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Analyses of both the *alkB* Gene Transcriptional Start Site and *alkB* Promoter-Inducing Properties of *Rhodococcus* sp. Strain BCP1 Grown on *n*-Alkanes▽†

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Rhodococcus **sp. strain BCP1, known for its capacity to grow on short-chain *n*-alkanes (C_2 to C_7) and to cometabolize chlorinated solvents, was found to also utilize medium- and long-chain *n*-alkanes (C_{12} to C_{24}) as energy and carbon sources. To examine this feature in detail, a chromosomal region which includes the *alkB* gene cluster encoding a non-heme di-iron monooxygenase (*alkB*), two rubredoxins, and one rubredoxin reductase was cloned from the BCP1 genome. Furthermore, the activity of the *alkB* gene promoter (P_{alkB}) was examined in the presence of gaseous, liquid, and solid *n*-alkanes along with intermediates of the putative *n*-alkane degradation pathway. A recombinant plasmid, $pTP_{alkB}LacZ$, was constructed by inserting the *lacZ* gene downstream of P_{alkB}, and it was used to transform *Rhodococcus* sp. strain BCP1. Measurements of β-galactosidase activity showed that P_{alkB} is induced by C_6 to C_{22} *n*-alkanes. Conversely, C_2 to C_5 and $>C_{22}$ *n*-alkanes and alkenes, such as hexene, were not inducers of *alkB* expression. The effects on P_{alkB} expression induced by alternative carbon sources along with putative products of *n*-hexane metabolism were also evaluated. This report highlights the great versatility of *Rhodococcus* sp. strain BCP1 and defines for the first time the *alkB* gene transcriptional start site and the *alkB* promoter-inducing capacities for substrates different from *n*-alkanes in a *Rhodococcus* strain.**

As *n*-alkanes are the major components of crude oils, they are commonly found in oil-contaminated environments (10, 19). Among the microbial enzymes that oxidize liquid *n*-alkanes (C_5 to C_{12}), the alkane hydroxylase system of *Pseudomonas putida* GPo1 (formerly *Pseudomonas oleovorans*) has been the most characterized (30). This enzyme represents the prototype of a variable set of related non-heme iron integral membrane oxygenases, namely, AlkB-like alkane hydroxylases, xylene monooxygenases, fatty acid desaturases, fatty acid monooxygenases, steroid oxygenases, and decarbonylases (27, 32).

The alkane hydroxylase system of *P. putida* GPo1, which is encoded by the *alkB* gene cluster, consists of three polypeptides: an alkane hydroxylase (AlkB), a rubredoxin (AlkG), and a rubredoxin reductase (AlkT) (2, 3). The regulation of *P. putida* GPo1 *alkB* gene expression is mediated by the LuxR-UhpA-like transcriptional regulator AlkS in response to a variety of inducers, such as *n*-alkanes, but also by dicyclopropylketone (2, 6) and other aliphatic compounds (30).

In recent years, *alkB* genes have been examined in a few Gram-positive bacteria, such as *Rhodococcus* sp. strains NRRL B-16531 and Q15 (33), while studies on *alkB* gene expression have been limited to *Rhodococcus opacus* strains B4 and TMP2 (23, 28). In both these strains, multiple copies of the *alkB* gene are present, and their expression seems to be regulated by different mechanisms.

Rhodococcus sp. strain BCP1 was isolated from an aerobic butane-utilizing consortium that had carried out a prolonged chloroform cometabolic transformation in batch slurry reactors (5). Strain BCP1 was grown on gaseous *n*-alkanes (C_2 to C_4) and on liquid *n*-alkanes (C_5 to C_7) as well as on intermediates of the most common *n*-alkane metabolic pathways. BCP1 was shown to degrade chloroform along with vinyl chloride and 1,1,2-trichloroethane by cometabolic growth on either *n*-butane or *n*-hexane, while the degradation of *n*-butane and chloroform was inhibited by acetylene. Based on the latter observation, a possible role of an iron-containing monooxygenase in *n*-alkanes and chloroform metabolism of *Rhodococcus* sp. strain BCP1 was suggested (4).

As AlkB hydroxylases play a key role in the catabolism of various *n*-alkanes, here we describe the isolation in *Rhodococcus* sp. strain BCP1 of a 10.5-kb genomic region that includes the *alkB* gene cluster and the *alkB*-flanking regions. The transcriptional start site was also detailed, while the putative regulatory regions in the *alkB* upstream region were obtained through the alignment of the *alkB* upstream regions from different *Rhodococcus* strains. The *alkB* promoter activity was determined through the use of a promoter probe vector, $pTP_{alkB}LacZ$, for analyses of gaseous, liquid, and solid *n*-alkanes. Finally, the effects on P_{alkB} promoter activity from alternative carbon sources to *n*-alkanes were examined. This is the first study on the *alkB* gene transcriptional start site and the

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant genotype, characteristics, or sequence[a]	Reference
Bacterial strains		
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	7
E. coli LE392	*supF supE hsdR galK trpR metB lacY tonA*	25
E. coli K-12 (W1130)	*E. coli* wild type, Lac^+	1
Rhodococcus sp. strain BCP1	Ability to grow on *n*-alkanes and cometabolize low-chlorinated solvents	4
Rhodococcus sp. strain BCP1 $pTP_{alkB}LacZ$	Derived from *Rhodococcus* sp. strain BCP1; contains promoter probe vector plasmid $pTipP_{alkB}LacZ$	This work
Plasmids		
pLFR5	Tc^r, conjugative plasmid and cosmid vector	9
pUC18	Amp^r, cloning vector	22
pAlk1	Tc^r, pLAFR5 containing the 10.5-kb DNA genomic fragment of *Rhodococcus* sp. strain BCP1 hybridizing *alkB* probe	This work
pMC1	Amp^r, pUC18 containing the KpnI 10.5-kb fragment of pAlk1	This work
pTipQT2	Amp^r (*E. coli*) Tc^r (*Rhodococcus*), *PtipA repAB* (pRE2895)	16
$pTipP_{alkB}LacZ$	Derived from pTipQT2; promoter probe vector containing P*alkB* (117 bp)-*lacZ* in the multiple-cloning site	This work
Primers		
TS2S	CGCCAGGGTTTTCCCAGTCACGAC	26
Deg1RE	TCACACAGGAAACAGCTATGAC	26
PrEx6	ACAGATACCGCTTGCGATC	This work
PrEx8	ATCAGCCCCATGAGCCACA	This work
RT1	CGGACGTGTCGTGCTGATC	This work
RT2	GATCACCACGCGAACCCGA	This work
F1	TACATCGAGCACAACCGCGGC	This work
R1	ACGTCGTTACGCAGGGTCCAC	This work
R2	TGACGATGTGGTCGGAGTT	This work
PLacF	ATCATCGCGACCACCATGATTACGGATTCACTGG	This work
PLacR	ATCAGCATGCTTATTTTTGACACCAGACCAACTG	This work
PalkLF	ACTTGTACAGTCGTTCCTTCCTGACGA	This work
PalkLR	ACTAGATCTTGCGCTGTTCCAGTTCTCCGT	This work

[a] Underlined portions in primer nucleotide sequences indicate incorporated restriction sites.

alkB promoter-inducing capacity by substrates different from *n*-alkanes in a *Rhodococcus* strain.

MATERIALS AND METHODS

Growth medium and culture conditions. The bacterial strains, plasmids, and cosmids used in this study are listed in Table 1. Lysogeny broth (here indicated as LB) medium was used as a complex medium for *Escherichia coli* and also for *Rhodococcus* in the case of DNA isolation. *Rhodococcus* sp. strain BCP1 was grown on either LB or in a mineral medium (MM) (4) supplemented with the suitable carbon source (as indicated in Results). When necessary, antibiotics were added to select *E. coli* and *Rhodococcus* transformants in the culture medium (ampicillin at 50 μg/ml and tetracycline at 20 μg/ml for *E. coli* and tetracycline at 10 μg/ml for *Rhodococcus*).

To test growth on plates at 30°C, BCP1 was streaked onto MM plates, and the liquid *n*-alkanes were added as a vapor (32). Solid alkanes were added as a thin powder directly to the MM. Growth on liquid alkanes was tested in 250-ml flasks containing 50 ml of MM and placed at 30°C on a rotary shaker (150 rpm). Alkanes were added either at 0.05% (vol/vol) for liquid *n*-alkanes or at 0.1% (wt/vol) for solid *n*-alkanes. BCP1 growth was evaluated both as turbidity and cell dry weight.

In both the β-galactosidase assay and RNA extraction procedures, *n*-alkanes were added to liquid MM at a final concentration of 0.1% (vol/vol) for C_5, C_6, and C_7, 0.05% (vol/vol) for C_{10}, C_{12}, C_{14}, C_{16}, C_{17}, and C_{18}, 0.1% (wt/wt) for C_{20}, C_{22}, and C_{28}, and 150 μM for *n*-butane (C_4). 1- and 2-hexanol, hexanal, and hexanoic acid at 0.1% (vol/vol), and succinate at 0.1% (wt/vol) were used. All substrates were added to cultures kept in sealed serum bottles to prevent volatilization. The headspace volume was sufficiently large to prevent any O_2 limitation during growth.

Nucleic acid extraction and manipulation. Genomic DNA was isolated from BCP1 cells essentially as described by Frascari et al. (5). Before lysis, cells of *Rhodococcus* sp. strain BCP1 were acetone homogenized for 30 min by using a Potter-Elvehjem homogenizer and then incubated in the presence of lysozyme (30 mg/ml), mutanolysin (100 U/ml), and proteinase K (1 mg/ml) (1 h, at 37°C) before the addition of 150 μl of a 10% SDS solution and a subsequent incubation period of 5 min at 65°C. Cells were then mechanically disrupted by transferring the culture into tubes containing 0.5 ml of nitric acid prewashed quartz beads (0.2- to 0.8-mm diameter; MERCK KGaA, Germany) and homogenized at maximum speed for 120 s in a bead beater (Precellys 24 bead beater; Bertin Technologies). DNA was finally purified via phenol-chloroform extraction, isopropanol precipitation, RNase treatment, and ethanol precipitation (22).

Total RNA was extracted from 100 ml of early-stationary-phase cultures (optical density at 600 nm of 0.6 to 0.7) of *Rhodococcus* sp. strain BCP1 grown at 30°C on MM enriched (MMenr) with yeast extract, Casamino Acids, peptone (0.5 g/liter each), and succinate at 1% (wt/vol). After cell washing in phosphate buffer (10 mM; pH 7.2), each culture was suspended in 30 ml of MM. Cell cultures were then incubated for 4 h in MM at 30°C in the presence of variable substrates. One-tenth volume of ice-cold phenol-ethanol solution was finally added to each bottle to stop the RNA degradation before harvesting and storing at −80°C for later use.

RNA extraction was performed as described for genomic DNA extraction. The total RNA was treated twice with 5 U of RNase-free DNase (Qiagen; 30 min at 30°C) and then cleaned by using an RNeasy kit (Qiagen) before being used for primer extension and reverse transcription-PCR (RT-PCR) experiments.

Genomic library construction. To construct a genomic library with DNA fragments of approximately 25 to 30 kb, genomic DNA (20 μg) was subjected to partial digestion with the restriction enzyme Sau3AI and fractionated on a linear 10-to-40% sucrose density gradient (22). DNA fragments of the desired sizes were recovered and purified, then ligated into pLAFR5 (9) digested by ScaI and EcoRI, and packaged by using the Packagene Lambda DNA packing system (Promega). Transduction was performed in *E. coli* LE392 cells, and tetracycline (15 μg/ml) was used for the selection.

Cloning a 10.5-kb DNA fragment comprising the *alkB* gene. To identify an *alkB*-like gene within the genomic DNA of strain BCP1, PCR was performed

with two degenerated primers, TS2S and DegR1 (Table 1), as described by Smits et al. (26). The PCR product, corresponding to the expected length of 550 bp, was cloned (Topo TA cloning kit; Invitrogen), sequenced, and used as a probe in a colony blot assay with the *Rhodococcus* sp. strain BCP1 genomic library (22). The cosmidic DNA from the *alkB* probe-hybridizing clone in the colony blot assay was prepared by using the Qiagen plasmid Midi kit.

Genomic DNA fragments of interest were cloned in the pUC18 cloning vector, and positive plasmids were sequenced at the BMR Genomics Service of Padova (Italy). Samples were prepared according to the recommended procedures (www.bmr-genomics.it), and oligonucleotide synthesis was done by Invitrogen Srl.

Nucleotide sequence analysis. Geneious Pro 4.7.6 software was used to process the nucleotide and amino acid sequences, while homology searches were performed with pBLAST. The amino acid alignment program Clustal W (http://www.ebi.ac.uk/clustalw/) was used for the amino acid comparative studies, and putative conserved domains were detected by using the Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (11). A phylogenetic tree was created by using Geneiuos Tree Builder with the following parameters: genetic distance model, Juke-Cantor; tree-building method, neighbor joining; *P. putida* GPo1 *AlkB* as outgroup.

Primer extension analysis and RT-PCR. Primer extension analysis was performed as described by Roncarati et al. (20). Transcription from the *alkB* promoter was assayed by using two 19-mer oligonucleotides named PrEx6 and PrEx8 (Table 1). To map the *alkB* promoter, a T7 sequencing kit (USB) was used to sequence plasmid pMC1 (Table 1) in parallel with the same oligonucleotide as the primer extension. The primer extension assays were performed on RNA isolated from early-stationary-phase BCP1 cells that were exposed for 4 h to butane, hexane, dodecane, and succinate as previously described.

Total RNA was extracted from *Rhodococcus* sp. strain BCP1 cells harvested in their early stationary phase in MMenr medium after incubation with hexane, as previously described. For cDNA synthesis, total RNA was incubated for 60 min at 37°C with Omniscript reverse transcriptase (Qiagen). Control reactions to assess the level of DNA contamination in the RNA samples were carried out by omitting only the reverse transcriptase. Primers RT1 and RT2 and pairs of primers F1-R1 and F1-R2 were used for the RT reaction and successive PCR amplification, respectively. The thermal cycling conditions were as follows: 1 min at 94°C, followed by 25 cycles of 30 s at 98°C and 10 min at 72°C with *Taq* polymerase (Roche).

Promoter activity analysis. The *lacZ* gene of the *E. coli* W1130 genome was amplified by PCR with primers P_{LacF} and P_{LacR} (Table 1), containing NruI and SphI restriction sites, respectively. The amplicon was digested by NruI and SphI and cloned into the pMC1 vector (Table 1), replacing in frame the 2,111-bp NruI-SphI fragment that included the *alkB* gene (from nucleotide 155), *rubA*, *rubB*, and part of the rubredoxin reductase encoding gene. The resulting vector was designated pMC1::LacZ and was used as the template in PCRs with the primers P_{alkL}F and P_{alkL}R, containing BsrGI and BglII restriction sites, respectively. The BsrGI-BglII-digested amplicon was cloned into pTipQT2 (Table 1), causing the elimination of both the pTipA promoter and multiple-cloning site. The resulting plasmid, named pTP_{alkB}LacZ (see Fig. S3 in the supplemental material), was introduced in *Rhodococcus* sp. strain BCP1 by electroporation as described by Treadway et al. (29). The transformed cells were selected with tetracycline (10 μg/ml) on trypticase soy agar plates.

pTP_{alkB}LacZ BCP1 cells were grown in 500 ml of LB with tetracycline (10 μg/ml). After phosphate buffer washing, the pTP_{alkB}LacZ cells were suspended in 500 ml of MM with tetracycline (10 μg/ml). Bottles (150 ml) were filled with 25 ml of cell suspension each, and after the substrate was added to each bottle, the cultures were sealed with butyl rubber stoppers and aluminum crimps; the cultures were incubated at 30°C on a rotary shaker at 200 rpm. After different incubation times (see below), 1-ml aliquots from each bottle were collected, suspended in 100 μl of breaking buffer (100 mM Tris-HCl [pH 7.5], 20% glycerol), and kept at −20°C for later use.

The pTP_{alkB}LacZ cells to be assayed were thawed on ice, and then 400 μl of buffer Z (40 mM $Na_2HPO_4 \cdot 7H_2O$, 60 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol; pH 7.0) was added to each aliquot. The β-galactosidase assays were performed by using the method described by M. Rose (21). pTP_{alkB}LacZ BCP1 cells were subjected to lysis by bead beater treatment. The reactions were then initiated by the addition of 0.2 ml of *ortho*-2-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg/ml in buffer Z) to each cell extract and stopped by the addition of 0.5 ml of Na_2CO_3 (1 M in H_2O). A blank sample was prepared every time in which buffer Z and ONPG were added, to a final volume of 1.2 ml, and no biomass was supplied.

Cell extract proteins were quantified by the bicinchoninic acid protein assay (ThermoScientific) using bovine serum albumin (BSA) as a standard. Each experiment was performed at least in triplicate.

Nucleotide sequence accession numbers. Sequences were submitted to the GenBank database and assigned the following accession numbers: *orfA*, HM771656; *orfB*, HM771655; *orfC*, HM771654; *orfD*, HM771653; *orfE*, HM771652; *orfF*, HM771651; *alkB*, HM771646; *rubA*, HM771647; *rubB*, HM771648; *rubR*, HM771649; and *tetR*, HM771650.

RESULTS

Growth of BCP1 on medium- and long-chain *n*-alkanes. In a previous study, *Rhodococcus* sp. strain BCP1 was described for its ability to grow on gaseous (C_2 to C_4) and liquid (C_5 to C_7) *n*-alkanes (4). Here, growth of BCP1 on MM plates and in liquid MM cultures supplemented with *n*-alkanes with carbon chain lengths of $>C_7$ was tested. *Rhodococcus* sp. strain BCP1 grew well on MM supplemented with *n*-alkanes ranging in length from C_{12} to C_{17}, but no growth was seen on MM supplemented with C_8, C_9, C_{10}, or C_{11} *n*-alkanes either on plates or in liquid medium. Although BCP1 growth on MM plates supplied with C_{18} to C_{28} *n*-alkanes was difficult to determine due to the insolubility of solid *n*-alkanes, a certain growth was indeed observed around the solid *n*-alkane agglomerates formed after the solidification of the medium. On the other hand, BCP1 cells grew significantly in liquid MM supplemented with C_{18} to C_{28} *n*-alkanes, despite the extremely low solubility of these compounds in water (10^{-5} to 10^{-3} mg/liter at 25°C).

Sequence analysis of the *alkB* gene cluster. The PCR amplification, conducted on *Rhodococcus* sp. strain BCP1 genomic DNA with degenerate primers designed by Smits et al. (26), resulted in one 550-bp PCR product. Its nucleotide sequence revealed a high degree of homology to other alkane hydroxylase sequences of Gram-positive bacteria available in the databases. The product was cloned, and the insert was used to screen the *Rhodococcus* sp. strain BCP1 genomic library. The clone of the genomic library, which was positive to the *alkB* probe hybridization in the colony blot assay, was named pAlk1 and further analyzed. The *Rhodococcus* sp. strain BCP1 DNA genomic fragment of pAlk1 was 10.5 kb long, and it was digested with KpnI to be ligated into the pUC18 cloning vector to create the pMC1 plasmid (Table 1). The restriction map of the 10.5-kb fragment was determined, and the discrete restriction fragments were inserted into pUC18 cloning vectors and introduced into *E. coli* DH5α. The nucleotide sequences of the subclones were obtained and ordered on the physical map (Fig. 1). Primer walking was then performed to cover the DNA sequence gaps. As a result, the whole 10.5-kb fragment was sequenced, covering each strand with a minimum of 2-fold redundancy.

Eleven putative open reading frames (ORFs) were assigned by taking into account the higher G+C contents in their codon regions and also by analyzing BLAST similarities of the amino acid and nucleotide sequences of each ORF. The downstream region revealed four consecutive ORFs homologues to the *alkB* gene cluster components: *alkB* (alkane monooxygenase), *rubA* (rubredoxin), *rubB* (rubredoxin), and *rubR* (rubredoxin reductase) (Fig. 1), covering a region of 2,893 bp.

The 11 putative ORFs were analyzed by using pBLAST, and the sequence similarity of each ORF with the homologues in the database was calculated by using the ClustalW program. Table S1 in the supplemental material summarizes the information regarding the identity values associated with each ORF

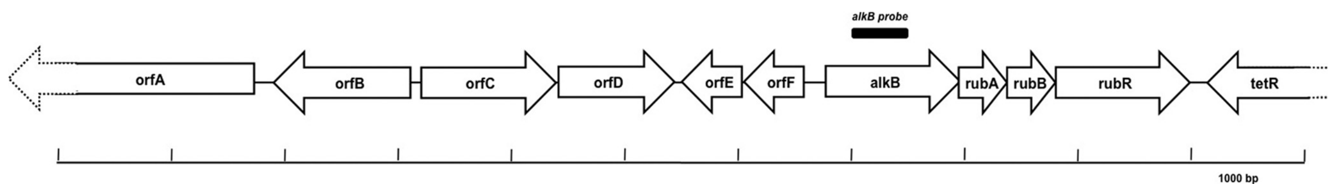

FIG. 1. Genetic organization of the *Rhodococcus* sp. strain BCP1 10.5-kb region, including the *alkB* gene cluster and the flanking regions. The ORF genes are shown as arrows, which indicate the corresponding direction of transcription. The location of the PCR fragment used as the *alkB* probe is also shown.

in the 10.5-kb fragment and the conserved domains identified in the amino acid sequences.

The only putative Shine-Dalgarno-like ribosome-binding site in the *alkB* region was found 8 bases upstream of the *alkB* start site (GGAGG). The genes *alkB*/*rubA* and *rubB*/*rubC* have 3′-end/5′-end overlaps that are TGATG for *alkB*/*rubA* and ATGA for *rubB*/*rubC*. Conversely, 1 nucleotide separates the stop codon of *rubA* and the start codon of *rubB*. The triplet GTG was found to be the *alkB* gene start codon, which is typical of the genetic code of *Rhodococcus* strains.

A dendrogram comparing the *Rhodococcus* sp. strain BCP1 AlkB sequence with the amino acid sequences of other alkane hydroxylases is provided in the supplemental material (see Fig. S1 and S2). In addition to the high full-length identity, the AlkB of the *Rhodococcus* sp. strain BCP1 protein contained three conserved histidines boxes (Hist1, HELXHK; Hist2, EH XXGHH; Hist3, LQRHSDHHA) plus an HYG motif (NYX EHYGL), which are typical of bacterial proteins belonging to the nonhemic iron integral membrane alkane hydroxylase family (27, 33).

Immediately downstream of the *alkB* gene, two consecutive rubredoxin genes (*rubA* and *rubB*) showed the nonheme iron-binding domain containing a $[Fe(SCys)_4]$ center. The 53-amino-acid RubA protein presents the highest sequence identity with the rubredoxin alkene monooxygenase rubredoxin of *Rhodococcus* sp. strain RHA1 (76%), while the 61-amino-acid RubB protein has the highest amino acid sequence identity with *Mycobacterium ulcerans* Agy99 rubredoxin RubB1 (86%). An additional large ORF in the *alkB* cluster, *rubR*, encodes a protein exhibiting significant full-length sequence identity to Gram-positive rubredoxin reductases (see Table S1 in the supplemental material).

The *alkB* gene cluster is flanked by two transcriptional regulators at both sides. The incomplete ORF following the *alkB* cluster encodes a protein with the highest amino acid sequence identity to a transcriptional regulator of *Rhodococcus* sp. strain RHA1 (80%), belonging to the TetR-like transcriptional regulator family. Because of this homology, the ORF immediately downstream of the *alkB* gene cluster was named *tetR*. In contrast, *orfF*, which is located upstream of the *alkB* gene cluster, encodes a transcriptional regulator belonging to ArsR family, while *orfE*, encoding an uncharacterized protein, is located upstream of *orfF*. These two consecutive genes have 3′-end/5′-end overlaps.

Analysis of the upstream flanking regions of *alkB* included in the 10.5-kb DNA fragment of *Rhodococcus* sp. strain BCP1 cloned in pMC1 was also performed. Based on comparison with the sequences in the database, the direction of transcription and the product of each ORF were predicted (see Table S1 in the supplemental material). While *orfA* encodes an uncharacterized protein, *orfB* and *orfC* encode an acetyl coenzyme A (CoA) acyltransferase and a 3-oxoacyl-[acyl-carrier protein] reductase, respectively. Protein OrfD was predicted to be a MaoC-like dehydratase.

Promoter analysis of *alkB*. The *alkB* gene start codon is separated from the *orfF* putative start codon by 210 bp. The transcriptional start site of the *alkB* gene, included in the 210 bp, was determined by primer extension analysis. Two different radioactively labeled oligonucleotides annealing at different positions in the 5′ region of the *alkB* gene were used (PrExt6 and PrExt8, which mapped to nucleotides 70 and 86, respectively) downstream of the GTG codon of the *alkB* gene.

Primer extension assays were conducted by using both these oligonucleotides with BCP1 cells exposed to *n*-dodecane, *n*-hexane, *n*-butane, and succinate. The primer extension product was detected by using only the total RNAs of *n*-dodecane-grown and *n*-hexane-grown cells, while no transcription product was revealed for *n*-butane-grown and succinate-grown cells (Fig. 2). The transcriptional start point as determined with both the oligonucleotides was a guanine 63 bp upstream of the *alkB* initiation codon (Fig. 2).

The *Rhodococcus* sp. strain BCP1 *alkB* upstream region (BCP1 P_{alkB}) was aligned with the corresponding 210-bp-long regions that are induced by *n*-alkanes in *Rhodococcus opacus* B4 and *Rhodococcus* sp. strain RHA1 (Fig. 3). The alignment of the *alkB* upstream regions showed the presence of nucleotide sequences, conserved among the rhodococcal *alkB* promoters, that are located around the regions suggested as putative −35 (TTGTCT) and −10 (TACTGT) regions separated by 22 bp in BCP1 P_{alkB} (Fig. 3).

Upstream of the BCP1 *alkB* putative −35 nucleotide region, a conserved region (Fig. 3, enlargement) includes two overlapping imperfect inverted repeat sequences that in BCP1 were named *invrepA* (CGTTTTacAAAttACG) and *invrepB* (ACAAAtTAcgaTAtTTTGT) (capital letters indicate the annealing part of the inverted repeat nucleotide strings). These two inverted repeat sequences share the motif ACAAATTACG. Another region showing a certain degree of conservation was identified downstream of the guanine mapping to the *alkB* transcriptional start site in *Rhodococcus* sp. strain BCP1. Conversely, the BCP1 P_{alkB} 11-bp sequence underlined in Fig. 3 did not align with any region of the other *Rhodococcus* strains.

To establish whether adjacent genes in the *alkB* gene cluster are cotranscribed, we conducted RT-PCR with RNA prepared from *Rhodococcus* sp. strain BCP1 grown in MM with *n*-hexane as a sole carbon source. When the RT1 primer, hybridizing to the 5′ end of *rubR*, and PCR primer pairs F1/R1 were used,

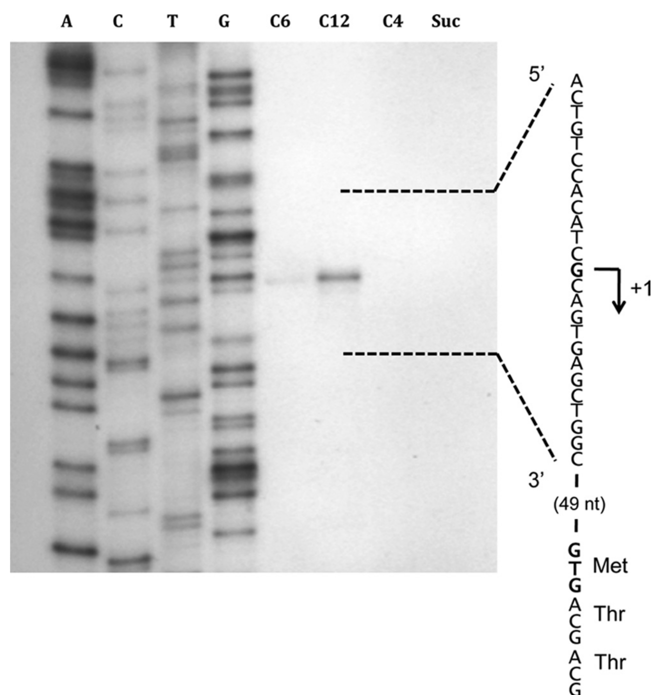

FIG. 2. Identification of the transcriptional start site of the *alkB* gene in *Rhodococcus* sp. strain BCP1. The polyacrylamide gel shows the primer extension products obtained from the total RNA isolated from BCP1 cells exposed for 4 h to *n*-hexane (C_6), *n*-dodecane (C_{12}), *n*-butane (C_4), and succinate (Suc). Lanes A, T, C, and G show the products of the sequencing reactions obtained with PrEx6 as primer. Lanes C6 and C12 show the primer extension signals with the same primer. The deduced transcriptional start site is shown in bold, and the direction of transcription is indicated by the arrow on the sequence. The distance between the start site and the start codon is also indicated in parentheses.

a PCR product of the expected size (250 bp) was obtained (Fig. 4). Utilizing the same cDNA (RT1-cDNA) as template, a 500-bp fragment was amplified with the other primer pair, F1/R2, confirming the result obtained with F1/R1 (Fig. 4). For the positive-control experiments, the RT2 primer (annealing the 3′ end of the *alkB* gene) was used in the reverse transcription step and the same primers pairs (F1/R1 and F1/R2) were used in the PCR. No amplification product was obtained when reverse transcriptase was omitted from the reaction mixture. These results, although not definitive, are taken as evidence that *alkB* is cotranscribed with the rubredoxin reductase; this suggests that the *alkB*, *rubA*, *rubB*, and *rubR* genes are transcribed as a polycistronic transcriptional unit.

***alkB* gene promoter activity.** To analyze the transcriptional regulation of the *alkB* gene cluster, the intergenic (*orfF-alkB*) DNA fragment (415 bp) also covering the 5′ end of the divergently oriented coding regions (200 bp and 117 bp in *orfF* and *alkB*, respectively) was cloned into the expression vector pTipQT2 fused with a promoterless *lacZ* gene (Table 1; see also Fig. S3 in the supplemental material). This promoter probe vector ($pTP_{alkB}LacZ$) (see Fig. S3) was transformed in *Rhodococcus* sp. strain BCP1 cells by electroporation, and the expression level of the promoter P_{alkB} in response to the presence of different substrates was measured in a β-galactosidase activity assay.

P_{alkB} activity as induced by different *n*-alkanes was markedly enhanced by incubation (24 h) with C_6 to C_{20} *n*-alkanes, while no induction was seen on either pentane (C_5) or the only gaseous *n*-alkane tested, *n*-butane (C_4) (Fig. 5). The activity of P_{alkB} was also weakly induced (three times over succinate) by C_{22}, while C_{28} was not a P_{alkB} inducer. Notably, the set of inducing alkanes also included *n*-decane, which is not used as

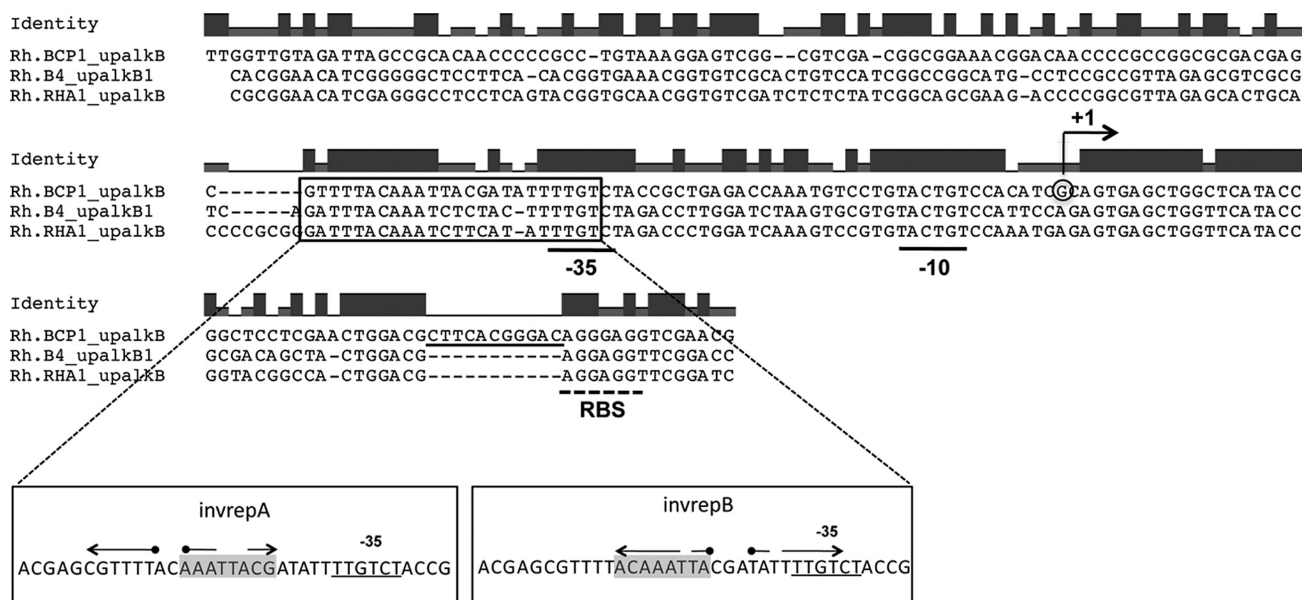

FIG. 3. Alignment of *Rhodococcus* sp. strain BCP1 P_{alkB} with the promoter regions of *alkB* genes inducible by *n*-alkanes in other *Rhodococcus* strains. The locus tags of the *alkB* genes taken into consideration are as follows: ro02534, *Rhodococcus jostii* RHA1; ROP 22570, *Rhodococcus opacus* B4. The identity is indicated. The deduced putative −10 and −35 promoter regions as well as the putative ribosome-binding site (RBS) are indicated below the alignment. The conserved region, including the two inverted repeat sequences, described in the text, is shown inside the black box (and in the enlarged section). The enlargement indicates the two imperfect inverted repeat sequences (*invrepA* and *invrepB*) in the promoter region of BCP1 *alkB*. The nucleotides forming the inverted repeats are indicated with divergently oriented arrows. The central sequence, which is present in both the inverted repeat sequences (see the text), is shaded in gray.

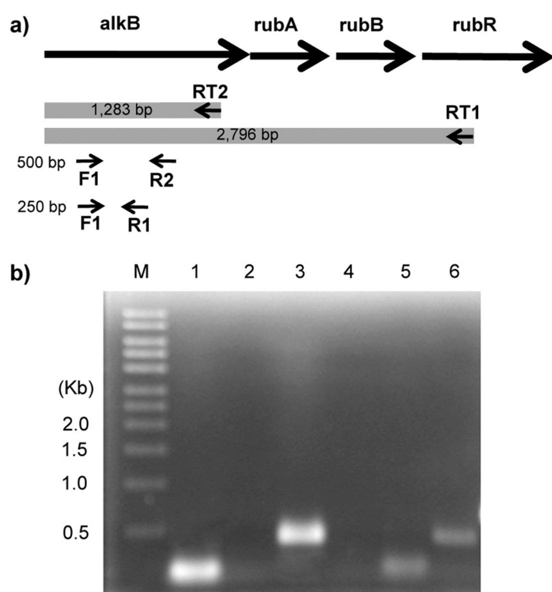

FIG. 4. Analysis of *alkB* gene cluster transcription by RT-PCR. (a) The genes, indicated above the large arrows, show the ORFs included in the *alkB* cluster. The oligonucleotides used for the RT-PCR experiment are indicated by small arrows below their annealing regions of the *alkB* cluster for RT1 and RT2 and below the annealing regions of the cDNA (shaded in gray) for F1, R1, and R2. The expected size of the PCR products obtained from the amplification with F1-R1 and F1-R2 (250 and 500 bp, respectively) are reported near each corresponding primer pair. (b) Lane 1, amplification with RT2, F1, and R1 (250 bp); lane 3, amplification with RT2, F1, and R2 (500 bp); lane 5, amplification with RT1, F1, and R1 (250 bp); lane 6, amplification with RT1, F1, and R2 (500 bp); lane M, molecular mass markers. Lanes 2 and 4 represent the negative controls.

a carbon source by *Rhodococcus* sp. strain BCP1. In summary, the highest inducing capacity was seen with C_{10} to C_{18} *n*-alkanes (600 to 1,300 nmol mg of protein^{-1} min^{-1}), while a prompt decrease in P_{alkB} induction resulted in switching to C_6/C_7 *n*-alkanes (around 400 nmol mg of protein^{-1} min^{-1}); an even lower inducing level was seen with C_{20}/C_{22} *n*-alkanes (100 to 250 nmol mg of protein^{-1} min^{-1}), while the expression activities with *n*-butane, *n*-pentane, and *n*-octacosane (C_{28}) were comparable to the basal activity given by succinate (20 to 40 nmol mg of protein^{-1} min^{-1}).

Table 2 shows the induction of P_{alkB} activities under three different concentrations of *n*-hexane after variable incubation times. Clearly, the P_{alkB} induction level after 24 h of incubation was proportional to the initial *n*-hexane concentration (R^2, 0.972). In addition, P_{alkB} induction by alternative carbon sources was measured in MM supplemented with *n*-hexane (0.1%, vol/vol) or dodecane (0.05%, vol/vol). As summarized in Table 2, the presence of these alternative carbon sources did not seem to repress the P_{alkB} induction promoted by *n*-alkanes. Additionally, P_{alkB} activities in the presence of a few key intermediates of the hydroxylation pathways of *n*-hexane are shown. Notably, P_{alkB} activity after 10, 19, and 24 h of exposure to *n*-hexane in the presence of hexanal (a putative aldehyde produced by alcohol dehydrogenase) or of hexanoic acid (produced by the aldehyde dehydrogenase) was strongly repressed. Furthermore, 1-hexanol enhanced the P_{alkB} activity by at least 2-fold, while 2-hexanol did not affect the P_{alkB} activity induced by *n*-hexane.

In Table 2, the effects on P_{alkB} activity by *n*-hexane metabolic products as used in the absence of *n*-hexane are also reported. Strikingly, the inducing capacity of 1-hexanol was almost 10 times higher than that obtained with 2-hexanol, although the latter alcohol is more soluble in water than 1-hexanol (5.9 g/liter and 14 g/liter, respectively). No effect on P_{alkB} activity was seen with hexanoic acid, and a very limited effect was seen with either hexanal or hexene (around 240 nmol mg of protein^{-1} min^{-1}).

DISCUSSION

The soil bacterium *Rhodococcus* sp. strain BCP1 was previously reported to grow on short-chain *n*-alkanes (C_2 to C_7) (4). In this present study we showed that strain BCP1 can also grow on medium- and long-chain alkanes ranging in length from C_{12}

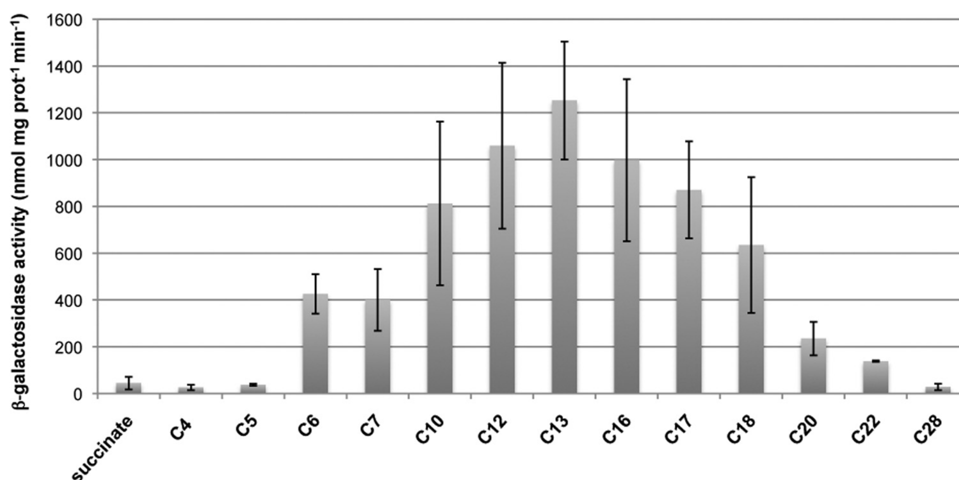

FIG. 5. *Rhodococcus* sp. BCP1 *alkB* promoter (P_{alkB}) activity induced by 24 h of incubation with *n*-alkanes having different chain lengths (the corresponding concentrations are indicated in Materials and Methods). Values represent the averages of three independent experiments with corresponding error bars.

TABLE 2. β-Galactosidase activities determined using PTP_{alkB}LacZ-BCP1[a]

Inducing substrate	Concn (%)[b] of inducing substrate	Addition	β-Galactosidase sp act[c] (± SD) after:		
			10 h	19 h	24 h
LB		None	92.7 (±43.2)	130.8 (±57.3)	106.4 (±48.5)
Succinate	0.1	None	48.5 (±17.3)	32.7 (±12.1)	44.3 (±26.6)
Glucose	0.1	None	56.0 (±20.5)	49.4 (±14.2)	55.9 (±13.8)
n-Hexane	0.05	None	81.9 (±38.7)	138.8 (±52.4)	140.9 (±35.7)
	0.1	None	228.7 (±98.6)	263.2 (±67.8)	425.7 (±84.3)
	0.1	LB	494.1 (±77.3)	484.2 (±48.7)	361.46 (±43.5)
	0.1	Succinate	425.6 (±102.5)	635.2 (±84.2)	360.6 (±172.9)
	0.1	Glucose	578.6 (±134.8)	721.3 (±109.1)	391.9 (±86.8)
	0.1	1-Hexanol	834.1 (±301.3)	947.9 (±83.2)	1,058.2 (±356.4)
	0.1	2-Hexanol	225.7 (±59.6)	241.3 (±67.5)	305.5 (±35.7)
	0.1	Hexanal	188.5 (±91.9)	130.3 (±14.4)	136.6 (±15.1)
	0.1	Hexanoic acid	155.8 (±5.8)	196.2 (±87.7)	178.9 (±35.3)
	0.5	None	1,019.1 (±180.4)	1,509.4 (±124.8)	1,363.1 (±174.7)
n-Dodecane	0.05	None	544.9 (±18.9)	739.2 (±126.1)	1,059.7 (±354.6)
	0.05	LB	582.3 (±278.9)	1,797.8 (±298.6)	1,726.0 (±198.0)
	0.05	Succinate	1,240.8 (±299.4)	1,851.9 (±274.4)	1,940.6 (±346.2)
	0.05	Glucose	954.8 (±154.2)	1,057.9 (±132.6)	1,007.5 (±241.4)
1-Hexanol	0.1	None	ND	ND	1,131.4 (±311.3)
2-Hexanol	0.1	None	ND	ND	110.8 (±30.0)
Hexanal	0.1	None	ND	ND	231.1 (±98.3)
Hexanoic acid	0.1	None	ND	ND	95.8 (±44.1)
Hexene	0.1	None	ND	ND	249.6 (±89.2)

[a] Cells were exposed to different substrates (the inducing substrate) for 10, 19, or 24 h with or without the addition of other substrates (indicated as additions; these substrates were added at the same concentration as the inducing substrate). Substrates were added to cell suspensions of pTP_{alkB}LacZ-BCP1 in MM. ND, not determined.

[b] The concentration refers to the percent (vol/vol) for liquid substrates and the percent (wt/vol) for solid substrates.

[c] β-Galactosidase values are reported as nmol/mg of protein/min. The values are the results of two independent experiments carried out in triplicate.

to C_{30}. Apparently, strain BCP1 does not grow in minimal medium supplemented with C_8 to C_{11} *n*-alkanes. This observation, which is presently unclear at the molecular level, is a feature similar to *Rhodococcus opacus* B4 cells, which are unable to grow on specific alkanes such as *n*-pentane (C_5) and *n*-hexane (C_6) (23).

Here we showed that the *alkB* gene in *Rhodococcus* sp. strain BCP1 is adjacent to genes encoding two rubredoxins and one rubredoxin reductase that are all required in the catalytic process as electron transfer proteins (31). Further, the phylogenetic analysis of BCP1 AlkB indicated the highest similarity with the homologous components in *Rhodococcus jostii* RHA1 and with the two *alkB* genes of *Rhodococcus opacus* B4 (see Fig. S1 and Table S1 in the supplemental material). The head-to-tail genetic organization of the *alkB* cluster displayed overlapping start-stop codons that suggest a translational coupling, as previously described by Whyte et al. (33). Similarly, the gene arrangement as an operon-like structure coding for the components of AlkB is mostly maintained among the few *alkB* gene clusters described for *Rhodococcus* strains. Only in *Rhodococcus erythropolis* PR4 does the final ORF of the cluster (rubredoxin reductase coding region) not follow the other three ORFs (*alkB* and the two rubredoxins) (24).

In the present analysis, the *Rhodococcus* sp. strain BCP1 *alkB* gene-flanking regions revealed the presence of two putative transcriptional regulators adjacent to *alkB*. In this respect, a putative TetR-type transcriptional regulator gene was previously found immediately downstream of the *alkB* gene of several rhodococcal strains as well as in actinomycetes (33). The conservation of the relative position of a TetR-like protein in proximity of the *alkB* gene cluster was taken as evidence of a role for TetR in the alkane-induced response, even though the actual function of this TetR-like transcriptional regulator is unclear (33). Notably, in rhodococcal strains closely related to *Rhodococcus* sp. strain BCP1, the TetR transcriptional regulator is transcribed in the same direction as the *alkB* gene cluster (14, 15, 24); conversely, the *Rhodococcus* sp. strain BCP1 transcriptional regulator is transcribed divergently from the *alkB* gene (Fig. 1). Moreover, unlike in other rhodococcal strains, *orfF*, which is located upstream of the BCP1 *alkB* operon, also encodes a transcriptional regulator. OrfF displays a helix-turn-helix (HTH) motif, suggesting a DNA-binding domain (ArsR HTH domain) typical of the ArsR-type transcriptional regulator family.

The 5′ region harbors *orfA*, encoding an unknown protein, which is followed by *orfB*, *orfC*, and *orfD* genes, encoding an acetyl-CoA acetyltransferase, a 3-oxoacyl-[acyl-carrier protein] reductase, and a MaoC-like dehydratase, respectively, that are putatively involved in fatty acid biosynthesis. Interestingly, although the location of *orfA*, *orfB*, *orfC*, and *orfD* is near *alkB* only in *Rhodococcus* sp. strain BCP1, their homologous genes are consecutive in all the related *Rhodococcus* strains and also in *Nocardia farcinica* IFM 10152 (8).

In this work we also localized the transcriptional start site upstream of the *alkB* gene of BCP1. Putative promoter elements were detected, and they appear to be conserved, based on comparisons with other rhodococcal strain regions upstream of *alkB* genes inducible by *n*-alkanes. According to these align-

ments, the presence of two imperfect inverted repeat sequences (*invrepA* [16 bp] and *invrepB* [19 bp]) is suggested, showing a common 10-bp sequence that can alternatively anneal with one of the two inverted sequences located at either side.

As the *alkB2* gene of *Rhodococcus opacus* B4 is not induced by *n*-alkanes (23), the conserved sequences obtained from the comparison of the *Rhodococcus* sp. strain BCP1 *alkB* upstream region and the one upstream of *alkB2* in *Rhodococcus opacus* B4 were tentatively assumed indicative of sequences not involved in the *n*-alkane-inducing transcription process. The region, including the two inverted repeat sequences (*invrepA* and *invrepB*), is conserved among rhodococcal P_{alkB} regions that are induced by *n*-alkanes (*Rhodococcus opacus* P_{alkB1}, *Rhodococcus* sp. strain RHA1 P_{alkB}, and *Rhodococcus* sp. strain BCP1 P_{alkB}) but is not maintained in *Rhodococcus opacus* P_{alkB2} (see Fig. S4 in the supplemental material). Further, the distinction between the BCP1 P_{alkB} nucleotide sequences conserved in the *n*-alkane-inducible P_{alkB}s and the BCP1 P_{alkB} nucleotide sequences conserved in *Rhodococcus opacus* B4 P_{alkB2} led us to suggest a role of the region, including the two inverted repeat sequences, as a regulatory element in the *n*-alkane-dependent response in *Rhodococcus* sp. strain BCP1.

This study shows that among the C_6 to C_{22} *n*-alkanes, those in the C_{12} to C_{16} range are the most efficient inducers of P_{alkB} activity. The latter evidence, along with the fact that *n*-butane, *n*-pentane, and *n*-octacosane (C_{28}) are not inducers of P_{alkB} activity, implies that the inducing capacities of specific *n*-alkanes are likely related to their aliphatic chain length (Fig. 5). The primary alcohol derived from *n*-hexane oxidation, 1-hexanol, showed a significant BCP1 P_{alkB}-inducing capacity, unlike the secondary alcohol, 2-hexanol. Although the reason for this phenomenon is still unknown, it is important to note that the P_{alkB}-inducing capacity of alcohols in BCP1 is different from that observed in *P. putida* GPo1 and *Burkholderia cenopacia* RR10 (6, 12). Indeed, in the latter organisms, no differences were seen in P_{alkB}-inducing capacity among the alcohol isomers derived from *n*-alkane oxidation. Moreover, unlike *Rhodococcus* sp. strain BCP1, both *Acinetobacter* sp. ADP1 and *P. aeruginosa* have alkane oxidation products that are not P_{alkB} inducers (13, 17, 18).

Finally, an important property of strain BCP1, compared to previously described *P. putida* GPo1 and *Burkholderia cenopacia*, is that in the latter bacteria the expression of *alkB* is subjected to catabolite repression, and therefore *n*-alkanes are not preferred growth substrates (12, 34). In contrast, the P_{alkB}-inducing capacity of *n*-alkanes in *Rhodococcus* sp. strain BCP1 (Table 2) is not affected by the presence of alternative carbon sources. This finding also underlines the unique feature of strain BCP1 as a good *n*-alkane degrader in the presence of more easily degradable carbon sources.

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