GC–MS Structural Characterization of Fatty Acids from Marine Aerobic Anoxygenic Phototrophic Bacteria

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ABSTRACT: The FA composition of 12 strains of marine aerobic anoxygenic phototrophic bacteria belonging to the genera *Erythrobacter, Roseobacter,* and *Citromicrobium* was investigated. GC–MS analyses of different types of derivatives were performed to determine the structures of the main FA present in these organisms. All the analyzed strains contained the relatively rare 11-methyloctadec-12-enoic acid, and three contained 12-methyloctadec-11-enoic acid, which has apparently never been reported before. High amounts of the very unusual octadeca-5,11-dienoic acid were present in 9 of the 12 strains analyzed. A FA containing a furan ring was detected in three strains. Analytical data indicated that this FA was 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid. A very interesting enzymatic peroxidation of the allylic carbon 10 of *cis*-vaccenic acid was observed in three strains. Deuterium labeling and GC–MS analyses enabled us to demonstrate that this enzymatic process involves the initial dioxygenase-mediated formation of 10-hydroperoxyoctadec-11(*cis*)-enoic acid, which is then isomerized to 10-hydroperoxyoctadec-11(*trans*)-enoic acid and converted to the corresponding hydroxyacids and oxoacids. Different biosynthetic pathways were proposed for these different compounds.

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It has long been known that various forms of bacteriochlorophyll (BChl) are present in prokaryotes, which perform anoxygenic photosynthesis under anaerobic conditions. In 1978, however, BChl *a* was detected in three strains of obligate aerobic bacteria (1,2). Since then, several strains of aerobic bacteria containing BChl *a* have been reported [for reviews see Shimada (3) and Yurkov and Beatty (4)]. The novel aspect of this increasingly large group of bacteria is that they are strict aerobes unable to grow under anaerobiosis. Apparently, these organisms, which are widely known as aerobic anoxygenic phototrophs (AAP) (4), might represent an evolutionary transient phase from anaerobic phototrophs to aerobic heterotrophs (3).

Until recently, AAP were thought to be confined only to specialized niches such as the surface of seaweed, coastal sands, cyanobacterial mats, and water in high tidal zones (5,6). However, in 1999, a significant activity of anoxygenic photosynthesis was discovered in the open ocean by BChl *a* fluorescence emission measurement and was attributed to the presence of AAP (7). Later, the presence of AAP was reported from the euphotic zone of various marine environments (8–11).

Little is known about AAP diversity. All the marine AAP isolates reported to date have been classified as members of four genera: *Erythrobacter*, *Roseobacter*, *Citromicrobium*, and *Roseibium* (6,12–15). Phylogenetically, the *Erythrobacter* and *Citromicrobium* genera belong to the Sphingomonadaceae family (α-4 subclass of the *Proteobacteria*), and the *Roseobacter* and *Roseibium* genera belong to the Rhodobacteriaceae family (α-3 subclass of the *Proteobacteria*).

In the present work, we analyzed the lipid composition of several isolates of marine AAP belonging to the *Erythrobacter*, *Roseobacter*, and *Citromicrobium* genera. The structures of FA were determined by GC–MS analysis of different derivatives.

MATERIALS AND METHODS

Organisms. Twelve strains containing functional bacterial photosynthetic reaction centers were isolated from diverse marine environments (Table 1). Isolation and characterization of these strains were described previously (10,15). The isolates were grown under fully aerobic conditions exposed to a light–dark cycle (12/12 h) at 23°C. The cultures were inoculated into Erlenmeyer flasks containing an organic enriched seawater medium supplemented with 0.5 g peptone and 0.1 g yeast extract per liter (15). The cells were harvested in a stationary phase by centrifugation at $10{,}000 \times g$.

Alkaline hydrolysis. Saponification was carried out on approximately 15 mg (dry weight) of wet cells of AAP; 25 mL of water, 25 mL of methanol, and 2.8 g of potassium hydroxide were added and the mixture was saponified by refluxing for 2 h. The content of the flask was acidified with hydrochloric acid (pH 1) after cooling and was subsequently extracted three times with dichloromethane. The combined dichloromethane extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated by rotary evaporation at 40°C to give the total solvent-extractable lipid compounds.

Acidic hydrolysis. The residues obtained after alkaline hydrolysis were treated with 2 M HCl by refluxing for 4 h. After cooling, the reaction mixture was extracted three times with dichloromethane. The combined extracts were then dried over

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Abbreviations: AAP, aerobic anoxygenic phototrophs; Bchl, bacteriochlorophyll; BSTFA, bis(trimethylsilyl)trifluoroacetamide.

TABLE 1
Genus and Origin of the Different Strains of Aerobic Anoxygenic Phototrophs (AAP) Analyzed

Strain	Genus	Origin	Temperature (°C)	Depth (m)
NAP1	*Erythrobacter*	NW Atlantic (39°36′N, 72°27′W)	12	0
MG3	*Erythrobacter*	SE Atlantic/W Indian (41°06′S, 19°25′E)	16	0
AT8	*Erythrobacter*	NE Pacific (48°15′N, 128°19′W)	16	0
BA13	*Erythrobacter*	SE Atlantic (48°33′N, 129°53′W)	16	22
B20	*Erythrobacter*	The Baltic Sea (55°17′N, 18°55′E)	18	0
COL2P	*Roseobacter*	Mediterranean coast (42°28′N, 3°11′E)	23	0
SYOP2	*Roseobacter*	Australia, Sydney coast	18	0
B09	*Roseobacter*	The Baltic Sea (59°39′N, 24°9′E)	17	0
B11	*Roseobacter*	The Baltic Sea (59°22′N, 22°36′E)	16	0
BS110	*Roseobacter*	Bosphorus (41°12.8′N, 29°07.4′E)	14	62
BS36	*Roseobacter*	The Black Sea (45°42.7′N, 31°05.7′E)	16	5
CV44	*Citromicrobium*	Central Atlantic (22°45′N, 46°04′W)	25	100

anhydrous Na_2SO_4, filtered, and concentrated by rotary evaporation at 40°C.

Lipid extraction. Approximately 15 mg (dry weight) of wet cells of AAP were extracted ultrasonically with chloroform/methanol/water (1:2:0.8, by vol) (16). Chloroform and deionized water were added to the combined extracts to obtain the final chloroform/methanol/water ratio of 1:1:0.9 (by vol) to initiate phase separation. The lipids were recovered in the lower chloroform phase, which was dried over anhydrous Na_2SO_4, filtered, and concentrated by rotary evaporation at 40°C.

Reduction. Reduction of allylic hydroperoxyacids and oxoacids to alcohols was performed in methanol (25 mL) by excess $NaBH_4$ or $NaBD_4$ (10 mg per 1 mg of extract). The mixture was agitated for 30 min at 20°C using magnetic stirring (17). After reduction, water and KOH were added and saponification was carried out as described in the *Alkaline hydrolysis* section.

Acetylation. Extracts were taken up in a mixture of 300 μL pyridine and acetic anhydride (2:1, vol/vol). The mixture was incubated overnight at 50°C and then evaporated to dryness under nitrogen. Under these conditions, hydroperoxides are quantitatively transformed to the corresponding ketones (18). After acetylation, saponification was carried out as described in the *Alkaline hydrolysis* section.

Osmium tetroxide oxidation. Lipid extracts and OsO_4 (1:2, w/w) were added to a pyridine/dioxane mixture (1:8, vol/vol; 5 mL) and incubated for 1 h at room temperature. Then 6 mL of Na_2SO_3 suspension (8.5 mL of 16% Na_2SO_3 in water/methanol, 8.5:2.5, vol/vol) was added and the mixture was again incubated for 1.5 h. The resulting mixture was gently acidified (pH 3) with HCl and extracted three times with dichloromethane (5 mL). The combined dichloromethane extracts were subsequently dried over anhydrous Na_2SO_4, filtered, and concentrated.

Formation of pyrrolidide derivatives. Lipid extracts were taken up in 2 mL of BF_3/methanol (14%) and heated at 80°C for 1 h. After cooling, an excess of water was added, and methyl esters were extracted three times with hexane, dried over anhydrous Na_2SO_4, filtered, and concentrated using rotary evaporation. The methyl esters obtained were dissolved in 1 mL of pyrrolidine. Then 0.1 mL of acetic acid was added and the mixture was heated at 100°C for 1 h. The excess pyrrolidine was removed under a stream of nitrogen at 50°C, and the residue was taken up in hexane/diethyl ether (1:1, vol/vol; 8 mL) and washed three times with water (4-mL portions). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated to obtain the required pyrrolidide derivatives.

Catalytic hydrogenation. Lipid extracts were hydrogenated overnight under magnetic stirring in methanol with $Pd/CaCO_3$ (10–20 mg/mg of extract) (Aldrich, St. Quentin Fallavier, France) as a catalyst. After hydrogenation, the catalyst was removed by filtration and the filtrate was concentrated by rotary evaporation.

Silylation. After evaporation of the solvent, each residue was taken up in 300 μL of a mixture of anhydrous pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, St. Quentin Fallavier, France) (2:1, vol/vol) and silylated for 1 h at 50°C. After evaporation to dryness under nitrogen, the residue was redissolved in ethyl acetate (2 mL/mg) and BSTFA (0.1 mL) and then analyzed by GC–MS.

GC–MS analysis. GC–EIMS analyses (under EI) were carried out with a HP 5890 Series II Plus gas chromatograph connected to an HP 5972 mass spectrometer. The following operating conditions were used: 30 m × 0.25 mm (i.d.) column coated with SolGel-1 (SGE, Ringwood, Australia; film thickness, 0.25 μm); oven temperature programmed from 60 to 130°C at a rate of 30°C min^{-1} and then from 250 to 300°C at 4°C min^{-1}; carrier gas (He) maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 to 1.5 bar at a rate of 0.04 bar min^{-1}; injector (on-column with retention gap) temperature, 50°C; electron energy, 70 eV; source temperature, 170°C; cycle time, 1.5 s.

Structural assignments were based on interpretation of the MS fragmentations and confirmed by comparison of retention times and mass spectra with those of authentic compounds when these were available.

Analyses of a blank treatment without cells and of AAP grown on glutamate (instead of yeast extract) were done in parallel to check for possible contamination.

TABLE 2
FA Compositions of the Different Strains of AAP Analyzed[a]

Acids	NAP1	MG3	AT8	BA13	B20	CV44	COL2P	SYOP2	B09	B11	BS110	BS36
Saturates												
12:0							3.6				0.1	
14:0	1.3	3.0	1.3	4.4	1.8	1.0	0.7	2.1	1.4	0.6	0.6	0.7
15:0	1.0	1.7	2.6	2.7	0.9	0.4	0.3	0.8	0.4	0.3	0.2	0.2
16:0	7.3	14.1	7.9	17.2	9.0	5.7	10.6	12.6	8.8	6.0	10.2	4.5
17:0	1.3	0.5	1.0	0.8	0.3	0.1	0.8	0.3	0.4	0.1	0.1	0.2
18:0	1.5	4.6	2.8	6.4	3.3	0.6	2.1	9.4	9.0	0.8	1.6	3.8
Monounsaturates												
12:1n-7										1.1	2.1	
16:1n-7	2.0	2.6	1.2	2.3	1.6	1.2	0.6	0.5	0.4	0.9	2.8	1.0
17:1n-8	2.2	0.3	1.5	0.5	0.5	Traces[b]					Traces	
17:1n-6	1.4	0.1	0.9	0.1	5.9	0.4					0.2	
18:1n-7	65.0	45.2	51.0	41.7	70.1	63.4	62.8	48.4	56.7	59.7	24.8	75.0
19:1n-8	1.8		0.4	0.4								
Polyunsaturates												
18:2n-7,13	9.4	7.8	12.9	9.2			[c]	12.3	6.8	11.4		5.4
Branched												
Me11,18:1n-6	3.1	10.5	9.8	7.1	Traces	10.9	2.8	9.4	10.1	10.5	3.0	4.7
Me12,18:1n-7	Traces		1.7	0.9								
Unknown							1.1			7.3		
Cyclic												
11,12-cyclo-18:0	Traces		Traces	0.4		0.1	3.4				25.1	
Furanoid							1.9				8.2	0.3
Hydroxyacids												
14:0 (2-OH)	0.5	5.8	1.5	2.6	1.6	4.0					1.4	
15:0 (2-OH)	0.5	0.6	2.4	1.5	0.8	Traces					0.4	
16:0 (2-OH)	0.4	0.4	0.3	0.3	3.1	11.9	4.1				6.4	
14:1n-7 (2-OH)											0.4	
16:1n-7 (2-OH)											2.2	
18:1n-7 (2-OH)											1.0	
10:0 (3-OH)							0.3			1.0	5.9	
12:0 (3-OH)							3.9		0.2	Traces	1.6	
13:0 (3-OH)											0.3	
14:0 (3-OH)							0.6					
12:1 (3-OH)								2.2	4.6			3.0
18:1n-9 (10-OH) *cis*		1.0									1.2	Traces
18:1n-9 (10-OH) *trans*		Traces									0.1	Traces

[a]For genera and origins of the bacterial strains see Table 1. See Table 1 for other abbreviation.
[b]<0.1%.
[c]Detected in cultures grown on glutamate.

RESULTS AND DISCUSSION

Saturated FA. The FA compositions of the different strains analyzed are summarized in Table 2. AAP contained a standard pattern of saturated FA ranging from C_{12} to C_{18}, which were rather uninformative. We failed to detect significant amounts of C_{15} and C_{17} branched saturated FA, which is in a good agreement with the well-known lack of such FA in most of the Gram-negative bacteria (19,20).

Monounsaturated FA. In EI mass spectra of monounsaturated FA methyl or trimethylsilyl esters, no feature permits the double bond to be located, because the double bond can migrate when the alkyl chain is ionized in the mass spectrometer. This phenomenon appeared to be particularly prominent in the case of branched FA (21). To avoid the migration problem, it was necessary to prepare specific derivatives of unsaturated FA that "fixed" the double bond. Two methods were used: OsO_4 oxidation (22) and pyrrolidide formation (23). The OsO_4 method involves the formation of diols by stereospecific oxidation of double bonds and subsequent analyses of the silylated diols by GC–MS. The position of the double bond can be deduced from the mass fragmentation patterns of these derivatized compounds. Pyrrolidide derivatives were chosen for this study since: (i) they are prepared under relatively mild conditions, (ii) they are stable chemically, and (iii) they are well suited to the structural characterization of branched unsaturated FA.

The AAP strains investigated contained monounsaturated FA ranging from C_{12} to C_{19} and were dominated by octadec-11(*cis*)-enoic acid (*cis*-vaccenic acid) (Table 2). Two interesting branched 19:1 FA were detected: the 11-methyloctadec-12-enoic and the 12-methyloctadec-11-enoic acids. The mass spectra of the bis-trimethylsilyloxy and pyrrolidide derivatives of the 11-methyloctadec-12-enoic acid are shown in Figure 1. The mass spectrum of the bis-trimethylsilyloxy derivative (obtained after OsO_4 oxidation of the double bond and subsequent silylation), exhibited two strong fragment ions at *m/z* 173 and

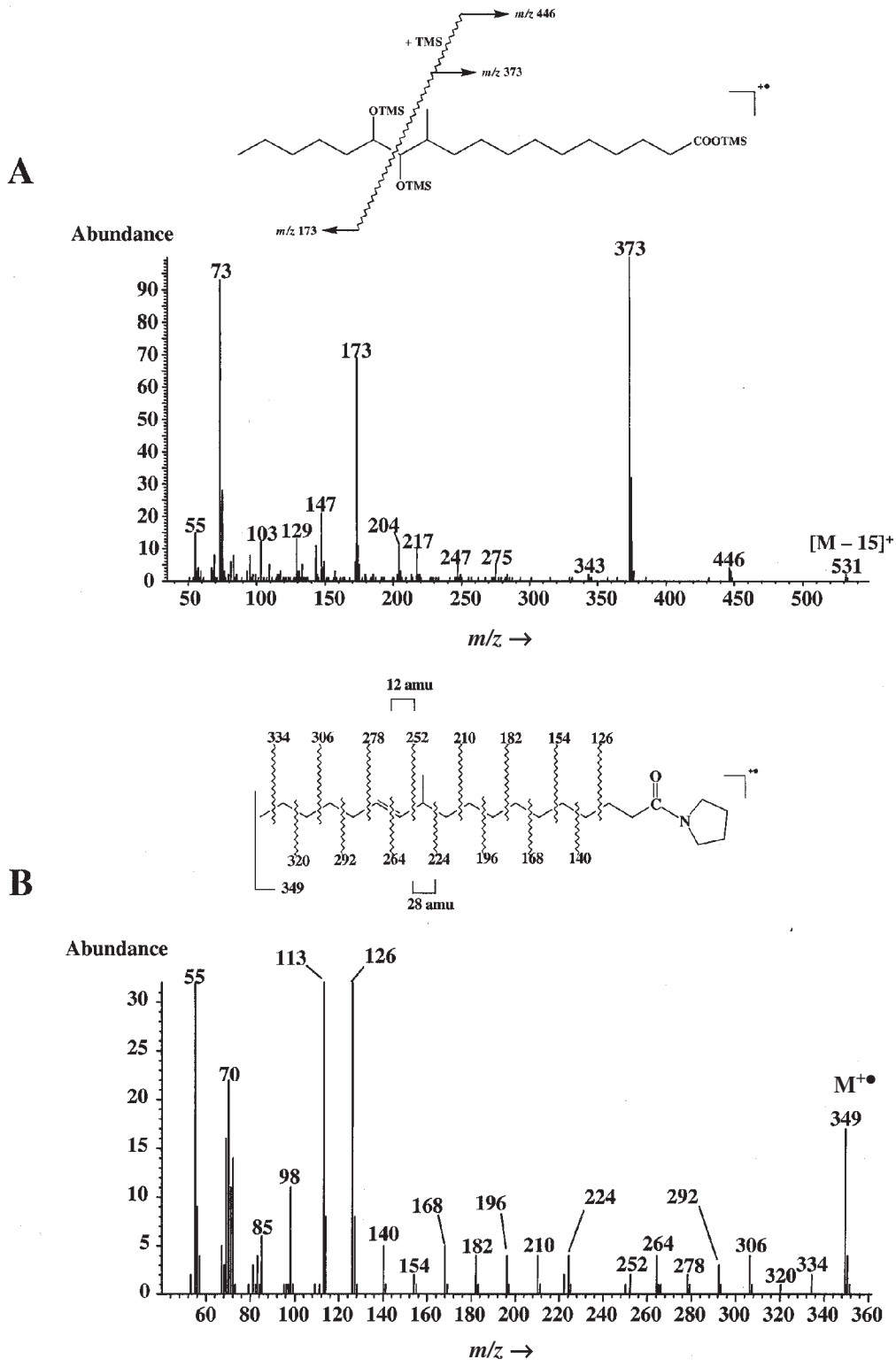

FIG. 1. EI mass spectra of the silylated OsO_4 (A) and pyrrolidide (B) derivatives of 11-methyloctadec-12-enoic acid. TMS, trimethylsilyl.

373 resulting from the cleavage of the bond between the two carbon atoms bearing the trimethylsilyloxy groups (Fig. 1A). This proved the presence of a double bond in position 12 in the parent FA. The useful ion at *m/z* 446 resulting from trimethylsilyl transfer toward the carboxylic group (24,25) confirmed that the fragment ion at *m/z* 373 contained the carboxylic group. In the mass spectrum of the pyrrolidide derivative (Fig. 1B), the gap of 28 amu between *m/z* 224 and 252 localized the methyl

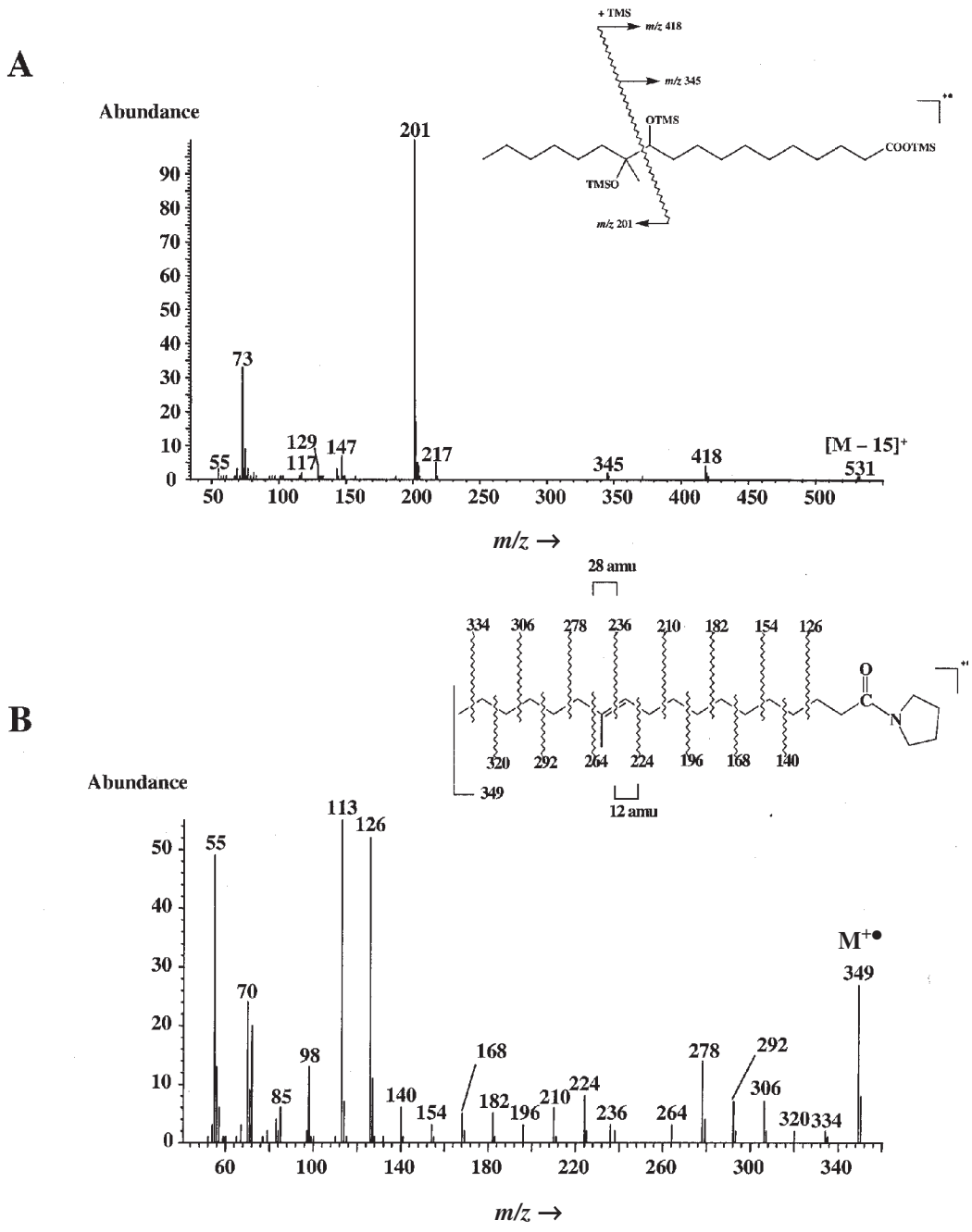

FIG. 2. EI mass spectra of the silylated OsO_4 (A) and pyrrolidide (B) derivatives of 12-methyloctadec-11-enoic acid. For abbreviation see Figure 1.

branch on the carbon C11 (26), whereas the gap of 12 amu between *m/z* 252 and 264 confirmed the presence of the double bond in position 12. Indeed, if an interval of 12 amu, instead of the regular 14, was observed between the most intense peaks of clusters of fragments containing n and n – 1 carbon atoms of the acid moiety, the double bond was located between carbon n and n + 1 in the molecule (23). 12-Methyloctadec-11-enoic acid was also identified from the mass spectra of its bis-trimethylsilyloxy and pyrrolidide derivatives (Fig. 2). In the mass spectrum of the pyrrolidide derivative (Fig. 2B), the gap of 12 amu between *m/z* 224 and 236 showed a double bond in position 11, whereas the gap of 28 amu between *m/z* 236 and 264 localized the methyl branch on the carbon C12. This assignment was also supported by the presence of fragment ions at *m/z* 201 (base peak), 345, and 418 in the mass spectrum of the bis-trimethylsilyloxy derivative (Fig. 2A).

11-Methyloctadec-12-enoic acid was present in all the AAP strains analyzed (Table 2). This acid was previously detected in other bacteria such as mycobacteria (27), *Shewanella putrefaciens* (28), *Thiobacillus* (29), *Thiohalocapsa halophila*, and

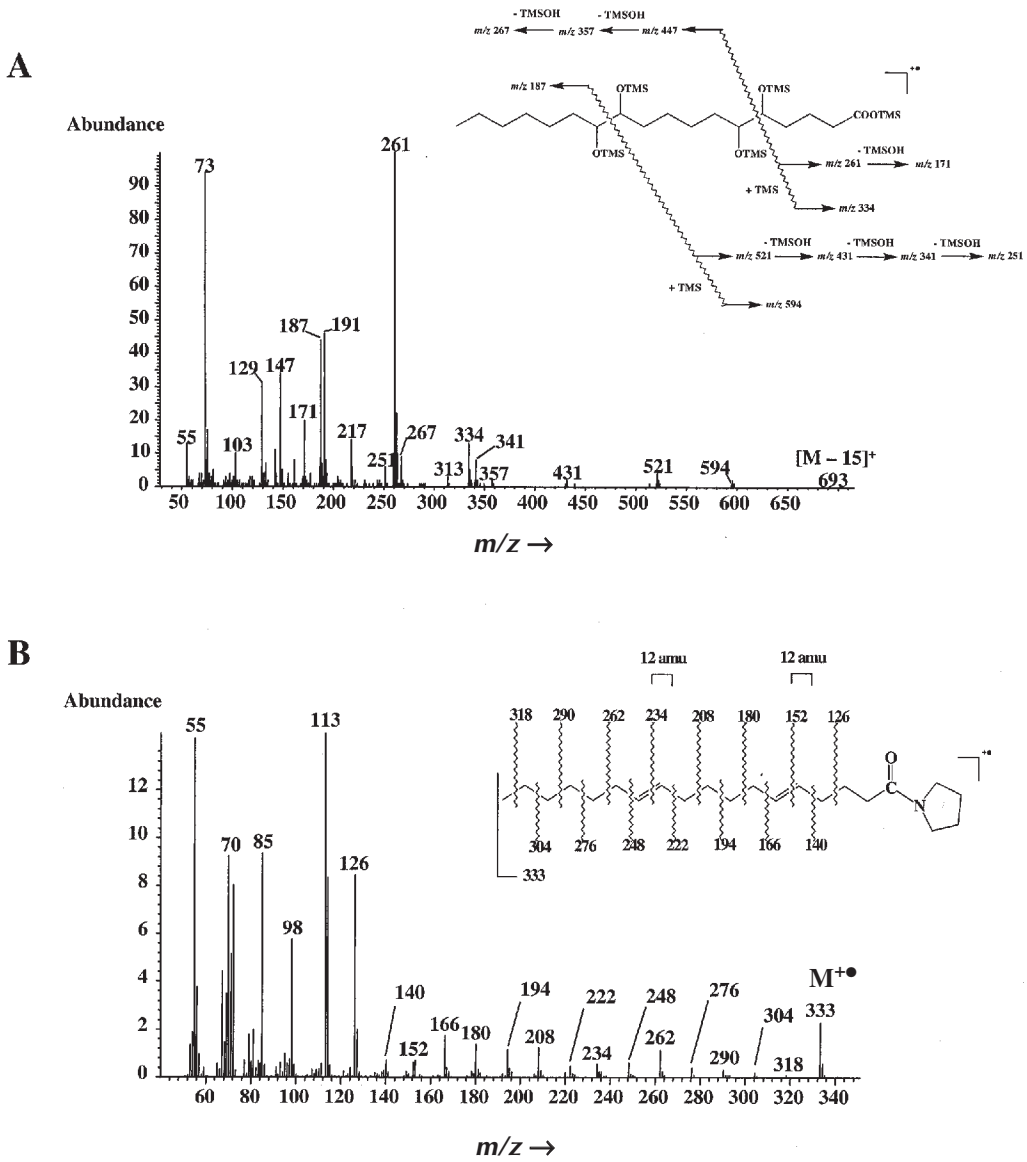

FIG. 3. El mass spectra of the silylated OsO_4 (A) and pyrrolidide (B) derivatives of octadeca-5,11-dienoic acid. For abbreviation see Figure 1.

Halochromatium salexigens (Rontani, J.-F., unpublished data). It seems to be a FA typical of bacteria. In contrast, 12-methyl-octadec-11-enoic acid was found in only three strains of AAP (Table 2). To our knowledge, this compound has never been reported before.

PUFA. Small amounts of octadeca-9,12-dienoic acid (linoleic acid) were detected in all the strains of AAP analyzed. This result is very surprising, since it is generally believed that bacteria are unable to synthesize methylene-interrupted PUFA (19). After careful examination, it appeared that this acid resulted from contamination during the analytical treatment.

Nine of the 12 strains of AAP analyzed contained a significant proportion of the unusual octadeca-5,11-dienoic acid (Table 2). The mass spectra of the tetra-trimethylsilyloxy and pyrrolidide derivatives of this acid are shown in Figure 3. The presence of double bonds in positions 5 and 11 is well supported by the intense fragment ions at *m/z* 187, 261, and 334 observed in the mass spectrum of the tetra-trimethylsilyloxy derivative (Fig. 3A) and by the gaps of 12 amu between *m/z* 140 and 152 and between *m/z* 222 and 234 in the mass spectrum of the pyrrolidide derivative (Fig. 3B).

Octadeca-5,11-dienoic acid was previously found in the seed oil of some gymnosperms, e.g., *Ginkgo biloba* (30), and occasionally in sponge lipids (Christie, W.W., unpublished results), always as a minor component. To our knowledge, the presence of this unusual FA has never been reported in phytoplankton (31–34) or in anaerobic photosynthetic bacteria (35). Even though this acid was not detected in all the strains analyzed

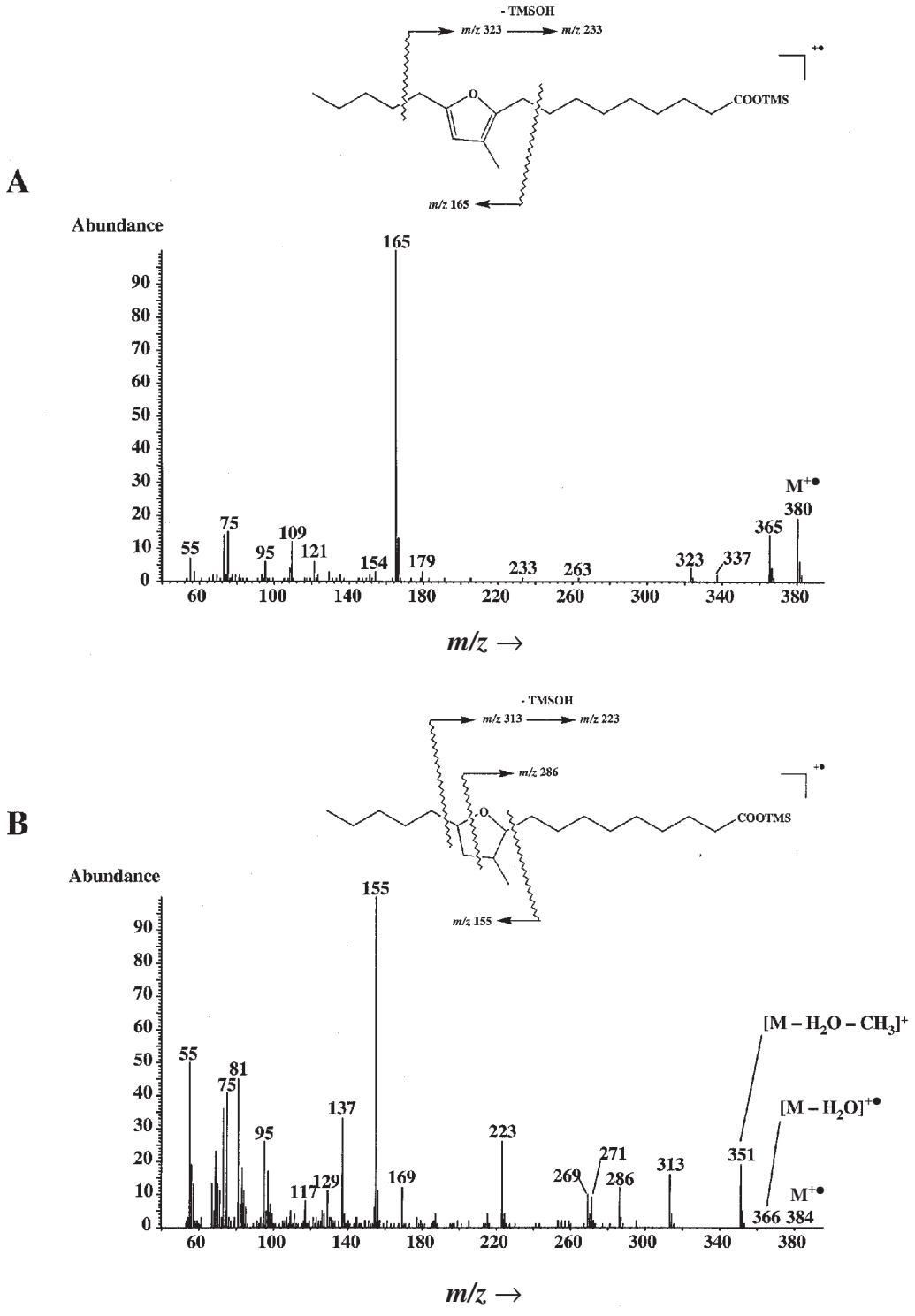

FIG. 4. EI mass spectra of silylated 10,13-epoxy-11-methyloctadeca-10,12-dienoic (A) and 10,13-epoxy-11-methyl-octadecanoic (B) acids. For abbreviation see Figure 1.

(Table 2), it could constitute a useful indicator of the presence of AAP because of its relative abundance in these organisms.

Cyclic FA. 11,12-Methyleneoctadecanoic acid (lactobacillic acid) was detected in six strains of AAP (Table 2). A particularly high proportion was present in the strain BS110. This cyclopropane FA has been differentiated from its corresponding olefinic isomers after catalytic hydrogenation (36). The first step in the biosynthesis of the cyclopropane ring in a FA is similar to that for certain methyl-branched FA, and involves initial addition of a methyl group from *S*-adenosylmethionine to a double bond and subsequent loss of a proton. Thus, lactobacillic acid must be formed by the addition of a methylene group

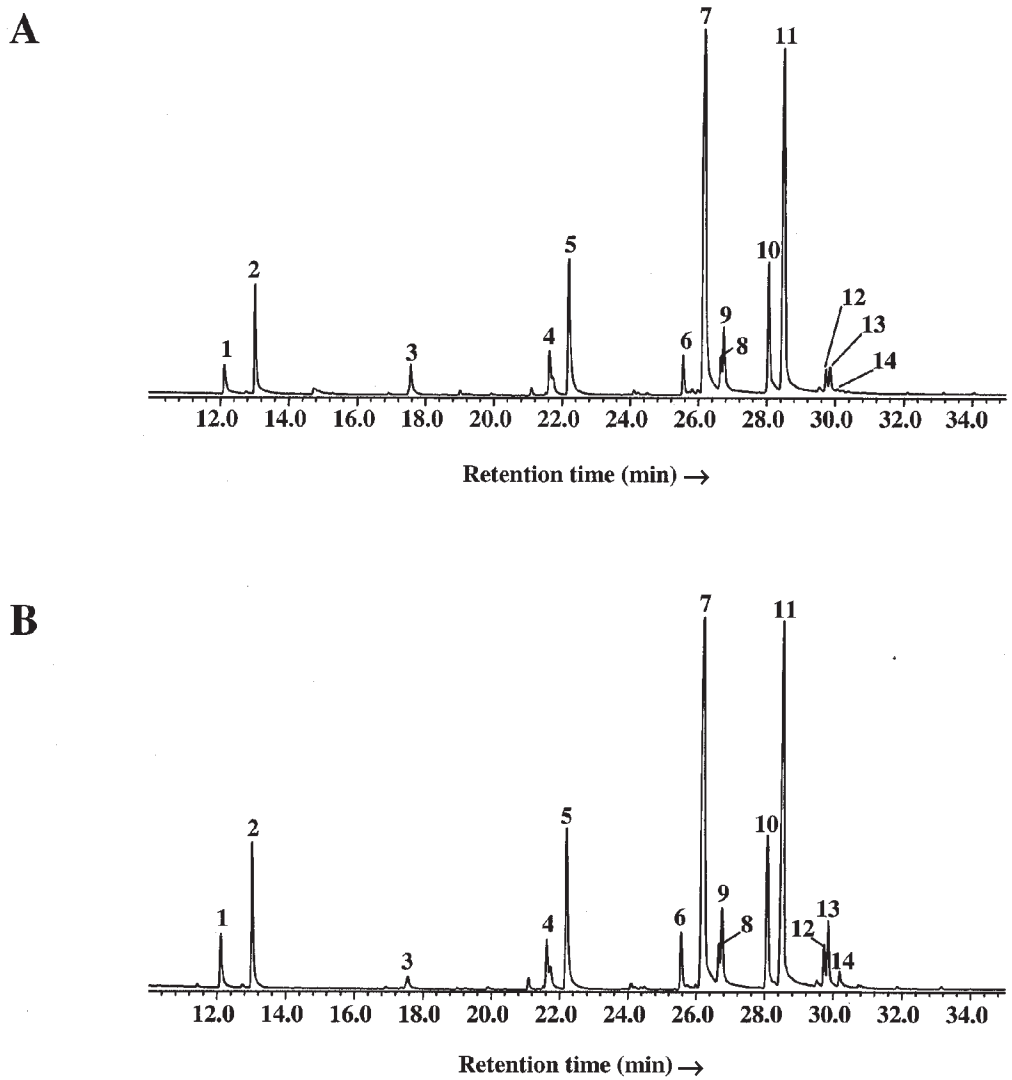

FIG. 5. Total ion current chromatogram of the silylated total lipid extract of the strain BS110 before (A) and after (B) $NaBH_4$ reduction. 1, Dodec-5-enoic acid; 2, 3-hydroxydecanoic acid; 3, 3-hydroxydodecanoic acid; 4, hexadec-9-enoic acid + 2-hydroxypentadecanoic acid; 5, hexadecanoic acid; 6, 2-hydroxyhexadec-9-enoic acid; 7, *cis*-vaccenic acid + 2-hydroxyhexadecanoic acid; 8, octadecanoic acid; 9, 11-methyloctadec-12-enoic acid; 10, 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid; 11, 11,12-methyleneoctadecanoic acid; 12, 2-hydroxyoctadec-11-enoic acid; 13, 10-hydroxyoctadec-11(*cis*)-enoic acid; 14, 10-hydroxyoctadec-11(*trans*)-enoic acid.

across the double bond of *cis*-vaccenic acid. This acid has been found in a wide range of bacterial species of many different types, both Gram-negative and Gram-positive, from strict anaerobes to obligate aerobes.

A furan FA, 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid, was detected in the strains BS110, BS36, and COL2P (Table 2). The mass spectrum of this silylated FA is shown in Figure 4A. Allylic cleavage of the alkylcarboxylic chain at the furan ring produced the base peak at *m/z* 165, whereas allylic cleavage of the alkyl chain yielded a fragment ion at *m/z* 323. The furan ring itself gave rise to a fragment ion at *m/z* 109 characteristic of trisubstituted furan acids (37). The position of the methyl group was determined after hydrogenation (in the presence of Pd as catalyst) (Fig. 4B), thanks to the THF-specific ring cleavage at *m/z* 286 enabling the unambiguous localization of the methyl substituent (38).

10,13-Epoxy-11-methyloctadeca-10,12-dienoic acid was previously detected in several strains of marine bacteria (*S. putrefasciens, Listonella anguillarum, Marinomonas communis, Pseudomonas fluorescens, Enterobacter agglomerans*) (28,37). A very interesting biosynthetic pathway of this acid starting from *cis*-vaccenic acid was proposed by Shirasaka *et al.* (28). The first step of this pathway is thought to be the formation of 11-methyloctadec-12-enoic acid through methylation of *cis*-vaccenic acid; 11-methyloctadeca-10,12-dienoic acid is then produced through desaturation. The next step involves a lipoxygenase-type oxidation, followed by ring closure. The involvement of such a process in BS110, BS36, and COL2P is well supported by the presence of relatively high proportions of 11-methyloctadec-12-enoic acid in these three strains (Table 2). However, the intermediate 11-methyloctadeca-10,12-dienoic acid, which was previously detected by Shirasaka *et al.* (28), has not been observed in these strains.

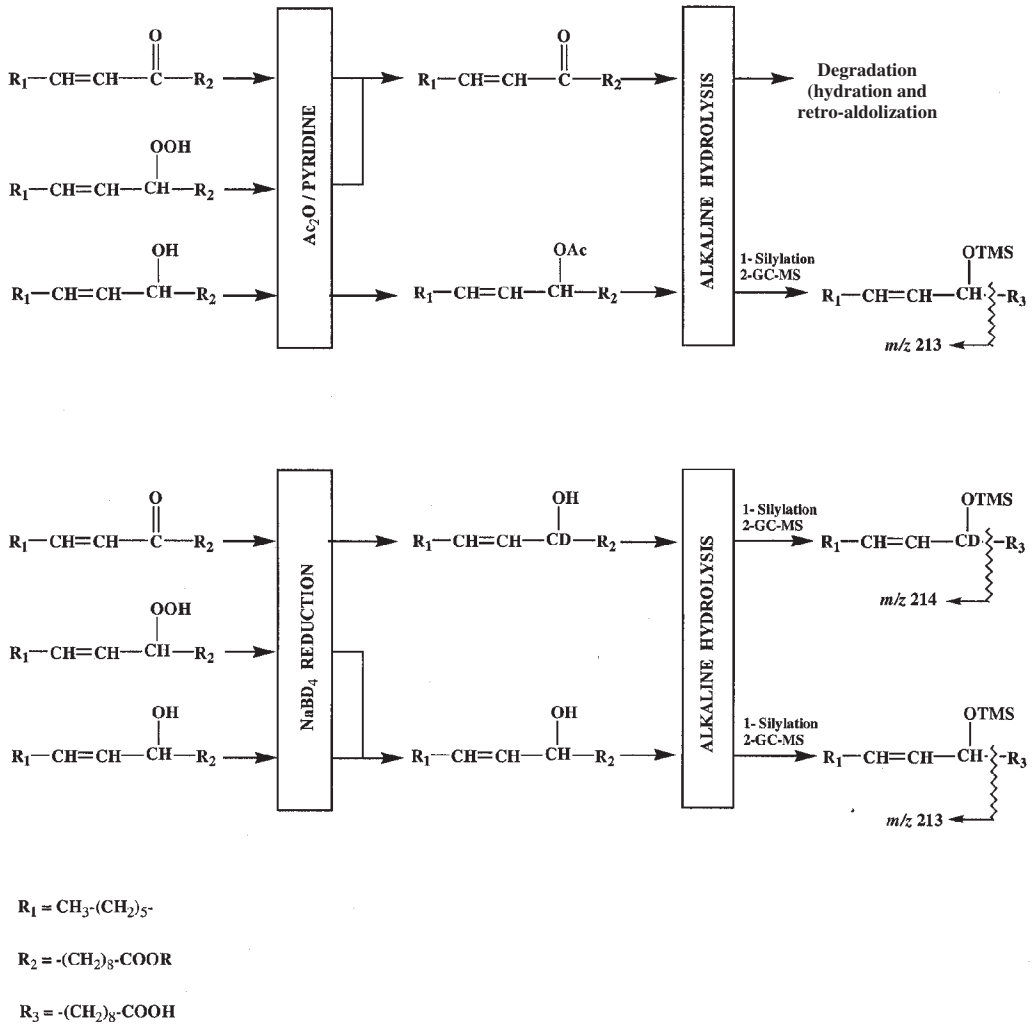

FIG. 6. Characterization of allylic hydroperoxy, hydroxy-, and oxoacids after parallel acetylation and $NaBD_4$ reduction. For abbreviation see Figure 1.

Hydroxyacids. Sphingomonadaceae are generally characterized by the presence of sphingolipids and 2-hydroxyacids and by the lack of 3-hydroxyacids (39). Indeed, all the tested *Erythrobacter*- and *Citromicrobium*-related strains belonging to the Sphingomonadaceae family contain 2-hydroxytetradecanoic, 2-hydroxypentadecanoic, and 2-hydroxyhexadecanoic acids (Table 2). The content of 2-hydroxyacids increases significantly when the acidic hydrolysis is carried out after saponification. This suggests that these compounds are components of sphingolipidic structures linked to sphingosine by amide bonds, which are only partially hydrolyzed during saponification. It is interesting to note that the hydrolysis of these amide bonds is induced during the natural senescence of AAP, when a significant increase in the 2-hydroxyacid content occurs (40).

3-Hydroxyacids were present only in *Roseobacter*-related AAP (Table 2). These compounds, which are typically produced as intermediates in the β-oxidation of monocarboxylic acids, constitute a classical component of the cell wall lipopolysaccharides of several Gram-negative bacteria (35,41). Interestingly, the *Roseobacter*-related strains COL2P and BS110, in addition to 3-hydroxyacids, also contained 2-hydroxyacids (Table 2).

Cis and *trans* 10-hydroxyoctadec-11-enoic acids were detected in the strains MG3, BS36, and BS110 (Fig. 5, Table 2). The mass spectra of their silylated derivatives were described previously (42). They exhibited a strong fragment ion at *m/z* 213 corresponding to the cleavage at the carbon-bearing $–OSiMe_3$ group. Determination of *cis*- and *trans*-isomers was based on comparison of the retention times with standards. A similar regiospecific oxygenation of the allylic carbon 10 of *cis*-vaccenic acid has been observed previously in senescent cells of the halophilic purple sulfur bacterium *Thiohalocapsa halophila* incubated under aerobic conditions in the dark (42). This enzymatic process was attributed to the involvement of a lipoxygenase. Lipoxygenases are nonheme iron-containing dioxygenases that catalyze the addition of molecular oxygen to

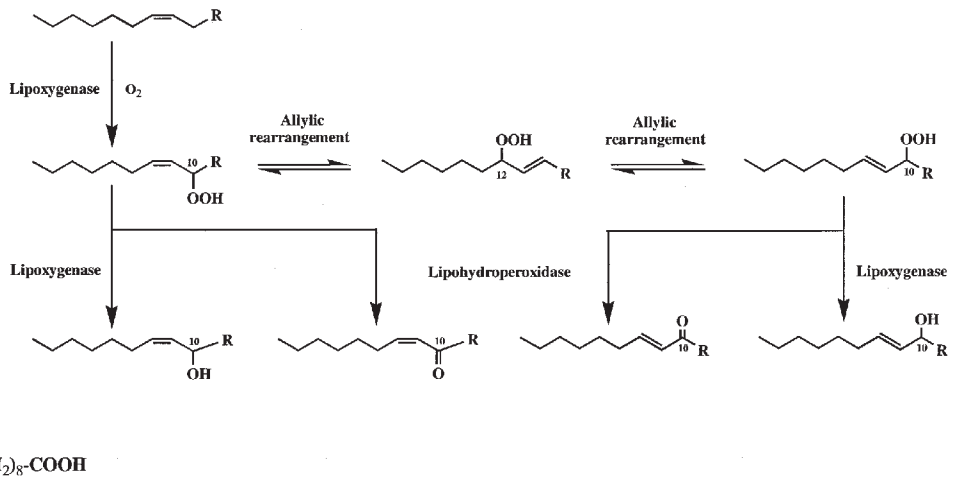

FIG. 7. Proposed pathways for the enzymatic oxygenation of *cis*-vaccenic acid in the strains BS110, BS36, and MG3.

PUFA with a (*cis,cis*)-1,4-pentadiene system to give an unsaturated FA hydroperoxide (43). Lipoxygenases may also catalyze the oxygenation of monounsaturated FA such as oleic (44–47), octadec-*cis*-12-enoic (47), or *cis*-vaccenic (42) acid. These processes afford mainly hydroxy-, hydroperoxy-, or oxoacids. Lipoxygenases are widely distributed in plants, fungi, and animals (48). Recently, these enzymes were also detected in bacteria (45,49). The presence of lipoxygenase in the strains BS110 and BS36 is consistent with their relatively high content of furan FA (Table 2). The amounts of *cis* and *trans* 10-hydroxyoctadec-11-enoic acids increased considerably when the samples were reduced with $NaBH_4$ before the alkaline hydrolysis (Fig. 5). Some tests involving deuterium labeling, acetylation, and GC–MS analyses (Fig. 6) were carried out to determine whether this additional production of hydroxyacids resulted from the reduction of the corresponding hydroperoxy- or oxoacids during the treatment. The organic extracts were divided into two fractions. One part was reduced by $NaBD_4$ and saponified, whereas the other was first acetylated (to dehydrate the hydroperoxides to ketones) and then saponified. Comparison of the amounts of unlabeled hydroxyacids present after acetylation (naturally occurring hydroxyacids) and after reduction (naturally occurring + hydroperoxide reduction-derived hydroxyacids) allowed us to estimate the proportion of hydroperoxyacids and hydroxyacids present in the samples, whereas quantification of the deuterated hydroxyacids after reduction (thanks to the fragment ion at *m/z* 214) (Fig. 6) corresponded to the amount of oxoacids. The allylic oxoacids could not be characterized directly since these compounds do not survive alkaline hydrolysis and are cleaved after hydration and retro-aldol reactions (50). These tests cannot be carried out on the strain BS36 since 10-hydroxyoctadec-11-enoic acids were present in only trace amounts in this strain (Table 2). The results obtained proved that strains BS110 and MG3 contained hydroperoxyacids, oxoacids, and hydroxyacids (Table 3). The presence of significant proportions of hydroperoxides is very surprising since they are extremely cytotoxic and cause damage to membranes and proteins in particular (51). Several enzymatic processes causing further reactions of the hydroperoxides and avoiding their accumulation have been described previously. These processes involve: (i) reduction to the corresponding hydroxyacids (51), (ii) homolytic cleavage of the O–O bond resulting in the formation of oxoacids (48), (iii) dehydration to allene oxides and subsequent hydrolysis of these unstable intermediates (52), and (iv) direct cleavage of the hydroperoxides to aldehydes and oxoacids (53). Only the first two degradation processes seem to act significantly in the strains BS110 and MG3. The reduction of hydroperoxyacids to the corresponding hydroxyacids is generally attributed to the lipoxygenases themselves (51), whereas the breakdown of hydroperoxyacids to oxoacids is induced by lipohydroperoxidases (48). Similar to enzymatic conversion, the hydroperoxides formed may also undergo chemical decomposition under mild conditions. The majority of these decomposition reactions involve free radicals and are promoted by heat, photolysis, metal ions, and metalloproteins. The products of these reactions are similar to those of enzymatic transformations or are either homologs or isomers of the enzymatically produced compounds (48). The quite distinct proportions of hydroperoxyacids, hydroxyacids, and oxoacids observed in the case of the

TABLE 3
Relative Proportions (%) of 10-Hydroperoxyoctadec-11-enoic Acids and Their Degradative Hydroxyacids and Oxoacids in the Strains BS110 and MG3

Compound	BS110	MG3
10-Hydroperoxyoctadec-11(*cis*)-enoic acid	35	35
10-Hydroxyoctadec-11(*cis*)-enoic acid	40	45
10-Oxooctadec-11(*cis*)-enoic acid	25	20
10-Hydroperoxyoctadec-11(*trans*)-enoic acid	20	—[a]
10-Hydroxyoctadec-11(*trans*)-enoic acid	15	—
10-Oxooctadec-11(*trans*)-enoic acid	65	—

[a]Not determined (only trace amounts). For genera and origins of the bacterial strains see Table 1.

cis and *trans* isomers (Table 3) allowed us to exclude the involvement of such abiotic processes during the degradation of *cis* and *trans* 10-hydroperoxyoctadec-11-enoic acid. Allylic hydroperoxides may also undergo highly stereoselective free radical allylic rearrangement (54). These processes, which can act on both *cis* and *trans* allylic hydroperoxides, afford only *trans* configurations (54). The detection of trace amounts of 12-hydroperoxyoctadec-10(*trans*)-enoic acid and its corresponding hydroxyacid and oxoacid in BS110 and MG3 strongly suggests that isomerization of 10-hydroperoxyoctadec-11(*cis*)-enoic acid results from such a rearrangement (Fig. 7).

In conclusion, the FA composition of AAP is characterized by saturated and monounsaturated FA ranging from C_{12} to C_{18} and from C_{12} to C_{19}, respectively, with *cis*-vaccenic acid as the major component. All the AAP analyzed contained the relatively rare 11-methyloctadec-12-enoic acid. This acid was previously detected in some marine bacteria (27–29). It seems to be a typical bacterial FA. The strains NAP1, AT8, and BA13 contained small amounts of the 12-methyloctadec-11-enoic acid, which apparently has never been reported before.

The presence of relatively high proportions of the octadec-5,11-dienoic acid in 9 of the 12 strains of AAP analyzed is very surprising since bacteria usually do not contain PUFA. This unusual acid could constitute a useful indicator of the presence of AAP in the marine environment.

An interesting regiospecific enzymatic peroxidation of the allylic carbon 10 of *cis*-vaccenic acid was observed in the strains BS110, BS36, and MG3. A similar oxidation process was previously described in the case of the purple sulfur bacterium *Thiohalocapsa halophila* (42) and attributed to the involvement of a lipoxygenase. This enzymatic attack of *cis*-vaccenic acid seems to be a characteristic of some (aerobic and anaerobic) phototrophic bacteria. The presence of a lipoxygenase in AAP is well supported by the detection of significant amounts of a furan FA, the 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid in the strains BS110, BS36, and COL2P.

All the strains analyzed belonging to the *Erythrobacter* and *Citromicrobium* genera contained 2-hydroxyacids linked by amide bonds in sphingolipidic structures. These compounds are released during the natural senescence of these organisms. In contrast, all the *Roseobacter*-like strains contained 3-hydroxyacids.

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