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Location of Double Bonds in Monounsaturated Fatty Acids of *Campylobacter cryaerophila* with Dimethyl Disulfide Derivatives and Combined Gas Chromatography-Mass Spectrometry

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Location of the double-bond position of monounsaturated fatty acids in *Campylobacter cryaerophila* was accomplished with combined gas chromatography-mass spectrometry analysis of dimethyl disulfide (DMDS) derivatives. The monoenoic fatty acids from whole bacterial cells were converted to methyl esters and then to DMDS adducts and analyzed by capillary gas chromatography-mass spectrometry. The mass spectra of DMDS adducts gave an easily recognizable molecular ion (M^+) and two major diagnostic ions attributable to fragmentation between the two CH_3S groups located at the original site of unsaturation. Two previously unidentified acids that distinguish *C. cryaerophila* from other bacteria were identified as $C_{14:1}\omega7$ and $C_{16:1}\omega7$ from their mass spectral fragmentation patterns. Resolution of *cis* and *trans* isomers by capillary column gas chromatography permitted assignment of the *trans* isomer to the $C_{16:1}\omega7$ acid.

For the past 8 years we have used fused silica capillary columns in our gas chromatography (GC) studies of the cellular fatty acid compositions of microorganisms. With these columns, it is possible to separate most of the long-chain fatty acids (as methyl esters), including positional isomers of monounsaturated acids known to occur in bacteria (2, 4). Often, closely related bacteria can be distinguished by differences in their monounsaturated acid content, as illustrated in a recent study which showed that oleic acid ($C_{18:1}\omega9c$) was a major component of all *Moraxella* species, whereas *cis*-vaccenic acid ($C_{18:1}\omega7c$) was the major acid of *Oligella urethralis* (4). Several relatively novel monounsaturated acids have been detected in other bacteria (3, 5). It has been reported that unidentified 14- and 16-carbon monounsaturated fatty acids are present in *Campylobacter cryaerophila* but absent from other *Campylobacter* species (2). Identification of these acids by combined GC-mass spectrometry (MS) analysis of dimethyl disulfide (DMDS) derivatives to determine double-bond position and geometry is described in this report.

MATERIALS AND METHODS

Preparation of bacterial fatty acids. Strains of *C. cryaerophila* were grown and processed for total cellular fatty acids as described previously (2). The resulting samples of fatty acid methyl esters were analyzed by GC before the DMDS derivatization procedure.

DMDS derivatization procedure. DMDS derivatives of unsaturated FAME were formed by an iodine-catalyzed reaction of DMDS with unsaturated bonds (1, 5). The method used for the formation of the DMDS adducts was identical to that of Nichols et al. (5), except that the reaction temperature was maintained at 35°C for 5 days. Briefly, approximately 50 μl of FAME contained in a standard 1-ml Wheaton vial was treated with 100 μl of DMDS (gold label; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 2 drops of iodine solution (6% [wt/vol] in diethyl ether); the vial was sealed with a crimp-style cap. After 5 days at 35°C the reaction mixture was transferred to a tube (13 by 100 mm) fitted with a Teflon-lined screw-cap lid. The vial was rinsed with 1 ml of hexane, which was then added to the reaction mixture. About 1 ml of 5% (wt/vol) aqueous sodium thiosulfate was added, and the mixture was shaken 20 to 25 times to remove iodine. The organic (hexane) layer was removed to a clean vial, and the aqueous layer was reextracted with hexane. The organic layers that contained FAME and FAME-DMDS adducts were combined and concentrated to 0.3 ml under a gentle stream of nitrogen before analysis by GC or GC-MS.

GC. Separation of FAME and FAME-DMDS adducts was performed with a fused silica cross-linked methyl silicone (OV-1) capillary column (50 m by 0.2 mm [inner diameter]) installed in a model 5880A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard Co., Avondale, Pa.). The column oven temperature was programmed from 170 to 290°C at 5.5°C/min, with hydrogen as the carrier gas at a flow rate of 0.6 ml/min. The injector temperature was maintained at 285°C, and the detector temperature was maintained at 300°C. Samples were injected in the split mode (1:50). Tentative peak identification of FAME was based on a comparison of retention times to those of standards from Supelco, Inc., Bellefonte, Pa. The length of the carbon chains and the number of double bonds in the unsaturated FAME samples were confirmed by hydrogenation and MS (3).

GC-MS. GC-MS analyses of FAME-DMDS adducts were performed on a model 4500 mass spectrometer equipped with a Superincos Data System (Finnigan MAT, San Jose, Calif.). Separation was done with a DB-5 capillary column (60 m by 0.25 mm [inner diameter]; J&W Scientific, Folsom, Calif.) which was interfaced directly into the ion source. Samples (1 μl) were injected in the splitless mode and vented from the injector after 0.5 min. The column temperature of 90°C at injection was maintained for 2 min and then programmed to 280°C at 25°C/min. The operating parameters of the mass spectrometer were as follows: electron multiplier,

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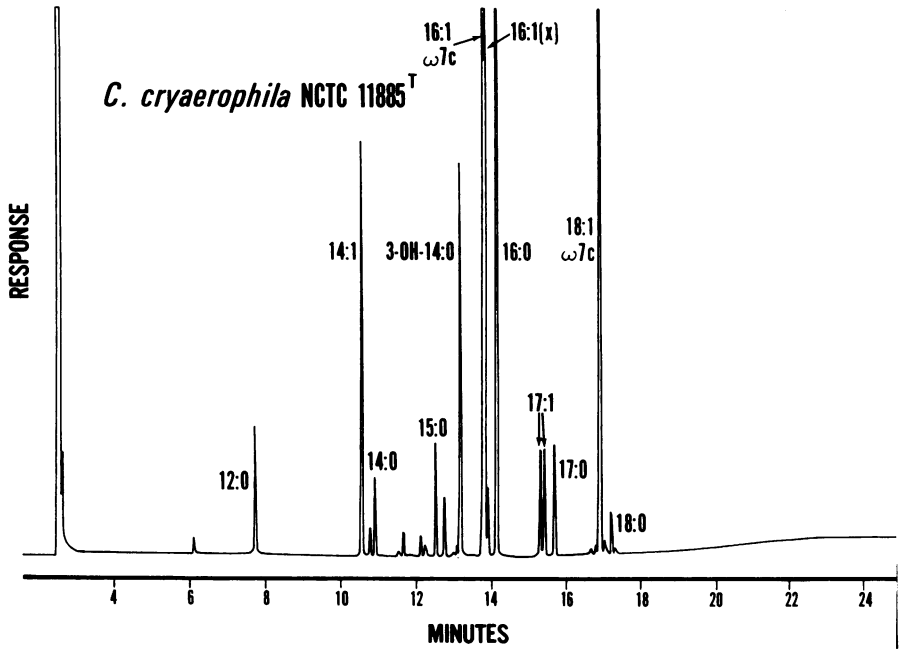

FIG. 1. Gas chromatogram of methylated fatty acids of *C. cryaerophila*. Fatty acids are represented by shorthand designations: number before the colon = number of carbon atoms; number after the colon = number of double bonds; ω7c = double-bond position from the hydrocarbon end of the *cis* isomer; (*x*) = double-bond position unknown; 3-OH = hydroxy group at carbon 3.

1,500 V; electron impact energy, 70 eV; source temperature, 200°C.

RESULTS AND DISCUSSION

C. cryaerophila contains two monounsaturated fatty acids that are absent in other *Campylobacter* species (2). These two acids [designated $C_{14:1}$ and $C_{16:1}(x)$], along with the other cellular acids (as methyl esters) of this organism, are shown in the chromatogram in Fig. 1. The length of the carbon chains and the number of double bonds for $C_{14:1}$ and $C_{16:1}(x)$ FAME as well as all other FAME were established by MS (3). Tentative assignments of double-bond position and geometry (*cis* or *trans*) to $C_{16:1}\omega7c$, $C_{16:1}\omega7t$ [16:1(*x*)], and $C_{18:1}\omega7c$ acids were made by comparing retention times with those of authentic standards. None of the available $C_{14:1}$ FAME standards, however, had a retention time that exactly matched that of the $C_{14:1}$ acid in *C. cryaerophila*.

Treatment of the FAME sample with DMDS as described above resulted in the addition of two CH_3S groups at the site of unsaturation. Upon subsequent GC analysis with the same column temperature program as that used for FAME, all peaks for monounsaturated FAME disappeared and a new peak for each FAME-DMDS adduct appeared later in the chromatogram. For example, the $C_{14:1}$ FAME peak at 10.5 min in Fig. 1 disappeared and a new peak for the DMDS adduct of this compound appeared at 19.5 min. Similar chromatographic results were obtained with the DB-5 column, as illustrated in Fig. 2, which shows the GC-MS total-ion chromatogram of the DMDS-treated FAME sample from *C. cryaerophila*. With the DB-5 column, FAME-DMDS adducts of $C_{14:1}$, $C_{16:1}$, and $C_{18:1}$ acids eluted after the saturated 16-carbon ($C_{16:0}$) FAME (Fig. 2).

The mass spectrum of the FAME-DMDS adduct of the $C_{16:1}(x)$ acid is shown in Fig. 3. The relative abundance of the molecular ion (M^+) at *m/e* 362 was approximately 10% of the base peak ion (*m/e* = 217). The two major ions at *m/e* 145 and *m/e* 217 represented fragmentation between the two CH_3S groups located at the original site of unsaturation in the molecule. The *m/e* 145 ion was the aliphatic fragment (ω), and the *m/e* 217 ion was the fragment containing the carboxyl end (△) of the molecule. The third major ion at *m/e* 185 resulted from the loss of methanol (CH_3OH) from the △ fragment (*m/e* 217 − 32 = *m/e* 185). This fragmentation pattern clearly showed the location of the double bond in $C_{16:1}(x)$ to be at the ω7 (or △9) position. Assignment of *cis* or *trans* geometry to $C_{16:1}\omega7$ was not possible by MS because the FAME-DMDS peak for $C_{16:1}\omega7c$ (which immediately preceded $C_{16:1}\omega7$ in Fig. 2) gave a fragmentation pattern identical to that shown in Fig. 3. However, discrimination between *cis* and *trans* geometry was possible by GC because the *cis* isomer eluted before the *trans* isomer for both the FAME derivative (Fig. 1) and the FAME-DMDS adduct (Fig. 2). Thus, the combined data from GC and GC-MS clearly established that the previously unidentified 16-carbon monounsaturated acid [16:1(*x*)] in *C. cryaerophila* was $C_{16:1}\omega7t$. The identity of the $C_{18:1}$ acid shown in Fig. 1 was also confirmed as $C_{18:1}\omega7c$ by these combined procedures.

The mass spectrum of the DMDS adduct of the $C_{14:1}$ FAME is shown in Fig. 4. The major ions at *m/e* 145 and *m/e* 189 were produced from fragmentation between the two CH_3S groups and showed the location of the double bond to be at the ω7 (or △7) position. The base peak ion at *m/e* 157 resulted from the loss of methanol from the △ fragment (*m/e* 189 − 32 = *m/e* 157). The molecular ion at *m/e* 334 was easily recognizable. Assignment of *cis* or *trans* geometry was not possible at this time because of the lack of $C_{14:1}\omega7$ reference standards.

Both $C_{14:1}\omega7$ and $C_{16:1}\omega7t$ are important marker fatty acids for distinguishing *C. cryaerophila* from other *Campylobacter* species and from other bacteria. For more than 150 bacterial species examined in this laboratory, the $C_{14:1}\omega7$

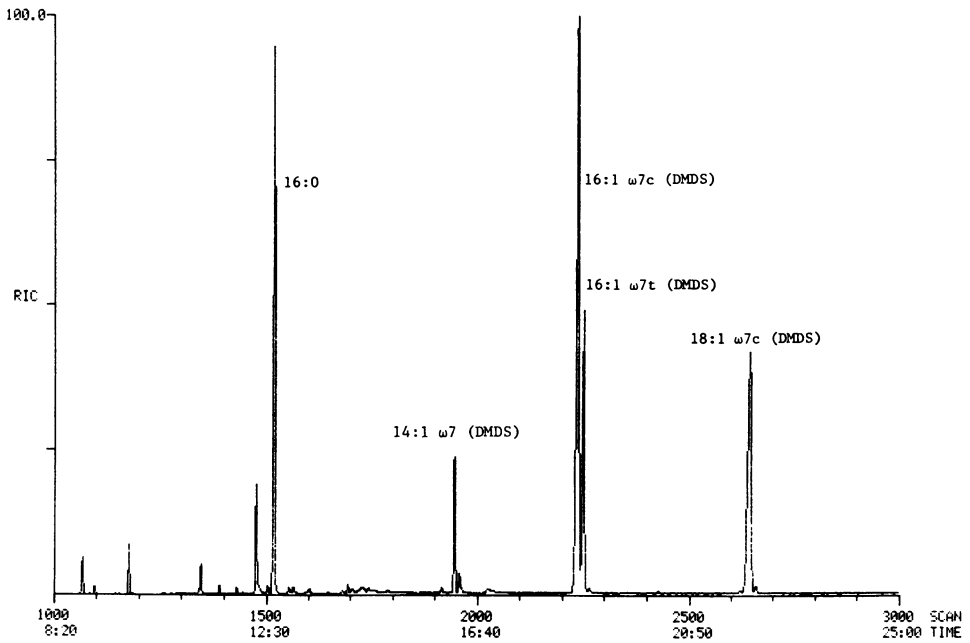

FIG. 2. Total-ion chromatogram of DMDS adducts from *C. cryaerophila* monounsaturated fatty acids. See the legend to Fig. 1 for shorthand designations of FAME.

acid has been detected in percentages greater than 1% in only 6 species (3 *Actinobacillus* species, 2 *Pastuerella* species, and *Pseudomonas diminuta*). Each of these six species contained from 1 to 2% $C_{14:1}\omega7$ and no $C_{16:1}\omega7t$, while *C. cryaerophila* contained 4% $C_{14:1}\omega7$ and 15% $C_{16:1}\omega7t$ (2). The high percentage (15%) of *trans* $C_{16:1}\omega7$ is another unique feature of *C. cryaerophila*, since most bacteria contain monounsaturated acids of the *cis* configuration, with few if any *trans* isomers. Reproducible separation with the GC capillary column and accurate calculation of equivalent chain lengths with the computer permit accurate resolution of geometric isomers. The observed difference in retention time between $C_{16:1}\omega7c$ and $C_{16:1}\omega7t$ in Fig. 1 was 5 s, and the equivalent chain length values were 15.818 ± 0.003 and 15.854 ± 0.003, respectively.

Use of the DMDS derivatization procedure followed by GC-MS analysis provides a simple and rapid method for determining monounsaturated double-bond positions. The

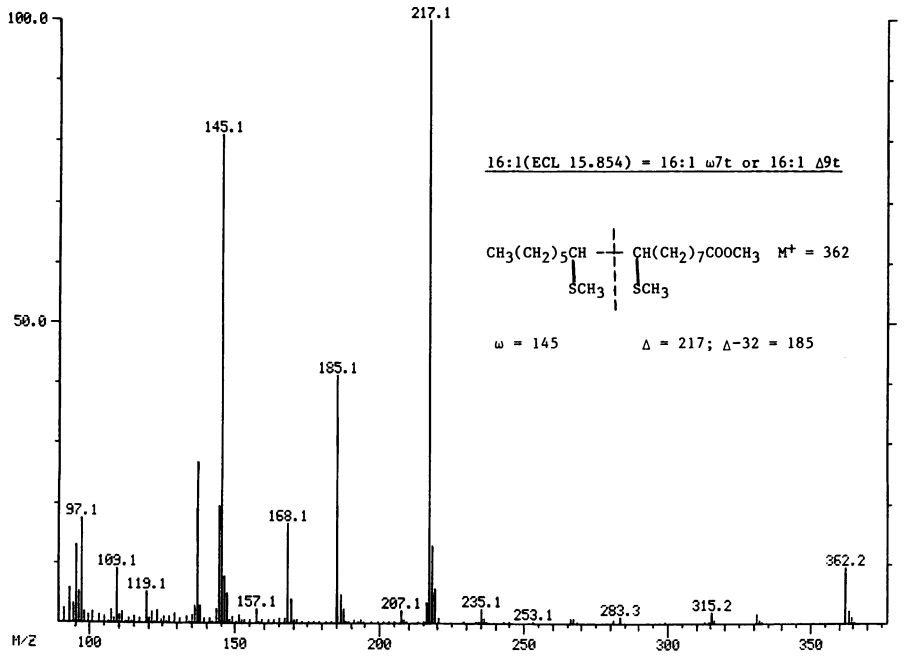

FIG. 3. Mass spectrum of the DMDS adduct of the $C_{16:1}\omega7t$ FAME from *C. cryaerophila*. The ω fragment is the aliphatic end of the molecule, and the Δ fragment is the carboxylic end of the molecule. M^+ = molecular ion; ECL = equivalent chain length value.

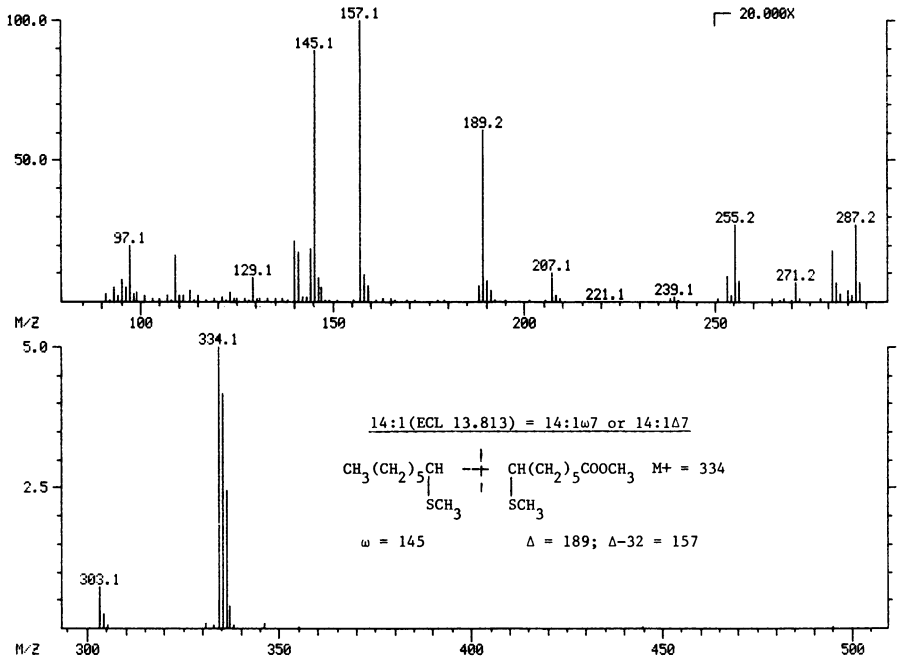

FIG. 4. Mass spectrum of the DMDS adduct of the $C_{14:1}\omega7$ FAME from *C. cryaerophila*. See the legend to Fig. 3 for explanations of ω, △, M^+, and ECL.

reaction is specific for both *cis* and *trans* isomers, yielding one adduct for each, with the *cis* isomer eluting before the *trans* isomer on nonpolar capillary columns. The mass spectra yield easily recognizable key fragments, including the molecular ion (M^+), the diagnostic ω and △ fragment ions, and the △ − 32 ion (1). Several other unusual monounsaturated acids that have been identified in other bacteria through routine application of this procedure will be the subjects of future reports.

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