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ORIGINAL ARTICLE

Identification of different alkane hydroxylase systems in *Rhodococcus ruber* strain SP2B, an hexane-degrading actinomycete

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Abstract

Aims: To investigate the alkane-hydroxylating system of isolate SP2B, closely related to *Rhodococcus ruber* DSM 43338^{T} and uncharacterized so far for its alkane degradation genes.

Methods and Results: Although isolate SP2B and reference strain can grow on by-products from hexane degradation, the type strain *R. ruber* was unable, unlike SP2B isolate, to use short-chain alkanes, as assessed by gas chromatography. Using PCR with specific or degenerated primers, inverse PCR and Southern blot, two alkane hydroxylase encoding genes (*alk*B) were detected in both bacteria, which is in agreement with their alkane range. The first AlkB was related to *Rhodococcus* AlkB7 enzymes and contains a nonbulky residue at a specific position, suggesting it might be involved in medium- and long-chain alkane oxidation. The second partial *alk*B gene potentially belongs to *alk*B5-type, which was found in bacteria unable to use hexane. Moreover, a partial P450 cytochrome alkane hydroxylase, thought to be responsible for the hexane degradation, was detected only in the isolated strain.

Conclusions: *Rhodococcus ruber* SP2B should prove to be a promising candidate for bioremediation studies of contaminated sites because of its large degradation range of alkanes.

Significance and Impact of the Study: This is the first thorough study on *R. ruber* alkane degradation systems.

Introduction

Alkanes are common environmental pollutants, constituting 20–50% of crude oil, and not surprisingly, alkane-degrading micro-organisms are widely distributed in nature. In spite of their relative inertness, *n*-alkanes, ranging from C_{12} to C_{18} and longer, are readily degraded in the presence of oxygen and support abundant growth of many different bacteria (Watkinson and Morgan 1990). Most of them are able to oxidize *n*-alkanes by terminal hydroxylases to the corresponding alcohols, themselves channelled via fatty acids into the β-oxidation cycle. For the initial step of this catabolic pathway, bacteria mainly use integral membrane nonheme diiron monooxygenases of the AlkB type. Two groups are distinguished depending on a single specific conserved amino acid that determines the substrate range for the enzyme. The type-1 integral membrane alkane hydroxylases (AH1), mainly found in pseudomonads and in some other *Gamma-Proteobacteria* (like *Alcanivorax borkumensis*), oxidize short- and medium-chain alkanes, while most members of the AlkB enzyme belonging to AH2 group oxidize long-chain

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alkanes (van Beilen and Funhoff 2007). All those AlkB enzymes function in a complex in which the active site of these AlkB receives electrons from NADH through a mononuclear iron rubredoxin reductase AlkT and a dinuclear iron rubredoxin AlkG. The most extensively studied AlkB (AH1 type) is that of *Pseudomonas putida* GPo1, a strain able to grow on C_3 to C_{12} *n*-alkanes (Baptist *et al.* 1963; Johnson and Hyman 2006).

Related *alk*B homologs have been found in *Actinobacteria* (GC-rich Gram-positive bacteria) such as *Gordonia* (Fujii *et al.* 2004; Quatrini *et al.* 2008) or *Mycobacterium tuberculosis* H37Rv (Smits *et al.* 1999). Whyte *et al.* (2002) have made the first report of a detailed characterization of alkane hydroxylase systems in *Rhodococcus*, a genus considered important in the microbial communities of contaminated soils and sediments. They have found at least four homologous alkane monooxygenase genes (*alk*B1, *alk*B2, *alk*B3 and *alk*B4), in two different rhodococcal strains (*Rhodococcus* strains Q15 and NRRL B16531). Two of these genes (*alk*B1 and *alk*B2) are parts of clusters containing rubredoxin (*rub*A) and rubredoxin reductase (*rub*B) genes (homologues to *alk*G and *alk*T in *Ps. putida*), whereas *alk*B3 and *alk*B4 were found as isolated genes in the genome. The multiplicity of *alk*B homologs in the genome is frequently observed in *Rhodococcus* spp. (Smits *et al.* 1999, 2002; Whyte *et al.* 2002).

In addition to the AlkB class, other alkane hydroxylase systems, involved in the initial oxidation of alkanes longer than *n*-C_5, have been described, such as the P450 cytochrome alkane monooxygenase systems (CYP153) that hydroxylate C_5–C_{16} *n*-alkanes (van Beilen *et al.* 2006) or the flavin-containing alkane dioxygenase with a proposed substrate range from C_{10} to C_{30} (Maeng *et al.* 1996). More recently, two oxidation systems have been discovered for long-chain alkanes: the soluble flavoprotein alkane monooxygenase LadA (Feng *et al.* 2007) and the flavin-binding monooxygenase AlmA (Throne-Holst *et al.* 2006). Detailed reviews summarize these systems (van Beilen and Funhoff 2007; Wentzel *et al.* 2007). In fact, for many *n*-alkane degraders, a combination of different alkane oxidation systems with overlapping substrate ranges, have been reported. For example, five CYP153 genes have been reported for *Sphingomonas macrogoltabida* HXN-200 (*Alpha-Proteobacteria*); *A. borkumensis* isolates (*Gamma-Proteobacteria*) possess two AlkB and three CYP153 (Sabirova *et al.* 2006); an *n*-alkane dioxygenase and two integral membrane alkane hydroxylases have been identified in the Gamma-Proteobacterium *Acinetobacter* sp. M1, while *Acinetobacter* sp. DSM 17874 harbours two AlkB type and one AlmA enzyme.

The strain SP2B, sharing 88·1% DNA–DNA identity with the type strain *R. ruber* DSM 43338^T, has previously been isolated from a gasoline-degrading consortium (Amouric *et al.* 2006). This aerobic, Gram-positive, non-motile bacterium, forming orange-pink colonies, was compared to the type strain by biochemical tests. Sugar uses and enzyme activities of the API ZYM test for strain SP2B were identical to those of the type strain *R. ruber* DSM 43338^T (Amouric *et al.* 2006). However, *Rhodococcus* sp. SP2B was able to degrade *n*-alkanes from C_5 to C_{21}, in opposition with the type strain that degrades *n*-alkanes only from C_8 to C_{21}.

Although several *Actinobacteria* described this last decade for their ability to use *n*-alkanes showed a very large diversity of alkane-degradative systems, few of these have yet been completely characterized. Here, we describe the occurrence of several genes encoding enzymes involved in the first step of alkane degradation in *Rhodococcus* sp. SP2B, able to degrade hexane and other short-chain alkanes, in comparison with the close type strain *R. ruber* DSM 43338^T, for which no alkane system characterization has been carried out so far.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

Rhodococcus ruber DSM 43338^T was supplied by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). The hexane-degrading strain SP2B (FJ705352, 16S rRNA gene sequence) used in this study was previously isolated from a gasoline-degrading consortium as described by Amouric *et al.* (2006). *Rhodococcus ruber* DSM 43338^T and *Rhodococcus* sp. SP2B were cultivated in Luria–Bertani (LB) medium or in minimal medium (g l^{-1}): K_2HPO_4 2; KNO_3 1; KH_2PO_4 2; $(NH4)_2SO_4$ 2; NaCl 1; $MgSO_4.7H_2O$ 0·2; $CaCl_2.2H_2O$ 0·02, pH 7 and 10 ml of trace element solution (Balch *et al.* 1979), supplemented with the appropriate carbon and energy source. Flasks (118 ml) containing 20 ml of medium were incubated with shaking at 30°C and sealed with Mininert teflon valves (Alltech, Carquefou, France) when a volatile alkane was supplied (for example, hexane: 2·5 μl, corresponding to about 0·5 mg l^{-1} of hexane dissolved in liquid phase). *Escherichia coli* JM109 cells (Stratagene, La Jolla, CA, USA) were cultivated in LB broth or on LB agar with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) when necessary, as a host strain for the cloning of DNA fragments. Plasmids pUC18 (Stratagene) and pGEM-T Easy (Promega, Charbonnieres, France) were used as cloning vectors.

Acid nucleic isolation

Standard methods for DNA and RNA manipulations were used. Total DNA from *Rhodococcus*, grown on LB

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medium, was isolated as previously described (Amouric *et al.* 2006). For recovery and purification of DNA fragments from agarose, the QIAquick Gel Extraction kit (Qiagen, Courtaboeuf, France) was used. Total RNA was extracted from 30 ml cultures after growth on alkane or on rich medium. Ten millilitres of RNA protect reagent (Qiagen) were added and incubated for 5 min at room temperature. After centrifugation, the pellet was resuspended with 500 μl of lysis solution (EDTA 1 mmol l^{-1}, sodium acetate 20 mmol l^{-1}, SDS 0·5%) and then was extracted twice with 500 μl of hot phenol (pH 4·3, 60°C) and washed with 500 μl of chloroform. The RNA was precipitated with ethanol and dissolved in 40 μl of diethylpyrocarbonate (DEPC)-treated water. Following extraction, the RNA was purified with the RNeasy mini kit according to the manufacturer's procedure (Qiagen). The extracted total RNA was treated with DNase I (Gibco BRL, Cergy Pontoise, France) for 15 min at 37°C, as suggested by the manufacturer. DNase I was inactivated by boiling at 70°C for 10 min.

Plasmids DNA were isolated with the NucleoSpin Plasmid QuickPure (Macherey-Nagel, Hoerdt, France).

Identification of partial *alk*B and CYP153 genes

All the primers used are listed in Table 1. PCR amplification of partial *alk*B genes on genomic DNA of the two actinomycetes was first carried out using primers Alk-3F and Alk-3R, described by Kohno *et al.* (2002). For other partial *alk*B homologs, amplification was carried out using degenerated primers Rhose2 and Rhoas2, described by Monot *et al.* (2002). For CYP153 gene, PCR was performed with highly degenerated primers P450fw1 and P450rv3 designed by van Beilen *et al.* (2006). The PCR were performed using the Expand High Fidelity PCR System (Roche Diagnostics, Meylan, France). Purified PCR products were cloned into PGEM-T Easy vector (Promega) and transformed into *E. coli* JM109 competent cells (Stratagene), according to manufacturers' instructions. Positive transformants were further screened by direct colony PCR method using primers (T7 and SP6) specific for the PGEM-T Easy vector. Selected clones were sequenced (GATC Biotech, Konstanz, Germany). The obtained sequences have been submitted to the GenBank database (accession numbers: FJ705347, FJ705348, FJ705349, FJ705350, FJ7053517, FJ705352, FJ705353).

For inverse PCR experiments, 4 μg of genomic DNA were digested with either *Pvu*II or *Bam*HI at 37°C for 5 h. The DNA was self-ligated in a 200 μl reaction volume, ethanol precipitated and used in subsequent inverse PCR with divergent primers based on SP2B nucleotide sequence (FJ705347). Standard PCR were carried out in 50 μl volume with 0·5 U Expand High Fidelity PCR (Roche Diagnostics) and 40 ng of circularized DNA.

Cloning of an *alk*B gene and flanking DNA

To construct an enriched gene library, all *Csp*45I-restriction fragments of about 3 kb (detected by Southern blot) were cut out from a gel, ligated in pUC18 resulting in a plasmid pUC18-C3 and transformed into *E. coli* JM109 cells (Stratagene). Before sequencing, transformants containing the targeted gene were identified by colony blotting and were further screened by colony PCR with

Table 1 Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Target	Source
PCR or RT-PCR experiments			
P450fw1	GTS GGC GGC AAC GAC ACS AC	CYP153 gene	Various bacteria (van Beilen *et al.* 2006)
P450rv3	GCA SCG GTG GAT GCC GAA GCC RAA		
Rhose2	ACG GSC AYT TCT ACR TCG	*alk*B3/*alk*B4 gene	*Rhodococcus* group (Monot *et al.* 2002)
Rhoas2	GTG YTC GAG RTA GTT KGC		
Alk-3F	TCG AGC ACA TCC GCG GCC ACC A	*alk*B gene	Bacteria group III (Kohno *et al.* 2002)
Alk-3F	CCG TAG TGC TCG ACG TAG TT		
F-1S	CCG ACG GGC AGA ATC CGC C	*alk*Ba gene	*Rhodococcus* sp. SP2B (this work)
R-2P	CTC GCG TAG CCG CTG GGC A		
F926	AAG CGG CTC ACC AGT TCG TCT CG		
F1009	TGC GCT CTG GGC TGC TTG TTC TCT		
R1848	ATC ACC TCG TCG GGC GGA TTC TGC		
F1348	TGA TTT GCC GGT CGG TCT TAT TCC	*alk*Ba promoter region	*Rhodococcus* sp. SP2B (this work)
F1556	CCG AAG ATC AGG GGA CAG		
R1942	CCA CAG GTA GCA GGC GAA GAC		
rubA1A2-R1718	GTG GTC TTC TCA GCG TC	Rubredoxin or rubredoxin reductase genes	*Rhodococcus* sp. SP2B (this work)
rubA1-F2848	GGA CGC GAT TCC CGA CGA CTG GTG		
rubB-R3348	GGC CGG TTC GAG TTG CGC CTT CTC		

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universal primers (M13 and M13reverse) located on the pUC18 or with specific primers (F-1S and R-2P) of the *alk*B gene (Table 1).

Southern and colony blotting

Aliquots of 10 μg genomic DNA were digested with restriction enzymes *Dde*I and *Csp*45I, respectively. The DNA was transferred onto nylon membranes, positively charged (Roche Diagnostics) and probed with a digoxygenin-11-dUTP-labelled DNA according to the DIG Application Manual for Filter Hybridization (Roche). This hybridization probe corresponds to the partial *alk*B sequence (828 bp) amplified with primers F-1S and R-2P (Table 1), using the PCR DIG Probe Synthesis kit (Roche Diagnostics). For immunological detection of DIG-labelled probes, we used the specific anti-digoxigenin-peroxydase (POD), Fab fragments (Roche Diagnostics) and a luminol-based solution from UptiLight HRP blot substrate (Uptima; Interchim, Montluçon, France) as chemiluminescent substrate for the horse-radish POD.

For colony hybridization, transformants were transferred from LB plates onto a nylon membrane for colony and plaque hybridization (Roche Diagnostics). Hybridization step was carried out using the same DIG-labelled probe and detection of colony blots was carried out using the chemiluminescence substrate, according to the manufacturer's instructions. Positive colonies were further picked from the original plates.

RT-PCR experiments

Reverse transcription was performed on 10μg total RNA extracted from *Rhodococcus* sp. SP2B and *R. ruber* DSM 43338^T, according to the Titan one tube RT-PCR kit (Roche). Primers used for RT-PCR are listed in Table 1. A cycling regimen of 50°C for 30 min (one cycle) for cDNA synthesis and 94°C for 2 min (one cycle), followed by 94°C for 30 s, 55°C for 20 s, 72°C for 1 min (35 cycles) and 72°C for 5 min (one cycle) for PCR was used. To confirm that cDNA synthesis occurred and that RNA preparation was free of genomic DNA, negative control RT-PCR was performed only with *Taq* polymerase.

DNA sequencing and phylogenetic analyses

DNA sequences were determined by GATC Biotech (Germany) with primers listed in Table 1, from double-stranded plasmid templates or PCR products. Nucleotide and protein sequences were analysed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Homologue searches were carried out using Blast (Altschul *et al.* 1990) at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/).

Three phylogenetic analyses were performed using, respectively, 30 CYP sequences, and 32 and 37 AlkB sequences. For each analysis, sequences were aligned using ClustalW, (Thompson *et al.* 1994) and the alignments were manually improved using the ED program from the MUST package (Philippe 1993). Only unambiguous amino acid positions were kept for phylogenetic reconstructions, i.e. a total of 170 (Fig. 1a) and 128 (Fig. 1b) for AlkB and 226 for CYP (Fig. 4).

Bayesian phylogenetic trees were constructed using MrBayes v3_0b4 (Ronquist and Huelsenbeck 2003) with a mixed model of amino acid substitution and a gamma correction (four discrete categories plus a proportion of invariant sites) to take into account among site rate variation. MrBayes was run with four chains for one million generations, and trees were sampled every 100 generations. To construct the consensus tree, the first 1500 trees were discarded as 'burnin'.

Alkane degradation activity assays in resting cells

To observe the induction of degradation genes, resting cell experiments were performed by inhibiting cell growth by the ionic strength of the buffer and by the absence of nutrients. Cultures of *Rhodococcus* sp. SP2B and *R. ruber* DSM 43338^T were prepared in basal medium in presence of liquid alkane (hexane, octane, decane, dodecane, hexadecane) as inducer with and without glucose (20 mmol l^{-1}) or in presence of peptones (1 g l^{-1}), yeast-extract (1 g l^{-1}) or LB. At the end of the growth phase, cells were collected by centrifugation, washed twice with a phosphate buffer 50 mmol l^{-1} pH 7·2 and were then resuspended in this buffer to a final cell concentration of 30 g l^{-1} wet weight. The microcosms contained 10 ml of phosphate buffer, 1 ml of concentrated cells (6 g l^{-1} final concentration), and hexane, heptane, octane, decane or dodecane (1·25–2·5 mmol l^{-1}). The reaction was monitored in microcosm carried out in duplicate and incubated on a rotary shaker at 150 rev min^{-1}, 30°C, for 20 h. Alkane concentration in the headspace of the microcosms was measured with a flame ionization detector gas chromatograph (Hewlett Packard HP6890 Series GC; Agilent, Massy, France) equipped with a HP-5 capillary column. The alkane degradation was confirmed by measuring the CO_2 release in the headspace of the microcosms, using thermal conductivity detection gas chromatograph (Shimadzu 8A, Champs-sur-Marne, France) with a concentric CTR1 column (Alltech). Operating conditions were as reported by Teran Perez *et al.* (2002). Maximal hexane degradation rate was calculated with the Gompertz model as described by Acuna *et al.* (1999).

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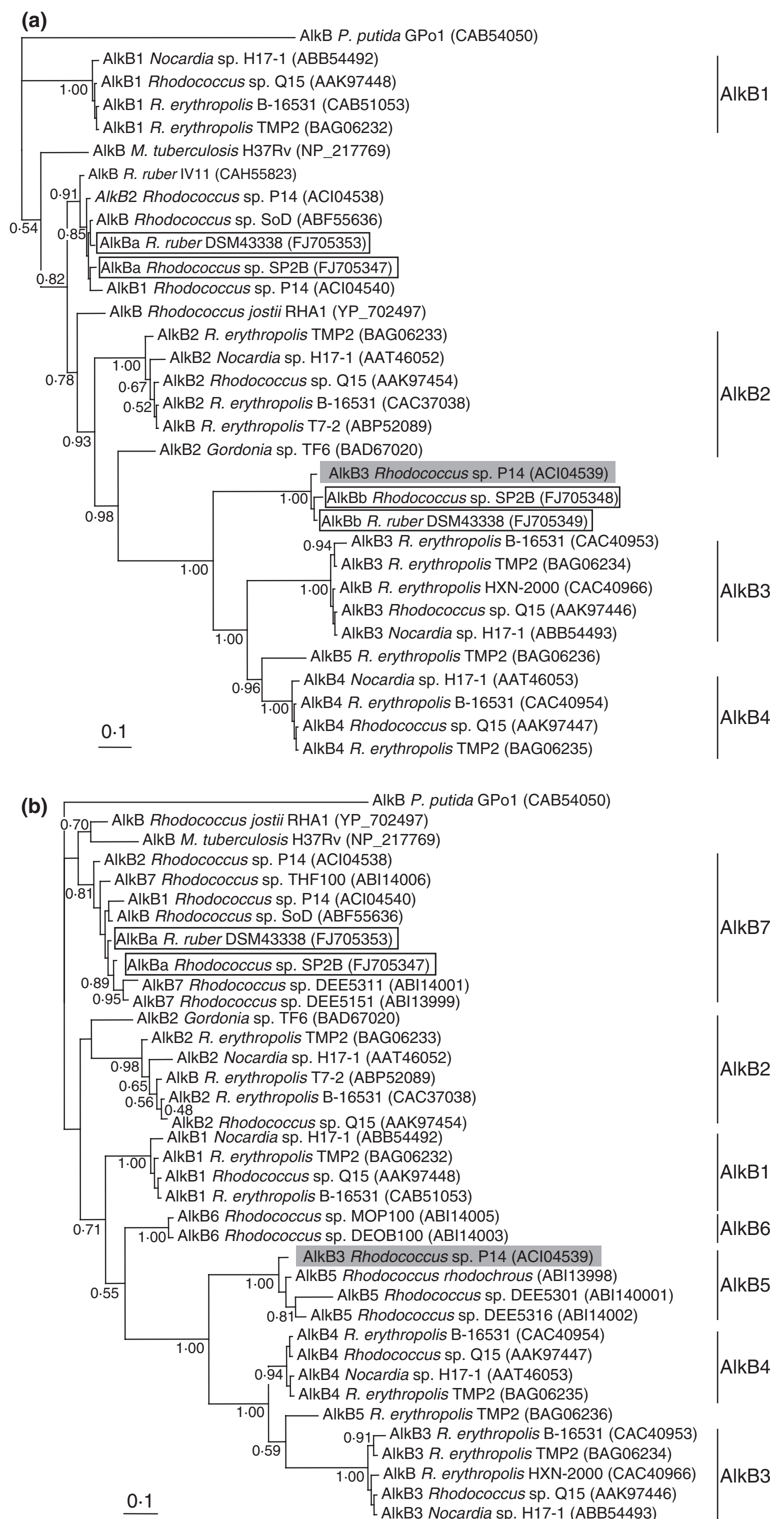

Figure 1 Bayesian trees of 31 (a) and 36 (b) AlkB protein sequences from actinomycetes. The tree is rooted with the AlkB from the gamma-proteobacterium *Pseudomonas putida* GPo1. Numbers at nodes represent the posterior probabilities PP (for clarity, only PP higher than 0·5 are shown). The accession numbers, given in bracket for each strain, correspond to protein sequences, except for our surrounded sequences for which the accession numbers refer to nucleotide sequences. An AlkB3 sequence, shaded in light grey, serves as landmark. The scale bar represents 0·1 substitutions per site.

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Results

Identification of an *alk*B gene

Many alkane degraders oxidize alkanes using the integral membrane nonheme iron monooxygenase from the Alk system. Using primers (Table 1), previously designed on conserved regions of rhodococcal *alk*B sequences (Kohno *et al.* 2002), we obtained a PCR product of the expected length of 330 bp from the isolated *Rhodococcus* sp. SP2B. This fragment was cloned and sequenced. A BlastP search indicated that it encodes a peptide having a high level of identity with alkane 1-monooxygenase proteins (Table 2). Using the inverse PCR method, the 5′ and 3′ flanking regions of this PCR product were cloned and sequenced. A putative gene of 1224 bp, annotated *alk*Ba (FJ705347), was obtained with a predicted ribosome-binding site AGGGAG located 8 bp upstream from the start codon. This is the first complete *alk*B sequence described for *R. ruber* species. Specific primers targeting the *alk*B-gene sequence (Table 1) were designed in this study and allowed to find by PCR amplification an identical *alk*B sequence in *R. ruber* DSM 43338^T (FJ705353). As for other complete *alk*B genes found in rhodococci (e.g. *Rhodococcus jostii* RHA1 *alk*B, *Rhodococcus* sp. Q15 *alk*B2 or *Rhodococcus erythropolis* T7-2 *alk*B), the predicted *alk*Ba translation initiation codon for *Rhodococcus* sp. SP2B and *R. ruber* DSM 43338^T is represented by GTG. This start codon has been reported to frequently occurring in GC-rich actinomycetes like *Rhodococcus* (Larkin *et al.* 1998).

The phylogenetic tree (Fig. 1a) shows that our AlkBa sequences emerged within a cluster (PP = 0·91) that includes all other AlkB from *R. ruber* strains SoD, IV11 and P14 (Heiss-Blanquet *et al.* 2005; Quatrini *et al.* 2008). Moreover, this cluster is positioned in between the AlkB2 and the AlkB1 clusters from *Actinobacteria*. The evolutionary distances between our sequences and AlkB2 sequences are shorter than with AlkB1 sequences, suggesting that AlkBa from *Rhodococcus* sp. SP2B and *R. ruber* DSM 43338^T might display a similar role than AlkB2. However, because the part of the tree comprising these three clusters is not fully resolved (weak PP), we cannot definitively conclude about the type of our AlkBa.

Identification of other genes in this *alk* cluster

The *alk*B gene is frequently flanked by other genes involved in alkane metabolism. However, this genetic context depends on the *alk*B type and on the considered strain. In *Rhodococcus* sp. SP2B, another open reading frame (*orf*) (FJ705347) was found downstream from the *alk*Ba gene (Fig. 2a), using inverse PCR method. Its

Table 2 Features of integral membrane alkane 1-monooxygenases from different *Rhodococcus* strains

	Complete AlkB				Partial AlkB from *Rhodococcus ruber* strains		
Name	AlkBa	AlkB2	AlkB	AlkB	AlkB	AlkB	AlkB7
Strains	*Rhodococcus* sp. SP2B	*Rhodococcus* sp. Q15	*Rhodococcus jostii* RHA1	*Rhodococcus erythropolis* T7-2	*Rhodococcus* sp. SoD	*R. ruber* IV11	*Rhodococcus* sp. DEE5151
Number of residues	407	408	410	408	167	98	85
Amino acid identity* (%)	100	81	82	81	98	97	98
Alkane range for this enzyme	C5–C21†	C12–C16	C3, ND†	C12–C36†	C12–C28†,‡	ND†	C16, ND†,‡
Amino acid for the alkane specificity	V (position 66)	V (position 66)	L (position 66)	V (position 66)	–	–	–
Reference	This study	(Whyte *et al.* 2002)	(Sharp *et al.* 2007)	(Huang *et al.* 2007)	(Quatrini *et al.* 2008)	(Heiss-Blanquet *et al.* 2005)	(Kim *et al.* 2007)

–, no sequence available; ND, not determined.
*With the AlkBa from *Rhodococcus* sp. SP2B.
†Alkanes used by the strain but not determined for the enzyme.
‡Hexane was not used by the strain.

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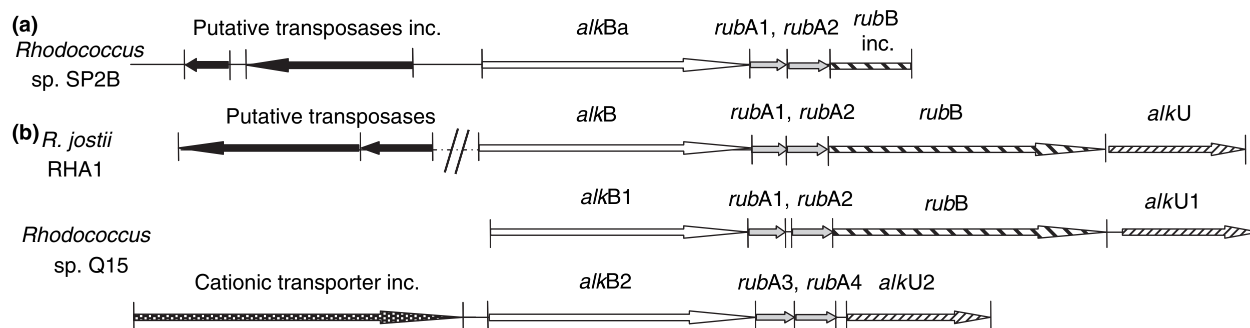

Figure 2 Comparison of cloned *alk* genes from *Rhodococcus* sp. SP2B (a) with the gene organization of other *Rhodococcus* alkane-degradative systems (b). *alk*B, alkane monoxygenase; *rub*A, rubredoxin; *rub*B, rubredoxin reductase; *alk*U, transcriptional regulatory protein. The directions of the arrows indicate the directions of transcription. inc., Incomplete *orf*.

putative product shows high sequence identity with the rubredoxin RubA of some *Rhodococcus* and *Mycobacterium* species (e.g. 84% identity with RubA3 of *Rhodococcus* sp. Q15). This rubredoxin annotated RubA1 constitutes one of the two physiological partners of alkane hydroxylases.

To keep on walking on the chromosome and therefore to find other genes flanking the *alk*Ba gene, an hybridization experiment was conducted on the *Rhodococcus* sp. SP2B genomic DNA by using a 0·8 kb DNA probe, complementary to the *alk*Ba internal region. This probe was designed to contain the four conserved histidine-rich regions, essential for activity, found in all integral alkane hydroxylases (van Beilen *et al.* 2003). They probably act as ligands for the nonheme dinuclear iron centre that participates in catalysis. According to *Rhodococcus* sp. SP2B *alk*Ba restriction analysis, among the fragments identified by Southern hybridization on *Csp*45I-digested genomic DNA, the 3-kb fragment contains the entire *alk*Ba gene. This fragment was cloned and sequenced. Sequence analysis revealed three *orf*s, in addition to the *alk*Ba and *rub*A1 genes already identified (Fig. 2a). One of them, annotated *rub*A2 (FJ705347), located immediately downstream from the *rub*A1 gene, codes for a peptide that shares 85% identity with the second rubredoxin of the *alk* cluster from *R. jostii* RHA1 and similar values with the rubredoxins RubA2 or RubA4 from *Rhodococcus* sp. Q15. Two *orf*s located directly upstream from *alk*Ba, encode proteins that share 55 and 77% identity with putative transposases of IS3 family from *R. jostii* RHA1. Using specific designed primers of this *alk* cluster, the same genetic organization was confirmed in *R. ruber* DSM 43338^T.

By a new inverse PCR experiment with DNA from *Rhodococcus* sp. SP2B digested by *Dde*I, we obtained, next to the *rub*A2 gene of the *alk* cluster (Fig. 2a), a new partial gene (FJ705347) potentially coding for a rubredoxin reductase (RubB), the second partner of integral membrane alkane hydroxylases. The deduced protein sequence (170 residues) shares 54% identity with the rubredoxin reductase of *R. jostii* RHA1 (424 residues) and 47% with that of *Rhodococcus* sp. Q15 (418 residues).

To analyse expression of the identified *alk* region, DNA-free total RNA was extracted from hexadecane-grown cells from *Rhodococcus* sp. SP2B and the type strain *R. ruber* DSM 43338^T. After RT reaction, the cDNA template was amplified with PCR primers (Table 1) located in *alk*Ba, *rub*A1 and *rub*B genes, yielding two products corresponding to the regions between *alk*Ba and *rub*A1 and between *rub*A1 and *rub*B (data not shown). Negative control treatments that omitted RT did not yield any amplified products. These results confirm, like in other strains, that these genes are cotranscribed. The *alk*Ba gene was thus organized in one operon in the two rhodococcal strains.

Identification of the promoter of the *alk* operon

Few studies have been performed on the regulatory regions of rhodococcal genes, and no highly-conserved sequences have been highlighted so far. However, we observed the presence of putative −35 and −10 conserved regions located within 55 bp upstream from the GTG initiation codon of *alk*Ba in *Rhodococcus* sp. SP2B (Fig. 3b). By sequence alignment, these regions seem to be highly conserved between *alk*B promoter regions of various actinomycetes (Fig. 3b). As the *alk*Ba promoter region was identical in both strains, the same conserved −35 and −10 regions were predicted for *R. ruber* DSM 43338^T.

RT-PCR was performed on RNA from *Rhodococcus* sp. SP2B to determine the approximate transcription start site of the *alk*Ba operon with four different forward primers (Table 1) located upstream from the 5′-end of *alk*B2 gene. With three primers located above the predicted promoter (235, 576 and 659 bp upstream from the translation start codon), no transcript was obtained. With a

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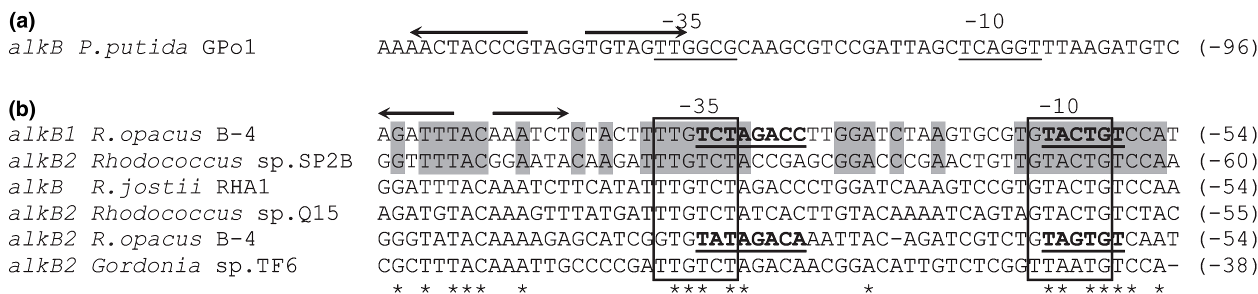

Figure 3 (a) Promoter region of *alk*B gene from *Pseudomonas putida* GPo1 with the 20 bp inverted repeat (arrows) proposed as AlkS binding site by van Beilen *et al.* (2001). (b) Alignment of various *alk*B promoter regions from actinomycetes. Boxes indicate putative −35 and −10 regions (this study) and underlined bold letters mark the regions suggested by Sameshima *et al.* (2008). Arrows mark the inverted repeat proposed as the AlkS binding site in *Rhodococcus opacus* B-4 *alk*B1 sequence. Asterisks indicate identical nucleotides between all the sequences. Shadowed boxes indicate the conserved sequences between *R. opacus* B-4 *alk*B1 and *Rhodococcus* sp. SP2B *alk*Ba. The distance to the initiation codon is given in brackets. The *alk*Ba promoter region of *Rhodococcus ruber* DSM 43338^{T} is identical to the one from *Rhodococcus* sp. SP2B.

primer located 24 bp after the predicted transcription start site, a band of the expected size was produced. This implies that the initiation of the transcription occurs between 30 and 235 bp upstream from the GTG start codon. This result is in agreement with the predicted promoter and with the few known rhodococcal transcription start sites that vary from 26 to 280 nucleotides upstream from the start codon (Li *et al.* 1996; Veselý *et al.* 2007). Our results are also supported by the recent report of the putative promoter regions of two *alk*B genes found in *Rhodococcus opacus* B-4 (Sameshima *et al.* 2008). They suggested possible −35 and −10 regions *c.* 60 bp upstream from each start codon (Fig. 3b). Sequence alignments showed that the upstream region of *alk*B1, rather than *alk*B2, from *R. opacus* B-4 was more similar to the promoter region of *alk*Ba from *Rhodococcus* sp. SP2B. However, contrary to the *R. opacus* B-4 *alk*B1 promoter region, we failed to find upstream from the −35 region of the *Rhodococcus* sp. SP2B *alk*Ba gene, an inverted repeat which could be the target of an AlkS regulator (Fig. 3b).

Identification of additional alkane-hydroxylating systems

To know whether other *alk* systems occurred in *Rhodococcus* sp. SP2B, a Southern experiment was performed using the 0·8-kb DNA probe described previously and the genomic DNA digested by *Csp*45I and *Dde*I. In both cases, two bands were highlighted with the probe: one of them corresponding to the already identified *alk*Ba (according to restriction analyses) and the other one corresponding, therefore, to another *alk*B homolog (data not shown). This result is not surprising because several *Rhodococcus* isolates have been described as containing up to five integral alkane hydroxylases of the AH2 group, able to hydroxylate long-chain alkanes (van Beilen *et al.* 2006; Takei *et al.* 2008).

To confirm this observation, Rhose2 and Rhoas2 primers (Monot *et al.* 2002) in Table 1, based on *alk*B3 or *alk*B4 sequences from some *Rhodococcus* strains, were used to amplify genomic DNA from *Rhodococcus* sp. SP2B. These primers were based approximately on the second conserved histidine box and the conserved HYG-motif of the integral membrane alkane hydroxylases and allowed the amplification of a PCR product with the expected length, annotated *alk*Bb (FJ705348). The deduced protein sequence (99 amino acids) shared 98% identity with a putative partial AlkB3 of *Rhodococcus* sp. P14 but only 60% identity in average with the other AlkB3 and 65% with AlkB5 from *R. erythropolis* TMP2. For *R. ruber* DSM 43338^{T}, the protein sequence deduced from the sequence FJ705349 amplified with the same primers, is very similar to the one of *Rhodococcus* sp. SP2B. In agreement with these observations, the phylogenetic tree (Fig. 1a) shows that the AlkBb from *Rhodococcus* sp. SP2B and *R. ruber* DSM 43338^{T} robustly group with the AlkB3 from *Rhodococcus* sp. P14 (PP = 1·00). However, they do not group with the other sequences annotated as AlkB3 and form a new cluster distinct from all the other AlkB type (PP = 1·00).

A new soluble P450 family (CYP153) responsible for the activation of alkane at the terminal position was recently identified in many bacteria. We have screened the rhodococcal genomic DNA using highly degenerate primers designed by van Beilen *et al.* (2006). The forward primer was based on the substrate-binding stretch in the helix 1, whereas the reverse primer was based on the sequence ending with the heme-binding cysteine. We amplified a partial CYP153 gene (FJ705350) in *Rhodococcus* sp. SP2B. The deduced CYP protein sequence presented very high identity (89–91%) with two closely related CYP153 from various *R. erythropolis*, such as

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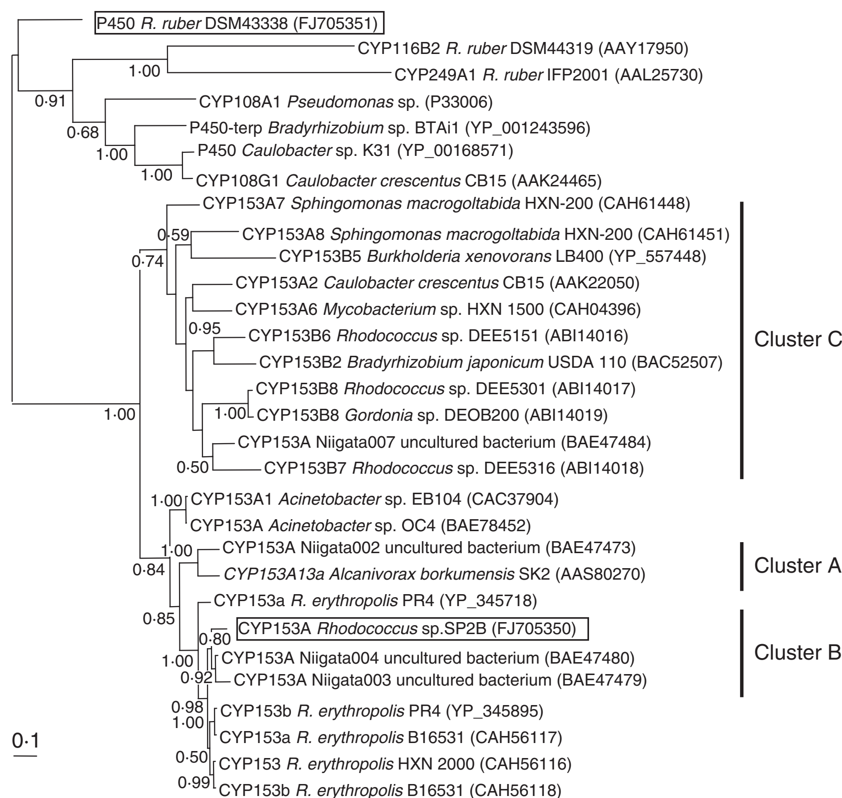

Figure 4 Bayesian tree of 30 CYP protein sequences from different bacterial groups. Numbers at nodes represent the posterior probabilities PP (for clarity, only PP higher than 0·5 are shown). For our surrounded sequences, the accession numbers refer to nucleotide sequences. The scale bar indicates 0·1 substitutions per site. CYP153a,b: two related copies from one strain.

strain HXN-2000 isolated from trickling-bed bioreactor used for hexane removal from airstreams or strain PR4, capable of degrading linear and branched alkanes as sources of carbon and energy (Komukai-Nakamura *et al.* 1996). The phylogenetic tree (Fig. 4) confirms that *Rhodococcus* sp. SP2B CYP153 groups with all the protein sequences mentioned above (PP = 1), in addition to two partial CYP153A – Niigata004 and Niigata003 – (from uncultured bacteria occurring in oil-contaminated soil), which are able to oxidize *n*-hexane, *n*-heptane and *n*-octane (Kubota *et al.* 2005).

A PCR product, obtained from the type strain *R. ruber* DNA (FJ705351) with the same primers, has no significant nucleotide similarity with that of *Rhodococcus* sp. SP2B, and their predicted protein sequences showed only 46% identity. Moreover, the best hit of identity obtained with other CYP153 proteins was only 51% with the CYP153A7 from *S. macrogoltabida* HXN-200. In the same way, among the other characterized P450 cytochromes from different CYP families, the best database match was only 56% identical with the CYP108A1 of *Pseudomonas* sp., which catalyses the hydroxylation of α-terpineol (Peterson *et al.* 1992). None of the other closely related CYP was described as involved in alkane degradation. The Bayesian tree (Fig. 4) is in agreement with these observations, because the CYP sequence from *R. ruber* DSM 43338^T is clearly positioned apart from all the CYP153 (PP = 1), including the one from *Rhodococcus* sp. SP2B, and is not close to some other families of P450 cytochromes (PP = 0·91). Nevertheless, this observation is not surprising, because the occurrence of nonalkane-degrading P450 cytochrome monooxygenases has already been described in other *R. ruber*, like a CYP249 responsible for ethyl *tert*-butyl ether (ETBE) oxidation in the strain IFP 2001 (Chauvaux *et al.* 2001).

Hexane degradation

Although hexane is a poor growth substrate because of its toxicity (log Pow = 3·9), *Rhodococcus* sp. SP2B was able to degrade it, with a maximum specific hexane degradation rate of 400 μmol g^{-1} h^{-1}. A similar rate was obtained for a cyclohexane-degrading *Rhodococcus* sp. EC1, which 16S rRNA gene shares 96% identity with that of *Rhodococcus* sp. SP2B (Lee and Cho 2008). In opposition, *R. ruber* DSM 43338^T was unable to grow on hexane or to degrade it (Amouric *et al.* 2006). To understand whether this absence of growth for *R. ruber* DSM 43338^T is a consequence of substrate toxicity, both strains were cultivated on rich medium with or without hexane. No significant difference in growth kinetics was observed. To localize the limiting step of hexane degradation in *R. ruber* DSM 43338^T, the growth on two hexane

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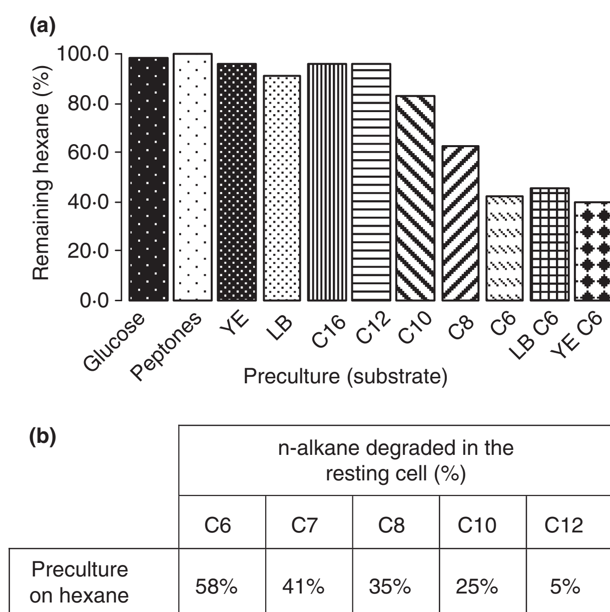
metabolic by-products, namely 1-hexanol and 1-hexanoate, was tested. Both strains are able to use these oxidized substrates as sole carbon and energy sources. Moreover, although catabolic repression by glucose was observed in *Ps. putida* GPo1 and in several other bacteria in presence of *n*-alkanes, *Rhodococcus* sp. SP2B, like several other rhodococcal strains (Warhurst and Fewson 1994), does not exhibit catabolic repression by glucose, when it grows on hexane in presence of this sugar (data not shown).

Finally, resting-cell experiments were performed in presence of various carbon sources, to specifically study the metabolism of hexane independently of *Rhodococcus* sp. SP2B growth. Hexane-grown cells oxidized hexane more rapidly than suspensions of octane-grown cells (Fig. 5a), whereas control experiments without cells indicated that the disappearance of the substrate was not abiotic. Hexane was not oxidized by suspensions of LB-, YE-, glucose- or peptone-grown cells, indicating that these nonalkane substrates did not induce hexane degradation, however, their presence does not impair hexane induction (Fig. 5a). Furthermore, hexane-grown cells were able to oxidize heptane, octane and decane but not dodecane (Fig. 5b). It thus seems that the presence of hexane allows inducing the degradation of other short-chain alkanes but not of medium-chain alkanes. Finally, the absence of hexane removal by dodecane- or hexadecane-grown cells suggests that these medium-chain alkanes



	n-alkane degraded in the resting cell (%)				
	C6	C7	C8	C10	C12
Preculture on hexane	58%	41%	35%	25%	5%

Figure 5 (a) Percentage of hexane not degraded within 20 h, by *Rhodococcus* sp. SP2B resting cells induced on different substrates. (b) Percentage of *n*-alkane degraded in resting cells by *Rhodococcus* sp. SP2B, after growth on hexane. C6–C16 = *n*-alkanes; YE, Yeast Extract.

failed to induce the hexane oxidation (Fig. 5a). Because *R. ruber* DSM 43338^T is unable to use hexane, resting cells in presence of hexane were not tested.

Discussion

SP2B isolate is closely related to the type strain *R. ruber* DSM 43338^T as attested by a high level of identity for 16S rRNA gene sequences and DNA–DNA hybridization. Both strains have a similar cellular fatty-acid profile and a similar lipophilicity response to alkane exposition (data not shown). Moreover, whatever the carbon number (odd or even) of the alkane substrate used, both strains degrade *n*-alkanes mainly by a monoterminal oxidation pathway (data not shown). The main difference found between two strains concerns the alkane degradation range, because among liquid and solid alkanes tested, *R. ruber* DSM 43338^T was recently described as degrading C_8 to C_{21} *n*-alkanes, whereas *Rhodococcus* sp. SP2B was able to degrade *n*-alkanes from C_5 to C_{21} (Amouric *et al.* 2006). Therefore, in spite of some little distinctions between both rhodococcal strains, *Rhodococcus* sp. SP2B could definitively be considered as *R. ruber* SP2B.

Like *R. ruber* DSM 43338^T, many other *R. ruber* strains, such as strain 219 (Bock *et al.* 1996), strain SoD (Quatrini *et al.* 2008) or strain IEGM231 (Philp *et al.* 2002), have been described as unable to use short-chain alkanes and especially hexane. When present in excess, short-chain alkanes are considered as toxic solvents, which can enter into cellular membranes and disturb them. Here, *R. ruber* DSM 43338^T was unable to metabolize this compound but appears to be tolerant to hexane (data not shown). Because *R. ruber* DSM 43338^T is able to degrade the first two by-products of the hexane degradation pathway (1-hexanol and 1-hexanoate), its inability to use hexane, in comparison to *R. ruber* SP2B, seems to be because of the lack of an alkane hydroxylase.

Although recently growing information was found about the genetic characteristics of the Gram-positive *Rhodococcus* alkane-degradative systems (Whyte *et al.* 2002; Sameshima *et al.* 2008), little is currently known about *R. ruber* ones. In the present study, through the identification of the alkane oxidation systems of *R. ruber* SP2B, we bring new information in the characterization of the type strain.

As expected by their capacity to grow on most *n*-alkanes, an identical integral membrane alkane hydroxylase AlkBa was found in both *R. ruber* strains studied here and, like for many organisms, the corresponding gene was flanked by other genes encoding enzymes involved in alkane degradation. This *alk* cluster (Fig. 2a) organized in operon in both *R. ruber* has a genetic organization observed in other *Actinobacteria*, such as the *alk*B1 cluster from *Rhodococcus* sp. Q15 (Whyte *et al.*

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2002), and shows a very high similarity in sequence and organization with the *alk* cluster of *R. jostii* RHA1 (Fig. 2b).

In several Gram-negative bacteria, expression of the *alk* system is under the control of the transcriptional regulator AlkS, belonging to the LuxR family. In *Actinobacteria*, a new TetR-type transcriptional regulator gene (*alk*U) involved in the *alk* cluster-expression regulation was sometimes found immediately downstream from the *alk* cluster (Whyte *et al.* 2002). It is the case for the *alk*B cluster in *R. jostii* RHA1 or in *Myco. tuberculosis* H37Rv and for the *alk*B1 and *alk*B2 clusters in *Rhodococcus* sp. Q15 (Fig. 2b). The genes identified so far in the *alk* cluster from *R. ruber* SP2B suggest a similar organization.

A putative binding site for the AlkS regulator, with low similarity with the target sequence recognized by the *Ps. putida* GPo1 AlkS (Fig. 3a,b), has been predicted upstream from the *alk*B1 gene in *R. opacus* B-4 and seems to be responsible for the activation of the expression by *n*-alkanes from C_5 to C_{16} (Sameshima *et al.* 2008). Because no binding site was found in the promoter region of *R. ruber* SP2B *alk*Ba gene, it is possible that it is not inducible by *n*-alkanes like *alk*B2 in *R. opacus* B-4. Not all expressions of *alk*B gene are regulated. Indeed, Takei *et al.* (2008) have shown that their *alk*B3, -4, -5 genes (in *Rhodococcus* sp. TMP2) are expressed whatever the substrate or the incubation temperature, and Whyte *et al.* (2002) have made the hypothesis that the *alk*B1 cluster in *Rhodococcus* sp. Q15 may have to be expressed constitutively. In the present study, an *alk*Ba transcript was obtained from glucose-grown *R. ruber* SP2B RNA (data not shown), showing that the expression of this gene could be constitutive.

According to van Beilen *et al.* (2005), a particular amino acid located in the middle of AlkB transmembrane helix 2 could be involved in substrate specificity, especially for strains with several *alk*B genes. When this amino acid, facing the central channel of the AlkB enzyme, has a bulky side chain like tryptophan, the hydroxylase belonging to the AH1 group cannot degrade long-chain alkanes. It is the case for *Ps. putida* GPo1 AlkB. If the residue has a small side chain, the AH2 hydroxylases degrade long-chain alkanes, probably because they are not restricted from entering further into the central channel. An alignment of AlkBa from *R. ruber* SP2B and *R. ruber* DSM 43338^T with other AlkB showed at this position a valine, sterically similar to the leucine found in the AlkB from *R. jostii* RHA1 (Table 2). Therefore, we can assume that this AlkBa, present in both *R. ruber*, belongs to the AH2 group and might be responsible for the medium- and long-chain alkane degradation. Moreover, AlkBa shares a high level of identity with published AlkB2 sequences such as the AlkB2 enzyme from *Rhodococcus* sp. Q15 (Table 2), enzyme for which the alkane oxidation range was determined from *n*-C_{12} to *n*-C_{16} (Whyte *et al.* 2002). This suggests that *R. ruber* SP2B AlkBa could oxidize the same alkane subset. In addition, a partial putative AlkB found in *Rhodococcus* sp. SoD has 98% identity with AlkBa of *R. ruber* SP2B or DSM 43338^T (Table 2). This strain is closely related to *R. ruber* DSM 43338^T (99% identity in their 16S rRNA gene sequences) and grows on *n*-alkanes from C_{12} to C_{28} without degrading shorter alkanes (Quatrini *et al.* 2008). No other AlkB homolog has been identified so far in *Rhodococcus* sp. SoD. This observation is in agreement with the fact that AlkBa in *R. ruber* SP2B or DSM 43338^T should be involved only in the oxidation of *n*-alkanes longer than C_{12}. Finally, our AlkBa and the other AlkB of the same cluster (Fig. 1a) also group with sequences annotated as AlkB7 (Fig. 1b, PP = 0·81), recently identified in strains closely related to *R. ruber* (such as strains DEE5311 or DEE5151). This suggests the affiliation of AlkBa and all other enzymes non or misannotated of the cluster to this new AlkB type. Although no substrate characterization was carried out for these enzymes, they were again found in bacteria that do not grow on volatile *n*-alkanes (Kim *et al.* 2007). This supports our hypothesis about the putative function of AlkB7 from our two *R. ruber* strains. Besides, our results obtained with resting cells suggest that hexane degradation in *R. ruber* SP2B is not a constitutive phenomenon and is proceeded by an enzymatic system inducible only by a small range of linear alkanes (<*n*-C10). Therefore, because the *alk*B7 gene in *R. ruber* SP2B seems to have a constitutive expression, another inducible gene should be responsible for hexane and other short-chain alkane oxidation.

The presence of multiple alkane hydroxylases may be a common feature of *Rhodococcus* strains (Whyte *et al.* 2002). This feature was illustrated in both *R. ruber* strains, by amplification of a partial *alk*Bb gene, coding for a protein that shows only 55% identity with the first AlkB7 identified. A similar AlkB sequence deposited in GenBank was recently found in *Rhodococcus* sp. P14. However, no more information is currently available about this enzyme or about the alkane degradation range of this strain. As illustrated in the phylogenetic tree (Fig. 1a), this second partial AlkBb from both *R. ruber* seems to belong to a new AlkB type (PP = 1). Although other partial *alk*B amplified from rhodococcal strains have been classified into three new subtypes *alk*B5, -B6 or -B7 (Kim *et al.* 2007), their sequence do not overlap with the known sequence of our *alk*Bb gene. However, the AlkB3 sequence from *Rhodococcus* sp. P14, which groups with our AlkBb, is long enough to be used as a landmark, because its sequence overlaps with all those partial sequences. Accordingly, a new phylogenetic analysis was performed

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without AlkBb sequences but containing the new AlkB5, -B6 and -B7 sequences (Fig. 1b). The topology of this second tree is similar to the first one (Fig. 1a,b). The marker AlkB3 from *Rhodococcus* sp. P14 groups robustly with the rhodococcal AlkB5 (PP = 1), suggesting that our partial AlkBb is an AlkB5. Therefore, both *R. ruber* contain a similar second putative integral membrane alkane hydroxylase, which is probably neither responsible of the hexane degradation, because AlkB5 enzymes were only detected, so far, in *Rhodococcus* strains unable to use short-chain alkanes.

Other bacterial systems, such as some P450 cytochromes belonging to different CYP families, are able to catalyse terminal alkane hydroxylation. In addition to the engineered CYP101, CYP102A1, -A2 and -A3, the soluble CYP153 constitutes a new family of P450 cytochrome alkane hydroxylases. A putative CYP153A was found in *R. ruber* SP2B. In the same way, CYP153A1, the first cloned member of this CYP family, was found to be involved in hexane oxidation in *Acinetobacter* sp. EB104 (Maier *et al.* 2001). Other CYP153 enzymes were also present in many other isolates selected for their ability to grow on C_5–C_{10} alkanes (van Beilen *et al.* 2006). In the type strain *R. ruber*, the sequence amplified with these same primers corresponds to another P450 cytochrome: the comparison with other CYP enzymes seems to indicate that it is not an alkane hydroxylase. Finding this enzyme is not surprising because Actinobacteria have several P450 cytochromes distributed over 220 families, such as *R. jostii* RHA1 that possesses 25 P450 cytochromes (McLeod *et al.* 2006). Both strains in this study contain two integral membrane alkane hydroxylases but *R. ruber* SP2B possesses an additional putative CYP153 gene not found in the type strain. Other *Rhodococcus* isolates, such as *R. erythropolis* NRRL B-16531 or *R. erythropolis* PR4, contain two related CYP153 and up to five AlkB (van Beilen *et al.* 2006). The authors have noted that most CYP153 were found in bacteria that do not possess AlkB enzymes acting on medium-chain alkanes, but have AlkB homologs acting on long-chain alkanes. Therefore, the CYP153 could be responsible for short-chain alkane oxidation. In the same way, the two AlkB studied here could be involved in the oxidation of medium- and/or long-chain alkanes, while the CYP153A of *Rhodococcus* sp. SP2B would oxidize hexane. It was suggested that CYP153A enzymes can be separated in three clusters depending on their biocatalytic activities (Kubota *et al.* 2005). As confirmed in the Bayesian tree (Fig. 4), the CYP153A from *R. ruber* SP2B belongs to the cluster B (PP = 1), for which *n*-hexane was determined to be the better substrate over the *n*-alkanes tested.

Alkane hydroxylase systems and their organization are highly diverse among alkane-degrading bacteria and especially in the Gram-positive strains, because of horizontal gene transfer (van Beilen *et al.* 2003). Genes encoding transposases were commonly found in the vicinity of the biodegradation operons and were proposed to play an important role in horizontal transfer of these catabolic genes between bacteria. These enzymes seem to be responsible for the genetic instability of some P450 cytochromes like the CYP249A1 from the ETBE-degrading *R. ruber* IFP2001 (Chauvaux *et al.* 2001) and could be responsible for the occurrence of the CYP153 in *R. ruber* SP2B. Therefore, we can suppose that the difference in alkane degradation observed between both related rhodococcal strains could result from the known plasticity of the *Rhodococcus* genome in favour of its adaptation to specific environmental niches or substrates.

In the present work, we have highlighted, through *R. ruber* SP2B, genetic systems involved in alkane oxidation also used by the type strain *R. ruber* DSM 43338^T. We have also pointed out that hexane degradation is an inducible phenomenon. We identified a gene coding for a P450 cytochrome alkane hydroxylase, only in the SP2B isolate. It has been shown that the expression of the CYP153 gene can be inducible (Cardini and Jurtshuk 1970), and the CYP153A found in *R. ruber* SP2B could be therefore responsible for the inducible hexane degradation. Genetic control of the pathways involved in alkane degradation has been studied in detail for the Gram-negative *Pseudomonas* species and related species but not in *Actinobacteria*. Further studies should clarify the implication of CYP153A in hexane degradation in *R. ruber*. *Rhodococcus* members are highly interesting microorganisms, for their degradation capacities, their hydrophobic cell surfaces, their production of biosurfactants and their resistance to harsh environments. *Rhodococcus ruber* SP2B should prove to be a promising candidate for microbial remediation studies of gasoline-contaminated sites because of its large degradation range of alkane substrates.

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