

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

PETER D. NICHOLS,^{1*} WILLIAM R. MAYBERRY,² CHRISTOPHER P. ANTWRORTH,¹ AND DAVID C. WHITE¹

Department of Biological Science, The Florida State University, Tallahassee, Florida 32306,¹ and Department of Microbiology, Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614²

Received 18 October 1984/Accepted 17 January 1985

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 24:1 ω 15c, 18:1 ω 9c, 22:1 ω 13c, 20:1 ω 11c, 16:1 ω 7c, 26:1 ω 17c, and 14:1 ω 7c. 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 (C₂₀ to C₂₆) 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.

* Corresponding author.

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	Δ ECL ^c	TMS	TFA	Δ ECL
14:1 ω 7 ^d	—	—	—	—	—	—	—	—	—	—	—
16:1 ω 7	187	259	495	337	409	19.6	16.5	3.1	19.4	16.6	2.8
18:1 ω 9	215	259	523	365	409	21.4	18.3	3.1	21.3	18.4	2.9
18:1 ω 7	187	287	523	337	437	21.5	18.4	3.1	21.4	18.5	2.9
19:1 ω 9	215	273	—	—	—	—	—	—	—	—	—
19:1 ω 6	173	—	—	—	—	—	—	—	—	—	—
20:1 ω 11	243	259	551	393	409	23.2	20.0	3.2	23.1	20.1	3.0
22:1 ω 13	271	259	579	421	409	25.1	21.8	3.3	25.0	22.0	3.0
24:1 ω 15	299	259	607	449	409	27.2	23.7	3.5	27.0	23.8	3.2
Branched											
25:1 ω 15	299	273	—	—	—	—	—	—	—	—	—
26:1 ω 17	327	259	—	—	—	—	—	—	—	—	—

^a All double bonds were determined to be *cis* configuration. Small amounts of *trans* 18:1 ω 9 and *trans* 24:1 ω 15 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 Δ ECL. 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 24:1 ω 15c, 18:1 ω 9c, 22:1 ω 13c, 20:1 ω 11c, 16:1 ω 7c, 26:1 ω 17c, and 14:1 ω 7c. 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 desatura-

tion 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 (22:1 ω 9, 24:1 ω 9, and 26:1) originate from 18:1 Δ 9 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
14:1 ω 7c	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
16:1 ω 7c	0.9
16:0	19.1
iso 17:0	tr ^a
anteiso 17:0	0.7
17:0	0.3
18:1 ω 9c	8.1
18:1 ω 7c	1.0
18:0	8.4
20:1 ω 11c	1.4
20:0	6.2
22:1 ω 13c	3.2
22:0	18.3
24:1 ω 15c	9.9
24:0	10.3
26:1 ω 17c	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, 18:1 ω 9t, and 24:1 ω 15t.

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

ACKNOWLEDGMENTS

This work was supported by contracts N0014-82-C0404 and N0014-83-K0056 from the Department of the Navy, Office of Naval Research.

LITERATURE CITED

1. Bobbie, R. J., and D. C. White. 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. *Appl. Environ. Microbiol.* **39**:1212-1222.
2. Calhoon, D. A., W. R. Mayberry, and J. Slots. 1981. Cellular fatty acids and soluble protein composition of *Actinobacillus actinomycetemcomitans* and related organisms. *J. Clin. Microbiol.* **14**:376-382.
3. Edelstein, P. H. 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* **14**:298-303.
4. Federle, T. W., M. A. Hullar, R. J. Livingston, D. A. Meeter, and D. C. White. 1983. Spatial distribution of biochemical parameters indicating biomass and community composition of microbial assemblages in estuarine mud flat sediments. *Appl. Environ. Microbiol.* **45**:58-63.
5. Hung, J. G. C., and R. W. Walker. 1970. Unsaturated fatty acids of mycobacteria. *Lipids* **5**:720-722.
6. Jantzen, E., B. P. Berdal, and T. Omland. 1979. Cellular fatty acid composition of *Francisella tularensis*. *J. Clin. Microbiol.* **10**:928-930.
7. Kidwell, D. A., and K. Blemann. 1982. Determination of double bond position and geometry of olefins by mass-spectrometry of their Diels-Alder adducts. *Anal. Chem.* **54**:2462-2465.
8. Mayberry, W. R. 1984. Relatively simple methodology for the determination of configuration of unsaturation of bacterial monounsaturated fatty acids: application to the unsaturates of *Legionella* spp. *J. Microbiol. Methods* **2**:177-187.
9. Mayberry, W. R. 1984. Monohydroxy and dihydroxy fatty acid composition of *Legionella* species. *Int. J. Syst. Bacteriol.* **34**:321-326.
10. Mayberry, W. R., D. W. Lambe, Jr., and K. P. Ferguson. 1982. Identification of *Bacteroides* species by cellular fatty acid profiles. *Int. J. Syst. Bacteriol.* **32**:21-27.
11. Perry, G. J., J. K. Volkman, R. B. Johns, and H. J. Bavor. 1979. Fatty acids of bacterial origin in contemporary marine sediments. *Geochim. Cosmochim. Acta* **43**:1715-1725.
12. Smith, D. J., G. Eglinton, and R. J. Morris. 1983. The lipid chemistry of an interfacial sediment from the Peru continental shelf: fatty acids, alcohols, aliphatic ketones and hydrocarbons. *Geochim. Cosmochim. Acta* **47**:2225-2232.
13. Van Vleet, E. S., and J. G. Quinn. 1979. Early diagenesis of fatty acids and isoprenoid alcohols in estuarine and coastal sediments. *Geochim. Cosmochim. Acta* **43**:289-303.
14. Volkman, J. K., and R. B. Johns. 1977. The geochemical significance of positional isomers of unsaturated acids from an intertidal zone sediment. *Nature (London)* **267**:693-694.
15. Walker, R. W., H. Baraket, and J. G. C. Hung. 1970. The positional distribution of fatty acids in the phospholipids and triglycerides of *Mycobacterium smegmetis* and *M. bovis* BCG. *Lipids* **5**:684-691.
16. White, D. C., R. J. Bobbie, J. S. Nickels, S. D. Fazio, and W. M. Davis. 1980. Non-selective biochemical methods for the determination of fungal mass and community structure in estuarine detrital microflora. *Bot. Mar.* **23**:239-250.