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Inactivation of Cytochrome *o* Ubiquinol Oxidase Relieves Catabolic Repression of the *Pseudomonas putida* GPo1 Alkane Degradation Pathway

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Expression of the alkane degradation pathway encoded by the OCT plasmid of *Pseudomonas putida* GPo1 is regulated by two control systems. One relies on the transcriptional regulator AlkS, which activates expression of the pathway in the presence of alkanes. The other, which is a dominant global regulation control, represses the expression of the pathway genes when a preferred carbon source is present in the growth medium in addition to alkanes. This catabolite repression control occurs through a poorly characterized mechanism that ultimately regulates transcription from the two AlkS-activated promoters of the pathway. To identify the factors involved, a screening method was developed to isolate mutants without this control. Several isolates were obtained, all of which contained mutations that mapped to genes encoding cytochrome *o* ubiquinol oxidase, the main terminal oxidase of the electron transport chain under highly aerobic conditions. Elimination of this terminal oxidase led to a decrease in the catabolic repression observed both in rich Luria-Bertani medium and in a defined medium containing lactate or succinate as the carbon source. This suggests that catabolic repression could monitor the physiological or metabolic status by using information from the electron transport chain or from the redox state of the cell. Since inactivation of the *crc* gene also reduces catabolic repression in rich medium (although not that observed in a defined medium), a strain was generated lacking both the Crc function and the cytochrome *o* terminal oxidase. The two mutations had an additive effect in relieving catabolic repression in rich medium. This suggests that *crc* and *cyo* belong to different regulation pathways, both contributing to catabolic repression.

The expression of bacterial catabolic pathways for the assimilation of aliphatic and aromatic hydrocarbons is frequently subject to complex control which links induction of the corresponding genes not only to the presence of the substrates to be degraded but also to the proper physiological status of the cell (11). This is the case of the alkane degradation pathway encoded by the OCT plasmid of *Pseudomonas putida* GPo1 (previously named *Pseudomonas oleovorans* GPo1; see reference 53). The genes of this pathway are grouped into two clusters, *alkBFGHJKL* and *alkST* (Fig. 1) (53, 54). The *alkBFGHJKL* operon is transcribed from a promoter, *PalkB*, whose expression requires the transcriptional activator AlkS and the presence of alkanes (31, 42). In the absence of alkanes, the *alkST* genes are expressed from promoter *PalkS1*, which is recognized by σ^S-RNA polymerase (7, 8). Therefore, during exponential growth with a carbon source other than alkanes, the gene for the AlkS regulator is essentially silent. Upon entry into the stationary phase, when σ^S-RNA polymerase becomes available, transcription of *alkS* from this promoter increases. AlkS acts as a repressor of *PalkS1*, allowing for low expression of the *alkST* genes. When alkanes are present, the AlkS protein bound to *PalkS1* activates the expression of promoter *PalkS2*, located 38 nucleotides (nt) downstream from *PalkS1*, and provides for high expression of the *alkST* genes (7). Therefore, the pathway is controlled by a positive feedback mechanism governed by AlkS (Fig. 1). The amount of AlkS protein in induced cells is, however, low since this protein seems to be very unstable (56, 57). The pathway is regulated by an additional control system, since the levels of the alkane degradation enzymes are modulated by catabolic repression depending on the carbon source being used (24, 47). This superimposed control occurs through a poorly characterized mechanism that ultimately regulates transcription from promoters *PalkB* and *PalkS2* (7, 56, 57). Activation of these promoters by AlkS and the alkane inducer is very efficient when cells are grown in a minimal salts medium containing citrate as carbon source but shows a three- to fourfold reduction when organic acids such as lactate, pyruvate, or succinate are used as the carbon source. Repression is much stronger (ca. 50-fold repression) when cells grow exponentially in a rich medium such as Luria-Bertani (LB) medium or in minimal salts medium supplemented with Casamino Acids. Repression in rich medium abruptly disappears as cells enter the stationary phase of growth, which suggests the existence of elements that assure low-level expression of promoters *PalkB* and *PalkS2* during exponential growth. The *P. putida* Crc protein plays an important role in the catabolic repression observed in rich medium, although not the one observed when cells grow in minimal salts medium containing lactate or succinate as a carbon source (57). Inactivation of the *crc* gene reduces catabolic repression in rich medium by about sixfold, although a fivefold repression still occurs in conditions of no catabolic repression, indicating that other elements besides Crc participate in this control (57). To

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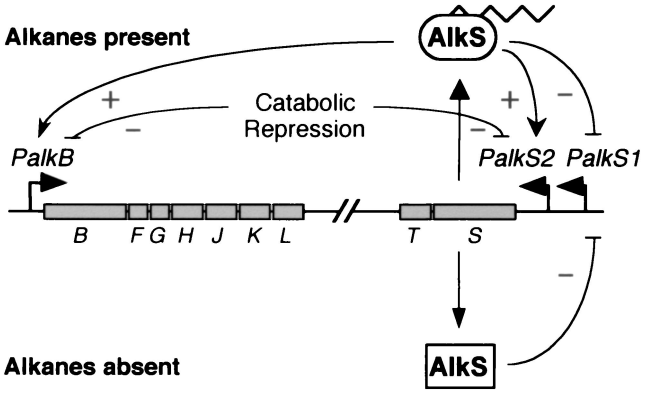

FIG. 1. *P. putida* GPo1 alkane degradation pathway. The genes are grouped into two clusters, *alkBFGHJKL* and *alkST*, both of which are regulated by the AlkS protein. In the absence of alkanes, AlkS is expressed from promoter *PalkS1*; AlkS acts as a repressor of this promoter, allowing for low expression levels. This promoter is recognized by σ^S-RNA polymerase, being active only in the stationary phase of growth. In the presence of alkanes, AlkS activates expression from the *PalkB* and *PalkS2* promoters, generating a positive amplification loop on *alkS* expression. Activation of these two promoters by alkanes is strongly repressed by catabolic repression when cells grow exponentially in rich LB medium. Growth in a minimal medium containing some organic acids (lactate or succinate) as a carbon source generates a milder catabolic repression effect.

identify additional factors involved in the modulation of this pathway, a screening method was developed to obtain mutants showing a reduced catabolic repression in rich medium. Nine mutants were isolated. In all cases, mutations mapped to genes encoding the cytochrome *o* ubiquinol oxidase, the main terminal oxidase of the electron transport chain under highly aerobic conditions. The aerobic respiratory chains of both *Escherichia coli* and *P. putida* include a number of membrane-bound dehydrogenases that transfer electrons to ubiquinone, reducing it to ubiquinol. This can then be oxidized by either of two respiratory ubiquinol oxidases: the cytochrome *o* complex or the cytochrome *d* complex (22). When cells grow exponentially with an ample supply of oxygen, cytochrome *o* oxidase (*cyo*) accommodates most of the electron flow. As the oxygen supply becomes limiting, cytochrome *d* oxidase (*cyd*) is synthesized as an alternative terminal oxidase (15, 16, 49). Strains that lack either *cyo* or *cyd* are fully capable of aerobic growth under normal laboratory conditions, since the function of the missing oxidase seems to be taken over by the one remaining (1). An analysis was made of the influence of cytochrome *o* ubiquinol oxidase in the AlkS-mediated activation of the *PalkB* and *PalkS2* promoters. The results suggest that catabolic repression of the *P. putida* GPo1 alkane degradation pathway is linked to the activity of the electron transport chain and/or to the redox state of the cell.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used are listed in Table 1. *P. putida* strains were grown at 30°C in LB medium or in M9 minimal salts medium (45), the latter supplemented with trace elements (4), and 30 mM citrate, lactate, or succinate as the carbon source. Expression of the *PalkB* or *PalkS2* promoters was induced was demontrated by the addition of 0.05% (vol/vol) dicyclopropylketone (DCPK), a nonmetabolizable inducer that mimics the effect of alkanes (24). *E. coli* cells were grown in LB medium at 37°C. Antibiotics were added when appropriate at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 8 μg/ml.

P. putida RC12 is derived from *P. putida* KT2442 by insertion of a *PalkB*::*kan* transcriptional fusion into its chromosome by using the suicide donor plasmid pALKRC1. This plasmid was generated as follows. A transcriptional fusion of the *PalkB* promoter to a kanamycin resistance (Km^r) determinant was generated by excising a DNA fragment containing the *PalkB* promoter (positions −525 to +66 relative to the transcriptional start site) from plasmid pPB7 with *Kpn*I (blunted with T4 DNA polymerase and deoxynucleoside triphosphates [dNTPs]) and *Hin*dIII and inserting it between the *Hin*cII and *Hin*dIII sites of plasmid pUCKm. The plasmid obtained was named pPBK10. The *PalkB*::*kan* fusion was excised from pPBK10 with *Kpn*I (blunted with T4 DNA polymerase and dNTPs) and *Eco*RI and inserted between the *Hin*dIII (blunted with T4 DNA polymerase and dNTPs) and *Eco*RI sites of plasmid pUJ8. The plasmid obtained was named pPBKJ1. The *PalkB*::*kan* fusion was excised from pPBKJ1 as a *Not*I DNA fragment and inserted at the *Not*I site of the mini-Tn*5* suicide donor plasmid pJMT6, generating plasmid pALKRC1. *P. putida* RC12S8 is derived from strain RC12 by insertion of the *alkS* gene into its chromosome by using the suicide delivery plasmid pTCS1. This plasmid is derived from mini-Tn*5*Tc by insertion at its *Not*I site of a *Not*I DNA fragment containing the *alkS* gene, which was obtained from plasmid pUJS1.

To generate the Δ*cyoB P. putida* strain PBS4B1, a 1,200-bp DNA fragment containing the *cyoB* gene was PCR amplified from *P. putida* KT2442 with the primers 5′-CGGGATCCACGAAGAAGCAGGCAGCA-3′ and 5′-CGGGATC CAGAACCAGAAGGCAG-3′, which contain *Bam*HI sites at their 5′ ends. The product was digested with *Bam*HI and cloned at the *Bam*HI site of plasmid pUC18, yielding plasmid pCYOB. A 2-kbp *Eco*RI fragment containing a tetracycline resistance (Tc^r) determinant was obtained from plasmid pUT-mini-Tn*5*Tc and inserted at the *Eco*RI site of pCYOB, located inside the *cyoB* gene. The plasmid generated was named pCYOBTc. Finally, a 3.2-kb *Bam*HI fragment from pCYOBTc containing the *cyoB*::*tet* allele was cloned into plasmid pKNG101, yielding pKCYOBTc. Plasmid pKNG101 is designed for marker exchange mutagenesis; it replicates in *E. coli* but not in *P. putida* and carries a streptomycin resistance (Sm^r) determinant and the *sacB* gene, which mediates sucrose sensitivity (29). Plasmid pKCYOBTc was transferred to *P. putida* PBS4 in triparental matings with plasmid pRK600 as donor of transfer functions, and Sm^r Tc^r sucrose-sensitive (8% [wt/vol]) exconjugants were selected. These cells were expected to contain plasmid pKCYOBTc integrated into the chromosome by a single recombination event at the *cyoB* gene, which generates a mutant and a wild-type *cyoB* gene. Cells having undergone a second recombination event leading to loss of the wild-type *cyoB* allele were selected by screening for Tc^r, streptomycin-sensitive (Sm^s), and sucrose-resistant colonies. The absence of the wild-type *cyoB* gene and the presence of the *cyoB*::*tet* allele were confirmed by PCR.

Inactivation of the *crc* gene in strain PBS4B1 was performed by marker exchange mutagenesis. The promoterless *xylE* reporter gene was excised from plasmid pXYLE10 with endonuclease *Sma*I and inserted at the *Nru*I site of the *crc* gene present in plasmid pCRC5. The plasmid obtained was named pCRC5A. The *crc*::*xylE* allele was excised from pCRC5A by partial restriction with *Bam*HI and cloned at the *Bam*HI site of plasmid pKNG101, yielding plasmid pCRC20. This plasmid was transferred to strain PBS4B1 and exconjugants in which the wild-type *crc* gene had been substituted by the *crc*::*xylE* allele were isolated as described above. A representative isolate was selected and named PBS4BC1.

Generation of mutant *P. putida* strains showing reduced catabolic repression in LB medium. *P. putida* RC12S8 (KT2442 with a *PalkB*::*kan* fusion and *alkS* in the chromosome) was mutagenized with minitransposon mini-Tn*5*Sm. This was delivered to the recipient cells in triparental matings as previously described (18) by using *E. coli* CC118λ*pir* containing the plasmid pUT-mini-Tn*5*Sm as a donor and *E. coli* HB101 containing the plasmid pRK600 as a helper for transfer functions. The exconjugants were plated on LB plates containing streptomycin, kanamycin, and 0.05% (vol/vol) DCPK and then incubated at 30°C for 24 to 48 h. Km^r colonies were streaked onto LB plates with kanamycin in the absence or presence of DCPK. Colonies resistant to kanamycin in the absence of DCPK were discarded (ca. 20%). Those selected were further tested for DCPK-dependent resistnace to kanamycin in LB broth. To identify the insertion point of the minitransposon, two approaches were used. For the mutant strains RCM4, RCM18, RCM2A, RCM3A, and RCM137A, chromosomal DNA was purified and digested with either *Kpn*I (strains RCM4 and RCM18), *Sac*I (strains RCM2A and RCM3A), or *Kpn*I+*Pst*I (strain RCM137A), none of which cut inside the minitransposon. The digested fragments were ligated to pUC18 excised with the same endonucleases and transformed into *E. coli* TG1. Plasmid DNA was extracted from Sm^r ampicillin-resistant (Ap^r) colonies, and the DNA insert was sequenced in both strands. In all other mutant strains analyzed, the

TABLE 1. Strains and plasmids

Strain or plasmid	Description or relevant phenotype[a]	Source or reference
Strains		
E. coli		
CC118(λpir)	CC118 lysogenized with λ*pir* phage	25
HB101	Host for plasmid pRK600	5
TG1	Host for DNA manipulations	45
P. putida		
KT2442	*hsdR* Rif	21
PBS4	KT2442 with *PalkB*::*lacZ* and *alkS* in the chromosome	56
PBS4B1	PBS4 with an inactivated *cyoB*::*tet* allele	This work
PBS4C1	PBS4 with an inactivated *crc*::*tet* allele	57
PBS4BC1	PBS4 with an inactivated *crc*::*xylE* and *cyoB*::*tet* alleles	This work
RC12	KT2442 with *PalkB*::*kan* in the chromosome	This work
RC12S8	KT2442 with *PalkB*::*kan* and *alkS* in the chromosome	This work
RCM4	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoC* gene	This work
RCM18	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoB* gene	This work
RCM2A	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoB* gene	This work
RCM3A	RC12S8 with a mini-Tn*5*Sm insertion at ORF2 of the *cyo* cluster	This work
RCM6A	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoA* gene	This work
RCM22A	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoE* gene	This work
RCM117A	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoB* gene	This work
RCM137A	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoA* gene	This work
RCM2B	RC12S8 with a mini-Tn*5*Sm insertion at the *cyoE* gene	This work
Plasmids		
pALKRC1	Ap^r Tel^r, *PalkB*::*kan* transcriptional fusion into pJMT6	This work
pCRC5	Ap^r; *P. putida* KT2442 *crc* gene cloned into pUC18	57
pCRC5A	Ap^r; *crc*::*xylE* allele into pUC18; derives from pCRC5	This work
pCRC20	Sm^r; *crc*::*xylE* allele inserted at the *Bam*HI site of pKNG101	This work
pCYOB	*P. putida* KT2442 *cyoB* gene cloned into pUC18	This work
pCYOBTc	*cyoB*::*tet* allele into pUC18	This work
pJMT6	Ap^r Tel^r; mini-Tn*5* suicide donor plasmid	46
pKCYOBTc	*cyoB*::*tet* allele into pKNG101	This work
pKNG101	Sm^r; suicide vector for marker exchange mutagenesis	29
pPB7	Ap^r; *PalkB* promoter into pUC19Ω	56
pPBK10	Ap^r; *PalkB*::*kan* fusion into pUCKm	This work
pPBKJ1	Ap^r; *PalkB*::*kan* fusion into pUJ8	This work
pRK600	Cm^r Mob$^+$ Tra$^+$; donor of transfer functions	30
pTCS1	Ap^r Tc^r; *alkS* in mini-Tn*5*Tc	This work
pTS1	Ap^r; *alkS* into pT7-12	56
pUC18	Ap^r; cloning vector	55
pUCKm	Ap^r; contains a promoterless Km^r cassette	13
pUJS1	Ap^r; *alkS* gene cloned into pUJ8	56
pUJ8	Ap^r; vector for transcriptional fusions to *lacZ*	18
pUT-mini-Tn*5*Sm	Ap^r Sm^r; mini-Tn*5* suicide donor	18
pUT-mini-Tn*5*Tc	Ap^r Tc^r; mini-Tn*5* suicide donor	18
pXYLE10	Km^r; *P. putida xylE* gene as an excisable cassette	48

[a] Tel^r, tellurite resistant; Rif^r, rifampin resistant.

point of insertion was identified by arbitrary PCR (6, 41) and was later verified by PCR with specific primers.

Assay for β-galactosidase. An overnight culture of the appropriate strain was diluted to a final turbidity (A_{600}) of ca. 0.04 in fresh LB medium, or in minimal salts M9 medium supplemented with the indicated carbon source. When turbidity reached to 0.08, the nonmetabolizable inducer DCPK (0.05% [vol/vol]) was added where indicated. Cultures were grown at 30°C. At different time points, aliquots were taken and β-galactosidase activity measured as described by Miller (33). At least three independent assays were performed in each case.

S1 nuclease protection assays. Total RNA was isolated from bacterial cultures as previously described (34). S1 nuclease reactions were also performed as previously described (3) with 50 μg of total RNA and an excess of a 5′-end-labeled single-stranded DNA (ssDNA) hybridizing to the 5′ region of the mRNA. This allows detection of the transcription start sites, as well as detection of the amounts of transcript generated. The ssDNA probe was obtained by linear PCR as described previously (56) by using either plasmid pTS1 linearized with *Hin*dIII (contains *alkS* and its promoter region) or plasmid pPB7 linearized with *Pst*I (contains the *PalkB* promoter) as a substrate. The primers used hybridized 70 nt downstream of promoter *PalkS2* or 73 nt downstream of promoter *PalkB* (the start sites were as described elsewhere [7]).

RESULTS

Generation of mutant strains showing reduced catabolic repression in rich LB medium. To obtain mutant strains showing a reduced catabolic repression on the *PalkB* promoter in LB medium, strain RC12S8 was constructed (see Materials and Methods). This strain, which derives from *P. putida* KT2442, contains in its chromosome a copy of the *alkS* gene and a *PalkB*::*kan* transcriptional fusion in which a Km^r determinant is expressed from the AlkS-dependent *PalkB* promoter. Strain RC12S8 was sensitive to kanamycin when grown in the absence of a positive effector of the AlkS regulator. In the

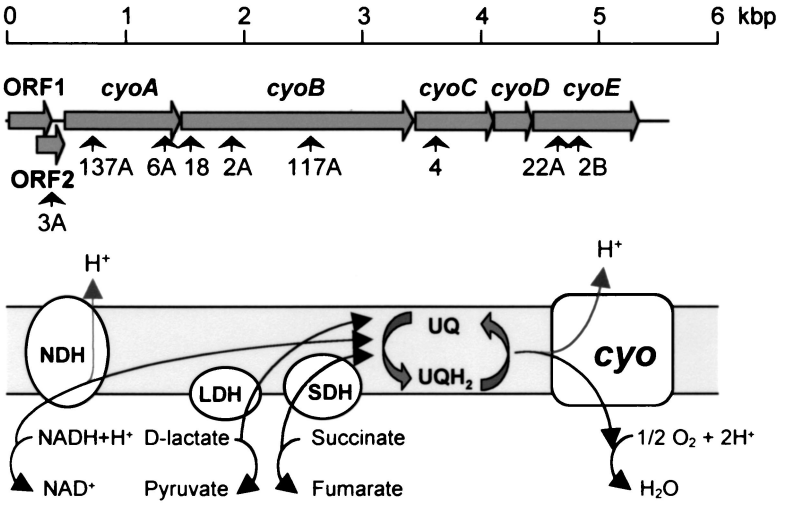

FIG. 2. Insertion points of mini-Tn*5*Sm in the *P. putida* catabolic repression mutants isolated. The upper scheme shows the genes encoding the *P. putida cyo* complex (28) and the positions of the mini-Tn*5*Sm insertions found in mutant strains RCM4, RCM18, RCM2A, RCM2B, RCM3A, RCM6A, RCM22A, RCM117A, and RCM137A (indicated by arrows). The *cyoA*, *cyoB*, *cyoC*, and *cyoD* genes encode subunits II, I, III, and IV of the oxidase complex, respectively; *cyoE* encodes the heme *o* synthase. The bottom scheme represents the electron transport chain. NDH, NADH dehydrogenase; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; UQ, oxidized ubiquinone; UQH_2, reduced ubiquinone; *cyo*, cytochrome *o* ubiquinol oxidase.

presence of such an effector (an alkane, or the nonmetabolizable analogue DCPK), strain RC12S8 was resistant to kanamycin when grown in a medium generating no catabolic repression (e.g., minimal salts medium containing citrate as carbon source). However, this strain was sensitive to kanamycin when grown in LB medium in the presence of the inducer DCPK, as might be expected owing to the strong catabolic repression that the LB medium imposes on *PalkB* induction. To search for mutants showing a reduced repression effect, strain RC12S8 was mutagenized with minitransposon mini-Tn*5*Sm. Nine mutant strains were isolated that were resistant to kanamycin in LB medium in the presence of inducer but not in its absence (see Materials and Methods). They were named RCM4, RCM18, RCM2A, RCM2B, RCM3A, RCM6A, RCM22A, RCM117A, and RCM137A. The sequence of the *PalkB* and *PalkS* promoters in all of these strains was found to be identical to that of the parental strain.

The point of insertion of the mini-Tn*5*Sm in these strains was localized either by cloning and sequencing of the chromosomal DNA segment containing the Sm^r determinant or by arbitrary PCR (see Materials and Methods). In mutant strain RCM4, the minitransposon was found inserted at an open reading frame showing 88% amino acid similarity to the *cyoC* gene of *P. putida* IH-2000 (28), which codes for subunit III (also known as subunit C) of cytochrome *o* ubiquinol oxidase (Fig. 2). In mutant strains RCM18, RCM2A, and RCM117A, the minitransposon was found to interrupt an open reading frame showing 96% amino acid similarity to the *cyoB* gene of *P. putida* IH-2000 encoding subunit I (or subunit B) of the same cytochrome (28). These two genes form part of the *cyoABCDE* cluster, which encodes subunits II, I, III, and IV of the *P. putida* cytochrome *o* oxidase complex (also known as the *cyo* complex) and the heme *o* synthase (28, 36) (see Fig. 2). Cytochrome *o* ubiquinol oxidase is one of the two terminal ubiquinol oxidases in the *P. putida* respiratory chain (36). In the other mutant strains analyzed, the minitransposon was found to map to other genes of the *cyo* cluster (Fig. 2), namely, the *cyoA* gene (strains RCM6A and RCM137A), and the *cyoE* gene (strains RCM22A and RCM2B), or at ORF2 (strain RCM3A).

Expression of the *PalkB* and *PalkS* promoters upon inactivation of the *cyoB* gene. To ascertain that the reduction of catabolic repression observed in the above mutant strains was due to the inactivation of cytochrome *o* ubiquinol oxidase rather than to another unknown mutation and to have a reliable system to measure the effect of this terminal oxidase on catabolic repression, a knockout mutation was performed at the *cyoB* gene of *P. putida* PBS4. This strain contains a *PalkB*::*lacZ* transcriptional fusion and the *alkS* gene inserted into the chromosome and was the host used in previous studies to analyze the catabolic repression of the *PalkB* promoter. Extensive work has shown that β-galactosidase levels in strain PBS4 faithfully reproduce the transcriptional activity of the *PalkB* promoter (56). The PBS4 *cyoB* gene was inactivated by marker exchange mutagenesis by using a plasmid containing a *cyoB* gene interrupted by a Tc^r determinant (the *cyoB*::*tet* allele; see Materials and Methods); the strain obtained was named PBS4B1. This strain showed normal growth in rich medium under the conditions used above and had essentially the same growth rate as the wild type. At least in *E. coli*, cells containing mutations in the *cyo* complex grow aerobically using the alternative *cyd* complex (2), which probably explains the normal growth of strain PBS4B1. When grown in defined medium with citrate, a compound that does not generate catabolic repression (56), activation of the *PalkB* promoter after the addition of the inducer was equally efficient in strains PBS4 and PBS4B1 (Fig. 3A). However, when cells were grown in LB medium, *PalkB* induction was different in the two strains (see Fig. 3B). As previously described (56), activation of *PalkB* in the wild-type strain PBS4 was very low during the exponential phase of growth and strongly increased at the onset of the stationary phase. This occurred at turbidity values (A_{600}) of ca. 1.2 to 1.4. In the case of the *cyo*-deficient strain PBS4B1,

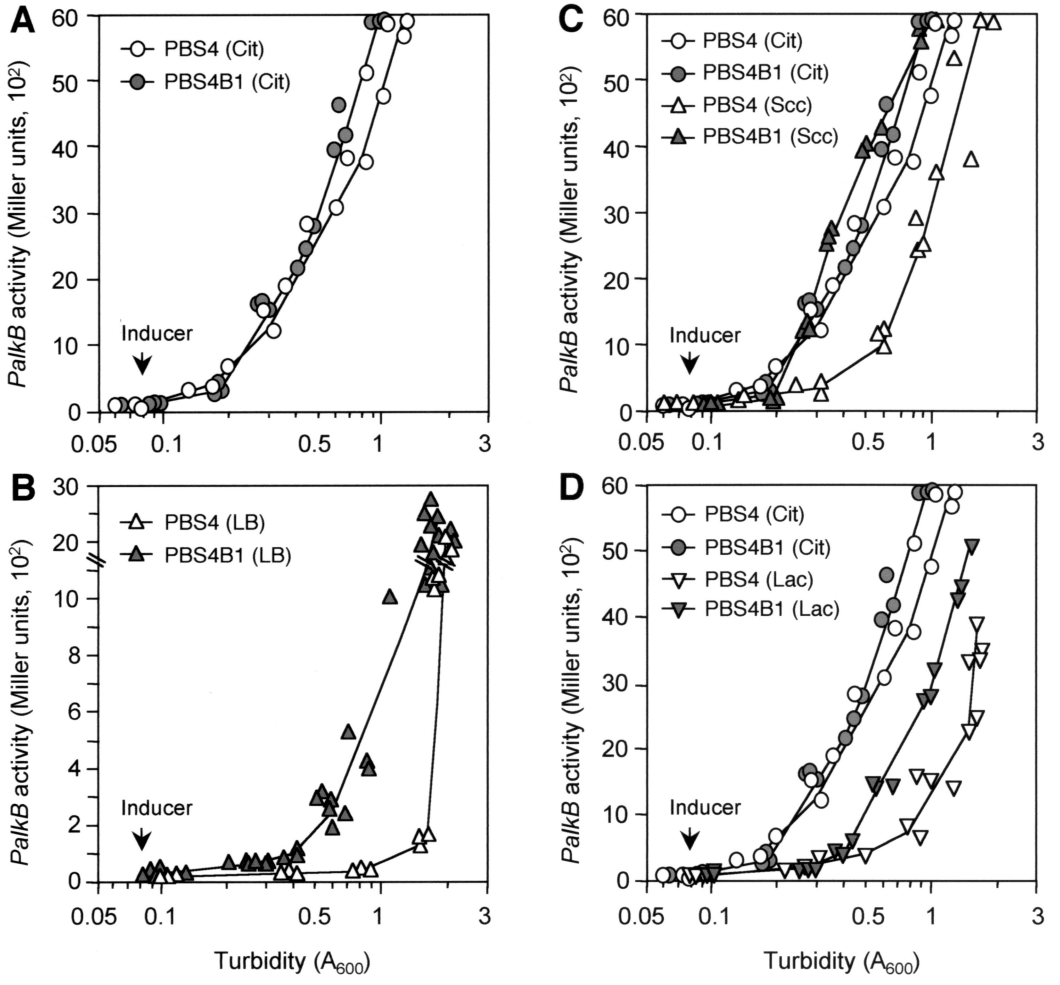

FIG. 3. Effect of the *cyoB* mutation on induction of the *PalkB* promoter in cells growing with different carbon sources. *P. putida* strains PBS4 and PBS4B1 (PBS4 with a knockout mutation at the *cyoB* gene) were grown in duplicate flasks either in LB medium (B) or in minimal salts medium containing either citrate (Cit) (A), succinate (Scc) (C), or lactate (Lac) (D) as the carbon source. At an A_{600} of 0.08, the nonmetabolizable inducer DCPK was added to one of the flasks; the other was left as a noninduced control. Aliquots were taken at different times, and the β-galactosidase activity was measured. The plots show the values observed for induced cultures (noninduced cultures had very low β-galactosidase activities [30 to 100 Miller units] and are not represented). The values shown correspond to three to six independent assays, all of which are represented on the same plot.

although *PalkB* activity was poor at the start of the exponential phase, repression was clearly relieved as growth proceeded. The β-galactosidase levels increased almost fourfold at mid-exponential phase (A_{600} of 0.5) and more than ninefold at late exponential phase (A_{600} of 1.0) compared to those with the wild-type strain PBS4 (Fig. 3B). If we consider the repression values observed for the wild-type strain, inactivation of cytochrome *o* ubiquinol oxidase therefore reduced the catabolic repression exerted by the LB medium by ~3.4-fold at the mid-exponential phase and by 7.5-fold at the late exponential phase of growth (Table 2). Since repression in the wild type is in the range of 50- to 70-fold (Table 2), it is clear that inactivation of cytochrome *o* ubiquinol oxidase only provides partial relief of the catabolic repression observed in LB medium.

The influence of the *cyoB*::*tet* mutation on the catabolic repression observed in cells growing in a minimal salts medium containing organic acids as the carbon source was also analyzed. In the case of the wild-type strain, the use of succinate or

TABLE 2. Catabolic repression of the *PalkB* promoter in strains PBS4 (wild type for *cyoB*) and PBS4B1 (*cyoB*::*tet*)

Medium	Activity (repression value)[a] in strain:				Repression relief[b]	
	PBS4		PBS4B1			
	ME phase	LE phase	ME phase	LE phase	ME phase	LE phase
Citrate	2,500 (1)	4,800 (1)	2,800 (1)	6,000 (1)	1	1
LB medium	50 (51)	70 (68)	190 (14.7)	670 (9)	3.4	7.5
Lactate	450 (5.5)	1,350 (3.5)	1,000 (2.8)	3,100 (1.9)	2	1.8
Succinate	750 (3.3)	3,000 (1.6)	3,900 (0.7)	6,000 (1)	3.3	1.6

[a] β-Galactosidase activities (in Miller units) in strains PBS4 and PBS4B1 at the mid-exponential (ME; A_{600} of 0.5) or late-exponential (LE; A_{600} of 1) phase of growth, growing in either LB medium or in a defined medium with citrate, lactate, or succinate as the carbon source, were obtained from Fig. 3. Repression values (indicated in parentheses) were obtained by dividing the activity observed when cells used citrate as a carbon source by that observed when LB medium, lactate, or succinate was used.

[b] That is, the quotient between the repression observed in strain PBS4 and that observed in the *cyo*-deficient strain PBS4B1.

lactate as the carbon source reduced *PalkB* induction at mid-exponential phase by about threefold in the case of succinate and by fivefold in the case of lactate versus the values observed when citrate was the carbon source (Fig. 3C and D; see also Table 2). Repression declined as cells approached the stationary phase of growth. The picture was different in the strain lacking a functional cytochrome *o* ubiquinol oxidase. In this case, succinate allowed an induction of the *PalkB* promoter as efficiently as citrate (Fig. 3C), showing that the *cyoB*::*tet* mutation had totally eliminated catabolic repression. When lactate was the carbon source, a reduction in repression was also evident, but the increase in *PalkB* expression was only of about twofold (Fig. 3D). Inactivation of cytochrome *o*, therefore, seems to affect not only the catabolic observed in LB medium but also the repression exerted by organic acids in a defined medium.

It was previously shown that the promoters *PalkB* and *PalkS2* are similarly regulated by the AlkS protein and are subject to the same catabolic repression effect (7, 57). To analyze whether inactivation of cytochrome *o* also eliminated catabolic repression of the *PalkS2* promoter, the activity of this promoter in cells growing in LB medium in the presence of the inducer DCPK was analyzed by S1 nuclease protection assays. As a control, the activity of the *PalkB* promoter was analyzed in parallel. As shown in Fig. 4, the levels of the transcripts originated at the *PalkS2* and *PalkB* promoters at the late exponential phase of growth were approximately sevenfold higher in strain PBS4B1 than in the wild-type strain PBS4. This is direct evidence that inactivation of cytochrome *o* ubiquinol oxidase diminishes catabolic repression at both the *PalkS2* and the *PalkB* promoters in cells growing in LB medium. It should be noted that when cells grow exponentially in the presence of DCPK, promoter *PalkS1* is inactive and is not influenced by catabolic repression (7).

Inactivation of the *crc* and *cyoB* genes has an additive effect in reducing catabolic repression. Inactivation of the *crc* gene decreases the catabolic repression of promoters *PalkB* and *PalkS2* in rich LB medium (57). If *crc* and *cyo* form part of independent regulation pathways, their simultaneous inhibition should have an additive effect. On the contrary, if they are components of the same regulation pathway, their simultaneous inhibition should cause a decrease in catabolic repression not greater than that exerted by any of the two elements alone. To distinguish between these two possibilities, a *P. putida* strain was constructed in which both the *crc* and *cyoB* genes had been inactivated by marker exchange mutagenesis (strain PBS4BC1). As shown in Fig. 5, at mid-exponential phase the β-galactosidase activity in the wild-type strain was of 50 Miller units; inactivation of the *crc* gene (strain PBS4C1) increased this activity to 220 U, whereas inactivation of the *cyoB* gene (strain PBS4B1) increased the activity to 190 U. Simultaneous inactivation of the *crc* and *cyoB* genes (strain PBS4BC1) led to β-galactosidase activities of 400 Miller units, a value that is about twice that observed in strains PBS4C1 or PBS4B1. This indicates that the catabolic repression effect exerted by the Crc and *cyo* functions is additive. It is likely, therefore, that Crc and *cyo* modulate induction of the *PalkB* promoter through different pathways.

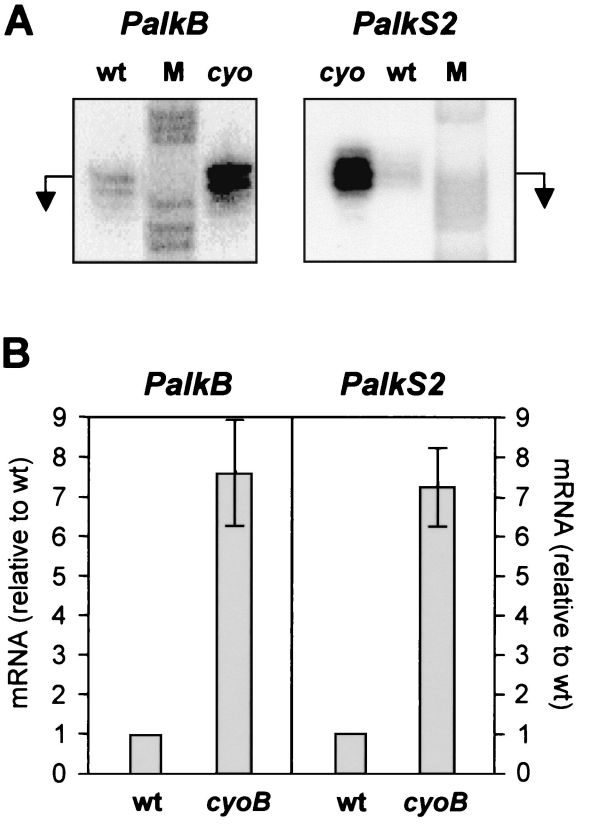

FIG. 4. Effect of the *cyoB* mutation on expression of the *PalkB* and *PalkS2* promoters. Strains PBS4 and PBS4B1 were grown in LB medium supplemented with the inducer DCPK (0.05% [vol/vol]). At an A_{600} of 0.8, cells were collected and the total RNA was obtained. The levels of mRNA originated at the *PalkB* and *PalkS2* promoters were measured by S1 nuclease protection assays in the presence of a large excess of the probe. Promoter *PalkS1* is inactive under these conditions (7) and gave no signal (results not shown). The cDNA resistant to S1 nuclease was resolved in a denaturing polyacrylamide gel, in parallel with a DNA size ladder obtained by chemical sequencing of the ssDNA used as probe.

DISCUSSION

The results presented here show that inactivation of cytochrome *o* ubiquinol oxidase reduces the catabolic repression that occurs at the *PalkB* and *PalkS2* promoters when cells grow exponentially in rich LB medium or in a defined medium with succinate or lactate as the carbon source. Repression reduction was only partial, since a clear repression effect was still observed. Inactivation of *cyo* did not affect growth rate significantly under the conditions used. Therefore, the lesser catabolic repression cannot be attributed to a decrease in the growth rate. Further, it is known that growth rate per se does not explain the catabolic repression of the alkane degradation pathway (56). Finally, mutations in many genes could have caused a decrease in growth rate, but the mutations obtained in subsequent screenings repeatedly mapped to the *cyo* cluster.

The observed link between a component of the electron transport chain and catabolic repression opens new ways to understand this global regulation process. There are some interesting examples of regulatory systems that monitor the flow of electrons through the electron transport chain and which

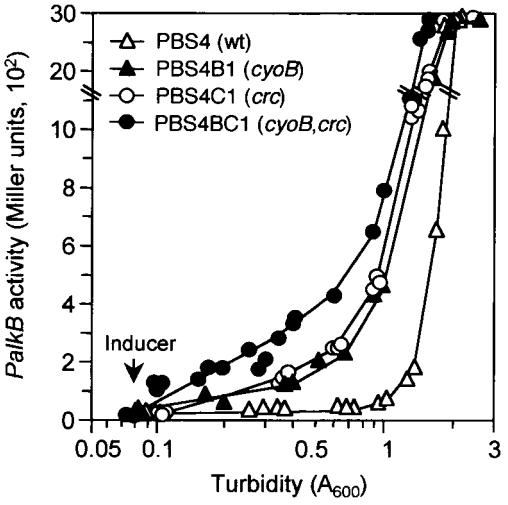

FIG. 5. Effect of the simultaneous inactivation of the *crc* and *cyo* genes on catabolic repression of the *PalkB* promoter. *P. putida* strains PBS4, PBS4B1 (PBS4 with a knockout mutation at the *cyoB* gene), PBS4C1 (PBS4 with a knockout mutation at the *crc* gene), and PBS4BC1 (PBS4 with knockout mutations at the *cyoB* and *crc* genes) were grown in duplicate flasks in LB medium. At an A_{600} of 0.08, the nonmetabolizable inducer DCPK was added to one of the flasks, whereas the other flask was left as a noninduced control. Aliquots were taken at different times, and the β-galactosidase activity was measured. The levels of β-galactosidase are represented as a function of cell growth. The plot shows the values observed for induced cultures (noninduced cultures had very low β-galactosidase activities [30 to 90 Miller units] and are not represented). The values shown correspond to several independent assays, all represented on the same plot.

use that information to regulate the activity of specific genes and integrate different metabolic activities. For example, in *Rhodobacter sphaeroides* the information obtained at two points of the electron transport chain is used to regulate the expression of photosynthesis genes (37, 38). A signal generated at the cbb_3 branch of the electron transport chain is transduced to a two-component activation system which directly regulates gene expression. In addition, the redox state of the quinone pool (the ubiquinol/ubiquinone ratio) is monitored by a redox-active antirepressor protein, which determines the functional state of a transcriptional repressor. The redox state of quinones is also used to regulate the transition from aerobic to anaerobic metabolism in *E. coli* by means of the Arc two-component system (23). In this case, the oxidized form of the quinones serves as a specific signal for the ArcB sensor kinase, silencing it and impeding phosphorylation of the ArcA global transcriptional regulator. It has also been proposed that the Aer protein responds to the cellular redox state to regulate aerotaxis, which guides cells to oxygen-rich environments (44). Therefore, it is clear that bacterial cells can monitor the activity of the electron transport chain and use this information to regulate gene expression. The results presented in the present study suggest that the flow of electrons through the electron transport chain, the redox state of the cell, or the amounts of *cyo* ubiquinol oxidase (the levels of which are known to depend on the oxygen tension [15, 16, 49]) could be one of the signals that *P. putida* uses to modulate catabolic repression of the alkane degradation pathway. It cannot be ascertained at present whether the *cyo* terminal oxidase has a direct or indirect role in this signal transmission process.

Inactivation of cytochrome *o* ubiquinol oxidase reduced, but did not eliminate, catabolic repression (except that generated by succinate). This suggests that catabolic repression depends on more factors than just the electron transport chain. It has recently been reported that the Crc protein has an important role in the catabolic repression of the alkane degradation pathway (57). Inactivation of the *crc* gene substantially reduced (although did not entirely eliminate) catabolic repression in rich medium but had no effect on the catabolic repression exerted by lactate or succinate in a defined medium. Crc appears to be an element of a signal transmission pathway connecting cell physiology to carbon metabolism, participating in catabolic repression of some sugar and amino acid pathways (14, 26, 27), as well as in cell adhesion (40). Simultaneous inactivation of the *cyoB* and *crc* genes generated a greater decrease in catabolic repression in rich medium than the individual inactivation of either *crc* or *cyoB*. This additive effect suggests that Crc and *cyo* form part of different signal transduction pathways, both of them contributing to catabolic repression. In both cases, the signal leads to a decrease in the AlkS-mediated induction of the *PalkB* and *PalkS2* promoters (57; the present study). The final consequence would be a decrease in the levels of the AlkS regulator, which appears to be present in the cell in limiting amounts (57). Keeping AlkS levels below those required for full induction of the pathway allows the cell to downmodulate expression of the alkane degradation genes when cells grow exponentially in medium containing a preferred carbon source in addition to alkanes. In agreement with this idea, overexpression of the *alkS* gene from a strong heterologous promoter totally eliminates catabolic repression (57).

Many other pathways for the degradation of linear or aromatic hydrocarbons are subject to physiological control (global regulation) in *Pseudomonas* spp. (17, 19, 32, 35, 39, 51), and the mechanisms involved often differ. Comparison of the factors implicated in the physiological control of the pWW0 toluene degradation pathway and of the *P. putida* CF600 phenol degradation pathway provides a remarkable example of how similar regulatory outcomes can be achieved by using different strategies (50). Both pathways are controlled by σ^{54}-dependent regulator-promoter pairs that are mechanistically and functionally similar. In the two cases, activation of the corresponding promoters is modulated by a dominant physiological control that generates a similar final effect. The host factors responsible for this physiological control differ in the two pathways (9, 12, 13, 50, 51, 52). The effect of the *cyo* terminal oxidase on the regulation of these pathways has not been specifically addressed. However, it is worth noting that the catabolic repression generated by excess succinate on expression of the pWW0 toluene degradation pathway decreases considerably when O_2 is limited (19, 20). In such conditions, the *cyo* terminal oxidase should decrease in favor of the *cyd* oxidase. This suggests the *cyo* terminal oxidase could also have some role in the repression of this pathway. Interestingly, the catabolic repression exerted by succinate on the expression of the phenol degradation pathway encoded in the *P. putida* H plasmid pPGH1 is reduced by inactivation of the *cyo* terminal oxidase (43). However, the effect of LB medium on the expres-

sion of this pathway has not been not reported. The role of the *cyo* terminal oxidase on the expression of other pathways has, to our knowledge, not been analyzed. The available data do not allow a unified picture of catabolic repression in *Pseudomonas* spp. to be drawn. However, the mechanisms used in each case seem to rely heavily on factors dictated by the promoters and regulators of the catabolic pathway (e.g., sigma factors involved in promoter recognition, regulator stability, etc.). It is likely that the physiological status of the cell is connected to gene expression in more than one way. Although certain mechanisms will probably be more suited to a particular pathway (or promoter-regulator pair) than to others, the evolutionary history of each pathway may be important in determining which factors participate in each case (10, 50). This could provide substantial diversity to physiological control mechanisms. However, more knowledge on the mechanisms mediating global control of the catabolic pathways of *Pseudomonas* sp. is required before final conclusions can be drawn.

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