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Dihydroxy and Monohydroxy Fatty Acids in *Legionella pneumophila*

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Five strains of *Legionella pneumophila* were examined for the presence of hydroxy fatty acid. The cellular distribution of the fatty acids was also determined, as was the variation of hydroxy acid production on five growth media. The strains tested all produced approximately 5 mol% of hydroxy fatty acid, most of which was found in the nonextractable, alkali-stable, acid-labile (wall-associated, amide-linked) fraction. Three major hydroxy acids were found, along with several minor components. The major hydroxy acids were analyzed by thin-layer chromatography, gas-liquid chromatography, mass spectrometry, and infrared spectrophotometry. These compounds were tentatively identified as 3-hydroxy-12-methyltridecanoate, 3-hydroxy-*n*-eicosanoate, and a novel dihydroxy acid, 2,3-dihydroxy-12-methyltridecanoate. The total amount of hydroxy acid produced, as well as the profile of the hydroxy acids, remained relatively unchanged with respect to strain and growth medium.

Legionella pneumophila has been designated the type species of the genus *Legionella* (2), which includes a number of fastidious organisms characterized by several properties, including fatty acid profiles comprised primarily of branched-chain fatty acids.

The fatty acid profiles of *L. pneumophila*, other members of the genus, and certain *Legionella*-like aquatic forms have been reported by Moss and co-workers (1-3, 6-8, 15, 17, 19, 20). Moss et al. have examined a large number of strains of *L. pneumophila* grown on several kinds of media and shown that the fatty acid profiles are relatively constant with respect to the species and are not strongly affected by growth medium (16). Finnerty et al. (5) have examined and characterized the lipids of several strains of *L. pneumophila*. Wong et al. (23) have analyzed the lipopolysaccharide of the organism.

In none of these reports is the presence of hydroxy acids mentioned, except as a comment that such compounds were not found, a surprising result in light of the fact that *L. pneumophila* and other members of the genus are gram-negative organisms and therefore might be expected to contain the hydroxylated compounds. An unidentified fatty acid, with chromatographic behavior suggesting hydroxylation, was reported by Finnerty et al. (5).

This report details the determination of the existence, identification, cellular distribution, and variation of production of low but significant levels of hydroxy fatty acids, including a novel dihydroxy fatty acid, in five strains of *L. pneumophila*.

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MATERIALS AND METHODS

Organisms. Five strains of *Legionella pneumophila* were obtained from the Biological Products Division of the Centers for Disease Control, Atlanta, Ga. The strains, including their Centers for Disease Control catalog numbers and, where applicable, the American Type Culture Collection number, were as follows: Philadelphia 1, BC1636, ATCC 33152; Knoxville 1, BC1640, ATCC 33153; Togus 2, BC1637; Bloomington 3, BC1638; and Los Angeles 1, BC1639, ATCC 33156. These strains represent four of the several known serogroups of *L. pneumophila* and include the type strain of the species. Strains were maintained by weekly subculture on charcoal-yeast extract agar plates and were tested periodically with specific fluorescent antisera obtained from the Centers for Disease Control.

Media and growth conditions. Charcoal-yeast extract (CYE) agar was prepared according to Feeley et al. (4). Yeast extract (YE) agar was prepared by the same procedure, with the omission of charcoal. Other solid media were prepared according to the directions of the manufacturer and supplemented with cysteine hydrochloride and ferric pyrophosphate to the levels suggested by Feeley et al. (4).

In general, suspensions were made in physiological saline, to a turbidity approximately that of a McFarland no. 1 standard, of 48- to 72-h growth from a

CYE plate. Plates of various media were inoculated with 0.2 ml of suspension spread with sterile glass rods. Plates were incubated at 37°C in a candle jar atmosphere.

To investigate possible variation of hydroxy fatty acid production with respect to strain or growth medium, two plates each of CYE, YE, Trypticase soy, brain heart infusion, and Mueller-Hinton agars were inoculated with each strain and incubated for 72 h.

To evaluate the possible variation in hydroxy fatty acid production as a function of strain or culture age, four plates of CYE agar were inoculated with each strain. After 48 h of incubation, two plates of each strain were removed for harvesting, and the remaining plates were returned to incubation for another 120 h.

To obtain sufficient material to examine the cellular distribution of fatty acids, six plates of CYE agar were inoculated with each strain and incubated for 72 h. Cells were harvested by scraping into distilled water and centrifuging at 2,600 × *g* for 30 min at 4°C. Pellets were washed once by suspension in and centrifugation from 15 ml of distilled water.

Hydrolysis and determination of total nonhydroxy and hydroxy fatty acids. Pellets from the medium variation and culture age variation series were treated with 2.5 ml of 2 N HCl, after addition of internal standards, and hydrolyzed overnight at 100°C in tubes with Teflon-lined screw caps. The hydrolysates were extracted once with 5 ml of chloroform. The chloroform phases were treated directly with 2.5 ml of 1 M HCl in methanol and esterified at 100°C for 1 h. The reaction mixtures were partitioned twice against 5-ml portions of water, and the chloroform phases were evaporated to dryness in vacuo.

The residues were taken up in 0.5 ml of dichloromethane and streaked to activated 0.5-mm plates of Silica Gel H. The plates were developed to 13 cm with chloroform. Zones of interest were located by spraying the plates to transparency with water. Zones containing nonhydroxy fatty acid esters (8 to 11 cm) and hydroxy fatty acid esters (0 to 2 cm) were scraped to sintered glass filters and eluted with 10 ml of chloroform-methanol (1:1, vol/vol). Solvents were removed in vacuo, and the fractions were analyzed by gas-liquid chromatography (GLC) after treatment with a trimethylsilylating mixture (11).

Determination of cellular distribution of fatty acids. Pellets were subjected to three successive 12-h static extractions with 15 ml of chloroform-methanol (2:1, vol/vol). After each extraction period, the mixtures were centrifuged at 2,600 × *g* for 30 min at 4°C. The pooled supernatant fractions for each strain were evaporated to dryness in vacuo and considered the extractable (lipid) fractions. The pellets were considered the nonextractable (bound) fractions.

All fractions, in the presence of appropriate internal standards, were subjected to overnight alkaline methanolysis in 0.5 M KOH in methanol at room temperature. Reaction mixtures were neutralized with acetic acid.

The methanolyzed lipid fractions were dried in vacuo, dissolved in chloroform, and separated into alkali-labile lipid fatty acid and partially deacylated lipid fractions by silicic acid chromatography.

The methanolyzed bound fractions were centrifuged, and the pellets were washed twice by centrifugation from 15 ml of methanol. The combined supernatant solutions were considered the alkali-labile bound fatty acid fractions.

The pellets, and the partially deacylated lipid fractions, in the presence of internal standards, were hydrolyzed overnight at 100°C in 2.5 ml of 2 N HCl. The hydrolysates were extracted into chloroform and esterified with acid methanol to yield the alkali-stable bound fatty acid and alkali-stable lipid fatty acid fractions, respectively.

The alkali-labile lipid fatty acid and alkali-labile bound fatty acid fractions, after re-esterification with acid methanol, were separated into nonhydroxy and hydroxy ester fractions by thin-layer chromatography with chloroform, as described above.

This sequence of procedures is summarized in the flow diagram in Fig. 1.

All fractions were treated with a trimethylsilylating mixture (11) before analysis by GLC.

Separation and identification of hydroxy fatty acids. Those fractions containing presumptive hydroxy acids were pooled, remethanolyzed, and subjected to a second thin-layer chromatography in chloroform to remove all traces of nonhydroxy acid methyl esters. Approximately 10% of the resulting hydroxy ester fraction was divided into equal portions which were analyzed as trimethylsilyl and trifluoracetyl derivatives. These fractions were then converted to isopropyl esters by esterification with acid isopropanol for 1 h at 100°C. After the isopropyl esters were extracted into chloroform and washed, they were analyzed as trimethylsilyl and trifluoroacetyl derivatives.

The anomalous behavior of one peak suggested that a polyhydric compound might be present. Consequently, the balance of the presumed hydroxy acid fraction was streaked to activated 0.5-mm Silica Gel H plates and subjected to thin-layer chromatography in diethyl ether-hexane (70:30, vol/vol) to 13 cm.

Compounds of interest were located by spraying the plates to transparency with water. Zones containing presumptive monohydroxy acid esters (7 to 9 cm) and presumptive dihydroxy acid esters (2 to 5 cm) were scraped, eluted, and dried as described above.

These fractions were converted to and analyzed as the trimethylsilyl, trifluoroacetyl, acetyl, and in the case of the presumed dihydroxy acid esters, the *n*-butylboronyl derivatives. The fractions were also tested for unsaturation by bromination.

To gain insight into the location of the hydroxyl groups, a portion of the presumed dihydroxy ester fraction and an approximately equal sample of methyl-9,10-dihydroxyoctadecanoate were dissolved in 1 ml of chloroform and treated with 1 ml of ethanol and 0.2 ml of a 2% solution of sodium metaperiodate. After incubation at room temperature for 30 min, the excess periodate was decomposed by the addition of 1 ml of 1% sodium bisulfite. After the iodine color had dissipated, the mixtures were acidified with 2 N HCl, extracted with 5 ml of chloroform, and washed once with water. The chloroform phases were evaporated to dryness, taken up in 0.2 ml of chloroform, and analyzed by GLC for aldehydic fragments. To the balance of each sample was added 0.5 ml of methanol, 0.5 ml of 0.05 M NaOH, and 0.1 ml of saturated

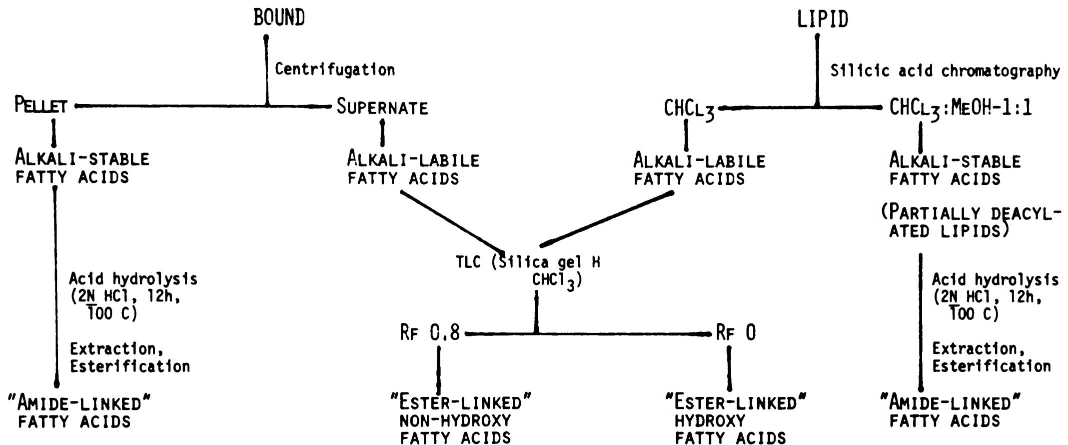

FIG. 1. *Flow diagram of determination of cellular distribution of fatty acids by separation of fractions after mild alkaline methanolysis. All fractions were analyzed by GLC on a 30-m WCOT capillary column of SP-1200. Samples were analyzed as methyl esters and trimethylsilyl ethers. TLC, Thin-layer chromatography.*

bromine water. After 15 min of incubation at room temperature, the mixtures were acidified with 2 N HCl and extracted with 3 ml of chloroform. The chloroform layers were esterified with acid methanol and washed with water. The esterified mixtures were analyzed by GLC directly and after treatment with trimethylsilylating and trifluoroacetylating agents.

Analytical methods. Column chromatography of lipid alkaline methanolysates was carried out on 1-g columns of silicic acid (Unisil, 100/120 mesh, Clarkson Chemical Co., Williamsport, Pa.) packed in chloroform. Neutralized methanolysis mixtures were applied in chloroform and eluted with 25 ml of chloroform to yield alkali-labile lipid fatty acid fractions, followed by 10 ml of chloroform-methanol (1:1, vol/vol) and 5 ml of methanol to yield partially deacylated lipid fractions. Fractions were evaporated to dryness in vacuo.

GLC was done on a Hewlett-Packard 5840A instrument equipped with flame ionization detectors. Samples were analyed on a 30-m wall-coated open tubular (WCOT) capillary column coated with the nonpolar polydimethylsiloxane liquid phase SP-2100 (Supelco, Inc., Bellefonte, Pa.), operated in the splitless mode, under temperature-programmed conditions. Fatty acid esters and their derivatives were tentatively identified by their equivalent chain length (ECL) values (9). Identifications were confirmed by comparison with standards when available, by determination of ECL values on a 30-m WCOT column coated with the polar polycyanopropylsiloxane liquid phase SP-2330 (Supelco, Inc.) and by gas-liquid chromatography–mass spectrometry.

Gas-liquid chromatography–mass spectrometry was carried out by using a Finnegan 4000 instrument equipped with a 25-m WCOT column of SP-2100. Chromatography was done in the splitless mode under temperature-programmed conditions. Mass spectrometry of peaks was done in the electron impact ionization mode at an ionization energy of 70 eV.

Infrared spectra were acquired by using a Perkin-Elmer 710B infrared spectrophotometer. Samples were analyzed as dried films between salt plates.

Standards. Straight-chain saturated nonhydroxy fatty acids of both even and odd carbon numbers were purchased individually (Sigma Chemical Co., St. Louis, Mo.) and weighed to provide a standard mixture of 8- to 24-carbon fatty acids. Unsaturated fatty acid standards were purchased individually (Sigma Chemical Co.) and as components of standard mixtures ANHI-D and GLC-10 (Supelco, Inc.). Branched-chain nonhydroxy fatty acids were obtained in standard mixtures BC-Mix L and BC-Mix 1 (Applied Science Laboratories, Inc., State College, Pa.) as well as from the previously characterized acid hydrolysates of *Bacteroides asaccharolyticus* and *B. fragilis* (9, 10). Straight-chain 3-hydroxy acids of 12 to 14 carbons were prepared from acid hydrolysates of *Escherichia coli*, and acids of 14 to 20 carbons were prepared from hydrolysates of *Acholeplasma axanthum* S743 (12). Branched-chain 3-hydroxy fatty acids of 15 to 17 carbons were prepared from acid hydrolysates of *B. asaccharolyticus* and *B. fragilis* (9, 10). Straight-chain 2-hydroxy acids of 10 and 12 carbons were prepared from acid hydrolysates of *Pseudomonas aeruginosa* (18).

Other standards include DL-12-hydroxyoctadecanoic acid and 9,10-dihydroxyoctadecanoic acid, both purchased from Applied Science Laboratories, Inc.

Since preliminary results, as well as published data (16, 19), indicated that tetradecanoic acid comprised less than 0.1 mol% of the total fatty acid profile, this compound was used as quantitative internal standard for nonhydroxy acids, and DL-12-hydroxyoctadecanoic acid was used as internal standard for hydroxy acids. 9,10-Dihydroxyoctadecanoic acid was used as a model compound in determining the structure of the *L. pneumophila* dihydroxy acid.

Chemicals. Chloroform, methanol, ethanol, isopropanol, and hexane were distilled before use. Pyridine, acetone, diethyl ether, heptane, and dichloromethane (Fisher Scientific Co., Fairlawn, N.J.) were used as received, as were the trimethylsilylating agents hexamethyldisilazane, trimethylchlorosilane, and *N,O*-bis(trimethylsilyl)trifluoroacetamide, which were pur-

chased from Sigma Chemical Co.

Other reagents which were used as received include *n*-butylboronic acid (Applied Science Laboratories, Inc.), trifluoroacetic anhydride (Eastman Kodak Co., Rochester, N.Y.), acetic anhydride, and bromine (J. T. Baker Chemical Co., Phillipsburg, N.J.).

Derivative formation. The procedures for forming the trimethylsilyl, trifluoroacetyl, and acetyl derivatives and for bromination of unsaturated compounds have been detailed previously (9). Reagents were removed under a stream of dry nitrogen, and the derivatives were reconstituted in chloroform for analysis. The *n*-butylboronyl derivatives were formed by dissolving the samples in 0.1 ml of a solution of 1 mg of *n*-butylboronic acid in 1 ml of acetone. After 30 min at room temperature, samples of the reaction mixture were analyzed directly by GLC.

RESULTS AND DISCUSSION

Total cellular fatty acids. Cells harvested from two plates yielded 8 to 20 μmol of total fatty acid, of which approximately 5 mol% (0.4 to 1 μmol) was hydroxy acid, including approximately 1 mol% (0.1 to 0.3 μmol) of dihydroxy acid. Although total cell yield and consequently total fatty acid yield varied with medium, strain, and culture age, the proportions of nonhydroxy to hydroxy fatty acid were relatively constant, as were the fatty acid profiles. The nonhydroxy fatty acid profiles were essentially those reported as the total fatty acid profile by a number of workers (2, 16, 19), confirming the stability of the nonhydroxy fatty acid profiles on various media, among several strains, as reported by Moss and Dees (16).

The hydroxy acid profiles were also essentially invariant with respect to strains, medium, and culture age and are discussed in the context of cellular distribution.

Cellular distribution of fatty acids. The distribution of fatty acids in various fractions of the five strains studied is shown in Table 1 as average values since the distribution in the five strains was quite similar. The bulk of the fatty acids, more than 90 mol%, were found in the extractable (lipid) fractions, with most of this in the ester-linked (alkali-labile) fraction.

TABLE 1. *Distribution of fatty acids in various fractions of five strains of L. pneumophila*

Fraction	Mol% of total fatty acid, mean (standard deviation)	
	Nonhydroxy	Hydroxy
Extractable (lipid)		
Ester-linked	88.4 (0.3)	0.1
Amide-linked	1.3 (0.6)	1.2 (0.7)
Total lipid	89.7 (0.8)	1.3 (1.0)
Nonextractable (bound)		
Ester-linked	4.2 (1.5)	0.3 (0.3)
Amide-linked	0.6 (0.1)	4.1 (1.0)
Total bound	4.7 (1.5)	4.3 (1.0)

Infrared spectrophotometry of the fractions labeled "partially deacylated lipid" showed negligible ester carbonyl absorption at 1,730 cm^{-1} and enhanced amide carbonyl absorption at 1,640 cm^{-1}, indicating that most, if not all ester-linked fatty acids had been removed by alkaline methanolysis. The fatty acids of these extractable, amide-linked fractions appeared to be evenly divided between hydroxy and nonhydroxy acids.

In the nonextractable or bound fractions, the fatty acids appeared to be evenly divided between the ester-linked (alkali-labile) fatty acids, which were predominantly nonhydroxy acids, and the amide-linked (alkali-stable, acid-labile) fatty acids, which were predominantly hydroxy acids. The bound fatty acid fractions comprised approximately 10 mol% of the total cellular fatty acids, a situation not uncommon in bacteria (9, 10).

The fatty acid profiles of the major fractions are listed in Table 2. The profile of the lipid, ester-linked fraction (88.5% of the total fatty acid) was quite similar to the total fatty acid profiles reported by Moss et al. (16, 19) and the lipid fatty acid profiles reported by Finnerty et al. (5).

In the lipid, amide-linked portion (2.5% of the total fatty acid), the hydroxy acid B was the major component. Component B and a small amount of component A were the only hydroxy acids found in this fraction. The relative amounts of the nonhydroxy acids differed from the lipid, ester-linked pattern, particularly at i16:0, 16:1, 16:0, and a17:0.

The profile of the bound, ester-linked fraction (4.4% of the total fatty acid) was similar to that of the lipid, ester-linked fraction, with the noticeable exception that the unsaturated fatty acid components were in extremely low concentration, if not absent entirely. This indicates that the nonhydroxy fatty acid contribution to the bound fatty acid profile was not a result of incomplete extraction of the lipids.

The profile of the bound, amide-linked fraction (4.6% of the total fatty acid) differed considerably from those of the other fractions, in both relative retention and relative composition. This fraction contained three major and numerous minor hydroxylated components.

Identification of hydroxy acids. Since the hydroxy acid profiles of the five strains were essentially invariant with respect to strain, me-

TABLE 2. *Fatty acid profiles of various fractions, as methyl esters, trimethylsilyl derivatives*

ECL	Identification	Mol% of fatty acid, mean (standard deviation)			
		Lipid, ester-linked	Lipid, amide-linked	Bound, ester-linked	Bound, amide-linked
13.6	i14:0[a]	5.0 (0.8)		3.7 (0.5)	
14.0	14:0		1.7 (0.7)	0.6 (0.3)	
14.6	i15:0	tr		tr	
14.7	a15:0	13.6 (3.8)	1.0 (0.7)	12.1 (2.8)	
14.8	15:1	1.7 (0.3)			
15.0	15:0	0.6 (0.2)	0.8 (0.5)	0.8 (0.3)	
15.2	A		1.4 (0.5)		1.2 (0.7)
15.4	i16:1	3.2 (0.8)			
15.6	i16:0	35.8 (4.8)	14.5 (2.8)	44.8 (5.6)	3.9 (2.3)
15.8	16:1	11.9 (1.9)	9.3 (2.1)	2.0 (1.0)	
15.9	B		42.8 (4.7)		27.4 (2.3)
16.0	16:0	5.1 (1.6)	14.6 (3.0)	11.1 (2.1)	2.1 (1.1)
16.1	C				2.4 (0.8)
16.6	i17:0	0.6 (0.3)			
16.7	a17:0	8.4 (1.7)	5.4 (1.0)	15.2 (3.4)	0.9 (0.2)
16.8	17cyc;D	2.4 (1.5)	3.0 (1.3)	0.5 (0.1)	1.2 (0.8)
17.0	17:0	1.4 (0.4)	0.7 (0.4)	1.0 (0.3)	
17.5	E				16.2 (3.6)
17.9	F				1.6 (0.7)
18.0	18:0	2.6(0.6)	3.9 (0.7)	4.5 (0.7)	
18.1	G				0.8 (0.2)
19.0	19:0	1.0 (0.4)		1.0 (0.5)	
19.7	H				0.8 (0.5)
20.0	20:0;J	3.1 (1.2)	0.7 (0.5)	2.8 (1.0)	2.3 (0.5)
20.6	K				0.9 (0.8)
21.0	21:0;L	tr			1.8 (0.3)
21.6	i22:0;M	tr			2.2 (1.2)
22.0	22:0;N	tr			14.6 (1.7)
22.7	O				1.8 (1.1)
23.0	P				5.1 (1.6)
23.6	R				3.2 (0.5)
23.9	S				3.2 (1.4)
% Total fatty acid		88.5	2.5	4.4	4.6
% Hydroxy fatty acid			44.2		93.1

[a] Number of carbons:number of double bonds; i, iso-branched; a, anteiso-branched; cyc, cyclopropane; tr, trace.

dium, or culture age, the combined hydroxy acids were examined for identification.

Upon conversion of the trimethylsilylated derivatives to trifluoroacetylated derivatives, the compounds designated E and F in Table 2 were found to move from ECL of 17.5 and 17.9 to ECL of approximately 14.0 and 14.4. This shift of approximately 3.5 ECL units, in contrast to a shift of approximately 1.5 ECL units for the other peaks, suggested that more than one and most likely two derivatizable groups were present in compounds E and F. Upon conversion of the pooled hydroxy acid sample to isopropyl esters, the trimethylsilyl and trifluoroacetyl patterns remained comparable to those of the corresponding methyl ester patterns, differing only in the increase of retention of all peaks by approximately 1 ECL unit. This shift indicated that all components contained only one carboxyl group. A shift of 2 or more ECL units would be expected from compounds containing any carbonyl (keto or aldehyde) group, or more than one carboxyl group.

On the assumption that the hydroxy acid fraction contained both mono- and dihydroxy monocarboxylic acids, the fraction was further separated by thin-layer chromatography in diethyl ether-hexane (70:30, vol/vol).

The gas chromatographic properties of the individual *L. pneumophila* monohydroxy esters and selected known 3-hydroxy esters are listed in Table 3 and summarized for the class in Table 4.

The chromatographic properties and mass spectral patterns strongly indicate that the monohydroxy acids are of the 3-hydroxy configuration. A large peak at m/z 103 was found in the mass spectra of the underivatized methyl

TABLE 3. *Identification of the methyl esters of the monohydroxy fatty acids of L. pneumophila*[a]

Compound	ECL on SP-2100 column			$(M-15)^+$	Identification
	Trimethylsilyl	Trifluoroacetate	Acetate		
3-OH-12:0	14.2	12.6	14.4	287	
A	15.2	13.6	15.4		3-OH-13:0
B	15.9	14.3	16.1	315	3-OH-i14:0
C	16.1	14.6	16.4		3-OH-14:0
3-OH-14:0	16.1	14.6	16.4	315	
D	16.8	15.3	17.1		3-OH-i15:0
3-OH-i15:0	16.8	15.3	17.1		
3-OH-15:0	17.1	15.6	17.4		
3-OH-i16:0	17.7	16.2	18.0		
G	18.1	16.6	18.4		3-OH-16:0
3-OH-16:0	18.1	16.6	18.4	343	
3-OH-i17:0	18.7	17.2	19.0	357	
3-OH-17:0	19.1	17.5	19.4	357	
H	19.7	18.1	20.0		3-OH-i18:0
J	20.0	18.4	20.3	371	3-OH-18:0
3-OH-18:0	20.0	18.4	20.3	371	
K	20.6	19.1	20.9		3-OH-i19:0
L	21.0	19.4	21.3		3-OH-19:0
M	21.6	20.0	21.9		3-OH-i20:0
N	22.0	20.4	22.3	399	3-OH-20:0
3-OH-20:0	22.0	20.4	22.3	399	
O	22.7	21.1	22.9		3-OH-i21:0
P	23.0	21.3	23.3		3-OH-21:0
R	23.6	21.9	23.9		3-OH-i22:0
S	23.9	22.3	24.2		3-OH-22:0

[a] From mass spectra of trimethylsilyl derivatives of methyl esters. The molecular weight of the underivatized ester can be calculated by subtracting 57 from the $(M-15)^+$ value listed. For abbreviations, see Table 2, footnote *a*.

TABLE 4. *Summary of chromatographic properties of monohydroxy fatty acids from L. pneumophila*

Fatty acid	R_f for TLC[a] of methyl esters (Silica Gel H; ether/hexane, 70:30)	Increase in ECL on GLC:		
		Methyl ester, *O*-trimethylsilyl (*O*-trifluoroacetate = 0)[b]	Methyl ester, *O*-acetate (*O*-trifluoroacetate = 0)	For all derivatives [isopropyl ester (methyl ester = 0)]
L. pneumophila	0.6	1.5	1.8	0.9
Mixture[c]	0.6	1.5	1.8	0.9

[a] TLC, Thin-layer chromatography.

[b] Chromatography was done on an SP-2100 capillary column.

[c] Straight- and branched-chain D-(−)-3-hydroxy fatty acids from *E. coli*, *A. axanthum*, and *Bacteroides* spp.

esters, whereas a large peak at m/z 175 was found in the spectra of the trimethylsilyl derivatives. These peaks represent the 3,4-cleavage product of 3-hydroxy-fatty acid methyl esters (13, 14, 21).

The gas chromatographic properties of the presumed dihydroxy esters and 9,10-dihydroxyoctadecanoic methyl ester are listed in Table 5 and summarized in Table 6.

The gas chromatographic behavior of compounds E and F strongly indicates that there are no more than two hydroxyl groups, which are most likely not located in the center of the carbon chain, as they are in 9,10-dihydroxyoctadecanoic ester. The fact that a derivative was formed with *n*-butylboronic acid suggests that the hydroxyl groups are either vicinal or methylene interrupted.

After periodate oxidation, neither 9,10-dihydroxyoctadecanoic ester nor compound E was recovered, indicating vicinal hydroxyl groups. Direct GLC of the reaction mixture of the model compound showed peaks at ECL 8.3 (nonanal) and 11.2 (9-oxo-nonanoic methyl ester) on the nonpolar column. After hypobromite oxidation and methylation, these peaks moved to ECL 9.0 (methyl nonanoate) and 12.1 (dimethyl nonanedioate), the expected fragments from 9,10-dihydroxyoctadecanoic methyl ester. Direct GLC analysis of the oxidation mixture of compound E yielded only one major peak at ECL 10.8, indicating an aldehyde of at least 11 carbons. Hypobromite oxidation, followed by methylation, yielded a single peak at ECL 11.6, the expected position of the isobranched compound methyl 10-methylundecanoate (methyl isolaurate). These findings strongly indicate compound E to be the methyl ester of 2,3-dihydroxy-12-methyltridecanoic acid.

TABLE 5. *Identification of the methyl esters of the dihydroxy fatty acids of L. pneumophila*

Compound	ECL on SP-2100 column					Identification
	Trimethylsilyl	Trifluoroacetate	Acetate	*n*-Butylboronate	IO_4^-, OBr^-, Me ester	
E	17.5	14.0	17.8	18.3	11.6	2,3-diOH-i14:0
F	17.9	14.4	18.2	18.7	—[a]	2,3-diOH-14:0
9,10-diOH-18:0	21.3	18.4	22.2	22.4	9.0, 12.1	

[a] —, Insufficient material for determination.

TABLE 6. *Summary of chromatographic properties of dihydroxy fatty acids from L. pneumophila*

Fatty acid	R_f for TLC[a] of methyl esters (Silica Gel H; ether/hexane, 70:30)	Increase in ECL on GLC:				ECL for IO_4^-/ OBr^-/ MeOH (H^+) products
		Methyl ester, *O*-trimethylsilyl (*O*-trifluoroacetate = 0)[b]	Methyl ester, *O*-acetate (*O*-trifluoroacetate = 0)	Methyl ester, *O*-*n*-butylboronate (*O*-trifluoroacetate = 0)	For all derivatives [isopropyl ester (methyl ester = 0)]	
L. pneumophila	0.3	3.5	3.8	4.3	0.8	11.6
9,10-Dihydroxystearic acid[c]	0.3	2.9	3.8	4.0	0.8	9.0, 12.1

[a] TLC, Thin-layer chromatography.
[b] Chromatography was done on an SP-2100 capillary column.
[c] From Applied Science, Inc.

None of the major peaks in either hydroxy acid class was affected by bromination, indicating that most of the hydroxy acids are saturated.

The characteristic mass spectral ions of the major dihydroxy fatty acid methyl ester are listed in Table 7. The proposed structure of this compound is shown in Fig. 2A. The structures of the derivatives and the proposed origin of the mass spectral ions are also demonstrated.

Common to all three derivatives is the $(M-184)^+$ ion. This ion appears to be a rearrangement product resulting from chain cleavage at site c and loss of $-OCH_3$, with concomitant transfer of two hydrogens to the ion, which contains the first three carbons of the parent acid as well as the derivatizing groups.

The trimethylsilyl derivative (Fig. 2B) shows only one other significant large ion in its spectrum, at m/z 257, most likely arising from cleavage at site b, with retention of charge on the fragment containing the alkyl chain.

The base peak of the spectrum of the acetyl derivative (Fig. 2C) is at m/z 132. This ion is possibly a rearrangement ion resulting from cleavage at site b with a transfer of one hydrogen to the ion, which contains the first two carbons of the parent acid as well as the ester and one of the acetate groups. The ions at m/z 256 and 197 appear to be a related series involving loss of one or more of the acetate groups and perhaps the C_3H_7 fragment from the branched alkyl terminus.

The cyclic *n*-butylboronate derivative (Fig. 2D) can show only two chain scissions: at sites a and c. Cleavage at site a is represented by the $(M-59)^+$ ion at m/z 281, whereas cleavage at site c is represented by both possible fragments at m/z 185 and 155.

TABLE 7. *Characteristic mass spectral ions of derivatives of the major dihydroxy fatty acid methyl ester*

Ion	Derivative		
	Trimethylsilyl	Acetyl	*n*-Butylboronyl
MW[a]	418	358	340
$(M-59)^+$			281 (6.0)[b]
$(M-59-43)^+$		256 (0.6)	
$(M-59-59-43)^+$		197 (0.9)	
Chain fragment	257 (11.4)		185 (8.2)
$(M-184)^+$	234 (23.0)	174 (17.2)	156 (61.2)
Chain fragment		132 (100.0)	155 (9.0)

[a] Molecular ions were not found.
[b] Relative abundance is indicated in parentheses.

The mass spectral, chromatographic, and periodate oxidation data are all consistent with the 2,3-dihydroxy structure.

The major monohydroxy fatty acids of *L. pneumophila* are therefore tentatively identified as 3-hydroxy-12-methyltridecanoic acid (β-hydroxyisomyristic acid) and 3-hydroxy-*n*-eicosanoic acid (β-hydroxyarachidic acid). The minor monohydroxy acids appear to be of the 3-hydroxy family as well and are tentatively identified as in Table 3. The major dihydroxy fatty acid is tentatively identified as 2,3-dihydroxy-12-methyltridecanoic acid (α,β-dihydroxyisomyristic acid), whereas the minor component appears

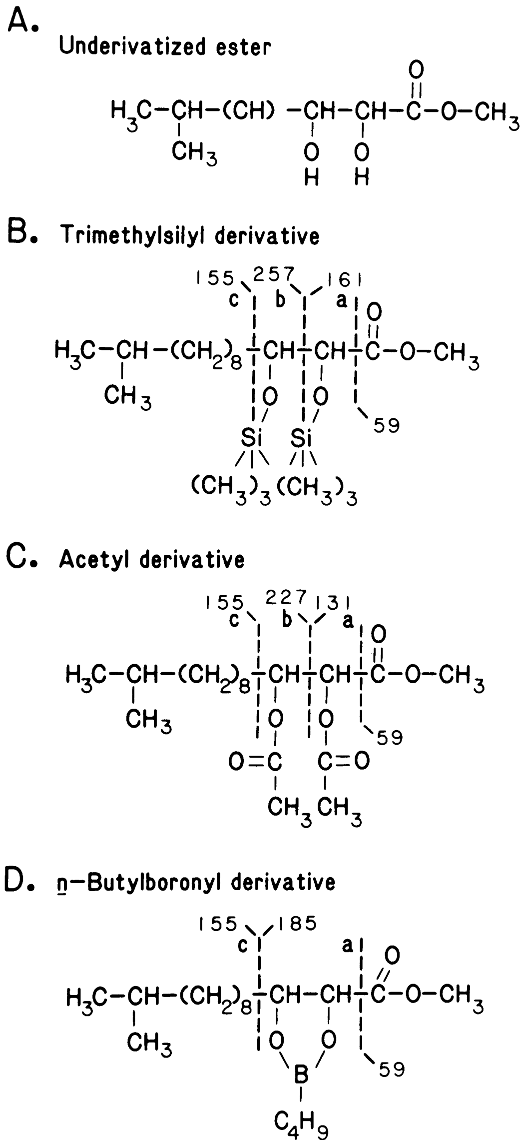

FIG. 2. *Tentative structure of the methyl ester of the dihydroxy fatty acid of L. pneumophila (A; molecular weight, 274) and its trimethylsilyl (B; molecular weight, 418), acetyl (C; molecular weight, 358), and n-butylboronyl (D; molecular weight, 340) derivatives, with proposed mass spectral fragmentation patterns.*

to be the straight-chain isomer. The steric configuration of these compounds has not yet been determined.

The difficulty of detecting the hydroxy acids in a total fatty acid analysis of *L. pneumophila* is apparent when one considers that the total concentration of hydroxy acid, some 5 to 6 mol%, is distributed over three major components and several minor components, so that any given hydroxy acid would comprise a maximum of approximately 1 mol% of the total. In addition, the underivatized esters (data not shown) and the commonly used derivatives tend to elute with or very near one of the relatively major nonhydroxylated components, as indicated by the ECL values listed in Tables 2 and 3. Thus, a change in peak area of 1% or less, or the appearance of a small new peak, as a result of derivatization, would most likely be discounted as experimental error or background.

Most of the hydroxy fatty acid and all of the dihydroxy acid are in the nonextractable, amide-linked fraction, suggesting a cell wall-associated site (Tables 1 and 2). Wong et al. (23), however, reported finding no hydroxy fatty acid in the lipopolysaccharide of *L. pneumophila*. It is possible that the cellular location of the hydroxy acids is on some moiety other than that isolated as lipopolysaccharide by these workers.

The biosynthetic relationships between the monohydroxy, dihydroxy, and nonhydroxy fatty acids are as yet unclear, although the preponderance of branched-chain compounds in all three groups seems to indicate such a relationship.

There appears to be no prior literature regarding dihydroxy fatty acids of moderate (>10 carbon) chain length in procaryotes, although such compounds are found in eucaryotic systems. The 2,3-dihydroxy configuration has not been reported for a moderate-chain fatty acid.

Umbarger et al. (22) reported two short-chain fatty acids: 2,3-dihydroxy-3-methylbutanoate (α,β-dihydroxyisovalerate) and 2,3-dihydroxy-3-methylpentanoate (α,β-dihydroxy-β-methylvalerate) as intermediates in the biosynthesis of valine and isoleucine, respectively, in *E. coli*. Thus, the 2,3-dihydroxy configuration is not without precedent in procaryotes.

It is unlikely that the 14-carbon compounds found in *L. pneumophila* arise from that pathway, which yields 2,3-dihydroxy-3-methyl fatty acids. Such a compound would have yielded a 12-carbon ketone (dodecane-2-one) on periodate oxidation, which would not have oxidized further to yield a 12-carbon fatty acid.

Thus, it appears that *L. pneumophila* produces novel 2,3-dihydroxy fatty acids, which appear to be unique among procaryotes and perhaps in nature. It remains to be seen if these acids are a common property of the genus *Legionella* or a marker unique to *L. pneumophila*.

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