INFECTION AND IMMUNITY, Apr. 1978, p. 215-220
0019-9567/78/0020-0215$02.00/0

Vol. 20, No. 1
Printed in U.S.A.

Lipid Metabolism of *Borrelia hermsi*

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Received for publication 3 November 1977

The synthesis of complex lipids by *Borrelia hermsi* while growing in Kelly medium was investigated by labeling cultures with D-[^{14}C]glucose or [^{14}C]palmitic acid. Labeled glucose was incorporated into phosphatidyl choline, phosphatidyl glycerol, monogalactosyl diglyceride, cholesteryl glucoside, and acylated cholesteryl glucoside. The fatty acid composition reflected that of the medium, suggesting that this spirochete directly incorporates acyl chains unaltered from its external milieu. Furthermore, the distribution of labeled carbon between acyl groups (lipid-soluble) and water-soluble moieties indicates that there is no metabolic exchange between these two pools. The relationship of *B. hermsi* to other spirochetes is discussed in terms of lipid metabolism as a generic characteristic.

We have previously shown that different patterns of lipid metabolism and composition exist within the order *Spirochaetales* and that these patterns can be used to differentiate the genera *Spirochaeta, Leptospira,* and *Treponema* (5). Unfortunately, we were unable to include any members of the genus *Borrelia* in our original survey of the order, since it was not possible to cultivate these spirochetes in a lipid-defined medium. However, work from Kelly's laboratory (4, 6) indicates that *Borrelia hermsi* requires a pair of fatty acids (a saturate and an unsaturate) for growth in his medium (3) and that it can dissimilate lysolecithin from the medium to obtain these. Because of the similarity of these findings to ours for members of the genus *Treponema,* but with obvious exceptions, we were prompted to investigate the lipid biosynthesis and composition of *B. hermsi* using radiolabeled precursors to circumvent the lack of a lipid-defined medium.

MATERIALS AND METHODS

Organisms and culture conditions. *B. hermsi* was maintained by passage in the medium developed by Kelly (3) at 35°C. Cells for lipid analyses were cultivated in 250-ml screw-capped bottles of the same medium. The organisms were harvested by centrifugation at 10,000 × *g* when the cell density reached 3 × 10^8 to 5 × 10^8 organisms per ml. The pellet was washed three times with a basal salt buffer and lyophilized.

Reagents and standards. All reagents and solvents used were of reagent grade or higher purity and purchased through local vendors. Standard lipids for chromatography were obtained through Sigma Chemical Co. (Saint Louis, Mo.), Supelco, Inc. (Bellefonte, Pa.), and Applied Science Laboratories (State College, Pa.). In addition, some standard lipids were derived from other spirochetes in which they had been previously characterized (5).

Radiolabeling experiments. When cells were to be radiolabeled, the precursor was added to 250 ml of growth medium at the time of inoculation at a level of approximately 1 mCi/ml. Cells were labeled with either [*U*-^{14}C]palmitic acid or D-[*U*-^{14}C]glucose obtained through New England Nuclear Corp. (Boston, Mass.). The palmitic acid had a specific activity of 750 mCi/mmol, and the glucose had a specific activity of 2.79 mCi/mmol. All counts were performed in a Packard Tri-Carb liquid scintillation spectrometer (model 3320, Packard Instrument Co., Inc., Downers Grove, Ill.) using the cocktail suggested by Kates (2) for polar lipids. The counting efficiency was 35%.

Lipid analysis. All of the methods used in lipid analysis were adopted or taken directly from the manual by Kates (2).

Extraction of cellular lipid. The total lipid was extracted by suspending the lyophilized cells (from 1 liter of medium) in 2 ml of distilled water, followed by 5 ml of methanol and 2.5 ml of chloroform. After 1 h at room temperature with intermittent mixing, the cellular residue was removed by centrifugation, and the supernatant liquid was transferred to another tube. A biphasic system was produced by adding 2.5 ml each of chloroform and water. The upper phase was aspirated off after centrifuging, and the chloroform layer was washed with two small portions of methanol-water, 10:9 (vol/vol). After diluting with benzene, the chloroform layer was evaporated to dryness in vacuo and made to volume with chloroform.

TLC. In thin-layer chromatography (TLC), separations were performed on 0.25-mm-thick layers of silica gel H containing an organic binder (silica gel HL Uniplates; Analtech, Inc., Newark, Del.). Chromatograms were developed by capillarity in solvent-saturated glass tanks.

Two-dimensional TLC was performed by eluting in

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the first dimension with chloroform-methanol-ammonium hydroxide, 65:35:5 (vol/vol), and, after air drying for 30 min, in the second dimension (rotated 90°) with chloroform-acetone-methanol-acetic acid-water, 10:4:2:2:1 (vol/vol). One-dimensional separation of polar lipids was accomplished with either of these solvent systems or with chloroform-methanol-water, 80:20:2 (vol/vol). Neutral lipids were separated in a solvent system containing petroleum ether-ethyl ether-acetic acid, 80:20:2 (vol/vol).

Lipid components were visualized on developed chromatograms with spray reagents specific for various functional groups, e.g., ninhydrin, Dragendorff, α-naphthol, Zinzadze reagent, etc. (2), or more generally either by spraying with 50% (wt/vol) sulfuric acid followed by charring at 140°C for 1 h or by exposing to iodine vapors in a closed container. Radiolabeled components were located on the chromatograms by autoradiography. The chromatographic plates were overlaid with a sheet of X-ray film (Kodak BB-54) and stored protected from light for a period of time proportional to the amount of ^{14}C present on the chromatogram (2). The developed film was then used to locate the areas on the chromatogram containing radioactivity by realigning reference marks made with radioactive ink. The gel was scraped into scintillation vials, suspended in cocktail, and counted to quantitate the relative amount of each component.

Preparative TLC was used to purify small amounts of individual components using the above solvent systems. The samples were applied to the plates as streaks instead of spots, and the separated bands were located on the developed chromatograms by brief exposure to iodine vapor. After the iodine had resublimed from the chromatogram, the gel containing each component was scraped into a column chromatographic tube and eluted with chloroform-methanol, 1:1 (vol/vol). The eluent was then taken to dryness after diluting with benzene and made to volume with chloroform.

GLC. All gas-liquid chromatography (GLC) separations were performed with a Perkin-Elmer Sigma 3 gas chromatograph equipped with flame ionization detectors. The carrier gas was nitrogen, and the flow rate maintained was approximately 30 ml/min.

Fatty acid methyl esters were analyzed on a stainless-steel column (1/8 inch by 6 feet, ca. 28.6 mm by 1.83 m) of 10% Silar 10-C on 100/120-mesh Gas- Chrom Q operated at 180 to 200°C. Steroids were also separated with this column, but the temperature was elevated to 225°C. A second column used for fatty acid esters consisted of 5% DEGS-PS (Supelco, Inc., Bellefonte, Pa.) on 100/120-mesh Supelcoport in a stainless-steel column (1/8 in by 6 feet, ca. 28.6 mm by 1.83 m) maintained at 200°C. Both Silar and DEGS affect polar separations, so, in addition to these liquid phases, nonpolar separation of fatty acid esters and analysis of the trimethyl silyl ethers of methyl glycosides were conducted on an aluminum column (1/8 inch by 6 feet, ca. 28.6 mm by 1.83 m) containing 3% OV-17 on 100/120-mesh Supelcoport. Separations of methyl glycosides were accomplished at 160°C, and fatty acid esters were analyzed at 180°C.

Quantitation of the separated components was accomplished by estimating peak areas (height times width at half height) and dividing by the total peak area to derive a relative percentage.

Column chromatography. The total lipid was fractionated into neutral lipid, glycolipid, and phospholipid fractions with silicic acid column chromatography, as previously described (2). A tube (1 cm ID by 30 cm) was packed with 10 g of SilicAR CC-4 (Mallinckrodt, Saint Louis, Mo.), and the total lipid was applied in chloroform. The column was then eluted in order with 100 ml of chloroform, 250 ml of acetone, and 150 ml of methanol. Each solvent was collected as a single fraction, with the neutral lipid eluted with chloroform, the glycolipid with acetone, and the phospholipid with methanol.

Paper chromatography. The water-soluble products of deacylation or acid hydrolysis were separated and identified with paper chromatography on Whatman no. 1 paper. Samples were applied as spots, and the papers were developed with phenol-water, 100:38 (wt/vol), for 16 to 24 h in the ascending direction. Radiolabeled components were located with autoradiography, while unlabeled compounds were detected with the periodate-*o*-tolidine dip reagent or the alkaline silver nitrate reagent (2).

Sequential degradation. To confirm the structure of the various lipid components, they were subjected to sequential degradation with chromatographic analysis of the fragments at each step. Fatty acid esters were first removed by mild alkaline deacylation, resulting in their transesterification to methyl esters (2). The methyl esters were then analyzed by GLC. The lipid was dissolved in order in 0.2 ml of chloroform, 0.3 ml of methanol, and 0.5 ml of 0.2 N methanolic NaOH. After 15 min at room temperature, 0.8 ml of chloroform, 0.2 ml of methanol, and 0.9 ml of water were added to produce a biphasic system. The aqueous upper phase was immediately removed after a brief centrifugation and passed through a small column of anion-exchange resin to remove excess base. The lower chloroform layer was washed with three small portions of methanol-water, 10:9 (vol/vol), which were also passed through the resin and combined with the upper phase.

A portion of both the base-stable (chloroform-soluble) and water-soluble deacylated lipid was hydrolyzed with 1 N HCl. The water-soluble fraction was hydrolyzed for 2 h at 100°C in aqueous acid, while the chloroform-soluble lipid was hydrolyzed in methanolic HCl at 80°C for 2 h. After cooling, the methanolic HCl was diluted with 10% water and extracted with several washes of petroleum ether. The hydrolysates (methanolic and aqueous) were taken to dryness in vacuo in the presence of KOH pellets to take up residual acid and made to volume before further analysis.

RESULTS

The autoradiogram of a two-dimensional TLC of the cellular lipids synthesized by *B. hermsi* in the presence of D-[^{14}C]glucose is reproduced in Fig. 1. Only five components were found to have incorporated the labeled precursor. Three of these, phosphatidyl choline (PC), phosphatidyl glycerol (PG), and monoglycosyl diglyceride

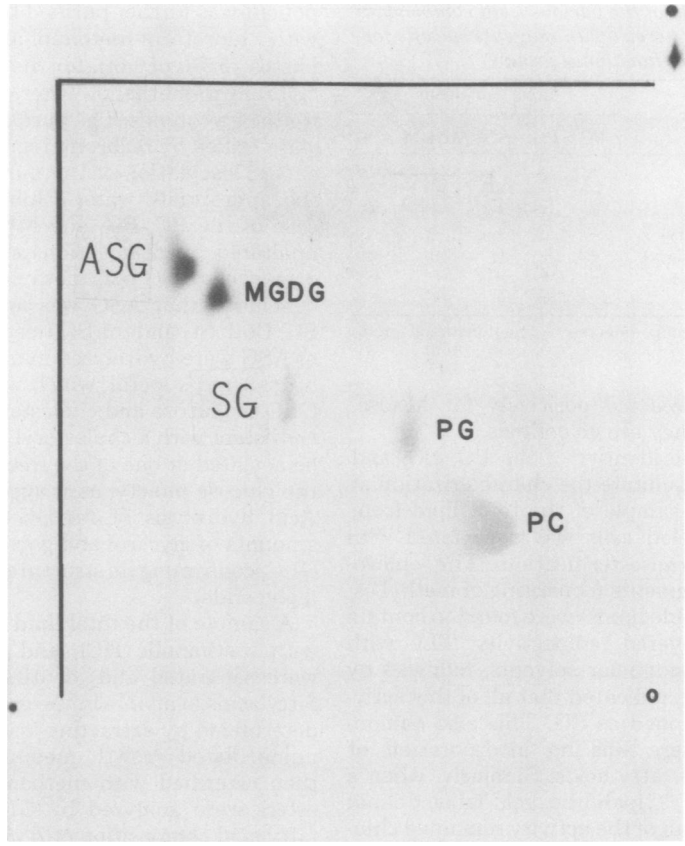

FIG. 1. *Autoradiogram of two-dimensional TLC of B. hermsi cellular lipids labeled with* D-[^{14}C]*glucose.* O, *Origin.*

(MGDG) were readily recognizable, since they were found in several other spirochetes previously examined (5). The other two, sterol glycoside (SG) and acylated sterolglycoside (ASG) (Fig. 1), had not been encountered before and were considered unknowns. Interestingly, similar autoradiograms of the lipids labeled by [^{14}C]palmitic acid contained PC, PG, MGDG, and ASG, but were completely devoid of any SG.

among these five components. Since the typical polar lipid contains two fatty acyl chains per molecule, the percentage of [^{14}C]palmitic acid incorporated into each lipid fairly accurately represents the molar amount synthesized when equilibrium has been reached. Autoradiograms of thin-layer chromatograms developed with neutral lipid solvent systems failed to show any radioactivity present in the neutral lipids, with the exception that a small amount of free fatty acid was found to contain radioactivity in the lipid extract from cells cultivated in [^{14}C]palmitic acid. This most probably represents carryover or hydrolysis.

TABLE 1. *Distribution of radioactivity among the various lipid components of B. hermsi*

Component	Precursor[a]	
	[^{14}C]palmitic acid[b]	D-[^{14}C]glucose[c]
PC	72	25
PG	17	17
SG	0	8
MGDG	6	24
ASG	5	26

[a] Distribution of labeled precursor as percentage of total recovered disintegrations per minute.
[b] Labeled with [*U*-^{14}C]palmitic acid.
[c] Labeled with D-[*U*-^{14}C]glucose.

Two-dimensional TLC of unlabeled total lipid extracts was also performed, and these chromatograms were visualized with specific reagents for various functional groups. The staining behavior of the five lipid components known to be synthesized by *B. hermsi* is presented in Table 2. The only phospholipids found were PC and PG; no other lipids reacted with the reagent for phosphorus. The remaining three lipids, MGDG,

TABLE 2. *Reaction of the various lipid components synthesized by B. hermsi with reagents specific for certain functional groups*[a]

Reagent	Lipid component				
	PC	PG	SG	MGDM	ASG
Phosphorus	+	+	−	−	−
Hexose	−	−	+	+	+
Choline	+	−	−	−	−
Amino groups (primary)	−	−	−	−	−
Amino groups (secondary)	−	−	−	−	−
Vicinal hydroxy groups	−	+	+	+	+
Sterol	−	−	+	−	+

[a] +, Functional group detected; −, no functional group detected.

ASG, and SG, reacted positively for hexose, indicating that they are glycolipids.

To confirm the identity of the PC, PG, and MGDG, and to facilitate the characterization of ASG and SG, a sample of the total lipid from [^{14}C]glucose-labeled cells was deacylated with mild alkaline transesterification. The chloroform-soluble fragments (consisting of methyl esters and base-stable lipids) were found to contain 35% of the recovered radioactivity. TLC with both polar and nonpolar solvents, followed by autoradiography, indicated that all of this activity chromatographed as SG. This also demonstrated that there was no incorporation of [^{14}C]glucose into fatty acids. Similarly, when a sample of the [^{14}C]palmitic acid-labeled lipid was deacylated, all of the activity remained chloroform soluble and chromatographed as methyl ester. From this, one may conclude that (i) there is no metabolic exchange of carbon between the water-soluble and fatty acyl precursors for complex lipid synthesis, and (ii) SG is base stable, since it does not contain any fatty acyl esters, and therefore does not incorporate [^{14}C]palmitic acid.

The water-soluble products of deacylation of the [^{14}C]glucose-labeled lipid were analyzed with paper chromatography and autoradiography. As expected, spots corresponding to glyceryl phosphoryl choline (derived from PC), glyceryl phosphoryl glycerol (derived from PG), and glycosyl glycerol (derived from MGDG) were identified; however, no fragment attributable to the deacylation of ASG could be located. Acid hydrolysis of the water-soluble deacylation products and paper chromatography of the hydrolysate demonstrated glycerol, glyceryl phosphate, choline, and hexose, compatible with PC, PG, and MGDG, but nothing that gave any indication of the composition of ASG.

The total cellular lipid was fractionated with silicic acid column chromatography into neutral lipid, glycolipid (MGDG, SG and ASG), and phospholipid (PC and PG) fractions. Each component was further purified by preparative TLC with chloroform-methanol-ammonium hydroxide, 65:35:5 (vol/vol), for the phospholipids and chloroform-methanol-water, 80:20:2 (vol/vol), for the glycolipids. The purity of each compound was verified by rechromatographing a portion of each. Deacylation of the purified lipids yielded the appropriate water-soluble products in the case of the PC, PG, and MGDG. The SG was unaltered by deacylation, while the ASG was converted to SG plus methyl esters. Thus it was concluded that ASG was an acylated form of SG. Both SG and the SG released by deacylation of ASG were hydrolyzed in methanolic HCl to a hexose and a sterol, which were identified with GLC as glucose and cholesterol. These data are consistent with a cholesteryl glucoside that may be acylated at one of the free hydroxy groups of the glucose moiety, as it appears to be in ASG. Acid hydrolysis of MGDG gave equal molar amounts of glycerol and galactose, identified by GLC, confirming its structure as monogalactosyl diglyceride.

A sample of the total lipid was transesterified with methanolic HCl, and the methyl esters were separated and identified with GLC. the fatty acids available in the growth medium were determined by extracting a small volume of the uninoculated growth medium. This lipid was then esterified with methanolic HCl, and the esters were analyzed by GLC (Table 3). The fatty acid composition of *B. hermsi* reflects that of the growth medium, with no acids being found in its complex lipids that are not available in the culture medium.

DISCUSSION

Since Kelly medium for *B. hermsi* contains serum (3), which is a source of fatty acids, cholesterol, and complex lipids, it is difficult to determine whether the lipids found in cells cultivated in this medium are synthesized de novo or merely incorporated unaltered from the external milieu. For this reason we chose to use both nonlipid and lipid precursors that were radiolabeled to distinguish between biosynthesis and simple uptake of preformed complex lipids.

TABLE 3. *Fatty acid composition of B. hermsi cellular and growth medium available lipids*

Lipids in:	Percent fatty acid:							
	14[a]:0[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
B. hermsi	2	49	1	5	33	8	1	Tr
Medium	Tr	23	1	11	42	19	2	2

[a] Chain length of the fatty acid.
[b] Number of double bonds in the carbon chain.

Previous work by Kelly indicated that *B. hermsi* requires a pair of fatty acids for growth, one saturated and the other unsaturated, and that the organism will not grow in a lipid-free medium unless so supplemented (4, 6). This suggests that *B. hermsi* does not have the metabolic ability to either synthesize or desaturate fatty acids. The data we have presented here from our studies of the incorporation of [^{14}C]palmitic acid and D-[^{14}C]glucose confirm this point. When cultivated in the presence of radiolabeled glucose, no radioactivity can be detected in the fatty acyl chains of the complex lipids, the label being restricted to the water-soluble backbone of these lipids. Conversely, cells cultivated with labeled palmitic acid have the label restricted to the acyl chains. This lack of exchange of carbon between the water-soluble and acyl precursors of complex lipids indicates that this organism is incapable of either chain elongation or β-oxidation of long-chain fatty acids. This is further substantiated by the similarity between the fatty acid profile of the cellular lipid and that of the available fatty acids of the medium. On the other hand, *B. hermsi* is fully capable of putting these two pools of precursors together in at least five ways, to account for the five different complex lipids that were labeled with [^{14}C]glucose. It is interesting to note that cells grown in unlabeled medium were not found to contain any species of complex lipid that were not synthesized by the cells. Apparently, direct incorporation of preformed complex lipids does not account for much of the lipid found in the cells.

Pickett and Kelly have reported that *B. hermsi* contains the enzymes for and can indeed dissimilate lysolecithin, but not other diacyl phospholipids or triacyl glycerols (6). Our data, while not contradicting this claim, suggest another explanation of their findings. We have demonstrated that this spirochete synthesizes PC as its major phospholipid with acyl groups drawn from the external milieu. It is conceivable that the lysolecithin, which is a rather toxic surfactant, is merely detoxified by acylating it to PC. This would account for its disappearance from the culture medium, but by an assimilatory rather than dissimilatory route.

Pickett and Kelly also reported that they were unable to detect α-glycerophosphate dehydrogenase activity in *B. hermsi* and that the lack of this activity would make it "more dependent upon its host for the provision of certain specific nutrients" (6). Our results suggest that *B. hermsi* can incorporate [^{14}C]glucose into its phospholipid. The incorporation of glucose into phospholipids, particularly into PC, indicates this enzyme is present in this spirochete.

The demonstration of the synthesis of cholesteryl glucoside and its acylated derivative by *B. hermsi* is a significant finding. This lipid is most commonly found in plants and has been reported in only one other bacterial genus. Cholesteryl-β-D-glucoside has been characterized in several mycoplasmas (8, 9), and recently the acylated cholesteryl glucoside had been identified (P. F. Smith, personal communication). This underscores the remarkable similarity in the lipid metabolism and composition of these two diverse groups of organisms.

The synthesis of cholesteryl glucoside by *B. hermsi* also helps to explain the selective removal of cholesterol from the culture medium that Pickett and Kelly have reported (6). Cholesterol has been reported to be an essential nutrient for *Treponema refringens* biotype Noguchi (7) and is also required by the other biotypes of this spirochete (unpublished data). It is quite possible that small, but significant, amounts of sterol glycosides are synthesized by these spirochetes also. Rothblat and Smith found that the cholesteryl glucoside synthesized by a non-sterol-requiring strain of mycoplasma was only found when the organism was supplied with exogenous cholesterol (8). Since our earlier survey of the genus *Treponema* utilized a lipid-defined medium devoid of steroids (5), a similar requirement would have resulted in the synthesis of sterol glycosides being overlooked. Further studies are necessary to determine the prevalence of these lipids within the genus *Treponema*.

We have demonstrated that the pattern of lipid composition and metabolism can be used to characterize and distinguish the genera *Leptospira, Spirochaeta,* and *Treponema* from one another (5). The data presented here also serve to characterize *B. hermsi* and to distinguish it from the other three genera. Since this organism does not have the ability to chain elongate or β-oxidize long-chain fatty acids, it is similar to the genus *Treponema*: *B. hermsi* also synthesizes monogalactosyl diglyceride, as do all of the treponemes. The major difference between these two genera is the appearance of cholesteryl glucoside in *B. hermsi,* which, as mentioned before, may not be unique to borreliae. No phosphatidylethanolamine could be detected in *B. hermsi,* although it is found in all the treponemes except *T. refringens* and *T. vincentii.* It is interesting to note that both of these species were once classified as *Borrelia.* Taken together, the similarities far outweigh the differences as compared with the other genera, *Leptospira* and *Spirochaeta.* Further studies including several more species of *Borrelia* will be necessary before the true taxonomic significance of these similarities can be evaluated.

ACKNOWLEDGMENTS

This investigation was supported by funding from the Oakland University Research Committee and the Biomedical Sciences Research Committee, awarded to B.P.L., and by Public Health Service grant AI-08124 from the National Institute of Allergy and Infectious Diseases, awarded to R.C.J.

LITERATURE CITED

1. **Felsenfeld, O., W. J. Decker, J. A. Wohlhieter, and A. Rafyi.** 1965. Studies on borreliae. II. Some immunolgoic, biochemical and physical properties of antigenic components of *Borrelia turicatae*. J. Immunol. **94**:805–817.
2. **Kates, M.** 1972. Techniques in lipidology. Isolation, analysis and identification of lipids, p. 269–610. *In* T. S. Work and E. Work (ed.), Laboratory techniques in biochemistry and molecular biology, vol. 3. North-Holland Publishing Co., Amsterdam.
3. **Kelly, R. T.** 1971. Cultivation of *Borrelia hermsi*. Science **173**:443–444.
4. **Kelly, R. T.** 1976. Cultivation and physiology of relapsing fever borreliae, p. 87–94. *In* R. C. Johnson (ed.), The biology of parasitic spirochetes. Academic Press Inc., New York.
5. **Livermore, B. P., and R. C. Johnson.** 1974. Lipids of the *Spirochaetales*: comparison of the lipids of several members of the genera *Spirochaeta, Treponema,* and *Leptospira*. J. Bacteriol. **120**:1268–1273.
6. **Pickett, J., and R. Kelly.** 1974. Lipid catabolism of relapsing fever borreliae. Infect. Immun. **9**:279–285.
7. **Rajkovic, A. D.** 1967. Cultivation of the Noguchi strain of *Treponema pallidum* in a lipid medium in the absence of serum. Z. Med. Mikrobiol. Immunol. **153**:297–312.
8. **Rothblat, G. H., and P. F. Smith.** 1961. Nonsaponfiable lipids of representative pleuropneumonia-like organisms. J. Bacteriol. **82**:479–491.
9. **Smith, P. F., T. A. Langworthy, and W. R. Mayberry.** 1973. Lipids of mycoplasma. Ann. N.Y. Acad. Sci. **225**:22–27.