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A *Mycobacterium* Strain with Extended Capacities for Degradation of Gasoline Hydrocarbons

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A bacterial strain (strain IFP 2173) was selected from a gasoline-polluted aquifer on the basis of its capacity to use 2,2,4-trimethylpentane (isooctane) as a sole carbon and energy source. This isolate, the first isolate with this capacity to be characterized, was identified by 16S ribosomal DNA analysis, and 100% sequence identity with a reference strain of *Mycobacterium austroafricanum* was found. *Mycobacterium* sp. strain IFP 2173 used an unusually wide spectrum of hydrocarbons as growth substrates, including *n*-alkanes and multimethyl-substituted isoalkanes with chains ranging from 5 to 16 carbon atoms long, as well as substituted monoaromatic hydrocarbons. It also attacked ethers, such as methyl *t*-butyl ether. During growth on gasoline, it degraded 86% of the substrate. Our results indicated that strain IFP 2173 was capable of degrading 3-methyl groups, possibly by a carboxylation and deacetylation mechanism. Evidence that it attacked the quaternary carbon atom structure by an as-yet-undefined mechanism during growth on 2,2,4-trimethylpentane and 2,2-dimethylpentane was also obtained.

Widely used petroleum products, such as gasoline, kerosene, and diesel oil, are common pollutants of the environment which have been shown to be biodegradable to some extent. It has been shown that the overall biodegradation of gasoline by microflorae from soil or groundwater of contaminated sites is efficient, and the level of biodegradation is as high as 90% (13, 23, 30, 32, 33). However, detailed information concerning the fate of components of gasoline is rather scarce; the only exceptions are some individual hydrocarbons. It has been demonstrated that benzene, toluene, ethylbenzene, *m*- and *p*-xylenes, and *n*-alkanes are readily biodegradable (7, 11, 14, 17, 20, 22, 26), whereas β-branched and quaternary branched alkanes, *o*-xylene, and cyclohexane have been found to be less susceptible to biodegradation (3, 4, 8, 15, 16, 20, 22).

Gasoline is a complex hydrocarbon mixture that can contain more than 200 individual compounds, as shown by gas chromatography (GC). Detailed analysis is tedious because only minor amounts of some components are present. In order to facilitate studies of biodegradation of individual hydrocarbons by microfloræ in the environment, we have developed specific methods in which hydrocarbon mixtures are used as substrates (20). The use of these gasoline model mixtures, which are simpler to handle than gasoline, has been validated by the finding that they yield results which are quite similar to the results obtained with gasoline (21). Degradation tests performed with gasoline model mixtures revealed that the degradation capacities of microfloræ obtained from various environments were usually high (total extent of degradation, at least 85%). However, the limitations of the microfloræ were apparent when degradation of compounds such as cyclohexane and trimethylpentanes, which appeared to be the most recalcitrant compounds in gasoline, was examined. For this reason, microfloræ that specifically degrade cyclohexane and 2,2,4-trimethylpentane were selected from samples obtained from the environment. When cyclohexane was the carbon source, no pure strain could be isolated (22), which is consistent with the accepted view that cometabolism and mutualism phenomena are involved in cyclohexane biodegradation (4, 15). The capacity to degrade isooctane (2,2,4-trimethylpentane) was found to be rare in natural samples, and only one microflora and one pure strain with this characteristic were obtained by Solano-Serena et al. (22).

In the present work, we investigated the degradation capacities of the new isolate obtained by Solano-Serena et al., which utilized isooctane, a component of gasoline that is usually considered recalcitrant, as a carbon source. Culture tests were performed under nonlimiting conditions. The carbon sources used were the main gasoline hydrocarbons, which were supplied individually or in mixtures. In order to simulate the possible bacterial strategies for attenuation of pollutants in the environment, the cometabolism capacities of the strain were also characterized by using cyclohexane as a cosubstrate.

MATERIALS AND METHODS

Culture media. Vitamin-supplemented mineral salt medium MSM (6) was used in this study. The mineral solution was autoclaved for 20 min at 120°C, and vitamins were added by sterile filtration (pore size, 0.22 μm). The only carbon sources used were individual hydrocarbons and two hydrocarbon mixtures that were representative of gasoline. For analytical purposes, two synthetic mixtures were prepared by using equal quantities of several commercial products. The first mixture was designated light fraction model 9 (LFM9), which contained the following nine hydrocarbons having 4 to 6 carbon atoms: butane, 2-methylpropane (isobutane), pentane, isopentane (2-methylbutane), 2- and 3-methylpentanes, 2,2- and 2,3-dimethylbutanes, and methylcyclopentane. LFM9 could be adequately analyzed by headspace sampling. The second mixture was designated gasoline model mixture 23 (GM23), which contained 23 hydrocarbons having 6 to 9 carbon atoms (20). This mixture could be appropriately analyzed after solvent extraction.

Isolation of 2,2,4-trimethylpentane- and cyclohexanone-degrading bacteria. 2,2,4-Trimethylpentane-degrading strain IFP 2173 was isolated from gasoline-contaminated groundwater (22). Cyclohexanone-degrading strain IFP 2149 was isolated from an activated sludge sample obtained from an urban wastewater

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treatment plant. Enrichment cultures were grown in 250-ml flasks containing 50 ml of medium MSM supplemented with 500 mg of cyclohexanone per liter and 300 mg (dry weight) of activated sludge per liter. After five subcultures at 30°C with shaking at 250 rpm, single loopfuls of the cell suspension were streaked onto agar plates containing the medium MSM supplemented with cyclohexanone and incubated at 30°C. Isolated colonies were used to inoculate medium MSM containing cyclohexanone in order to confirm that this carbon source was utilized.

Degradation tests. The degradation capacities of strain IFP 2173 were determined in flasks inoculated (10%, vol/vol) with a preculture of the isolate grown on isooctane. The flasks were incubated for 28 days at 30°C with shaking at 250 rpm.

The culture tests in which individual hydrocarbons were used were carried out in 120-ml flasks containing 10 ml of inoculated medium MSM. The flasks were closed with butyl rubber stoppers. Five-microliter portions of liquid substrates (hydrocarbons or ethers) were dispensed into the sealed flasks with a 10-μl syringe. Gaseous hydrocarbons (1.5 ml) were introduced into the sealed flasks with a gas-tight syringe.

The degradation tests in which LFM9 was used were carried out under the same conditions by using 4-μl portions of a liquid mixture containing equal volumes of the seven liquid hydrocarbons (pentane, isopentane, 2- and 3-methylpentanes, 2,2- and 2,3-dimethylbutanes, methylcyclopentane) and 250 μl of each gaseous hydrocarbon (butane, isobutane).

The degradation tests in which GM23 was used were performed in 500-ml flasks having side arms equipped with Mininert valves (Pierce, Oud-Beijerland, The Netherlands) and containing 50-ml portions of inoculated culture medium and 25-μl portions of GM23 as previously described (20).

After incubation, the total amount of CO_2 produced was determined by GC by obtaining 250 μl of gas from the headspace with a gas-tight syringe after HNO_3 (5 μl of 68% HNO_3 per 10 ml of culture medium) was added. For analysis, samples of the LFM9 hydrocarbons (either individually or in a mixture) were obtained from the flask headspace with a gas-tight syringe and analyzed by GC by using an external standard method. Two GM23 hydrocarbons, hexadecane and pristane, were extracted in a CH_2Cl_2 phase (1 ml of CH_2Cl_2 per 10 ml of culture medium) containing 600 mg of an internal standard (dodecane) per liter and used for GC analysis. Residual ether contents were determined by performing a direct GC analysis of the culture medium after filtration (pore size, 0.22 μm). All experiments were performed in duplicate. Abiotic controls containing 1 g of $HgCl_2$ per liter were examined under similar conditions. Inoculated flasks that did not contain a carbon source were incubated to estimate the endogenous respiration of the culture.

Cometabolism and commensalism experiments. Cultures were grown in 155-ml flasks that were hermetically closed and contained 10 ml of inoculated culture medium. In cometabolism experiments, the flasks were inoculated (10%, vol/vol) with a preculture of strain IFP 2173 that had been grown on isooctane, whereas in commensalism experiments, the flasks were inoculated with both a preculture of strain IFP 2173 that had been grown on isooctane and a preculture of strain IFP 2149 that had been grown on cyclohexanone (5% [vol/vol] each).

Before incubation, 5 μl of the hydrocarbon used as the growth substrate and 2.5 μl of cyclohexane (used as a cosubstrate) were introduced into the sealed flasks with a 10-μl syringe. Acetate (1 g/liter) and ethanol (1 g/liter) were also used as substrates. After incubation, the residual amounts of the hydrocarbon, of the cosubstrate, and of the metabolites produced (cyclohexanone and cyclohexene) were extracted with CH_2Cl_2 as described above and were analyzed by GC. Ethanol contents were determined by performing a direct GC analysis of the culture, and acetate contents were determined by enzymatic analysis (TC Acetic acid; Boehringer, Mannheim, Germany).

Cometabolism experiments were performed in duplicate. Commensalism experiments were performed in triplicate, and control experiments for each strain were performed in duplicate. Substrate-free flasks and abiotic controls were incubated and analyzed under similar conditions.

Chromatographic analyses. CO_2 contents were determined with a chromatograph equipped with a thermal conductivity detector and a Porapak Q column (80/100 mesh; length, 2 m) by using an external standard method. The carrier gas was helium, and the column temperature was 50°C (20).

Hydrocarbons were analyzed with a model 3400 chromatograph (Varian, Sugarland, Tex.) equipped with a flame ionization detector and a CP-Sil Pona CB column (0.25 mm by 100 m) obtained from Chrompack (Raritan, N.J.). The carrier gas was helium. The temperature of the injector was 250°C, and the temperature of the detector was 300°C. For headspace analysis (LFM9 hydrocarbons), the column temperature was set at 40°C. For other hydrocarbons, the column temperature was first set at 35°C for 10 min; then it was increased to 114°C at a rate of 1.1°C/min and to 280°C at a rate of 1.7°C/min and finally was set at 280°C for 40 min.

Ethers and alcohols were analyzed by the method of Fayolle et al. (9) by using a model 3400 chromatograph (Varian) equipped with a flame ionization detector and a DB 624 column (0.32 mm by 30 m; J & W Scientific, Rancho Cordova, Calif.).

Degradation and mineralization yields. Mineralization yields were calculated by determining the molar ratios of the difference between the amount of carbon in the total CO_2 produced in a test flask and the amount of carbon in the total CO_2 produced in a substrate-free flask to the amount of carbon in the substrate consumed.

The extents of degradation of individual substrates were calculated by determining the ratio of the amount of substrate degraded in test flasks to the amount of substrate recovered in abiotic controls.

GC-MS analyses. The metabolites were analyzed by performing electron impact mass spectrometry (MS) with an Autospec Ultimas apparatus (Micromass, Manchester, United Kingdom). The mass spectrometer was operated at 70 eV, and the temperature of the ion source was 250°C. For GC-MS analyses, we used a CP-Sil Pona CB column obtained from Chrompack (0.25 mm by 100 m). The temperature program was the same as the temperature program described above.

16S rDNA PCR and DNA sequencing. Strain IFP 2173 was grown on medium MSM plates containing isooctane as a carbon source. A colony was resuspended in 20 μl of sterile demineralized water. Five microliters of the cell suspension were directly used for 16S ribosomal DNA (rDNA) amplification. The sequence of forward primer fD1 was 5′-AGAGTTTGATCCTGGCTCAG-3′, and the sequence of reverse primer rD1 was 5′-AAGGAGGTGATCCAGCC-3′, as described by Weisburg et al. (27). The thermal profile was as follows: 30 cycles consisting of 94°C for 30 s, primer annealing at 53°C for 30 s, and extension at 72°C for 1.30 min. All experiments were performed in triplicate. The amplification products were purified after gel electrophoresis with a Gene Clean Kit II (Bio 101, Vista, Calif.), and both strands were sequenced (ESGS, Evry, France). The sequences were compared to sequences in the EMBL/GenBank database by using the BLAST alignment tool (1).

Phylogenetic analysis. 16S rDNA sequences (length, about 1,380 bp) of isolate IFP 2173 and 14 other *Mycobacterium* strains were aligned by using the CLUSTALV software (12). An unrooted tree was constructed by using the neighbor-joining method (18). A bootstrap analysis of the tree was performed with 1,000 resamplings (10).

Chemicals. Cyclohexane, benzene, toluene, xylenes, ethylbenzene, and trimethylbenzenes were purchased from Prolabo (Fontenay-sous-Bois, France). Other hydrocarbons, ethers, and vitamins were obtained from Sigma-Aldrich Chimie (Saint-Quentin-Fallavier, France).

Nucleotide sequence accession numbers. The nucleotide sequence of strain IFP 2173 has been deposited in the GenBank database under accession no. AF190800. The GenBank accession numbers of the other sequences used in the analyses are as follows: *Mycobacterium aichiense*, X55598; *M. asiaticum*, X55604; *M. aurum*, X55595; *M. austroafricanum*, X93182; *M. fallax*, M29562; *M. flavescens*, X52932; *M. fortuitum*, X52933; *M. gadium*, X55594; *M. komossense*, X55591; *M. nonchromogenicum*, X52929; *M. obuense*, X55597; *M. vaccae*, X55601; *M. xenopi*, X52928; and *Mycobacterium* sp. strain PYR-I, X84977.

RESULTS

Identification of strain IFP 2173. Using isooctane (2,2,4-trimethylpentane) as the sole carbon and energy source, Solano-Serena et al. isolated strain IFP 2173 from gasoline-contaminated groundwater by preparing successive enrichment cultures (22). This organism was tentatively identified as a *Corynebacterium urealyticum* strain by using classical methods, and it was deposited in the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, Paris, France) as strain CIP-I-2126. A comparison of its complete PCR-amplified 16S rDNA sequence to previously published 16S rDNA sequences revealed high levels of similarity to the rapidly growing species of the genus *Mycobacterium* (Fig. 1). The level of 16S rDNA sequence identity between strain IFP 2173 and a reference strain of *M. austroafricanum* (ATCC 33464) was 100%.

Hydrocarbon utilization. The degradative capacities of *Mycobacterium* sp. strain IFP 2173 were assessed by using a bacterial test in which growth on individual hydrocarbons was measured by measuring hydrocarbon consumption and production of CO_2 (mineralization). Most of the hydrocarbons tested were compounds detected in commercial gasoline (Table 1). We found that *Mycobacterium* sp. strain IFP 2173 degraded and mineralized *n*-alkanes with 5 to 16 carbon atoms. Propane was not degraded, and butane was only partially consumed. An analogous effect of carbon chain length on biodegradation was also observed with 2-methylalkanes; hydrocarbons with 5 to 8 carbon atoms in the main carbon chain were completely degraded, whereas isobutane (2-methylpropane) was only partially degraded (41%). Monomethylalkane isomers (2-methyl and 3-methyl alkanes) were degraded similarly.

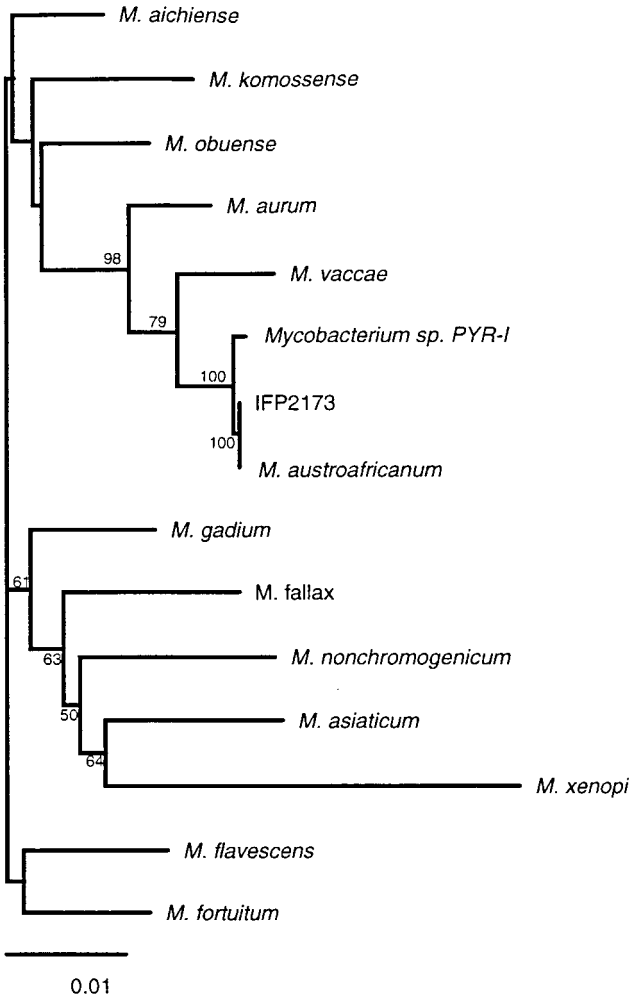

FIG. 1. Unrooted evolutionary distance tree based on the 16S rDNA sequence studied and sequences of bacteria belonging to the genus *Mycobacterium*. Bootstrap values greater than 50% are indicated at the nodes. Bar = 0.01 nucleotide difference per sequence position.

TABLE 1. Degradation of individual hydrocarbons by *Mycobacterium* sp. strain IFP 2173

Hydrocarbon structure	Compound	Extent of degradation (%)[a]	Mineralization yield (%)[b]
n-Alkanes	Propane	0	0
	Butane	47	14
	Pentane	100	57
	Heptane	100	41
	Octane	100	36
	Decane	100	42
	Hexadecane	88	48
Methylalkanes	2-Methylpropane	41	4
	2-Methylbutane	57	83
	2-Methylpentane	100	59
	3-Methylpentane	100	61
	2-Methylhexane	100	58
	3-Methylhexane	100	55
	2-Methylheptane	100	46
	3-Methylheptane	100	39
	2-Methyloctane	100	37
Dimethylalkanes	2,2-Dimethylbutane	2	0
	2,3-Dimethylbutane	14	39
	2,2-Dimethylpentane	100	52
	2,3-Dimethylpentane	100	45
	2,4-Dimethylpentane	100	42
	3,3-Dimethylpentane	34	<1
	2,2-Dimethylhexane	27	7
	2,4-Dimethylhexane	100	39
Dimethylalkanes	2,5-Dimethylhexane	100	55
	3,4-Dimethylhexane	100	48
Trimethylalkanes	2,2,4-Trimethylpentane	100	46
	2,3,4-Trimethylpentane	87	5
	2,2,4-Trimethylhexane	28	<1
Other isoalkanes	Pristane	89	60
Cyclic alkanes	Methylcyclopentane	33	5
	Cyclohexane	0	0
Aromatic compounds	Benzene	0	0
	Toluene	98	30
	Ethylbenzene	0	0
	n-Propylbenzene	0	0[c]
	o-Xylene	0	0[c]
	m-Xylene	100	50
	p-Xylene	97	59
	2-Ethyltoluene	0	0[c]
	3-Ethyltoluene	0	0[c]
	4-Ethyltoluene	0	0[c]
Ethers and alcohol	Ethyl *tert*-butyl ether	81	7
	Methyl *tert*-butyl ether	32	<1
	Methyl *tert*-amyl ether	51	<1
	tert-Butyl alcohol	0	0

[a] The values are means based on the values obtained for two test flasks. Extents of degradation were calculated as described in Materials and Methods. Hydrocarbons were quantitatively recovered in abiotic flasks as the amounts of hydrocarbons in abiotic controls were within ±10% of the theoretical amounts present in the 5 μl initially supplied.

[b] The values are means based on the values obtained for two test flasks. Mineralization yields were determined with respect to the substrate degraded (see Materials and Methods).

[c] The amount of CO_2 produced was smaller than the amount in substrate-free flasks.

Of the dimethylpentane and dimethylhexane isomers examined, those with no quaternary carbon atom were completely degraded and mineralized to similar extents (40 to 50%); this was true even for isoalkanes with methyl anteiso structures, which are known to be resistant to biodegradation. Indeed, 2,3-dimethylpentane and 3,4-dimethylhexane were metabolized. By contrast, dimethylalkanes with a quaternary carbon atom (3,3-dimethylpentane, 2,2-dimethylbutane, and 2,2-dimethylhexane) were only slightly degraded or were not degraded; the only exception to this was 2,2-dimethylpentane. Trimethylpentanes were also degraded; 2,2,4-trimethylpentane was mineralized, but 2,3,4-trimethylpentane was not mineralized. 2,2,4-Trimethylhexane was degraded only slightly. When quaternary carbon atoms were considered, the results revealed the significance of the localization of these atoms and the significance of the carbon chain length for the biodegradation process. It is worth noting that pristane (2,6,10,14-tetramethylpentadecane), a large isoalkane having several substitutions, was extensively degraded and mineralized.

We found that the kinetics of CO_2 production from C_8 hydrocarbon isomers was similar to the kinetics of CO_2 production from *n*-octane, 2-methylheptane, and 2,4-dimethylhexane. Over time, CO_2 production was linear at first and then stopped. The production rates during the linear phase were 74.7, 70.7, and 54.1 μmol/(liter · h) for *n*-octane, 2-methylheptane, and 2,4-dimethylhexane, respectively. The limited hydro-

TABLE 2. Biodegradation of LFM9 by *Mycobacterium* sp. strain IFP 2173

Hydrocarbon	Initial concn (mg/liter)[a]	Extent of degradation (%)[b]
Butane	120.5	6
Pentane	67.9	86
2-Methylpropane	120.5	0
2-Methylbutane	66.7	42
2-Methylpentane	72.9	100
3-Methylpentane	74.4	100
2,2-Dimethylbutane	74.4	96
2,3-Dimethylbutane	75.2	88
Methylcyclopentane	84.1	99
LFM9	756.6	39

[a] Amount of hydrocarbon supplied per liter of culture medium.
[b] The values are means based on the values obtained for two test flasks. When each hydrocarbon in abiotic flasks was considered, the amount recovered was 85% ± 3% of the amount supplied.

TABLE 3. Biodegradation of GM23 by *Mycobacterium* sp. strain IFP 2173

Hydrocarbon	Initial concn (mg/liter)[a]	Extent of degradation (%)[b]
n-Heptane	13.7	100
n-Octane	15.2	100
2-Methylhexane	13.3	93
3-Methylhexane	14.0	92
3-Methylheptane	14.9	95
2,4-Dimethylhexane	15.2	83
2,5-Dimethylhexane	14.2	74
2,2,4-Trimethylpentane	14.2	97
2,3,4-Trimethylpentane	15.2	100
Cyclohexane	15.3	57
Benzene	18.1	13
Toluene	18.9	93
Ethylbenzene	20.2	100
n-Propylbenzene	19.6	100
o-Xylene	19.6	90
m-Xylene	19.1	92
p-Xylene	19.7	100
2-Ethyltoluene	21.5	100
3-Ethyltoluene	20.2	100
4-Ethyltoluene	18.6	100
1,2,3-Trimethylbenzene	19.0	50
1,2,4-Trimethylbenzene	20.2	100
1,3,5-Trimethylbenzene	20.2	53
GM23	400.1	86

[a] Amount of hydrocarbon supplied per liter of culture medium.
[b] The values are means based on the values obtained for two test flasks. When each hydrocarbon in abiotic flasks was considered, the average amount recovered was 89% ± 4% of the amount supplied.

carbon transfer from the gaseous phase to the aqueous phase could probably explain the zero-order kinetics observed.

Mycobacterium sp. strain IFP 2173 could not use cyclohexane as a sole carbon source. Methylcyclopentane was degraded only slightly. In this experiment, methylcyclopentanone was detected by a GC-MS analysis in the culture medium.

Some aromatic compounds (toluene, *m*- and *p*-xylenes) were mineralized, whereas others were not. Inhibition of endogenous respiration was observed with *n*-propylbenzene, ethyltoluene isomers, and, to a lesser extent, *o*-xylene. Benzene and ethylbenzene were not degraded but had no inhibitory effect.

When we examined utilization of ethers as sole carbon sources, we found that the extents of degradation were measurable, but the mineralization yields were very low. The degradation observed probably resulted from activity of the inoculum acting as resting cells.

Degradation capacities with hydrocarbon mixtures. The degradative capacities of *Mycobacterium* sp. strain IFP 2173 were also evaluated by using hydrocarbon mixtures in order to allow substrate interactions (competition and cometabolism) in cultures. As described above, two model gasoline mixtures were used.

The overall extent of degradation of LFM9 was 39%, which was moderate since butane and 2-methylpropane (isobutane) were not degraded (Table 2). A comparison with the extents of degradation of these compounds when they were provided individually suggested that competitive inhibition between substrates might have occurred during degradation of LFM9. Pentane and methylpentane isomers were degraded at extents similar to those observed when these compounds were supplied individually. Degradation of 2,2-dimethylbutane, 2,3-dimethylbutane, and methylcyclopentane was even enhanced when these compounds were supplied in a mixture, possibly because of cometabolism.

The overall extent of degradation of GM23 was 86% (Table 3). Of the 23 components, those that were degraded when they were supplied individually were also degraded when they were supplied in the mixture, indicating that no inhibition took place. We found that compounds which inhibited endogenous respiration in individual incubation preparations (*n*-propylbenzene, ethyltoluene isomers, and *o*-xylene) were completely degraded in the mixture. These results suggested that the concentration used in the individual hydrocarbon tests (350 mg/liter, compared with 13 to 20 mg/liter in the mixture) was toxic for the bacterial cells. Of the hydrocarbons which were not degraded in individual preparations, benzene was not degraded when it was supplied in the mixture, but ethylbenzene and cyclohexane were consumed. Cometabolism accounted for degradation of the latter two compounds in the mixture. Furthermore, a metabolite shown in Fig. 2 (retention time, 41.3 min) was detected in test flasks. This metabolite was identified by GC-MS analysis as cyclohexanone (Fig. 3), which indicated that cooxidation of cyclohexane occurred.

Cometabolism capacities. We confirmed that cometabolic degradation of ethylbenzene occurred. We found that during incubation with isooctane as the growth substrate, ethylbenzene was converted into acetophenone, as shown by GC-MS analysis, but not into phenylacetic acid, implying that there was preferential attack on the CH_2 group.

To examine cyclohexane cooxidation, we performed experiments in which we used various hydrocarbons as growth substrates. CO_2 production was monitored in order to detect the degradation end points. The overall extents of degradation of the substrate and cosubstrate were determined (Table 4). The data for the growth substrate were similar to the data obtained for the substrate alone (Table 1), indicating that cyclohexane did not influence the biodegradation process. Degradation of cyclohexane took place with all of the hydrocarbons tested, as well as with acetate or ethanol, and the extent of degradation was usually high.

In addition to cyclohexanone, we found another coproduct, which was identified as cyclohexene by GC-MS analysis. The data indicated that cyclohexane was transformed mainly into cyclohexanone and that cyclohexene was a by-product. The high rates of recovery in mass balance experiments suggested that no further degradation of cyclohexanone took place.

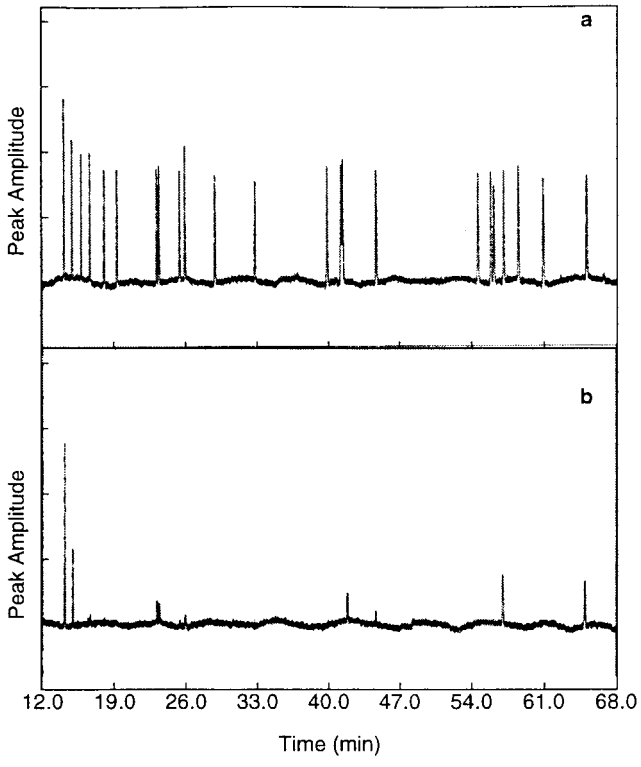

FIG. 2. Chromatographic patterns of residual hydrocarbons of GM23 after 28 days of incubation. (a) Abiotic flask. (b) Test flask.

Syntrophic biodegradation of cycloalkanes. A cyclohexanone-degrading strain, designated IFP 2149, was isolated from activated sludge by using cyclohexanone as the sole carbon and energy source. This bacterium was identified by classical methods as an *Acinetobacter lwoffii* strain. Interactions between strains IFP 2173 and IFP 2149 during degradation of cyclohexane were investigated by using isooctane as an energy source (Table 5). *A. lwoffii* was not able to degrade isooctane or cyclohexane. Degradation of cyclohexane with *Mycobacterium* sp. strain IFP 2173 alone was similar to degradation of cyclohexane with strain IFP 2173 in association with *A. lwoffii* IFP 2149, although cyclohexanone accumulated in the former preparation but not in the latter preparation, in which it appeared to be used by strain IFP 2149. However, the final amount of cyclohexene was not different, confirming that cyclohexene was an end product of a side reaction. The amounts of CO_2 produced in the different cultures are shown in Fig. 4. The amount of CO_2 produced with both strains was significantly larger than the amount of CO_2 produced with *Mycobacterium* sp. strain IFP 2173 alone. Mineralization of cyclohexanone by *A. lwoffii* probably accounted for the increase in CO_2 production.

DISCUSSION

Mycobacterium sp. strain IFP 2173 was isolated from a gasoline-polluted groundwater sample because of its ability to degrade isooctane. In several ways, this strain biodegraded an unusual spectrum of hydrocarbon compounds.

First, *Mycobacterium* sp. strain IFP 2173 could degrade various molecular structures, such as *n*-alkanes, isoalkanes, aromatic compounds, or oxygenates, which are commonly introduced into gasoline formulations. Second, it could also attack a wide range of compounds with chain lengths ranging from 4 to 16 carbon atoms. The degradation of short alkanes is noteworthy since these compounds are usually considered toxic to bacterial cells because of their solubility in culture media (7). Moreover, it is known that propane and butane are degraded by bacterial strains that specialize in short-chain alkane metabolism (2, 25, 29).

The most spectacular feature of strain IFP 2173 was its ability to degrade branched alkanes. Recently, workers have described quite notable advances in degradation of *n*-alkanes, and bacteria from specific habitats, such as marine (31) or cold (28) environments, have been characterized, but less effort seems to have been devoted to degradation of isoalkanes. We found that *Mycobacterium* sp. strain IFP 2173 was able to degrade monomethylated alkanes and almost all dimethylated alkanes. Even β-methyl-branched (anteiso) structures, which usually prevent or hinder normal β-oxidation (19), were found to be susceptible to biodegradation. In fact, 3-methylacyl co-

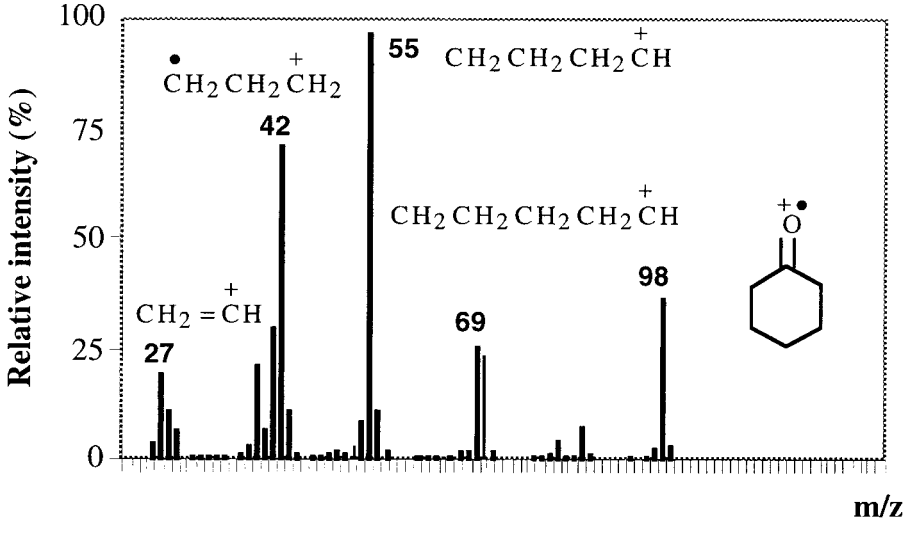

FIG. 3. Mass spectrum of the metabolic compound produced during incubation with GM23 (retention time, 41.3 min).

TABLE 4. Effect of substrate on cometabolism of cyclohexane by *Mycobacterium* sp. strain IFP 2173

Substrate	Incubation period (days)	Extent of degradation (%) for:		Final amt (μmol) of:			Recovery rate[c]
		Substrate[a]	Cosubstrate[b]	Cyclohexane	Cyclohexanone	Cyclohexene	
Heptane	21	100	76	5.8	15.5	0.8	0.93
Octane	21	100	76	5.8	14.8	0.7	0.89
2-Methylhexane	21	100	77	5.6	16.0	0.9	0.94
3-Methylheptane	21	100	80	4.8	16.8	0.8	0.94
2,4-Dimethylhexane	21	99	80	4.7	16.8	0.6	0.93
2,5-Dimethylhexane	21	100	92	2.0	18.7	0.3	0.87
2,2,4-Trimethylpentane	21	100	85	3.5	17.9	0.9	0.93
2,3,4-Trimethylpentane	33	89	39	14.5	7.2	0.9	0.95
Toluene	33	98	58	10.0	9.9	0.9	0.87
m-Xylene	33	98	31	16.5	5.7	0.1	0.93
p-Xylene	33	98	76	5.8	15.6	0.9	0.93
Acetate	11	98	43	13.6	9.0	1.2	0.99
Ethanol	4	69	60	9.5	10.6	1.3	0.90

[a] See Table 1, footnote *a*.
[b] The values are means based on the values obtained for two test flasks. The degradation rates were calculated as described in Materials and Methods. The amount of cyclohexane recovered in abiotic flasks was 23.9 ± 1.3 μmol.
[c] The recovery rate was the molar ratio of the sum of the amount of the residual cosubstrate and the amount of the coproducts to the amount of cosubstrate recovered in abiotic flasks.

enzyme A intermediates which cannot undergo β-oxidation might be degraded through a decarboxymethylation mechanism. In this pathway, which has been elucidated in some specialized microorganisms, CO_2 is first enzymatically fixed on the lateral methyl group, and the acetyl group formed is subsequently removed from the molecule. As a result, the initial methyl group on carbon 3 is eliminated from the molecule (8). The existence of such a pathway is strongly suggested by our results. Not surprisingly, β-dimethyl-branched alkanes (3,3-dimethylpentane) could not be degraded through the decarboxymethylation process. The most remarkable property of strain IFP 2173, however, is its ability to degrade some isoalkanes that have a quaternary carbon structure (e.g., 2,2-dimethylpentane and 2,2,4-trimethylpentane). As the high mineralization yields determined for degradation of these isoalkanes indicated that the quaternary structure was attacked, the presence of a relevant unknown mechanism may be postulated.

We found that as a consequence of the bacterial activity, some methyl groups, such as the terminal CH_3 of the main hydrocarbon chains or substituents of branched alkanes or aromatic compounds, could be attacked. Furthermore, methylene groups, such as CH_2 included in aliphatic or alicyclic chains, were also attacked. In contrast, the CH groups of benzene and several quaternary carbon structures were found to be refractory, as pointed out previously by McKenna (16).

Mycobacterium sp. strain IFP 2173 also exhibited a substantial capacity for cometabolism. Cyclohexane was attacked by the initial oxidation system of this microorganism, which produced cycloalkanone. A similar cometabolism capacity has been found in *M. vaccae* JOB5, which produces cycloalkanones from cycloalkanes after growth on propane (5). With *Mycobacterium* sp. strain IFP 2173, cyclohexanone was produced when the energetic requirements were met by utilization of an appropriate substrate. We found that many hydrocarbon compounds, particularly isooctane, could satisfy the energetic needs of the strain. In addition to cyclohexanone, cyclohexene was identified as one of the cometabolic products. Hence, it seems likely that a common precursor of the cyclohexanone and cyclohexene that accumulated was cyclohexanol, although the latter compound was not identified. When we compared degradation of the gasoline mixtures by strain IFP 2173 and degradation of individual hydrocarbons, we observed a positive cometabolism effect for several compounds. In the standard test in which gasoline model mixtures were used, cyclohexane, methylcyclopentane, and 2,2- and 2,3-dimethylbutanes appeared to be cometabolized. Finally, up to 86% of GM23 was

TABLE 5. Syntrophic degradation of cyclohexane

Microorganism(s)	Carbon source(s)	Extent of degradation for cyclohexane (%)[a]	Final amt (μmol) of:		
			Cyclohexane	Cyclohexanone	Cyclohexene
A. lwoffii IFP 2149	Cyclohexane	<5[b]	23.0[b]	<0.1[b]	<0.1[b]
	2,2,4-Trimethylpentane + cyclohexane	<5[b]	24.7[b]	<0.1[b]	<0.1[b]
Mycobacterium sp. strain IFP 2173	Cyclohexane	<5[b]	23.4[b]	1.2[b]	<0.1[b]
	2,2,4-Trimethylpentane + cyclohexane	62 ± 4[c]	9.2 ± 0.7[c]	12.4 ± 0.8[c]	1.2 ± 0.2[c]
Mycobacterium sp. strain IFP 2173 + *A. lwoffii* IFP 2149	Cyclohexane	<5[b]	24.9[b]	<0.1[b]	<0.1[b]
	2,2,4-Trimethylpentane + cyclohexane	65 ± 5[c]	8.4 ± 0.9[c]	<0.1[c]	1.2 ± 0.1[c]

[a] The degradation rates were calculated as described in Materials and Methods. The amount of cyclohexane recovered in abiotic flasks was 23.9 ± 1.3 μmol.
[b] The value is the mean based on the values obtained for two test flasks.
[c] The values are the mean ± standard deviation based on the values obtained for three test flasks.

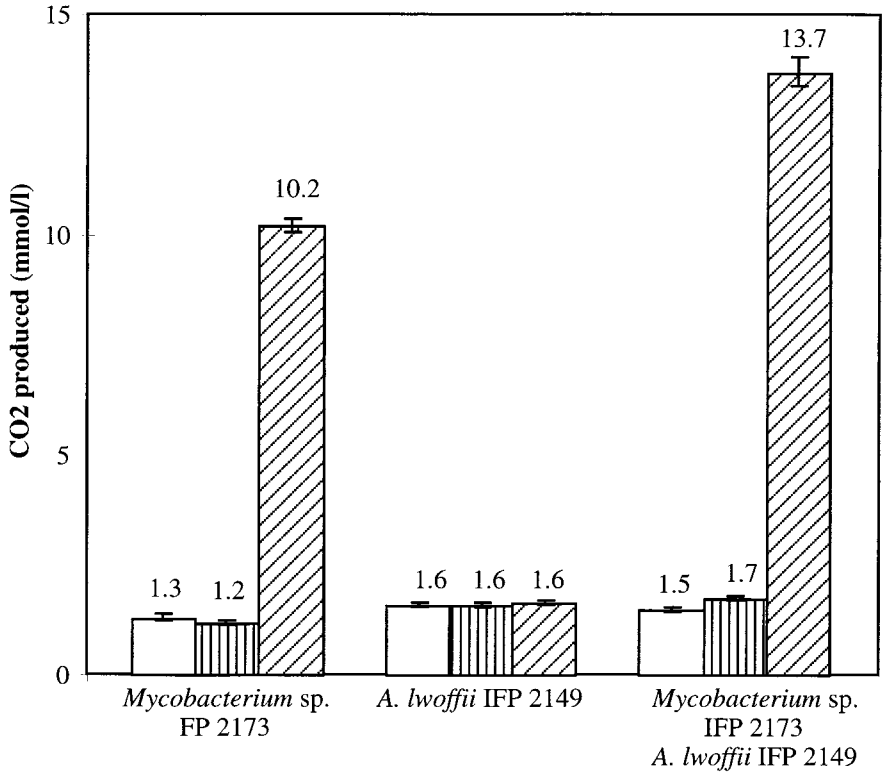

FIG. 4. Production of CO_2 during cyclohexane biodegradation. Open bars, hydrocarbon-free flasks; vertically striped bars, flasks containing cyclohexane; cross-hatched bars, flasks containing cyclohexane and isooctane. The average values (usually based on three replicates) are shown above the bars, and the error bars indicate standard deviations.

found to be biodegraded by strain IFP 2173. Therefore, the extent of degradation obtained with a pure strain was similar to the extent of degradation obtained with a complex microbial population obtained from a soil sample (i.e., 89%) (20).

The efficiency of using two selected strains together for cyclohexane degradation by cometabolism illustrates the extended potential of complex natural microfloræ in the environment for hydrocarbon attenuation. The synergistic relationship in the mixed culture containing strains IFP 2173 and IFP 2149 was clearly shown by the fact that strain IFP 2149 could utilize cyclohexanone produced by *Mycobacterium* sp. strain IFP 2173. Accordingly, the synergistic actions of the two bacterial populations resulted in mineralization of cyclohexane instead of cyclohexanone production.

In summary, *Mycobacterium* sp. strain IFP 2173, which was isolated from gasoline-polluted groundwater, is at present the only strain that has been described which can degrade isooctane, one of the major octane boosters in gasoline. This strain belongs to the rapidly growing *Mycobacterium* species group which has been shown to play an important role in the environment, particularly in xenobiotic compound biodegradation (24).

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