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Spiroplasma Membrane Lipids

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Membranes of six spiroplasma strains belonging to different *Spiroplasma* species and subgroups were isolated by a combination of osmotic lysis and sonication in the presence of EDTA to block endogenous phospholipase activity. Analysis of membrane lipids showed that in addition to free and esterified cholesterol the spiroplasmas incorporated exogenous phospholipids from the growth medium. Sphingomyelin was preferentially incorporated from phosphatidylcholine-sphingomyelin vesicles or from the serum used to supplement the growth medium. Palmitate was incorporated better than oleate into membrane lipids synthesized by the organisms during growth. The major phospholipid synthesized by the spiroplasmas was phosphatidylglycerol. The positional distribution of the fatty acids in phosphatidylglycerol of *Spiroplasma floricola* resembled that found in *Mycoplasma* species, in which the saturated fatty acids prefer position 2 in the glycerol backbone and not position 1 as found in *Acholeplasma* species and elsewhere in nature. Electron paramagnetic resonance analysis of spin-labeled fatty acids incorporated into *S. floricola* membranes exhibited homogeneous single-component spectra without immobilized regions. The *S. floricola* membranes were more rigid than those of *Acholeplasma laidlawii* and less rigid than those of *Mycoplasma gallisepticum*.

Spiroplasmas, the helical wall-less procaryotes included in the class *Mollicutes*, are widely distributed in arthropods and plants. Our knowledge on their cell biology is still very fragmentary. The few spiroplasmas tested so far resemble the classical animal mycoplasmas in that they require cholesterol for growth, but differ in having a larger genome (10^9 daltons) resembling in this respect the sterol-nonrequiring acholeplasmas (5, 24). As with mycoplasmas and acholeplasmas, it is relatively easy to isolate the cell membrane of spiroplasmas by osmotic shock (16). The spiroplasma membrane, like that of other mollicutes, is built essentially of protein and lipid (16). Data on membrane lipid composition is available for only the citrus pathogen *Spiroplasma citri* and for the genetically related corn-stunt spiroplasma (8, 12–14, 16). The discovery of numerous new spiroplasmas which constitute an enormous group of microorganisms exhibiting diverse biological, pathological, and ecological properties (4, 5, 24, 25) has prompted us to carry out a comparative analysis of membrane lipids in several *Spiroplasma* species and strains representing different serogroups. Emphasis was put on the examination of several properties, such as the positional distribution of fatty acids in the glycerol backbone of membrane phospholipids and the ability to incorporate exogenous phospholipids, since these properties distinguish the sterol-requiring *Mycoplasma* species from the sterol-nonrequiring *Acholeplasma* species.

MATERIALS AND METHODS

Organisms and growth conditions. *S. citri* (Maroc R8A2) from citrus suffering from stubborn disease, *Spiroplasma* sp. BC-3 from honey bee (serogroup I-2), *Spiroplasma floricola* BNR1 from flowers (serogroup III), *Spiroplasma apis* (serogroup IV) from honey bees suffering from May disease, *Spiroplasma* sp. PPS1 (serogroup IV) from flowers, and *Spiroplasma* sp. MQ-1 (serogroup VII) from the Monobia wasp were kindly provided by R. F. Whitcomb, Beltsville, Md. The organisms were grown for 24 to 48 h at 30°C in the medium of Saglio et al. (22) supplemented with 10% (vol/vol) horse serum. In some experiments the horse serum was replaced with 1.0% delipidated bovine serum albumin and a mixture of cholesterol (20 μg/ml of medium, Sigma Chemical Co., St. Louis, Mo.), oleic and palmitic acids (10 μg/ml, Sigma), and lipid vesicles containing various ratios of egg phosphatidylcholine (PC) and egg sphingomyelin (SPM) (Sigma). The vesicles were prepared by dispersing dried phospholipid mixtures in the growth medium by ultrasonic irradiation for 3 min at 0°C in a W-350 Heat Systems sonicator at 50% duty cycle at 160 W. The phospholipid dispersions were added to the medium containing albumin, cholesterol, and fatty acids to give a final phospholipid concentration of 25 μg/ml. To label membrane lipids, 0.002 μCi of either [1-^{14}C]palmitic acid (50 to 60 Ci/mol) or [1-^{14}C]oleic acid (50 to 60 Ci/mol) per ml (both products of the Radiochemical Center, Amersham, United Kingdom) were added to the medium. To monitor endogenous phospholipase A activity, *S. floricola* BNR1 cells were grown with 0.002 μCi of [1-^{14}C]palmitic acid per ml and 0.02 μCi of [9,10^{3}H]oleic acid per ml. The organisms were grown to the mid-exponential phase of growth (pH 5.7 to 6.1; absorbance at 640 nm, 0.11 to 0.13). EDTA was added to the culture to a final concentration of 20 mM, and the cells were collected by centrifugation at 12,000 × *g* for 15 min. The cells were washed once with and resuspended in a solution containing 20 mM EDTA and 25 mM Tris-hydrochloride (pH 7.5) in 250 mM NaCl at 4°C. Membranes were prepared by injecting 1 volume of washed cells into 50 volumes of 20 mM EDTA. After 5 min at 4°C, the preparation was briefly sonicated for 1 min in a W-350 Heat Systems sonicator at 160 W, and membranes were harvested by centrifugation at 34,000 × *g* for 30 min at 4°C and washed with 20 mM EDTA at 4°C. After protein was determined (10), the membranes were resuspended in a solution of 20 mM EDTA–25 mM Tris (pH 7.5) and stored at −20°C. Membranes of *Mycoplasma gallisepticum* and *Acholeplasma laidlawii* were prepared as described before, from cells grown in Edward medium (18) supplemented with 10% horse serum.

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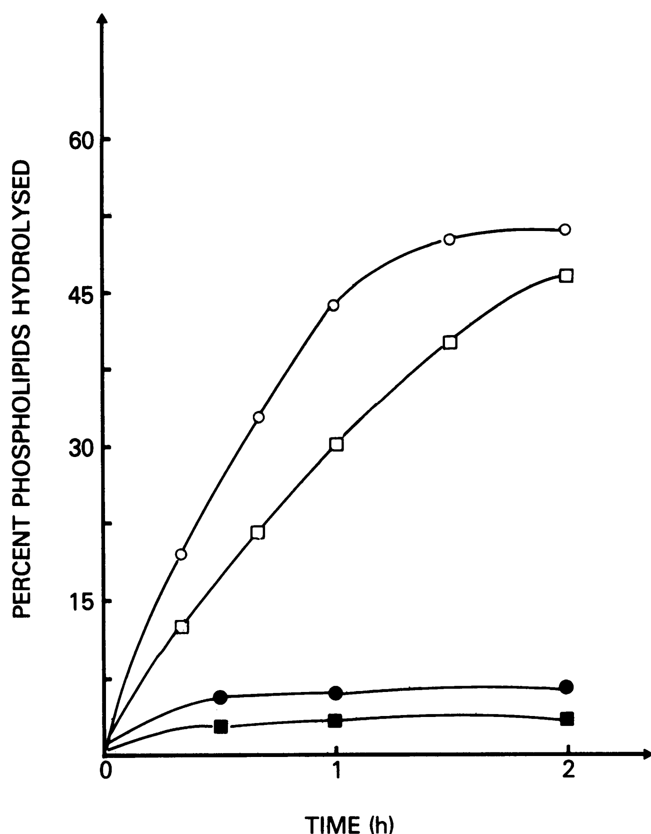

FIG. 1. Endogenous hydrolysis of membrane phospholipids of *S. floricola* BNR1. Isolated membranes of *S. floricola* labeled during growth with radioactive palmitate (○) or oleate (□) were incubated at 37°C in 20 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM $MgCl_2$. Percent phospholipids hydrolyzed was assessed by the amount of labeled free fatty acids produced in the presence (open symbols) or absence (filled symbols) of 20 mM EDTA.

Lipid analyses. Lipids were extracted by the procedure of Bligh and Dyer (2). Polar lipids were separated from neutral lipids by silicic acid column chromatography. Neutral lipids were eluted with 10 bed volumes of chloroform, and phospholipids were eluted with 10 bed volumes of chloroform-methanol (1:1; vol/vol), followed by 5 bed volumes of methanol. The lipid fractions were evaporated to dryness under a stream of nitrogen and redissolved in chloroform. The phospholipid fractions were applied to silica gel HR thin-layer plates (0.8 mm), and the plates were developed in chloroform-methanol-water (65:25:4; vol/vol/vol) at 4°C. Lipid spots were detected by exposing the plates to iodine vapor. The radioactivity of the cell suspensions, lipid extracts, column fractions, and lipid spots was measured in 5 ml of scintillation liquid (40% Lumax; Lumac, Schaesberg, The Netherlands) with a Packard Tricarb liquid scintillation spectrometer. Phosphorus in the lipid extracts, fractions, and lipid spots was determined by the method of Ames (1) after digestion with $Mg(NO_3)_2$.

Positional distribution of fatty acids. Phosphatidylglycerol (PG) from *S. floricola* BNR1 was scraped from thin-layer chromatography plates into test tubes containing 1 ml of 50 mM Tris-hydrochloride (pH 7.4), 25 mM $CaCl_2$, and 1.25% bovine serum albumin. Phospholipase A_2 (100 μg) from porcine pancrease (Boehringer Manneheim, Federal Republic of Germany) was added, and the reaction mixture was incubated at 37°C for 2 h. Hydrolysis was stopped by adding 1 ml of 100 mM EDTA, and the lipids were extracted. Lyso-PG and free fatty acids were separated by thin-layer chromatography. Methyl esters of the fatty acids of PG, lyso-PG, and free fatty acid fraction were prepared by heating the lipids in 14% boron trifluoride in methanol (Sigma) at 72°C for 15 min. The methyl esters were extracted with *n*-hexane and analyzed in a Packard model 437 gas-liquid chromatograph equipped with a polar column (200 by 1.0 mm; 10% SP 2330 on 100/120 Chromosorb W).

Electron paramagnetic resonance spectrometry. Membranes were labeled with the *N*-oxyl-4,4-dimethyloxazolidine derivative of 5-ketostearic acid (5-doxylstearate) and 12-ketostearic acid (12-doxylstearate), purchased from Syva, Palo Alto, Calif. A 5-μl volume of a 2.5-mM solution of the spin label in ethanol was added to the membrane preparations containing 2 mg of protein in 1 ml of 0.25 M NaCl. The mixture was shaken at 37°C for 15 min, 4 ml of 0.25 M NaCl was added, and the labeled membranes were collected by centrifugation at 34,000 × *g* for 30 min at 4°C. The membrane pellets were suspended in 50 μl of 0.25 M NaCl solution and analyzed in a Varian E-4 spectrometer equipped with a temperature control accessory. The order parameter (*S*) was calculated as described before (9). In our hands, repeated determinations of *S* on a given preparation yielded a standard deviation of ± 0.01.

Endogenous phospholipase activity. Endogenous phospholipase activity in double-labeled *S. floricola* BNR1 sonicated cell preparations was determined in a reaction mixture containing 3 mg of cell protein per ml in 25 mM Tris buffer (pH 7.0) containing 50 mM $MgCl_2$. The reaction was carried out at 37°C for up to 2 h. At various time intervals, 100-μl samples were withdrawn and transferred to test tubes containing 50 μl of 50 mM EDTA. Free fatty acids were extracted by the method of Dole (7), and radioactivity was determined.

RESULTS

Membranes of the spiroplasmas tested in the present study were obtained by osmotic lysis of the organisms, followed by a brief ultrasonic treatment. Preliminary analysis of some of these membranes revealed an extremely low phospholipid content (ca. 150 nmol of lipid phosphorous per mg of membrane protein) and high amounts of free fatty acids, suggesting endogenous phospholipase A activity. Incubating *S. floricola* (BNR1) membranes at 37°C in 20 mM Tris-hydrochloride buffer (pH 7.5) containing 50 mM $MgCl_2$ resulted in a rapid breakdown of a significant portion of membrane phospholipids, as judged by the release of radioactive fatty acids into the free fatty acid fraction (Fig. 1). Both [^{14}C]palmitate and [^{3}H]oleate were released, but the release of palmitate was somewhat faster. Endogenous phospholipase activity exhibited a very broad pH optimum. Thus, the rate of hydrolysis of membrane phospholipids was almost the same between pH 5.5 and 8.0. No endogenous phospholipase activity was found in the presence of 20 mM EDTA. EDTA was therefore added routinely to the culture before harvest, as well as to the lysing and washing solutions.

Table 1 shows the polar lipid composition of the spiroplasmas tested. Essentially all of the polar lipids were located in the cell membrane. The polar lipid content varied to some extent, being the lowest in *Spiroplasma* sp. MQ1 (310 nmol/mg of membrane protein) and the highest in *S. citri*. The molar ratios of cholesterol to phospholipid were 0.80 ± 0.20 for all of the strains, except for *Spiroplasma* sp. MQ1. Thin-layer chromatography of the polar lipid fraction

TABLE 1. Lipid composition of *Spiroplasmas*[a]

Organism	Polar lipids: Total nmols/mg of membrane protein	% of total: SPM	PC	PG	DPG	Ratio of cholesterol to phospholipid (mol/mol)
S. citri	560	33	14	38	15	0.80
Spiroplasma sp. BC-3	520	37	7	43	12	0.82
S. floricola BNR1	410	29	11	56	<1	0.82
S. apis B31	470	31	3	65	<1	0.78
Spiroplasma sp. PPS1	580	39	4	56	<1	0.79
Spiroplasma sp. MQ1	310	39	4	32	9	0.68

[a] The organisms were grown in a medium containing 10% horse serum. Lipid extraction and analyses were performed as described in the text.

of all the strains grown in a medium containing horse serum consisted of four major spots. The spots were identified as SPM, PC, PG, and diphosphatidylglycerol (DPG) according to their comigration on the thin-layer plates with commercially available standards, specific spraying reagents, and sensitivity to phospholipase A_2 and to mild alkaline hydrolysis. In *Spiroplasma* sp. MQ1, an unidentified phospholipid (PL-X) migrating in chloroform-methanol-acetic acid-water (65:25:1:4; vol/vol/vol/vol) with an R_f value of 0.60 (as compared with R_f's of 0.44 for PG and 0.77 for DPG) was detected (not shown in Table 1). According to the R_f, PL-X possibly can be identified as lyso-DPG previously detected in spiroplasmas (8, 14). The absence of substantial amounts of PC was apparent, whereas relatively large amounts of SPM were detected.

Table 2 shows the incorporation of radioactive fatty acids into the lipid fraction of the spiroplasmas tested. In all strains the level of labeled palmitate incorporated was higher than that of oleate. The difference in the incorporation of [^{14}C]palmitic and [^{14}C]oleic acids persisted also when the organisms were grown in a medium supplemented with 10 μg of unlabeled palmitate and oleate per ml, ruling out the possibility that the differences in labeling were due to lower concentrations of free palmitate present in the growth medium. The SPM and PC fraction remained unlabeled, whereas the PG, DPG, and the unidentified phospholipid (PL-X) fractions were labeled. This suggests that SPM and PC are taken up unchanged from the growth medium and are not synthesized by the cells. The major de novo synthesized phospholipid in all strains tested was PG. In *S. citri* and in the related *Spiroplasma* sp. BC3, substantial labeling was found in the DPG fraction. The percentage of labeled fatty acid incorporated into the neutral lipid fraction, though much smaller than that incorporated into the polar lipid fraction, was still significant, especially that of palmitic acid. Most of the radioactivity in the neutral lipid fraction was recovered as free fatty acids.

TABLE 3. Selective incorporation of exogenous phospholipids by *S. floricola* BNR1[a]

Exogenous lipid added to medium (μg/ml)	Phospholipid incorporated (nmol/mg of membrane protein): SPM	PC	SPM/PC ratio
SPM (16.5) + PC (8.5)	139	29	4.8
SPM (12.5) + PC (12.5)	150	34	4.4
SPM (8.5) + PC (16.5)	142	42	3.4

[a] Cells were grown in a medium containing 1% delipidated albumin, oleic and palmitic acids (10 μg of each per ml), 20 μg of cholesterol per ml, and 25 μg of exogenous phospholipids (SPM and PC at various proportions) per ml. Lipid analysis was performed as described in the text.

As mycoplasmas grown in a medium containing horse serum incorporate large amounts of PC (up to ca. 35% of total membrane phospholipids; 17) and to a lower extent SPM, we examined the possibility that spiroplasmas preferentially incorporate SPM. The experiment was carried out with the fast-growing *S floricola* BNR1 grown in a serum-free medium that contained 0.5% bovine serum albumin, oleic and palmitic acids (10 μg of each per ml), 20 μg of cholesterol per ml, and vesicles containing various proportions of SPM and PC (final concentration of exogenous phospholipid was maintained at 25 μg/ml of medium). *S. floricola* selectively incorporated SPM (Table 3). The ratio of SPM to PC in the cell membrane was high even when the cells were grown with an excess of PC.

The positional distribution of fatty acyl chains in the PG of *S. floricola* BNR1 is presented in Table 4. The positional distribution was studied with the aid of phospholipase A_2, which catalyzes the hydrolysis of fatty acid ester linkages exclusively at position 2. Unsaturated fatty acids were found predominantly in position 1, and saturated fatty acids were more abundant in position 2 (Table 4). Therefore, the ratio of saturated to unsaturated fatty acids was high in the free fatty acid fraction (5.2, representing fatty acids occupying position 2) and low (1.3) in the lyso-PG fraction containing fatty acids linked to position 1.

The physical state of spiroplasma membranes was assessed by measuring S in the hydrophobic core of *S.*

TABLE 2. Incorporation of labeled fatty acids into spiroplasmas[a]

Organism	[^{14}C]oleate: Intact cells (cpm/mg of protein)	Lipids (% of total cpm): Polar	Neutral	% of total cpm in polar lipids: PG	PL-X	DPG	[^{14}C]palmitate: Intact cells (cpm/mg of protein)	Lipids (% of total cpm): Polar	Neutral	% of total cpm in polar lipids: PG	PL-X	DPG
S. citri	49,400	94	5	70	<1	26	65,000	84	16	69	<1	16
Spiroplasma sp. BC3	35,900	96	3	65	2	30	53,200	80	18	74	3	22
S. floricola BNR1	61,000	89	11	89	<1	2	57,500	65	35	85	<1	5
S. apis B31	27,700	88	12	94	<1	1	57,500	65	32	95	<1	1
Spiroplasma sp. PPS1	31,000	86	14	93	<1	1	55,600	64	36	94	<1	2
Spiroplasma sp. MQ1	10,500	77	21	70	22	6	32,700	67	32	78	15	8

[a] Cells were grown in a medium containing 10% horse serum supplemented with either [^{14}C]oleate or [^{14}C]palmitate. Lipid analysis was performed as described in the text.

floricola BNR1 membranes. *S* was calculated from electron paramagnetic resonance spectra recorded at 10 and 45°C. At both temperatures, the spectra of both 5-doxylstearate and 12-doxylstearate in the membranes were homogenous single-component spectra showing no immobilized regions, suggesting that in *S. floricola* BNR1 the lipids form a continuous bilayer with relatively little lipid-protein interactions. Table 5 shows *S* of 5-doxylstearate in *S. floricola* BNR1 membranes as compared with *S* in *A. laidlawii* and *M. gallisepticum* membranes. At 10°C, *S* of the spiroplasma membrane was similar to *S* of *M. gallisepticum* and significantly higher than *S* of *A. laidlawii*. At 45°C, *S* of *S. floricola* membranes was again higher than *S* obtained with membranes of *A. laidlawii* but significantly lower than *S* of *M. gallisepticum*. The values of *S* are related to the mean angular deviations of the labeled fatty acid chain from its average orientation in the membrane. Low values of *S* are associated with higher fluidity and lower order of membrane lipids (9).

DISCUSSION

The spiroplasmas included in our study belong to several serogroups (serogroups I, III, IV, and VII) which show no antigenic relationship, as determined by serological tests, and no genetic relatedness as assessed by DNA-DNA hybridization tests and by electrophoretic patterns of cell proteins (3, 6, 11, 25). Hence, despite the limited number of spiroplasmas included in our study, preliminary conclusions as to spiroplasma lipid composition can be reached with some caution. Synthesis of PG as a major membrane lipid appears to be a property shared by all the spiroplasmas included in our study and by other mollicutes (15, 19). The ability of spiroplasmas to incorporate exogenous phospholipids, combined with their capacity to take up substantial amounts of free and esterified cholesterol from the growth medium (8, 12, 13, 16), relates the spiroplasmas to the sterol-requiring mycoplasmas, rather than to the acholeplasmas.

The higher incorporation of palmitate as compared with oleate noticed in our study was reported previously for *S. citri* (8, 12, 13). Moreover, we have demonstrated that in *S. floricola*, the major de novo synthesized phospholipid, PG, shows an unusual distribution of fatty acids. Saturated fatty acids were found more abundantly in position 2, and unsaturated fatty acids were more abundant in position 1. Unusual positional distribution of fatty acids was previously shown in *Mycoplasma* but not in *Acholeplasma* species that exhibit the configuration usually found elsewhere in nature (20, 21).

The spiroplasmas tested incorporate preferentially SPM from serum or from SPM-PC vesicles added to the growth medium. Significant amounts of SPM were observed in *S. citri* before (8). Some *Mycoplasma* species grown with serum incorporated more PC than SPM, whereas others resemble the spiroplasmas in incorporating more SPM (17).

The resemblance in lipid composition of *Spiroplasma* sp. BC3 to *S. citri* (Tables 1 and 2) is in line with the marked genetic relatedness (more than 60% DNA homology) of these two organisms (3). Similarly, the lipid composition of *S. citri* was found to be almost identical to that of the corn-stunt spiroplasma (13, 14), an organism showing ca. 50% DNA homology with *S. citri* (3). In fact, these three spiroplasmas belong to the same serogroup, serogroup I (3). Our electron paramagnetic resonance measurements revealed a lower degree of order in the lipid domain of *S. floricola* membranes as compared with that of membranes of *M. gallisepticum*, despite their similar cholesterol content (20). This may suggest that in *S. floricola* membrane proteins do not restrict the freedom of motion of membrane lipids. Indeed the spectra of spin-labeled fatty acids incorporated into *S. floricola* membranes were single-component spectra exhibiting order characteristics of a lipid bilayer without any indications for the presence of label immobilized by its binding to lipid-protein interface (23).

TABLE 4. Positional distribution of fatty acids in PG from *S. floricola* BNR1[a]

Chain length: no. of double bonds	Fatty acid content (mol%)		
	PG	Lyso-PG (position 1)	FFA (position 2)
14:0	1.0	<1.0	6.0
16:0	40.0	39.0	42.0
18:0	25.0	18.0	36.0
18:1	29.0	37.0	14.0
18:2	5.0	6.0	2.0
Saturated/unsaturated ratio[b]	1.9	1.3	5.2

[a] PG was obtained from cells grown in a medium supplemented with 10% horse serum. The positional distribution was estimated by hydrolyzing the PG preparations with phospholipase A_2 as described in the text. The fatty acid content of lyso-PG represents the fatty acyl chains at position 1, whereas the fatty acid content of the free fatty acids (FFA) represents the fatty acyl chains at position 2.

[b] Molar ratio of saturated to unsaturated fatty acids.

TABLE 5. Values of *S*. for 5-doxylstearate in cell membranes of *S. floricola* (BNR1), *A. laidlawii*, and *M. gallisepticum*[a]

Membranes	*S* of spectra recorded at:	
	10°C	45°C
S. floricola BNR1	0.84	0.57
A. laidlawii	0.77	0.49
M. gallisepticum	0.83	0.65

[a] Cells were grown in a medium supplemented with 10% horse serum. Isolation of membranes and calculations of *S* were performed as described in the text.

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