Genes Involved in the Degradation of Ether Fuels by Bacteria of the *Mycobacterium/Rhodococcus* Group

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Résumé — **Gènes impliqués dans la dégradation des éthers carburants par des bactéries du groupe *Mycobacterium/Rhodococcus*** — Un cluster de gènes codant pour un système de monooxygénase à cytochrome P-450 impliqué dans l'utilisation de l'éthyl *tertio* butyl éther (ETBE) a été cloné chez *Rhodococcus ruber* IFP 2001. Ce cluster comprend *ethR*, un gène régulateur putatif de la famille des régulateurs transcriptionnels *araC/xylS* ; *ethA*, codant pour une ferrédoxine réductase ; *ethB*, codant pour un cytochrome P-450 ; *ethC*, codant pour une ferrédoxine ; et *ethD*, indispensable au bon fonctionnement du système, mais dont le rôle exact est inconnu. Le cluster *ethRABCD* est flanqué de part et d'autre de deux copies identiques d'un transposon de classe II, ce qui explique qu'il soit perdu à haute fréquence par recombinaison homologue quand les bactéries sont cultivées en conditions non sélectives. Des clusters de gènes *eth* extrêmement conservés ont été mis en évidence chez deux autres souches utilisant l'ETBE, *Rhodococcus zopfii* IFP 2005 et *Mycobacterium* sp. IFP 2009. Dans tous les cas, le contexte génomique flanquant le locus *eth* est différent, ce qui suggère que celui-ci peut être transféré horizontalement d'une espèce à l'autre et s'insérer à différents endroits du génome. Par ailleurs, la copie aval du transposon comporte une délétion de 117 nucléotides chez *R. zopfii* IFP 2005. Chez *Mycobacterium* sp. IFP 2009, cette même copie est insérée 2771 nucléotides plus en amont que chez les deux autres bactéries, et la copie amont est absente.

Abstract — *Genes Involved in the Degradation of Ether Fuels by Bacteria of the* Mycobacterium/ Rhodococcus *Group* — *A cluster of genes encoding a cytochrome P-450 monooxygenase system involved in the utilisation of ethyl* tert-*butyl ether (ETBE) was cloned in* Rhodococcus ruber *IFP 2001. This cluster includes* ethR, *a putative regulator gene of the* araC/xylS *family;* ethA, *encoding a ferredoxin reductase;* ethB, *encoding a cytochrome P-450,* ethC, *encoding a ferredoxin; and* ethD, *which is required for the function of the monooxygenase system, but whose exact role is unknown. The* ethRABCD *cluster is flanked on either side by two identical copies of a class II transposon, which explains that it is lost at high frequency by homologous recombination when the strain is grown under non selective conditions. Two other, highly conserved clusters of* eth *genes were detected in the ETBE-utilizing strains* Rhodococcus zopfii *IFP 2005 and* Mycobacterium *sp. IFP 2009. In all cases, the eth locus is inserted in a different genomic context, suggesting that it may be transferred horizontally between different species and inserted at different sites in the genome. In addition, in* R. zopfii *IFP 2005, the downstream copy of the transposon carries a* 117-bp *(base pairs) deletion; in* Mycobacterium *sp. IFP 2009, the upstream copy is absent and the downstream copy is inserted 2771 bp closer to the ethRABCD cluster.*

INTRODUCTION

Methyl *tert*-butyl ether (MTBE), and ethyl *tert*-butyl ether (ETBE), commonly referred to as ether fuels, are currently used on a large scale as anti-knocking additives in unleaded gasoline. As a consequence, these compounds have become a cause of concern owing to their introduction into the environment due to spills and seepage from leaky tanks. Ether fuels are highly soluble in water and poorly adsorbed by soil particles, and they are readily transported away from polluted sites by water run-off, contaminating aquifers; furthermore, they are generally regarded as poorly biodegradable.

In recent years, however, several studies have shown that bacterial [1-7] and fungal [8] isolates are capable of breaking the ether bond of ether fuels, generating *tert*-butyl alcool (TBA) in the process. TBA can then be further metabolized, either by the same microorganism [2, 5-7] or by other microorganisms living in the same natural or artificial consortium [9-11]. Therefore, strategies can be developed for the bioremediation of pollution by ether fuels, based on the addition of selected strains (bioaugmentation), on the optimization of growth conditions (biostimulation), or both. For this, a complete picture of the bioremediation process is desirable in order to assess its efficiency. Analytical chemistry provides generally adequate tools to follow the concentration of pollutants in environmental samples (although biosensors may prove an interesting alternative). However, gathering relevant data about the microflora involved in bioremediation remains quite difficult, owing to the large diversity of microorganisms present in natural environments.

Two types of tools based on nucleic acid probes are currently available to follow specific populations within microbial consortia. One is the detection of specific sequences of ribosomal RNA by hybridization or Polymerase Chain Reaction (PCR) amplification and sequencing. The other is the detection and quantification of genes encoding specific functions required for the metabolism of a given pollutant. Ribosomal RNA (rRNA) is present in large amounts in bacterial cells, which makes it readily detectable with a high sensitivity. In addition, rRNA genes being less likely than other genes to be deleted or acquired through horizontal transfer, the method provides important information about the taxonomic status of the microorganisms detected. However, rRNA sequences generally provide little information about the metabolic pathways present in a given organism. The same pathway may be present in microorganisms that are taxonomically very distant, and, conversely, some functions may be present or absent in very closely related strains, owing to gene loss or acquisition due to horizontal transfer. To address this problem, hybridisation or quantitative PCR amplification using appropriate oligonucleotide primers offers an interesting alternative for the quantification of genes encoding specific functions within

environmental samples [12]. With a view to developing tools for monitoring bacterial populations that participate in the biodegradation of ether fuels, we set out to clone and sequence the genes involved in the metabolism of ETBE.

1 CLONING OF GENES REQUIRED FOR THE DEGRADATION OF ETHER FUELS BY *RHODOCOCCUS RUBER* IFP 2001

Rhodococcus ruber IFP 2001 was isolated by Fayolle *et al.* [3] after enriching cultures from activated sludge on medium containing ETBE as a sole carbon source. The strain is able to split the ether bond of ETBE and utilize the resulting two-carbon compound as a source of carbon and energy. TBA, however, is not metabolized and accumulates in stoichiometric amounts [13]. Resting cells of *R. ruber* IFP 2001 grown on ETBE are also capable of cleaving MTBE and TAME (*tert*-amyl methyl ether), but these substrates do not support growth, presumably because the organism is not able to grow on C_1 carbon sources. In the presence of ethanol or isopropanol as a growth substrate, co-cultures of *R. ruber* IFP 2001 and of another organism that degrades TBA (*e.g. Burkholderia cepacia* IFP 2003) can mineralize MTBE completely [11].

1.1 Evidence for a Cytochrome P-450 Monooxygenase Involved in the Cleavage of Ether Fuels

Spectrophotometric analysis of crude extracts of *R. ruber* IFP 2001 revealed the presence of a cytochrome P-450 that was inducible by growth on ETBE, suggesting that it was involved in the metabolism of this substrate [13]. This was confirmed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis showing the presence of an inducible polypeptide of 43 kDa [14], whose microsequencing yielded a partial sequence sharing 66 % identity with a putative cytochrome P-450 from *Mycobacterium tuberculosis*. A second inducible polypeptide of 10 kDa turned out to be similar to an open reading frame of unknown function present in a cytochrome P-450 gene cluster of *Rhodococcus erythropolis* involved in the *N*-dealkylation of thiocarbamates [15].

1.2 Instability of the ETBE-Degrading Phenotype Due to a Chromosomal Deletion

It was soon noticed that the ETBE-degrading phenotype of *R. ruber* IFP 2001 was unstable. After culturing the strain for 60 generations in LB broth, some 20-100 % of the reisolated clones were found to be unable to grow in the presence of ETBE as a sole carbon source. The mutants lost the ability to degrade TAME and MTBE concomitantly, and did not revert with a detectable frequency. Five independent mutants were

characterized by subjecting their genomic DNA to pulse-field electrophoresis after digestion with *Xba*I. All mutants displayed the same deletion of a 15 kilobase (kb) segment located within a 125 kb *Xba*I fragment [14].

1.3 The Region Deleted in the ETBE-Mutants Encodes the Cytochrome P-450 System Inducible by ETBE

The 125 kb wild type *Xba*I fragment was purified, labeled with ^{32}P, and used as a probe to compare the hybridization pattern of wild type and ETBE⁻ mutant genomic DNA digested by *Bam*HI. Two wild type *Bam*HI fragments of 7.4 and 16.3 kb, respectively, were found to be replaced in the mutant by a single fragment of 9.3 kb. These fragments were cloned and subjected to sequence analysis [14]. Figure 1a shows that the wild type region comprises a cluster of five open reading frames, termed *ethRABCD*, encoding the components of a cytochrome P-450 system. The sequence of EthB matches the partial sequence obtained from the inducible cytochrome P-450 detected in crude extracts. EthA

and EthC correspond to a ferredoxin reductase and a ferredoxin, respectively, which presumably transfer the electrons required for the monooxygenase reaction to the cytochrome P-450. EthD corresponds to the inducible 10 kDa polypeptide of unknown function detected in crude extracts. EthR belongs to the AraC/XylS transcriptional regulator family and presumably participates in the regulation of the other genes in the cluster [14].

1.4 Two Identical 5.6-kb Repeats Flanking the *ethRABCD* Cluster Explain the Instability of the ETBE-Degrading Phenotype

As shown in Figure 1a, the *ethRABCD* gene cluster is flanked on either side by two perfectly identical repeats of 5.6 kb. Each repeat consists of a class II transposon comprising a terminal repeat of 38 base pairs (bp), a transposase gene (*tnpA*) and a resolvase gene (*tnpR*) that is interrupted by an insertion sequence of the IS3 family. Analysis of the corresponding region in the ETBE-mutant
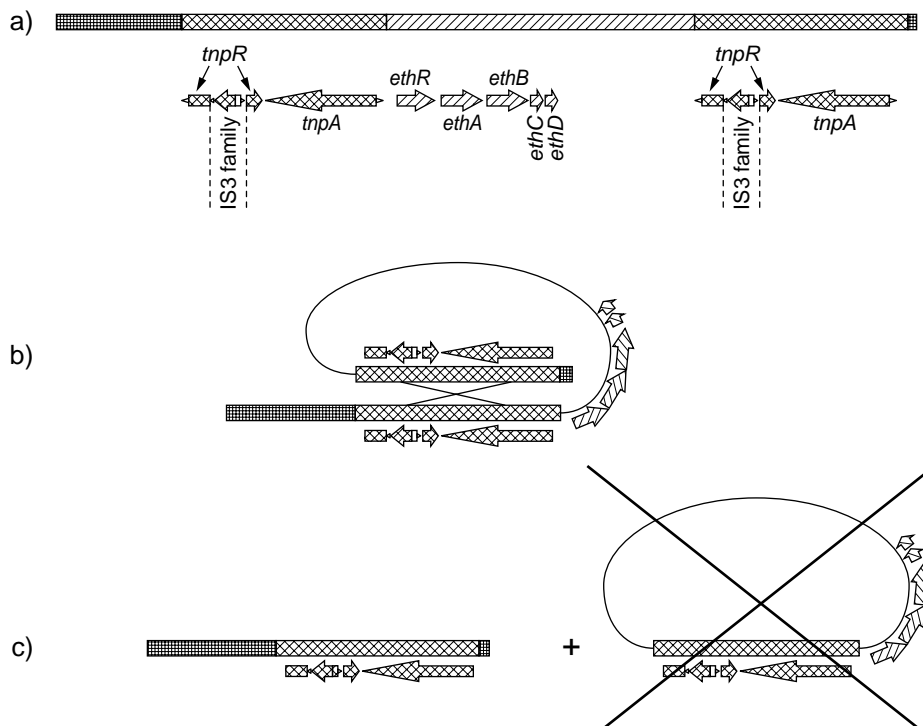

Figure 1

a): structure of the *eth* locus in *Rhodococcus ruber* IFP 2001. The region containing the genes *ethR* (putative regulator), *ethA* (ferredoxin reductase), *ethB* (cytochrome P-450), *ethC* (ferredoxin), and *ethD* (auxiliary gene of unknown function) are shown with oblique hatching. The regions corresponding to the two copies of the transposon and containing the genes *tnpA* (transposase), *tnpR* (interrupted resolvase) and the IS3 insertion sequence are shown with oblique cross-hatching. The region of the genome into which the *eth* locus is inserted is shown with dark cross-hatching. b) and c): diagram showing how a single crossing over between the two copies of the transposon leads to the excision of the region containing the *eth* genes, forming a circle that is not further replicated and leaving a single copy of the transposon in the genome.

shows the presence of a single copy of the transposon, the second copy and the region located in between being deleted (*Fig. 1c*). This deletion may be readily explained by a recombination event occurring between the two repeats (*Fig. 1b*).

1.5 The *ethRABCD* Cluster is Sufficient to Confer the Ability to Grow on ETBE

In order to confirm that the *ethRABCD* gene cluster was indeed responsible for the utilization of ETBE, the corresponding DNA fragment was inserted into the pRE-7 vector and the resulting recombinant plasmid, termed pGT222, was introduced into the ETBE-negative, deleted mutant. The transformed strain recovered the ability to grow on minimal medium containing ETBE as a sole carbon source with the same doubling time as the wild-type strain. In addition, resting cells of the transformed strain produced TBA from ETBE at a rate that was 38% of that measured for the wild-type [14]. Further proof that *ethRABCD* gene cluster was sufficient to confer the ability to utilize ETBE was obtained by introducing pGT222 into *Mycobacterium smegmatis*. In contrast to wild-type *M. smegmatis*, the transformed strain became capable of growth on ETBE as a sole carbon source, and TBA was shown to be produced in the process [16].

1.6 *EthD* is an Essential Component of the Cytochrome P-450 System

The *ethD* gene has few identified counterparts in the sequence databases, and none whose function has been experimentally determined. Nevertheless, like its closest relative *orf4* of *R. erythropolis*, it is part of a gene cluster encoding the components of a cytochrome P-450 system. Evidence that it is indeed involved in the activity of the *eth*-cytochrome P-450 system was obtained by transforming the ETBE-negative mutant with a pRE-7 derivative harbouring only the *ethRABC* genes, but not *ethD*. The transformed strain failed to grow on ETBE and the ETBE-degrading activity of resting cells was only 3% of that of the wild-type [14]. Further characterization of the role of *ethD* will require reconstituting the full system *in vitro*.

2 EVIDENCE FOR GENES SIMILAR TO THE *ethRABCD* CLUSTER IN OTHER ETBE-DEGRADING BACTERIA

Three other strains isolated from activated sludge for their ability to utilize ETBE were found to carry genes similar to the *ethRABCD* cluster characterized in *R. ruber* IFP 2001. One of them, IFP 2008, turned out to be an independent isolate of the same strain as IFP 2001, as shown by the identity of colony phenotypes and of restriction patterns

obtained for digests of total genomic DNA. Consequently, it was not studied further. The two other strains, IFP 2005 and IFP 2009, had distinct colony phenotypes. The rRNA sequence of IFP 2005 was 100% identical to that of the type strain of *Rhodococcus zopfii*; the rRNA sequence of IFP 2009 did not fully match any of the available type strains, but showed that IFP 2009 belongs to the *Mycobacterium* genus (unpublished data).

2.1 The *ethRABCD* Cluster is Highly Conserved in *Rhodococcus zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009

Preliminary attempts using PCR amplification and DNA hybridization were made to detect in *R. zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009 the presence of *eth* genes similar to those characterized in *R. ruber* IFP 2001. The results showed that both strains contained conserved *eth* genes as well as sequences similar to the transposons flanking the *ethRABCD* cluster in *R. ruber* IFP 2001. The two newly identified *eth* loci were further characterized by sequencing. The sequence of DNA regions that were highly similar to the *R. ruber* sequence were amplified by PCR and each amplified segment was sequenced using a mixture of six independent amplification reactions as a template and oligonucleotides derived from the sequence of *R. ruber eth* genes as primers. Restriction fragments overlapping the ends of the similarity region were amplified by reverse PCR [17], cloned and sequenced. The sequence was then verified by resequencing a mixture of six independent PCR reactions made from the original genomic DNA.

Figure 2 shows that the structure of the *eth* loci in *R. ruber* IFP 2001 and *R. zopfii* IFP 2005 is virtually identical with two copies of the transposon flanking the *ethRABCD* cluster. Within the boundaries indicated in Figure 2, the sequence is extremely well conserved, with a rate of mutations not exceeding 0.1% throughout the locus. However, the 3' end of the transposon located downstream of the *eth* cluster is shorter by 117 nucleotides. Sequence conservation ends abruptly at the extremities of the locus, indicating that the locus is inserted at unrelated sites in the genomes of the two bacteria.

The same approach was used to characterize the structure of the *eth* locus in *Mycobacterium* sp. IFP 2009. As shown in Figure 2, the locus differs from the pattern found in *R. ruber* IFP 2001 in that the copy of the transposon present upstream of the *ethRABCD* cluster in *R. ruber* IFP 2001 is missing in *Mycobacterium* IFP 2009, and the transposon located downstream of the *eth* genes is inserted 2771 bp closer to the cluster. The latter is not deleted at the 3' end, unlike the downstream transposon of *R. zopfii* IFP 2005. As is the case with *R. zopfii* IFP 2005, the regions similar to the *eth* locus of *R. ruber* are 99.9% conserved, and sequence conservation ends abruptly at the extremities of the locus.
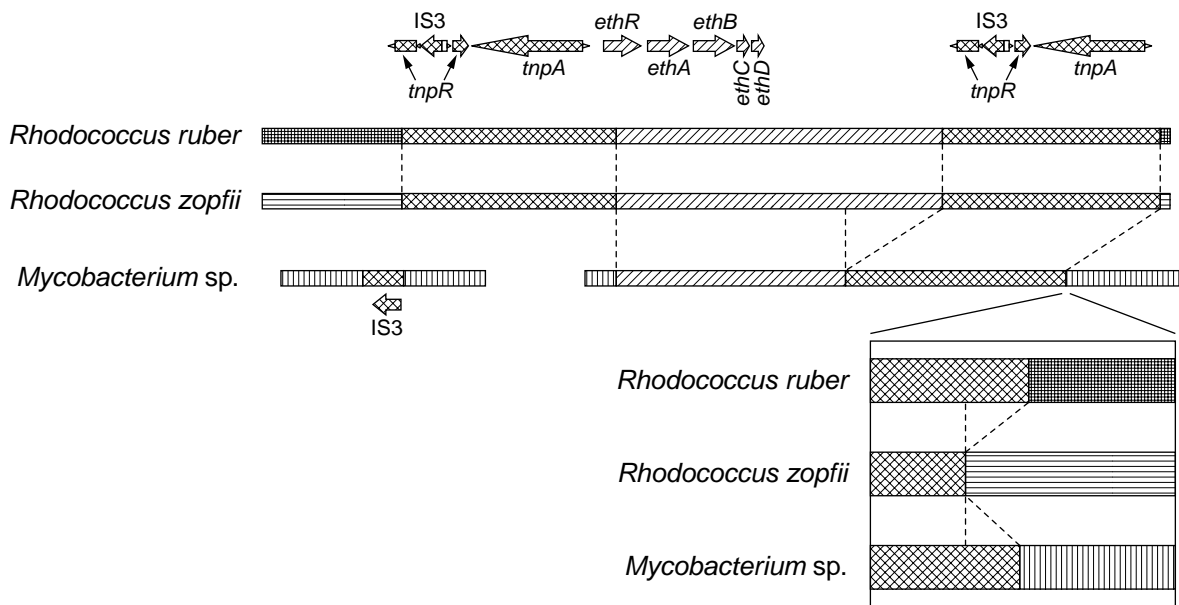
Figure 2

Comparison of the structure of the *eth* locus in *R. ruber* IFP 2001, *R. zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009. The insert shows that the rightmost extremity of the right transposon is deleted in *R. zopfii* IFP 2005. An IS3 insertion sequence, which is not linked to the *eth* locus is present within the genome of *Mycobacterium* sp. IFP 2009.

2.2 Instability of the ETBE-Degrading Phenotype in *Rhodococcus zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009

Four independent cultures of *R. zopfii* IFP 2005 and three cultures of *Mycobacterium* sp. IFP 2009 were subjected to subculturing in LB for 30 generations in order to check for the stability of the ETBE-degrading phenotype. Out of the reisolated clones (70 for each culture), none was found to retain the ability to grow on ETBE as a sole carbon source. Colony hybridization using a probe derived from the sequence of *R. ruber ethB* indicated that in each case, the cytochrome P-450 gene had been deleted in the mutants. In the case of *R. zopfii* IFP 2005, two out of four mutants tested still hybridized to a probe consisting of the transposon sequence, as expected if the mutations resulted from homologous recombination between the two transposons flanking the *ethRABCD* genes. The two other mutants did not contain any sequence hybridizing to the transposon sequence and resulted from a larger deletion event. In the case of *Mycobacterium* sp. IFP 2009, the deletion extended beyond the limits of the segment characterized and shown in Figure 2. The only segment still hybridizing to the transposon sequence turned out to correspond to an IS3 sequence with no close linkage to the *eth* locus.

CONCLUSIONS

This study confirms that a cytochrome P-450 mono-oxygenase system is responsible for the utilization of ETBE by *R. ruber* IFP 2001, as had been proposed by Hernandez-Pérez *et al*. [13]. An unusual feature of the system is the presence of EthD, a component whose exact function is not defined, but which plays an important role in the oxidation of ETBE. Further characterization of EthD will require isolation of the EthABCD components and *in vitro* reconstitution of an active enzyme system for the degradation of ETBE, a difficult task since activity is readily lost upon rupturing bacterial cells (unpublished data).

Although the *ethRABCD* cluster is located on the chromosome of *R. ruber* IFP 2001, it is clearly located within a genetically unstable structure, which explains why it is lost with a high frequency when the strain is grown under non-selective conditions. At the same time, the recombination event leading to the excision of the *eth* genes should generate a circular structure bearing a transposon, which could promote its insertion into other replicons and, therefore, participate in the horizontal transfer of *eth* genes. Indeed, the presence of extremely conserved *eth* loci located amongst otherwise unrelated sequences in *R. zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009 strongly suggests that

interspecific gene transfer does occur. In the latter two strains, rearrangements and deletions have altered the general structure of the locus relative to *R. ruber* IFP 2001, *Mycobacterium* sp. IFP 2009 being more extensively modified.

Like *R. ruber* IFP 2001, *R. zopfii* IFP 2005 and *Mycobacterium* sp. IFP 2009 are genetically unstable. In the case of *R. zopfii* IFP 2005, which bears two copies of the transposon flanking the *eth* genes, a recombination event similar to that proposed for *R. ruber* IFP 2001 probably accounts for the appearance of mutants that still hybridize to the transposon probe. However, in the case of the *R. zopfii* mutants no longer hybridizing to the transposon probe, and of the *Mycobacterium* sp. mutants, the deletion event encompasses a larger region and completely removes the flanking transposons. One possible explanation could be that in these strains, the *eth* locus is borne by an independent replicon that is lost upon growth under nonselective conditions. We are currently checking this hypothesis by using pulse-field gel electrophoresis to look for the presence of large plasmids that would be lost in the mutants.

What are the perspectives for using the *eth* genes characterized in the three strains discussed above as probes to assess the degrading potential for ETBE and MTBE of the microflora present in polluted environments? The fact that the genes are part of structures that can promote horizontal transfer would suggest that they are probably present in yet other ether fuel-degrading bacteria. At this point, however, we know little about their geographic or taxonomic distribution. The three strains we studied originated from the Seine Basin, although they were isolated at different locations: *Rhodococcus ruber* IFP 2001 and *Rhodococcus zopfii* came from the water treatment plant of Achères, while *Mycobacterium* sp. IFP 2009 came from the plant in Valenton. In addition, the three strains are very closely related taxonomically, and nothing is known about the presence of similar genes in more distantly related groups. Indeed, indirect evidence suggests that oxidative cleavage of ether bonds may be performed by enzyme systems whose structure and original function may differ. Among inducing substrates, lower alkanes [2, 4, 18], but also cyclohexane [19] have been identified. Thus, ether fuel-degrading enzymes may have been recruited from several hydrocarbon-degrading pathways. This hypothesis is further supported by the observation that the well-known camphor-oxidizing cytochrome P-450 cam is able to degrade MTBE partially [2]. In addition, even if indirect evidence suggests that cytochrome P-450 systems participate in the degradation of ether fuels by several microorganisms [2, 10, 19], this may not be always the case: the cleavage of MTBE by *Mycobacterium austroafricanum* IFP 2012 appears to involve a nonheme monooxygenase [7]. Owing to this diversity, it is likely that more genes from different ether fuel-degrading microorganisms will have to be cloned in order to get a fully representative panel of probes for monitoring the MTBE- and ETBE-degrading potential of the microflora.

REFERENCES

1 Salanitro, J. P., Diaz, L. A., Williams, M. P. and Wisniewski, H.L. (1994) Isolation of a Bacterial Culture that Degrades Methyl *t*-butyl ether. *Appl. Environ. Microbiol.*, **60**, 7, 2593-2596.

2 Steffan, R.J., McClay, K., Vainberg, S., Condee, C.V. and Zhang, D. (1997) Biodegradation of the Gasoline Oxygenates Methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by Propane-Oxidizing Bacteria. *Appl. Environ. Microbiol.*, **63**, 11, 4216-4222.

3 Fayolle, F., Hernandez, G., Le Roux, F. and Vandecasteele, J.P. (1998) Isolation of Two Aerobic Bacterial Strains that Degrade Efficiently ethyl *tert*-butyl ether. *Biotechnol. Lett.*, **20**, 3, 283-286.

4 Liu, C.Y., Speitel, G.E.J. and Georgiu, G. (2001) Kinetics of Methyl *t*-butyl ether Cometabolism at Low Concentration by Pure Cultures of Butane-Degrading Bacteria. *Appl. Environ. Microbiol.*, **67**, 5, 2197-2201.

5 Hatzinger, P.B., McClay, K., Vainberg, S., Tugusheva, M., Condee, C.W. and Steffan, R.J. (2001) Biodegradation of Methyl-*tert*-butyl ether by a Pure Bacterial Culture. *Appl. Environ. Microbiol.*, **67**, 12, 5601-5607.

6 Hanson, J.R., Ackerman, C.R. and Scow, K.M. (1999) Biodegradation of Methyl *tert*-butyl ether by a Bacterial Pure Culture. *Appl. Environ. Microbiol.*, **65**, 11, 4788-4792.

7 François, A., Mathis, H., Godefroy, D., Piveteau, P., Fayolle, F. and Monot, F. (2002) Biodegradation of Methyl *tert*-butyl ether and Other Fuel Oxygenates by a New Strain, *Mycobacterium austroafricanum* IFP 2012. *Appl. Environ. Microbiol. 2754-2762*, **68**, 6, 2754-2762.

8 Hardison, L.K., Curry, S.S., Ciuffetti, L.M. and Hyman, M.L. (1997) Metabolism of Diethyl Ether and Cometabolism of MTBE by a Filamentous Fungus, a *Graphium* sp. *Appl. Environ. Microbiol.*, **63**, 8, 3059-3067.

9 Fortin, N.Y. and Deshusses, M. A. (1999) Treatment of Methyl *tert*-butyl ether Vapors in Biotrickling Filters. 1. Reactor Start-Up, Steady-State Performance, and Culture Characteristics. *Environ. Sci. Technol.*, **33**, 17, 2980-2986.

10 Kharoune, M., Kharoune, L., Lebeault, J.-M. and Pauss, A. (2001) Isolation and Characterization of Two Aerobic Strains that Completely Degrade ethyl *tert*-butyl ether (ETBE). *Appl. Microbiol. Biotechnol.*, **55**, 3, 348-353.

11 Piveteau, P., Fayolle, F., Le Penru, Y. and Monot, F. (2000) Biodegradation of MTBE by Cometabolism in Laboratory-scale Fermentations, in *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds*, (G.B. Wickramanayake, A. Gavaskar, B.C. Alleman and V.S. Magar, Eds.), Battelle Press, Columbus, Ohio.

12 Grüntzig, V., Nold, S.C., Jizhong, Z. and Tiedje, J.M. (2001) *Pseudomonas stutzeri* Nitrite Reductase Gene Abundance in Environmental Samples Measured by Real-Time PCR. *Appl. Environ. Microbiol.*, **67**, 2, 760-768.

13 Hernandez-Perez, G., Fayolle, F. and Vandecasteele, J.P. (2001) Biodegradation of Ethyl-*t*-butyl ether (ETBE), Methyl-*t*-butyl ether (MTBE) and *t*-amyl-methyl ether (TAME) by *Gordonia terrae*. *Appl. Microbiol. Biotechnol.*, **55**, 1, 117-121.

14 Chauvaux, S., Chevalier, F., Le Dantec, C., Fayolle, F., Miras, I., Kunst, F. and Beguin, P. (2001) Cloning of a Genetically Unstable Cytochrome P-450 Gene Cluster Involved in Degradation of the Pollutant Ethyl *tert*-butyl ether by *Rhodococcus ruber*. *J. Bacteriol.*, **183**, 22, 6551-6557.

15 Nagy, I., Schoofs, G., Compernolle, F., Proost, P., Vanderleyden, J. and De Mot, R. (1995) Degradation of the Herbicide EPTC (*S*-ethyl dipropylcarbamothioate) and Biosafing by *Rhodococcus* sp. Strain NI86/21 Involve an Inducible Cytochrome P-450 System and Aldehyde Dehydrogenase. *J. Bacteriol.*, **177**, 3, 676-687.

16 François, A. (2002) Dégradation d'un composé xénobiotique récalcitrant : métabolisme du méthyl *tert*-butyl éther (MTBE) par *Mycobacterium austroafricanum* IFP 2012, *Ph.D. Thesis*, Institut national d'agronomie Paris-Grignon.

17 Ochman, H., Ayala, F.-J. and Hartl, D.L. (1993) Use of Polymerase Chain Reaction to Amplify Segments Outside Boundaries of Known Sequences. *Methods in Enzymology*, **218**, 309-321.

18 Hyman, M., Taylor, C. and O'Reilly, K. (2000) Cometabolic Degradation of MTBE by *iso*-Alkane-Utilizing Bacteria from Gasoline-Impacted Soil. In: *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds* (G.B. Wickramanayake, A. Gavaskar, B.C. Alleman and V.S. Magar, Eds.), Battelle Press, Columbus, Ohio.

19 Corcho, D., Watkinson, R.J. and Lerner, D.N. (2000) Cometabolic Degradation of MTBE by a Cyclohexane-Oxidising Bacteria. In: *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds* (G.B. Wickramanayake, A. Gavaskar, B.C. Alleman and V.S. Magar, Eds.), Battelle Press, Columbus, Ohio.