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Aerobic and Anaerobic Metabolism of 6,10,14-Trimethylpentadecan-2-one by a Denitrifying Bacterium Isolated from Marine Sediments

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This report describes the metabolism of 6,10,14-trimethylpentadecan-2-one by a denitrifying bacterium (*Marinobacter* sp. strain CAB) isolated from marine sediments. Under aerobic and denitrifying conditions, this strain efficiently degraded this ubiquitous isoprenoid ketone. Several bacterial metabolites, 4,8,12-trimethyltridecan-1-ol, 4,8,12-trimethyltridecanal, 4,8,12-trimethyltridecanoic acid, *Z*-3,7-dimethylocten-2-oic acid, *Z*-3,7,11-trimethyldodecen-2-oic acid, and 6,10,14-trimethylpentadecan-2-ol, were formally identified, and different pathways were proposed to explain the formation of such isoprenoid compounds.

Isoprenoid compounds are well suited as biological markers since they are often abundant and are widely distributed in the geosphere. Also, the relatively stable isoprane skeletal unit is readily identified and allows these compounds to be used as tracers over long periods of geological time (45).

There are numerous reports of 6,10,14-trimethylpentadecan-2-one (compound 1) in sediment (11, 22, 39) and water column particulate matter (44) samples. In the water column, this isoprenoid ketone can be produced in several ways (Fig. 1): (i) from free phytol, by photosensitized oxidation (31) and aerobic bacterial degradation (5, 6); (ii) by photosensitized oxidation of some isoprenoid alkanes such as pristane (30) and phytane (32); and (iii) by hydrolysis of chlorophyll photoproducts (33). Moreover, in sediments, early diagenesis of intact (11, 12) and photooxidized (35) chlorophyll phytyl chain constitutes a nonnegligible source of this compound.

Since the formation of 6,10,14-trimethylpentadecan-2-one in the marine environment involves essentially oxidative pathways, this isoprenoid compound has been proposed as an indicator of oxic conditions during sedimentation (22).

Results recently obtained during a study of early diagenesis of intact and photooxidized chlorophyll phytyl chain in a recent temperate sediment (36) strongly suggested that biotic processes act intensively on this isoprenoid ketone under aerobic and anoxic conditions and play a key role in its short-term fate in aquatic sediments. Unfortunately, in the literature, there are relatively few studies dealing with the microbial degradation of acyclic isoprenoid compounds other than phytol, pristane, and phytane (45), and these works essentially concern aerobic microorganisms.

The purpose of this study was to describe the aerobic and anaerobic metabolism of 6,10,14-trimethylpentadecan-2-one by a denitrifying bacterium isolated from marine sediments.

MATERIALS AND METHODS

Procedure for isolation of the strain. The strain was isolated from hydrocarbon-polluted marine coastal sediments collected near a petroleum refinery (Lavera, Gulf of Fos, France). Portions (10 ml) of the sediments were used to inoculate 100-ml portions of an enrichment medium consisting of artificial seawater (ASW) (2) supplemented with KNO_3 (20 mM) as the electron acceptor and 6,10,14-trimethylpentadecan-2-one (3 mM) as the sole carbon and energy source (PN medium). Anaerobic enrichment cultures were incubated in 150-ml serum flasks sealed with a black rubber stopper. Anaerobic conditions were obtained by flushing nitrogen through the flask for 1 h. After an incubation of 12 days at 30°C, the culture was subcultured on solid TBA medium (1 g of KNO_3 per liter, 15 g of Trypticase Soja [Mérieux] per liter, 15 g of agar [Difco] per liter, and 5 g of Biosoyase [Mérieux] per liter in ASW). The plates were incubated anaerobically in an atmosphere of H_2-CO_2 for 15 days at 30°C. Colonies were picked off and replated on the same medium to check purity. Each pure isolate was tested for anaerobic growth on 6,10,14-trimethylpentadecan-2-one as sole carbon source.

Phenotypic analysis of the strain. The type strain of *Marinobacter hydrocarbonoclasticus* (ATCC49840) was used for comparison of taxonomic characteristics (16). Biolog GN microplates (Biolog TNC, Hayward, Calif.) were used to investigate the carbon source pattern of the strains. Routine tests (Gram staining, oxidase, catalase, gelatinase, DNase, esculinase, tweenase, amylase, agarase, and urease activities) were done as previously described by Smibert and Krieg (40). Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activities were determined by the techniques of Stolp and Gadkari (41). Sodium chloride and temperature tolerance were tested aerobically in ASW medium supplemented with lactate (2 g per liter) and containing appropriate concentrations of NaCl (0 to 3.5 M). Growth rates were determined over a temperature range from 10 to 45°C in the presence of 0.2 M NaCl. Detailed cell shapes and flagella were examined by transmission electron microscopy. The cells were negatively stained with uranyl acetate (1%) and observed with a Zeiss EM 912 transmission electron microscope at 100 kV.

PCR amplification of the 16S RNA gene. PCR amplification of the 16S RNA gene of the strain was performed with primers EU3 (5′ AAG-GAG-GTG-ATC-CAG-CC 3′) and EU5 (5′ AGA-GTT-TGA-TKM-TGG-CTC-AG 3′ [where K is G or T and M is A or C]), which are known to amplify most eubacterial 16S RNA genes (46), under PCR conditions specified by the *Taq* polymerase manufacturer (Boehringer). The single DNA band of approximately 1.5 kb as detected by agarose gel electrophoresis was purified with an agarose gel DNA extraction kit (Boehringer). Its nucleotide sequence was determined directly from the PCR amplification products on an Applied Biosystems sequencer (model 373A).

Phylogenetic analysis and alignment. The phylogenetic data described below were obtained by using regions through all the rRNA sequences that were definitely aligned and did not include gaps. Only full-length ribosomal DNA (rDNA) genes were included in this study. Sequences were aligned with the sunMASE program (15). The number of substitutions was calculated with the DIFFCOUNT program (19), and the neighbor-joining algorithm was used for phylogenetic analysis (37). A bootstrap analysis with 1,000 replications was performed to check the robustness of the tree. Finally, the tree was plotted with a Macintosh computer and a program (njplot) (27) that allows the transformation of a formal tree representation (Newick's format) into a MacDraw drawing.

Growth conditions. The basic growth medium consisted of ASW supplemented with iron sulfate (0.1 mM), potassium phosphate (0.33 mM), and sodium bicarbonate (20 mM), with 6,10,14-trimethylpentadecan-2-one (3 mM) as the carbon source (ϕ medium). Except where otherwise indicated, cultures were grown in ϕ medium and incubated at 30°C. Aerobic growth experiments (50-ml cultures) were performed in 250-ml Erlenmeyer flasks agitated on a reciprocal shaker at 96 rpm. Anaerobic growth experiments were performed in 125-ml serum flasks containing 70 ml of ϕ medium supplemented with KNO_3 (20 mM). Anaerobiosis was obtained as described above, and cultures were magnetically stirred. For each experiment, two serum flasks were inoculated: the first for

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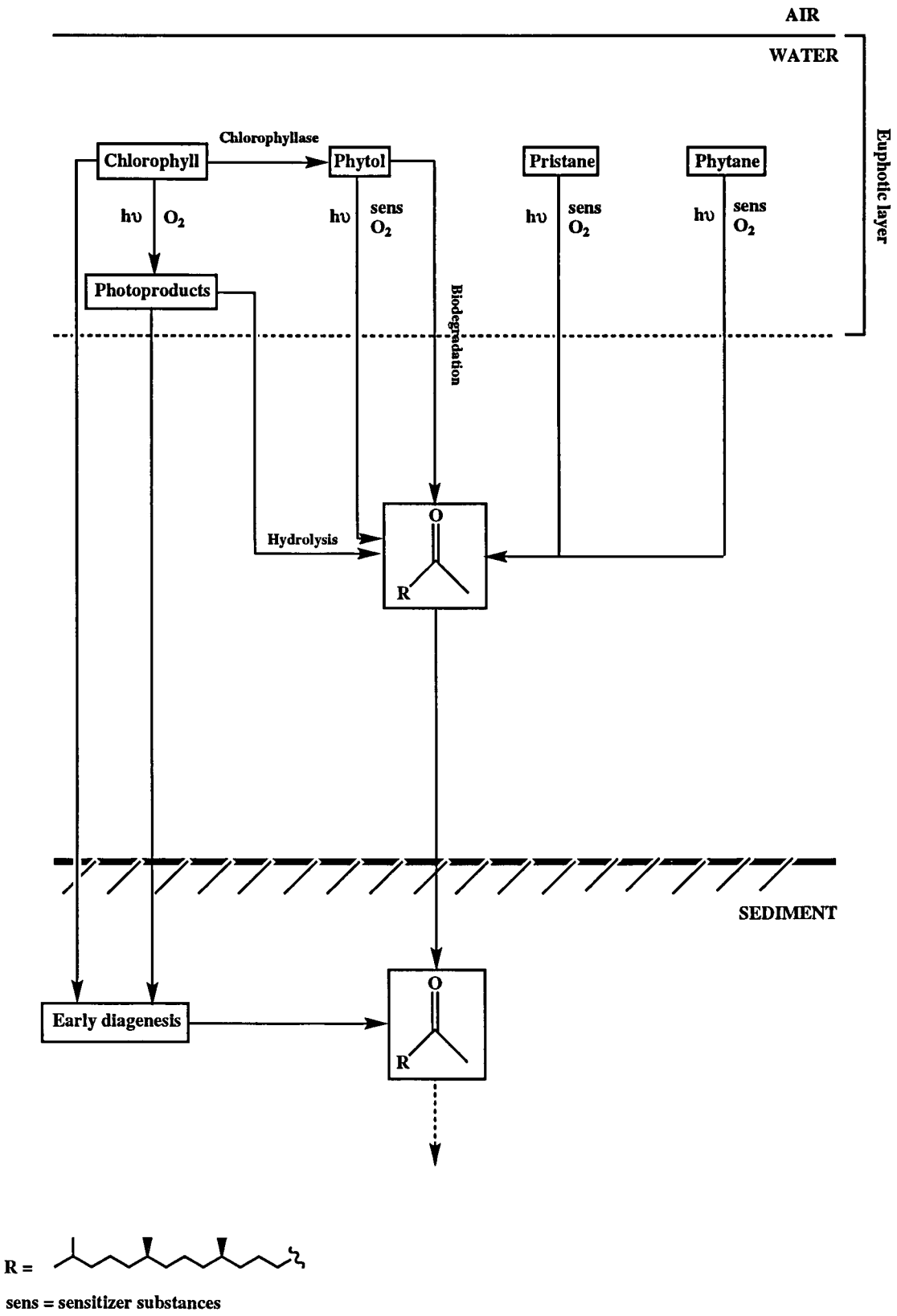


FIG. 1. Production of 6,10,14-trimethylpentadecan-2-one in the marine environment.

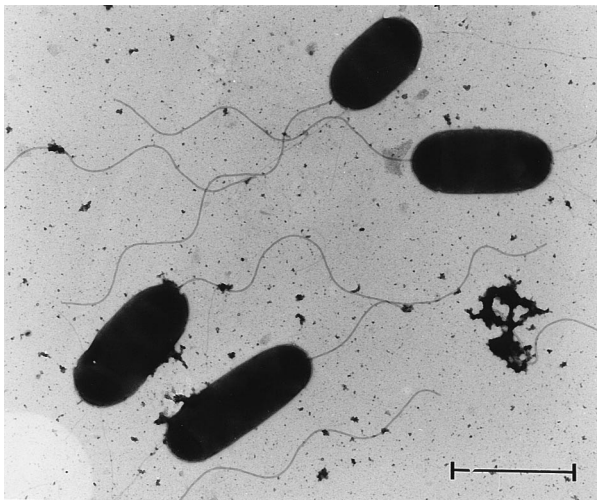


FIG. 2. Transmission (negative-staining) electron micrograph of *Marinobacter* sp. strain CAB grown on ASW medium containing yeast extract (5 g per liter), Bacto Peptone (Difco; 5 g per liter), and sodium chloride (0.2 M). The cells were stained at the mid-exponential phase of growth. Bar, 1.5 μm.

monitoring of growth and nitrate reduction and the second for estimation of substrate (6,10,14-trimethylpentadecan-2-one) degradation and identification of metabolites. Sterile control experiments were carried out in parallel. Growth was monitored by measuring the optical density at 450 nm with a Shimadzu UV 240 spectrophotometer.

Analytical assays. Assays of NO_3^- and NO_2^- concentrations were performed by the procedures of Tréguer and Le Corre (43). The detection limits of NO_3^- and NO_2^- were 0.5 and 0.1 μM, respectively. Denitrification was determined as N_2O accumulation during anaerobic growth with NO_3^-. The last step of denitrification (N_2O reduction) was blocked with acetylene (0.1 atm) (1). N_2O accumulation was determined with a Girdel series 30 gas chromatograph equipped with an electron capture detector (3). N_2O from the liquid phase was extracted by the procedure of Chan and Knowles (9) modified by the multiple-equilibrium technique (25).

Isolation and characterization of bacterial metabolites. At the end of the growth period, the contents of the flask were extracted by performing continuous extraction with chloroform for 24 h; this resulted in a neutral extract, E_1. After acidification to pH 1 with hydrochloric acid, the aqueous layer was extracted with chloroform for 24 h to give an acidic extract, E_2. Extracts E_1 and E_2 were dried with anhydrous Na_2SO_4, filtered, and concentrated by means of rotary evaporation. After evaporation of the solvent, the extracts were taken up in 250 μl of a mixture of pyridine and *N*,*O*-bistrimethylsilyl-trifluoroacetamide (BSTFA) (Supelco) and allowed to silylate at 40°C for 1 h. Following evaporation to dryness under nitrogen, the residue was taken up in hexane and analyzed by gas chromatography-electron impact mass spectrometry.

Bacterial metabolites were identified by comparison of their retention times and mass spectra with those of synthesized reference compounds. Gas chromatography-electron impact mass spectrometry analyses were carried out with an HP 5890 series II plus gas chromatograph connected to an HP 5972 mass spectrometer (Hewlett-Packard). The following operating conditions were used: 15-m by 0.22-mm (inner diameter) capillary column coated with BPX35 (SGE); oven temperature programmed from 60 to 150°C at 30°C · min^{-1} and then from 150 to 320°C at 3°C · min^{-1}; carrier gas pressure (He), 0.48 bar; injector temperature, 320°C; electron energy, 70 eV; source temperature, 170°C.

Chemicals. 6,10,14-Trimethylpentadecan-2-one was produced by oxidation of phytol (Riedel de Haën) with $KMnO_4$ in acetone (8). Reduction of this ketone with lithium aluminum hydride gave 6,10,14-trimethylpentadecan-2-ol. 4,8,12-Trimethyltridecanoic acid was obtained from isophytol (Interchim) by a previously described procedure (33). Reduction of 4,8,12-trimethyltridecanoic acid with lithium aluminum hydride in anhydrous diethyl ether gave 4,8,12-trimethyltridecan-1-ol. 4,8,12-Trimethyltridecanal was produced by oxidation of the corresponding alcohol with CrO_3-pyridine in dry CH_2Cl_2 (17). The synthesis of *Z*- and *E*-3,7,11-trimethyldodecen-2-oic acids from geranylacetone (Aldrich) required four steps: (i) hydrogenation of geranylacetone in CH_3OH with a Pd-$CaCO_3$ catalyst, (ii) sonically accelerated condensation of the foregoing 6,10-dimethylundecan-2-one with α-bromoethyl acetate in the presence of zinc and iodine (Reformatsky reaction) (21), (iii) dehydration of the resulting β-hydroxy ester in benzene catalyzed by *para*-toluene sulfonic acid, and (iv) saponification of the produced unsaturated esters (5% KOH in 50% CH_3OH). Further purification of the crude acids by column chromatography on silica gel afforded a mixture of *Z*- and *E*-3,7,11-trimethyldodecen-2-oic acids which were characterized by ^1H and ^{13}C nuclear magnetic resonance. *Z*- and *E*-3,7-Dimethylocten-2-oic acids were similarly produced in four steps from 6-methyl-5-hepten-2-one (Aldrich).

Nucleotide sequence accession number. Nucleotide sequence data have been deposited with GenBank under the accession number U61848.

RESULTS AND DISCUSSION

Morphological, physiological, and biochemical characteristics. The new isolate was examined during the exponential phase of growth in stock culture (0.2 M NaCl). The cells appeared to be regular, gram-negative motile rods. Negative staining, showed that they were 1.5 to 1.8 μm long and 0.5 μm in diameter with a single polar flagellum (Fig. 2). The isolate was able to grow between 10 and 45°C; the optimal temperature for growth (30°C) was approximately the same as that reported for *M. hydrocarbonoclasticus* (16). Both strains (our isolate and *M. hydrocarbonoclasticus*) exhibited positive responses in oxidase, catalase, and tweenase tests. For both strains, the arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, gelatinase, starch hydrolysis, agarase, amylase, urease, esculinase, and DNase tests were negative. The strains appeared to be nonfermentative but denitrifying. They did not require growth factors. The ability of the isolate to use different organic compounds as the sole source of carbon and energy was studied. Of the 29 carbohydrates tested, any substrate could be used as a source of carbon and energy. Only 4 (β-hydroxybutyric acid, α-ketobutyric acid, DL-lactic acid, and succinic acid) of the 27 organic acids tested and 3 (Glu, Leu, and Pro) of the 20 amino acids tested permitted growth. In contrast to *M. hydrocarbonoclasticus*, the isolate was able to grow in the absence of NaCl. The optimal sodium chloride concentration was around 0.6 M, and no growth was

TABLE 1. rDNA sequence relatedness[a]

Species	% Difference between sequences of:						
	Marinobacter sp. strain CAB	*E. coli*	*H. denitrificans*	*H. variabilis*	*M. hydrocarbonoclasticus*	*O. linum*	*P. aeruginosa*
Marinobacter sp. strain CAB							
Escherichia coli	14.3						
Halomonas denitrificans	10.6	14.5					
Halovibrio variabilis	10.8	14.5	7.8				
Marinobacter hydrocarbonoclasticus	0.6	14.7	10.9	11.1			
Oceanospirillum linum	8.7	13.7	9.3	10.4	9.1		
Pseudomonas aeruginosa	9.7	13.8	11.6	12.0	9.8	10.8	
Pseudomonas mendocina	8.9	13.6	10.6	11.5	9.5	10.3	2.9

[a] Relatedness of rDNA sequences for the bacterial species shown in Fig. 3.

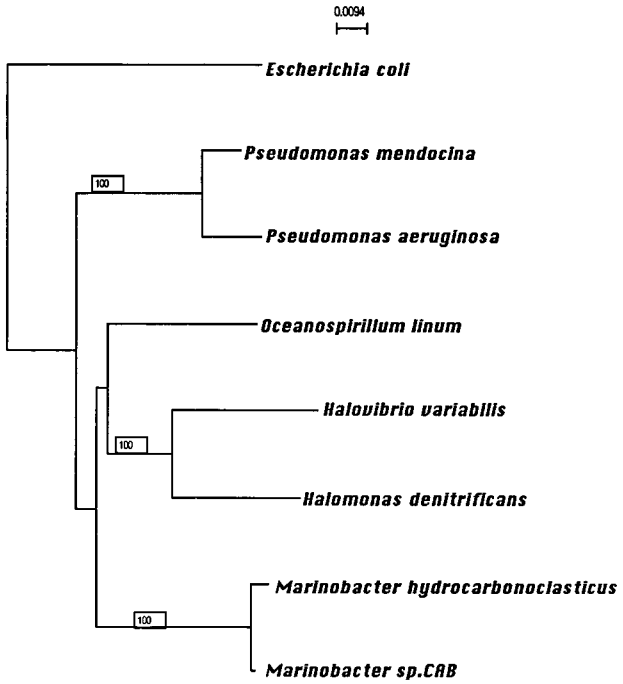


FIG. 3. Unrooted phylogenic tree obtained by distance matrix analysis by the neighbor-joining method (37). The bar represents the number of substitutions per site. Boxed numbers are the results of the bootstrap analysis.

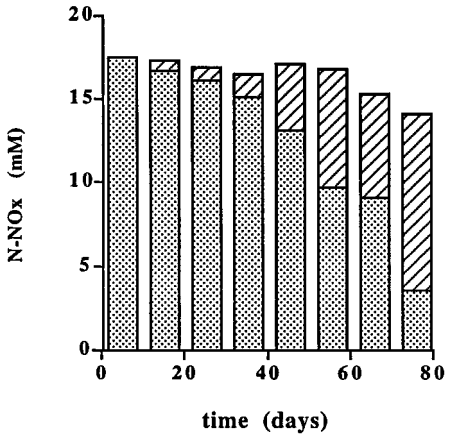


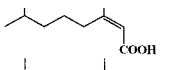
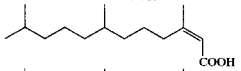
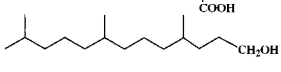
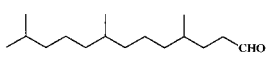
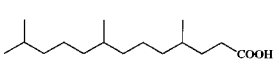
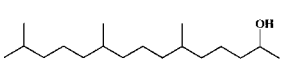
FIG. 4. Kinetics of nitrogen oxide consumption of *Marinobacter* sp. strain CAB on 6,10,14-trimethylpentadecan-2-one in the presence of nitrate. Symbols: ▨, NO_3^- (mM); ▨, N_2O (mM) (calculated as NO_3^- equivalents).

observed for NaCl concentrations higher than 2.5 M, whereas *M. hydrocarbonoclasticus* continued to grow up to 3.5 M.

Small-subunit rRNA sequence and phylogenetic analyses. The rDNA sequence of the isolate was compared to sequences available from public databases. The closest strain appeared to be an *M. hydrocarbonoclasticus* strain (ATCC 49840) that showed 99.2% similarity to the rDNA sequence of the isolate (Table 1; Fig. 3). Despite their genetic similarities, the two clearly showed differences at the phenotypic level mainly regarding their response to Na^+ concentration. In contrast to the isolate, Na^+ is absolutely necessary for growth of *M. hydrocarbonoclasticus*, which does not possess a flagellum when grown in medium with 0.2 M NaCl and is still able to grow in medium with 3.5 M NaCl. The genetic results seem to indicate that the isolate could be classified in the genus *Marinobacter*, and the phenotypic results show that the two strains are not identical. For this study the isolate was named *Marinobacter* sp. strain CAB.

Growth on 6,10,14-trimethylpentadecan-2-one. The isolate was able to grow on 6,10,14-trimethylpentadecan-2-one as the sole carbon and energy source under aerobic and anaerobic conditions in the presence of nitrate, with doubling times of 18 and 72 h, respectively. In anaerobiosis, the kinetics of nitrogen oxide production shown in Fig. 4 indicates that nitrate was consumed without a transient accumulation of nitrite. The production of N_2O was observed from the beginning of growth and increased throughout growth up to a final level of 5.25 mM. At the end of the culture, nitrate was stoichiometrically converted to N_2O in the presence of acetylene, indicating that a denitrification process had occurred. About 90% substrate degradation had occurred after 10 days of growth under aerobic conditions, and about 75% degradation had occurred after 2 months of growth under denitrifying conditions. Unexpectedly, in the presence of nitrate as the electron acceptor, the theoretical stoichiometry of 6,10,14-trimethylpentadecan-2-one oxidation and nitrate loss to N_2O was not experimentally

TABLE 2. Metabolites detected during growth of *Marinobacter* sp. strain CAB on 6,10,14-trimethylpentadecan-2-one

Compound	Compound no.	Detection of compound during:			
		Anaerobic growth (2 mo)	Anaerobic control (2 mo)	Aerobic growth (15 days)	Aerobic control (15 days)
	2	+	−	+	−
	3	+	−	+	−
	4	−	−	+	−
	5	−	−	+	−
	6	−	−	+	−
	7	+	−	−	−

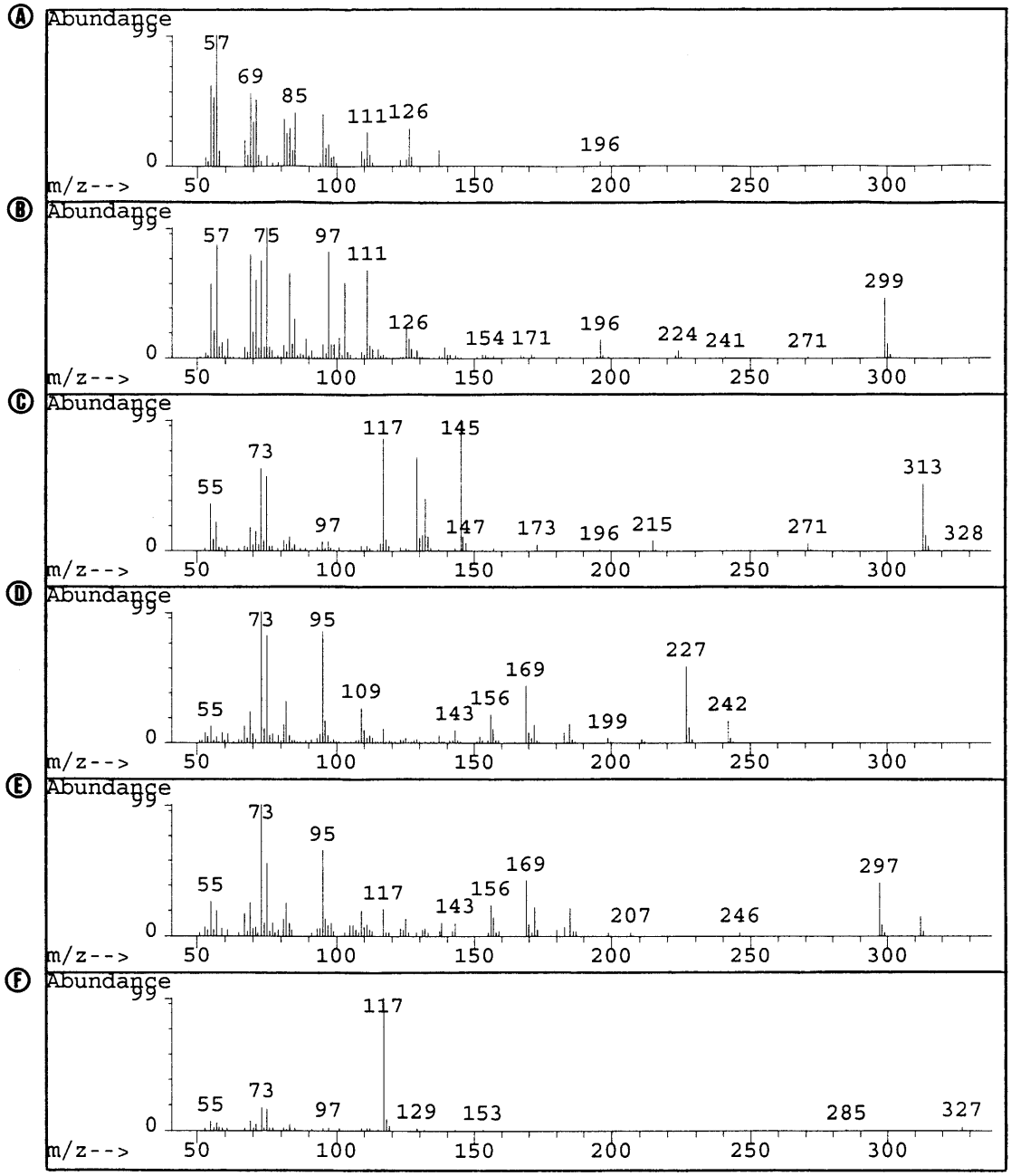


FIG. 5. Electron impact mass spectra of 4,8,12-trimethyltridecanal (A), 4,8,12-trimethyltridecan-1-ol (silylated) (B), 4,8,12-trimethyltridecanoic acid (silylated) (C), *Z*-3,7-dimethylocten-2-oic acid (silylated) (D), *Z*-3,7,11-trimethyldodecen-2-oic acid (silylated) (E), and 6,10,14-trimethylpentadecan-2-ol (silylated) (F).

obtained. Thus, we cannot assess the complete mineralization coupled to denitrification.

Metabolism of 6,10,14-trimethylpentadecan-2-one by *Marinobacter* sp. strain CAB. We detected *Z*-3,7-dimethylocten-2-oic acid (compound 2), *Z*-3,7,11-trimethyldodecen-2-oic acid (compound 3), 4,8,12-trimethyltridecan-1-ol (compound 4), 4,8,12-trimethyltridecanal (compound 5), and 4,8,12-trimethyltridecanoic acid (compound 6) during aerobic growth (Table 2), whereas only compounds 2 and 3 and 6,10,14-trimethylpentadecan-2-ol (compound 7) were present under anaerobic conditions (Table 2). These different isoprenoids were formally identified by comparison of their retention times and mass spectra (Fig. 5) with those of synthesized reference compounds.

The production of compounds 4 to 6 during aerobic growth can be attributed to an oxidation sequence involving the transformation of ketone 1 to 4,8,12-trimethyltridecan-1-ol acetate. Subsequent hydrolysis of this ester affords alcohol 4, which can be metabolized (after oxidation to the corresponding acid 6) via a classical β-oxidation sequence (Fig. 6, pathway II). Such enzymatic oxidation of ketones to esters, analogous to the Baeyer-Villiger oxidation with peracids (4), was previously observed for numerous microorganisms (13, 26, 34).

To explain the production of acids 2 and 3, we propose a second pathway involving initial oxidation of the keto terminal methyl group (18). The α-keto acid thus formed can easily be decarboxylated to 5,9,13-trimethyltetradecanoic acid (com-

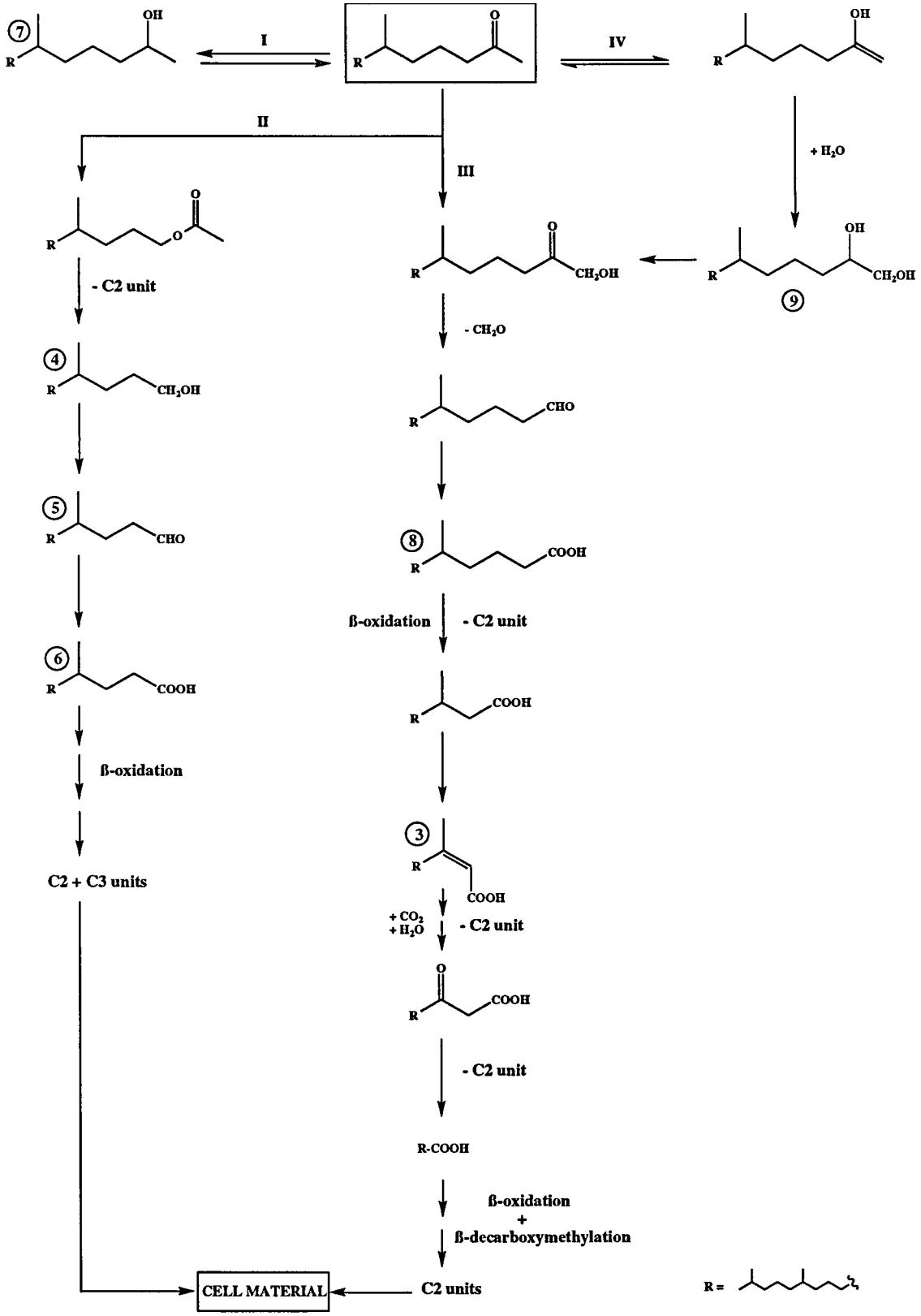


FIG. 6. Proposed pathways for the metabolism of 6,10,14-trimethylpentadecan-2-one by *Marinobacter* sp. strain CAB.

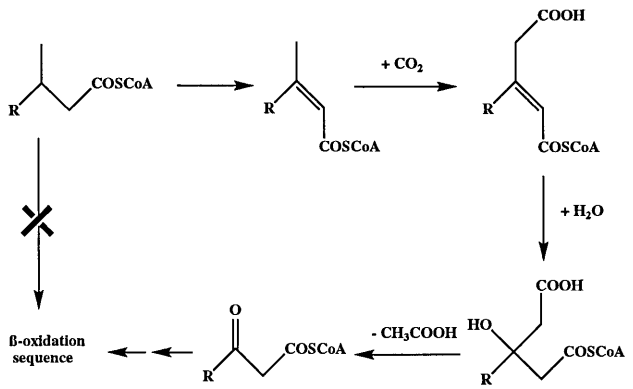


FIG. 7. β-Decarboxymethylation sequence.

pound 8), which is subsequently metabolized via alternative β-oxidation and β-decarboxymethylation reactions (Fig. 6, pathway III). The ability of microorganisms to carry out β-decarboxymethylation was originally established by Seubert (38) during a study of citronellol metabolism by *Pseudomonas citronellolis*. The net effect of this process is to replace a β-methyl substituent (which prevents β-oxidation) with a carbonyl oxygen (Fig. 7) (7, 14), generating a suitable substrate for this classical oxidation sequence. The involvement of such a mechanism is supported by the detection of only the *Z* isomers of acids 2 and 3. Activation of allylic methyl groups via carboxylation in fact occurs only in the case of *Z* isomers (7).

Acids 2 and 3 are also produced under anaerobic conditions with nitrate as the electron acceptor. Since an oxygenase cannot be involved in this case, another pathway must intervene. For the anaerobic degradation of ketone 1, we propose a mechanism involving the hydration of the enol form of this ketone to 6,10,14-trimethylpentadecan-1,2-diol (compound 9), with this diol being then metabolized to 5,9,13-trimethyltetradecanoic acid (compound 8) (Fig. 6, pathway IV). Such a pathway was proposed for the metabolism of acetone by *Mycobacterium smegmatis* (24) and soil bacteria (42). The formed acid 8 may be subsequently metabolized via alternative β-oxidation and β-decarboxymethylation reactions as described above (Fig. 6, pathway III).

Aerobic and anaerobic degradation of acetone and higher ketones by different strains related to the genus *Pseudomonas* involved an initial carboxylation reaction (28). In the case of *Marinobacter* sp. strain CAB, we excluded such a mechanism, because CO_2 is not essential for growth. The use of cultures where CO_2 is continuously captured (flasks with potassium hydroxide in a central well) revealed that the degradation rate of ketone 1 is not affected by the lack of CO_2. Furthermore, we detected a quantity of acid 3 12 times greater than that obtained in the presence of CO_2. This accumulation can be easily explained by the slowing of carboxylation in the β-decarboxymethylation sequence (Fig. 7).

Small amounts of 6,10,14-trimethylpentadecan-2-ol (compound 7) were formed in anaerobic cultures, probably by a dehydrogenase (28). The involvement of this apparent "blind-alley" pathway (Fig. 6, pathway I) suggests that this reaction results from nonspecific enzyme activity that is not related to ketone 1 degradation (23). Brooks et al. (6) previously showed that a microbial population enriched from a lake sediment and grown on phytol under anaerobic conditions produced the C_{18} alcohol 7. These authors suggest that the likely route for the formation of this isoprenoid alcohol present in Green River shale (10) is via reduction of the corresponding ketone produced during phytol biodegradation. This hypothesis is supported by the results obtained in the present study.

The available data concerning the bacterial degradation of isoprenoids are still very limited, and more studies of the processes which modify isoprenoid distributions in microbially active sediments are essential (45). The results obtained during this investigation suggest that denitrifying bacteria must play a role in the short-term fate of these compounds. Although 6,10,14-trimethylpentadecan-2-one is produced during early diagenesis of intact (11, 12) and photooxidized (35) chlorophyll, it does not accumulate in marine sediments (36). This can be easily explained by the quick aerobic and anaerobic biodegradation of this substrate observed in this study, which calls into question its role as an indicator of oxic conditions during sedimentation. It is indeed essential for a biomarker to be preserved during the sedimentary record. The degradation of 6,10,14-trimethylpentadecan-2-one by denitrifying bacteria can constitute a nonnegligible source of 4,8,12-trimethyltridecan-1-ol (compound 4), 4,8,12-trimethyltridecanoic acid (compound 6), and 6,10,14-trimethylpentadecan-2-ol (compound 7), which are present in sediments (10, 20, 29). Moreover, the identification of the *Z* isomers of 3,7-dimethylocten-2-oic and 3,7,11-trimethyldodecen-2-oic acids is a very interesting result, since there is a real need to identify bacterial metabolites that are specific enough to act as biological markers for microbial degradation in the aquatic environment.

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