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Lipids of the *Spirochaetales*: Comparison of the Lipids of Several Members of the Genera *Spirochaeta*, *Treponema*, and *Leptospira*

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The lipid compositions of 17 spirochetes belonging to the genera *Spirochaeta* and *Treponema* were investigated and compared with data previously derived from 11 strains of *Leptospira*. The lipid compositions and lipid metabolism of any of these genera is sufficiently different to be characteristic of that genus and to differentiate it from the other two genera. Members of the genus *Leptospira* are characterized by their ability to beta-oxidize long chain fatty acids as their major carbon and energy source. With few exceptions, they are incapable of synthesizing fatty acids de novo. The major phospholipid found was phosphatidyl ethanolamine. No glycolipid or phosphatidyl choline was found in these organisms. Members of the genus *Treponema* studied were incapable of beta-oxidation as well as de novo synthesis of fatty acids. Phosphatidyl choline is the major phospholipid of this genus. The glycolipid, monogalactosyl diglyceride, is a major component of the *Treponema*. Members of the *Spirochaeta* did synthesize fatty acids de novo. Although these spirochetes contain a monoglycosyl diglyceride, the hexose content of the glycolipid varied from species to species. Neither phosphatidyl ethanolamine nor phosphatidyl choline was found in the *Spirochaeta*.

The bacterial order *Spirochaetales* is subdivided into five genera: *Spirochaeta*, *Cristispira*, *Treponema*, *Borrelia*, and *Leptospira*. Until recently, these distinctions were based primarily upon ecological and morphological considerations, since comparative biochemical studies were hampered by the inability to culture many of these organisms in vitro. With recent advances in the isolation and cultivation of *Spirochaeta*, *Treponema*, and *Leptospira*, conventional methods of bacterial taxonomy have been employed to clarify the relationships of these three genera to each other. These studies are summarized in the review by Smibert (17), and the changes that have been recommended will be appearing in the forthcoming edition of *Bergey's Manual of Determinative Bacteriology*.

The spirochetes are rich in lipid as compared with other bacteria, approximately 20% of their dry weight being lipid (8, 9, 11). Although lipids play a major role in the metabolism of the *Treponema* (7) and the *Leptospira* (10), little is known of the lipid composition and lipid metabolism of these bacteria from a comparative standpoint. Early work from this laboratory with the lipids of the *Leptospira* (9, 14) and of *Treponema phagedenis* biotype kazan 5 (8, 13), as well as the work of Meyer and Meyer with *T. phagedenis* biotype reiterii and *Spirochaeta zuelzerae* (15), revealed significant genera differences in lipid composition and lipid metabolism of these three species. It was of interest, therefore, to see if these differences were characteristic of each genus and could serve as useful taxonomic markers for the characterization of these three genera. This paper reports the lipid composition and lipid metabolism of several representative members of the genera *Spirochaeta* and *Treponema* as compared with each other and with previously published data for *Leptospira* (9).

MATERIALS AND METHODS

Organisms. *Spirochaeta litoralis* strain R1 was kindly provided by R. B. Hespell, Department of Bacteriology, U.C.L.A., Los Angeles, Calif. J. A. Breznak, Department of Bacteriology, University of Wisconsin, Madison, supplied the J1 and J4T strains of *S. aurantia*. *S. zuelzerae* ATCC 19044 was purchased from the American Type Culture Collection, Rockville, Md. E. Canale-Parola, Department of

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Microbiology, University of Massachusetts, Amherst, provided *S. stenostrepta* strain Z1. A. W. Hanson, V.D.R.L., Center for Disease Control, Atlanta, Ga., furnished cultures of *T. phagedenis* biotypes kazan 5, phagedenis, and reiterii; *T. refringens* biotypes refringens, nichols, and noguchi; *T. ambiguum*; and *T. scoliodontum*. *T. refringens* biotype calligyrum, *T. denticola* biotype denticola strain T32-A, and *T. denticola* biotype comondonii came from R. M. Smibert, The Anaerobe Lab, Virginia Polytechnic Institute and State University, Blacksburg.

Media and culture conditions. The facultative anaerobes *S. aurantia* strains J1 and J4T were cultivated aerobically at 30 C. The J1 strain was grown in a medium composed of 0.5% Trypticase (BBL), 0.2% yeast extract, and 0.3% maltose (2). The medium for the J4T strain had the composition: 0.2% Trypticase, 0.2% yeast extract, and 0.2% glucose (J. A. Breznak, personal communication). Sterile M phosphate buffer (pH 7.4) was added aseptically to the sterilized media prior to inoculation to a final concentration of 10^{-2} M.

S. zuelzerae was cultured anaerobically at 30 C in the medium recommended by the American Type Culture Collection. The medium contained 0.1% yeast extract, 0.1% agar, 0.1% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.004% $CaCl_2$, and 0.000125% $FeCl_3 \cdot 6H_2O$. Just prior to inoculation the following solutions were added aseptically: 5% filter-sterilized $NaHCO_3$, 20 ml/liter; 10% glucose (autoclaved), 10 ml/liter; and 10% $Na_2S \cdot 9H_2O$ (autoclaved), 5 ml/liter.

Spirochaeta stenostrepta, *S. litoralis*, and all of the *Treponema* were cultured anaerobically in prereduced media prepared and inoculated as described in the manual of the Anaerobe Laboratory (5). The medium for *S. litoralis* contained 0.3% tryptone, 0.25% yeast extract, 0.5% glucose, 10 ml of 5% sodium thioglycolate per liter, 4 ml of 0.025% resazurin solution per liter, and 10 ml of a salts solution per liter. The salts solution consisted of the following: 0.2% tetrasodium ethylenediaminetetraacetic acid, 0.75% $CaCl_2 \cdot 2H_2O$, 2.5% $MgCl_2 \cdot 6H_2O$, and 0.1% $FeSO_4 \cdot 7H_2O$ (3). Sterile M phosphate buffer (pH 7.4) was added aseptically to the medium at the time of inoculation to a final concentration of 2×10^{-2} or 6×10^{-2} M. Incubation was at 30 C.

S. stenostrepta was cultured at 35 to 37 C in a medium composed of 0.2% Trypticase, 0.2% yeast extract, 0.5% glucose, 10 ml of 5% sodium thioglycolate per liter, 4 ml of resazurin per liter, 1 ml of M phosphate buffer per liter (pH 7.4), and 10 ml of M tris(hydroxymethyl)aminomethane per liter (pH 7.4). This medium was adapted from Hespell and Canale-Parola (4).

All of the *Treponema* were cultivated at 35 to 37 C in a prereduced basal medium appropriately supplemented. The basal medium was composed of 1% Trypticase, 1% yeast extract, 1% glucose, 0.08% L-cysteine·HCl, 4 ml of 0.025% resazurin per liter, and 10 ml of a salts solution per liter. The salts solution consisted of 0.2% $MgSO_4 \cdot 7H_2O$, 0.13% $CaCl_2 \cdot 2H_2O$, 1% NaCl, and 5% $(NH_4)_2SO_4$. Prior to inoculation, a phosphate-bicarbonate buffer (10 ml/liter) (pH 7.4) was added aseptically. This buffer was prepared by dissolving 27.3 g of KH_2PO_4, 139.2 g of K_2HPO_4, and 50.0 g of $NaHCO_3$ in 1 liter of distilled water, followed by filter sterilization.

Long chain fatty acids were supplied to the treponemal medium by adding 10% heat-inactivated rabbit serum or 1% bovine serum albumin complexed with 2×10^{-4} M each palmitic and oleic acids. The albumin-fatty acid supplement was prepared with chloroform-methanol-extracted albumin and was essentially lipid free except for the added fatty acids (7). The supplement was filter sterilized.

Batch cultures of the spirochetes for lipid analyses were harvested by sedimentation at $12,000 \times g$ for 45 min in a Sorvall RC-2B refrigerated centrifuge, or at $40,000 \times g$ in a Beckman J-21 refrigerated centrifuge equipped with a JCF-Z continuous-flow head. Cells were washed with a salts solution of the same qualitative and quantitative composition as the medium from which they were derived. The washed cells were then resuspended in distilled water, lyophilized, and stored at 4 C.

Reagents and standards. All solvents were redistilled prior to use in glass equipment as previously described (20). Individual phospholipid standards were purchased from Supelco, Inc. (Bellefonte, Pa.). Neutral lipid and phospholipid standard mixtures were obtained through the Hormel Institute (Austin, Minn.). Standard fatty acid methyl esters were also purchased from the Hormel Institute, as well as from NuChek Prep (Elysian, Minn.).

Lipid analysis. The lipids were extracted by a modification of the method of Bligh and Dyer (1). Lyophilized cells were resuspended in 4.0 ml of distilled water by vortexing. A 10-ml amount of methanol and 5.0 ml of chloroform were added in order, and the suspension was vigorously mixed. After extracting 4 to 5 h at room temperature, the suspensions were centrifuged, and the supernatant liquids were collected by decanting carefully into another tube. The pellets were reextracted briefly with 9.5 ml of chloroform-methanol-water (1:2:0.8, vol/vol/vol) and centrifuged, and the supernatant liquids were combined with the first extracts. A biphasic system was formed by adding 7.5 ml of chloroform and 7.4 ml of 0.73% saline, making the final ratio of chloroform-methanol-water 1:1:0.9 (vol/vol/vol). After centrifuging to break any emulsions formed, the chloroform phase was removed, diluted with benzene, and taken just to dryness on a rotary evaporator. The lipid was washed into a volumetric flask with several portions of benzene-absolute ethanol (4:1, vol/vol) and made up to volume. Samples were taken for gravimetric analyses, and the lipid extracts were stored at −10 C in tubes sealed with Teflon-lined screw caps.

Samples of each lipid extract containing 1.00 mg of total lipid were hydrolyzed in 0.75 N methanolic HCl for 24 h at 80 C. Prior to hydrolysis mannitol and heneicosanoic acid were added as internal standards. This hydrolysis procedure yielded fatty acids as their methyl esters and hexoses as their glycosides. The methyl esters were extracted with several washes of petroleum ether, and the remaining hydrolysate was taken to dryness in vacuo over P_2O_5 and KOH pellets.

The residue was made up to volume with distilled water, and a sample was taken for hexose analysis. HCl was added to yield a final concentration of 2 N, and the solution was hydrolyzed at 120 to 125 C for 24 h to yield inorganic phosphate. The hydrolysate was then neutralized with an equal volume of 2 N NaOH and titrated to pH 6.8 to 7.2 with ammonia gas, as indicated by adding bromothymol blue as a pH indicator. Inorganic phosphate was assayed spectrophotometrically by the method of Townsend et al. (20).

Fatty acid methyl esters and methyl glycosides were separated, identified, and quantitated by gas liquid chromatography using liquid phases of 3% silar-5cp (Applied Science) and 3% SE-30 silicone. The fatty acid esters were prepared for gas liquid chromatography by saponifying them in 0.5 N NaOH in 80% methanol at 80 C for 2 h. The non-saponifiable material was extracted with petroleum ether, and the free fatty acids were extracted after acidifying. The free fatty acids were converted to methyl esters with 5% methanolic HCl at 80 C for 2 h. An equal volume of water was added to the methanol, and the esters were extracted with petroleum ether and chromatographed on both liquid phases. Identification was based upon comparison of the retention time of each fatty acid ester relative to that of the heneicosanoic acid standard to authentic standards. The presence of unsaturates was verified by rechromatographing the ester mixtures after catalytic hydrogenation. Quantitation was achieved by calculating the area of each peak as the height times the width at half the height.

Methyl glycosides were chromatographed as their trimethylsilyl ethers (18, 19) on the SE-30 liquid phase. Identification was based on the comparison of the multiple peaks obtained for the various anomers of each hexose with retention times for authentic standards. The nanomoles of each sugar were calculated from the ratio of their peak areas to that of the internal standard of mannitol (times 1.25 as the correction factor) (18).

The total lipid extracts were separated into their component lipid classes by thin-layer chromatography. Chromatography was conducted on 0.25-mm layers of Silica Gel G coated onto glass plates (Brinkman Instruments, Inc., Westbury, N.Y.) in glass tanks lined with filter paper to saturate the atmosphere. Neutral lipids were developed with petroleum ether-diethyl ether-glacial acetic acid (90:10:1 and 70:30:2, vol/vol/vol). Polar lipids were separated with the solvent systems: chloroform-methanol-water (65:25:4, vol/vol/vol), chloroform-ethanol-glacial acetic acid (7:2:1, vol/vol/vol), chloroform-methanol-glacial acetic acid-water (65:25:4:2, vol/vol/vol/vol), and chloroform-methanol-concentrated ammonium hydroxide (65:30:8, vol/vol/vol).

The various lipid classes were visualized by spraying the plates with 50% (wt/vol) sulfuric acid and heating at 125 C until charred. Lipids containing various functional groups were detected with specific spray reagents. Glycolipids were detected with the alpha-naphthol reagent of Jacin and Mishkin (6). Phospholipids were detected with the molybdenum reagent used for the inorganic phosphate assay diluted with water until green (20). Ninhydrin was used to detect lipids containing primary amino groups (16), and choline-containing lipids were visualized with Dragendorff's reagent (16).

Individual lipid classes were identified by comparing their chromatographic mobility with that of authentic lipid standards in several solvent systems. This identification was further established by the demonstration of characteristic functional groups when possible.

RESULTS

The spirochetes were found to contain relatively large amounts of lipid. Extractable lipids accounted for 14.6 to 30.0% of the dry weight of the organisms studied, with the mean being 21.8%. This lipid was essentially all polar lipid except for the biotypes of *T. refringens*. Only small amounts of free fatty acid, probably due to nonspecific adsorption from the medium, were found on thin-layer chromatography of the neutral lipid. Since *T. refringens* would not grow in the lipid-defined medium, it was cultivated in media containing serum. These cells contained significant amounts of cholesterol ester, and triglycerides, all of which are abundant in serum. In one isolated instance, it was possible to achieve limited multiplication of *T. refringens* biotype refringens in the lipid-defined medium. These cells did not contain the neutral lipid components found when cells were derived from the serum-containing medium.

The polar lipid of the *Treponema* and *Spirochaeta* consisted of approximately equal amounts of phospholipid and glycolipid. The mean mole percentage composition was 51.3% phospholipid and 48.7% glycolipid. Notable exceptions were *S. aurantia*, which contained a large amount of glycolipid (85%), and *T. denticola* biotype ambiguum, which contained the largest amount of phospholipid (78%).

All of the *Spirochaeta* and *Treponema* examined contained the glycolipid monoglycosyl diglyceride. However, the hexose content of the glycolipid was found to vary from species to species. Table 1 lists the various sugars found in the total lipid extracts. In some instances more than one hexose occurs. Since only the monoglycosyl form of the glycolipid was found despite efforts to find the diglycosyl form, it is presumed that in these instances the glycolipid is a pair of homologues. Perhaps these homoloques result from altered or multiple glycosyl transferases involved in the synthesis of this lipid.

The various phospholipids found in the spirochetes are listed in Table 2. All of the organisms studied contained the acidic phospholipids

phosphatidyl glycerol and cardiolipin (i.e., bisphosphatidyl glycerol). In addition, the *Treponema* were found to contain phosphatidyl choline.

The fatty acid compositions of the five strains of the genus *Spirochaeta* are given in Table 3. Chain lengths of the fatty acids varied from 12 to 18 carbon, with the major acids being C_{15}. All of the *Spirochaeta* contained iso-branched chain fatty acids. In addition, *S. stenostrepta* and *S. zuelzerae* also contained the anteiso isomers of these acids. Unsaturated fatty acids were confined to *S. aurantia* and *S. zuelzerae*.

Fatty acids found in the genus *Treponema* are listed in Table 4. Since these organisms require long chain fatty acids for growth, it is not surprising that they generally only contain palmitic and oleic acids, the fatty acids supplied in the albumin-fatty acid supplement. Thus, it appears that the *Treponema* are incapable of chain modification. *T. refringens*, which must be grown in the serum-containing medium, contained the major fatty acids found in serum. In the one instance that this organism was able to grow in the lipid-defined medium, it contained only palmitic and oleic acids.

TABLE 1. *Distribution of various sugars in total lipid*

Organism[a]	Total carbohydrates (%)		
	Glucose	Galactose	Mannose
S. litoralis			100
S. aurantia J1	100		
S. aurantia J4T	100		
S. stenostrepta		100	
S. zuelzerae	100		
T. phagedenis			
btp. *phagedenis*		100	
btp. *kazan* 5		100	
btp. *reiter*		100	
T. refringens			
btp. *refringens*	15	85	
btp. *nichols*	18	82	
btp. *noguchi*	17	83	
btp. *calligyrum*	8	92	
T. denticola			
btp. *denticola*	38	62	
btp. *comondonii*	38	62	
btp. *ambiguum*		100	
T. scoliodontum		100	
T. vincentii		100	

[a] btp., Biotype.

DISCUSSION

With the exception of *T. refringens*, which was cultivated in a serum-containing medium, all of the organisms in this study were grown in lipid-defined media. Therefore, any lipid isolated from these organisms that was not present in the medium must be the result of biosynthesis. Following this logic, the *Spirochaeta* synthesize all of their lipids de novo. *Treponema*, on the other hand, require long chain fatty acids supplied in the medium. Although they cannot synthesize their own fatty acids, they are capable of utilizing the acids provided to them to synthesize all of their own complex polar lipids. *Treponema* require a saturated fatty acid of at least 14 carbon and a *cis*-unsaturated fatty acid

TABLE 2. *Phospholipids present in spirochetes*[a]

Organism	Phospholipid[a]					
	PC	PE	PS	PG	CL	PA
S. litoralis	–	–	–	+++	+	++
S. aurantia	–	–	–	+++	++	–
S. stenostrepta	–	–	+	++	++	+
S. zuelzerae	–	–	–	++	++	–
T. phagedenis	++	++	–	+	+	–
T. refringens	+++	–	+	+	+	–
T. denticola	+++	++	–	+	+	–
T. scoliodontum	+++	++	–	+	+	–
T. vincentii	+++	–	–	+	+	–

[a] +++, Phospholipid is a major component; ++, phospholipid is a significant component; +, phospholipid is a minor component; –, phospholipid is not present. Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PG, phosphatidyl glycerol; CL, cardiolipin; PA, phosphatidic acid.

TABLE 3. *Relative percentage of fatty acid compositions of Spirochaeta*

Organism	Fatty acid composition[a]												
	12:0	i-13	i-14	14:0	i-15	ai-15	i-16	16:0	16:1	i-17	ai-17	18:0	18:1
S. litoralis	0	0	0	13.0	56.7	0	0	19.0	0	11.3	0	0	0
S. aurantia J1	0	Trace	0	5.1	49.4	0	0	19.7	12.4	1.5	0	Trace	11.9
S. aurantia J4T	0	Trace	0	6.6	79.6	0	0	4.4	3.2	0.7	0	Trace	5.5
S. stenostrepta	0	0	1.6	2.8	70.7		2.0	8.9	0	12.1		1.8	0
S. zuelzerae	1.5	11.1	14.6	19.2	42.9		2.3	3.5	3.3	Trace		Trace	1.6

[a] The chain length of the fatty acid is indicated by the number preceding the colon; the number of double bonds is indicated by the number following the colon. i preceding the chain length indicates that the fatty acid is an iso-branched chain acid. a preceding the chain length indicates an anteiso-branched chain.

with 15 or more carbon (7), since they can neither alter the chain length nor desaturate fatty acids (8). *Leptospira* beta-oxidize long chain fatty acids as their major carbon and energy source (17) and, with few exceptions, they require fatty acids for growth (10, 17). They require a chain length of at least 15 carbon (10), since they cannot chain elongate the acids provided them (9). Most of the *Leptospira* do not require unsaturated fatty acids because they can directly desaturate palmitic and stearic acids to their corresponding monounsaturated fatty acids (9). Like the *Treponema*, the *Leptospira* synthesize all of their complex polar lipids with the fatty acids provided in the medium (9).

The types of polar lipid synthesized by the organisms from these genera are substantially different from each other. Table 5 demonstrates how lipid composition and lipid metabolism can be used to distinguish and characterize the spirochetes of these genera. As can be seen from the table, the differences are significant. Because they are consistent with the data derived by classical methods of bacterial taxonomy (17), they may well serve as useful taxonomic markers to differentiate and characterize new isolates. Furthermore, the observations necessary to make these distinctions can be made quickly with small amounts of material because the spirochetes are so rich in lipid. All that is necessary to distinguish the genus of an unknown isolate is to separate the total lipid extract of the organism on thin-layer chromatography and to determine the presence or absence of monoglycosyl diglyceride and phosphatidyl choline using the appropriate spray reagents.

Differences in the fatty acids synthesized by the *Spirochaeta* also allow the species of these oganisms to be differentiated. Table 6 presents

TABLE 5. *Differentiation of spirochetes by lipid composition and lipid metabolism*

Lipid composition and metabolism	*Spirochaeta*	*Treponema*	*Leptospira* (9)
Monoglycosyl diglyceride	Present	Present	Absent
Phosphatidyl choline	Absent	Present	Absent
Phosphatidyl ethanolamine	Absent	Some	Present
Beta-oxidation of fatty acids as major source of carbon and energy	No	No	Yes
De novo fatty acid synthesis	Yes	No	Rare
Synthesis of unsaturated fatty acids	Some	No	Yes

TABLE 6. *Differentiation of Spirochaeta by fatty acid composition*

Organism	Unsaturated fatty acids	Anteiso-branched acids
S. litoralis	Absent	Absent
S. stenostrepta	Absent	Present
S. aurantia	Present	Absent
S. zuelzerae	Present	Present

TABLE 4. *Relative percentage of fatty acid compositions of Treponema*

Organism[a]	Fatty acid composition[b]											
	12:0	i-14	14:0	i-15	15:0	i-16	16:0	16:1	18:0	18:1	18:2	18:3
T. refringens												
btp. refringens (defined medium)	0	0	0	0	0	0	71.7	0	0	28.3	0	0
btp. refringens (serum medium)	Trace	0	1.5	0	1.5	0	62.0	2.8	4.0	6.4	21.5	0.9
btp. nichols	Trace	0	1.8	0	0.8	0	50.6	3.4	7.9	4.9	25.1	3.8
btp. noguchi	Trace	0	1.6	0	0.9	0	50.3	4.2	7.8	6.3	23.3	3.8
btp. calligyrum	Trace	0	1.7	0	1.2	0	67.9	4.4	6.8	4.9	11.3	0.8
T. phagedenis												
btp. phagedenis	0	0	0	0	0	0	49.2	0	0	50.8	0	0
btp. kazan 5	0	0	0	0	0	0	58.4	0	0	41.6	0	0
btp. reiteri	0	0	0	0	0	0	51.3	0	0	48.7	0	0
T. scoliodontum	0	0	0	0	0	0	70.5	0	0	29.5	0	0
T. vincentii	0	0	8.6	0	0	0	66.9			24.6	0	0
T. denticola												
btp. denticola	Trace	1.8	10.0	0.9	3.6	0.8	53.4	0	0	29.6	0	0
btp. comondonii	0	0	0	0	0	0	79.7	0	0	20.3	0	0
btp. ambiquum	0	0	0	0	0	0	74.8	0	0	25.2	0	0

[a] btp., Biotype.
[b] See footnote to Table 3.

the distribution of unsaturated and anteiso-branched chain fatty acids within this genus. Each species can be characterized by the presence or absence of one or both types of fatty acid. With the sensitivity of modern gas chromatographs, extremely small amounts of material can be assayed. In this instance, whole cells could be saponified directly and the fatty acids could be extracted, derivatized, and analyzed without the need of extracting the total lipid by normal methods. Such a procedure could be performed within a day using as little as 5 to 10 mg (dry weight) of cells.

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