Journal of Bacteriology

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J. Bacteriol. **2005, 187(11):3678. DOI: 10.1128/JB.187.11.3678-3686.2005.**

JOURNAL OF BACTERIOLOGY, June 2005, p. 3678–3686 Vol. 187, No. 11
0021-9193/05/$08.00+0 doi:10.1128/JB.187.11.3678–3686.2005

Levels and Activity of the *Pseudomonas putida* Global Regulatory Protein Crc Vary According to Growth Conditions

Ana Ruiz-Manzano, Luis Yuste, and Fernando Rojo*

Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Received 12 January 2005/Accepted 25 February 2005

The global regulatory protein Crc is involved in the repression of several catabolic pathways for sugars, hydrocarbons, and nitrogenated and aromatic compounds in *Pseudomonas putida* and *Pseudomonas aeruginosa* when other preferred carbon sources are present in the culture medium (catabolite repression), therefore modulating carbon metabolism. We have analyzed whether the levels or the activity of Crc is regulated. Crc activity was followed by its ability to inhibit the induction by alkanes of the *P. putida* OCT plasmid alkane degradation pathway when cells grow in a complete medium, where the effect of Crc is very strong. The abundance of *crc* transcripts and the amounts of Crc protein were higher under repressing conditions than under nonrepressing conditions. The presence of *crc* on a high-copy-number plasmid considerably increased Crc levels, but this impaired its ability to inhibit the alkane degradation pathway. Crc shows similarity to a family of nucleases that have highly conserved residues at their catalytic sites. Mutation of the corresponding residues in Crc (Asp_{220} and His_{246}) led to proteins that can inhibit induction of the alkane degradation pathway when present at normal or elevated levels in the cell. Repression by these mutant proteins occurred only under repressing conditions. These results suggest that both the amounts and the activity of Crc are modulated and support previous proposals that Crc may form part of a signal transduction pathway. Furthermore, the activity of the mutant proteins suggests that Crc is not a nuclease.

Expression of many bacterial catabolic pathways is controlled not only by the presence or absence of the compound to be assimilated (a specific control response) but also by several global regulatory proteins that link the induction of the pathway genes to the physiological status of the cell (a global control response). Global control is usually dominant over the specific control. In pseudomonads, this kind of global control has been studied for some pathways responsible for the assimilation of sugars, amino acids, hydrocarbons, and aromatic compounds (for reviews, see references 8, 10, 33, and 35). Although the molecular mechanisms responsible for these global regulatory processes are still not well understood, it is clear that there are several factors involved. Which global regulator, or combinations of regulators, controls a particular catabolic pathway depends not only on the signal to be transmitted but also on the characteristics of the promoters and of the specific transcriptional regulators of the pathway (33, 35). The signals sensed include nutrient or oxygen availability and the presence of alternative carbon sources in addition to that assimilated by the pathway considered. The factors involved include the alarmone (p)ppGpp (7, 37, 39), integration host factor (38, 40), components of the electron carbon chain (12, 13, 32), the FtsH chaperone/protease (6), and the Crc protein (18, 25, 29, 45).

Among these factors, Crc (catabolite repression control) is a master regulator of carbon metabolism in *Pseudomonas*. It is particularly important for catabolite repression, a complex regulatory response that allows the cell to preferentially use a particular carbon source over a mixture of several other potentially assimilable, but less preferred, compounds. Crc is involved in the catabolite repression generated by succinate or lactate on the expression of a number of genes implicated in the metabolism of several sugars and nitrogenated compounds in both *Pseudomonas aeruginosa* (10, 25, 43) and *P. putida* (18, 19). Crc also controls the assimilation of hydrocarbons and aromatic compounds. When cells grow in a complete medium, Crc inhibits the expression of the alkane degradation pathway encoded in the *P. putida* OCT plasmid (45) as well as that of the homogentisate, catechol, and protocatechuate pathways (29), which direct the assimilation of many aromatic compounds in *P. putida* (21). Available data suggest that Crc is a component of a signal transduction pathway modulating carbon metabolism and other phenomena such as biofilm development (19, 26, 30). Crc ultimately affects the expression of the target genes, although the precise molecular mechanism underlying this effect remains to be elucidated. The scarce information obtained so far suggests that Crc does not bind DNA, so it would not be a classical DNA-binding repressor (10, 19, 25). Its precise target is not known.

We have analyzed how Crc modulates gene expression using the alkane degradation pathway (*alk* pathway) encoded in the *P. putida* OCT plasmid as a model system (41, 42; Fig. 1). Induction of the pathway genes by alkanes is negatively modulated by a dominant global control when cells grow in a complete medium or in a minimal salts medium containing succinate as a carbon source (44). If citrate substitutes for succinate as a carbon source, inhibition does not occur. The global control observed in a complete medium, which is very strong, depends on the additive effects of Crc (45) and the cytochrome *o* ubiquinol oxidase (Cyo), a component of the

* Corresponding author. Mailing address: Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Phone: (34) 91 585 45 39. Fax: (34) 91 585 45 06. E-mail: frojo@cnb.uam.es.

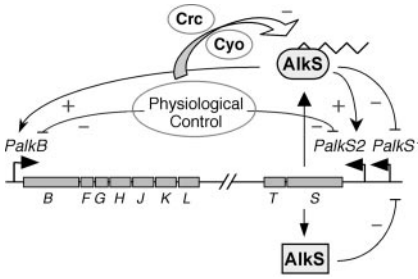

FIG. 1. The *P. putida* OCT plasmid alkane degradation pathway. The genes are encoded in two clusters (*alkBFGHJKL* and *alkST*), both of which are regulated by the AlkS protein. In the absence of alkanes, *alkS* is expressed from promoter *PalkS1*. AlkS negatively modulates this promoter, allowing for a low expression. In the presence of alkanes (or of the nonmetabolizable analogue DCPK), AlkS activates transcription from promoters *PalkB* and *PalkS2*, which leads to a self-amplification of *alkS* expression. Activation of these two promoters is negatively modulated by a dominant global control, a process mediated by Crc and Cyo (see the text for details). Inhibition of *PalkS2* leads to a decrease in AlkS levels, an unstable protein present in limiting amounts (modified from data in references 12 and 45).

electron transport chain (12, 13). In contrast, the inhibition imposed by succinate in a defined medium depends mainly on Cyo, with Crc playing a very minor role (12). At least in a complete medium, the final effect of the inhibition process is a strong decrease in the levels of the AlkS transcriptional activator, an unstable protein present in the cell in limiting amounts even under inducing conditions (45). Keeping AlkS levels below those required for maximal induction of the pathway allows down-modulation of the expression of the two clusters of the pathway in a simple and coordinated way (Fig. 1).

In a complete medium, repression of the *alk* pathway is particularly strong during exponential growth but rapidly disappears when cells enter the stationary phase of growth. This can be explained in part by the decrease in Cyo levels that occurs upon entry into the stationary phase (12). However, the repression relief in stationary phase is still detected in cells in which Cyo has been inactivated (13). This suggests that either the levels or the activity of the Crc protein could be regulated, decreasing when cells enter the stationary phase of growth. To investigate this possibility, we have analyzed whether transcription of *crc*, the levels of the Crc protein, or its activity is modulated throughout the growth phase and in different growth mediums. The results indicate that Crc levels, and probably its activity, are modulated.

MATERIALS AND METHODS

Bacterial strains and culture media. The strains and plasmids used are listed in Table 1. Cells were grown at 30°C in LB medium or in M9 minimal salts medium (34), with the latter supplemented with trace elements (4) and 30 mM citrate or succinate as the carbon source. Where indicated, a spent LB medium was used, which was obtained by growing cells to stationary phase, eliminating the cells by centrifugation and subsequent filtration, and sterilizing the medium after adjusting the pH to 7. Expression of promoter *PalkB* was induced by the addition of 0.05% (vol/vol) dicyclopropylketone (DCPK), a nonmetabolizable inducer that mimics the effect of alkanes (16). Antibiotics were added when appropriate at the following concentrations (in μg/ml): ampicillin, 100; kanamycin, 50; streptomycin, 50; tetracycline, 8. Cell growth was followed by measuring turbidity at 600 nm.

P. putida PBS4 is derived from *P. putida* KT2442 (15) by insertion of a *PalkB*::*lacZ* transcriptional fusion and the *alkS* gene in its chromosome (44). *P. putida* PBS4C1 derives from strain PBS4 by inactivation of the *crc* gene (contains a *crc*::*tet* allele [45]). To obtain strain KT2442-C1, the *crc* gene of strain KT2442 was inactivated by marker exchange mutagenesis as described previously (45) by using plasmid pCRC10, which contains a *crc*::*tet* allele. Construction of all other strains is described further on in the text in the appropriate section.

S1 nuclease protection assays. Cells were collected by centrifugation and chilled, and total RNA was obtained using the phenol/guanidine thiocyanate mix Tri Reagent LS (Molecular Research Center, Inc.). S1 nuclease protection assays were performed as described previously (5), using equal amounts of RNA in each sample. The single-stranded DNA (ssDNA) probe used, which was added in excess to titrate the mRNA, was generated by linear PCR as described previously (5). The plasmid used as substrate, pCRC5 (45), contains the *crc* gene and upstream sequences and was linearized with BamHI. The oligonucleotide used as primer hybridized to positions 33 to 13 (5′→3′ direction) relative to the *crc* translational start site. The size of the nondigested double-stranded DNA was determined by denaturing gel electrophoresis using a size ladder obtained by chemical sequencing (27) of the ssDNA used as a probe. Band intensities were quantitated by densitometry (QuantityOne software) after digitalizing the image with the VersaDoc imaging system (Bio-Rad).

Transcriptional fusions to *lacZ*. To obtain a transcriptional fusion of promoter *Pcrc* to the *lacZ* reporter gene, a 303-bp DNA fragment containing the promoter was PCR amplified using plasmid pCRC5 as a substrate and primers 5′-GCCG GATCCATAAATCTCGTGCGTGT and 5′-GGTTTTCCCAGTCACGACGT. The DNA fragment obtained was cloned into plasmid pGEM-T-Easy, sequenced to verify the absence of mutations generated through the amplification process, and excised as an EcoRI-BamHI fragment that was cloned between the same sites of plasmid pUJ8. The plasmid obtained was named pCRCR1. The *Pcrc*::*lacZ* transcriptional fusion was excised from pCRCR1 as a NotI DNA fragment and cloned into the suicide delivery plasmid pUT-mini-Tn*5*-Km, generating plasmid pCRCR1K. This plasmid was introduced into *Escherichia coli* CC118(λ*pir*) and used to deliver the *Pcrc*::*lacZ* fusion into the chromosome of KT2442 or of its *crc*-deficient strain, KT2442-C1, by triparental matings using plasmid pRK600 as a donor of transfer functions. Four isolates in which the mini-Tn*5* containing the fusion had mobilized to the chromosome were selected and tested for expression of the *lacZ* gene. Representative strains were chosen and named CFR2 (derived from KT2442) or CFR2C1 (derived from KT2442-C1).

Assay for β-galactosidase. A culture of the strain of interest which was grown overnight was diluted to a final turbidity (A_{600}) of 0.04 in the appropriate medium. When turbidity reached 0.08, the nonmetabolizable inducer DCPK (0.05% [vol/vol]) was added, where indicated, to induce expression of the *alk* pathway promoters. Growth was continued at 30°C, and at different time points, aliquots were taken and β-galactosidase activity was measured as described previously (28) by using *o*-nitrophenyl-β-D-galactoside as a substrate. At least three independent assays were performed.

Crc derivatives (His_6)*crc*, *crc(H246E)*, and *crc(D220A)*. To introduce a His_6 tag at the N terminus of Crc, the *crc* gene was PCR amplified using pCRC5 as substrate and primers 5′-CACGGATCCATGCGGATCATCAGTGTG and 5′-GAACCATGGCCGATCAAATAACCA. The DNA fragment obtained, which contains the entire *crc* gene without its native promoter, was cloned into pGEM-T-Easy, generating pGHCRC. This plasmid was digested with BamHI and PstI, and the fragment containing *crc* was cloned between the equivalent sites of pQE30, generating pHCRC. A HindIII-XhoI DNA fragment containing (His_6)*crc* was excised from pHCRC and cloned between the equivalent restriction sites of plasmid pKT231, generating pHCRC11.

Crc residues Asp-220 and His-246 were changed to Ala (D220A mutation) or to Glu (H246E mutation) by PCR, as described previously (9), using plasmid pCRC5 as a substrate. The final PCR product obtained was cloned into pGEM-T-Easy and sequenced. Plasmids with the appropriate sequence were named pGCRC-CA (D220A mutation) or pGCRCH_{246}E (H246E mutation). A 420-bp DNA segment containing the mutagenized *crc* region was excised from pGCRCH_{246}E with KpnI and NruI and used to substitute the equivalent segment in plasmid pCRC5, generating pCRC5H_{246}E. The complete *crc(D220A)* and *crc(H246E)* genes, including their own promoters, were excised with BamHI from pGCRC-CA and pCRC5H_{246}E, respectively, and cloned into plasmid pKT231, generating pCRC11-D_{220}A and pCRC11H_{246}E, respectively.

To insert *crc* or its mutant derivatives into the *P. putida* chromosome, the corresponding genes were cloned into the suicide delivery plasmid pUT-mini-Tn*5*Sm. Wild-type *crc*, including its own promoter, was excised from pCRC5 as an EcoRI-HindIII fragment and cloned between the EcoRI and HindIII sites of pUJ8, generating plasmid pUJ-CRC. A NotI DNA fragment containing *crc* was excised from pUJ-CRC and cloned at the NotI site of pUT-mini-Tn*5*Sm, generating pSMC1. The *crc(D220A)* gene, including its own promoter, was excised

TABLE 1. Strains and plasmids

Strain or plasmid	Description or relevant phenotype	Reference or source
Strains		
E. coli		
CC118 (λ*pir*)	CC118 lysogenized with λ*pir* phage	17
HB101	Host for plasmid pKR600	34
M15	Host for pQE30-derived plasmids	QIAGEN, Inc.
TG1	Host for DNA manipulations	34
P. putida		
CFR2	KT2442 with a *Pcrc*::*lacZ* transcriptional fusion in the chromosome	This work
CFR2C1	CFR2 with an inactivated *crc* allele (*crc*::*tet*)	This work
KT2442	*hdsR;* Rif^r derivative of KT2440	15
KT2442-C1	KT2442 derivative with an inactivated *crc* allele (*crc*::*tet*)	This work
PBS4	KT2442 with a *PalkB-lacZ* fusion and *alkS* in the chromosome	44
PBS4C1	PBS4 with an inactivated *crc* allele (*crc*::*tet*)	45
PBS4C1C	PBS4C1 with an added wild-type *crc* allele in the chromosome	This work
PBS4C1-HC	PBS4C1 with an added (His_6)*crc* allele in the chromosome	This work
PBS4C1-CE	PBS4C1 with an added *crc*(*H246E*) allele in the chromosome	This work
PBS4C1-CA	PBS4C1 with an added *crc*(*D220A*) allele in the chromosome	This work
Plasmids		
pCRC5	Ap^r; *P. putida crc* gene into the BamHI site of pUC18	45
pCRC5H_{246}E	Ap^r; *P. putida crc*(*H246E*) gene into the BamHI site of pUC18	This work
pCRC10	Sm^r; *P. putida crc*::*tet* gene cloned at the SmaI site of pKNG101	45
pCRC11	Km^r Sm^r; *P. putida crc* gene cloned at the BamHI site of pKT231	45
pCRC11D_{220}A	Km^r Sm^r; *crc(D220A)* gene cloned at the BamHI site of pKT231	This work
pCRC11H_{246}E	Km^r Sm^r; *crc(H246E)* gene cloned at the BamHI site of pKT231	This work
pCRC21	Km^r Tet^r; *crc* gene cloned at the Bg/II site of pRK2501	This work
pCRCR1	Ap^r; contains a *Pcrc*::*lacZ* transcriptional fusion; derived from pUJ8	This work
pCRCR1K	Ap^r Km^r; *Pcrc*::*lacZ* transcriptional fusion cloned into pUT-mini-Tn5Km	This work
pGCRCH$_{246}$E	Ap^r; *crc(H246E)* gene cloned into pGEM-T-Easy	This work
pGCRC-CA	Ap^r; *crc(D220A)* gene cloned into pGEM-T-Easy	This work
pGEM-T-Easy	Ap^r, cloning vector	Promega
pGHCRC	Ap^r; *P. putida crc* gene cloned into pGEM-T-Easy	This work
pHCRC	Ap^r; pQE30 with the (His_6)*crc* gene	This work
pHCRC11	Sm^r; (His_6)*crc* gene cloned into pKT231	This work
pKT231	Sm^r Km^r; broad-host-range RSF1010-derived vector	2
pQE30	Ap^r; expression vector for His-tagged proteins	QIAGEN
pRK2501	Km^r Tet^r; low-copy-number broad-host-range cloning vector	22
pRK600	Cm^r ColE1*onV* RK2*mob*$^+$ *tra*$^+$; donor of transfer functions	24
pSMC1	Ap^r Sm^r; *crc* gene cloned at the NotI site of pUT-mini-Tn*5*Sm	This work
pSM-HC	Ap^r Sm^r; (His_6)*crc* gene cloned at the NotI site of pUT-mini-Tn*5*Sm	This work
pSMC2-E	Ap^r Sm^r; *crc(H246E)* gene cloned at the NotI site of pUT-mini-Tn*5*Sm	This work
pSMCRC-CA	Ap^r Sm^r; *crc(D220A)* gene cloned at the NotI site of pUT-mini-Tn*5*Sm	This work
pUC18Not	Ap^r; pUC18 with a polylinker flanked by NotI sites	17
pUC-E	Ap^r; *P. putida crc(H246E)* gene into the BamHI site of pUC18Not	This work
pUJ8	Ap^r; vector to make transcriptional fusions to *lacZ*	11
pUJ-CRC	Ap^r; *crc* gene cloned between the EcoRI and HindIII sites of pUJ8	This work
pUT-mini-Tn*5*Km	Ap^r Km^r; mini-Tn5 suicide donor plasmid	11
pUT-mini-Tn*5*Sm	Ap^r Sm^r; mini-Tn5 suicide donor plasmid	11
pVTRA	Cm^r; cloning vector to express recombinant genes	31
pVTRAd	Cm^r; derives from pVTRA by elimination of the NcoI site	J. M. Sánchez-Romero and F. Rojo, unpublished data
pVTR-HC	Cm^r; *crc* gene cloned at the EcoRI site of pVTRAd	This work

from pGCRC-CA as a NotI fragment and cloned into pUT-mini-Tn*5*Sm, generating pSMCRC-CA. The *crc(H246E)* gene, including its own promoter, was excised from pCRC5H_{246}E as a BamHI fragment and cloned into the BamHI site of pUC18Not, generating plasmid pUC-E, from which it was recovered as a NotI DNA segment and cloned at the NotI site of pUT-mini-Tn*5*Sm. The plasmid obtained was named pSMC2-E. The (His_6)*crc* gene was excised from pHCRC with EcoRI and cloned at the EcoRI site of pVTRAd, generating pVTR-HC. In this plasmid, (His_6)*crc* is expressed from the *Ptrc* promoter of the vector. Plasmid pVTRC-HC was digested with NotI, and the fragment containing *Ptrc*-(His_6)*crc* was cloned at the NotI site of pUT-mini-Tn*5*Sm, generating pSM-HC.

The suicide delivery plasmids pSMC1, pSM-HC, pSMCRC-CA, and pSMC2-E, which contain the *crc*, (His_6)*crc*, *crc(D220A)*, and *crc(H246E)* genes, respectively, were introduced into *E. coli* CC118(λ*pir*) and subsequently transferred to *P. putida* PBS4C1 (which contains an inactive *crc*::*tet* allele) by triparental matings using plasmid pRK600 as a donor of transfer functions. Selected isolates in which the mini-Tn*5* containing either the wild-type *crc*, (His_6)*crc*, *crc(D220A)*, or *crc(H246E)* gene had been mobilized to the chromosome were named PBS4C1C, PBS4C1-HC, PBS4C1-CA, and PBS4C1-CE, respectively.

Protein purification. *E. coli* M15 containing plasmid pHCRC was grown in LB medium at 28°C. At a turbidity of 0.9 (A_{600}), expression of (His_6)Crc was induced by the addition of isopropyl-β-D-thiogalactopyranoside up to 1 mM. After 3 h, cells were centrifuged; resuspended in 10 mM Tris-HCl, pH 8, 100 mM sodium phosphate, and 8 M urea; and disrupted by sonication. After elimination of cell debris, the supernatant was loaded onto a column containing Ni-NTA resin (QIAGEN Inc.) equilibrated in the same buffer. The column was washed with 20

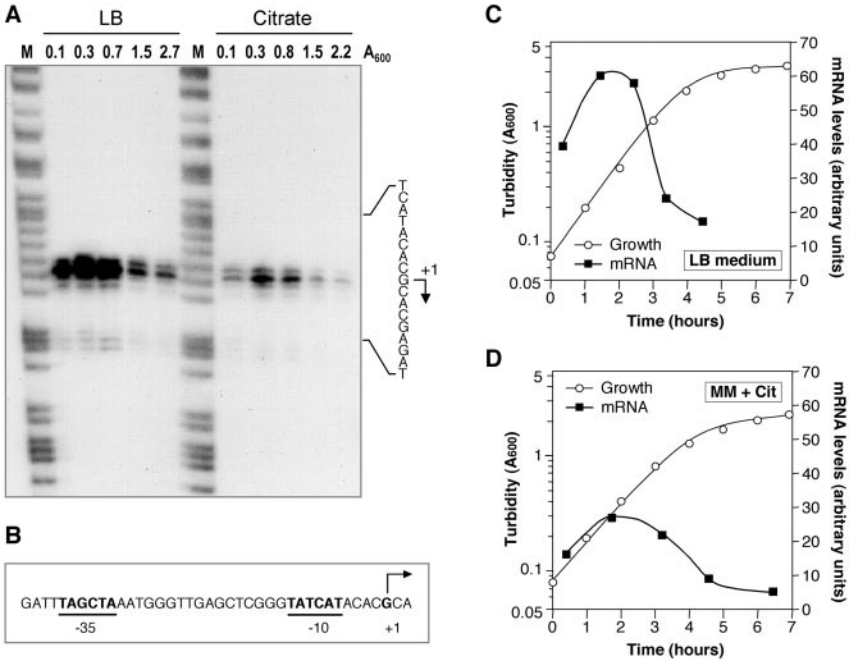

FIG. 2. Characterization of the promoter for the *crc* gene. (A) *P. putida* KT2442 was grown in rich LB medium or in minimal salts medium supplemented with citrate as a carbon source; at different moments of the growth curve (A_{600} values in the figure), cells were collected and total RNA was purified. The transcription start site and the amounts of transcripts present were determined by S1 nuclease protection assays. A size ladder obtained by chemical sequencing (27) of the ssDNA used as a probe was run in parallel (lane M). (B) Sequence of the *Pcrc* promoter. The −10 and −35 consensus boxes for σ^{70}-RNA polymerase are indicated. The transcription start site (+1) is indicated with an arrow. (C, D) The signal obtained from promoter *Pcrc* in panel A was quantitated by densitometry and represented as a function of cell growth.

volumes of Tris-HCl, pH 6.3, 100 mM sodium phosphate, and 8 M urea and 4 volumes of 10 mM Tris-HCl, pH 5.9, 100 mM sodium phosphate, and 8 M urea and eluted with 4 volumes of 10 mM Tris-HCl, pH 4.5, 100 mM sodium phosphate, and 8 M urea. The (His_6)Crc protein was detected by immunoblotting with a His_6 monoclonal antibody (Clontech).

Immunological techniques. Polyclonal antibodies against Crc were obtained by immunizing rabbits with purified (His_6)Crc. The polyclonal antiserum obtained was purified by immunoadsorption to enrich it in antibodies directed towards Crc, essentially as described previously (34). Crc protein was detected by immunoblotting with the antiserum obtained using standard techniques (34). (His_6)Crc was revealed by either the Crc antiserum or a monoclonal anti-His_6 antibody (Clontech, Inc). Membranes were treated with a secondary antibody conjugated to peroxidase (anti-rabbit for the polyclonal antibody and anti-mouse for the monoclonal anti-His_6 antibody; Amersham-Pharmacia Biotech). The blot was developed by chemiluminescence using the ECL+Plus system from Amersham-Pharmacia Biotech, as recommended by the supplier. To analyze and calibrate the linearity of the response of the Western blots, serial dilutions of PBS4 cell extracts (40, 20, 10, and 5 μg of total protein) were resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the amounts of Crc protein were revealed with the polyclonal antiserum as indicated above.

RESULTS

Expression of the *crc* gene under different growth conditions. The transcription start site of *crc* was determined by S1 nuclease protection assays performed with total RNA obtained from exponentially growing *P. putida* KT2442. Assays were performed with cells grown in rich LB medium or in minimal salts medium containing citrate as a carbon source and with samples collected at different moments of the growth curve. A single start site was observed (Fig. 2) and was located 23 nucleotides upstream from the predicted translation initiation codon. The −10 region of the promoter contained a sequence similar to the consensus recognized by the vegetative σ^{70}-RNA polymerase holoenzyme. Seventeen base pairs upstream from the −10 box, a moderately conserved −35 box was present (three out of six matches).

In cells grown in complete LB medium, expression of *crc* was highest during exponential growth and decreased when the growth rate declined before cells entered into stationary phase (Fig. 2). In stationary-phase cells, transcript levels were three- to fourfold lower than those of cells in mid-exponential phase. When cells were grown in minimal salts medium with citrate as a carbon source, a substrate that does not induce catabolite repression on the alkane degradation pathway, expression of *crc* was two- to threefold lower than in LB-grown cells and also declined in stationary phase (Fig. 2).

A transcriptional fusion of promoter *Pcrc* to the *lacZ* reporter gene was constructed and inserted into the chromosome of *P. putida* KT2442 and its *crc*-deficient derivative, KT2442-C1, generating strains CFR2 and CFR2C1, respectively. Expression of the reporter gene was analyzed in cells cultivated in several growth media. At mid-exponential phase (turbidity of 0.5 to 0.6), transcription from promoter *Pcrc* in strain CFR2 was almost twofold higher in cells grown in LB (448 ± 37 Miller units [MU]) than in cells grown in minimal salts medium containing citrate as a carbon source (250 ± 32 MU). When citrate was substituted with succinate, β-galactosidase levels increased to 360 ± 30 MU. It should be noted that succinate generates catabolite repression on the alkane degradation pathway, but this repression is signaled mainly by Cyo, with Crc having a very little role in it (12). When cells were grown in a

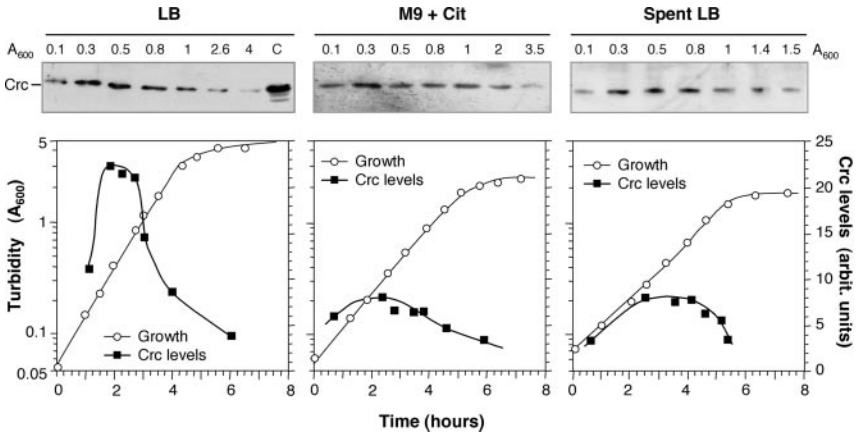

FIG. 3. Crc levels along the growth phase in cells grown in different media. Strain PBS4 was grown in LB medium, in minimal salts medium containing citrate as carbon source (M9 + Cit), or in spent LB. At different time points, aliquots were taken, cellular proteins were resolved in SDS-polyacrylamide gels, and the amounts of Crc present were revealed by immunoblotting. The Western blots (top) show the Crc signal obtained at different turbidity values (A_{600}); "C" corresponds to a control lane containing (His_6)Crc. The graphs below show the Crc levels observed (squares) and the turbidity of the cultures (circles) as a function of time. Crc levels were calculated by dividing the signal intensity (densitometric values) obtained in each lane by the sum of the signal intensities corresponding to all lanes for a given growth medium. Since Crc levels were 2.5-fold lower in minimal salts medium and in spent LB medium than in LB medium (see the text), the values shown for these two media are corrected accordingly.

spent LB medium (a medium already used for growth and filtered and sterilized), which generates little catabolite repression (44), expression of the *Pcrc*::*lacZ* transcriptional fusion was similar to that observed in minimal salts medium containing citrate as a carbon source (300 ± 25 MU). Inactivation of the *crc* gene (strain CFR2C1) did not affect the expression of promoter *Pcrc* in LB medium (402 ± 48 MU), indicating that Crc does not control the expression of its own gene under the conditions tested.

Levels of the Crc protein under different growth conditions. A Crc derivative with a His_6 tag in its N terminus was constructed, purified, and used to obtain a polyclonal antiserum, which was used to analyze the levels of Crc in cells grown in different growth media and at different moments of the growth phase. The growth media used were LB, where Crc-mediated inhibition of *alk* pathway induction is very strong; minimal salts medium with citrate as carbon source, in which there is no inhibition of the *alk* pathway; and spent LB medium, where inhibition of *alk* pathway induction is very low (44). As shown in Fig. 3, Crc levels varied significantly as growth proceeded in the three media used. These levels increased at the start of the exponential phase of growth, reached a maximum at mid-exponential phase (turbidity of 0.3 to 0.6), and declined at turbidity values above 0.8 to 1. To compare Crc levels in cells grown in each of the three growth media, cells grown in each of them were collected at a turbidity of 0.6, and proteins were resolved by SDS-polyacrylamide gel electrophoresis on adjacent lanes of the gel. Probing Crc with the antiserum showed that Crc levels are about 2.5-fold higher in LB medium than in minimal salts or spent LB medium (not shown).

Expression of Crc over its physiological levels decreases its repressing effect. If regulation of Crc activity relies on adjusting its levels in the cell, an artificial increase in the amount of Crc may lead to a much stronger inhibition of the *alk* pathway. To test this possibility, the *crc* gene including its own promoter was cloned into the broad-host-range high-copy-number plasmid pKT231, which has about 15 to 20 copies per cell (2). The resulting plasmid, named pCRC11, was introduced into strains PBS4 and PBS4C1. PBS4 is an indicator strain that contains a transcriptional fusion of the *alk* pathway *PalkB* promoter to the *lacZ* reporter gene. The strain also harbors *alkS*, which encodes the transcriptional regulator that activates *PalkB* in the presence of alkanes. Strain PBS4C1 is derived from *P. putida* PBS4 by inactivation of its *crc* gene. When strain PBS4 is grown in LB medium containing alkanes (or the nonmetabolizable analog DCPK), the activity of promoter *PalkB* remains very low during exponential growth due to the strong inhibition imposed by Crc and Cyo (44; Fig. 4B). Inhibition ceases at the onset of stationary phase, when β-galactosidase levels abruptly increase. Inactivation of the *crc* gene (strain PBS4C1) partially relieves this inhibitory effect (45; Fig. 4B). Introduction of *crc* (including its own promoter) into the chromosome of strain PBS4C1 via a minitransposon (strain PBS4C1C) restored normal *crc* expression levels (Fig. 4A) as well as the inhibitory effect on *PalkB* induction (Fig. 4B). Introduction of the multicopy plasmid pCRC11 (which contains *crc*) into strain PBS4C1 led to a considerable increase in the Crc levels (Fig. 4A) but did not restore the inhibitory effect (Fig. 4B). The amount of Crc detected in the Western blots for strain PBS4C1 containing pCRC11 was about 20-fold higher than that detected in the wild-type strain, which fits well with the copy number of the vector. The protein remained soluble, since after disrupting the cells, it was detected in the soluble fraction rather than in the particulate fraction (not shown). Introduction of plasmid pCRC11 into strain PBS4, which has a wild-type copy of *crc* in the chromosome, severely interfered with normal Crc function, although a partial repression was still observed during the early exponential phase (Fig. 4A and C). Altogether, the results suggest that the presence of Crc at high levels impairs its inhibitory effect on *PalkB* induction. Alternatively, the effect may result from a mutation in the *crc* copy present in pCRC11 or from an unpredicted negative influence

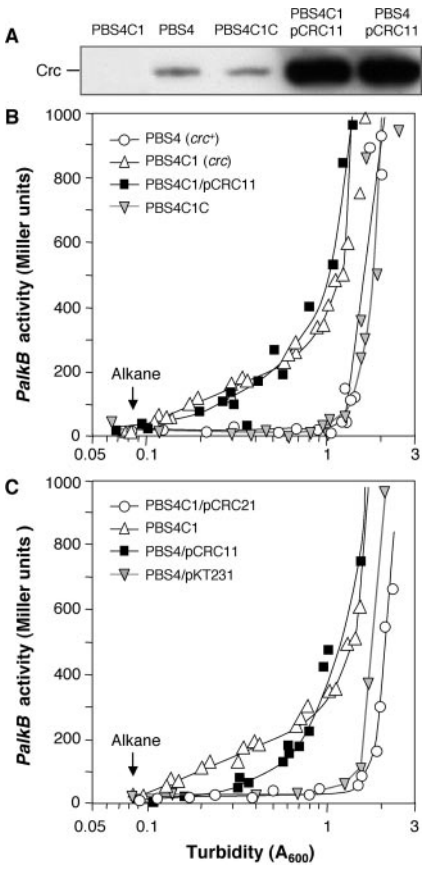

FIG. 4. Effect of an increased gene dosage on the ability of Crc to inhibit induction of the *alk* pathway. (A) Levels of Crc in strains PBS4 (wild type for *crc*; contains a *PalkB*::*lacZ* fusion and the *alkS* gene in the chromosome), PBS4C1 (derived from PBS4 by inactivation of *crc*), PBS4C1C (derived from PBS4C1 by insertion of a wild-type *crc* gene in its chromosome), PBS4C1 containing plasmid pCRC11 (a multicopy plasmid with a wild-type *crc* gene), and PBS4 containing plasmid pCRC11. Cells were grown in LB medium, collected at mid-exponential phase (A_{600} of 0.6), and disrupted by sonication; proteins were resolved in an SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane, and the presence of Crc was revealed with and antiserum directed against (His_6)Crc. (B, C) Activity of Crc in cells containing different Crc levels. Cells of the indicated strains were grown in duplicate flasks in rich LB medium, and at a turbidity of 0.08, DCPK was added up to 0.05% (vol/vol) to one of the flasks to allow the AlkS activator to induce expression of *lacZ* from promoter *PalkB*. The levels of β-galactosidase were determined at different time points as indicated by Miller (28). β-Galactosidase levels in the absence of DCPK were very low and are not indicated.

of the plasmid vector used (pKT231). The *crc* gene present in pCRC11 was sequenced, and no mutations were observed. Plasmid pKT231 was introduced into strain PBS4, but it did not interfere with normal Crc function (Fig. 4C). To further analyze this problem, the *crc* gene was excised from pCRC11 as a BamHI DNA fragment and cloned into the BglII site of the low-copy-number broad-host range plasmid pRK2501, which has about three to four copies per cell (22). Introduction of the resulting plasmid (pCRC21) into strain PBS4C1 led to Crc levels very similar to those of wild-type strain PBS4, as judged by Western blot assays (not shown). Repression of the *alk* pathway in the presence of plasmid pCRC21 was as efficient as that in strain PBS4 (Fig. 4C). We conclude that the presence of 3 to 4 copies of the *crc* gene does not interfere with its function, while increasing the copy number to about 15 to 20 leads to a loss of its repression efficiency. In other words, the inhibitory effect of Crc is reduced or lost when the protein is present in amounts that exceed a certain level.

Mutations at Crc residue Asp_{220} or His_{246} generate active proteins. The use of BLAST (1) and PFAM (3) software (http://ncbi.nlm.nih.gov/BLAST/) shows that Crc is related to the endonuclease-exonuclease-phosphatase family of proteins, which includes Mg^{2+}-dependent endonucleases and a large number of phosphatases involved in intracellular signaling (14). The highest similarity scores correspond to exonuclease III from several bacterial species (67%, 54%, and 43% similarity, respectively, to that of *Coxiella burnetii*, *Bacillus subtilis*, and *E. coli*). Proteins from this family have a striking conservation of the catalytic residues of apurinic/apyrimadinic endonucleases (14), which correspond to Asp_{220} and His_{246} in Crc. To investigate the role of these residues, Asp_{220} was changed to Ala by site-directed mutagenesis, while His_{246} was changed to Glu. The genes encoding these mutant proteins, named *crc(D220A)* and *crc(H246E)*, were individually introduced into the chromosome of the *crc*-deficient strain PBS4C1 via the mini-Tn*5*Sm transposon. The strains obtained were named PBS4C1CA and PBS4C1CE, respectively. Induction of promoter *PalkB* in these two strains by alkanes was severely inhibited during exponential growth in LB medium, indicating that *crc(D220A)* and *crc(H246E)* can complement the *crc*::*tet* mutation present in PBS4C1 (Fig. 5B). Therefore, these two residues are not essential for Crc function. Interestingly, mutant proteins Crc(D220A) and Crc(H246E) were also active when their corresponding genes were introduced into PBS4C1 via a multicopy plasmid, which led to elevated levels of the two proteins (Fig. 5A). It should be noted, however, that mutant protein Crc(D220A) appeared to be less stable than the wild-type Crc, since the protein levels detected were lower and some degradation bands were detected (Fig. 5A). Introduction of the multicopy plasmids expressing these mutant proteins into strain PBS4, which contains a wild-type *crc* allele, did not interfere with the normal function of wild-type Crc, since inhibition of *PalkB* induction during exponential growth was efficient (Fig. 5D). However, inhibition ceased when cells entered into the stationary phase (at turbidity values above 1.5), in spite of the fact that the levels of the mutant Crc proteins were still high. As will be discussed below, these results suggest that repression by Crc depends not only on its levels in the cell but also on its activity and/or on its cooperation with additional factors.

To analyze whether Crc(H246E) could inhibit induction of the *alk* pathway in a nonrepressing medium, strain PBS4C1CE, which contains *crc(H246E)* inserted in the chromosome, or PBS4C1 containing plasmid pCRC11H_{246}E was grown in a minimal salts medium with citrate as a carbon source. Expression of β-galactosidase from the *PalkB* promoter after the addition of an inducer of the *alk* pathway was very efficient in the two strains, reaching levels similar to those observed in strain PBS4 grown in the same medium (not shown). Therefore, mutant protein Crc(H246E) cannot repress the *alk* pathway in this nonrepressing medium.

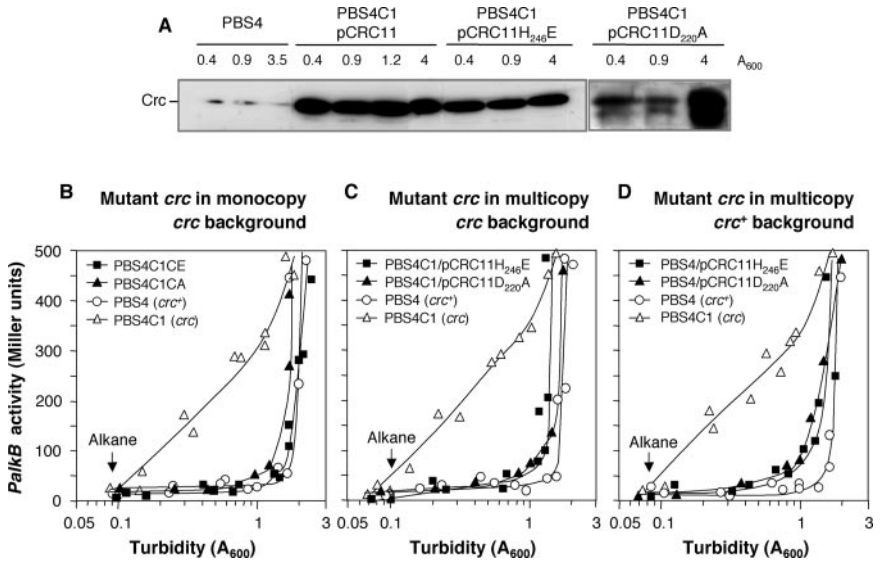

FIG. 5. Ability of mutant proteins Crc(H246E) and Crc(D220A) to inhibit the induction of promoter *PalkB*. (A) The levels of Crc in strain PBS4 and strain PBS4C1 containing plasmid pCRC11, pCRC11H_{246}E, or pCRC11D_{220}A were determined by Western blots as indicated in Fig. 4. (B, C, D) Strains PBS4 (wild type for *crc*), PBS4C1 (*crc* derivative of PBS4), PBS4C1CE [PBS4C1 containing *crc(H246E)* inserted into the chromosome], PBS4C1CA [PBS4C1 containing *crc(D220A)* inserted in the chromosome], and PBS4C1 containing the multicopy plasmid pCRC11H_{246}E [which bears the *crc(H246E)* gene] or pCRC11D_{220}A [which bears the *crc(D220A)* gene] were grown in LB medium. At a turbidity of 0.08, DCPK was added to 0.05% (vol/vol) to induce expression of *lacZ* from promoter *PalkB*. The levels of β-galactosidase were determined at different time points as indicated by Miller (28). β-Galactosidase levels in the absence of DCPK were very low and are not represented.

DISCUSSION

Previous reports have shown that induction of the *alk* pathway by alkanes is severely inhibited when cells grow exponentially in rich LB medium, repression that is partly dependent on Crc and which rapidly diminishes when cells reach stationary phase (44, 45). The results presented here show that in cells growing in rich LB medium, transcription of the *crc* gene varies according to the growth phase, being three- to fourfold more efficient during exponential growth than in stationary phase. Similarly, the levels of the Crc protein are four- to fivefold higher in mid-exponential phase than in the stationary phase of growth. Therefore, Crc levels are high during the phase of growth in which there is a high repression of the *alk* pathway and decrease when repression is no longer observed. When cells were grown in minimal salts medium containing citrate as a carbon source, where there is no repression of the *alk* pathway, transcription of *crc* and the amounts of Crc protein observed were also higher during exponential growth and decreased at the onset of the stationary phase. However, maximal Crc levels in this growth medium were about 2.5-fold lower than that in rich LB medium. A similar result was observed when cells were grown in spent LB medium, in which catabolic repression is very weak (44). Therefore, under the conditions analyzed, there is a clear correlation between the Crc levels and the degree of repression of the *alk* pathway.

High Crc levels, however, do not guarantee an efficient repression of the *alk* pathway. The mutant *crc*::*tet* allele present in strain PBS4C1 could be complemented by a wild-type *crc* allele if the gene was introduced into the chromosome of this strain via a minitransposon or a low-copy-number plasmid. However, no complementation was observed if the *crc* gene was introduced by means of a high-copy-number plasmid, which led to Crc protein levels about 20-fold higher than when the gene was present in just one copy. Interestingly, introduction of the *crc*-containing multicopy plasmid into strain PBS4, which has a wild-type *crc* allele in the chromosome, interfered with the normal Crc function, considerably reducing the repression effect. In other words, the inhibitory effect of Crc is reduced or lost when the protein is present in amounts that greatly exceed its normal levels. It has been proposed that Crc is a component of a signal transduction pathway that senses nutritional signals (30). Taking this into account, the abovementioned results can be explained if we assume that Crc needs to be activated by a signal, or an upper element of the pathway, to inhibit induction of the *alk* pathway, the activating mechanism being a bottleneck that is overloaded or titrated out when large amounts of Crc are present. In this model, Crc would exist in two forms, one active and the other one inactive. When present in high amounts, the inactive form would predominate. Competition of both forms for its target under conditions in which the inactive form is more abundant would result in poor or no inhibition of the *alk* pathway. An Ala-for-Asp substitution at Crc position 220, or a Glu-for-His substitution at Crc position 246, yields mutant proteins that can inhibit induction of the *alk* pathway even when present in high amounts. The behavior of these Crc derivatives suggests that they no longer need to be activated to achieve their function. Alternatively, the levels of these mutant proteins, albeit high, do not reach levels high enough to disturb signal transduction. When present at high levels, these two mutant proteins could efficiently inhibit the *alk* pathway only during exponential growth but not when cells reached the stationary phase, where Crc(D220A) and Crc(H246E) levels were still very high. In addition, Crc(H246E) was active only in cells growing expo-

nentially in LB medium but not in cells grown in a nonrepressing medium. It is clear that under nonrepressing conditions, Crc cannot exert its function even if it contains mutations at residue D_{220} or H_{246}. This would be an expected behavior if Crc has not received a proper signal under these conditions or if it needs to transmit a signal to another component of the transmission pathway that is not present or is not active under nonrepressing conditions, or if Crc does not work alone at the final target. The nature of this hypothetical factor or signal is at present unknown.

The role of residues Asp_{220} and His_{246} in Crc activity is not clear at present. Similarity searches place Crc in a family that includes Mg^{2+}-dependent endonucleases and a divergent set of prokaryotic and eukaryotic proteins that include phosphatases involved in cell cycle regulation and signal transduction (14, 25). It has been proposed that this protein superfamily has an ancient phosphoesterase fold which served as a scaffold for a large set of functionally diverse proteins (14). Some residues are highly conserved in domains believed to surround the catalytic pocket. Crc residues Asp_{220} and His_{246} align with the catalytic residues of the Mg^{2+}-dependent endonucleases, which are invariably conserved in the family. However, our results clearly show that these residues are not essential for Crc function, suggesting that although Crc may have kept the folding characteristic of these endonucleases, it does not conserve an endonuclease activity. The similarity to endonucleases suggests that Crc may either bind to or act on nucleic acids. Purified Crc does not seem to bind DNA (10, 25). Its effect on the mRNAs generated at the *PalkB* and *PalkS2* promoters has not been studied in detail.

Crc similarity to phosphatases involved in regulation and signal transduction suggests that it may form part of a signal transmission cascade that includes components activated by phosphorylation or dephosphorylation. Protein kinase cascades are widely used in bacteria to sense and respond to external and internal signals (20). However, Crc shows no obvious similarity to known components of these transmission pathways, such as the classical two-component and His-Asp phosphorelay systems (20), the transcriptional regulators controlled by components of the PTS system through phosphorylation of a histidine residue (36), and the HWE histidine kinases (23). Therefore, the precise role of Crc residues Asp_{220} and His_{246}, as well as the molecular mode of action of Crc, cannot be predicted at this time.

In summary, our results indicate that the levels of Crc fluctuate according to growth conditions, although this cannot fully explain the changes in Crc activity observed. Available information suggests that Crc must receive a signal and probably transmit it to another unknown factor or must work in combination with this factor to repress gene expression.

ACKNOWLEDGMENTS

We are grateful to J. L. Martínez and G. Morales for critical reading of the manuscript.

This work was supported by grants BMC2003-00063 and GEN2001-4698-C05-01 from the Spanish Ministry of Education and Science. A.R.-M. was a recipient of a predoctoral fellowship from the Government of the Basque Country.

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