

# Microbial Degradation of Monoterpenes in the Absence of Molecular Oxygen

JENS HARDER\* AND CHRISTINA PROBIAN

*Abteilung Mikrobiologie, Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen, Germany*

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**Anaerobic degradation of natural monoterpenes by microorganisms was evaluated by using *Pseudomonas citronellolis* DSM 50332 and enrichment cultures containing nitrate as an electron acceptor. *P. citronellolis* grew anaerobically on 3,7-dimethyl-1-octanol and citronellol but not on geraniol, nerol, and alicyclic monoterpenes. In contrast, several  $\alpha$ -, mono-, and bicyclic monoterpenes supported microbial growth and denitrification in enrichment cultures. We found that consumption of linalool, menthol, menth-1-ene,  $\alpha$ -phellandrene, limonene, 2-carene,  $\alpha$ -pinene, and fenchone in enrichment cultures depended on the presence of living microorganisms and nitrate. In these experiments, the ratios of number of electrons derived from complete substrate oxidation to number of electrons derived from nitrate reduction ranged from 1.2:1 to 2.9:1. Microbial degradation was accompanied by the formation of small traces of monoterpenes, which were characterized by gas chromatography-mass spectroscopy. The formation of geraniol and geranial from linalool suggested that a 3,1-hydroxyl- $\Delta^1$ - $\Delta^2$ -mutase reaction initiates linalool degradation. Seven strains of motile, oval to rod-shaped, facultatively denitrifying bacteria were isolated on agar bottle plates by using linalool, menthol, menth-1-ene,  $\alpha$ -phellandrene, 2-carene, eucalyptol, and  $\alpha$ -pinene as sole carbon and energy sources.**

Nonmethane hydrocarbons in the atmosphere, which are released from man-made and natural sources, are a major component of the global carbon cycle. A major natural source of these compounds is the terpenes emitted from trees; the estimated emission rate is  $4.8 \times 10^{14}$  g/year (21). A large fraction of the nonmethane hydrocarbons is oxidized abiotically in the atmosphere with nitric oxides and molecular oxygen by light activation (8). Biological degradation of monoterpenes has been studied under aerobic conditions (3, 17, 18). The utilization pathway for aliphatic monoterpene primary alcohols (e.g., citronellol) is independent of molecular oxygen. In contrast, it has been observed that microbial oxidation of monoterpenes with carbon-carbon double bonds as functional group or cyclic structures requires molecular oxygen as a co-substrate for monoterpene transformation into degradable compounds. Therefore, such monoterpenes might be considered recalcitrant in anoxic environments.

In recent years, isolation of anaerobic bacteria by using alkanes (1, 14) and aromatic hydrocarbons (6, 13) as sole carbon and energy sources has revealed that anaerobic biodegradation of many natural low-molecular-weight carbon compounds previously considered recalcitrant is possible. The main objectives of this study were (i) to examine whether a broad range of natural monoterpenes that have recalcitrant structures can serve as carbon and energy sources for microbial growth in the absence of oxygen and (ii) to isolate bacteria by using monoterpenes as sole substrates.

## MATERIALS AND METHODS

**Sources of organisms and chemicals.** Enrichment cultures were inoculated with activated sludge obtained from a local wastewater plant (Lintel, Osterholz-Scharmbeck, Germany) or with a water-mud mixture obtained from a ditch in a mixed forest near Bremen, Germany. The ditch was sampled in December and

contained black mud below fallen autumn leaves; the leaves were mainly beech and larch leaves. *Pseudomonas citronellolis* DSM 50332 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. The chemicals used were analytical grade. Monoterpenes were obtained from Fluka, Neu-Ulm, Germany.

**Media and enrichment conditions.** Anoxic media and cultivation techniques were used in this study (20). The medium contained (per liter of distilled water) 1 g of NaCl, 0.1 g of  $MgCl_2 \cdot 7H_2O$ , 0.04 g of  $CaCl_2$ , 0.5 g of KCl, 0.125 g of  $NH_4Cl$ , 0.2 g of  $Na_2SO_4$ , 0.4 g of  $KH_2PO_4$ , 1.2 g of  $K_2HPO_4$ , and 0.85 g of  $NaNO_3$ . After autoclaving, 2 ml of a nonchelated trace element mixture (20), 2 ml of a selenite-tungstate solution (20), vitamins (1), and 20 ml of a 1 M  $NaHCO_3$  solution were added, and the pH was adjusted to 7.0. To make sure that the process occurred not only in oxygen-free media but also in chemically reduced media, anoxic media used in control experiments were pre-reduced with either 4 mM ascorbate or 1 mM ferrous sulfide. A 0.5 M FeS solution was prepared from  $FeSO_4 \cdot 7H_2O$  and  $Na_2S$  (9). In FeS-reduced media, the reduced state was confirmed by decolorization of resazurin ( $1 \text{ mg liter}^{-1}$ ).

The growth of *P. citronellolis* DSM 50332 under nitrate-reducing conditions was examined in 21-ml culture tubes containing 15 ml of anoxic medium and 20  $\mu$ l of liquid substrate or 20 mg of solid substrate in 1 ml of degassed anoxic 2,2,4,4,6,6,8,8-heptamethylnonane (HMN) as the carrier phase under an  $N_2$ - $CO_2$  (90:10, vol/vol) atmosphere.

Enrichment cultures were prepared by using 2-ml portions of sewage sludge in 0.5-liter bottles that contained 400 ml of medium, 4 ml of HMN, and 200  $\mu$ l of liquid substrate or 200 mg of solid substrate under an  $N_2$ - $CO_2$  (90:10, vol/vol) atmosphere at 28°C in the dark. A 100-ml portion of the water-mud mixture from the ditch was inoculated into each bottle together with 350 ml of medium, 8 ml of HMN, and 400  $\mu$ l or 400 mg of substrate.

The overpressure due to gas formation was measured with a syringe, and 10 mM nitrate was added when the electron acceptor was depleted. The gas formed was analyzed by gas chromatography. Enrichment cultures were maintained and monoterpene consumption was measured in 21-ml glass tubes containing 15 ml of anoxic medium (which contained 10 mM nitrate), 0.5 ml of HMN, and 4 mM monoterpene. The preparation used in the menthone experiment (see Table 1) contained 2 mM menthone. For pasteurization, inoculated enrichment cultures were heated for 1 h at 80°C. Most cultures received a second charge of an electron acceptor (10 mM). Bacterial growth was observed daily and required between 3 days (linalool) and 22 days (fenchone). Our analysis started with a determination of the overpressure and sampling of the water phase for a nitrate-nitrite analysis. Then 4 ml of hexane was added to each culture tube, and a sample was removed from the organic phase and used for gas chromatography and gas chromatography-mass spectrometry analyses.

**Isolation.** Pure cultures were isolated anaerobically on agar bottle plates in which the substrate was provided by diffusion from a reservoir through the gas phase to the agar surface (10, 19). During all manipulations, the bottles containing the agar were flushed with  $N_2$ - $CO_2$  (90:10, vol/vol) by using a Hungate syringe. A 20-ml portion of liquid anoxic medium containing 1.5% washed agar was prepared with chelated trace elements instead of nonchelated trace elements (20) and with 50 mM carbonate instead of the phosphate-carbonate buffer

\* Corresponding author. Mailing address: Abteilung Mikrobiologie, Max-Planck-Institut für Marine Mikrobiologie, Fahrenheitstr. 1, D-28359 Bremen, Germany. Phone: 49-421-2208-126. Fax: 49-421-2208-130. Electronic mail address: jens@postgate.mpi-mm.uni-bremen.de.

TABLE 1. Amounts of monoterpenes consumed by nitrate-reducing enrichment cultures

Substrate	Amt of monoterpene consumed ( $\mu\text{mol}$ )	Amt of nitrate consumed ( $\mu\text{mol}$ )	Bacterial growth <sup>a</sup>	Amt of gas formed (ml)	No. of electrons released from complete substrate oxidation to $\text{CO}_2$ per no. of electrons consumed by nitrate reduction (%)
Linalool	31.7	300	+0.157	3.6	118
<i>R</i> -(+)-Limonene	44.9	256	+0.170	3.5	203
(+)- <i>p</i> -Menth-1-ene	36.9	300	+0.431	3.0	138
Menthone	22.6	150	+0.254	ND <sup>b</sup>	167
<i>R</i> -(-)- $\alpha$ -Phellandrene	43.2	300	+0.419	3.2	161
(+)-2-Carene	47.7	300	+0.165	4.4	178
(+)-Fenchone	40.0	150	+0.173	1.6	288
(-)- $\alpha$ -Pinene	40.3	300	+0.547	3.2	150

<sup>a</sup> Expressed as the change in units of optical density at 578 nm.

<sup>b</sup> ND, not determined.

mentioned above, dispensed into a 100-ml bottle, and solidified quickly by placing the bottle on a plastic bag filled with ice. After inoculation, the agar bottles were inverted, and 75  $\mu\text{l}$  of monoterpene in 3 ml of HMN was added to each bottle. During incubation, the bottles were kept horizontal with the agar aloft. Direct contact between the organic phase and the agar was avoided. The enrichment procedure was monitored by transferring grown colonies (i) onto new anoxic bottle plates, (ii) onto oxic plates containing nutrient agar (which contained [per liter] 5 g of peptone, 3 g of meat extract, and 15 g of agar; pH adjusted to 7.0), (iii) into anoxic medium containing monoterpene, (iv) into anoxic medium containing ascorbate, and (v) into anoxic medium containing monoterpene and ascorbate. The denitrifying bacteria isolated did grow on monoterpene as a sole carbon and energy source in anoxic and chemically reduced media, formed uniform colonies, and were pure as determined microscopically.

**Chemical analyses.** Nitrate and nitrite were separated by high-performance liquid chromatography (HPLC) by using a type A09 micro anion-exchange column (3 by 125 mm; Sykam, Gilching/Munich, Germany) at 65°C and 70 mM NaCl at a flow rate of 1 ml  $\text{min}^{-1}$  as the eluent. The HPLC system included a model S 3200 UV detector (Sykam) and an autosampler (Jasco, Tokyo, Japan). The gas phases of enrichment cultures were analyzed with a Shimadzu model GC-8A gas chromatograph equipped with a thermal conductivity detector. Compounds were separated by using a Poraplot Q column (3 mm by 2 m) at 40°C and  $\text{N}_2$  as the carrier gas at a flow rate of 32 ml  $\text{min}^{-1}$ . Monoterpene contents were determined with a gas chromatograph equipped with flame ionization detectors and connected to a digital data-analyzing system (Perkin Elmer, Überlingen, Germany). Compounds were separated by using a type PVMS 54 column (0.32 mm by 50 m; Perkin Elmer),  $\text{N}_2$  at a flow rate of 1.7 ml  $\text{min}^{-1}$ , and the following temperature programs: injection port temperature, 220°C; column temperature, 100°C for 5 min, increasing to 220°C for 10 min at a rate of 10°C  $\text{min}^{-1}$ ; and flame ionization detector temperature, 250°C. For calculations, we assumed that the monoterpene content in the organic phase after hexane was added represented the total monoterpene content. The amounts of monoterpene dissimilated were calculated from the differences in monoterpene contents between the pasteurized controls and the grown enrichment cultures. A model MAT ITS 40 ion trap system (Finnigan MAT, Bremen, Germany) was used for the gas chromatography-mass spectrometry analysis. Transformation products extracted into the hexane phase were separated on a type NB-54 column (0.32 mm by 25 m; Nordion, Helsinki, Finland) by using a temperature gradient (the injection port and transfer line temperature was 250°C, the initial column temperature was 50°C, and the column temperature was increased to 200°C at a rate of 5°C  $\text{min}^{-1}$ ) and helium as the carrier gas. Mass spectra were obtained in the EI mode

by using 50 to 80 eV, 10- $\mu\text{A}$  emission, and a manifold temperature of 180°C. Compounds were identified by comparing them with injected standards, compounds in the library at the National Institute of Standards and Technology, and compounds in a terpene library.

## RESULTS

**Anaerobic growth of *P. citronellolis* on monoterpenes.** *P. citronellolis* was originally isolated from soil obtained from under pine trees and grew on citronellol by using oxygen or nitrate as an electron acceptor. Nitrate was reduced to nitrite by this organism (15). The original strain could not be kept alive, and another strain was isolated and deposited. The strain that is available now utilizes a range of acyclic monoterpenes under aerobic conditions (4). To evaluate whether *P. citronellolis* can grow on recalcitrant terpenes by using nitrate as an electron acceptor, we determined whether this strain, which is maintained by the Deutsche Sammlung von Mikroorganismen, could grow under anaerobic conditions in the presence of a broad range of a-, mono-, and bicyclic monoterpenes. Citronellol and 3,7-dimethyl-1-octanol were utilized under nitrate-reducing conditions. The organism did not grow on 2,6-dimethyloctane, 3,7-dimethyl-1-octene, citronellene, geraniol, linalool, nerol, phytol, cyclohexanol, (+)-camphoric acid, (1*R*)-(+)-*trans*-isolimonene, (+)-isomenthol, isophronone, (-)-isopulegol, *R*-(+)-limonene, (+)-*p*-menth-1-ene, menthol, *R*-(-)- $\alpha$ -phellandrene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, (-)-borneol, (+)-2-carene, (+)-3-carene, eucalyptol, (+)-fenchol, (+)-fenchone, (+)-isopinocampheol, (+)-*trans*-myrtanol, (1*S*)-(-)-*trans*-pinane, (-)- $\alpha$ -pinene,  $\alpha$ + $\beta$ -thujone, squalene, or  $\beta$ -carotene as a sole carbon and energy source.

**Denitrifying enrichment cultures growing on monoterpenes.** We used two strategies to obtain anoxic enrichment cultures

TABLE 2. Monoterpenes formed by enrichment cultures in the presence and in the absence of nitrate<sup>a</sup>

Substrate	Monoterpenes formed	
	In the presence of nitrate	In the absence of nitrate
Linalool	Geraniol, geraniol	Geraniol
<i>R</i> -(+)-Limonene	$\alpha$ -Terpinene	2-Carene
<i>R</i> -(-)- $\alpha$ -Phellandrene	$\alpha$ -Terpinene, $\gamma$ -terpinene, $\beta$ -phellandrene	
(+)-2-Carene	$\alpha$ -Terpinene, cymene, limonene	
(+)-3-Carene	Cymene, limonene, fenchone	Fenchone
Eucalyptol	$\alpha$ -Terpinene	
(+)- <i>p</i> -Menth-1-ene	None	
(-)- $\alpha$ -Pinene	$\alpha$ -Terpinene, cymene, limonene, eucalyptol	

<sup>a</sup> The compounds were identified by gas chromatography-mass spectrometry.

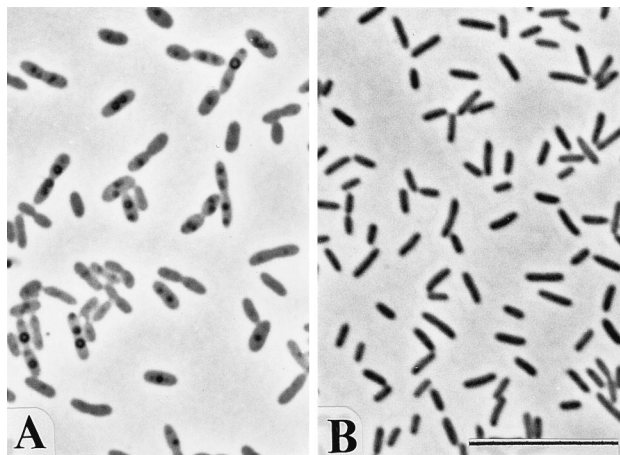


FIG. 1. Phase-contrast photomicrographs of newly isolated strains 21Mol (A) and 51Men (B) grown on menthol and menth-1-ene, respectively. Bar = 10  $\mu$ m.

growing on monoterpenes under nitrate-reducing conditions. Activated sludge contains a high number of bacteria per volume. Therefore, we used a small inoculum to reduce the amount of endogenous electron donors. Microbial growth on (-)- $\beta$ -citronellol, 3,7-dimethyl-1-octanol, geraniol, linalool, nerol, (+)-*p*-menth-1-ene, (-)-menthol, menthone, (-)- $\alpha$ -pinene, and  $\alpha$ + $\beta$ -thujone was observed within 10 days to 3 weeks. Longer incubation times were required for cultures containing (-)- $\beta$ -citronellene and (-)- $\beta$ -fenchone (6 and 12 weeks, respectively). The compounds 2,6-dimethyloctane, 2,6-dimethyloctene, (+)-campher, (+)-camphoric acid, (-)-borneol, and (+)-fenchol were not utilized as sole carbon and

energy source within 4 months, as determined visually and by overpressure measurements. In enrichment cultures containing water and mud obtained from a forest ditch, a large mud inoculum was used. Initially, the denitrification rates were similar in the enrichment cultures and a control culture containing no added monoterpene. Then 10 mM nitrate was added after 1 week. After 2 weeks, the denitrification rate in the control culture decreased slowly, but the denitrification rates in the presence of (1*R*)-(+)-*trans*-isolimonene, *R*-(+)-limonene, (+)-*p*-menth-1-ene, *R*-(-)- $\alpha$ -phellandrene, (+)-2-carene, (+)-3-carene, eucalyptol, (-)- $\alpha$ -pinene, and  $\alpha$ + $\beta$ -thujone increased. Denitrification was stimulated by the presence of (+)-*trans*-myrtanol after 8 weeks and by the presence of (+)-isomenthol after 12 weeks. The enrichment cultures prepared with samples obtained from a ditch in a mixed forest did not utilize (+)-campher, (+)-camphoric acid, (-)-isopulegol, (-)-borneol, (+)-fenchol, (+)-fenchone, (+)-isopinocampheol, and (1*S*)-(-)-*trans*-pinane within 6 months. A gas analysis of enrichment cultures revealed that traces of nitrogen dioxide were present, but no methane was formed. In subsequent passages, microbial growth and denitrification in enrichment cultures were also observed in media that were reduced chemically with ferrous sulfide, which resulted in anaerobic reactions.

The disappearance of monoterpenes in mud-free enrichment cultures was quantified (Table 1). Pasteurized samples and inoculated preparations without nitrate or monoterpene were used as controls (data not shown). A gas chromatography analysis of standards and hexane extracts obtained from inoculated cultures that were pasteurized prior to inoculation revealed that monoterpenes were chemically stable under the enrichment culture conditions used. Bacterial growth was limited in the absence of either nitrate or monoterpenes; the increase in optical density at 578 nm was less than 0.020 U. The increase in the optical density of the culture containing men-

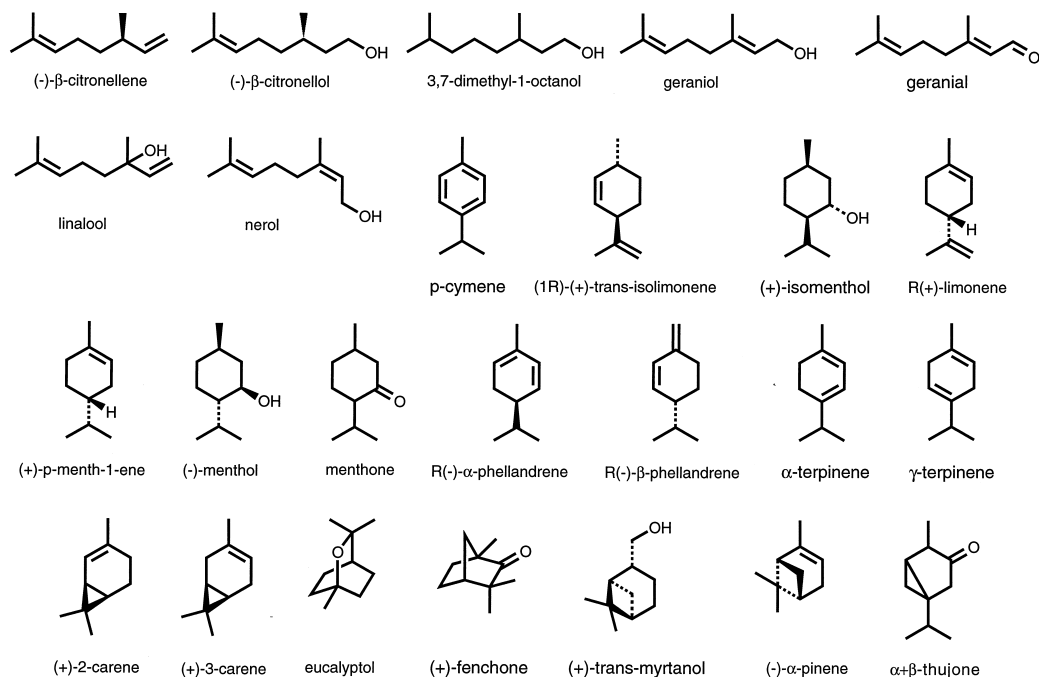


FIG. 2. Monoterpene structures.

there but no nitrate was 0.077 U. In contrast, the optical densities of complete preparations increased by between 0.157 and 0.547 U. In these cultures, 53 to 80% of the monoterpenes disappeared (100% was equivalent to 60  $\mu$ mol, except in the menthone experiment, in which 100% was equivalent to 30  $\mu$ mol). In the absence of nitrate, the decrease in the amount of monoterpene was less than 8%. Nitrate was completely consumed in all of the complete preparations except the limonene-containing cultures. Nitrite was found only in inoculated controls that did not contain an organic substrate. The formation of up to 0.75 mM nitrite coincided with the disappearance of up to 1.1 mM nitrate. The amounts of gas formed by growing cultures were 1.6 and between 3.0 and 4.4 ml in the presence of 10 and 20 mM nitrate, respectively. In control experiments, between 0.0 and 0.8 ml of gas was formed, except in the preparation without limonene (in which 1.4 ml of gas was formed). The ratios of number of electrons derived from complete substrate oxidation to numbers of electrons derived from nitrate reduction ranged from 1.2:1 to 2.9:1 for the enrichment cultures, values which are similar to the degradation balances observed with denitrifying isolates degrading aromatic compounds (2, 5). A monoterpene gas chromatography analysis revealed that additional substances were present in hexane extracts of living enrichment cultures. Traces of biological transformation products were reproducibly found at the end of incubation, and a gas chromatography-mass spectroscopy analysis revealed that the compounds were monoterpenes (Table 2). The concentrations found ranged from 4 to 20  $\mu$ M.

#### Isolation of monoterpene-utilizing denitrifying bacteria.

Anaerobic bacteria are usually isolated by using repeated agar dilution series (20). The growth of isolated colonies in an agar dilution series that was overlaid with menthol in HMN was extremely slow (it took 3 months). Therefore, we determined whether the microorganisms grew better on agar than in agar by using anoxic bottle plates (10, 19). This approach yielded single colonies after 2 to 3 weeks of incubation. We tried to isolate denitrifying bacteria with linalool, menthol, menthene,  $\alpha$ -phellandrene, 2-carene, eucalyptol, fenchone, and  $\alpha$ -pinene as sole carbon and energy source. Five passages in agar bottles were required to obtain pure cultures with menthene,  $\alpha$ -phellandrene, 2-carene, eucalyptol, and  $\alpha$ -pinene. When menthol and linalool were used, the bacteria isolated from the third agar bottle plate passages were not able to grow anaerobically on monoterpenes in liquid cultures. Therefore, isolation was completed by using one anoxic liquid dilution series (menthol) or two anoxic liquid dilution series (linalool) with colonies grown in the second agar bottle plate passage. Bacteria that utilize fenchone as a sole carbon and energy source were not isolated. Several attempts in which anoxic bottle plates, anoxic liquid dilution series, or nutrient agar plates and anoxic selective media were used failed. After 18 months of cultivation and several passages, the enrichment culture did not continue to grow and was lost. All of the strains isolated are motile, facultatively denitrifying bacteria, and the cells are oval to rod shaped (Fig. 1).

## DISCUSSION

In this study we found that in addition to acyclic monoterpenes mono- and bicyclic monoterpenes were oxidized anaerobically by microorganisms. Figure 2 shows the chemical structures of the monoterpenes which supported microbial growth or were detected by gas chromatography-mass spectrometry as biological transformation products. Degradation of acyclic terpenes can occur by the pathway described previously for *P. citronellolis* (15, 18). In the case of linalool, the formation of

geraniol and the formation of geranial, which is formed only in the presence of nitrate, suggest that linalool degradation is initiated by rearrangement of geraniol and then continues by oxidation on the pathway mentioned above. The enzyme required for this rearrangement should be a 3,1-hydroxyl- $\Delta^1$ - $\Delta^2$ -mutase which has not been described previously. The known biochemistry of the degradation pathways suggests that the cleavage of a ring carbon-carbon bond is required for complete oxidation of cyclic monoterpenes. Aerobic ring cleavages are catalyzed by insertion of an oxygen atom through monooxygenases and subsequent ester hydrolyses (17). Several pathways by which carbon ring cleavage occurs under anoxic conditions are known. Many aromatic substances are transformed into benzoate, and after ring reduction a  $\beta$ -oxo-carboxyl-coenzyme A ester is formed; this results in  $\beta$ -cleavage analogous to the  $\beta$ -oxidation of fatty acids (12). Cyclohexanol and aromatic di- and polyhydroxybenzenes are modified into  $\beta$ -diketones (5, 11). This structure also allows  $\beta$ -cleavage to occur. Degradation of monocyclic monoterpenes containing a hydroxyl group (e.g., menthol) may occur by a similar pathway, assuming that the isopropyl side chain is not an impediment. Anaerobic utilization of compounds containing nonactivated carbon-carbon double bonds (menthene, phellandrene) has been observed previously when hexadecene was used as a substrate for nitrate- and sulfate-reducing bacteria (1, 7). Degradation may be initiated by adding water to the double bond, and the alcohol formed may be channelled into a  $\beta$ -cleavage pathway. Rearrangements of the carbon skeleton are typical of the chemistry of bicyclic terpenes. However, the reactions require light or heat activation. Cultures were inoculated in the dark at mesophilic temperatures, and the substrates were chemically stable under these conditions. Therefore, the possibility that there was an abiotic reaction that preceded biological utilization can be eliminated. The transformation products observed suggest that oxidation of bicyclic monoterpenes may occur via monocyclic intermediates. Enzymatic catalysis may result in cleavage of the strained ring structures via cationic or radical intermediates, similar to the enzymatic catalysis of cyclopropane derivatives (16). The isolation of motile, oval to rod-shaped, denitrifying bacteria by using monoterpenes as single carbon and energy sources should allow us to study the degradation pathways. In addition to the scientific interest created by new insights into biochemical transformations, our results may have practical applications. The ability of anaerobic microorganisms to degrade monoterpenes could be used for anaerobic treatment and purification of intensely acrid wastewaters in the food industry, especially the flavor industry, which could reduce the release of nonmethane hydrocarbons into the atmosphere.

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