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The Alkane Hydroxylase Gene of *Burkholderia cepacia* RR10 Is under Catabolite Repression Control

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In many microorganisms the first step for alkane degradation is the terminal oxidation of the molecule by an alkane hydroxylase. We report the characterization of a gene coding for an alkane hydroxylase in a *Burkholderia cepacia* strain isolated from an oil-contaminated site. The protein encoded showed similarity to other known or predicted bacterial alkane hydroxylases, although it clustered on a separate branch together with the predicted alkane hydroxylase of a *Mycobacterium tuberculosis* strain. Introduction of the cloned *B. cepacia* gene into an alkane hydroxylase knockout mutant of *Pseudomonas fluorescens* CHAO restored its ability to grow on alkanes, which confirms that the gene analyzed encodes a functional alkane hydroxylase. The gene, which was named *alkB*, is not linked to other genes of the alkane oxidation pathway. Its promoter was identified, and its expression was analyzed under different growth conditions. Transcription was induced by alkanes of chain lengths containing 12 to at least 30 carbon atoms as well as by alkanols. Although the gene was efficiently expressed during exponential growth, transcription increased about fivefold when cells approached stationary phase, a characteristic not shared by the few alkane degraders whose regulation has been studied. Expression of the *alkB* gene was under carbon catabolite repression when cells were cultured in the presence of several organic acids and sugars or in a complex (rich) medium. The catabolic repression process showed several characteristics that are clearly different from what has been observed in other alkane degradation pathways.

Alkanes are major components of crude oil and are also present in many organisms, such as green plants (43, 54). It has long been recognized that many microorganisms can use medium- or long-chain *n*-alkanes as sources of carbon and energy (27), which has stimulated many studies on the usefulness of these organisms in the bioremediation of oil spills and contaminated sites (reviewed in references 2 and 44). In addition, the enzymes involved in alkane degradation have proven to be useful and versatile biocatalysts, opening the possibility of their use in the industrial production of fine chemicals (16, 57, 59).

Alkane degradation by bacteria usually occurs through the sequential oxidation of one or both terminal methyl groups of the molecule, first to an alcohol, then to an aldehyde, and finally to a fatty acid, which is assimilated through the β-oxidation pathway (reviewed in references 7 and 43). The best-characterized alkane degradation pathway is that encoded by the OCT plasmid of *Pseudomonas putida* (*Pseudomonas oleovorans*) GPo1 (reviewed in references 56 and 58). In this case, initial oxidation is performed by an alkane monooxygenase system composed of a membrane-bound nonheme iron monooxygenase (a hydroxylase) and two soluble proteins, rubredoxin and rubredoxin reductase, which act as electron carriers between NADH and the hydroxylase (40). The study of alkane hydroxylase diversity could provide useful information about the mechanism of action and about the regions involved in substrate specificity. Sequencing of small PCR products from several alkane-degrading bacteria suggested that alkane hydroxylases related to that of *P. putida* GPo1 are widely distributed (49). However, only a few bacterial alkane hydroxylases have been cloned and sequenced in addition to that of strain GPo1, namely those of *P. putida* P1 (49), *Stenotrophomonas maltophilia* (28), and some *Acinetobacter* spp. strains (41, 49, 53).

Regulation of the expression of alkane degradation enzymes has received much less attention. In the case of *P. putida* GPo1, the expression of alkane degradation genes is controlled by the AlkS protein (39), a transcriptional regulator that controls its own levels through a positive feedback mechanism (8). Expression of the pathway is also modulated by catabolite repression, depending on the carbon source being used (8, 20, 50, 62). In the case of *Acinetobacter* sp. ADP1, expression of the alkane hydroxylase gene is controlled by the transcriptional activator AlkR, a protein unrelated to AlkS (42). In *Acinetobacter* sp. strain M-1, which has two alkane hydroxylases of different substrate specificities, two regulators have been predicted (53). To our knowledge, regulation has not been studied in other alkane degradation pathways.

The isolation and characterization of a number of bacterial strains which can grow at the expense of residues obtained as end products of crude oil processing has recently been described (63). These residues show a considerable toxicity because they contain large amounts of high-molecular-mass polyaromatic compounds and heavy metals. The isolated strains did not degrade the polyaromatic compounds but rather degraded the high-molecular-mass alkanes present in the residue.

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TABLE 1. Strains and plasmids

Strain or plasmid	Description or relevant phenotype	Reference or source
Bacterial strains		
E. coli CC118(λpir)	CC118 lysogenized with λpir phage	23
E. coli HB101	Host for transfection with cosmid pLAFR3	6
E. coli TG1	Host for DNA manipulations	45
B. cepacia RR10	Degrades alkanes containing 12 to 30 carbon atoms	63
B. cepacia CPBC2	Strain RR10 with a *PalkB*::*lacZ* fusion in the chromosome	This work
P. putida KT2442	*hsdR*, Rif; host for DNA manipulations	15
P. fluorescens KOB2Δ1	*P. fluorescens* CHAO with a deletion in the *alkB* gene	J. B. van Beilen
Plasmids		
pALK12	Ap^r, contains a 3.4-kbp *Pst*I DNA segment from pALK8 including the 5′ end of the *B. cepacia alkB* gene	This work
pALK14	Ap^r, contains a 3.2-kbp *Bam*HI fragment from pALK7 cloned into the *Bam*HI site of pUC18; contains the 3′ end of the *alkB* gene	This work
pALK301	Km^r, contains the *B. cepacia* RR10 *alkB* gene in pNM185	This work
pALK31	Ap^r, contains a *PalkB*::*lacZ* transcriptional fusion; derives from pUJ8	This work
pALK32	Ap^r Tc^r, contains a *PalkB*::*lacZ* fusion into mini-Tn*5*Tc	This work
pALK7	Tc^r, contains a 22-kbp DNA segment including the *B. cepacia alkB* gene	This work
pALK8	Tc^r, contains a 20-kbp DNA segment including the 5′ end of the *B. cepacia alkB* gene	This work
pGEM7-Zf(+)	Ap^r, cloning vector	Promega
pLAFR3	Tc^r, broad-host-range cosmid vector	51
pNM185	Km^r, Mob^+, broad-host-range expression vector	36
pRK2013	Km^r, Mob^+, Tra^+, donor of transfer functions	14
pRR10	Ap^r, contains a 550-bp DNA segment of the *B. cepacia alkB* gene	This work
pUC18	Ap^r, cloning vector	61
pUJ8	Ap^r, vector to make transcriptional fusions to *lacZ*	13
pUT-mini-Tn*5*Tc	Ap^r Tc^r, mini-Tn*5* suicide donor plasmid	13

We describe here the characterization of a gene coding for an alkane hydroxylase in one of these strains, *Burkholderia cepacia* RR10. Strain RR10 is interesting because it can grow at the expense of alkanes containing 12 to at least 30 carbon atoms (63), a range that is broader than that reported for most bacterial isolates (43, 58). The alkane hydroxylase gene, which was named *alkB*, is related to that of *P. putida* GPo1. Its promoter was identified, and its expression was analyzed under different growth conditions. The regulation pattern showed both differences and similarities to that of other alkane hydroxylases characterized so far.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. Plasmid pRR10 derives from pGEM7-Zf(+) by insertion between its *Sac*I and *Eco*RI restriction sites of a 550-bp DNA fragment from *B. cepacia* RR10, which was obtained by PCR using degenerated oligonucleotides designed to amplify conserved regions of alkane hydroxylases related to the *P. putida* GPo1 AlkB protein, as described previously (49). Plasmids pALK7 and pALK8 were selected from a gene bank of *B. cepacia* RR10 total DNA in the cosmid vector pLAFR3 on the basis of their positive hybridization to the 550-bp DNA fragment cloned into pRR10. They contain overlapping DNA inserts of about 22 and 20 kbp, respectively. Plasmid pALK12 was constructed by cloning a 3.4-kbp *Pst*I DNA segment from pALK8, hybridizing with the 550-bp probe, in the *Pst*I site of pUC18. This DNA fragment includes the 5′ end of the *B. cepacia* RR10 *alkB* gene. A 3.2-kbp *Bam*HI fragment from plasmid pALK7, including the 3′ end of the *B. cepacia alkB* gene, was cloned into the *Bam*HI site of pUC18 to produce plasmid pALK14. To obtain a transcriptional fusion of the promoter for the *alkB* gene to the *lacZ* reporter gene, a 624-bp DNA fragment including positions −574 to +51 relative to the *alkB* transcription start site (the *alkB* translation start is located at position +117) was inserted between the *Eco*RI and *Bam*HI sites of plasmid pUJ8. The fragment was obtained by PCR using plasmid pALK12 as template and oligonucleotides 5′-aaggtcgaattcatgcaggc and 5′-ttttgt gcggatcctcgtcg as primers, which include restriction sites for *Eco*RI or *Bam*HI. The resulting plasmid was named pALK31. Subsequently, the 4.9-kbp *Not*I fragment with the *PalkB*::*lacZ* fusion was cut from plasmid pALK31 and inserted in the *Not*I site of Mini-Tn5-Tc, obtaining plasmid pALK32. Plasmid pALK301 contains the complete *B. cepacia alkB* gene obtained by PCR using total *B. cepacia* DNA as template; the DNA fragment obtained was digested with *Eco*RI and cloned into the *Eco*RI site of plasmid pNM185.

Media and culture conditions. Cells were grown at 37°C in Luria-Bertani (LB) medium or in minimal salts M9 medium (45), the latter supplemented with trace elements (4) and a carbon source (30 mM for organic acids or sugars and 1% [vol/vol] for alkanes, alkanols, or fatty acids). To prepare spent LB medium (46), *B. cepacia* RR10 was grown in fresh LB to stationary phase; cells were eliminated by centrifugation and subsequent filtration through a 0.45-μm-pore-size filter, the pH was adjusted to 7.0, and the medium was filter sterilized. Antibiotics were used at the following concentrations (in μg/ml): ampicillin, 100; kanamycin, 50; tetracycline, 12; streptomycin, 50.

Construction of a gene bank of *B. cepacia* chromosomal DNA. Total DNA from *B. cepacia* RR10, obtained as previously described (3), was partially digested with endonuclease *Sau*3AI and fractionated on a 10 to 40% linear sucrose gradient. DNA fragments 15 to 30 kbp in length were cloned into the *Bam*HI site of cosmid pLAFR3, using *Escherichia coli* HB101 as the host for transfection. About 7,000 independent clones were obtained, which were pooled and stored at −70°C in LB medium containing 30% glycerol.

Recombinant DNA techniques. General methods for DNA manipulation and Southern blots were performed as described previously (45). DNA was sequenced on both strands with an Applied Biosystems DNA sequencer by the S.I.D.I-U.A.M. Sequencing Service. The primers used to PCR amplify a 550-bp internal region of the *B. cepacia* alkane hydroxylase as well as PCR conditions have been described previously (49). All other amplifications were performed using standard protocols (annealing temperature, 55 to 65°C; elongation temperature, 70°C; 30 cycles). Plasmids were introduced into *B. cepacia* RR10 by conjugation using plasmid pRK2013 as the donor of transfer functions in triparental matings (13). In the case of *Pseudomonas fluorescens*, plasmids were introduced by electroporation as described previously (25).

Assay for β-galactosidase. An overnight culture of a *B. cepacia* strain harboring a *PalkB*::*lacZ* transcriptional fusion was diluted to a final turbidity of about 0.04 either in LB medium (fresh or spent, as indicated) or in minimal salts M9 medium supplemented with the specified carbon source and in the absence or presence of the indicated inducer. Cultures were grown at 37°C, and at different cell densities aliquots were taken and β-galactosidase activity was measured as

described by Miller (37). At least three independent assays were performed in each case.

S1 nuclease analyses of mRNAs. Total RNA was isolated from bacterial cultures as described earlier (38). S1 nuclease reactions were performed as described previously (3), using 25 μg of total RNA and an excess of a 5′-end-labeled single-stranded DNA (ssDNA) hybridizing to the 5′ region of the mRNA. The ssDNA probe was generated by linear PCR (62) using plasmid pALK12 cut with endonuclease *Not*I as substrate. This plasmid contains the 5′ end of the *B. cepacia* RR10 *alkB* gene and about 2.3 kbp upstream from it; the *Not*I target is located 213 nucleotides upstream of the RR10 *PalkB* translation start site.

Nucleotide sequence accession number. The nucleotide sequence of the 6,237-bp *B. cepacia* RR10 chromosomal region containing the alkane hydroxylase gene was submitted to the EMBL data bank under accession no. AJ293306.

RESULTS AND DISCUSSION

Identification of a gene coding for an alkane hydroxylase in *B. cepacia* RR10. A set of degenerated oligonucleotides has been described which allows the PCR amplification of a small internal region of genes related to the *P. putida* GPo1 and *Acinetobacter* sp. ADP1 alkane hydroxylases (49). Using these oligonucleotides, a 550-bp DNA fragment was obtained from *B. cepacia* RR10 coding for a polypeptide which shows 53% identity (71% similarity) to the corresponding internal region of the *P. putida* GPo1 alkane hydroxylase. The amplified DNA fragment was used as a probe to screen a gene bank of total *B. cepacia* RR10 DNA in cosmid pLAFR3. Two clones, named pALK7 and pALK8, yielded a positive signal and contained partially overlapping DNA inserts more than 20 kbp in length. A 3.4-kbp *Pst*I DNA segment from pALK8 and a 3.2-kbp *Bam*HI DNA segment from pALK7, both hybridizing to the probe, were cloned in pUC18, obtaining plasmids pALK12 and pALK14, respectively. The inserts in these two plasmids were sequenced. They were found to contain partially overlapping sequences spanning a 6,237-bp contig. A BLASTX (1, 19) analysis of this sequence revealed the presence of a 1,158-bp open reading frame (ORF) coding for a polypeptide showing high similarity scores to known or predicted alkane hydroxylases. The highest scores were obtained for the putative alkane hydroxylase from *Mycobacterium tuberculosis* H37Rv (54% identity), followed by the alkane hydroxylases from *P. putida* GPo1 (44% identity), *P. putida* F1 (44% identity), *Acinetobacter* sp. ADP1 (44% identity), and *S. maltophilia* (22% identity). A BLASTX search on the *Pseudomonas aeruginosa* PAO1 genome (http://www.pseudomonas.com) showed the presence of two putative alkane hydroxylases with 39 and 41% identity, respectively, to the *B. cepacia* RR10 predicted alkane hydroxylase. A similar search at the unfinished sequencing project of the *Burkholderia pseudomallei* genome (http://www.sanger.ac.uk) showed the presence of a polypeptide with 80% identity to the *B. cepacia* RR10 protein. A phylogenetic tree showing the relationships among these proteins is shown in Fig. 1. The *B. cepacia* RR10 alkane hydroxylase clustered in a separate group together with the predicted hydroxylases of *M. tuberculosis* and *B. pseudomallei*. The presence of the *M. tuberculosis* alkane hydroxylase in this group is surprising, considering that gram-positive bacteria are evolutionarily very distant from the *Burkholderia* group. The similarity between the different alkane hydroxylases was distributed throughout the entire polypeptide (not shown), being particularly strong at a series of invariant histidine boxes which have been found to be important and highly conserved in integral membrane non-heme iron proteins such as hydrocarbon hydroxylases and desaturases (26, 48, 49). The size (386 amino acids) and molecular weight (43,953) of the polypeptide are also similar to those of known alkane hydroxylases. Therefore, the cloned ORF most likely corresponds to an alkane hydroxylase and was named *alkB*, following the nomenclature used for *P. putida* GPo1, the most thoroughly characterized alkane hydroxylase. It is worth noting that among the reported PCR-amplified fragments of genes related to *P. putida* GPo1 alkane hydroxylase obtained from several alkane-degrading bacteria (49), the *B. cepacia* RR10 *alkB* gene showed highest similarity to that of *B. cepacia* ATCC 25416 (92.3% identity over the 182 amino acids in the PCR fragment).

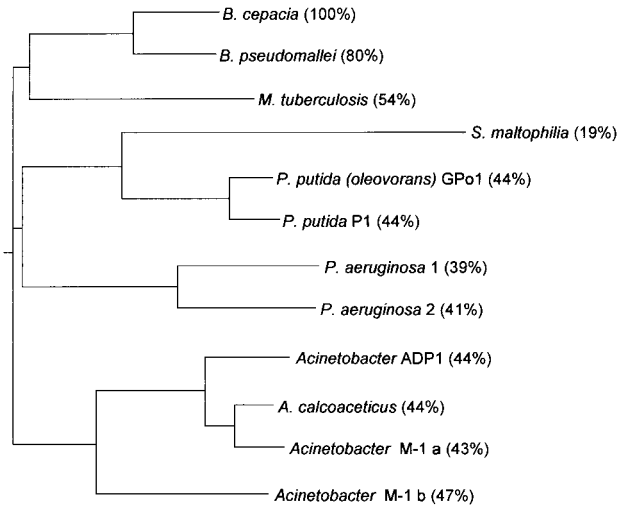

FIG. 1. Similarity of the *B. cepacia* RR10 alkane hydroxylase to other known or predicted bacterial alkane hydroxylases. Proteins were aligned with ClustalW (55) at the European Bioinformatics Institute web site (http://www2.ebi.ac.uk). The data obtained were used to generate a phylogenetic tree with the Phylodendron application at the IUBio Archive for Biology web site (http://iubio.bio.indiana.edu). The identity of each alkane hydroxylase to that of *B. cepacia* RR10 is indicated in parentheses.

A BLASTX analysis of the regions sequenced upstream and downstream of the *B. cepacia* RR10 *alkB* gene did not reveal the presence of other ORFs showing similarity to genes known to play a role in alkane oxidation. A detailed description is presented in Fig. 2. An inverted repeat (15-bp stem, 4-bp loop) followed by a run of T's, similar to a Rho-independent terminator, was found 19 bp downstream from the stop codon of the *B. cepacia alkB* gene, suggesting that transcription of *alkB* is not coupled to that of any other ORF located downstream of it. Altogether, these observations indicate that the gene coding for the membrane component of the *B. cepacia* RR10 alkane hydroxylase is not clustered with other genes required for alkane degradation. Present knowledge suggests that there is a large variability in the clustering of alkane degradation genes in bacteria. For example, the genes coding for alkane oxidation in the OCT plasmid of *P. putida* GPo1 are grouped in two clusters (58). In *S. maltophilia* a gene coding for a protein with similarity to rubredoxins but named rubredoxin reductase is located adjacent to that coding for the hydroxylase (28), while the two analyzed *Acinetobacter* sp. alkane hydroxylase genes

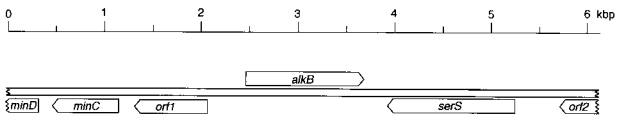

FIG. 2. Analysis of the ORFs surrounding the *B. cepacia* RR10 alkane hydroxylase gene. The 6,237-bp *B. cepacia* DNA region sequenced was analyzed for the presence of ORFs homologous to described genes of known function at the website services of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and of the European Bioinformatics Institute (http://www2.ebi.ac.uk) using the program BLASTX (19). The 1,158-bp ORF coding for a polypeptide showing high similarity scores to known or predicted alkane hydroxylases was named *alkB* (see the text). A polypeptide showing 30% identity to the *Methanococcus jannaschii* hypothetical protein MJ1207, which belongs to the acetyltransferase family of proteins, was found upstream of *alkB*; it is indicated as *orf1*. Downstream of *orf1,* an ORF was present encoding a protein 43% identical to *E. coli* MinC, followed by part of another ORF coding for a polypeptide homologous to *E. coli* MinD (80% identity in the region analyzed, which covers just the 112 N-terminal residues). *E. coli* MinC and MinD are known to play an important role in the process of cell division (reviewed in reference 31). Downstream of *alkB* and oriented in the opposite direction, an ORF was present encoding a protein 61% identical to the *E. coli* seryl-tRNA synthetase (*serS* gene). Upstream from *serS* and in the same orientation, there is an ORF coding for a polypeptide homologous to part of an *E. coli* hypothetical protein named YcaJ (84% identity in the region analyzed, which covers only the C-terminal half of the protein), the gene of which is also located upstream of *serS* in *E. coli*. It is indicated as *orf2*. The nucleotide sequence of the analyzed segment was deposited at the EMBL data bank under accession no. AJ293306.

are not linked to those encoding the rubredoxin and rubredoxin reductases (17, 18, 41, 53). Finally, the two putative alkane hydroxylases of *P. aeruginosa* PAO1 are located on separate sites of the chromosome and are not clustered with other genes of the alkane degradation pathway (unpublished observations).

Expression of the *B. cepacia* RR10 *alkB* gene in a heterologous host. To show that the cloned *alkB* gene indeed encodes a functional alkane hydroxylase, the gene was transferred to *P. fluorescens* KOB2Δ1, a derivative of the alkane-degrading strain *P. fluorescens* CHAO in which part of the alkane hydroxylase gene has been deleted (T. H. M. Smits, S. Balada, B. Witholt, and J. B. van Beilen, unpublished data). For this purpose, the *B. cepacia* RR10 *alkB* gene was cloned into the broad-host-range expression vector pNM185 under the influence of the *Pm* promoter. This promoter is activated by the plasmid-encoded XylS activator in the presence of an appropriate inducer, such as 3-chlorobenzoate, a compound that is not used by *P. fluorescens* KOB2Δ1 as a carbon source. Transfer of the resulting plasmid, named pALK301, to *P. fluorescens* KOB2Δ1 restored the ability of this strain to grow on alkanes. Growth was faster in the presence of the inducer 3-chlorobenzoate (doubling time of about 40 h, compared to about 24 h for *P. fluorescens* CHAO), although it was also evident in its absence (doubling time of about 70 h), probably because of a low basal expression of the *B. cepacia alkB* gene in the absence of the inducer. The recombinant strain-degraded alkanes have between 12 and 22 carbon atoms, a range similar to that observed for the parental strain *P. fluorescens* CHAO in parallel control assays. All these results show that the *B. cepacia alkB* gene encodes a functional alkane hydroxylase.

Characterization of the promoter of the *B. cepacia* RR10 alkane hydroxylase gene. To analyze the expression of the *B. cepacia* RR10 *alkB* gene in its original host, total RNA was purified from *B. cepacia* RR10 cells grown in minimal salts medium using tetradecane as the carbon source. The transcription initiation site as well as the levels of expression at different stages of the culture growth were determined by S1 nuclease protection assays. As shown in Fig. 3, a single transcription start site was detected, located 118 bp upstream of the *alkB* translation initiation codon. A clear −10 recognition sequence for the vegetative RNA polymerase (RNAP) was present upstream of the start site (Fig. 3B). A moderately conserved −35 box for the vegetative RNAP could also be recognized 17 bp upstream of the 5′ end of the −10 box. The amount of transcripts detected was reproducibly higher in cells collected at the start of the stationary phase than in cells collected in the exponential or in the late stationary phase. This behavior, which has not been observed in the few alkane degraders whose regulation has been studied to date, is reminiscent of the *E. coli* promoters recognized by σ^S-RNAP (21, 22, 30). However, the consensus for this form of RNAP in *E. coli* (5), which is probably conserved in other gram-negative bacteria as well (9), is not present in the *PalkB* promoter. Alternatively, promoter activity could be under the control of a quorum-sensing mechanism which is known to be present in *B. cepacia* (29). Despite several attempts, however, ethyl-acetate extracts obtained from tetradecane-grown stationary-phase cultures failed to stimulate expression of the *PalkB* promoter in exponential phase. Addition to a fresh culture of up to 10% (vol/vol) of a spent M9 medium obtained from tetradecane-grown cells did not have a positive effect either. The behavior of the *B. cepacia PalkB* promoter could be explained by assuming that it is regulated by a factor that is more active or that is present at higher levels in stationary-phase cells. In fact, when the *PalkB*::*lacZ* fusion was transferred to *E. coli* CC118(λpir) or to *P. putida* KT2442, only low β-galactosidase levels were observed (in the range of 150 to 200 Miller units), which were constant under all growth conditions. It is likely, therefore, that this promoter requires a transcriptional activator for induction.

To gain further insight into the regulation of the *PalkB* promoter, its expression was investigated in cells of strain RR10 growing either in a rich LB medium or in a minimal salts medium using citrate as a carbon source, in the absence or presence of tetradecane. No expression of the promoter could be detected in LB medium independent of the absence or presence of tetradecane and of the growth phase (Fig. 3A). Similar results were found with cells grown in minimal salts medium using citrate as a carbon source or using a mixture of citrate and tetradecane (not shown). This indicates that expression of *alkB* is regulated. Furthermore, these results suggest that alkanes are not preferred carbon sources for *B. cepacia*, so that when other more favorable carbon sources are used, expression of the *PalkB* promoter is downregulated by a strong catabolite repression. To further investigate this possibility, a transcriptional fusion of promoter *PalkB* to the *lacZ* reporter gene was cloned into the suicide donor plasmid pUT-mini-Tn*5*Tc and transferred to the *B. cepacia* RR10 chromosome. A representative transconjugant, named CPCB2, was selected for

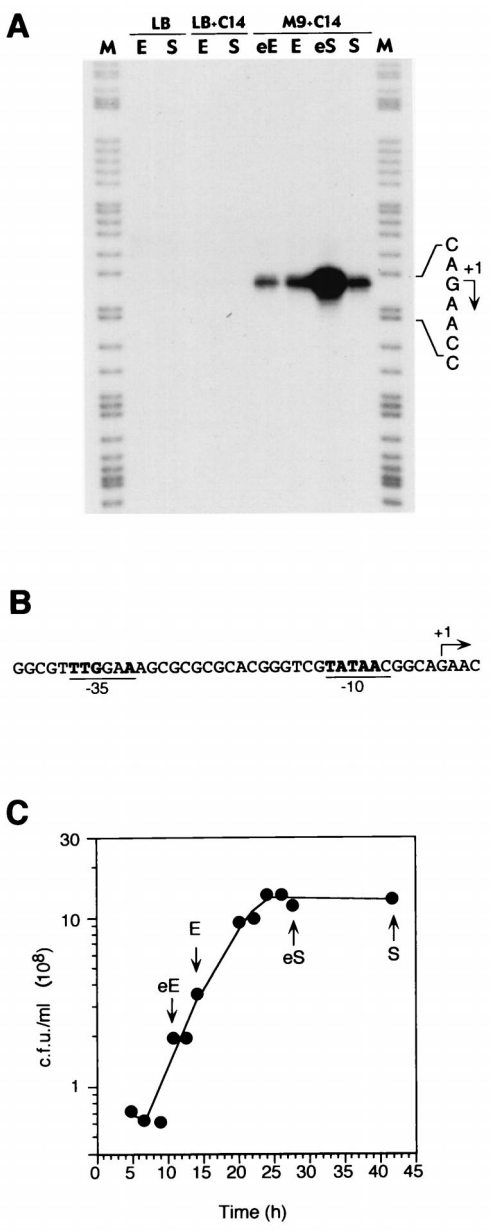

FIG. 3. Identification of the promoter for the *B. cepacia* alkane hydroxylase gene. (A) *B. cepacia* RR10 was grown in either M9 minimal salts medium in the presence of tetradecane (indicated as C_{14}) or in LB medium in the absence or presence of tetradecane. At different time points (early exponential phase, eE; exponential phase, E; early stationary phase, eS; or stationary phase, S), samples were collected and processed to obtain total RNA. Transcripts originating upstream of *alkB* were analyzed by S1 nuclease protection assays using equal amounts of total RNA and an excess of ssDNA probe in all cases. Samples were electrophoresed in parallel with a DNA size ladder obtained by chemical sequencing (35) of the same ssDNA used as a probe (lane M). The transcription start site is indicated by an arrow. (B) Sequence of the promoter for the *alkB* gene. The transcription start site observed in the S1 nuclease protection assay is indicated with an arrow. Positions at the −10 and −35 regions identical to those recognized by the vegetative RNA polymerase at promoters in most eubacteria (60) are highlighted in bold face. (C) The growth curve of *B. cepacia* RR10 grown in M9 minimal salts medium with tetradecane. Arrows indicate the time points when samples were collected to obtain the total RNA used for the S1 nuclease protection assays described for panel A. Growth was followed by measuring the increase in CFU on LB plates.

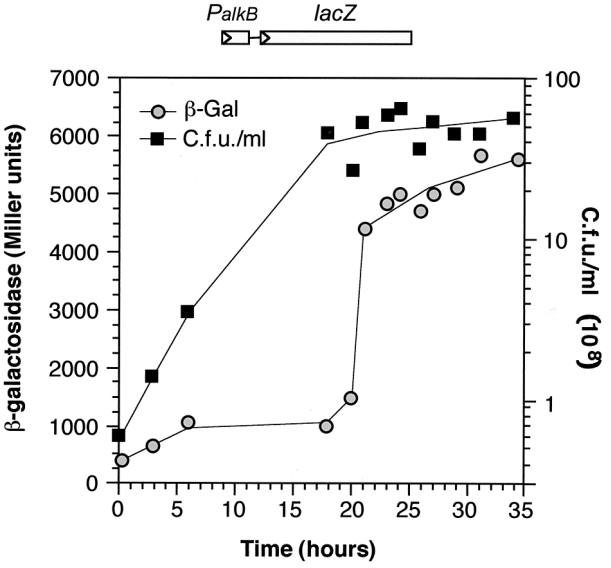

FIG. 4. Expression of the *B. cepacia* RR10 *PalkB* promoter in cells growing in a minimal salts medium using tetradecanol as the carbon source. *B. cepacia* strain CPBC2, a derivative of RR10 containing a *PalkB*::*lacZ* transcriptional fusion integrated into the chromosome, was grown in minimal salts medium containing tetradecanol as the sole carbon source. Samples were taken at different times, and the amount of β-galactosidase present in the cells was measured (represented as gray circles). Growth was followed in parallel by counting CFU on solid media (dark rectangles).

further analyses. Measurements of turbidity values in cultures of *B. cepacia* RR10 growing on alkanes proved to be unreliable due to the fine emulsification of the alkane, a problem which impaired a confident determination of β-galactosidase activities. However, we observed that the *PalkB*::*lacZ* fusion was efficiently induced by tetradecanol, which is a good growth substrate for *B. cepacia* RR10 and does not present the emulsification problem. Therefore, expression of the *PalkB*::*lacZ* fusion was monitored by measuring β-galactosidase activity throughout the growth curve in cells of strain CPCB2 grown in minimal salts medium containing tetradecanol as a carbon source. As shown in Fig. 4, β-galactosidase activity remained at moderate levels (about 1,000 Miller units) during the exponential phase of growth and rapidly increased as cells entered into stationary phase, eventually reaching about sixfold higher values. Higher expression of the *PalkB* promoter in stationary-phase tetradecanol-grown cells agrees with the *PalkB* activity values observed in tetradecane-grown cells with S1 nuclease protection assays (Fig. 3).

Having established the behavior of the *PalkB*::*lacZ* fusion, we proceeded to test the effect of a number of carbon sources on the expression of the *PalkB* promoter. Promoter activity in cells grown in LB medium in the presence of tetradecanol was low and very similar to that of cells grown in its absence (Table 2). In exponentially growing cells, β-galactosidase levels in minimal salts cultures where tetradecanol was the sole source of carbon were about 12-fold higher than those in LB cultures in the presence of tetradecanol, a value that increased to 63-fold in stationary-phase cultures. These results agree with the repressing effect of the LB medium observed by the S1 nucle-

TABLE 2. Expression of the *B. cepacia PalkB* promoter in strain CPBC2 grown at the expense of different carbon sources

Carbon source[a]	*PalkB* promoter expression during:			
	Exponential phase		Stationary phase	
	Activity[b]	Repression[c]	Activity[b]	Repression[c]
TetOH	1,160	1	6,280	1
LB	74	NA	97	NA
LB+TetOH	100	12	99	63
Spent LB	74	15	115	54
Spent LB+TetOH	53	22	105	60
TetOH+citrate	82	14	102	61
TetOH+fumarate	64	18	80	78
TetOH+lactate	71	16	83	76
TetOH+pyruvate	101	11	97	65
TetOH+succinate	62	19	88	71
TetOH+glutamate	70	16	89	70
TetOH+acetate	129	9	100	62
TetOH+glycerol	140	8	80	78
TetOH+glucose	69	17	73	86
TetOH+fructose	79	15	123	51
TetOH+lactose	97	12	100	63
TetOH+arabinose	90	13	88	71
TetOH+myristic acid	292	4	379	16

[a] Cells were grown in fresh or spent LB medium in the absence or presence of tetradecanol (1% [wt/vol]) or in minimal salts medium in the presence of either tetradecanol (1% [wt/vol]) or a combination of tetradecanol and the indicated compound (30 mM for organic acids or sugars, 1% for myristic acid). TetOH, tetradecanol.

[b] Activity of the *B. cepacia PalkB* promoter (in Miller units) under each growth condition in exponentially growing or in stationary-phase cells. Values represent the averages of three independent assays; the standard deviation was less than 20%.

[c] Repression (in folds) indicates the decrease in promoter activity observed in each case relative to the activity observed when tetradecanol was the sole carbon source. NA, nonapplicable.

ase protection assays in cells growing in the presence of tetradecane (Fig. 3A). A similar repressing effect of the LB medium has been observed in the expression of the alkane hydroxylase of *P. putida* GPo1, as well as in other catabolic pathways for hydrocarbons (24, 33, 52), and is probably due to the amino acids in LB (8, 62). However, the repression effect on the *P. putida* GPo1 pathway vanishes when cells reach the stationary phase of growth. The behavior of the *B. cepacia PalkB* promoter was different. As shown in Table 2, stationary-phase cultures of strain CPBC2 grown in LB medium containing tetradecanol as the inducer showed essentially the same β-galactosidase levels as exponential cultures. This agrees with the transcription levels of the parental strain when grown in LB medium in the presence of tetradecane (Fig. 3). Another interesting characteristic of the catabolic repression effect of LB medium on the promoters of the *P. putida* GPo1 alkane degradation pathway is that repression is no longer observed when cells are grown on spent LB medium (a medium that has already supported cell growth) (62). Again, the behavior of the *B. cepacia PalkB* promoter was different, since cells grown in a spent LB medium did not show an increase in promoter activity in the presence of an inducer (Table 2). This distinct behavior suggests mechanistic differences in the way the metabolic status of the cell is coupled to the expression of the alkane hydroxylase in *P. putida* GPo1 and in *B. cepacia* RR10.

The effects of several organic acids and sugars in cells cultured in minimal salts medium containing tetradecanol were also analyzed. As shown in Table 2, all the organic acids tested, as well as glucose, fructose, lactose, and arabinose, strongly repressed expression of the *PalkB* promoter in the presence of tetradecanol, both in the exponential and stationary phases of growth. The effect of myristic acid, the product of tetradecanol oxidation, was also analyzed. This fatty acid decreased the induction effect of tetradecanol by about 4-fold in exponential phase and by about 16-fold in stationary phase. This repression is interesting, since fatty acids are the product of alkane oxidation. It is tempting to speculate that accumulation of fatty acids may serve as a checkpoint to limit oxidation of alkanes when cells sense that the β-oxidation pathway is overloaded. In summary, the behavior of the *PalkB*::*lacZ* fusion is in full agreement with the mRNA analyses performed with S1 nuclease protection assays. Both assays indicate that expression of the *PalkB* promoter is induced by hydrocarbons and that the induction is strongly repressed by catabolite repression in the presence of other compounds that cells probably metabolize preferentially.

Expression of the *alkB* gene in cells grown at the expense of alkanes of different chain lengths. Some alkane-degrading bacterial strains are believed to contain only one alkane hydroxylase, while others have two different (albeit related) alkane hydroxylases. Examples of the former possibility are *P. putida* GPo1 (56, 58) and *Acinetobacter* sp. ADP1 (41). *P. aeruginosa* PAO1 contains two related alkane hydroxylases, although their individual contribution to alkane metabolism has not been elucidated yet. *Acinetobacter* sp. strain M-1 has two related alkane hydroxylases with different substrate specificities; one of them preferentially oxidizes alkanes of 12 to 16 carbon atoms, while the other shows higher activity with alkanes of more than 20 carbon atoms (32). It has been shown that each hydroxylase is differentially regulated by a specific transcription factor, each one responding to the presence of alkanes of the chain length recognized by the corresponding hydroxylase (53). To investigate the range of alkanes able to induce the *B. cepacia* RR10 *alkB* gene, cells were grown in minimal salts medium containing alkanes of either 12, 14, 16, 18, 20, 24, 26, or 30 carbon atoms as the sole carbon and energy source. The expression of the *PalkB* promoter was analyzed in each case by S1 nuclease protection assays. As shown in Fig. 5, all these alkanes induced the *PalkB* promoter to similar levels. Unless we assume a gratuitous induction by some of these alkanes, this suggests that the hydroxylase may oxidize all these substrates. Since the inherent difficulties in the genetic manipulation of *B. cepacia* RR10 has precluded us from obtaining a mutation in the *alkB* gene, it is at present unclear whether *B. cepacia* RR10 contains one or several alkane hydroxylases. However, several data are compatible with the idea that the isolated *alkB* gene is the only alkane hydroxylase gene present. First, the PCR amplification strategy to isolate the probe used for cloning, based on degenerated primers directed towards conserved regions of known alkane hydroxylases, identified a single gene in several independent assays. Second, the amplified DNA fragment afforded a single hybridization band in Southern blots performed with total *B. cepacia* RR10 chromosomal DNA (data not shown). Finally, the complementation analyses described above showed that transfer of the *B. cepacia alkB* gene to *P. fluorescens* KOB2Δ1 (lacking the alkane hydroxylase that allows it to grow in C_{12} to C_{18} alkanes) allowed it to grow at the expense of

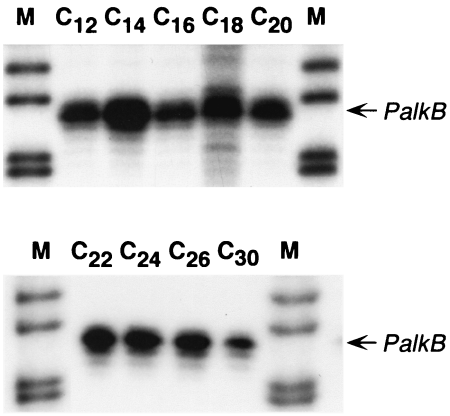

FIG. 5. Expression of the *B. cepacia* RR10 *alkB* gene in cells growing at the expense of alkanes of different chain lengths. *B. cepacia* RR10 was grown in minimal salts medium containing either dodecane (C_{12}), tetradecane (C_{14}), hexadecane (C_{16}), octadecane (C_{18}), eicosane (C_{20}), docosane (C_{22}), tetracosane (C_{24}), hexacosane (C_{26}), or triacontane (C_{30}) as the sole carbon source. Samples were taken at the end of the exponential phase, and expression of the *PalkB* promoter was analyzed as described in the legend to Fig. 3. Lane M corresponds to the DNA size ladder. The two panels correspond to gels containing equivalent amounts of sample exposed for the same period of time.

alkanes having from 12 to 22 carbon atoms, a range similar to that assimilated by the parental *P. fluorescens* CHAO strain in parallel control tests. In this case, factors other than the substrate range of the alkane hydroxylase possibly impede this strain to assimilate longer alkanes (J. B. van Beilen, unpublished observations).

Conclusions. The work presented here shows that *B. cepacia* RR10 contains an alkane hydroxylase which is induced by alkanes of very diverse chain lengths, its expression being fivefold higher at the onset of the stationary phase than during exponential growth. Transcription of the *alkB* gene was strongly repressed by catabolite repression by many carbon sources that *B. cepacia* seems to prefer over alkanes. Many degradation pathways for diverse hydrocarbons are controlled by catabolite repression (reviewed in reference 10). However, the repression exerted on the *B. cepacia alkB* gene is significantly stronger than that observed in other alkane degradation pathways. For example, expression of the *P. putida* GPo1 alkane hydroxylase is also repressed by several organic acids, but the effect is much milder (about fourfold [50, 62]). Similarly, citrate generates a strong repression in *B. cepacia* but has no effect in strain GPo1. The repression observed in LB medium is also different in the two strains. In *P. putida* GPo1, expression of *alkB* is strongly repressed during exponential growth, but repression ceases abruptly at the onset of the stationary phase (62). Repression of the *B. cepacia alkB* gene in LB medium was tightly maintained during the stationary phase. Similarly, repression is not observed when *P. putida* GPo1 is grown in a spent LB medium (62), although it is strong in the case of *B. cepacia*. This may be due to metabolic and/or mechanistic differences in the catabolic repression strategies of these two bacterial species.

Hydrocarbons, and alkanes in particular, seem to be unfavorable growth substrates for bacteria. This could be due at least in part to the fact that many hydrocarbons show a certain level of toxicity for bacteria. For example, despite providing abundant growth, alkane degradation is known to impose a stress on cell physiology in *P. putida* GPo1 (11, 12). Solvents like toluene activate multiple responses in gram-negative bacteria (47), while methyl benzoates switch on a stress response in *E. coli* and probably in *P. putida* as well (34). Alternatively (or in addition), the low solubility of hydrocarbons and the high metabolic cost of biosurfactant production limit their usefulness as carbon sources. Catabolite repression, which is probably advantageous for bacterial fitness in their natural environments, can pose limitations on the use of hydrocarbon-degrading bacteria for diverse biotechnological applications. A detailed knowledge of the global and specific regulation mechanisms of bacterial pathways for hydrocarbons will surely help to optimize their applications.

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