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trans-Monounsaturated Acids in a Marine Bacterial Isolate

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A sedimentary bacterial isolate has been shown to contain *trans*-monounsaturated fatty acids (6% of the total fatty acids). The ratio of *trans*- to *cis*-acids in this isolate was in the range 3.2 to 7.6. The identification of *trans*-monounsaturated acids in a marine bacterium implied that the *trans*-acids which have been reported in recent sediments could derive, in whole or part, from direct bacterial input.

In the past, *trans*-monounsaturated acids have often been reported in recent sediments; it has been proposed that these acids originate from microbial or abiological degradation, or both, of *cis*-mono- and polyunsaturated fatty acids (2, 13–15) or from direct bacterial input (9; J. K. Volkman, R. B. Johns, F. T. Gillan, G. J. Perry, and H. J. Bavor, Geochim. Cosmochim. Acta, in press). The presence of *trans*-acids in bacteria has been confirmed in the case of rumen bacteria, where derivation from *Z,Z*-Δ9,12-18:2 has been demonstrated (6). (Fatty acids are denoted as *X,X,* . . . -Δ*x,y,* . . . -*M*:*n*, where *X,X,* . . . details the geometrical configurations of the double bonds [*E* is equivalent to *trans*, and *Z* is equivalent to *cis*], *x,y,* . . . indicates the positions of the double bonds from the carboxyl end, M is the number of carbon atoms, and *n* is the number of double bonds.) Traces of these acids have also been tentatively identified in mixed marine bacterial isolates, with *trans/cis* ratios between 0.01 and 0.03 (9; Volkman et al., in press). This paper reports the finding of a marine bacterial isolate containing monounsaturated acids with principally *trans* geometry, a finding of considerable geochemical and biochemical significance to the understanding of the origins of these geolipids.

MATERIALS AND METHODS

Cultures. The two bacterial isolates (LIAB 3 and LIAB 7, both gram-negative aerobic rods and facultative anaerobes) used in this study were isolated from sediment samples from a mangrove stand on the Low Isles, North Queensland, Australia (16°23′ S, 145°34′ E), and grown aerobically on modified Zobell 2216E medium as previously described (9).

Extraction procedures. Cell pellets were obtained from the cultures by centrifugation and then saponified (5% KOH–80% aqueous methanol; 24 h, ambient temperature). After filtration to remove the cell residue, the neutral lipids were extracted from the basic aqueous fraction with $CHCl_3$–*n*-heptane (1:4, vol/vol). Acidification of the residual aqueous layer to pH 2 was followed by extraction of the fatty acids into $CHCl_3$–*n*-heptane (1:4, vol/vol). This acidic fraction was concentrated by evaporation of the solvent under reduced pressure and then methylated (14% BF_3–methanol; 15 min, under reflux).

Chromatographic analysis. After methylation, the resultant mixtures of fatty acid methyl esters were analyzed by capillary gas-liquid chromatography on a nonpolar glass SCOT SE 30 (50 m by 0.5-mm inside diameter; SGE, Australia; N_{eff} = 60,000) column and a polar glass WCOT SIL-47-CNP (47% cyanopropyl silicone, 45 m by 0.20-mm inside diameter; Chromalytic Technology; N_{eff} = 140,000) column. Splitless injection and temperature programing (SE 30, 140 to 280°C at 2.5°C/min; SIL-47-CNP, 140 to 240°C at 3°C/min) were used for both analyses. Fractional chain lengths (FCLs) were calculated from peak retention times by interpolation, using the polynomial: ECL = $a + bT_R + cT_R^2 + dT_R^3 + eT_R^4$, where ECL is the equivalent chain length (= K + FCL, where K is the ECL of the corresponding saturated fatty acid), T_R is the retention time, and a, b, c, d, and e are derived constants.

The constants a, b, c, d, and e were derived from the T_R values and known ECLs of a coinjected, straight-chain fatty acid methyl ester mixture (containing $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{20:0}$ fatty acids). The reproducibility of FCL for a given acid was +0.012 (2σ). Double-bond positions were estimated from FCL-carbon number plots.

Monounsaturated fatty acids: positional isomer identification. The monounsaturated fatty acids in the mixture were converted to the corresponding dihydroxy acids by oxidation with osmium tetroxide (5% OsO_4 in pyridine [1]). These dihydroxy acids were subsequently converted to the corresponding ditrimethylsilyl ethers by reaction with *N,O*-bistrimethylsilylacetamide (10 min, 40°). The trimethylsilyl ethers were analyzed by probe mass spectral (MS) analysis (VG Micromass 7070F mass spectrometer with VG System 2000 data system; ion source at 70 electron

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volts, 6-s scan repetition rate). The mass spectra were intensity averaged as the sample volatilized in the ion source and were subjected to background subtraction normalized to m/e 32 (O_2^+). Standards of the fatty acids observed in this study were generally unavailable commercially, and thus all the probe MS data are presented uncorrected for relative response factors. The intensities of the major ions corresponding to the carboxyl fragments (fragment A in Fig. 1) were summed and normalized to 100%. The terminal fragment ions (fragment B in Fig. 1) were treated similarly.

In the case of LIAB 7, the fatty acid ditrimethylsilyl ethers were further analyzed by capillary gas chromatography (GC)-MS (Finnigan 4000 GC-MS with INCOS data system; column [OV-1] 20 m by 0.3-mm inside diameter; electron energy, 35 electron volts; scan rate, 1.0 s).

Fatty acid class separation. Total fatty acid methyl esters were separated into saturated fatty acids and *cis*- and *trans*-monounsaturated fatty acids by argentation thin-layer chromatography as described previously (9). Dihydroxy acid methyl esters were separated from saturated fatty acid methyl esters by thin-layer chromatography on silica gel (*n*-heptane–diethyl ether–methanol, 80:10:1 [vol/vol]) (Volkman et al., in press) or by high-pressure liquid chromatography. High-pressure liquid chromatographic analyses employed an Altex 110A pump, a Spherisorb 5-μm, CN-bonded column (25 cm by 4.6-mm inside diameter) and a refractive index detector (Varian Proprietary Ltd.). The mobile phase was *n*-heptane–1-propanol (97:3, vol/vol) at a flow rate of 1.0 cm^3/min.

Procedural blank. An aliquot of the sterile culture medium was extracted twice with chloroform. The organic extract was concentrated and saponified as for the bacterial samples. Completion of the work-up on this sample revealed only traces of the acids 16:0, 18:0 and *Z*-Δ9-18:1.

RESULTS AND DISCUSSION

Chromatographic analysis and identification of fatty acids. Analysis of sample LIAB 7 on the polar capillary column revealed the presence of monounsaturated acids with FCLs much smaller than those of known *cis*-monounsaturated fatty acids (9) (Table 1). Since *trans*-isomers eluted before the corresponding *cis*-isomers (11, 12) on low-resolution packed columns with the polar cyanopropyl silicones (SILAR-10CP and OV-275) as liquid phases, it was probable that these esters of lower FCL than typical *cis*-isomers were of *trans* configuration. Some support for this conclusion could be derived from the report by Pearce and Stillway (8) of a *cis-trans* isomer separation for a fish lipid-fatty acid mixture on a similar capillary column (SILAR-5CP; 50% cyanopropyl silicone). Their paper reported the separation of *E*-Δ6-16:1 (the *trans*-acid) and *Z*-Δ9-16:1 (the *cis*-acid), quoting FCLs of 0.22 and 0.33, respectively. The FCLs of the monounsaturated fatty acids of sample LIAB 7 were much smaller (0.08), suggesting that our column may have been much more selective for double-bond geometry or that the fatty acids observed for LIAB 7 were not monounsaturates. Argentation thin-layer chromatography revealed that these unusual acids chromatographed at an R_f between that of typical *cis*-monounsaturated fatty acids (*Z*-Δ9-18:1 as standard) and that of saturated acids, as expected for *trans*-monounsaturated fatty acids. In addition, after osmium tetroxide oxidation to the corresponding dihydroxy acids, the products cochromatographed on high-pressure liquid chromatography and thin-layer chromatography with an authentic dihydroxy acid standard (9, 10-dihydroxystearic acid methyl ester). It was evident from these results that the polar capillary column used in this study was highly selective for *trans-cis* geometry in monounsaturated fatty acids. The fatty acids extracted from LIAB 3, in contrast to those from LIAB 7, all had FCLs in the usual *cis*-monounsaturated fatty acid range. For these acids, FCL-carbon number plots were used to provide tentative identifications of the positional isomers present (5). LIAB 3 was thus considered to contain 16.3% *Z*-Δ9-monounsaturated fatty acids, with smaller amounts of *Z*-Δ11- and *Z*-Δ7-acids also present, based only on GC data. In the case of LIAB 7, traces of *cis*-isomers dominated by *Z*-Δ9-18:1 and *Z*-Δ6-16:1 were tentatively identified: the predominant monounsaturated fatty acids (with FCLs < 0.12) could not even be tentatively identified; however, the close concordance of the FCLs for these isomers suggested that unsaturation occurred at the same position in each case.

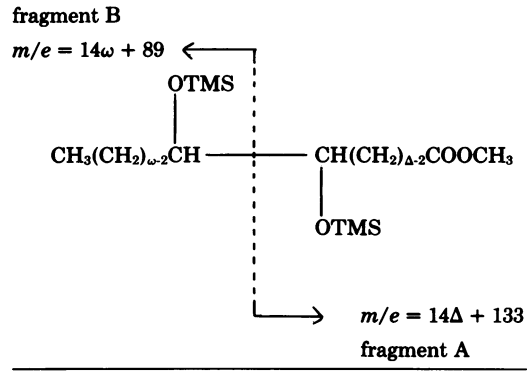


FIG. 1. *Characteristic mass fragmentations of the monounsaturated acid derivatives.*

The mass spectra of the ditrimethylsilyl ethers of vicinal dihydroxy fatty acid methyl esters showed a characteristic cleavage between the

TABLE 1. *Relative percentages of fatty acids of two marine bacterial isolates*[a]

Fatty acid type	Fatty acid methyl ester[b]	FCL[c]	Relative % in:	
			LIAB 7	LIAB 3
Saturated	i12:0		—[d]	0.2
	12:0		0.07	—
	i13:0		—	0.5
	i14:0		1.46	3.6
	14:0		0.66	0.1
	i15:0		21.2	28.8
	a15:0		37.6	—
	15:0		0.29	—
	i16:0		3.84	30.9
	16:0		5.66	1.7
	i17:0		7.67	1.0
	a17:0		12.9	—
	17:0		0.07	—
	i18:0		0.07	0.3
	18:0		0.64	1.5
	i19:0		0.06	—
	a19:0		0.05	—
Monounsaturated[e]	*E*-Δ5-i16:1	0.08	3.73	—
	E-Δ5-16:1	0.08	1.02	—
	E-Δ5-i17:1	0.11	0.28	—
	E-Δ5-a17:1	0.10	1.05	—
	Z-Δ5-i16:1	0.26	0.12	—
	Z-Δ5-16:1	0.25	0.12	—
	Z-Δ6-16:1	0.22	0.41	—
	E-Δ7-18:1	ND[f]	0.07	—
	Z-Δ7-i15:1	0.27	—	0.3
	Z-Δ7-i16:1	ND	—	0.2
	Z-Δ8-18:1	0.24	0.16	—
	Z-Δ9-i15:1	0.37	—	3.4
	Z-Δ9-i16:1	0.33	—	1.8
	Z-Δ9-16:1	0.32	—	1.2
	Z-Δ9-i17:1	0.27	—	8.6
	Z-Δ9-17:1	0.28	—	0.3
	Z-Δ9-i18:1	ND	—	0.1
	Z-Δ9-18:1	0.25	1.14	0.9
	Z-Δ11-i17:1	ND	—	0.3
	Z-Δ11-18:1	0.33	—	3.5

[a] For LIAB 7 components, <0.05% was not reported; for LIAB 3 components, <0.1% were not reported.

[b] *E* and *Z* are equivalent to *trans* and *cis*, respectively. i, Iso-; a, anteiso-.

[c] FCL were determined on SIL-47-CNP capillary columns.

[d] —, None detected.

[e] LIAB monounsaturates included traces of *Z*-Δ6-i16:1, *Z*-Δ6-a17:1, and *Z*-Δ6-17:1.

[f] ND, Not determined.

two trimethylsilyl groups (see Fig. 1), resulting in a very simple mass spectrum (e.g., Fig. 2) dominated by the two cleavage fragments and *m/e* 73 [$Si(CH_3)_3^+$] (3). The additional fragment ion at *m/e* 185 in Fig. 2 was derived by loss of methanol from the carboxyl fragment (*m/e* 217). Since the two major cleavage ions were of different masses from those of any fragment ions derived from saturated fatty acid methyl esters and since the carboxyl-derived fragments and terminal-derived fragments (Fig. 2) differed in mass by at least 2 atomic mass units, it was possible, in principle, to deduce the distribution of double-bond positional isomers in a sample of the derived ditrimethylsilyl ethers from the mass spectrum of the mixture. Uncorrected single-scan mass spectra of the ditrimethylsilyl ethers of the dihydroxy fatty acid methyl esters derived from the fatty acids of LIAB 7 and LIAB 3 are shown in Fig. 3. After background subtraction and averaging, the relative abundances of individual carboxyl and terminal fragments were calculated. Table 2 lists the results for samples from LIAB 3 and LIAB 7 after normalization to $\Sigma\Delta = 100\%$ and $\Sigma\omega = 100\%$, where Δ and ω define the chain lengths of the carboxyl and terminal fragments, respectively. As no suitable standards were available for comparison, the data are presented uncorrected; it was expected that this assumption would result in errors of ±30% in absolute abundance for individual fragment ions.

The mass spectrum of the sample derived from isolate LIAB 3 showed a predominant ion due to Δ9 unsaturation and major fragment ions at masses corresponding to ω7 and ω8. The major acids thus had unsaturation at Δ9 and ω7 and at Δ9 and ω8, although the data did not allow the assignment of positions of branching or the geometry of the double bond. These acids were thus "Δ9-16:1" and "Δ9-17:1" isomers. GC analysis (FCL data) supported the assignment of *cis* geometry and the presence of predominantly branched fatty acid isomers. Final assignments and abundances are listed in Table 1. The calculated Δ and ω abundances based on the GC analysis are listed in Table 2 for comparison with the MS results. Excellent agreement is observed for all data except the ω6 fragment. Without suitable standards, no explanation for this result can be proposed.

A similar analysis of the sample derived from LIAB 7 revealed the presence of largely Δ5 unsaturation characterized by an intense ion at *m/e* 203 (see Table 2 and Fig. 3). The major terminal fragments were ω11 and ω12, indicating that the predominant monounsaturated fatty acids present in LIAB 7 were "Δ5-16:1" and "Δ5-17:1." The double-bond geometry and fatty acid branching were, again, not determinable from the MS data. GC analysis demonstrated that the major unsaturated fatty acids had the same FCLs and were not of a *cis* configuration. These data indicate that the major unsaturated fatty acids in LIAB 7 were *E*-Δ5 unsaturated (i.e., Δ5-*trans*-acids). The GC identification of the presence of *Z*-Δ9-18:1 is also supported by the presence of Δ9 and ω9 fragment ions at approximately the expected abundances. GC and MS analyses agree extremely well in this case (Table 2). Final confirmation of the double-bond positional isomers present in LIAB 7 was obtained by capillary GC-MS. Figures 4 and 5 illustrate typical mass fragmentograms obtained when the ditrimethylsilyl ethers were analyzed by capil-

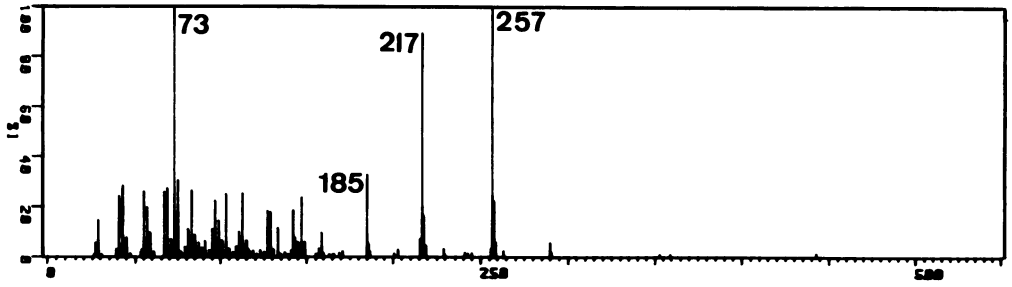
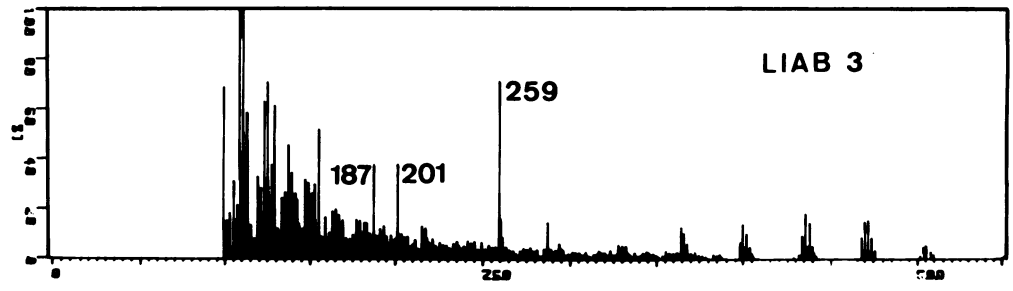


FIG. 2. *Probe mass spectrum of the ditrimethylsilyl ether derived from the Z-Δ6-18:1 fatty acid methyl ester (methyl petroselenate).*



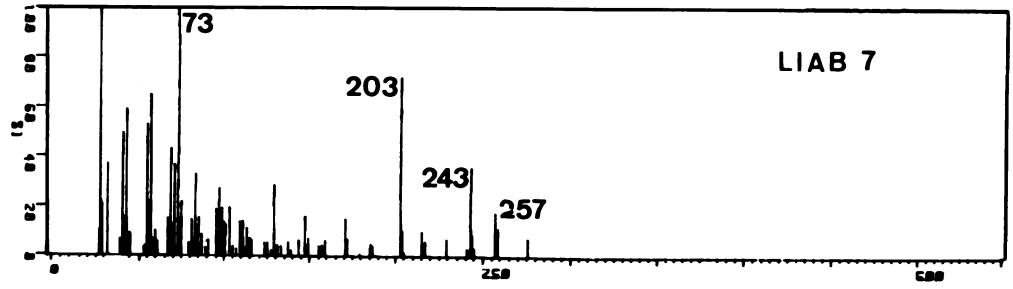


FIG. 3. *Probe mass spectra of the mixtures of ditrimethylsilyl ethers derived by oxidation (OsO_4) and derivatization (bovine serum albumin) of the fatty acid methyl ester mixtures from LIAB 3 and LIAB 7.*

TABLE 2. *Relative percentages of positional isomers of monounsaturated esters by mixed MS and GC analyses*

Isolate	Method of determination	Relative % of:										
		Δ5 (*m/e* 203)	Δ6 (*m/e* 217)	Δ9 (*m/e* 259)	Δ11 (*m/e* 287)	ω6 (*m/e* 173)	ω7 (*m/e* 187)	ω3 (*m/e* 201)	ω9 (*m/e* 215)	ω10 (*m/e* 229)	ω11 (*m/e* 243)	ω12 (*m/e* 257)
LIAB 3	MS	—[a]	—	83	13	—	46	47	7	—	—	—
	GC[b]	—	—	79	18	18	31	43	6	—	—	—
LIAB 7	MS	87	3	10	—	—	1	—	12	7	56	24
	GC[b]	78	5	14	—	—	—	—	14	7	62	16

[a] —, Not observed.
[b] Assignments as in Table 1.

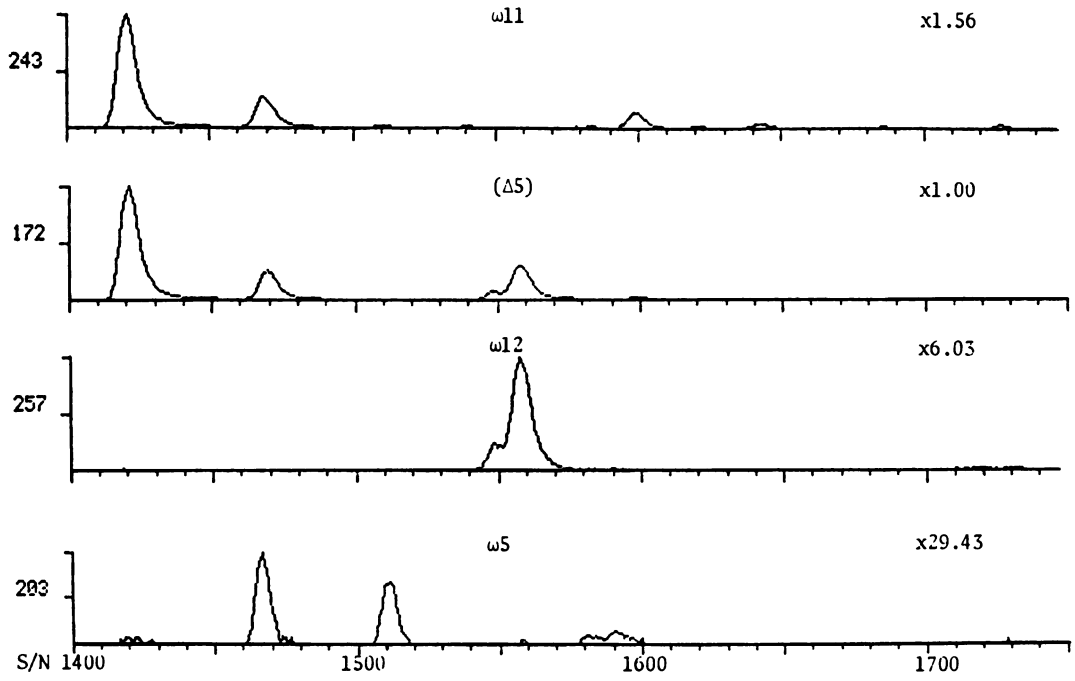


FIG. 4. *Single-ion chromatograms, normalized to the major peak, of m/e 243, m/e 172, m/e 257, and m/e 202 (from LIAB 7), scan numbers (S/N) 1,400 to 1,750. Scan time was 1.0 s. ×1.56, ×1.00, ×6.03, and ×29.43 indicate the relative gains of electron multiplier required to produce the observed chromatograms.*

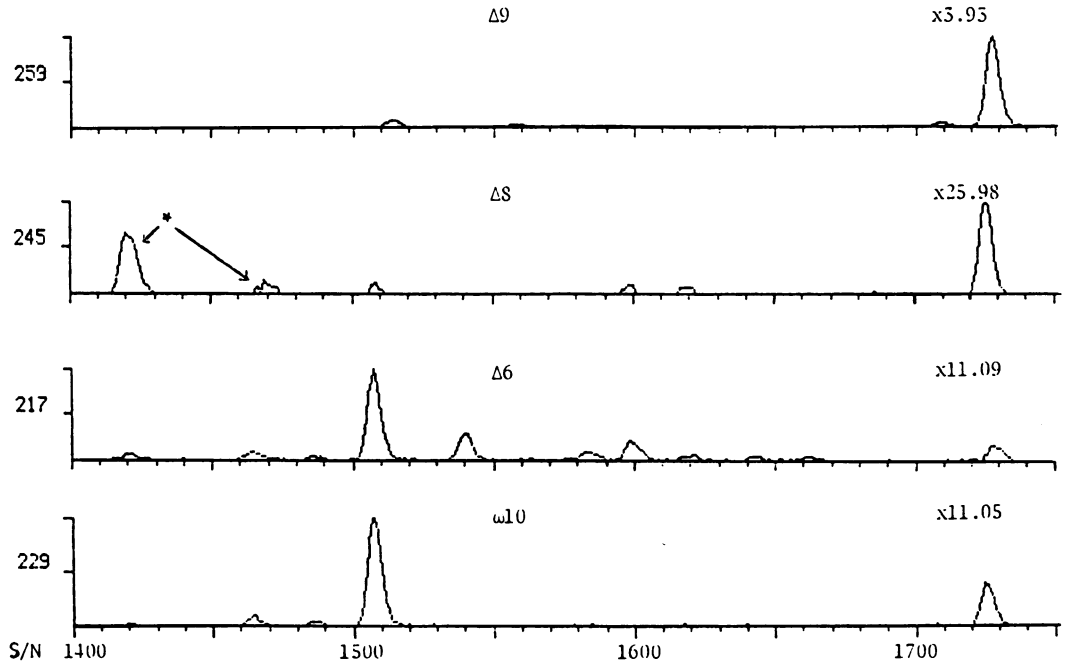


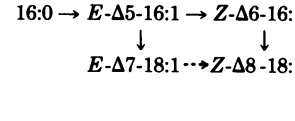
FIG. 5. *Single-ion chromatograms, normalized to major peak, of m/e 259, m/e 245, m/e 217, and m/e 229, scan numbers (S/N) 1,400 to 1,750. Scan time was 1.0 s. ×3.95, ×25.98, ×11.09, and ×11.05 indicate the relative gains of electron multiplier required to produce the observed chromatograms.*

lary GC-MS. Unexpectedly, *m/e* 203 was extremely weak, requiring a relative gain of 29 when compared with a major fragment ion at *m/e* 172. The spectrum of the major component (scan 1,421) is shown in Fig. 6. The major fragment ions were *m/e* 73, *m/e* 243 (as expected for Δ5 and ω11), and *m/e* 172. The latter fragment probably arose via loss of a methoxyl radical from *m/e* 203. This process was not of importance for the other isomers (i.e., other than Δ5 isomers) in LIAB 7, as shown in the cases of Δ8-18:1 and Δ9-18:1 (scan 1,728, Fig. 6), where the demethoxylated fragment ions (at *m/e* 214 and *m/e* 228) were relatively less important than their precursors (*m/e* 245 and *m/e* 259). It is interesting to note that the probe mass spectrum had the expected *m/e* 203 as the dominant fragment ion, not *m/e* 172 as observed in the capillary GC-MS run (compare Fig. 3 and 6).

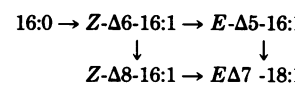
Many minor isomers were identified in LIAB 7 by concordance of the peaks in single-ion chromatograms in the capillary GC-MS run. *m/e* 245 and *m/e* 229 (Fig. 5), for example, both displayed peaks at scan 1,726 of approximately equal intensities. Using these data, the presence of a small amount of the unusual acid Δ8-18:1 was inferred. Similarly, the presence of concordant ions at *m/e* 217 and *m/e* 229 indicated that the initial sample contained Δ6-16:1. Assignment of the double-bond geometry of these acids was based on the original polar column data. Further confirmation of the *trans* geometry for the major isomers was based on the observation that the *erythro*-ditrimethylsilyl ethers of the dihydroxy acids (derived from the *Z* [*cis*]-isomers) elute after the corresponding *threo*-isomer on nonpolar phases (2): traces (<0.5% total fatty acids) of Δ5-isomers were observed eluting approximately 45 s after the predominant isomer in the capillary GC-MS run. These minor components were assigned *erythro* configuration, and, hence, the corresponding monounsaturated fatty acids were of *cis* geometry. The major isomers had *trans* geometry, as previously suggested on the basis of polar capillary column data. Final assignments are listed in Table 1.

Fatty acids of isolates LIAB 3 and LIAB 7. The composition of LIAB 3 was characterized by a preponderance of iso-branched acids with the *cis*-isomers *Z*-Δ7-, *Z*-Δ9-, and *Z*-Δ11-monounsaturated fatty acids being present. The mixture of isomers of the C_{15} and C_{16} acids suggested a typical anaerobic biosynthetic pathway. Many of the iso-branched monounsaturated fatty acids reported here have been previously reported in *desulfovibrio desulfuricans* (3); however, the composition of LIAB 3 differs in containing only iso-branched acids of the same carbon number. The presence of these iso-branched unsaturated fatty acids in an aerobic bacterium questions their use by Boon et al. (2) as specific markers of *Desulfovibrio* spp. in marine sediments.

LIAB 7 contained both iso- and antiiso-branched acids, and largely *trans*-Δ5-monounsaturated fatty acids. *Bacillus megaterium* has been reported to contain predominantly (93%) branched saturated fatty acids with branched- and straight-chain monounsaturated fatty acids (*cis*-Δ5) present in small quantities (4). The fatty acid composition of LIAB 7 was explicable in terms of a *trans*-Δ5 desaturase. The unusual minor components *Z*-Δ6- and *Z*-Δ8-monounsaturated fatty acids were probably derived from the *E*-Δ5 isomers. A stereospecific hydration-dehydration sequence from *E*-Δ5, similar to the conversion from *E*-Δ2 to *Z*-Δ3 in the common anaerobic pathway (10), and subsequent chain elongation to *Z*-Δ8 is possible. Conversely, the observed *Z*-Δ6 and *Z*-Δ8 unsaturated fatty acids may be intermediates in the biosynthesis of the *E*-Δ5, *E*-Δ7 series by analogy with the *Z*-Δ6 desaturase system of higher plants (2 and references therein). In this case, conversion from the *cis*-isomer to the *trans*-acid could occur via the action of an enzyme similar to the *Z*-Δ12/*E*-Δ11 isomerase found in *Butyrivibrio fibrisolvens* (7). If the desaturase is oxygen dependent, then the two pathways will result in different fatty acid compositions under anaerobic growth conditions. As yet, this is only conjecture. The monounsaturated fatty acid composition of LIAB 7 was consistent with the following biosynthetic payway:



or



The presence of Δ6-16:1 in LIAB 7 is of interest, as its occurrence in sediments has been previously explained as resulting from biohydrogenation of fatty acids derived from algal origins (2). The results presented here suggest that the trace of this acid observed may result from direct input, although the associated Δ5-acids in the isolate have not been observed in the sediment analyses (2).

This study has shown that single-ion chromatograms of *m/e* 203 may be unsatisfactory for the detection of the ditrimethylsilyl ether derivatives of the *E*-Δ5-monounsaturated fatty acids. This would readily explain the lack of reports of

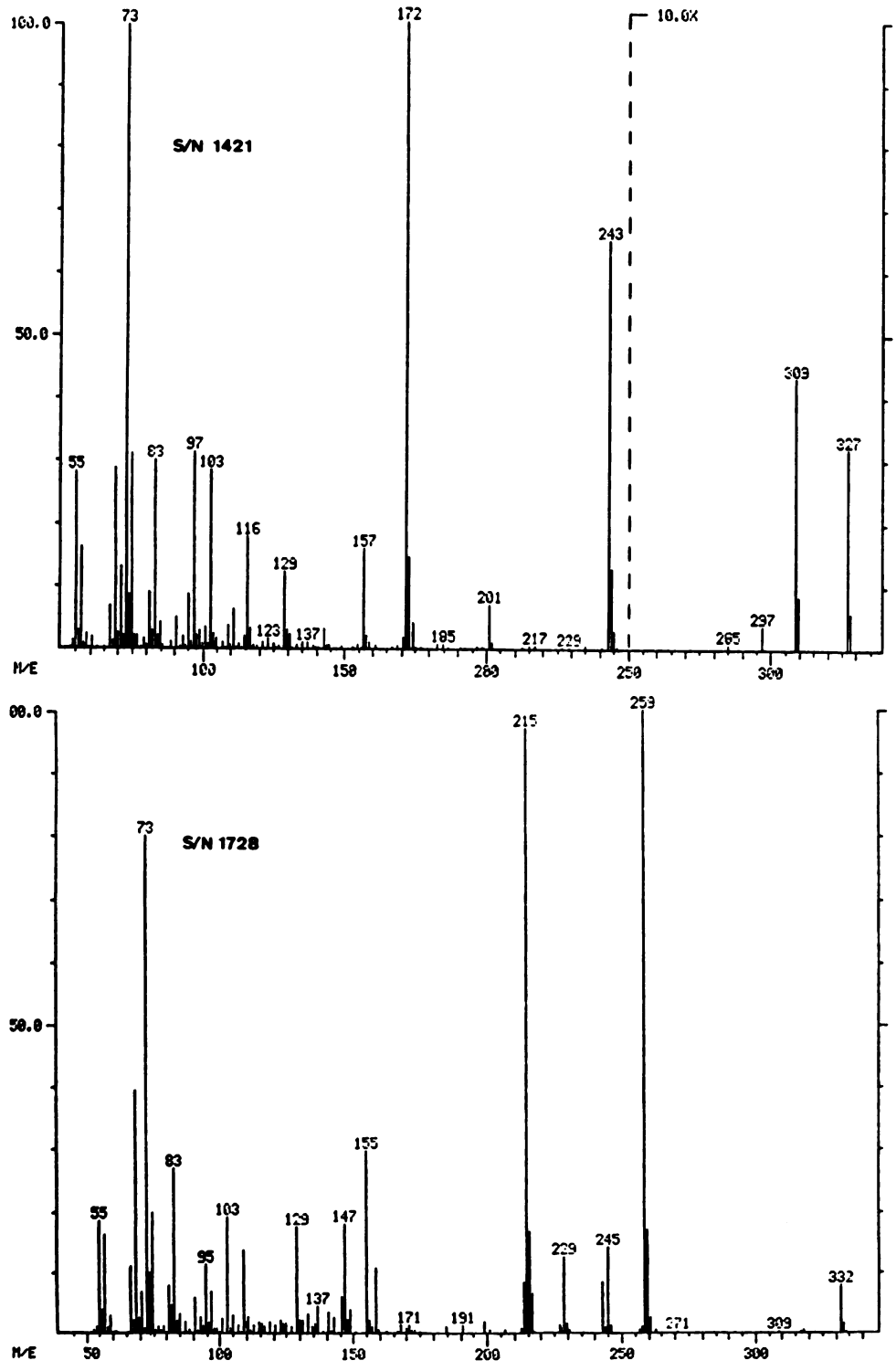


FIG. 6. *Capillary GC-MS mass spectra, scan numbers 1,421 and 1,728. Assignment of ions: m/e 215, ω9; m/e 229, ω10; m/e 243, ω11; m/e 203, Δ5; m/e 217, Δ6; m/e 231, Δ7; m/e 245, Δ8; m/e 259, Δ9.*

these acids in sediments with known high bacterial inputs. Clearly, it would be advantageous to run an additional single-ion chromatogram at *m/e* 172 in any study of sedimentary fatty acids.

The *trans/cis* ratios for LIAB 3 and LIAB 7 were 0.0 and 3.2, respectively. Since *Z*-Δ9-18:1 was identified as a contaminant introduced into our samples from either the culture medium or the analytical reagents, the quantity of this acid biosynthesized by isolate LIAB 7 (or LIAB 3) is probably lower than that observed. *Z*-Δ9-18:1 was the major *cis*-acid in LIAB 7: the *trans/cis* ratio biosynthesized by LIAB 7 is likely to be much higher than 3.2 and could be as high as 7.6. Sedimentary *trans/cis* ratios are commonly less than 0.2 (9, 15; Volkman et al., in press): minor contributions from such bacteria as isolate LIAB 7 could fully explain this level. However, the chain length and positional isomer distribution of the *trans*-monounsaturated fatty acids in LIAB 7 was not typical of the sediments we had previously analyzed. In the light of our earlier results in which we had demonstrated the presence of a variety of *trans*-monounsaturated fatty acids in mixed aerobic bacterial cultures (9), it seemed probable that other bacteria produced different positional isomers of the *trans*-monounsaturates. If bacteria producing *trans*-monounsaturated fatty acids with different positional specificities could be isolated from marine sediments, it would further question the current view that these acids derive largely from biohydrogenation or in situ isomerization. Indeed, these acids may not result from biological or chemical diagenetic processes, but, rather may be useful as chemical markers for a living biomass of specific bacterial species: the *trans*-monounsaturated fatty acids present in sediments could derive principally from bacteria constituting only a small proportion of the total microbial biomass.

ACKNOWLEDGMENTS

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