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The *Pseudomonas putida* Crc Global Regulator Controls the Expression of Genes from Several Chromosomal Catabolic Pathways for Aromatic Compounds

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The Crc protein is involved in the repression of several catabolic pathways for the assimilation of some sugars, nitrogenated compounds, and hydrocarbons in *Pseudomonas putida* and *Pseudomonas aeruginosa* when other preferred carbon sources are present in the culture medium (catabolic repression). Crc appears to be a component of a signal transduction pathway modulating carbon metabolism in pseudomonads, although its mode of action is unknown. To better understand the role of Crc, the proteome profile of two otherwise isogenic *P. putida* strains containing either a wild-type or an inactivated *crc* allele was compared. The results showed that Crc is involved in the catabolic repression of the *hpd* and *hmgA* genes from the homogentisate pathway, one of the central catabolic pathways for aromatic compounds that is used to assimilate intermediates derived from the oxidation of phenylalanine, tyrosine, and several aromatic hydrocarbons. This led us to analyze whether Crc also regulates the expression of the other central catabolic pathways for aromatic compounds present in *P. putida*. It was found that genes required to assimilate benzoate through the catechol pathway (*benA* and *catBCA*) and 4-OH-benzoate through the protocatechuate pathway (*pobA* and *pcaHG*) are also negatively modulated by Crc. However, the pathway for phenylacetate appeared to be unaffected by Crc. These results expand the influence of Crc to pathways used to assimilate several aromatic compounds, which highlights its importance as a master regulator of carbon metabolism in *P. putida*.

Expression of bacterial catabolic pathways is usually tightly controlled. Regulation can respond to the presence or absence of the compound to be assimilated (a specific control response) or to signals that link the induction of the pathway genes to the physiological status of the cell (a global control response). The global control is normally dominant over the specific control. One such global control mechanism is the so-called catabolic 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. Catabolic repression seems to operate through different mechanisms in different bacterial species. In pseudomonads, this process has been studied for some pathways responsible for the assimilation of sugars, amino acids, hydrocarbons and aromatic compounds (for reviews, see references 4, 5, and 27). However, the molecular mechanisms underlying catabolic repression in pseudomonads remain mostly unknown. The metabolism of hydrocarbons and aromatic compounds has attracted special attention because many of them are responsible for important pollution problems in the environment. Available evidence indicates that expression of the pathways for the assimilation of hydrocarbons and aromatic compounds is modulated by different kinds of global control signals, with catabolic repression being just one of them (8, 27).

Very few proteins have been shown to participate in catabolic repression in pseudomonads. The first to be described, Crc (for "catabolite repression control"), is involved in the catabolic repression generated by succinate or lactate on the expression of a number of genes implicated in the metabolism of some sugars and nitrogenated compounds. In *Pseudomonas aeruginosa*, genes regulated by Crc include those encoding glucose-6-phosphate dehydrogenase, glucokinase, 6-phosphogluconate dehydratase, 2-keto-3-deoxy-6-phosphogluconate aldolase, amidase, and the branched-chain keto acid dehydrogenase (5, 11, 20, 34). At least some of these genes (glucose-6-phosphate dehydrogenase, amidase, and branched-chain keto acid dehydrogenase) are controlled by Crc in *Pseudomonas putida* as well (11). When cells grow in a rich medium such as 2× YT or Luria-Bertani (LB), Crc also exerts a strong repression on the induction of the *P. putida* branched-chain keto acid dehydrogenase (11, 12) and of the alkane degradation pathway encoded in the OCT plasmid from *P. putida* GPo1 (36). Available data suggest that Crc would be a component of a signal transduction pathway modulating carbon metabolism as well as other phenomena such as biofilm development (12, 21, 25). Crc ultimately affects the expression of the regulated genes, although the precise molecular mechanism underlying this effect remains to be elucidated. Crc does not appear to bind DNA, suggesting that it is not a classical DNA-binding repressor, but its target has not been identified (5, 12, 20). At least in *P. putida*, the effect of Crc is particularly important in cells growing exponentially in a rich medium (8, 12, 36).

In an effort to better understand the role of the Crc protein on the regulation of bacterial metabolism, we have compared

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the proteome profile of a *P. putida* strain to that of an isogenic derivative in which the *crc* gene had been inactivated. This kind of global analysis had not been done before. The results indicate that Crc is involved in, among other things, the expression of genes belonging to several of the central chromosomal pathways for the assimilation of aromatic compounds.

MATERIALS AND METHODS

Bacterial strains and culture media. Strain PBS4 derives from *P. putida* KT2442 (9) by insertion in its chromosome of a *PalkB*::*lacZ* transcriptional fusion and of the *alkS* gene (35). *P. putida* KT2442 is a rifampin-resistant derivative of *P. putida* KT2440 (9). *P. putida* PBS4C1 derives from strain PBS4 by inactivation of the *crc* gene (contains a *crc*::*tet* allele) (36). Strains were grown at 30°C in LB medium (29) supplemented where indicated with 5 mM 4-hydroxybenzoate, 5 mM benzoate, or 5 mM phenylacetate. Cell growth was monitored by measuring turbidity at 600 nm.

Two-dimensional (2-D) electrophoresis and mass spectrum analysis. Twenty milliliters of exponentially growing cells (A_{600} of 0.6) were spun down at 4°C; resuspended in 60 μl of 0.3% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β-mercaptoethanol, and 50 mM Tris-HCl (pH 8); and boiled for 2 min. Samples were treated for 30 min on ice with a DNase I-RNase solution (final concentrations, 15 mg of DNase I/ml, 75 mg of RNase A/ml, 1 mM $MgCl_2$). Finally, 240 μl of a lysis buffer containing 6 M urea, 2 M thiourea, 4% (vol/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% (vol/vol) precast pH gradient buffer (pH 4 to 7; Amersham Biosciences, Uppsala, Sweden) and 2 mM Tris carboxy ethyl phosphine-HCl was added.

2-D electrophoresis was performed as described previously (10, 26) with precast immobilized pH 4 to 7 gradient (IPG) strips (18 cm in length; Amersham Biosciences) for the first dimension (isoelectric focusing [IEF]). Briefly, 100-μg protein samples were applied by anodic cup-loading of IPG strips previously rehydrated with 350 μl of rehydration buffer (6 M urea, 2 M thiourea, 2% [wt/vol] CHAPS, 0.5% [vol/vol] of the corresponding carrier ampholytes, 1 mM Tris carboxy ethyl phosphine-HCl, and minute amounts of bromophenol blue) for at least 10 h. Focusing was carried out with the IPG Phor (Amersham Biosciences) by applying an increasing voltage as follows: 200 V for 1 h; gradient increases from 200 to 3,000 V for 3 h, 3,000 to 6,000 V for 2 h, and 6,000 to 8,000 V for 1 h; and constant 8,000 V until a total of 60,000 V · h was reached. After IEF separation, the strips were equilibrated two times for 20 min with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, and trace amounts of bromophenol blue. The first equilibration solution contained 2% (wt/vol) dithiothreitol. The second equilibration solution contained 4% (wt/vol) iodoacetamide. The second-dimension (SDS-polyacrylamide gel electrophoresis) was performed with 1-mm-thick, 16- by 15-cm, 12.5, 10, or 8% (wt/vol) homogeneous polyacrylamide gels, and electrophoresis was carried out overnight at constant current (5 mA/gel) and temperature (5°C). After electrophoresis, gels were stained with the mass spectrometry-compatible modified PlusOne silver-staining protein kit (Amersham Biosciences).

In-gel digestion of proteins and matrix-assisted laser desorption ionization (MALDI) peptide mass fingerprinting. Protein spots were excised manually and then processed automatically with an Investigator ProGest protein digestion station (Genomic Solutions, Huntingdon, Cambridgeshire, United Kingdom) (16). The digestion protocol used was as described earlier (30) with minor variations. Gel plugs were washed with 25 mM ammonium bicarbonate and acetonitrile prior to reduction with 10 mM dithiothreitol in 25 mM ammonium bicarbonate, and alkylation was done with 100 mM iodoacetamide in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, Wis.) at a final concentration of 16 ng/μl in 25 mM ammonium bicarbonate was added to the dry gel pieces, and the digestion proceeded at 37°C for 12 h. Peptides were eluted with 25 mM ammonium bicarbonate and 0.1% (vol/vol) trifluoroacetic acid for a final extraction volume of 50 μl.

For MALDI peptide mass fingerprinting, a 0.3-μl aliquot of matrix solution (5 g of 2,5-dihydroxybenzoic acid/liter in 33% [vol/vol] aqueous acetonitrile and 0.1% [vol/vol] trifluoroacetic acid) was manually deposited onto a 400-μm-diameter AnchorChip MALDI target and allowed to dry at room temperature. Then, 0.3 μl of the above-described extraction solution was added and allowed to dry at room temperature. Samples were measured on a Reflex IV MALDI-time of flight mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT source in positive-ion reflector mode with delayed extraction. The ion acceleration voltage was 20 kV. The equipment was first externally calibrated by employing protonated mass signals from a peptide mixture covering the 1,000 to 3,500 *m/z* range, and thereafter, every spectrum was internally calibrated by using selected signals arising from trypsin autoproteolysis to reach a typical mass measurement accuracy of ±30 ppm. The measured tryptic peptide masses were transferred through the BioTools program as inputs to search either the National Center for Biotechnology Information nonredundant database or a *P. putida* KT2440 database (23) by using Mascot software (Matrix Science, London, United Kingdom). No restrictions were placed on the species of origin of the protein, and the allowed protein molecular mass was 1 to 200 kDa. Up to one missed tryptic cleavage was considered, and a mass accuracy of 50 ppm was used for all tryptic mass searches.

RT-PCR assays. Exponentially growing cells (A_{600} of 0.6) were collected, spun down at 4°C, and frozen in dry ice at −80°C. Total RNA was extracted by using the phenol-guanidine thiocyanate mix Tri Reagent LS (Molecular Research Center, Inc.). Residual DNA was removed by treatment with DNase I. Reverse transcriptase (RT)-PCR assays were performed by using Ready-To-Go RT-PCR beads (Amersham Biosciences) as indicated by the manufacturer, primers specific for the desired genes, and serial 10-fold dilutions of the RNA (1, 0.1, and 0.01 μg) to ensure a linear response. To ascertain that no residual DNA was present in the RNA preparations, a PCR was performed with the same primers and overall conditions, except that no RT was added.

Determination of benzoate, 4-OH-benzoate, and phenylacetate. Benzoate, 4-OH-benzoate, and phenylacetate utilization by whole cells was monitored by measuring their concentrations in culture supernatants by high-performance liquid chromatography (HPLC). The column used was a reverse-phase octyldecyl silane hypersil C_{18} (124 by 4 mm), and the mobile phase contained 60% (vol/vol) 11 mM H_3PO_4 and 40% (vol/vol) methanol. The absorbance of the eluate was monitored at 254 nm.

RESULTS AND DISCUSSION

Influence of Crc on the proteome of *P. putida* cells growing exponentially in LB medium. To have a global view of the effect of the Crc regulator on the expression of the *P. putida* proteome, the total proteins of *P. putida* strain PBS4 and of its isogenic *crc* mutant derivative PBS4C1 were analyzed by 2-D electrophoresis. Cells were collected while actively growing in rich medium (A_{600} = 0.6), since, at least in the case of the OCT plasmid alkane degradation pathway, the repression effect of Crc is observed only during the exponential phase of growth (36). Inactivation of the *crc* gene led to a clear increase in the intensity of 11 protein spots in the 2-D gels and to a decrease in the amount of two proteins (of 315 spots detected). Figure 1 shows selected areas of the 2-D gels where such changes were detected; only those spots whose intensity reproducibly changed in at least three independent assays are marked. To identify the nature of the spots whose intensities varied by inactivation of *crc*, each spot was excised from the stained gels and digested with trypsin, and the peptides generated were resolved by MALDI-time of flight mass spectrometry. The peptide patterns were compared to those of a virtual digestion of each protein encoded by the *P. putida* KT2440 genome, whose sequence has been determined (23) (www.tigr.org), or to digestions of the proteins included in the National Center for Biotechnology Information nonredundant database by using the MASCOT software.

As detailed in Table 1, spots 1 and 2, which where present in the *crc*$^+$ strain but absent in the *crc* mutant strain, showed the highest scores with *P. putida* KT2440 cysteinyl-tRNA synthetase (CysS) and malate-quinone oxidoreductase 2 (Mqo-2), respectively. The *P. putida* KT2440 genome encodes three malate-quinone oxidoreductases (Mqo-1, Mqo-2, and Mqo-3), which are very similar in sequence (23). Malate-quinone oxidoreductase is an enzyme of the citric acid cycle-glyoxylate cycle, and it transforms malate into oxaloacetate. In *Esche-*

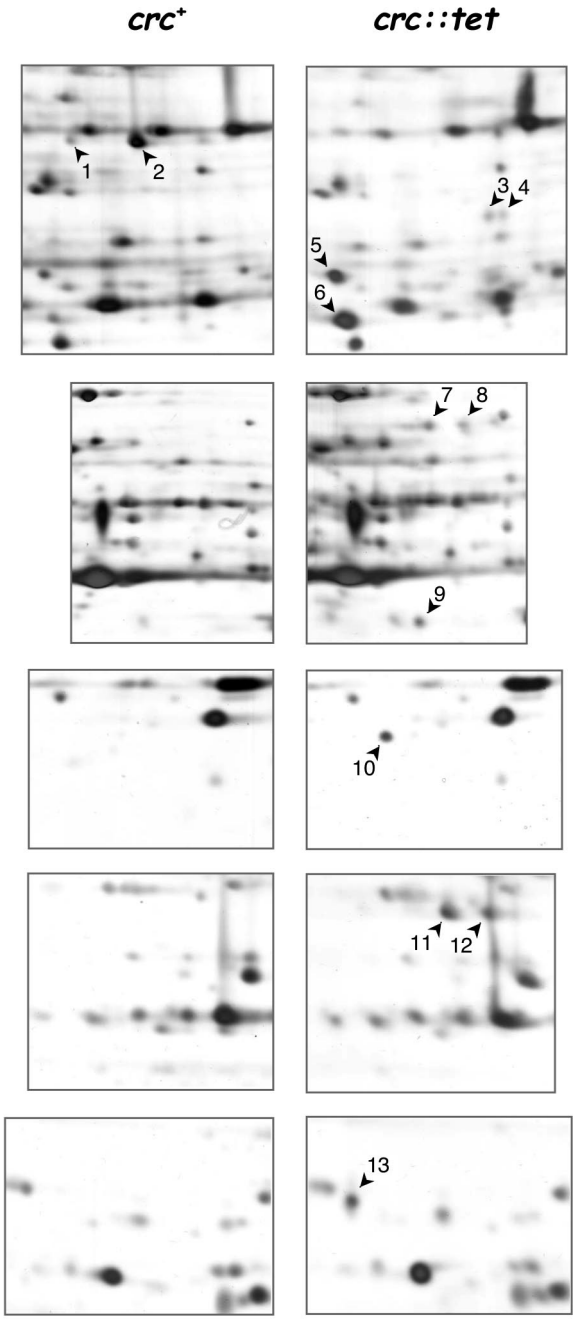

FIG. 1. Proteomic analysis of the effect of Crc in *P. putida* cells growing exponentially in LB medium. Total proteins obtained from strains PBS4 (wild type for *crc*) and PBS4C1 (contains an inactivated *crc* allele), exponentially growing in LB medium, were resolved by 2-D electrophoresis. Proteins were first separated according to their isoelectric point by IEF and then resolved through a 12.5% (wt/vol) denaturing polyacrylamide gel. Protein spots were revealed by silver staining. The figure shows areas of the 2-D gels containing spots whose intensity reproducibly decreased or increased by inactivation of *crc* in at least three independent assays. These proteins were excised from the gel and identified by mass spectrometry; the results are indicated in Table 1. Panels on the left correspond to strain PBS4 (*crc*$^+$) while those on the right correspond to strain PBS4C1 (*crc*::*tet*).

TABLE 1. Identification of the protein spots that increase or decrease in intensity upon inactivation of the *crc* gene[a]

Spot(s)	Protein (gene) with best homology[b]	Effect of Crc[c]
1	Cysteinyl-tRNA synthetase (*cysS*)	Stimulation
2	Malate-quinone oxidoreductase 2 (*mqo-2*)	Stimulation
3 and 4	Homogentisate 1,2 dioxygenase (*hmgA*)	Repression
5	Branched-chain amino acid ABC transporter (*braC*)	Repression
6 and 9	Sugar ABC transporter (PP1015)	Repression
7 and 8	Oxaloacetate decarboxylase alpha (*oadA*)	Repression
10	Probable CoA transferase, subunit A (PP3122)	Repression
11 and 12	4-Hydroxyphenylpyruvate dioxygenase (*hpd*)	Repression
13	Probable CoA transferase, subunit B (PP3123)	Repression

[a] Spots indicated in Fig. 2 were excised from the gel and subjected to MALDI peptide mass fingerprinting.

[b] Protein showing the best score. Score is $-10 \cdot \mathrm{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores higher than 50 are significant. Only those higher than 80 were taken into account. All proteins belong to *P. putida* KT2440.

[c] The intensity of spots 5 and 6 increased by about three- and eightfold, respectively, upon inactivation of *crc*. Spots 1 and 2 were undetectable in the strain lacking Crc, and spots 3, 4, and 7 to 13 were undetectable in the strain having a wild-type *crc* gene.

richia coli, the activity of malate-quinone oxidoreductase is regulated by the global regulator ArcA, the carbon source used, and by the growth phase (33). Its regulation in pseudomonads is not so well characterized. In the case of *P. aeruginosa*, this enzyme has been shown to be essential for growth at the expense of ethanol or acetate (18). Our results show that the levels of Mqo-2 in *P. putida* are under the influence of Crc.

Spots 3 and 4, which were visible only when the *crc* gene was inactivated, were both identified as homogentisate 1,2 dioxygenase (HmgA). Therefore, the two spots are probably isoforms of the same enzyme with a slightly different pI. HmgA is the key enzyme of the homogentisate pathway, one of the central pathways for the catabolism of aromatic compounds in *P. putida* and several other bacteria (17) (Fig. 2). Interestingly, spots 11 and 12, which were also visible only in the gel corresponding to the *crc* mutant strain, were identified as two isoforms of 4-hydroxyphenylpyruvate dioxygenase (Hpd). This enzyme hydroxylates 4-hydroxyphenylpyruvate to render homogentisate, which is then cleaved by HmgA (Fig. 2). The *hpd* and *hmgA* genes map separately in the *P. putida* KT2440 chromosome (17). It is worth noting that the aromatic amino acids phenylalanine and tyrosine, which are potential carbon and nitrogen sources considering that cells were grown in LB medium, are metabolized through the homogentisate pathway after conversion to 4-hydroxyphenylpyruvate (Fig. 2) (17).

Spot 5, whose intensity increased about threefold upon inactivation of *crc*, showed the highest homology to BraC. In *P. aeruginosa*, this protein has been characterized as the periplasmic amino acid-binding component of the high affinity LIV-I transport system for alanine, threonine, and branched-chain amino acids (13). This transport system is encoded by the *braCDEFG* operon (14). In both *P. putida* and *P. aeruginosa*, growth in the presence of branched-chain amino acids induces the expression of the *bkd* operon, which encodes a keto acid dehydrogenase that allows their assimilation (32). Expression of this operon is regulated by catabolite repression, an effect that is at least in part mediated by the Crc protein (11). Induction of the *bkd* operon in a *crc* mutant strain grown in a rich

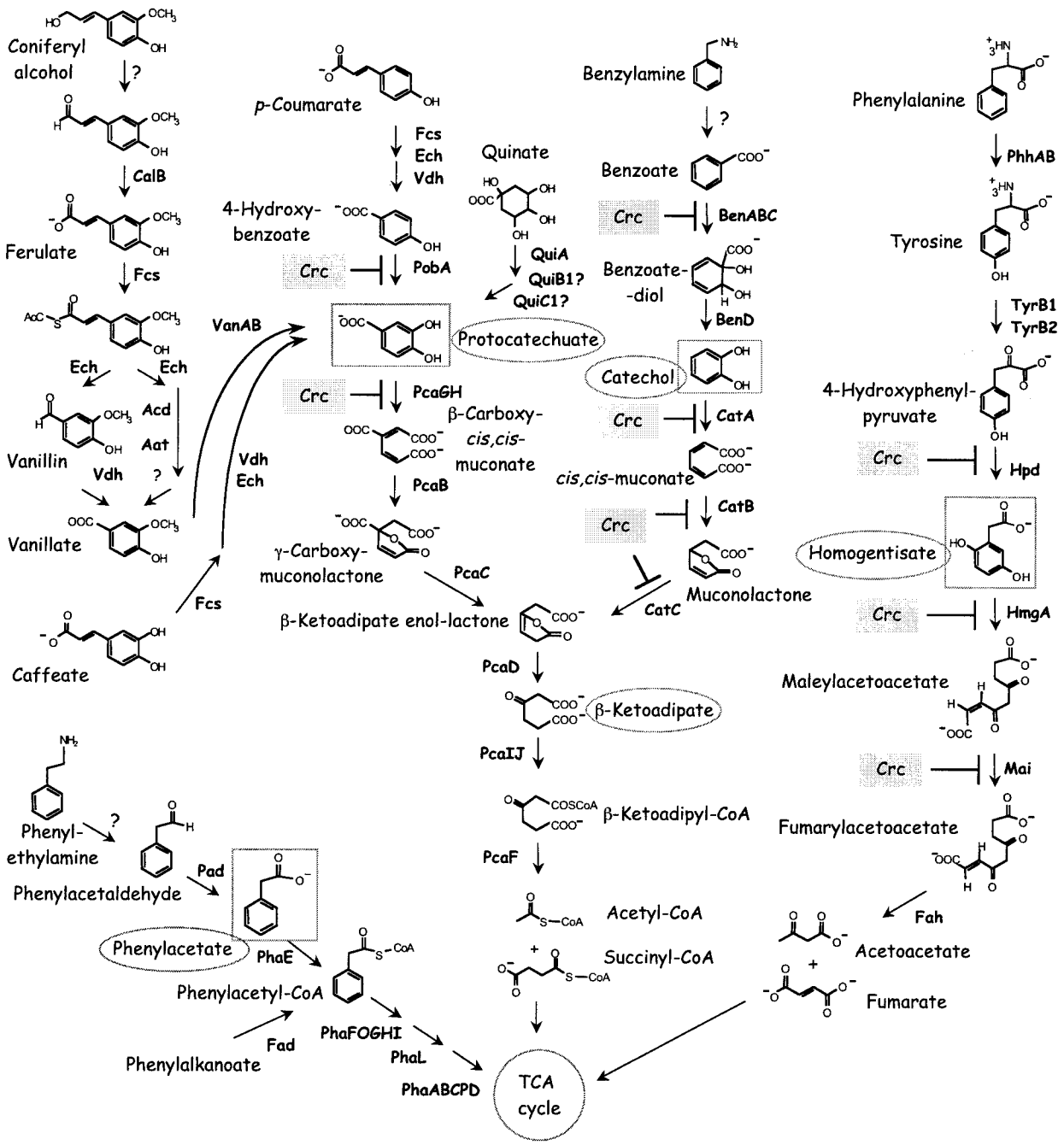

FIG. 2. Catabolic pathways for aromatic compounds identified in *P. putida* KT2440: effect of Crc. The pathways are those reported in reference 17. The key intermediate compounds of the homogentisate, catechol, protocatechuate, and phenylacetate pathways are highlighted. Genes whose expression is repressed by Crc as shown in this report are indicated. The scheme was modified from reference 17 with permission.

medium is, however, low, even if the medium is supplemented with valine and isoleucine (12). This probably explains why we were unable to detect the polypeptides of the branched-chain keto acid dehydrogenase in our 2-D gels. However, our results clearly show that Crc controls the expression of the transport system for branched-chain amino acids, an observation that, to our knowledge, had not been reported before.

Spots 6 and 9 corresponded to two isoforms of open reading frame PP1015, identified as the periplasmic sugar-binding component of a sugar ABC transporter. Spot 6 increased by about eightfold upon inactivation of *crc*, whereas spot 9 was almost undetectable in the strain containing a wild-type *crc* allele. As detailed in the introduction, Crc is involved in the repression of a number of genes implicated in the oxidation of some sugars in *P. aeruginosa* and *P. putida*. Our finding that Crc also regulates the expression of components of the sugar transporters is consistent with these observations and highlights the importance of Crc in the regulation of carbohydrate

metabolism in *P. putida*. The precise role of regulation of open reading frame PP1015 has, to our knowledge, not been reported.

Spots 7 and 8 were identified as two isoforms of OadA, the alpha subunit of oxaloacetate decarboxylase. This enzyme catalyzes the decarboxylation of oxaloacetate to pyruvate and CO_2 (7). Oxaloacetate decarboxylase is formed by three subunits, alpha, beta, and gamma, encoded by the *oadGAB* genes (19). This enzyme has been studied mainly in *Klebsiella pneumoniae*, where it participates in citrate fermentation under anaerobic conditions. Expression of the oxaloacetate decarboxylase in *K. pneumoniae* is subject to catabolite repression by the CRP protein (22). A protein showing high similarity to *E. coli* and *K. pneumoniae* CRP is present in *P. aeruginosa* and in *P. putida* and has been called Vfr (1). Evidence gathered to date indicates that Vfr is a global regulator of gene expression. However, it is not involved in catabolite repression but in regulation of the quorum-sensing response (1, 31). It is interesting that expression of oxaloacetate decarboxylase is regulated by catabolic repression in both *P. putida* and *K. pneumoniae* but through different global regulatory proteins. This observation agrees with the idea that there are probably diverse alternative strategies for reaching the same final regulatory response, with the only important requisite being that they all allow for a suitable responsiveness to the proper specific and global regulation signals (3).

Spots 10 and 13 corresponded to subunits A and B, respectively, of a probable coenzyme A (CoA) transferase, whose role is unknown at present.

Among the spots whose intensity varied upon inactivation of *crc*, we did not detect those of several proteins that are known to be regulated by Crc, such as branched-chain keto acid dehydrogenase, glucose-5-phosphate dehydrogenase, and amidase (11, 12). This result is to be expected for proteins which are present in amounts below detection limits, which are not induced under the growth conditions used, or which have a pI or a molecular mass that falls outside the range resolved by the 2-D gels used.

Influence of Crc on expression of the *P. putida* aromatic catabolic pathways. The aerobic catabolism of aromatic compounds follows a number of convergent pathways that lead to formation of a few key central intermediates that are subsequently cleaved by specific dioxygenase enzymes (Fig. 2). In *P. putida* KT2440, the identified chromosomally encoded aromatic pathways are the homogentisate pathway, the catechol pathway, the protocatechuate pathway, and the phenylacetate pathway (17). The catechol pathway eventually converges into the protocatechuate pathway (Fig. 2). The proteomic analysis described above indicated that Crc represses the expression of Hpd and HmgA from the homogentisate pathway. It is conceivable that Crc could also affect other catabolic pathways for aromatic compounds. Expression of these pathways is induced by the corresponding substrates (or their metabolites). The cells utilized in the proteomic analyses described above were grown in LB medium, so that the amino acids phenylalanine and tyrosine that can be obtained from it allow induction of the homogentisate pathway (Fig. 2). However, the catechol, protocatechuate, and phenylacetate pathways are not expected to be active in this growth medium unless the proper substrates are added, making it unlikely that spots corresponding to these pathways can be visualized in the 2-D gels shown in Fig. 1.

To analyze whether the catechol, protocatechuate, and phenylacetate catabolic pathways are also under the influence of Crc, strains PBS4 and PBS4C1 were grown in LB medium in the absence or presence of either 5 mM benzoate (catabolized through the catechol pathway) (Fig. 2), 5 mM 4-hydroxybenzoate (catabolized through the protocatechuate pathway), or 5 mM phenylacetate (catabolized through the phenylacetate pathway). When cultures reached a turbidity of 0.6 (mid-exponential phase), total RNA was obtained and the level of expression of the genes encoding key enzymes of the mentioned catabolic pathways was analyzed by RT-PCR. To compare the expression levels of each gene in the two strains used, the RT-PCR was performed with serial dilutions of the total RNA purified, and the RNA levels of a *crc*-independent gene were analyzed in parallel as an external control. The *npt* gene encoding resistance to kanamycin was used for this purpose, since it is present in both strains and is expressed at constant levels from a −10 extended promoter recognized by the vegetative RNA polymerase. As a first approach, the mRNA levels of the *hpd* and *hmgA* genes were analyzed in cells growing in LB medium. The proteomic analyses described above had indicated that the levels of the Hpd and HmgA proteins are undetectable in the strain containing a functional Crc protein but increase considerably upon inactivation of the *crc* gene. However, it was not known whether Crc should influence transcription of *hpd* and *hmgA*. The RT-PCR analysis showed that the mRNA levels corresponding to *hpd* and *hmgA* were clearly higher in the *crc* mutant strain than in the parental strain (Fig. 3). This result suggests that Crc regulates the levels of Hpd and HmgA proteins by interfering directly or indirectly with the transcription of the corresponding genes. The *hmgA* gene maps immediately upstream from the *fah* and *mai* genes, which encode enzymes that transform the product of homegentisate cleavage into acetoacetate and fumarate (Fig. 2 and 3). RT-PCR assays showed that Crc controls *mai* expression as well (Fig. 3). To our knowledge, a detailed analysis of the expression of the *hmgA*, *fah*, and *mai* genes has not been reported. However, it would not be surprising to find that they are cotranscribed. If this was the case, Crc controls expression of the three genes.

Growth of *P. putida* PRS2000 in a minimal salts medium containing benzoate as a carbon source leads to the induction of the *benABC*, *benD*, and *catBCA* genes (15). *BenABC* and *benD* encode a benzoate dioxygenase and a dehydrogenase that converts benzoate into catechol, which is further transformed by the products of the *catBCA* genes to render β-ketoadipate-enol-lactone (Fig. 2). Expression of *benABC* is induced by benzoate by means of the BenR transcriptional activator (6), and expression of *catBCA* is activated by the CatR activator in the presence of *cis*,*cis*-muconate, which is produced from catechol by the action of CatA. These genes are also present in the *P. putida* KT2440 genome (17). The *benA*, *catA*, *catB*, and *catC* genes were selected for RT-PCR analysis. As shown in Fig. 3, the mRNA levels corresponding to these four genes were considerably higher in the *crc* mutant strain PBS4C1 than in the parental strain PBS4, suggesting that Crc regulates their expression, exerting an inhibitory effect when cells are grown in LB medium containing benzoate.

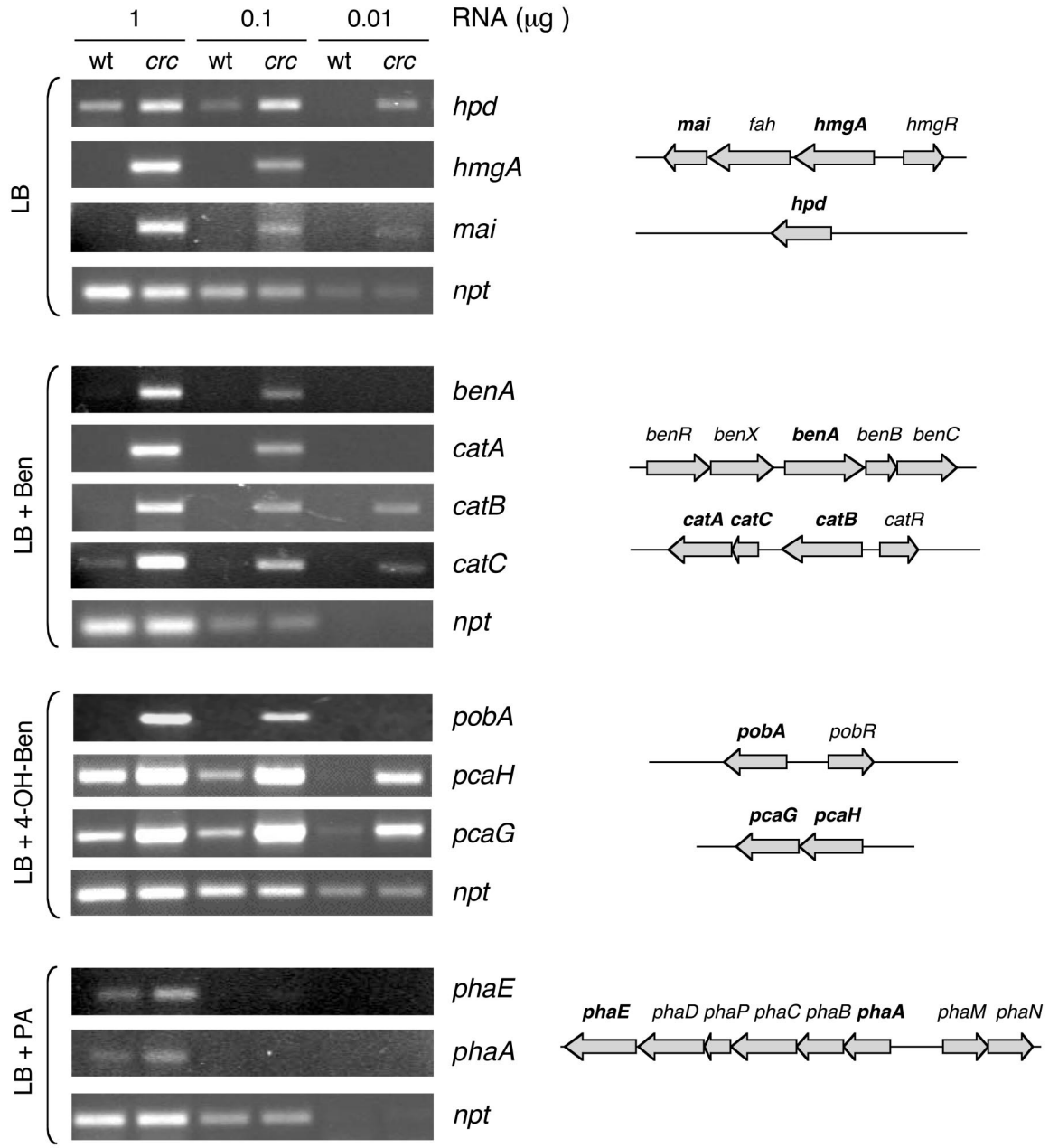

FIG. 3. RT-PCR analysis of the effect of Crc on the expression of the homogentisate, benzoate, protocatechuate, and phenylacetate pathways. Serial dilutions of total RNA isolated from strains PBS4 or PBS4C1 growing exponentially in LB medium or in LB medium supplemented with 5 mM benzoate (LB+Ben), 4-hydroxybenzoate (LB + 4-OH-Ben), or 5 mM phenylacetate (LB+PA) were subjected to RT-PCR with primers designed to detect the mRNA from the *hpd*, *hmgA*, *mai*, *benA*, *catA*, *catB*, *catC*, *pobA*, *pcaH*, *pcaG*, *phaE*, or *phaA* gene. Expression of the *npt* gene, specifying resistance to kanamycin (present in the chromosome of PBS4 and PBS4C1), was analyzed as a control to ensure both the linearity of the response and that the amounts of RNA from the two strains were comparable. Control reaction mixtures to which no RT was added yielded no amplification product (data not shown). wt, wild type.

Assimilation of 4-hydroxybenzoate by *P. putida* PRS2000 requires the expression of *pobA*, which encodes a hydroxylase that transforms 4-hydroxybenzoate into protocatechuate; the enzymes encoded by the *pca* genes further transform protocatechuate into acetyl-CoA and succinyl-CoA (28) (Fig. 2). The *pobA* gene is present in many *Pseudomonas* and *Acinetobacter* strains, where its expression is activated by the PobR (or PobC) activator in the presence of 4-hydroxybenzoate (reference 2 and references therein). The *pca* genes are arranged in four different clusters, *pcaHG*, *pcaBDC*, *pcaIJ*, and *pcaF* (reference 28 and references therein). With the exception of *pcaHG*, which is induced by protocatechuate, the remaining genes of the regulon are induced by β-ketoadipate through the PcaR transcriptional regulator (28). All these genes are present in *P. putida* KT2440 (17). As shown in Fig. 3, the mRNA levels corresponding to *pobA*, *pcaH*, and *pcaG* were clearly

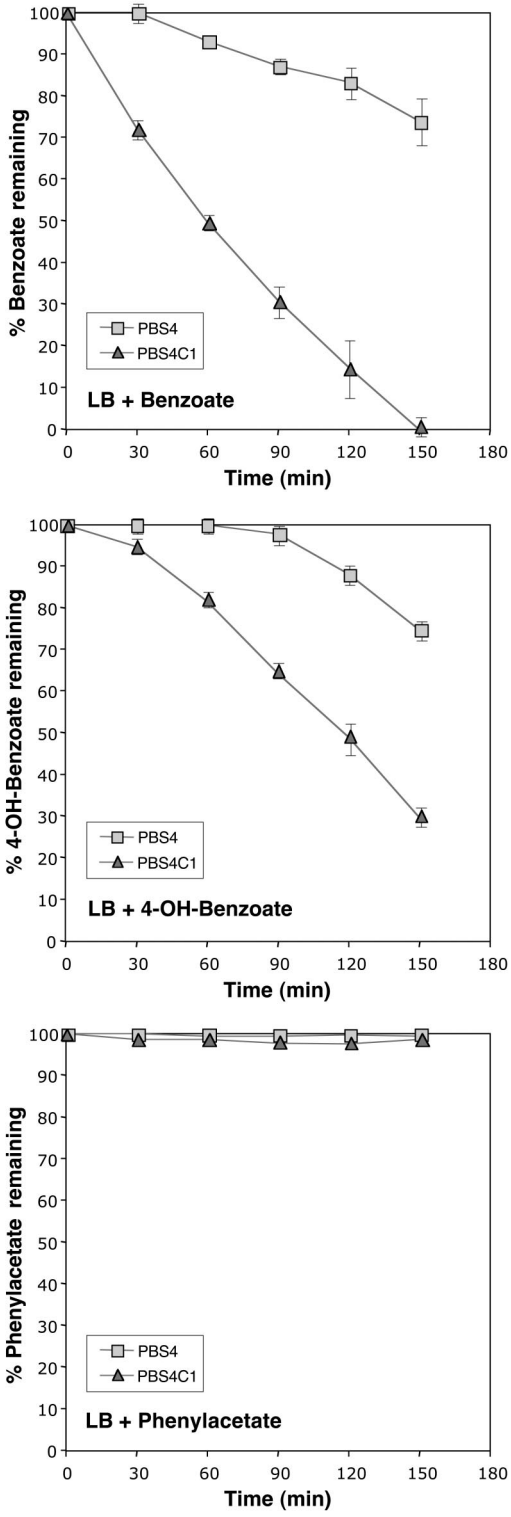

FIG. 4. Utilization of benzoate, 4-hydroxybenzoate or phenylacetate by cells of strains PBS4 and PBS4C1 grown in LB medium. Cells were grown in LB medium in the presence of 5 mM benzoate, 5 mM 4-hydroxybenzoate, or 5 mM phenylacetate. At a turbidity of 0.8 (A_{600}), cells were collected and resuspended in LB medium containing the corresponding aromatic compound at a concentration of 5 mM. Incubation was continued with aeration at 30°C. At different times, samples were collected and the amount of the aromatic compound remaining in the culture supernatant was determined by HPLC. Values correspond to the averages of the results from two independent assays in which the amount of the indicated compound at each time point was measured at least three times.

higher in the *crc* mutant strain PBS4C1 than in the parental strain PBS4. Therefore, *pobA* and *pcaHG* apparently belong to the Crc regulon as well.

Phenylacetate is produced from the oxidation of several other related compounds by a number of genes that conform to the phenylacetyl-CoA catabolon (24). Assimilation of phenylacetate requires 14 genes organized in three contiguous operons, the expression of which is induced in the presence of phenylacetate (24). The possible influence of Crc on expression of the phenylacetate pathway was also investigated, monitoring the mRNA levels of the *phaE* and *phaA* genes, which specify the phenylacetyl-CoA ligase and the enoyl-CoA hydratase isomerase I, respectively. Expression of these two genes was rather similar in both the absence and presence of Crc (Fig. 3), which suggests that Crc does not control their expression. However, the mRNA levels detected under the experimental conditions used were very low. Therefore, conclusions on the expression of these genes should be made with caution (see below).

Influence of Crc on the assimilation of benzoate, 4-OH-benzoate, and phenylacetate. As a final way to investigate the influence of Crc on the catechol, protocatechuate, and phenylacetate pathways, the ability of cells containing a wild-type or an inactivated *crc* allele to assimilate these compounds was determined. To this end, strains PBS4 and PBS4C1 were grown in LB medium supplemented with benzoate (assimilated through the catechol pathway), 4-hydroxybenzoate (assimilated through the protocatechuate pathway), or phenylacetate. Exponentially growing cells were collected and resuspended in LB supplemented with the corresponding aromatic compound at a concentration of 5 mM. The consumption of each aromatic compound was monitored as a function of time by HPLC. As shown in Fig. 4, the wild-type strain PBS4 was very inefficient at removing benzoate from the culture medium, since 87% of the benzoate still remained in the culture supernatant after a 90-min incubation. However, in the case of the *crc* mutant strain PBS4C1, only 30% of the initial benzoate could be detected after the same incubation time. One hour later (minute 150), all benzoate had been consumed by strain PBS4C1, whereas in the case of the wild-type strain, about 75% of the compound remained unused in the culture supernatant. This result is consistent with the RT-PCR assays, which indicated that in LB medium, the presence of benzoate leads to efficient activation of the *benA* and *catA* genes only in the *crc* mutant strain and not in the parental strain.

Inactivation of Crc clearly also stimulated the removal of 4-OH-benzoate from the culture media, although its consumption was somewhat slower (Fig. 4). This is again consistent with the RT-PCR assay results shown in Fig. 3. Under the same conditions, phenylacetate was metabolized by neither the wild-type nor the *crc* mutant strain (Fig. 4). Both of them, however, could efficiently grow in a minimal salts medium containing phenylacetate as the sole carbon source. These results suggest that the phenylacetate pathway is induced poorly, if at all, in

cells growing in LB supplemented with phenylacetate, an idea that agrees with the low expression of the *phaA* and *phaE* genes observed in the RT-PCR assays whose results are shown in Fig. 3. In summary, the metabolism of phenylacetate in LB medium seems to be inhibited, possibly by catabolite repression, although Crc does not seem to be involved in the process.

The work presented here shows that the expression of key genes to assimilate 4-hydroxyphenyl pyruvate, benzoate, and 4-OH-benzoate through the homogentisate, catechol, and protocatechuate pathways, respectively, is controlled by the Crc global regulatory protein in *P. putida*. However, the pathway for phenylacetate does not seem to be regulated by Crc. The assimilation of many different aromatic compounds converges to the homogentisate, catechol, and protocatechuate pathways, both in *P. putida* KT2440 (17) and in many other *Pseudomonas* strains. These compounds add to the increasing list of hydrocarbons (36), sugars (5), and amino acids (11, 12) that are not preferred carbon sources for *P. putida* and whose metabolism is inhibited when other preferred carbon sources are available. Crc stands, therefore, as a master regulator of carbon metabolism in *P. putida* in response to physiological and environmental inputs.

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