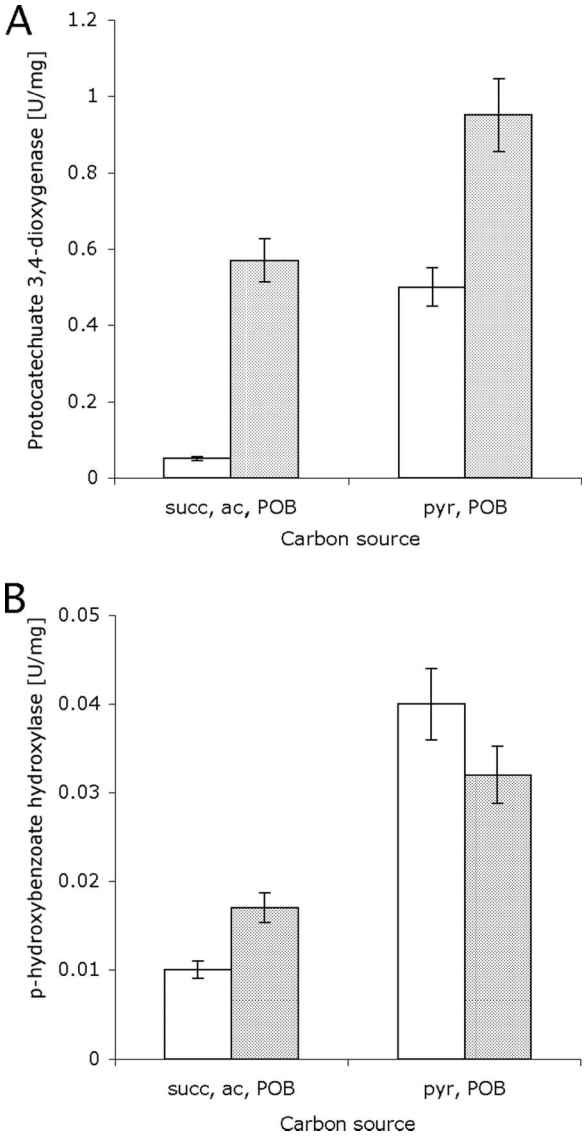

FIG. 5. Effects of Crc on activities of protocatechuate 3,4-dioxygenase (A) and *p*-hydroxybenzoate hydroxylase (B) under conditions of carbon catabolite repression (succinate [succ], acetate [ac], and *p*-hydroxybenzoate [POB]) or neutral conditions (pyruvate [pyr] or *p*-hydroxybenzoate). White bars, wild-type strain ADP1; gray bars, *crc* mutant strain ADPU44. The error bars indicate standard deviations.

marized in Fig. 5. In the *crc* strain, protocatechuate 3,4-dioxygenase activity under conditions of catabolite repression was as high as in the nonrepressed wild type. Furthermore, under neutral conditions with respect to carbon catabolite repression (addition of pyruvate to the aromatic carbon source), protocatechuate 3,4-dioxygenase activity was significantly higher than the wild-type activity under the same growth conditions. Thus, carbon catabolite repression in the *crc* mutant (1.7-fold) was significantly lower than in the wild type (10-fold). We also measured *p*-hydroxybenzoate hydroxylase, an enzyme known to show the same catabolite repression pattern as the dioxygenase (9). In contrast to the results with the dioxygenase, the repressed activity of this enzyme increased only slightly in the strain without a functional *crc* gene.

The steady-state *pca-qui* and *pobA* transcript levels are slightly increased in the absence of Crc. One explanation for the increase of the protocatechuate 3,4-dioxygenase activity is an increase of the transcript level caused either by increased transcription or by a posttranscriptional regulatory mechanism. Quantitative Northern blot analysis was applied to answer the question of whether the transcript level of the *pca-qui* operon was increased in the *crc* strain. Strains ADP1 and ADPU44 were grown under repressing conditions (*p*-hydroxybenzoate, acetate, and succinate) and nonrepressing conditions (*p*-hydroxybenzoate or quinate), and the *pca-qui* transcript level was determined in samples throughout growth using a *pcaC* or *-H* probe. Under repressing conditions, the *pca-qui* transcript was present in small amounts (5 to 10%) compared to nonrepressing conditions during the growth phase. Strain ADPU44 displayed an increased *pca-qui* transcript level during growth under repressing conditions (a two- to threefold increase in comparison to the wild type). Under nonrepressing conditions, *pca-qui* transcript levels were slightly higher than in the wild type (110 to 120% of the wild-type level). Corresponding observations were made for the transcript abundance of *pobA*, encoding *p*-hydroxybenzoate hydroxylase. In summary, the absence of the *crc* gene led to a moderate increase of the *pca-qui* transcript level under carbon catabolite repressing conditions, as well as under nonrepressing conditions.

Crc is not involved in carbon catabolite repression of the *pca-qui* operon at the transcriptional level. To evaluate the involvement of the *pca-qui* promoter (*pcaIp*) in the changes in protocatechuate 3,4-dioxygenase levels, a *pcaIp* reporter strain was constructed that did not contain a functional *crc* gene. The *crc* mutation described above was introduced into a strain containing a transcriptional *pcaIp-luc* fusion on the chromosome. The resulting strain, ADPU49, was grown with a number of different carbon sources to compare the resulting level of expression at *pcaIp* (Fig. 6A). The known regulatory patterns (specific induction by protocatechuate and carbon catabolite repression by succinate and acetate) were clearly present in the wild type, as well as in the *crc* mutant strain, demonstrating that the *crc* gene product is not a player in transcriptional regulation of carbon catabolite repression of the *pca-qui* operon. Corresponding measurements of *A. baylyi* strains containing the luciferase gene in a transcriptional fusion with the *pobA* promoter (with and without Crc) revealed a similar expression pattern. Pyruvate allowed the highest induction, lactate led to a slight reduction, and succinate and acetate caused strong catabolite repression (Fig. 6B). For both operons (*pca-qui* and *pobA*), withdrawal of Crc resulted in a slight but significant increase of expression under all tested growth conditions.

Absence of Crc dramatically enhances the stability of the *pca-qui* transcript. Since the strongly differing protocatechuate 3,4-dioxygenase activities between the wild type and the *crc* strain could not be explained by an effect of Crc on initiation of transcription, we addressed the posttranscriptional level. We therefore determined the half-life of the *pca-qui* transcript by Northern blot analysis. Cells of the wild-type strain, ADP1, and the *crc* strain, ADPU44, were grown on mineral medium with 60 mM lactate and 5 mM quinate, allowing the culture to reach a turbidity of 3 to 4. Quinate is converted to protocatechuate by *Acinetobacter* and then degraded via the β-ketoadipate pathway and thus is an efficient inducer. At the same time,

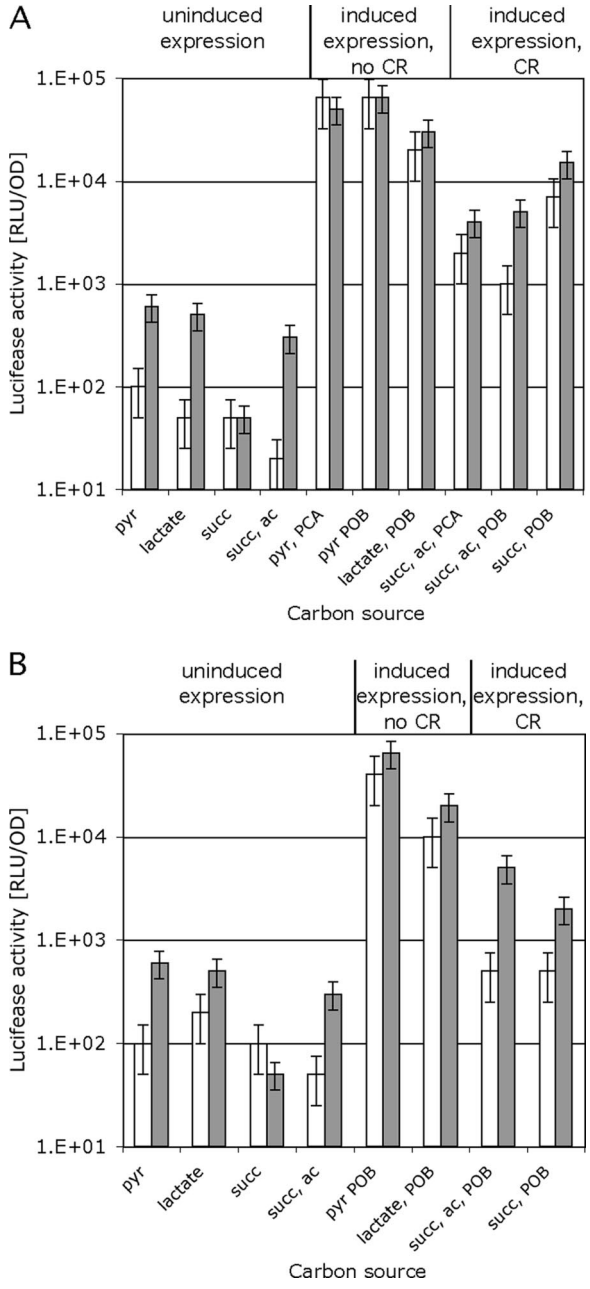

FIG. 6. (A) Activity of the *A. baylyi pcaIp* promoter measured with a chromosomal reporter gene fusion (transcriptional level) after growth on a number of different carbon sources in mineral medium in the wild-type background (strain ADPU47 [open bars]) and the *crc* background (strain ADPU49 [filled bars]). (B) Corresponding measurements with a *pobAp* fusion strain in a wild-type background (strain ADPU53 [open bars]) and the *crc* background (strain ADPU54 [filled bars]). The carbon sources were pyruvate (pyr), succinate (succ), acetate (ac), protocatechuate (PCA), *p*-hydroxybenzoate (POB), and catabolite repression (CR). The error bars indicate standard deviations.

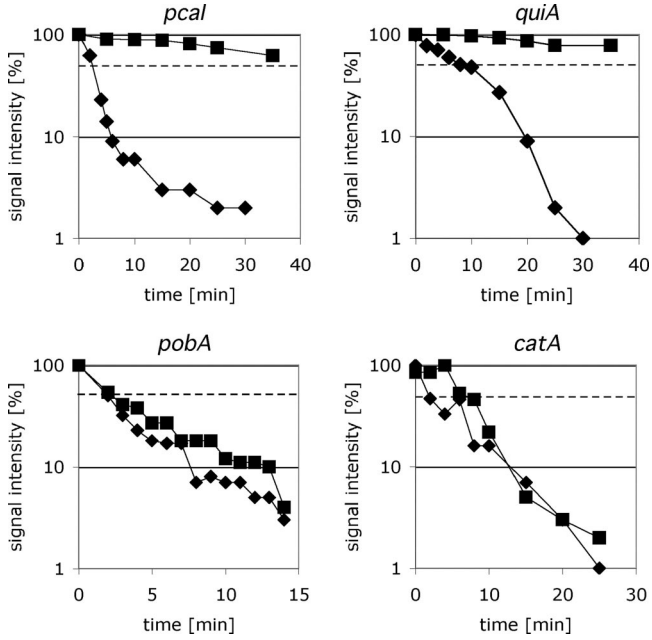

FIG. 7. Determination of the half-lives of the *A. baylyi pca-qui* transcript (investigated with two different probes, *pcaI* and *quiA*) and the *pobA* and the *catA* transcripts. The data are derived from Northern blot analysis performed with radioactive probes. Total RNA (10 μg) from samples withdrawn from cultures of the wild type (diamonds) and the *crc* strain (squares) taken immediately before and after addition of rifampin at time zero were analyzed. After signal detection using a phosphorimager, the total signal per lane was determined. The highest signal for each culture was set to 100%, and other data were related to that value.

it is a better growth substrate than protocatechuate itself. These conditions do not represent catabolite repression conditions, but we decided to use cells with the greatest possible amount of *pca-qui* transcript to have optimal conditions for the quantification; up to this point, it was unknown whether the effect of Crc is specific to carbon catabolite repression. At a turbidity of 1.5 to 2, quinate was added to the culture to ensure the presence of the inducer (final concentration, 3 mM). One hour later (and thus still in the logarithmic growth phase), the antibiotic rifampin was added to prevent subsequent transcriptional initiation and therefore to allow the determination of the stability of the *pca-qui* transcript. With a probe targeting the 5′ area of the large *pca-qui* transcript (14 kb), the half-life was 2.45 min for the wild type and >35 min for the *crc* strain. Applying a probe targeting the 3′ end of the transcript (*quiA*), the half-life for the wild type was 8 min, and for the *crc* strain it was >35 min (Fig. 7). Thus, in both cases, a dramatic stabilization was observed for the *pca-qui* transcript when Crc was missing (>14-fold for the 5′ end and >4-fold for the 3′ end). The difference between the different stabilities at the 5′ and the 3′ ends was probably due to the unknown degradation dynamics of the transcript. Also, a change in the degradation rate of the wild-type transcript was observed (lower after an initial faster decay for the 5′ end and vice versa for the 3′ end), which was probably also based on a complex degradation mechanism.

The half-lives of two other transcripts involved in catabolism of aromatic compounds, *pobA* and *catA*, are Crc independent. Given the dramatic effect of Crc on the stability of the *pca-qui* transcript and the predicted global nature of Crc, at least in *Pseudomonas*, we wanted to test whether Crc also affected the stability of other transcripts. We chose to test the

pobA gene (encoding *p*-hydroxybenzoate hydroxylase) and *catA* (encoding catechol dioxygenase), both involved in aromatic compound degradation in *A. baylyi*. The experiments were performed as described above except for the inducer, which was *p*-hydroxybenzoate for the investigation of the *pobA* transcript and benzoate for the investigation of the *catA* transcript (at a lower concentration: 2 mM at the start of growth and 2 mM 1 h before the addition of rifampin). In both cases, the half-lives of the transcripts did not differ significantly between the wild type and the *crc* strain (*pobA* transcript, 2.0 min for the wild type and 2.25 min for the *crc* strain; *catA* transcript, 3.5 min for the wild type and 3.0 min for the *crc* strain) (Fig. 7).

DISCUSSION

***A. baylyi* contains a *crc* gene.** Significant sequence identity between the *P. aeruginosa* Crc gene and ACIAD3526, referred to as *crc*, suggested a similar function of the *A. baylyi* gene product. Support for this idea came from similar chromosomal neighborhoods around the *crc* genes in several *Pseudomonas* species and *A. baylyi* (Fig. 2A). The gene products neighboring *crc* do not appear to be in a functional relationship to catabolite repression as judged from the annotation by Genoscope (arsenate reductase and orotate phosphoribosyltransferase). A monocistronic nature of *crc* is indicated by the Northern blot analysis. Proteins similar to Crc apparently occur in all domains of life (the endonuclease/exonuclease/phosphatase family). Worth mentioning is the yeast protein Ccrp4, originally described as a carbon catabolite repressor protein (54). This protein (837 residues) is similar to Crc in its C-terminal part and plays a role as an mRNA deadenylase. The fact that *A. baylyi* Crc, the inactivation of which leads to a strong stabilization of a transcript, is related by sequence similarity to a protein involved in a related area (an mRNA deadenylase accelerating mRNA degradation) may be indicative of related functions of these proteins (15).

Effect of Crc on gene expression of the *pca-qui* operon. The observation of the two enzymes protocatechuate 3,4-dioxygenase and *p*-hydroxybenzoate hydroxylase (which are encoded on separate operons [11, 53]) indicates that Crc is not globally involved in all cases of catabolite repression in *A. baylyi*. At the level of RNA abundance, as well as at the level of transcription activity for the two operons, the known patterns of gene expression (specific induction and strong carbon catabolite repression by acetate and succinate) were present in the *crc* strain as in the wild type. These results put forward the idea that Crc affects gene expression of the *pca-qui* operon at a posttranscriptional level. We found a tremendous effect of Crc withdrawal on *pca-qui* RNA stability: whereas its half-life was consistent with an average bacterial mRNA half-life in the wild type, *pca-qui* mRNA stability was dramatically increased in the *crc* strain, and this result was observed in independent experiments using probes for the 5′ end or for the 3′ end of the long *pca-qui* transcript (14 kb). In contrast to this very clear effect, two other transcripts also involved in aromatic compound catabolism did not show any difference in terms of their degradation rates between the wild type and the *crc* mutant strain.

From the data presented here, Crc could be a specific nuclease (or a protein mediating the contact with a known nuclease) initiating the degradation of certain transcripts (*pca-qui*) but not of others (like the *pobA* and the *catA* transcripts). These results also lead to the conclusion that in *A. baylyi* components other then Crc must be responsible for the transcriptional response to the repressing conditions of succinate and acetate by the main promoter of the *pca-qui* operon shown here (Fig. 6) and elsewhere (9, 47). Crc has a modulating function, and the inactivation of the *crc* gene results in an increase of gene expression and, at the same time, a significant reduction of carbon catabolite repression. The thorough analysis of different levels of gene expression revealed that transcriptional regulation was not affected, but instead, a later level (transcript stability) was. As the data presented further revealed, the effect of Crc is not the same for different operons. Therefore, one of the next questions to answer will certainly be how specifically or globally Crc affects other transcripts. It is possible that Crc is not specifically involved in carbon catabolite repression of the aromatic catabolic pathway but that it is more globally involved in different networks of gene expression and in different physiological processes. A hint in this direction comes from the observation that Crc in *P. putida* and *P. aeruginosa* is involved in carbon catabolite repression of a large number of different catabolic pathways, but also in the seemingly completely unrelated process of biofilm formation (40). Not to be overlooked is the slight but significant increase of promoter activity of the *pca-qui* and *pobA* operon under most conditions tested, indicating an involvement of Crc in transcription, as well.

***Acinetobacter* and *Pseudomonas* Crc proteins.** A comparison of the current results with data describing the function of Crc in *Pseudomonas* species revealed individual differences and common qualities. Both proteins play a role in carbon catabolite repression of aromatic degradative pathways (and many more pathways are documented in the *Pseudomonas* species). Crc levels are regulated in *Pseudomonas*, and this is also the case in *Acinetobacter*, as described above. The DNA binding quality of *P. aeruginosa* Crc has been addressed, and no Crc-DNA interaction could be detected (8, 33). The lack of a helix-turn-helix domain in the amino acid sequence indicates that Crc proteins indeed do not bind DNA. Binding of DNA in general cannot be excluded, since Crc is related to proteins that have DNA as a substrate (for example, exonuclease III) (35). Several lines of evidence indicated that Crc acts posttranscriptionally. *P. aeruginosa* Crc was shown to regulate the amount of the BkdR regulator on a level after transcription (27). In a similar fashion, the amount of the regulator AlkS of *P. putida* is responsible for catabolite repression by complex medium, and it depends on the presence of Crc (56). Recently, *P. putida* Crc was shown to repress AlkS expression at the translational level (8, 39, 56). The authors demonstrated that Crc(His_6) binds to the 5′ area of the *alkS* RNA, including the translation initiation region, and suggested that Crc modulates AlkS levels by blocking translation initiation. The regulator level, in turn, would determine the efficiency of structural-gene expression; thus, in this instance, Crc regulates enzyme expression indirectly. The connection of this mechanism to the presence of the complex medium (LB) remains to be explored. A corresponding finding has been described for the BenR transcriptional regulator from *P. putida* (38). For *A. baylyi* Crc, it is not known yet whether it affects the expression of the specific regulators in the case of the *pca-qui* operon of PcaU. Instead,

a strong effect on transcript stability has been found, which distinguishes *A. baylyi* Crc from the *P. putida* protein. In *P. putida*, the two transcripts starting at *PalkB* and at *PalkS2* (mentioned before) displayed only small differences in their half-lives depending on the presence of Crc. It is worth mentioning that in both cases the absence of Crc increased *alkB* and *alkS2* transcript stability, as was observed here for the *A. baylyi pca-qui* transcript (but to a much smaller extent) (56). Thus, in summary, in both organisms Crc modulates gene expression at a level after transcription. Whereas it inhibits efficient translation of regulators in *P. putida* by binding to the translation initiation region, it destabilizes the target transcript in *A. baylyi*.

Conclusion. In this report, for the first time, data are presented dealing with the Crc protein from an organism outside of the genus *Pseudomonas*. Common qualities, as well as differences, were found. *A. baylyi* Crc acts posttranscriptionally and is involved (directly or indirectly) in the degradation of at least one mRNA. Future investigations will address how Crc affects mRNA stability and which other genes are affected by Crc. It will be challenging to unravel whether its function is limited to carbon catabolite repression or includes more, possibly unrelated, processes.

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