

## Cellular Fatty Acid Composition of Isolates from Legionnaires Disease

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The cellular fatty acids of four isolates from Legionnaires disease and two antigenically related isolates were identified by gas chromatography, mass spectrometry, and associated techniques. The six isolates had essentially the same fatty acid composition, which was characterized by large amounts (>80%) of branched-chain acids.

In July and August 1976, an outbreak of febrile respiratory illness with several deaths occurred in Philadelphia, Pa. (Morbidity and Mortality Weekly Report [MMWR], vol. 25, no. 30, 33, and 34, Center for Disease Control [CDC], Atlanta, Ga.). Most of the cases were in persons associated with a state American Legion convention—hence the designation Legionnaires disease (MMWR, vol. 26, no. 14). The isolation of a bacterium that is thought to be the cause of Legionnaires disease was made by J. E. McDade and C. C. Shepard of the Leprosy and Rickettsia Branch, Virology Division, Bureau of Laboratories, CDC (MMWR, vol. 26, no. 2). Subsequently, these workers have isolated other bacteria that apparently are related to the organism of Legionnaires disease. The source, morphology, growth and staining characteristics, and putative relationships to respiratory disease indicated a similarity or identity of these cultures. To assess their potential chemical relatedness, we analyzed six different cultures of this apparently unusual gram-negative organism for cellular fatty acids by gas-liquid chromatography (GLC); the identity of the fatty acids was confirmed by mass spectrometry.

### MATERIALS AND METHODS

Cultures designated Philadelphia 1, 2, 3, and 4 were isolated by McDade and Shepard from the lungs of four persons with fatal cases of Legionnaires disease or Broad Street pneumonia occurring in Philadelphia, Pa., during July and August 1976 (MMWR, vol. 26, no. 2). They isolated these organisms by inoculating human lung tissue into guinea pigs and subsequently culturing the infected guinea pig tissues in the yolk sacs of embryonated eggs of hens. A detailed description of the isolation of these organisms is in preparation (J. E. McDade et al., personal communication). One of us (R.E.W.) has successfully cultured the yolk sac isolate on Mueller-Hinton agar supplemented with 1% hemoglobin and 1% (vol/vol) IsoVitaleX (BBL) enrich-

ment.

Culture "Flint" was obtained from the pleural fluid of a patient in Flint, Mich., by direct growth on enriched chocolate agar; it was submitted by Morris Dumoff through the Bureau of Laboratories, Michigan Department of Health, Flint, Mich., to CDC for identification. Culture "Pontiac" was isolated from lung tissue of a guinea pig that had been exposed to the environment of the Pontiac, Mich., Department of Health during an outbreak of respiratory illness among occupants of that building in 1968 (MMWR, vol. 17, no. 34). The guinea pig lung tissue had been maintained frozen at  $-70^{\circ}\text{C}$  since that time.

Each of the six cultures was inoculated onto a plate of Mueller-Hinton agar supplemented with 1% hemoglobin and 1% (vol/vol) IsoVitaleX (BBL) and incubated in a candle jar at  $35^{\circ}\text{C}$  for 72 h. After the addition of about 3 ml of sterile distilled water, the heavy cell growth on the plates was removed with glass rod spreaders. Smears were prepared for Gram staining to determine the purity of the cultures. The cells were saponified, and the fatty acids were methylated by the procedure described previously (6). *Pseudomonas cepacia*, an organism of known fatty acid composition (6), was used as a control; it was grown and processed under the same conditions as the six test cultures.

Methyl esters were analyzed on a model 900 gas chromatograph (Perkin-Elmer, Norwalk, Conn.) equipped with a flame ionization detector and a disc integrator recorder. The instrument contained a coiled glass column (0.16 inch by 12 feet [ca. 4.06-mm inner diameter by 3.66 m]) packed with 3% OV-101 methyl silicone that was coated on 100- to 120-mesh Gas-Chrom Q (Applied Science Laboratory, State College, Pa.). Helium was used as the carrier gas at a flow rate of 60 ml/min. The column temperature was  $160^{\circ}\text{C}$  and, after injection of the sample, it was increased to  $265^{\circ}\text{C}$  at a rate of  $5^{\circ}\text{C}/\text{min}$ . Fatty acid methyl ester peaks were tentatively identified by comparing their retention times to those of methyl ester standards (Applied Science Laboratory; Analabs, North Haven, Conn.; Supelco, Bellefonte, Pa.). Final identification was established by hydrogenation (6) and mass spectrometry (6, 10, 12). Combined gas chromatography-mass spectrometry of methyl esters was done with a DuPont instrument

type 21-491B equipped with a combination electron impact (EI)-chemical ionization (CI) source. Isobutane was used as the reagent gas for CI.

### RESULTS AND DISCUSSION

Gram stains of the cell suspensions used for GLC analyses revealed thin, gram-negative rods with occasional long filaments; the morphology was very similar in all six cultures and was entirely consistent with that previously observed for these organisms. The morphology and cultural and biochemical characteristics of these bacteria will be described in detail elsewhere.

The fatty acid profile (as methyl esters) of one of the six cultures examined is shown in the chromatogram in Fig. 1. The single most abundant acid in the chromatogram was a saturated, branched-chain 16-carbon acid (i-16:0) with the methyl branch at the iso (penultimate) carbon atom. The next most abundant were a mono-unsaturated, 16-carbon straight-chain acid (16:1), a 15-carbon branched-chain acid (a-15:0) with the methyl branch at the anteiso (antipenultimate) carbon atom, a saturated, 14-carbon branched-chain acid (i-14:0), a saturated, 17-carbon branched-chain acid (a-17:0), and a mono-unsaturated, 16-carbon iso-branched-chain acid (i-16:1). With the exception of 16:1, the normal straight-chain saturated (15:0, 16:0, etc.) and unsaturated (14:1) acids were present in only small to trace amounts. The identity of the labeled fatty acid methyl esters in the chromatogram was con-

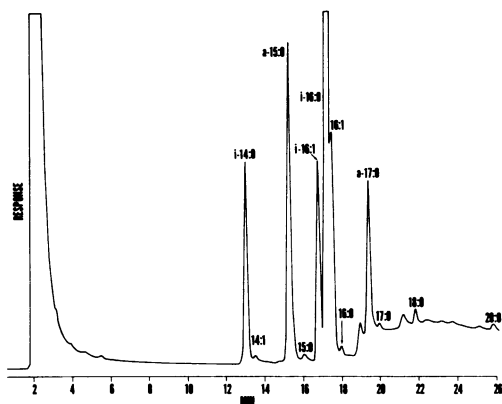


FIG. 1. Gas chromatogram of esterified fatty acids from saponified whole cells of an isolate from Legionnaires disease. Analysis was made on a 3% OV-1 column. Peak designation: the number before the colon refers to the number of carbon atoms; the number to the right refers to the number of double bonds; *i*- indicates a branched-chain acid with the methyl group at the penultimate carbon atom; and *a*- indicates a branched-chain acid with the methyl group at the antipenultimate carbon.

firmed by both EI and CI mass spectrometry. The EI spectra of anteiso branched methyl esters were clearly distinguished from iso branched- and normal straight-chain esters by comparing the ratio of the  $m/e = M-29$  and  $m/e = M-31$  peaks in the spectra. With anteiso esters, the  $M-29$  is equal to or greater than the  $M-31$  peak, whereas the  $M-31$  peak is approximately twice the size of the  $M-29$  peak in iso-branched and normal straight-chain esters (10, 12). Unsaturation was confirmed by hydrogenation of the methyl ester sample (6), which resulted in the disappearance of the *i*-16:1 and 16:1 peaks, with concomitant increases in the size of the *i*-16:0 and 16:0 peaks, respectively. The absence of hydroxy acids was confirmed by the fact that there was no change in the retention time of any peak in the chromatogram when the methyl ester sample was treated with trifluoroacetic anhydride (6). The profiles of the other five cultures were strikingly similar to that shown in Fig. 1. The fatty acid profile of the control culture, *P. cepacia*, was essentially the same as that reported previously (6).

The cellular fatty acid compositions of each of the six cultures are shown in Table 1. Peak areas from GLC were determined with the disc integrator, and the percentage of each acid was calculated from the ratio of the area of its peak to the total area of all peaks. Relative response factors were determined for each acid, and these were used in the calculation. The data clearly show the similarity of the fatty acid compositions of the six cultures. In each culture the *iso*-16:0 acid was the major component and was present in concentrations ranging from 32 to 43% of the total acids. With the exception of 16:1, the other four major acids in each culture were also branched-chain acids. These acids (*i*-14:0, *a*-15:0, *i*-16:1, and *a*-17:0) were present in each culture but in different proportions. The total percentage of branched-chain acids in each of the six cultures ranged from 81 to 90%. The only qualitative difference in the fatty acids of the six cultures was the presence of relatively small amounts (2 to 4%) of a 17-carbon cyclopropane acid (17  $\Delta$ ) in the Pontiac and the Philadelphia 2, 3, and 4 cultures that was not detected in the other two cultures. The fatty acid composition of each culture was essentially the same when tested a second time through the entire procedure (growth, saponification, extraction, GLC), as shown by the data for trial 2 in Table 1.

In general, branched-chain fatty acids are characteristic of gram-positive bacteria (4, 5, 11), but they are present in major amounts (>50% of total) in relatively few genera such as *Bacillus* (2, 3), *Listeria* (9), *Propionibacterium*

TABLE 1. Cellular fatty acid composition of isolates from Legionnaires disease (Philadelphia 1, 2, 3, and 4) and two related organisms (Flint and Pontiac)

Fatty acid <sup>a</sup>	% of total acids											
	Philadelphia								Flint		Pontiac	
	1		2		3		4		1	2	1	2
	1	2 <sup>b</sup>	1	2	1	2	1	2	1	2	1	2
i-14:0	11	12	13	11	14	14	10	13	11	11	11	12
14:1	T <sup>c</sup>	T	T	T	T	T	T	T	T	T	T	T
14:0	T	T	T	T	T	T	T	T	T	T	T	T
a-15:0	20	20	13	14	14	11	12	13	20	20	11	12
15:0	T	T	T	T	T	T	T	T	T	T	T	T
i-16:1	11	11	11	11	13	9	12	10	12	12	11	10
i-16:0	34	32	39	42	35	43	40	40	36	32	40	40
16:1	13	14	14	15	16	14	15	14	10	14	14	14
16:0	T	T	T	T	T	T	T	T	T	T	T	T
a-17:0	11	11	8	5	5	5	7	6	11	11	9	8
17Δ			2	2	3	4	4	4			4	4
17:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 18:0	T	T	T	T	T	T	T	T	T	T	T	T
18:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 19:0	T	T	T	T	T	T	T	T	T	T	T	T
19:0	T	T	T	T	T	T	T	T	T	T	T	T
Br 20:0	T	T	T	T	T	T	T	T	T	T	T	T
20:0	T	T	T	T	T	T	T	T	T	T	T	T
Total of branched-chain acids	87	86	84	83	81	82	81	82	90	86	82	82

<sup>a</sup> The number to the left of the colon refers to the number of carbon atoms; the number to the right refers to the number of double bonds; i- indicates a methyl branch at the iso carbon atom, and a- indicates a methyl branch at the anteiso carbon; Br indicates a branched-chain acid.

<sup>b</sup> Data obtained from a second experiment of cells processed through the entire procedure of growth, saponification, extraction, and GLC analysis.

<sup>c</sup> T = less than 2%.

(7), *Micrococcus* (1), and *Staphylococcus* (1). Only two gram-negative organisms, *Thermus aquaticus* and a thermophilic *Flavobacterium*, are known to contain major amounts of these acids (8). As shown in the above data, a striking feature of the cellular fatty acids of these six cultures is their high content of branched-chain acids (81 to 90%). Although these acids have been found in other bacteria, because of the presence and relative concentration of these acids and the absence of other acids that are generally present in other bacteria, these six cultures have a unique fatty acid profile. In view of the limited data from conventional cultural and biochemical tests with these cultures (J. E. McDade et al., submitted for publication), it appears that data from cellular fatty acid analysis will provide valuable information for assessing the relationship of future isolates to those in this study. It should be noted that the cellular fatty acid composition of bacteria has been shown to be influenced by the composition of growth media, age of culture, temperature of incubation, and other factors (3, 5). It is essential, therefore, that media, condi-

tions of growth, and methods of analysis be standardized for all strains to be compared. With the cultures reported in this study, special attention must be given to growth medium since it is known that the relative abundance of branched-chain cellular fatty acids is affected by the availability of precursors of the terminal portion of these acids, such as branched-chain amino acids (3, 7).

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