INFECTION AND IMMUNITY, Sept. 1980, p. 990-994
0019-9567/80/09-0990/05$02.00/0

Vol. 29, No. 3

Endotoxin-Like Activities of Mycoplasmal Lipopolysaccharides (Lipoglycans)

ROBERT C. SEID, JR.,[1] PAUL F. SMITH,[2] GABRIEL GUEVARRA,[1] H. DONALD HOCHSTEIN,[3] AND MICHAEL F. BARILE[4]*

Department of Bacterial Diseases, Walter Reed Institutes of Research, Washington, D.C. 20012[1]; Department of Microbiology, University of South Dakota, Vermillion, South Dakota 57069[2]; Division of Control Activities[3] and Division of Bacterial Products,[4] Bureau of Biologics, Bethesda, Maryland 20205

Lipoglycans (previously designated lipopolysaccharides) from several species of *Acholeplasma* and from *Thermoplasma acidophilum* were examined for endotoxin-like activities as measured by the standard rabbit fever test and the *Limulus* amoebocyte lysate assay. The lipoglycans from *Acholeplasma granularum*, *Achloplasma laidlawii*, *Acholeplasma modicum*, and *Acholeplasma oculi* caused a febrile response at concentrations of 1 ng/ml per kg or greater, whereas with control *Escherichia coli* EC-2 lipopolysaccharides, 6.25 ng/ml per kg was required. Similar results were obtained in the *Limulus* amoebocyte lysate test. The minimum concentrations in nanograms per milliliter required to stimulate formation of a solid clot were: *Acholeplasma axanthum*, 0.22; *A. granularum*, 0.85; *A. modicum*, 0.51; *A. laidlawii*, 1.05; *A. oculi*, 0.74. Standard *E. coli* 1B lipopolysaccharide required a concentration of 0.125 ng/ml. *Thermoplasma* lipoglycan was least active, requiring 4.25 ng/ml. Clotting of the *Limulus* lysate proceeds by the activation by lipopolysaccharide plus Ca^{2+} of a proenzyme which cleaves an arginine-lysine peptide bond of the coagulogen. The clotting and amidase activities are inactivated by deoxycholate and can be reactivated by addition of lipopolysaccharide and Ca^{2+}. As with *E. coli* 1B lipopolysaccharide, acholeplasmal lipoglycans were shown to restore both clotting and amidase activities of the deoxycholate-inactivated *Limulus* clotting enzyme. The degree of restoration of amidase activity by mycoplasmal lipoglycans relative to *E. coli* lipopolysaccharide (1.00) were: *A. axanthum*, 1.71; *A. modicum*, 1.22; *A. granularum*, 0.61; and *Thermoplasma*, 0.37. The coagulating enzyme, restored with either *E. coli* lipopolysaccharide or mycoplasmal lipoglycans, was able to react with the synthetic peptide benzoyl-Ile-Glu-(γ-OCH_3)-Gly-p-nitroaniline (an analog of the coagulogen) or with the purified coagulogen itself to form the clot. The mycoplasmal lipoglycans alone were incapable of promoting these reactions when incubated with the synthetic peptide or with the purified coagulogen, thereby ruling out the contamination of these lipoglycans with proteases capable of cleaving the same Arg-Lys peptide bond of the coagulogen. These results show that acholeplasmal lipoglycans possess endotoxin-like activities. Their passive or active role in disease remains to be established.

Lipopolysaccharides (LPS) play an important role in the pathogenesis of diseases caused by gram-negative bacteria (3). These outer membrane components of bacteria are partially responsible for the fever observed during infectious diseases. Lipopolysaccharides are also constituents of organisms belonging to the class Mollicutes, including the *Acholeplasma*, *Anaeroplasma*, and *Thermoplasma* species (11, 13). Due to the confusion caused by naming these compounds LPS, a term commonly used for gram-negative bacterial LPS, the mycoplasmal polymers are now called lipoglycans (LG). The mycoplasmal LG are structurally different from LPS of gram-negative bacteria. They are components of the cytoplasmic membrane and have long oligosaccharide chains covalently attached to a diglyceride (13). The mycoplasmal LG lack heptoses, ketodeoxyoctanoate, and phosphoryl ethanolamine components found in the core oligosaccharide of gram-negative bacteria (12). The ester and amide-linked fatty acids typically found in LPS of gram-negative bacteria are present also in mycoplasmal LG. The LG from *Acholeplasma axanthum* is distinct because 90% of its fatty acids exists in *N*-acyl linkage to amino sugars, and only 10% of the fatty acid is esterified to glycerol (11, 12).

Scanning electron microscopic studies indicate that both the acholeplasmal and thermo-

plasmal LG have structural similarities to gram-negative bacterial LPS; for example, the purified mycoplasmal LG can aggregate into particles of several million daltons, forming ribbon-like structures 5 nm in width. In the presence of detergents, the mycoplasmal LG break down to form vesicular-shaped particles (9, 12). The lipoglycans are immunogenic, bind to sheep erythrocytes and to rabbit epithelial cell cultures, and produce other biological effects (14). The biological activity of mycoplasmal LG, especially as compared with that of the gram-negative bacterial LPS, has not been investigated. Therefore we examined purified mycoplasmal LG in the *Limulus* lysate and rabbit pyrogenicity test systems. These mycoplasmal LG evoked a febrile response in rabbits and activated the *Limulus* amoebocyte lysate system. Some of the mycoplasmal LG were more effective in stimulating the amidase and clotting activities of the *Limulus* coagulating enzyme than were the reference *Escherichia coli* LPS.

MATERIALS AND METHODS

***Limulus* lysate.** Pyrogen-free water was purchased from Abbott Laboratories, (Baltimore, Md.), and the tetrapeptide S-2222 (benzoyl-Ile-Glu-(γ-OCH_3)-Gly-*p*-nitroaniline) was purchased from Ortho Diagnostics (Raritan, N.J.). The *E. coli* EC-2 and 1B LPS used in these studies were the standard references used currently at the Bureau of Biologics. Intact, live *Limulus polyphemus* (horseshoe crabs) were collected from beaches of Long Island, New York. *Limulus* lysate was obtained by the method of Jorgensen and Smith (2). *Limulus* coagulogen was prepared by gel filtration of *Limulus* lysate in 20% acetic acid (15), and the semi-purified, inactivated *Limulus* coagulating enzyme was prepared by gel filtration of a lysate clot extract in sodium deoxycholate buffer (R. C. Seid, Jr., and T.-Y. Liu, *in* K. Yasonubu and T.-Y. Liu (ed.), *Frontiers in Protein Chemistry*, in press).

LG. The mycoplasmal LG were prepared from *Thermoplasma acidophilum* strain 122-1B2, *A. axanthum* strain S-743, *Acholeplasma granularum* strain BTS 39, *Acholeplasma laidlawii* strain PG9, *Acholeplasma oculi* strain 19L, and *Acholeplasma modicum* strain PG49. The procedure used for preparation and chemical analysis of LG has been reported elsewhere (11, 13). In brief, hot aqueous phenol extracts were treated with nuclease followed by filtration through columns of Corning controlled-pore-size glass beads (11). The purity of these preparations exceeded 98%, and each proved to be homogeneous as to antigenic specificity and size. The trace impurities were shown to be nucleic acids, based on both phosphorus content and absorbance at 260 nm. The presence of protein was not detectable (at levels of 0.01%) either by using the method of Lowry et al. (8) or by using an amino acid analyzer and *o*-phthalaldehyde as the fluorescent detection reagent (5). The mycoplasmal LG were dried in vacuo over phosphorus pentoxide at 56°C for 12 h. The properties of the mycoplasmal LG are summarized in Table 1.

Rabbit pyrogenicity tests. One milligram of the dried mycoplasmal LG or bacterial LPS was dissolved in 10 ml of pyrogen-free normal saline. After sonication for 3 min in an Ultra-Met II sonic bath, serial 10-fold dilutions were made with normal saline, and 1 ml/kg was injected intravenously into rabbits. A modified pyrogen test as described in *United States Pharmacopeia* was used (17). Healthy albino rabbits were conditioned in rooms of controlled temperature, background noise, light, and humidity, and three animals were used for each data point. A positive fever test was read as a rise in rectal temperature of 0.6°C within 3 h after injection.

***Limulus* amoebocyte lysate test.** One to three milligrams of the LG or LPS was dissolved in 1 ml of pyrogen-free water; the mixtures were sonicated for 3

TABLE 1. *Qualitative composition of mycoplasmal LG*

Mycoplasmal LG	Neutral sugars	Amino sugars[a]	Glycerol[b]	Fatty acids
T. acidophilum	Mannose, Glucose	None	+	C_{40} isopranyl diols
A. axanthum	Glucose, galactose	Galactosamine, fucosamine, quinovosamine	+	Saturated, unsaturated, branched, 3-hydroxy
A. granularum	Glucose, galactose	Glucosamine, fucosamine, quinovosamine	+	Saturated, unsaturated, branched
A. modicum	Glucose, galactose	Galactosamine, fucosamine, quinovosamine	+	Saturated, unsaturated, branched
A. laidlawii PG9	Glucose, mannose	Glucosamine, galactosamine, fucosamine, quinovosamine	+	Saturated, unsaturated, branched
A. oculi	Galactose, glucose, fucose	None	+	Saturated, unsaturated, branched

[a] All amino groups are *N*-acetylated.
[b] +, Contains glycerol.

min, and serial 10-fold dilutions were made for use in the test as described above. From these 10-fold dilutions, serial 2-fold solutions were made to determine the final endpoints in the lysate test. LPS and LG maintain endotoxin activities best when stored in a dried state. After rehydration, these endotoxins, especially at dilute concentrations, lose potency within a few weeks unless they are stored properly. The potency of diluted preparations is best maintained at −70°C. Before each test, the endotoxin preparations must be solubilized by shaking vigorously with a Vortex vibrator or sonicator.

The *Limulus* assay was performed by adding 0.1 ml of the LG dilution to 0.1 ml of lysate in a pyrogen-free tube (10 by 75 mm). After gentle mixing, the tubes were placed in an incubator at 37°C and maintained undisturbed for 1 h. The clotting reactions were read by gently inverting the tube 180°. If a solid clot formed, the dilution of LG was recorded as positive. The dilution of LG was considered negative when a clot did not occur or when the clot did not hold on tube inversion. Positive control dilutions of the reference *E. coli* 1B LPS were assayed at the same time, and pyrogen-free water was used as a negative control. The rabbit pyrogenic and *Limulus* lysate assays were done independently by two or more investigators, and similar or identical results were obtained.

Reactivation of deoxycholate-inactivated *Limulus* coagulating enzyme: preparation of deoxycholate-inactivated *Limulus* clotting enzyme from lysate. Limuli were bled by cardiac puncture, and the blood was collected in ice-cold, 250-ml siliconized bottles and centrifuged at 1,000 × *g* for 15 min at 4°C. After removal of the blue plasma by decantation, the amoebocytes (5 to 10 ml) were washed twice with pyrogen-free 3% NaCl. Pyrogen-free water in a 1:3 ratio was added, and the amoebocytes were lysed by blending in a Vortex mixer at 4°C. After centrifugation (1,000 × *g*, 30 min) to remove cellular debris, the supernatant was induced to clot by the addition of 1 ml of *E. coli* endotoxin solution (0.5 mg/ml) and 0.1 ml of 1 M $CaCl_2$. After 4 h of incubation at 37°C, the solid gel formed was broken with a stirring rod and centrifuged at 5,000 × *g* for 30 min at 4°C. The resulting supernatant (15 ml), containing the clotting enzyme, was recovered, and the precipitate was discarded. To inactivate the clotting enzyme, deoxycholate was added to the supernatant to a final concentration of 0.2%. After the addition of NaCl to a final concentration of 0.5 M, the resulting solution was immersed in an Ultramet II sonic bath for 30 s, and 10 ml was applied to a Bio-Gel A-5 m column equilibrated with 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, and 0.1 M NaCl containing 0.2% deoxycholate (Fig. 1). As described previously (Seid and Liu, in press), both endotoxin and calcium were added to samples of the collected fractions to establish the elution pattern of the deoxycholate-inactivated clotting enzyme.

Amidase activity measurement. Fifty microliters of deoxycholate-inactivated enzyme (obtained from tube number 55 in Fig. 1) was added to 1 ml of a 0.1 mM S-2222 solution prepared in 0.05 M Tris-hydrochloride buffer, pH 8.0. For the reactivation process, 50 μl of 0.1 M $CaCl_2$ solution and 200 μl of the mycoplasmal LG (1 mg/ml) were added. After incubation at 37°C for 6 h or less, based on the intensity of the yellow color produced, 0.5 ml of glacial acetic acid was added to quench the enzymatic reaction. The amount of *p*-nitroaniline released was measured by absorbance at 405 nm. Appropriate controls, with and without standard *E. coli* LPS and with and without the inactivated enzyme, were performed at the same time periods.

Clotting activity measurement. Fifty microliters of the deoxycholate-inactivated enzyme was added to 1 ml of the purified coagulogen solution (5 mg/ml) in 0.01 M Tris-hydrochloride buffer, pH 7.5, followed by calcium chloride (50 μl, 1 M) and 200 μl of the mycoplasmal LG (1 mg/ml). After incubation at 37°C for 6 h, the tubes were inverted and examined for clot formation as described for the *Limulus* lysate test procedure.

RESULTS

Rabbit thermal induction. The four acholeplasmal LG tested were as effective as the reference *E. coli* LPS in eliciting a specific febrile response in rabbits (Table 2). All four of the acholeplasmal LG caused fever at a concentration of 1 ng/ml per kg of rabbit body weight. The *E. coli* LPS induced fever at 6.25 ng/ml per kg.

***Limulus* lysate assay.** The effects of the mycoplasmal LG on the *Limulus* lysate are shown in Table 3. Each LG tested promoted

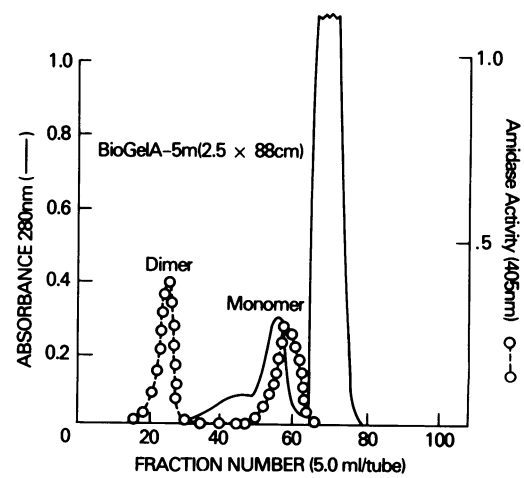

FIG. 1. *Elution of amidase activity in enzyme fraction from a Bio-Gel A column (2.5 by 88 cm) with 0.2% deoxycholate buffered with 50 mM Tris-hydrochloride and 0.1 M NaCl. Amidase activity was stimulated by E. coli LPS reference and calcium: samples (0.1 ml) were withdrawn from each fraction and added to 0.1 ml of buffer solution containing 0.40 mg of LPS and 0.22 mg of $CaCl_2$. After 30 min incubation at 37°C, 0.5 ml of 0.1 mM S-2222 substrate was added. The amount of p-nitroaniline released was measured by absorbance at 405 nm.*

TABLE 2. *Ability of mycoplasma LG to produce a positive rabbit pyrogen test*[a]

Mycoplasmal LG	Test results at concn of LG (ng/ml/kg):			
	100	10	1	0.1
A. granularum	+	+	+	−
A. laidlawii, PG9	+	+	+	−
A. modicum	+	+	+	−
A. oculi	+	+	+	−
A. axanthum	+	+	+	−
T. acidophilum	+	+	−	−

[a] The reference *E. coli* lipopolysaccharide EC-2 was positive at 6.25 ng/ml per kg and negative at 3.125 ng/ml per kg. +, Positive; −, negative.

TABLE 3. *Ability of mycoplasmal LG to produce a positive Limulus amoebocyte lysate test*

Mycoplasmal LG	Minimum concn to produce positive test (ng/ml)
T. acidophilum	4.25
A. axanthum	0.22
A. granularum	0.85
A. modicum	0.51
A. laidlawii, PG9	1.05
A. oculi	0.74
E. coli 1B LPS[a]	0.125

[a] Reference LPS used at Bureau of Biologics.

lysate gelation. The LG of *A. axanthum* was the most potent preparation and caused gelation at 0.22 ng/ml; the thermoplasmal LG was the least active.

Assay with semi-purified *Limulus* coagulating enzyme. A typical gel filtration chromatogram of the elution pattern of the *Limulus* coagulating enzyme, inactivated with sodium deoxycholate, is shown by the circled line in Fig. 1. As discussed elsewhere (Seid and Liu, in press), both the amidase and clotting activities of the coagulating enzyme can be restored by the addition of both *E. coli* LPS and calcium. Without these two components, no measurable enzymatic activities can be detected. Thus, the circled line in Fig. 1 represents restored amidase activity when the deoxycholate-enzyme fractions were supplemented with *E. coli* LPS and calcium. Mycoplasmal LG were shown to stimulate the deoxycholate-inactivated enzyme in the same manner (Table 4), i.e., incubation of the inactive enzyme with 200 μg of mycoplasmal LG plus calcium also caused reactivation of the amidase and clotting enzymatic activities. Thus, the acholeplasmal LG had activities similar to those of *E. coli* LPS reference in reactivating the amidase and clotting activities. Based on the amount of recoverable amidase activity, thermoplasmal LG had the lowest activity.

TABLE 4. *Reactivation of deoxycholate-inactivated Limulus clotting enzyme by mycoplasmal LG*[a]

Mycoplasmal LG	Amidase activity recovered[b]	Clotting activity[c]
A. axanthum	0.339 (1.71)[d]	+
A. modicum	0.241 (1.22)	+
A. granularum	0.121 (0.61)	+
T. acidophilum	0.073 (0.37)	+
E. coli 1B LPS[e]	0.198 (1.00)	+

[a] Controls: LG + S-2222 solution, no *p*-nitroaniline absorbance at 405 nm; LG + Coagulogen, no clot formation; Inactivated enzyme + S-2222, no *p*-nitroaniline absorbance at 405 nm; Inactivated enzyme + coagulogen, no clot formation.

[b] Amidase activity expressed as absorbance at 405 nm after 6 h of incubation.

[c] +, clot formation after 6 h of incubation.

[d] Numbers within parentheses indicate amidase activity relative to activity restored by *E. coli* reference LPS.

[e] Reference LPS used at Bureau of Biologics.

DISCUSSION

These findings indicate that the recently described class of LG (LPS) (11, 13) isolated from mycoplasmas have rabbit pyrogenic and *Limulus* amoebocyte lysate activities. When compared on a weight basis, the LG from the *Acholeplasma* species tested were slightly more effective in eliciting a febrile response in rabbits and almost as effective in *Limulus* lysate clotting activity than was the standard *E. coli* LPS.

The biochemical mechanism underlying the coagulation process appears to be a simple enzymatic cascade system (7). The process involves the activation of a proclotting enzyme by the bacterial LPS in the presence of calcium to an activated coagulating enzyme which in turn cleaves a single arginine-lysine peptide bond of the coagulogen. This limited proteolysis directly results in clot formation of the coagulogen (15). Our findings indicate that the acholeplasmal LG were as effective in concentrations ranging from 0.22 to 1.05 ng/ml as the *E. coli* LPS reference in activating *Limulus* lysate. However, at least a 10-fold variation in lysate activity was noted in several lots of LG tested.

The activation of the *Limulus* amoebocyte lysate system by the mycoplasmal LG appeared similar to that induced by gram-negative bacterial LPS (6); for example, the mycoplasmal LG can also reactivate the deoxycholate-treated *Limulus* coagulating enzyme. The coagulating enzyme, having both the amidase and clotting activities restored, reacts with the synthetic S-2222 peptide (10), an analog of the coagulogen, or with the purified coagulogen itself, to form the clot. The mycoplasmal lipoglycans were unable to promote these reactions when incubated with the synthetic S-2222 peptide alone or with

the purified coagulogen. These findings are similar to those obtained with *E. coli* LPS (Fig. 1) (Seid and Liu, in press) and indicate that the mycoplasmal LG are free of contaminating proteases. Proteases or trypsin-like enzymes are known to effect *Limulus* lysate gelation by cleaving the same Arg-Lys peptide bond of the coagulogen (15). If contaminating proteases were present, we would have expected the release of *p*-nitroaniline or clot formation to have occurred in the absence of the coagulating enzyme.

Among the mycoplasmal LG, the thermoplasmal LG appeared to be the least effective in promoting *Limulus* lysate gelation and in reactivating the deoxycholate-treated *Limulus* coagulating enzyme. The structural differences of the acholeplasmal and thermoplasmal LG may account for these differences. The thermoplasmal LG have been reported to contain a polymannosyl chain linked to glucose which is glycosidically bonded to a diglycerol tetraether. The long-chain alkyl groups are ether linked, rather than ester linked, to the glycerol backbone (4). The *A. axanthum* LG is a polymer consisting of neutral sugars, amino sugars (with the deoxyamino sugars predominating), and glycerol (11, 13). *A. axanthum* LG also contain long-chain fatty acids, with a high percentage of 3-hydroxy acids that are linked by amide bonds to amino sugars; thus, these LG are more akin to lipid A of gram-negative bacterial LPS (1, 11). Structural information on the other acholeplasmal LPS is not available (11, 13).

The pyrogenic activity of gram-negative bacterial LPS is believed to reside in the lipid A region of the molecule (18). It is likely that the lipoidal region of the mycoplasmal LG is also responsible for pyrogenicity and *Limulus* lysate gelation. The finding that mycoplasmal LG have rabbit pyrogenic and *Limulus* clotting activities similar to those of *E. coli* LPS may provide an opportunity to study pathogenic mechanisms of fever-inducing diseases by mycoplasmas. Endotoxins or LPS of *E. coli* cause abortion in animals and prematurity in humans. The possible role of LG in the pathogenesis of mycoplasmal disease merits consideration. Although acholeplasmas are isolated from tissues of infected animals, their role as causative agents of disease is not well defined (16). However, whereas the acholeplasmal endotoxin-like activities may have potential implications relevant to disease, this is not likely for thermoplasmal LG. These latter organisms are not found in animals and only survive at high temperatures and low pH in self-heating coal-refuse piles, a habitat which is man-made and generated from waste materials in the coal recovery process (4). Studies are in progress to examine whether the known pathogenic species of *Mycoplasma* contain LG and whether such LG are involved in the pathogenesis of mycoplasmal diseases.

LITERATURE CITED

1. **Galanos, C., O. Luderitz, E. T. Rietschel, and O. Westphal.** 1977. Chapter title, p. 239-335. *In* T. W. Goodwin (ed.), International review of biochemistry, biochemistry of lipids II, vol 14. University Park Press, Baltimore.
2. **Jorgensen, J. H., and R. F. Smith.** 1973. Preparation, sensitivity, and specificity of *Limulus* lysate for endotoxin assay. Appl. Microbiol. **26**:43-48.
3. **Kass, E. H., and S. M. Wolff (ed.).** 1973. Bacterial lipopolysaccharides. University of Chicago Press, Chicago.
4. **Langworthy, T. A.** 1979. Special features of Thermoplasmas, p. 495-513. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas. Academic Press, Inc., New York.
5. **Lee, H. M., D. J. Bucher, and R. C. Seid, Jr.** 1979. A highly sensitive and inexpensive amino acid analyzer, I&EC Prod. Res. Dev. **18**:122-126.
6. **Levin, J., and F. B. Bang.** 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. Bull. Johns Hopkins Hosp. **115**:265-274.
7. **Liu, T.-Y., R. C. Seid, Jr., J. Y. Tai, S. M. Liang, T. P. Sakmar, and J. B. Robbins.** 1979. Studies on the *Limulus* amoebocyte lysate coagulating system, p. 147-158. *In* E. Cohen (ed.), Progress in clinical and biological research, vol 29. Alan R. Liss, Inc., New York.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
9. **Mayberry-Carson, K. J., I. L. Roth, and P. F. Smith.** 1975. Ultrastructure of lipopolysaccharide isolated from *Thermoplasma acidophilum*. J. Bacteriol. **121**:700-703.
10. **Nakamura, S., T. Morita, S. Iwanaga, M. Niwa, and K. Takahasi.** 1977. A sensitive substrate for the clotting enzyme in horseshoe crab hemocytes. J. Biochem. **81**:1567-1569.
11. **Smith, P. F.** 1977. Homogeneity of lipopolysaccharides from *Acholeplasma*. J. Bacteriol. **130**:393-398.
12. **Smith, P. F.** 1979. The composition of membrane lipids and lipolysaccharides, p. 231-257. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol 1. Academic Press, Inc., New York.
13. **Smith, P. F., T. A. Langworthy, and W. R. Mayberry.** 1976. Distribution and composition of lipopolysaccharides from mycoplasmas. J. Bacteriol. **125**:916-922.
14. **Smith, P. F., and R. J. Lynn.** 1978. Biological effects of acholeplasmal lipopolysaccharides. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **241**:180-181.
15. **Tai, J. Y., R. C. Seid, Jr., R. D. Huhn, and T.-Y. Liu.** 1977. Studies on *Limulus* amoebocyte lysate II. J. Biol. Chem. **252**:4773-4776.
16. **Tully, J. G.** 1979. Special features of the Acholeplasmas, p. 431-449. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas. Academic Press, Inc. New York.
17. **United States Pharmacopeia, 19th ed.** 1975. Pyrogen test, p. 613; Suppl. 4, p. 19; and the National formulary; 1975, 14th ed., p. 948. Mack Publishing Co., Easton, Pa.
18. **Yin, E. T., C. Galanos, S. Kinsky, R. A. Bradshaw, S. Wessler, O. Luderitz, and M. E. Sarmiento.** 1972. Picogram-sensitive assay for endotoxins: gelatin of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from gram-negative bacteria. Biochim. Biophys. Acta **261**:284-289.