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Determination of Monounsaturated Double-Bond Position and Geometry in the Cellular Fatty Acids of the Pathogenic Bacterium *Francisella tularensis*

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The nonhydroxy fatty acid composition of *Francisella tularensis* is reported in detail. The double-bond configuration of the monounsaturated acids has been determined by capillary gas chromatography-mass spectrometry of the derivatized fatty acids. The monounsaturated fatty acids detected, in decreasing order of abundance, were 24:1ω15c, 18:1ω9c, 22:1ω13c, 20:1ω11c, 16:1ω7c, 26:1ω17c, and 14:1ω7c. The fatty acid profile found in *F. tularensis*, in particular the double-bond positions, represents a valuable taxonomic characteristic of this pathogenic bacterium.

Analysis of fatty acids has been used widely in the fields of microbial ecology (1, 4, 16), organic geochemistry (12–14), and medical microbiology (2, 10). The necessity for precise structural determinations (positional and geometrical isomers) of key biological marker fatty acids has been stressed (11). Although isomer separation and characterization techniques have been known for some time, microbiologists and organic geochemists have rarely applied them to sediment or microorganism extracts. Two relatively simple methods for the determination of fatty acid double-bond position and geometry in extracts of microorganisms and sediments have recently been reported (8; P. D. Nichols, Ph.D. dissertation, University of Melbourne, Australia, 1983; P. D. Nichols, P. M. Shaw, and R. B. Johns, J. Microbiol. Methods, in press).

In this report we present data obtained from the use of these derivatization procedures with the cellular nonhydroxy fatty acids of the etiological agent of tularemia in humans and animals, *Francisella tularensis*. The cellular fatty acids of this bacterium have been previously reported (6). In addition to nonhydroxy acids of 10, 12, 14, 16, and 18 carbon atoms, saturated and monounsaturated nonhydroxy fatty acids (C_{20} to C_{26}) accounted for significant proportions of the fatty acid profile (6). Double-bond positions were not, however, determined in the previous report.

MATERIALS AND METHODS

The bacterium analyzed in this study, originally isolated on *Legionella* medium from a clinical specimen, had been submitted to one of us (W.R.M.) as a presumptive *Legionella* sp. The organism was grown for 72 h on plates of buffered charcoal yeast extract agar containing α-ketoglutarate, cysteine, and ferric pyrophosphate (3). Cells were harvested and extracted, and the nonhydroxy and hydroxy esters were separated as described previously (9). The extractable cellular fatty acid profile agreed closely with that reported by Jantzen et al. (6) for strains of *F. tularensis* rather than with that for known *Legionella* spp. Subsequent fluorescent antibody testing by W. F. Bibb at the Centers for Disease Control, Atlanta, Ga., showed a strong positive reaction with anti-*F. tularensis* conjugate (H. W. Wilkinson, personal communication).

Initial identification of individual components was performed by high-resolution capillary gas chromatography (GC) with a Hewlett Packard 5880A gas chromatograph equipped with a flame ionization detector. Fatty acid methyl esters were injected at 50°C in the splitless mode on a nonpolar cross-linked methyl silicone fused silica capillary column (50 m by 0.2 mm [inner diameter]; Hewlett Packard). The oven was temperature programmed from 50 to 160°C at 5°C per min and then at 2°C per min to 300°C. Hydrogen was used as the carrier gas (1.0 ml/min). Uniform response has been assumed for individual components, and fatty acid compositional data are expressed as percentage of the total fatty acids.

GC-mass spectrometric (MS) analyses were performed on a Hewlett Packard 5995A system fitted with a direct capillary inlet. The same column type as the one described above was used for the analyses. Fatty acid methyl esters were injected in the splitless mode at 100°C, and the oven was programmed from 100 to 300°C at 40°C per min. Hydrogen was used as the carrier gas (0.7 ml/min). MS operating parameters (peak finder mode) were as follows: electron multiplier, 1,600 V; transfer line, 300°C; source and analyzer, 250°C, autotune file DFTPP normalized; optics tuned at *m/z* 502; MS peak detect threshold of 300 triggered on total ion abundance; electron impact energy, 70 eV.

Determination of double-bond configuration. Double-bond configuration was determined by capillary GC-MS analysis of the derivatives formed by reaction of the fatty acid methyl esters with (i) either Fenton or Woodward reagent, resulting in *trans*- and *cis*-dihydroxylation, respectively (8); or (ii) the diene 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene, to form Diels-Alder adducts of the unsaturated esters. Woodward reagent (silver acetate and iodine in acetic acid) produced *cis*-hydroxylations, and Fenton reagent (hydrogen peroxide and ferrous sulfate in acetic acid) produced *trans*-hydroxylations of the unsaturated fatty acid esters. These were analyzed by GC-MS as the trimethylsilyl (TMS) or trifluoroacetyl (TFA) derivatives. The Diels-Alder adducts were prepared by addition of the diene to the fatty acid methyl ester. Detailed descriptions of these derivatization procedures have been reported elsewhere (7; Nichols, Ph.D.

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TABLE 1. Monounsaturated fatty acids of *F. tularensis*, characteristic ion fragments, and GC behavior of derivatized dihydroxylation products and Diels-Alder adducts

Parent acid[a]	Ion fragments (*m/z*)					Equivalent chain length[b] of:					
	Dihydroxy-trimethylsilyl ether		Diels-Alder adducts			Woodward product			Fenton product		
	ω-Fragment	Δ-Fragment	[M-HCl]$^+$	ω-Fragment	Δ-Fragment	TMS	TFA	ΔECL[c]	TMS	TFA	ΔECL
14:1ω7[d]	—	—	—	—	—	—	—	—	—	—	—
16:1ω7	187	259	495	337	409	19.6	16.5	3.1	19.4	16.6	2.8
18:1ω9	215	259	523	365	409	21.4	18.3	3.1	21.3	18.4	2.9
18:1ω7	187	287	523	337	437	21.5	18.4	3.1	21.4	18.5	2.9
19:1ω9	215	273	—	—	—	—	—	—	—	—	—
19:1ω6	173	—	—	—	—	—	—	—	—	—	—
20:1ω11	243	259	551	393	409	23.2	20.0	3.2	23.1	20.1	3.0
22:1ω13	271	259	579	421	409	25.1	21.8	3.3	25.0	22.0	3.0
24:1ω15	299	259	607	449	409	27.2	23.7	3.5	27.0	23.8	3.2
Branched											
25:1ω15	299	273	—	—	—	—	—	—	—	—	—
26:1ω17	327	259	—	—	—	—	—	—	—	—	—

[a] All double bonds were determined to be *cis* configuration. Small amounts of *trans* 18:1ω9 and *trans* 24:1ω15 were also detected.
[b] ECL, Equivalent chain length (Hewlett Packard 50 m by 0.2 mm [inner diameter] cross-linked methyl silicone fused silica capillary column). Products were formed by Woodward and Fenton reactions, respectively (8).
[c] ΔECL, Difference in equivalent chain length between the two derivatives (TMS − TFA).
[d] Double-bond position determined by GC retention time data alone. Insufficient sample was available for GC-MS analysis.

dissertation; Nichols et al., in press). GC-MS analysis of the bis-trimethylsilyl ether derivatives of the dihydroxy fatty acids showed major ions attributable to fragmentation between derivatized hydroxyl groups (Table 1). Equivalent chain length values of TMS and TFA derivatives (8) were calculated to determine double-bond geometry (Table 1). Mass spectra of monounsaturated fatty acid Diels-Alder adducts were characterized by prominent ion fragments at [M-HCl]$^+$, with Δ-fragmentation (carboxyl end of the molecule) and ω-fragmentation (aliphatic end of the molecule) (7; Nichols, Ph.D. dissertation; Nichols et al., in press). Characterization of the geometrical isomers from the Diels-Alder adducts is due to the separation of the *trans*- and *cis*-derived adducts on the capillary column. Further confirmation is provided by the differing ratios of the ion fragments noted above (7; Nichols, Ph.D. dissertation; Nichols et al., in press). The methodology described here is suitable for the definitive assignment of fatty acid double-bond configuration in clinical isolates as analyzed in this study and for confirmation of double-bond assignments in more complex environmental sedimentary samples.

RESULTS AND DISCUSSION

Monounsaturated acids and characteristic ion fragments obtained by using the two derivatization procedures are listed in Table 1. The monounsaturated fatty acids detected in *F. tularensis*, in decreasing order of abundance, were 24:1ω15c, 18:1ω9c, 22:1ω13c, 20:1ω11c, 16:1ω7c, 26:1ω17c, and 14:1ω7c. Saturated fatty acids and trace amounts of other monounsaturated fatty acids were also detected (Table 2).

The phospholipids of *F. tularensis* are unusual, as they contain high proportions of both monounsaturated and hydroxyl fatty acids (6). As shown in this study, the position of the unsaturation in the monoenoic fatty acids is still more unusual, producing a phospholipid fatty acid profile sufficiently unique to serve as a signature for this pathogenic bacterium in environmental samples.

The long-chain monounsaturated fatty acids of *F. tularensis* contain the double bond at the Δ^9 position. These data suggest that chain elongation is occurring before desaturation of the fatty acids in *F. tularensis*, in contrast to that reported for *Mycobacterium smegmatis* (5, 15). In *M. smegmatis*, it was proposed that the long-chain monounsaturated fatty acids (22:1ω9, 24:1ω9, and 26:1) originate from 18:1Δ9 followed by subsequent C-2 chain elongation (5, 15).

It is of interest to note that the unsaturated fatty acids of *Legionella* spp. are also reported to be of the *cis* Δ^9 family (8).

The unique distribution of fatty acids in *F. tularensis*, particularly when confirmation of double-bond configuration

TABLE 2. Fatty acid composition of *F. tularensis*

Acid	Composition (%)
10:0	0.2
12:0	0.2
iso 14:0	0.2
14:1ω7c	0.1
14:0	6.7
iso 15:0	0.1
anteiso 15:0	0.7
15:0	0.6
iso 16:0	0.8
16:1ω7c	0.9
16:0	19.1
iso 17:0	tr[a]
anteiso 17:0	0.7
17:0	0.3
18:1ω9c	8.1
18:1ω7c	1.0
18:0	8.4
20:1ω11c	1.4
20:0	6.2
22:1ω13c	3.2
22:0	18.3
24:1ω15c	9.9
24:0	10.3
26:1ω17c	0.3
26:0	0.5
Others[b]	0.7

[a] tr, Trace; less than 0.1%.
[b] Included small amounts of branched 15:1, 17:1, 19:1, 25:1, 21:0, 23:0, 25:0, 18:1ω9t, and 24:1ω15t.

has been achieved, represents a valuable taxonomic signature of this pathogenic bacterium.

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