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A Tricarboxylic Acid Cycle Intermediate Regulating Transcription of a Chloroaromatic Biodegradative Pathway: Fumarate-Mediated Repression of the *clcABD* Operon

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The *ortho*-cleavage pathways of catechol and 3-chlorocatechol are central catabolic pathways of *Pseudomonas putida* that convert aromatic and chloroaromatic compounds to tricarboxylic acid (TCA) cycle intermediates. They are encoded by the evolutionarily related *catBCA* and *clcABD* operons, respectively. Expression of the *cat* and *clc* operons requires the LysR-type transcriptional activators CatR and ClcR, respectively, and the inducer molecules *cis,cis*-muconate and 2-chloro-*cis,cis*-muconate, respectively. The regulation of the *cat* and *clc* promoters has been well studied, but the extent to which these operons are repressed by growth in TCA cycle intermediates has not been explored. We demonstrate by transcriptional fusion studies that the expression from the *clc* promoter is repressed when the cells are grown on succinate, citrate, or fumarate and that this repression is ClcR dependent and occurs at the transcriptional level. The presence of these organic acids did not affect the expression from the *cat* promoter. In vitro transcription assays demonstrate that the TCA cycle intermediate fumarate directly and specifically inhibits the formation of the *clcA* transcript. No such inhibition was observed when CatR was used as the activator on either the *cat* or *clc* template. Titration studies of fumarate and 2-chloromuconate show that the fumarate effect is concentration dependent and reversible, indicating that fumarate and 2-chloromuconate most probably compete for the same binding site on ClcR. This is an interesting example of the transcriptional regulation of a biodegradative pathway by the intracellular sensing of the state of the TCA cycle.

Pseudomonas putida is able to use a wide variety of chlorinated and nonchlorinated aromatics as sole sources of carbon and energy (15, 17). Nonchlorinated aromatics such as aniline, benzene, phenol, cinnamate, anthranilate, tryptophan, benzoate, and mandelate are all converted to catechol, which is catabolized to tricarboxylic acid (TCA) cycle intermediates by the central *ortho*-cleavage pathway (15). The catechol pathway is encoded by the *catBCA* and *pcaD* genes. Likewise, chlorinated aromatics such as chloroaniline, chlorophenoxyacetate, chlorophenol, chloronaphthalene, chlorosalicylate, chlorotoluene, chlorobiphenyl, chlorobenzoate, and chlorobenzene are funneled to chlorocatechol, which is converted to TCA cycle intermediates by the central, modified *ortho* pathway (17). Three operons encoding plasmid-encoded chlorocatechol pathways have been identified: the *clcABD* operon, isolated from plasmid pAC27 of *P. putida* (11) and plasmid pWR1 of *Pseudomonas* sp. strain B13 (36); the *tcbCDEF* operon, isolated from plasmid pP51 of *Pseudomonas* sp. strain P51 (38); and the *tfdCDEF* operon, isolated from plasmid pJP4 of *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus* JMP134) (35, 41). The catechol and chlorocatechol operons are regulated by LysR-type transcriptional activators that are typically divergently transcribed from their structural genes (6, 21, 37, 38). Because these pathways have similar enzymes, substrates, and genetic organizations (see Fig. 1), it has been proposed that the chlorocatechol operon may have evolved from the catechol operon (10, 29).

Expression of the *catBCA* operon requires the transcriptional activator CatR and an intermediate of the catechol pathway, *cis,cis*-muconate, as the inducer (37). Likewise, we have demonstrated that the expression of the *clcABD* operon requires a similar transcriptional activator, ClcR, and an analogous intermediate of the 3-chlorocatechol pathway, 2-chloro-*cis,cis*-muconate, as the inducer (6, 25). CatR and ClcR are 32.5% identical and 43% similar in their predicted amino acid sequences, and their operator regions are ~50% identical (6). Our laboratory has previously shown that these similarities have functional implications. Studies have demonstrated that *catR* can functionally replace *clcR* for growth on 3-chlorobenzoate but that *clcR* cannot replace *catR* for growth on benzoate (33). DNase I footprinting, DNA bending, and in vitro transcription analyses with RNA polymerase mutants indicate that ClcR and CatR activate transcription of the *clcABD* promoter via the same mechanism (24, 33).

The regulation of the *cat* and *clc* operons in response to their respective inducers has been extensively studied. However, the effect of the availability of other carbon sources, i.e., carbon catabolite control, has not been investigated. Growth on succinate and other TCA cycle intermediates has been reported to cause carbon catabolite repression in pseudomonads (8, 22, 28). In this paper, we report that the expression of the *clcABD* operon is repressed during growth with TCA cycle intermediates and that this repression is at the transcriptional level. The expression of the *catBCA* operon is unaffected by growth with TCA cycle intermediates. By in vitro transcription analyses, we demonstrate that fumarate specifically inhibits the formation of the *clcA* transcript, probably by directly interacting with the ClcR protein.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
E. coli TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)	23
P. putida		
PRS2000	Wild-type strain	39
PRS4020	*catR*::Gm^r Ben$^-$	32
Plasmids		
pDC15	17-kb *Bgl*II insert *clcR-clcABD* in pLAFR1, Tc^r, 3-CBA$^+$	14
pQF50	Broad-host-range *lacZ* promoter probe vector, Ap^r	9
pSMM50R-B′	2.5-kb *Sph*I-*Hin*dIII *clcR-B′* insert from pDC15, *clcAB-lacZ* in pQF50	25
pSMM50R′AB′	1.9-kb *Eco*RV-*Hin*dIII *clcR′-B′* insert from pDC15 in the *Sma*I-*Hin*dIII site of pQF50, *clcAB-lacZ* in pQF50	This study
pSMM50-CAT	245-bp *Sal*I-*Hin*dIII PCR-generated fragment containing the *catBCA* promoter in pQF50, *catB-lacZ* in pQF50	This study
pJRD215	Broad-host-range vector, Km^r Sm^r	7
pKR215-13	2-kb *Eco*RI-*Bam*HI *catR* insert in pJRD215	33
pSMM215RA	2.8-kb *Xba*I *clcR-AB′* insert in pJRD215	33
pMP7	In vitro transcription vector, Ap^r	18
pSMM7-C1	428-bp *Sst*I-*Hin*dIII PCR-generated fragment containing the *clcABD* promoter in pMP7	25
pIVTACH2	304-bp *Bam*HI-*Sal*I PCR-generated fragment containing the *catBCA* promoter in pMP7	4

[a] Gm^r, gentamicin resistant; Tc^r, tetracycline resistant; Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant.

MATERIALS AND METHODS

Bacteria, plasmids, media, and chemicals. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria broth at 37°C. *P. putida* strains were grown in Luria broth, *Pseudomonas* isolation agar (Difco Laboratories, Detroit, Mich.), TYP (16 g of tryptone, 16 g of yeast extract, 5 g of NaCl, and 2 g of K_2HPO_4 per liter), or basal salts medium at 30°C as described previously (1). Media and agar plates were supplemented with antibiotics for selection as follows (in milligrams per liter): for *E. coli*, ampicillin, 100; kanamycin, 100; streptomycin, 100; and tetracycline, 20; for *P. putida*, carbenicillin, 1,000; streptomycin, 1,000; kanamycin, 1,000; and gentamicin, 10. Benzoate was purchased from Aldrich Chemicals (Milwaukee, Wis.); 3-chlorobenzoate, succinate, citrate, pyruvate, malic acid, maleic acid, fumarate, and *trans,trans*-muconate were purchased from Sigma Chemical Co. (St. Louis, Mo.); 3-chlorocatechol was purchased from Helix Biotech (Richmond, British Columbia, Canada); and *cis,cis*-muconate was kindly provided by Celgene Corp. (Warren, N.J.). 2-Chloromuconate was produced by the enzymatic conversion of 3-chlorocatechol as described previously (24). ClcR was purified by the method of Coco et al. (5), and CatR was purified by the method of Parsek et al. (34).

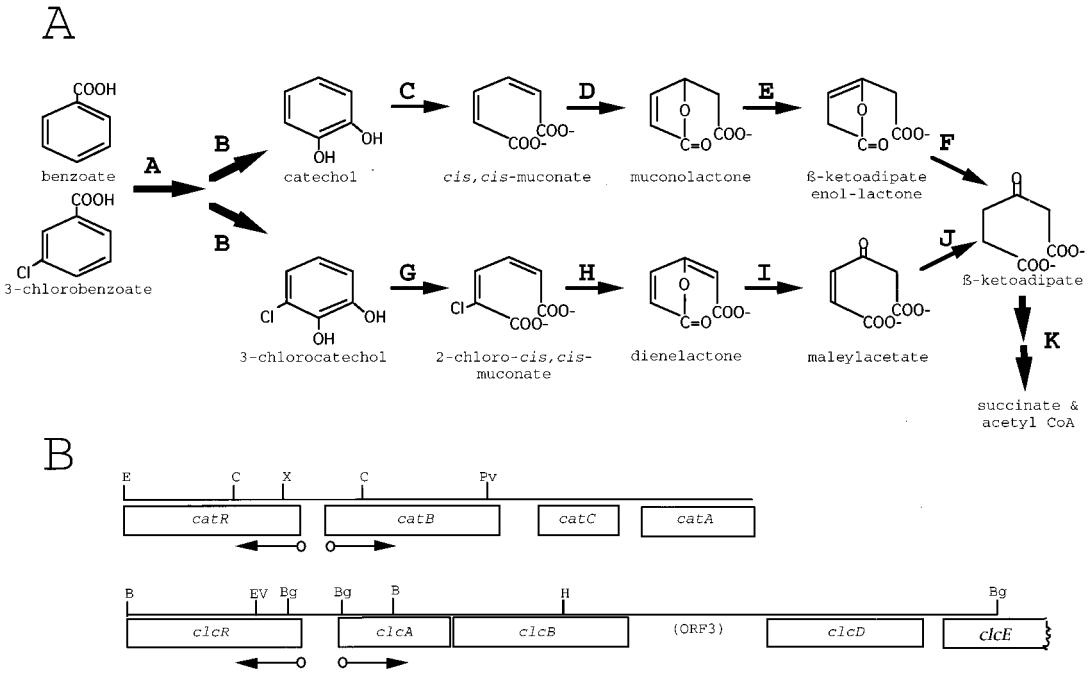

FIG. 1. (A) The *P. putida* benzoate and 3-chlorobenzoate pathways. Steps A and B, benzoate dioxygenase and benzoate diol dehydrogenase (*benABCD*); step C, catechol 1,2-dioxygenase (*catA*); step D, *cis,cis*-muconate lactonizing enzyme (*catB*); step E, muconatelactone isomerase (*catC*); step F, β-ketoadipate enol-lactone hydrolase (*pcaD*); step G chlorocatechol 1,2-dioxygenase (*clcA*); step H, chloromuconate lactonizing enzyme (*clcB*); step I, dienelactone hydrolase (*clcD*); step J, maleylacetate reductase (*clcE*); step K, β-ketoadipate coenzyme A transferase (*pcaIJ*) and β-ketoadipyl coenzyme A thiolase (*pcaF*). (B) Genetic arrangement of the *catR-catBCA* and *clcR-clcABD* loci with relevant restriction endonuclease cleavage sites. Abbreviations: E, *Eco*RI; C, *Cla*I; X, *Xho*I; Pv, *Pvu*I; B, *Bam*HI; EV, *Eco*RV; Bg, *Bgl*II; H, *Hin*dIII. The *catR*, *catBCA*, *clcR*, and *clcABD* promoters are indicated by circles attached to arrows noting the direction of transcription.

E. coli holoenzyme was purchased from Epicentre Technologies (Madison, Wis.).

DNA manipulations. Recombinant DNA techniques were performed by standard procedures (23). The Qiagen (Chatsworth, Calif.) Plasmid Midiprep kit was used for plasmid purification for use in electroporation and in vitro transcription assays. Recombinant *Pfu* polymerase for PCR was purchased from Stratagene (La Jolla, Calif.), and restriction enzymes were purchased from Gibco BRL (Gaithersburg, Md.). All the enzymes were used as recommended by the manufacturers. Agarose gel electrophoresis and ligations were performed as described previously (23). DNA fragments for subcloning were gel purified with a Qiaex II gel extraction kit from Qiagen. The broad-host-range plasmid pQF50 and its derivatives were introduced into *P. putida* strains via electroporation (25).

Plasmid constructions. Plasmid pSMM50R′AB′ was constructed by inserting the 1.9-kb *Eco*RV-*Hin*dIII fragment from pDC15 (14) containing truncated *clcR*, the intragenic promoter region, and an intact *clcA* gene to form a *clcAB-lacZ* fusion in the promoterless *lacZ* vector pQF50 (9). Plasmid pSMM50-CAT was constructed by using PCR to generate a 245-bp *catBCA* promoter fragment containing a truncated *catR* gene, the intragenic promoter region, and a truncated *catB* gene to form a *catB-lacZ* promoter fusion. The primers used were CH1(SAL) (5′GGGTCGACCAGCAGCTCGGCGGCGCGAGT) and N1B (HINDIII) (5′GGAAGCTTTTGCCTCGATACGTTCAAT). Supercoiled templates, i.e., pSMM7-C1 (25), which contains a *clcABD* promoter fragment spanning −245 to +86 with respect to the *clcA* gene, and pIVTACH2 (4), which contains a *catBCA* promoter fragment spanning −148 to +156 with respect to the *catB* gene, were used for in vitro transcription assays.

Promoter activity assays. Quantitative determination of β-galactosidase activity was performed by the method of Miller (26). To determine if carbon catabolite repression influenced the expression of the *clc* or *cat* operons, *P. putida* PRS4020 (*catR*) cells containing pSMM50R-B′ or PRS2000 (wild-type) cells containing pSMM50-CAT were grown for 16 h at 30°C in basal salts medium supplemented with 10 mM glucose, 10 mM gluconate, 10 mM pyruvate, 10 mM succinate, or 10 mM citrate with or without 5 mM 3-chlorobenzoate or 5 mM benzoate, respectively. To determine if the repression was specific for ClcR-activated expression of the *clcABD* promoter, *P. putida* PRS4020 cells containing pSMM50R′AB′ and either pJRD215 (vector control), pRK215-13 (*catR*), or pSMM215RA (*clcR*) were grown for 16 h at 30°C in 10 mM glucose or 10 mM succinate with or without the addition of 5 mM 3-chlorobenzoate or 5 mM benzoate. To demonstrate that fumarate also repressed the expression of the *clc* operon in vivo, PRS4020 cells containing pSMM50R-B′ were grown for 16 h at 30°C in glucose, succinate, or fumarate with and without 5 mM 3-chlorobenzoate. Each experiment was performed in triplicate. The protein concentrations were determined with a Bradford protein assay kit from Bio-Rad Laboratories (Hercules, Calif.).

In vitro transcription assays. In vitro transcription assays were performed as described previously (18, 25). The assays were conducted by initially incubating ClcR or CatR and appropriate inducer and/or repressor compounds with template at 37°C for 15 min. To the initial binding reaction mixture was added 2.5 μl of 1× transcription buffer containing 0.6 pmol of *E. coli* holo RNA polymerase and 4 U of RNase inhibitor (Boehringer Mannheim, Indianapolis, Ind.), and the mixture was incubated for an additional 15 min at 37°C. Transcription was initiated by the addition of 2.5 μl of 1× transcription buffer containing GTP, ATP, CTP (250 μM each), UTP (25 μM), and [α-^{32}P]UTP (10 μCi). The reactions were allowed to proceed for 10 min and terminated by the addition of 10 μl of loading dye. The reaction mixtures were heat denatured at 70°C for 5 min, loaded onto an 8 M urea–6% polyacrylamide gel, and electrophoresed for approximately 4 h. The gel was then soaked for 20 min in a solution of 20% methanol and 10% acetic acid and dried. The radioactive bands were detected with a PhosphorImager and quantified with ImageQuant 1.1 software (Molecular Dynamics). Transcript production was normalized to the production of RNA-1 transcript from the ColE1 ori of the supercoiled plasmid pMP7 (18).

RESULTS

Expression of the *clcABD* operon is regulated by catabolite repression. It has been widely reported that growth in succinate and other TCA cycle intermediates represses the expression of many operons at the transcriptional level in pseudomonads (8, 22, 28). To examine the regulation of the catechol and 3-chlorocatechol pathways in vivo, we typically provide the cells with benzoate and 3-chlorobenzoate, respectively, as growth substrates (Fig. 1). To determine if succinate-mediated regulation occurs at the *clcABD* promoter, *P. putida* PRS4020 (*catR*) cells harboring pSMM50R-B′ (a *clcAB-lacZ* transcriptional fusion) were grown with different carbon substrates with or without 5 mM 3-chlorobenzoate, the cells were harvested, and β-galactosidase assays were performed. Cells grown with glucose, gluconate, or pyruvate in the presence of 3-chlorobenzoate showed 25- to 50-fold-induced levels of β-galactosidase activity over those grown without 3-chlorobenzoate (Fig. 2A). The cells grown with citrate or succinate plus 3-chlorobenzoate showed only a four- to fivefold increase in activity over cells grown with citrate or succinate without 3-chlorobenzoate. This study indicated that growth with succinate or citrate repressed the expression of the *clcABD* operon in the presence of 3-chlorobenzoate. A similar study was done to determine if the *catBCA* promoter was also repressed when the cells were grown with succinate or citrate. Wild-type *P. putida* PRS2000 cells harboring pSMM50-CAT, a *catB-lacZ* fusion, were grown with the same growth substrates as above, supplemented with 5 mM benzoate or not supplemented. No repression was observed with the *catBCA* promoter. Very similar levels of β-galactosidase activity were detected regardless of the growth substrate (Fig. 2B).

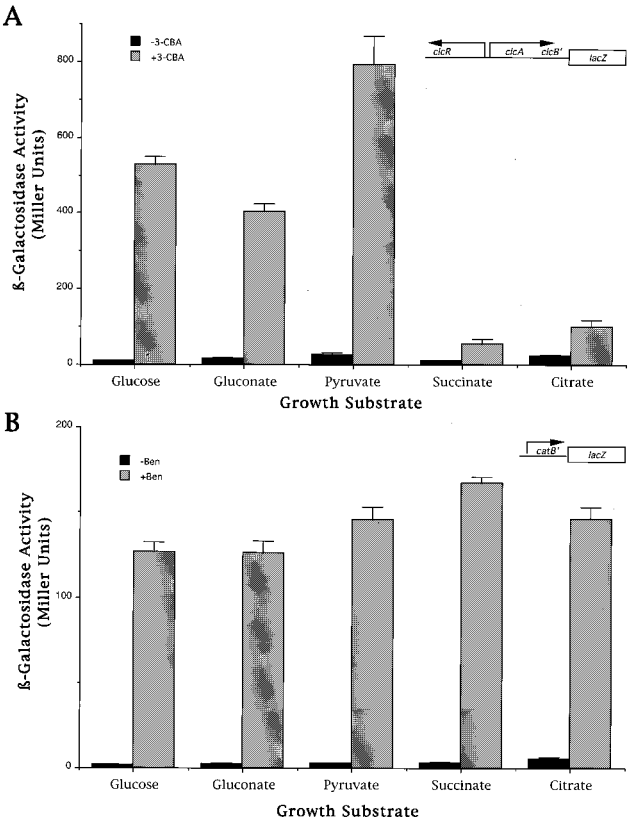

FIG. 2. (A) Transcriptional fusion assays demonstrating that the *clcABD* operon (*clcR-AB-lacZ* construct shown in the upper right-hand corner) is regulated by carbon catabolite control. (B) Transcriptional fusion assays demonstrating that the *catBCA* operon (*catB-lacZ* construct shown in the upper right hand corner) is not regulated by carbon catabolite control. Each assay was performed in triplicate. Miller units are nanomoles of nitrophenol generated per minute per milligram of protein. Abbreviations: 3-CBA, 3-chlorobenzoate; Ben, benzoate.

Repression of the *clcABD* operon is at the transcriptional level and requires *clcR*. We have previously demonstrated that *catR* activates the *clcABD* promoter in response to growth with benzoate (33). To determine if the organic acid-induced catabolite repression was specific for *clcR*, pSMM50R′AB′, a *clcAB-lacZ* fusion that lacks functional *clcR*, was introduced into CatR$^-$ PRS4020 cells that contain a vector expressing either CatR or ClcR. Succinate was used as the repressing substrate because it had been widely reported to be the catabolite-repressing molecule in pseudomonads (22, 40). Cells containing pJRD215 (vector control) had a low level of β-galactosidase activity under any growth condition tested (Fig. 3). Cells con-

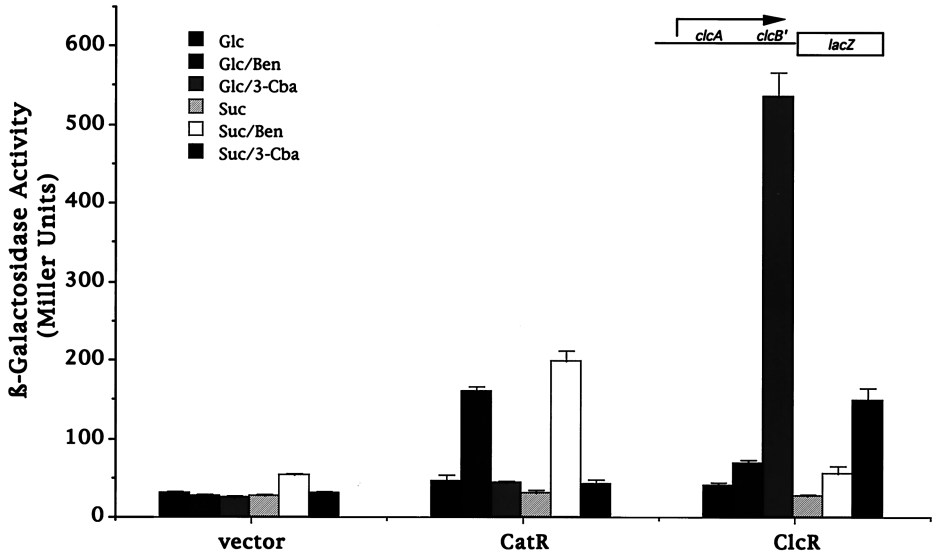

FIG. 3. Transcriptional fusion assays demonstrating that the catabolite repression of the *clcABD* operon is *clcR* dependent and occurs at the transcriptional level. *P. putida* PRS4020 cells harbored pSMM50R′AB′ (a *clcAB-lacZ* construct shown in the upper right hand corner) and either pJRD215 (vector control), pKR215-13 (*catR*), or pSMM215RA (*clcRA*). Each assay was performed in triplicate. Miller units are nanomoles of nitrophenol generated per minute per milligram of protein. Abbreviations: Glc, glucose; Ben, benzoate; 3-Cba, 3-chlorobenzoate; Suc, succinate.

taining *catR* (pKR215-13) showed similar levels of expression in glucose, glucose plus 3-chlorobenzoate, succinate, and succinate plus 3-chlorobenzoate. We have previously demonstrated that 2-chloromuconate cannot act as an inducer of CatR-activated transcription (24). A four- to sixfold increase in β-galactosidase activity was observed when the cells were grown in either glucose plus benzoate or succinate plus benzoate, demonstrating no repression of the *clcABD* promoter when *catR* was provided as the activator. Cells containing *clcR*(pSMM215RA) had similar levels of expression when they were grown with either glucose or succinate. There was an ~twofold increase when the cells were grown with glucose plus benzoate or succinate plus benzoate. When they were grown with glucose plus 3-chlorobenzoate, there was a 13-fold increase in β-galactosidase activity, whereas when they were grown with succinate plus 3-chlorobenzoate, there was only a 5.5-fold increase. This study demonstrates that succinate-mediated repression is specific for *clcR*-activated *clcABD* operon expression and that this regulation is at the transcriptional level.

Fumarate inhibits the transcription of the *clcA* gene in vitro. To determine if any TCA cycle intermediates directly inhibited the expression of the *clcABD* operon, in vitro transcription assays were performed with 0.31 pmol of ClcR, 250 μM 2-chloromuconate, and pSMM7-C1 (a supercoiled *clc* promoter template) plus either 1 mM acetate, 1 mM pyruvate, 1 mM citrate, 1 mM succinate, 1 mM fumarate, or 1 mM malate (Fig. 4). Lanes 1 to 3 demonstrate that both ClcR and 2-chloromuconate were required for full activation of the *clcA* promoter, as has been previously reported (6, 25). Fumarate was the only metabolite that caused specific inhibition of the formation of the *clcA* transcript (lane 8). Similar in vitro transcription studies were performed with CatR, *cis,cis*-muconate, pIVTACH2 (a *cat* promoter template), and the potentially inhibiting compounds. No inhibition of *catB* transcript production was observed with any of the added compounds (data not shown). Therefore, as in the *lacZ* transcriptional fusion studies, only the *clcABD* operon activated by ClcR is subject to transcriptional repression.

It appeared that fumarate inhibited the expression of the *clc* transcript, most probably by interacting directly with ClcR. Because fumarate and 2-chloromuconate have some structural similarities (see Fig. 6), it was presumed that these two compounds may compete for the inducer binding pocket of ClcR. To test this hypothesis, we first determined the minimum concentration of fumarate that would cause full inhibition of *clcA* transcript formation when 250 μM 2-chloromuconate was added as an inducer. Figure 5A demonstrates that as increasing concentrations of fumarate were added to the assay mixtures, less *clcA* transcript was observed. We found that 250 μM fumarate was required to inhibit the production of the *clcA* transcript under the experimental conditions used. In Fig. 5B,

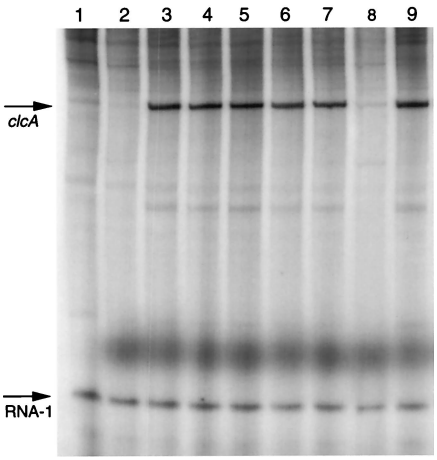

FIG. 4. In vitro transcription assay demonstrating that fumarate specifically inhibits the production of the *clcA* transcript. Lane 1 contains no ClcR and no inducer. Lane 2 contains 0.31 pmol of ClcR and no inducer. Lanes 3 to 9 contain 0.31 pmol of ClcR and 250 μM 2-chloromuconate with or without potential repressor molecules: lane 3, no repressor; lane 4, 1 mM acetate; lane 5, 1 mM pyruvate; lane 6, 1 mM citrate; lane 7, 1 mM succinate; lane 8, 1 mM fumarate; lane 9, 1 mM malate. The *clcA* and RNA-1 transcripts are indicated by arrows. RNA-1 transcripts were used as internal controls as described previously (24, 25).

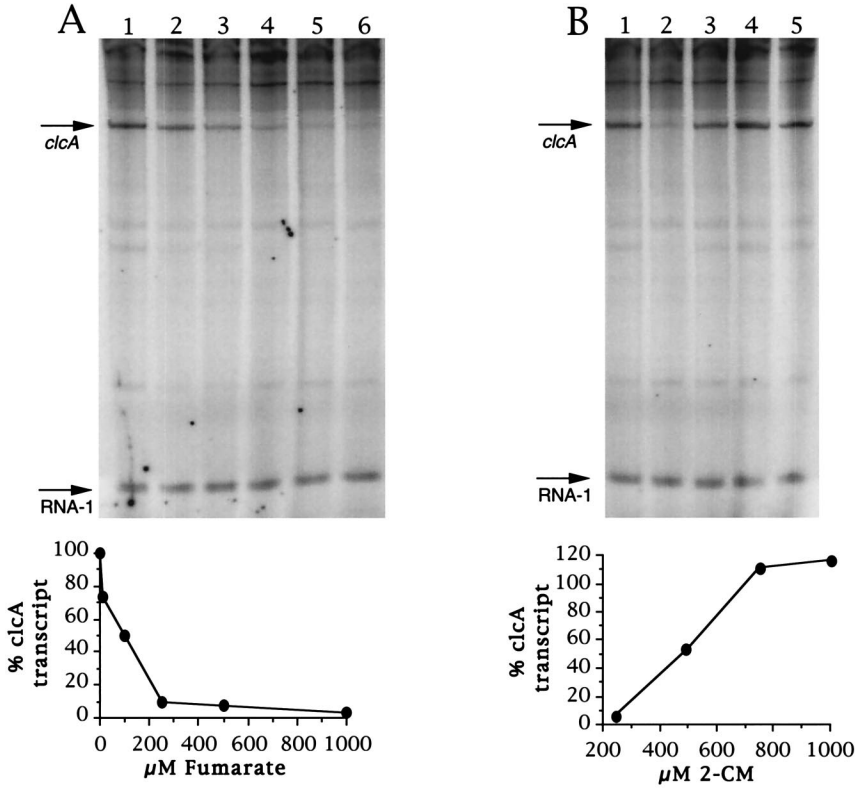

FIG. 5. In vitro transcription assays demonstrating the effect of increasing concentrations of fumarate (A) and 2-chloromuconate (B) on the production of *clcA* transcript. (A) The lanes contain 0.31 pmol of ClcR and 250 μM 2-chloromuconate with increasing concentrations of fumarate: lane 1, no fumarate; lane 2, 10 μM; lane 3, 100 μM; lane 4, 250 μM; lane 5, 500 μM; lane 6, 1 mM. These data are shown graphically below the gel. The value for the *clcA* transcript produced without fumarate (lane 1) was set at 100%. (B) Lanes 1 to 5 contain 0.31 pmol of ClcR. Lane 1 contains 250 μM 2-chloromuconate (2-CM) and no fumarate. Lanes 2 to 5 contain 250 μM fumarate with increasing concentrations of 2-chloromuconate: lane 2, 250 μM; lane 3, 500 μM; lane 4, 750 μM; lane 5, 1 mM. These data are shown graphically below the gel. The value for the *clcA* transcript produced without fumarate (lane 1) was set at 100%. Transcript production was normalized to production of the RNA-1 transcript from the ColE1 ori of the supercoiled plasmid pMP7. The radioactive bands were detected with a PhosphorImager and quantified with ImageQuant 1.1 software.

lane 1 contains 250 μM 2-chloromuconate and 0.31 pmol of ClcR with no fumarate. The transcript level produced here was set arbitrarily at 100%. In lanes 2 to 5, the fumarate concentration was held at 250 μM and the 2-chloromuconate concentration was increased from 250 μM to 1 mM. As the 2-chloromuconate concentrations increased, so did the production of the *clcA* transcript, demonstrating that fumarate does not disable ClcR irreversibly and that fumarate and 2-chloromuconate most probably compete for the same ClcR binding site. Thus, fumarate may be considered an anti-inducer.

Fumarate inhibits the expression of the *clcABD* operon in vivo. Because fumarate had a direct effect on the expression of the *clcA* gene in vitro, PRS4020 (*catR*) cells harboring plasmid pSMM50R-B′ were grown in 10 mM glucose, 10 mM succinate, or 10 mM fumarate with or without 5 mM 3-chlorobenzoate. After 16 h of growth, the cells were harvested and β-galactosidase assays were performed. Cells grown in glucose plus 3-chlorobenzoate showed a 54-fold induction of β-galactosidase activity, and cells grown with succinate plus 3-chlorobenzoate showed only a 6-fold induction. These results are very similar to those presented in Fig. 2. However, cells grown with fumarate plus 3-chlorobenzoate showed a nearly 16-fold induction of β-galactosidase activity. Therefore, fumarate does provide catabolite repression in vivo, albeit to a lesser extent than succinate under the growth conditions used in this study.

DISCUSSION

Many inducible catabolic pathways of pseudomonads have been demonstrated to be repressed by growth in the presence of TCA cycle intermediates (40). In this paper, we demonstrate that the expression of the *clcABD* operon but not the *catBCA* operon is reduced when cells are grown with succinate, citrate, or fumarate. When cells were grown for longer than 24 h in the presence of succinate plus 3-chlorobenzoate, they experienced an enhanced death rate as measured by viable-colony formation (unpublished observation). A pinkish brown color, which was presumed to be due to an oxidized polymer of 3-chlorocatechol which has been demonstrated to be toxic to *Pseudomonas* sp. strain RHO1, accumulated in the medium (12). Many chlorinated aromatics like chlorophenols or polychlorinated biphenyls are broken down to chlorocatechols, which are catabolized via *ortho*-cleavage pathways (17, 19). Therefore, TCA cycle intermediate repression of chlorocatechol pathways could prove to be a significant problem in the biodegradation of recalcitrant compounds. Inhibition of *clcABD* expression is observed when as little as 4 mM succinate is added to the growth medium (unpublished observations).

Although the evolutionarily related *clcABD* and *catBCA* operons appear to have the same transcriptional activation mechanisms (24), they are differentially regulated by growth in TCA cycle intermediates. What is the difference between these

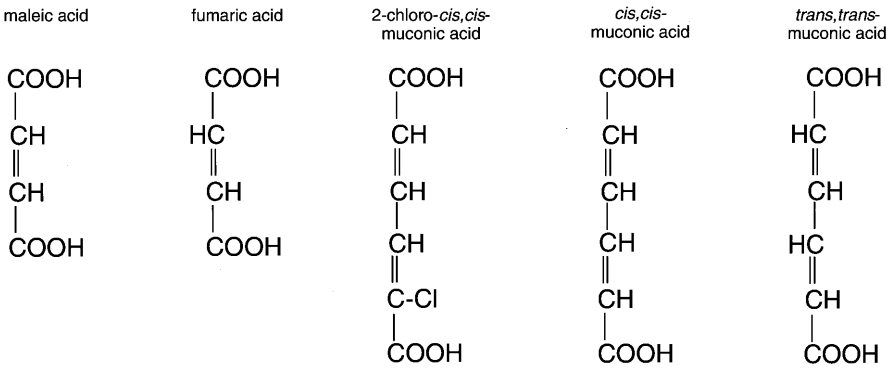

FIG. 6. Fischer projection structures of maleic acid, fumaric acid, 2-chloro-*cis,cis*-muconic acid, *cis,cis*-muconic acid, and *trans,trans*-muconic acid.

two pathways that makes it advantageous for the cell to catabolite repress the expression of the chlorocatechol pathway but not the catechol pathway? Three recent reports, one about *P. putida* and two about closely related bacteria, *R. eutropha* and *A. calcoaceticus*, indicate that benzoate degraded via the *ortho*-cleavage pathway of catechol is a preferred carbon substrate for these strains (2, 13, 30). Catechol and *cis,cis*-muconate have been identified as key regulatory intermediates in the preferred degradation of benzoate over 4-hydroxybenzoate and acetate. The mechanism of this regulation is unknown, and the hierarchy of carbon utilization in pseudomonads is also unknown. Nevertheless, it appears that the *catBCA* operon is not repressed in cells grown on TCA cycle intermediates, because benzoate or other compounds that are degraded via the *ortho*-cleavage pathway of catechol are preferred carbon substrates.

In vitro transcription assays demonstrate that fumarate specifically inhibits the expression of the *clc* transcript, most probably by interacting directly with ClcR as an anti-inducer, and that this inhibition is reversible. This is the first report, to our knowledge, that proposes a mechanism for catabolite repression of transcription by a TCA cycle intermediate in pseudomonads. The power of the in vitro transcription assay is that it allows the unequivocal identification of fumarate as the effector molecule. Another LysR-type transcriptional regulator, CysB, the regulator of the cysteine regulon which includes genes necessary for the synthesis of *O*-acetyl-L-serine, the uptake and reduction of sulfate to sulfide, and the reaction of sulfide with *O*-acetyl-L-serine to form L-cysteine in *Salmonella typhimurium*, has also been shown to have anti-inducers (20, 31). Full expression of the cysteine regulon requires *cysB*, the inducer *N*-acetyl-L-serine, and sulfur limitation. It has been demonstrated that sulfide and thiosulfate act as anti-inducers for CysB-activated transcription from the *cysP* and the *cysJIH* promoters in vitro. The *clc* system is analogous to the *cys* system because a product of the pathway, in this case fumarate, which is a TCA cycle intermediate, directly inhibits the transcription of the upper pathway, the *clcABD* operon.

The mechanism by which fumarate blocks transcriptional activation of the *clcABD* promoter is unknown. Fumarate does not prevent the binding of ClcR to the *clc* operator, as demonstrated by gel retardation assays (unpublished observation). Both fumarate and 2-chloromuconate are dicarboxylic acids with a double bond, but fumarate has a *trans* configuration and 2-chloromuconate has a *cis* one. We thought it possible that fumarate binds in the inducer binding pocket of ClcR and that other similar compounds also affect the formation of the *clcA* transcript either positively or negatively. To determine if other related compounds could play either an activating or a repressing role in the expression of the *clc* operon, the *cis* isomer of fumarate, maleic acid, *cis,cis*-muconate, and *trans,trans*-muconate were added to in vitro transcription assay mixtures with or without 2-chloromuconate (the structures are shown in Fig. 6). None of these compounds modulated the expression level of the *clcA* transcript (unpublished observations). Therefore, the action of fumarate as an anti-inducer does not appear to be an accident of chemical structure. Rather, it implies that fumarate plays a true metabolic role in the transcriptional regulation of the *clcABD* operon.

Regulation of bacterial cellular processes by intracellular fumarate levels is not unique to this system. Alginate production in *Pseudomonas aeruginosa* (16) and phosphorylation-independent flagellar motor switching in *E. coli* (3, 27) are both regulated by intracellular fumarate levels. It cannot be ruled out that the catabolite repression observed in vivo and the in vitro inhibition of *clcA* transcript production are unrelated. The facts that the in vivo observed repression is at the transcriptional level and is *clcR* dependent do correlate well with the in vitro transcription results. It would be interesting to determine the effect on *clcABD* operon expression of varying the levels of intracellular fumarate. The chemical structure of fumarate has a *trans* double bond which is unique among the TCA cycle intermediates. This difference may allow fumarate to act as a key signaling molecule to indicate the metabolic status of the cell. If the levels of fumarate are high, the transcription of the *clcABD* operon is repressed and, by extension, the cell is not able to catabolize a myriad of chloroaromatic compounds. If the levels of fumarate were low, the *clcABD* operon would be transcribed and a variety of chloroaromatics could be used as carbon and energy sources. This provides the cell with an elegant mechanism to regulate the utilization of a variety of substrates at one central point.

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