JOURNAL OF BACTERIOLOGY, Dec. 1984, p. 994–1002
0021-9193/84/120994-09$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 160, No. 3

Chemical and Immunological Characterization of Lipopolysaccharides from Phase I and Phase II *Coxiella burnetii*

KEN-ICHI AMANO† AND JIM C. WILLIAMS‡*

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

Received 5 July 1984/Accepted 15 September 1984

Lipopolysaccharides (LPSs) isolated from phase I and phase II *Coxiella burnetii* (LPS I and LPS II, respectively) were analyzed for chemical compositions, molecular heterogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunological properties. The yields of crude phenol-water extracts from phase I cells were roughly three to six times higher than those from phase II cells. Purification of LPSs by ultracentrifugation gave similar yields for both LPS I and LPS II. Purified LPS I and LPS II contained roughly 0.8 and 0.6% protein, respectively. The fatty acid constituents of the LPSs were different in composition and content, with branched-chain fatty acids representing about 15% of the total. β-Hydroxymyristic acid was not detected in either LPS I or LPS II. A thiobarbituric acid-periodate-positive compound was evident in the LPSs; however, this component was not identified as 3-deoxy-D-mannooctulosonic acid by gas and paper chromatographies. LPS II contained D-mannose, D-glucose, D-glyceromannoheptose, glucosamine, ethanolamine, 3-deoxy-D-mannooctulosonic acid-like material, phosphate, and fatty acids. LPS I contained the unique disaccharide galactosaminuronyl glucosamine and nine unidentified components in addition to the components of LPS II. The hydrophobic, putative lipid A fraction of LPS I and LPS II contained the above constituents, but the hydrophilic fraction was devoid of ethanolamine. The LPS I disaccharide galactosaminuronyl glucosamine was found in both fractions of the acetic acid hydrolysates. Analysis of LPSs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining indicated that LPS II was composed of only one band, whereas LPS I consisted of six or more bands with irregular spacing. Ouchterlony immunodiffusion tests demonstrated that LPS I reacted with phase I but not with phase II whole-cell hyperimmune antibody, and LPS II reacted neither with phase I nor phase II hyperimmune antibody. From these results, it was concluded that the chemical structures of LPSs from *C. burnetii* were different from those of the LPSs of gram-negative bacteria; however, the LPS structural variation in *C. burnetii* may be similar to the smooth-to-rough mutational variation of saccharide chain length in gram-negative bacteria.

The rickettsial agent of Q fever, *Coxiella burnetii*, undergoes a transition from virulent (phase I) to avirulent (phase II). This phase transition correlates with some of the biological characteristics of the "smooth-to-rough" lipopolysaccharide (LPS) variation observed for gram-negative *Enterobacteriaceae* (10, 20, 31). *C. burnetii* in phase I may be converted to phase II by serial passage in the yolk sacs of embryonated eggs (8, 32). This phase variation detected by serological techniques with polyclonal sera (32) and monoclonal antibodies (40) is due to differences in the antigenic surface of cells. Additionally, the phase variants exhibit various differences such as exposure of immunogenic surface proteins (42), surface charge (16), cell density (12), virulence (5, 13), and resistance to phagocytosis by macrophages and lymphocytes (14, 15).

LPS extracted with hot phenol-water from phase I *C. burnetii* (LPS I) exhibits some biological activities associated with the endotoxic LPS from gram-negative *Enterobacteriaceae* (4, 6, 27). LPS I is considered to be a major determinant of virulence expression and infection (4, 5, 13). Recent studies (3, 29) of the structures of LPS I and phase II LPS (LPS II) of *C. burnetii* produced results which were somewhat contradictory. The results of Baca et al. (3) suggest that LPS I and LPS II have similar sugar compositions and serological activities. However, the more recent study by Schramek and Meyer (31) confirms previous indications (29) of LPS sugar components but reveals distinctly different sugar compositions.

Studies in our laboratory of the structures of LPS I and LPS II suggested that the chemical composition and antigenic structures of LPS I and LPS II were distinctly different. In this paper, we compare some chemical and immunological properties of LPS I and LPS II derived from two different phase I isolates and a cloned pure phase II isogenic derivative of phase I cells. Our studies corroborated the results of Schramek and Meyer (31) and expanded our knowledge of the chemical properties, molecular heterogeneity, and immunological properties of *C. burnetii* LPSs.

(Preliminary reports of this work were presented at the 82nd Annual Meeting of the American Society for Microbiology, Atlanta, Ga., 7–12 March 1982 [Williams, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B88, p. 32], and the Third National Meeting of the Society for Rickettsiology and Rickettsial Diseases, Atlanta, Ga., 12–14 March 1982.)

MATERIALS AND METHODS

Organisms. *C. burnetti* strains were in various passage levels in guinea pigs, chicken embryo fibroblast tissue culture, and chicken embryo yolk sacs (41). Clones were

* Corresponding author.
† Present address: Department of Bacteriology, Hirosaki University School of Medicine, Hirosaki, Aomori 036, Japan.
‡ Present address: Rickettsial Diseases Laboratory, Aerobiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21701.

isolated by M. Peacock by selecting plaques on chicken embryo fibroblast monolayers (22). Phase determinations of each strain were carried out as described by Stoker and Fiset (32). The Ohio (CBOI) (5EP/2GP/2EP) and Nine Mile (CB9MI) (307GP/1TC/1EP, clone 7) isolates were phase I, no phase I activity was demonstrated with the Nine Mile phase II isolate (CB9MII) (90EP/1TC/4EP, clone 4). Cultivation of rickettsial cells, purification by Renografin density gradient centrifugation, and inactivation by formaldehyde were described previously (41). All preparations were tested for contamination by autonomously growing bacteria on blood agar base plates and thioglycolate medium. Media were inoculated with 0.1 ml of freshly purified *C. burnetii*, and incubation was carried out at 36°C in a humidified incubator for 15 days.

CM extraction of whole cells. Lyophilized whole cells (500 mg in 100 ml of chloroform-methanol [CM] [4:1, vol/vol]) were refluxed at 53°C for 6 to 8 h (39). After cooling to room temperature, the CM residue (CMR) was separated from the CM extract by filtration through Whatman no. 1 filter paper. The CMR was extracted three more times with 100 ml of CM. Finally, the CMR was dried in vacuo.

Preparation of LPS and putative lipid A. LPS was extracted from formaldehyde-inactivated *C. burnetii* with hot phenol-water by the method of Westphal and Jann (38) with slight modification. Whole cells or CMR (500 mg each) were suspended in 50 ml of 67 mM phosphate-buffered saline (pH 7.4), and 50 ml of 90% (vol/vol) redistilled phenol at 68°C was added. The suspension was vigorously shaken for 20 min in a water bath at 68°C. After cooling and centrifugation at 2,500 rpm for 20 min, the aqueous phase was collected. The interphase and phenol phase were extracted twice with an equal volume of phosphate-buffered saline, and the aqueous phases were pooled and dialyzed against distilled water for 7 days with daily changes of distilled water. The crude phenol-water extract (CPWE) was centrifuged at 10,000 × *g* for 20 min at 4°C. This low-speed supernatant was centrifuged at 100,000 × *g* for 15 h. The LPS precipitate was washed twice with demineralized water and lyophilized. The high-speed supernatant was dialyzed against demineralized water by using endotoxin-free tubing of 8,000-dalton restriction. The lipid A fraction (10) from LPS I and LPS II was prepared by hydrolysis of LPS with 2% acetic acid at 100°C for 2 h and then centrifugation at 100,000 × *g* for 15 h. The polysaccharide portion in the supernatant and the putative lipid A in the precipitate were analyzed for chemical components.

Analytical methods. Amino acids and amino sugars were analyzed in a Beckman 118-CL amino acid analyzer with samples hydrolyzed in 6 N HCl at 100°C for 15 h in sealed glass ampoules. No corrections for the loss of amino acids and amino sugars during hydrolysis were made. Amino sugars were also determined by the method of Tsuji et al. (36) with glucosamine as a reference standard. Protein contents were determined by amino acid analysis. Neutral sugars were measured by the phenol-H_2SO_4 method (7) with glucose as a reference standard. Heptose was determined by the cysteine-H_2SO_4 method (23) with glycero-D-glucoheptose (Sigma Chemical Co., St. Louis, Mo.) as a reference. Total phosphorus was determined by the method of Lowry et al. (19). The concentration of the 3-deoxy-D-mannooctulosonic acid (KDO)-like compound (Sigma) was analyzed by the method of Osborn (23). Reducing groups were determined by the method of Park and Johnson (26) with glucose as a reference standard.

Fatty acids were analyzed as methyl esters in a Perkin-Elmer 900 gas chromatograph on a Gas-Chrom P column (100 to 120 mesh) containing 10% ethylene glycol succinate (Applied Science Laboratories, Park Ridge, Ill.) as previously described (2). Quantitative analysis of neutral sugars was determined by alditol acetates. Samples (4 mg) were hydrolyzed by 4 N HCl at 100°C for 4 h. Dried hydrolysates were reduced with excess $NaBH_4$ in 0.5 ml of distilled water at room temperature overnight. Samples were then applied to short columns of Dowex 50W-X4 (200 to 400 mesh; bed volume, 1 ml) and eluted with 3 bed volumes of distilled water. The eluates were lyophilized, dissolved with 1 ml of methanol, and evaporated to remove borate. This process was repeated twice. Reduced samples were incubated with 0.1 ml of pyridine and 0.1 ml of acetic anhydride at 100°C for 1 h. Dried alditol acetates were dissolved with 0.1 ml of chloroform and analyzed on a 3% ECNSS-M (ethylene succinatecyanoethyl silicone polymer) Gas-Chrom Q stainless column with a temperature program of 160 to 210°C at 2°C per min. Reference alditol acetate derivatives were prepared from authentic samples.

Paper chromatography was conducted by the descending method on Whatman no. 1 filter paper by employing *n*-butanol–pyridine–acetic acid–water (60:40:3:30, vol/vol/vol/vol). Reducing sugars and amino acids were visualized on the developed chromatograms with alkaline $AgNO_3$ (34) and with ninhydrin, respectively.

***N*-Acetylation and reduction of compound X.** *N*-Acetylation of compound X was carried out as described by Amano et al. (1). Compound X (0.5 μmol, calculated by using glucosamine as a standard) was dissolved in a mixture containing 10 mg of $NaHCO_3$ and 6 μl of acetic anhydride in 200 μl of distilled water. The solution was shaken, kept at 0°C overnight, and then added to a column of Dowex 50W-X4 (H^+ form). The column was eluted with 3 bed volumes of distilled water, and the eluate was lyophilized.

The reduction of compound X with $NaBH_4$ was carried out as described previously (1). The reduction of carboxyl groups in compound X was performed by the method of Taylor and Conrad (33). To an aqueous solution (0.5 ml) of *N*-acetylated saccharide (0.5 μmol), 2 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was added, and the pH of the solution was adjusted to 4.8 with HCl. After the solution was stirred for 3 h at room temperature, 0.3 ml of $NaBH_4$ solution was added slowly, and stirring was continued overnight at pH 9.7. The reaction mixture was applied to a Dowex 50W-X4 column. Constitutents were eluted with 3 bed volumes of distilled water. After lyophilization, boric acid was removed from the eluate by repeated evaporation with methanol.

SDS-PAGE. Samples were analyzed by sodium dodecyl

TABLE 1. Yields of LPS fractions extracted with phenol-water from whole cells and CMRs

Extraction from[a]:	Yield of LPS fraction[b]	
	CPWE[c]	LPS
CB9MI WC	8.3	1.9
CB9MI CMR	10.7	2.4
CBOI WC	12.3	4.3
CBOI CMR	11.4	4.0
CB9MII WC	3.4 (40)	3.2 (168)
CB9MII CMR	2.0 (19)	2.0 (83)

[a] WC, Whole cells.
[b] Dry weight percentage of WC or CMR.
[c] CPWE and LPS represent before and after purification by ultracentrifugation, respectively. The percentage of the isogenic phase I variant is shown within parentheses.

TABLE 2. Chemical analysis of LPSs from CMRs

LPS from CMR of:	Chemical component[a]					
	Protein	Neutral sugar[b]	Heptose	Phosphate	KDO-like compound	Fatty acid
CB9MI	0.8 (1.0)[c]	1,500 (1.0)	336 (1.0)	303 (1.0)	41 (1.0)	2.2 (1.0)
CBOI	0.9 (1.1)	1,500 (1.0)	338 (1.0)	443 (1.5)	54 (1.3)	0.6 (0.3)
CB9MII	0.6 (0.8)	1,000 (0.7)	588 (1.8)	916 (3.0)	132 (3.2)	1.2 (0.5)

[a] Protein and fatty acid expressed as percentages; all other components expressed as nanomoles per milligram.
[b] Total sugar minus heptose.
[c] Chemical component ratios of strain CB9MI compared with the CBOI isolate and CB9MII isogenic derivative.

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (17) but with a 5% polyacrylamide spacer gel and 12.5% separating slab gels, which were 1.5 mm thick. Each sample (20 μg [dry weight]) was boiled for 5 min in sample buffer (Laemmli buffer plus 0.1 M dithiothreitol) and immediately applied to each slot (20 μg per slot). After electrophoresis of samples, the separated components were fixed with an aqueous solution of 25% isopropyl alcohol and acetic acid. Slab gels were stained with Coomassie brilliant blue R250 (Sigma) and by the silver staining method described by Tsai and Frasch (35).

Immunological detection of antigen-antibody interaction. Immunodiffusion assays were done with 1% agarose gels and Veronal buffer (24). Hyperimmune sera were raised in guinea pigs by injecting them with Formalin-killed whole cells of strains CB9MI, CB9MII, and CBOI (39).

RESULTS

Isolation and chemical analysis of LPS fractions. The yields of CPWE from phase I cells (strains CB9MI and CBOI) were roughly three to six times higher than those of CPWE from phase II cells (strains CB9MII). However, when the LPSs were purified by ultracentrifugation, the yields of phase I and II were similar (Table 1). All of the LPS II from strain CB9MII whole cells and strain CB9MII CMR was easily precipitated by ultracentrifugation at 100,000 × *g* for 2 h. However, the precipitation of LPS I from CB9MI whole cells, CB9MI CMR, CBOI whole cells, and CBOI CMR required ultracentrifugation at 100,000 × *g* for 15 h and gave yields of 22 and 35%, respectively, of the CPWE.

The amounts of protein, neutral sugar, and heptose were not significantly different in LPS fractions derived from CB9MI and CBOI CMRs (Table 2), although the phosphate and KDO-like constituents were slightly higher in the LPS of strain CBOI. However the fatty acid content of the LPS from CB9MI CMR was 3.7-fold greater than that of the LPS of CBOI CMR. In contrast, the protein, neutral sugar, and fatty acid concentrations of LPS II were less than those of the isogenic LPS I. However, increases in contents of heptose, phosphate, and the KDO-like compound were noted in LPS II as compared with the isogenic LPS I. Comparable differences in the concentrations of chemical components were obtained for the CPWE of whole cells and CMR (data not shown). The thiobarbituric acid-periodate-positive compound was identified in the CPWE of whole cells, CMR, LPS I, and LPS II; however, this component did not comigrate with standard KDO acid by gas and paper chromatographies (data not shown).

The fatty acid compositions of LPSs derived from CMRs of *C. burnetii* isolates were compared (Table 3). Differences in fatty acid composition were obtained for the LPSs from CMRs of the isogenic pairs of strains CB9MI and CB9MII. The predominant fatty acids (>1.0%) associated with LPSs were C_{11} (absent in LPS II), C_{12}, C_{14}, C_{16}, C_{18}, $C_{18:1}$ (absent in LPS II), $C_{20:1}$ (absent in LPS I of strain CBOI), C_{21}, C_{22}, $C_{22:1}$ C_{23}, C_{14} (iso), C_{15} (anteiso; absent in LPS I of strain CBOI), and C_{17} (anteiso; absent in LPS I). On the other hand, we could not detect β-hydroxymyristic acid, which is a constituent of enteric lipid A of LPS (10). Other hydroxy fatty acids were not examined in this study.

Amino acid and amino sugar compositions of LPS fractions. Amino acid analysis of LPS I and LPS II derived from CMRs indicated that the purified LPSs contained aspartic acid, glutamic acid, lysine, threonine, serine, glycine, alanine, and leucine (Table 4). LPS I fractions contained three amino compounds (glucosamine, ethanolamine, and an unidentified amino compound referred to as compound X), whereas LPS II contained only two amino compounds (glucosamine and ethanolamine) in addition to the amino acids. Although diaminopimelic acid was detected in CPWE, it was not found in the purified LPS fractions.

Neutral sugar compositions of LPSs. Hydrolysates of LPS I and LPS II were converted to alditol acetates and analyzed by gas chromatography. The relative retention times com-

TABLE 3. Fatty acid analysis of LPSs derived from CMRs

Constituent	LPS derived from[a]:		
	CB9MI	CBOI	CB9MII
Unbranched			
C_{11}	2.0	0.4	—[b]
C_{12}	2.2	1.3	1.8
C_{14}	6.8	7.2	8.1
C_{16}	12.0	13.9	20.4
C_{18}	4.1	6.1	8.1
$C_{18:1}$	7.8	5.2	—
$C_{20:1}$	3.7	—	4.7
C_{21}	26.3	31.4	21.7
C_{22}	4.6	6.4	8.5
$C_{22:1}$	8.5	8.0	8.4
C_{23}	2.8	5.5	2.7
Branched[c]			
C_{14I}	13.7	14.6	11.8
C_{15A}	1.7	—	1.6
C_{17A}	—	—	2.3

[a] Expressed as a percentage of total fatty acids.
[b] —, Not detected.
[c] I, Iso; A, anteiso.

TABLE 4. Amino acid and amino sugar compositions of LPSs from CMRs

Constituent	LPS derived from[a]:		
	CB9MI	CBOI	CB9MII
Acidic			
Aspartic acid	11	18	10
Glutamic acid	5	15	6
Basic			
Lysine	4	6	2
Uncharged hydrophilic			
Threonine	3	8	Tr
Serine	16	17	12
Glycine	11	22	17
Hydrophobic			
Alanine	Tr	13	Tr
Leucine	4	14	3
Other compounds			
Diaminopimelic acid	—[b]	—	—
Glucosamine	108	143	31
Ethanolamine	31	77	203
Compound X[c]	93	116	—

[a] Expressed as nanomoles per milligram of LPS (wt/wt).
[b] —, Not detected.
[c] Compound X was calculated as the same intensity of glucosamine by amino acid analysis.

pared with hexaacetylmannitol are summarized in Table 5. At least 12 compounds, including arabinose, mannose, and D-glyceromannoheptose as major sugars and glucose as minor sugar, were detected in LPS I, although only mannose and D-glyceromannoheptose as major sugars and glucose as a minor sugar were detected in LPS II. On the basis of the data of Tables 2 to 5, LPS II consisted of mannose, glucose, D-glyceromannoheptose, phosphate, a KDO-like compound, glucosamine, ethanolamine, and fatty acids. The ratio of mannose to D-glyceromannoheptose for LPS I and LPS II was about 1:1 (Table 5). LPS I contained many unidentified components and an amino compound X.

Isolation and partial characterization of compound X. To determine the structure of compound X, 45 mg of LPS I from strain CB9MI whole cells was hydrolyzed in 6 N HCl at 100°C for 15 h, evaporated to dryness, and then dissolved in sterile demineralized distilled water. The hydrolysate solution was applied to a Dowex 50W-X4 column (bed volume, 10 ml), and the column was washed with 60 ml of distilled water and then eluted stepwise with 60 ml each of 0.6, 1.2, and 2 N HCl. The four eluates were lyophilized and analyzed for amino acids. Compound X was detected in the 1.2 N HCl elutate with traces of diaminopimelic acid, leucine, and lysine (Fig. 1A). Glucosamine was detected in the 0.6 N HCl eluate. Quantitative analysis of compound X showed that this compound was an amino sugar with a reducing group-amino sugar-neutral sugar-phosphate molar ratio of 1:1.26:0.03:0.03. Further, the retention time of compound X (80.1 min) in amino acid analysis was different from those of glucosamine, galactosamine, and mannosamine (60.5, 65.0,

TABLE 5. Analysis of alditol acetates of LPSs extracted from CMRs

No.	Relative retention time[a]	Carbohydrate	LPSs derived from[b]:		
			CB9MI	CBOI	CB9MII
1	0.55	Arabinose	4.7	4.9	Tr
2	0.61		3.0	4.3	0
3	0.81		9.2	10.2	0
4	0.94		2.3	2.7	0
5	1.00	Mannose	20.1	20.6	46.8
6	1.23	Glucose	1.7	1.5	0.5
7	1.36		3.3	4.2	0
8	1.48		2.4	3.0	0
9	1.64	D-Glyceromannoheptose	24.0	26.5	51.3
10	2.43		15.4	13.8	0
11	2.64		6.7	5.7	0
12	3.01		3.2	1.3	0

[a] Retention time of mannose was 11.2 min under the conditions described in the text.
[b] Expressed as area percentages.

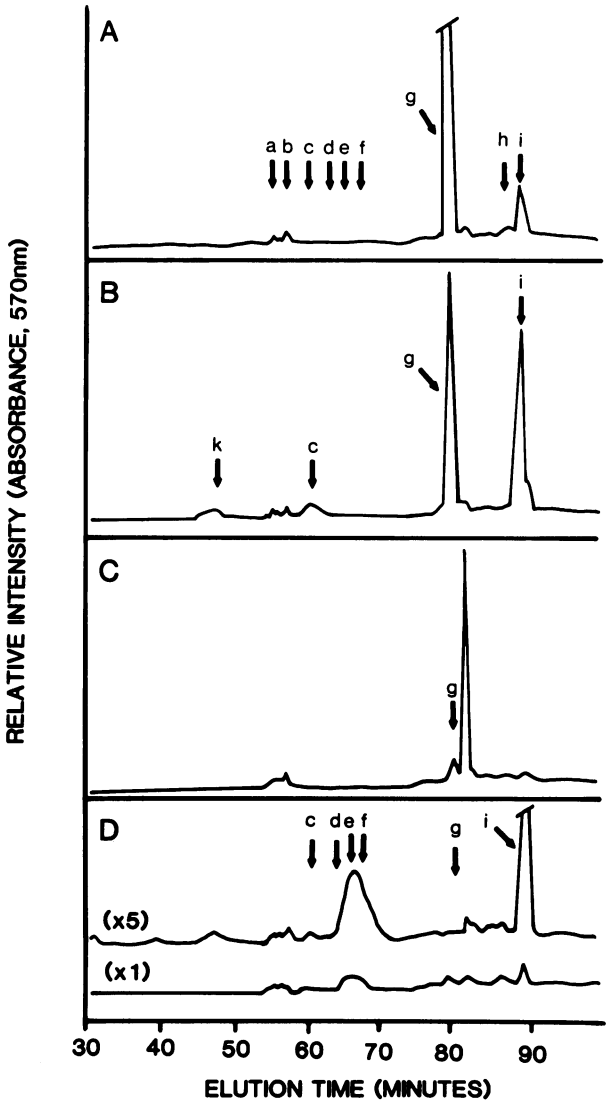

FIG. 1. Amino acid analysis of compound X and its derivatives. A, Compound X; B, acid hydrolysate of compound X in 6 N HCl at 100°C for 15 h; C, reduced compound X with $NaBH_4$; D, reduced compound X with 1-ethyl-3-(3-dimenthylamino propyl)carbodiimide and $NaBH_4$. Standards and compound X: a, diaminopimelic acid; b, leucine; c, glucosamine; d, mannosamine; e, glucosaminitol; f, galactosamine; g, compound X; h, lysine; i, NH_3; k, hexosaminuronic acid (44).

TABLE 6. Chemical analysis of the putative O-specific saccharide and lipid A fractions of LPSs

Chemical component[a]	Fraction of LPS from[b]:					
	CB9MI		CBOI		CB9MII	
	PPT	SUP	PPT	SUP	PPT	SUP
Reducing group	10	324	3	269	10	276
Neutral sugar[c]	330	1,930	160	1,920	380	1,640
Phosphate	133	90	116	133	452	239
Glucosamine	27	106	15	141	6	28
Compound X[d]	21	75	11	93	—[e]	—
Ethanolamine	64	—	55	—	153	—
Protein	399	40	55	20	38	6

[a] Expressed as nanomoles per milligram of LPS (wt/wt). The fatty acid compositions were not determined. Protein content was determined by amino acid analysis.

[b] Acid condition is 2% acetic acid at 100°C for 2 h. PPT, High-speed precipitate which correlated with lipid A; SUP, high-speed supernatant which correlated with the saccharide portion.

[c] Total neutral sugar content includes the heptose content.

[d] Compound X was calculated as the same color intensity of glucosamine by amino acid analysis.

[e] —, Not detected.

and 66.9 min, respectively); however, the retention times were similar to those of basic amino acids (histidine and lysine, 82.7 and 85.4 min, respectively [Fig. 1A]).

When compound X was hydrolyzed in 6 N HCl at 100°C for 15 h, two ninhydrin-positive compounds corresponding to glucosamine and hexosaminuronic acid (43) were released (Fig. 1B), but the major portion of compound X was still present. This result suggested that the unknown compound consisted of two amino sugars (glucosamine and hexosaminuronic acid). Several laboratories (18, 43, 44) have reported that the hexosaminuronic acid-containing disaccharides are highly resistant to acid hydrolysis. For example, galactosaminuronyl fucosamine is not completely hydrolyzed in 6 N HCl at 100°C for 96 h (18). When compound X was reduced with $NaBH_4$ and applied to the amino acid analyzer, the retention time (81.7 min) differed from that of the original compound (Fig. 1C).

Compound X was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and $NaBH_4$ after *N*-acetylation to quantitatively reduce carboxyl groups. This method also promoted reduction of the reducing end of the sugars at the same time. The reduced compound was hydrolyzed in 6 N HCl at 100°C for 15 h, evaporated to dryness, and applied to the amino acid analyzer. The broad peak corresponded to either galactosamine or glucosaminitol in the reduced samples; however, when the sample was subjected to amino acid analysis, two peaks corresponding to the elution times of the two amino sugars described above appeared (Fig. 1D). It was possible that galactosamine and glucosaminitol were derived from the galactosaminuronic acid and glucosamine, respectively, of the original compound X (Fig. 1B and 1D). Furthermore, it seemed likely that compound X was a disaccharide galactosaminuronyl glucosamine. Further studies are required to establish the complete structure of compound X.

Acetic acid hydrolysis of LPS I and LPS II. Hydrolysis of LPSs was carried out to generate analogs of O-specific side chains and lipid A fractions described for other bacterial LPSs (10). Supernatant and precipitate fractions after centrifugation of acetic acid-hydrolyzed LPSs were putatively O-specific side chains and lipid A fractions, respectively. A comparison of chemical compositions of the supernatant and precipitate fractions revealed similarities and differences between these fractions (Table 6). The preciptates of LPS I, although containing the same constituents, showed differences in relative concentrations of chemical components. The precipitate of strain CB9MI LPS contained 2- and 7.2-fold greater concentrations of neutral sugar and protein, respectively, than did the precipitate of strain CBOI LPS, whereas the concentrations of the other constituents were very similar. Precipitates from the isogenic pairs of strain CB9MI and CB9MII LPSs showed marked differences in chemical components. Phosphate and ethanolamine concentrations of the precipitates of LPS II were 3.3- and 2.3-fold greater than those of the precipitate of CB9MI LPS, whereas the glucosamine and protein concentrations of the precipitates of LPS II were 4.5- and 10.5-fold less than those of the precipitates of LPS I from strain CB9MI. Moreover, compound X, which was detected in both precipitates and supernatants of LPS I, was not detected in the precipitates or supernatant of LPS II. In contrast, the supernatant fractions of LPS I and LPS II contained similar concentrations of reducing groups, neutral sugars, and phosphate, but LPS II contained 3.7- and 6.6-fold-less glucosamine and protein, respectively, than did LPS I from CB9MI. Ethanolamine was the only component that was clearly fractionated into the precipitates since the component was not detected in any supernatant fractions.

The neutral sugar compositions of precipitates and supernatants of LPS I and LPS II were analyzed as alditol acetates (Table 7). Mannose and D-glyceromannoheptose were detected as major neutral sugars from the precipitate and supernatant of LPS I and LPS II hydrolysates in a molar ratio of about 1:1. The contents of mannose and D-glyceromannoheptose were less in the supernatant fraction derived from LPS I, but the concentrations of these two carbohydrates were very similar in the precipitate and supernatant of LPS II. Arabinose was detected in 8.8-fold-higher concentrations in the precipitate as compared with the supernatant of LPS II, whereas the concentration of arabinose in the supernatant of LPS I was 1.8- to 2.2-fold greater than that in the precipitate. Glucose was detected in the supernatant of LPS I but not in the supernatant of LPS II, and the majority of the glucose was clearly fractionated into the precipitates of LPS I and LPS II. Unknown carbohydrates were fractionated into either or both fractions. Six unidentified minor components and four unidentified major (Table 7) components were detected from the supernatant of LPS I hydrolysates. Seven minor components were detected from the precipitate of LPS I hydrolysates.

SDS-PAGE of LPS fractions. Since we were interested in comparing the molecular weights and banding profiles of LPSs from the three isolates, SDS-PAGE of these samples was carried out after boiling each sample in sample buffer (Fig. 2). Several components were resolved in silver-stained SDS-polyacrylamide gels. No Coomassie brilliant blue-stainable bands were detected in the LPS fractions (data not shown). Gel patterns of SDS-PAGE indicated that LPS fractions from strain CB9MI (Fig. 2, lanes 2 to 4) and from strain CBOI (Fig. 2, lanes 6 to 8) were composed of similarly distributed molecular weight components. In addition, the gel patterns of the CB9MI and CBOI whole cells were also similar (Fig. 2, lanes 5 and 9, respectively). However, a comparison of the gel patterns of LPS II fractions (Fig. 2, lanes 10 to 12) and LPS I fractions (Fig. 2, lanes 2 to 4 and 6 to 8) indicated that these two LPSs were completely different. These differences in gel patterns were reflective of the banding patterns of the whole cells of strains CB9MII (Fig. 2, lane 1) and CB9MI (Fig. 2, lanes 5 and 9).

TABLE 7. Alditol acetates of acetic acid hydrolysates of LPSs isolated from CMRs

No.[a]	Relative retention time	Carbohydrate	Fraction of LPS from[b]:					
			CB9MI		CBOI		CB9MII	
			PPT	SUP	PPT	SUP	PPT	SUP
N-1	0.18		—[c]	2.5	—	5.7	—	—
N-2	0.20		2.1	4.6	1.7	8.2	—	—
N-3	0.37		—	2.7	—	4.7	—	—
1	0.54	Arabinose	4.0	7.2	4.0	8.9	4.4	0.5
2	0.62		—	1.7	—	2.0	—	—
3	0.81		—	14.1	—	16.0	—	—
N-4	0.85		1.2	—	3.0	—	—	—
N-5	0.90		1.4	—	1.8	0	1.1	—
4	0.93		—	1.9	—	2.3	—	—
5	1.00	Mannose	30.6	16.1	30.2	14.9	49.0	51.9
6	1.23	Glucose	6.4	0.7	10.6	0.9	1.2	—
7	1.35		—	2.1	—	2.0	—	—
N-6	1.38		1.7	2.1	1.0	1.9	—	—
9	1.63	D-Glyceromannoheptose	43.0	33.1	40.5	25.5	44.4	46.8
10	2.51		3.9	9.1	2.2	4.0	—	—

[a] N-1 to N-6 are new components not found in untreated LPS (Table 5).
[b] Expressed as area percentages.
[c] —, Not detected.

LPS I fractions (CPWE, Fig. 2, lanes 2 and 6; supernatant of CPWE, lanes 3 and 7; LPS, lanes 4 and 8) consisted of a major component with an apparent molecular weight of 16.1 $\times 10^3$ (16.1K) and five minor components with apparent molecular weights of 22, 21.1, 20, 18.6, and 14.7K. The phase II CPWE and LPS II (Fig. 2, lanes 10 and 12, respectively) were composed of one component (apparent molecular weight, 10.5K), although phase II CPWE had only a faint band of the 10.5K component.

We applied the LPSs of *Salmonella typhimurium* wild strain LT-2 and Re mutant strain G30/C21 to the same gel (Fig. 2, lanes 13 and 14, respectively) with the LPS fractions from *C. burnetii*. As described by Tsai and Frasch (35), many bands of doublet character from wild-type LPS were revealed by silver staining (11, 25). LPS from the Re mutant revealed only one major band and a few minor bands. A comparison of LPSs from *C. burnetii* and *S. typhimurium* with SDS-PAGE suggested that LPSs from *C. burnetii* phase II and the Re mutant of *S. typhimurium* were similar in molecular weight. However, the banding profiles from *S. typhimurium* wild-type LPS showed orderly spacing of the bands, whereas LPS I gave roughly six major bands which were not regularly spaced.

Immunological properties of LPSs. Antigenicities of LPS I and LPS II were examined by immunodiffusion, using LPS I, and LPS II, and hyperimmune antisera against *C. burnetii* Formalin-killed whole cells from both phases (Fig. 3). LPSs obtained from strain CB9MII whole cells and CMR did not react with hyperimmune guinea pig sera containing antibodies against phase I and II antigens, whereas LPSs from strains CB9MI and CBOI reacted with the guinea pig antisera used above (Fig. 3A). LPS I and LPS II did not react with the guinea pig sera containing phase II antibody (Fig. 3B). The precipitin patterns showed immunological identity between LPSs of strains CB9MI and CBOI.

DISCUSSION

In this study, we examined the chemical composition, electrophoretic profile in SDS-PAGE, and immunological properties of LPSs from strains CB9MI, CBOI, and CB9MII. The CB9MII isolate used here was cloned in primary chicken embryo cells after over 90 serial egg passages. Upon inoculation of cloned CB9MII into laboratory mice or guinea pigs, phase I cells cannot be isolated (Williams and Peacock, unpublished data). The sugar compositions of LPS I and LPS II were similar to those described by Schramek and Meyer (31) but greatly different from those described by Baca et al. (3). Only three sugars (D-glucose, D-mannose, and D-glyceromannoheptose) were detected in LPS II by gas chromatography and paper chromatography (Table 5), whereas 12 components, including the above sugars, were detected in LPS I in addition to a KDO-like component and glucosamine (Tables 2 and 4). The KDO-like component was present in the LPS fraction as measured by a positive reaction with thiobarbituric acid-periodate. However, this KDO-like component was not identified as standard KDO by gas and paper chromatographies. Thus, our data and the results of Schramek and Meyer (31) indicate that the thiobarbituric acid-periodate-positive material may not be KDO. On the other hand, three ninhydrin-positive compounds were found in LPS I (Table 4), whereas only two were found in LPS II.

Acetic acid hydrolysis of LPS has been used to prepare O-specific saccharide and lipid A moieties (10). This method was used by Schramek and Galanos (30) to prepare a lipid A fraction of *C. burnetii* LPS. These investigators studied the biological and immunological properties of the lipid A fraction. However, they did not analyze the chemical components of the lipid A fraction. Our results suggest that acetic acid hydrolysis does not produce a pure lipid A fraction, and for this reason, we refer to our preparation as the putative lipid A fraction of *C. burnetii* LPS. Since *C. burnetii* LPS contains an analog of the acid-labile KDO (10), this may lead to incomplete hydrolysis by acetic acid. Glucosamine, a constituent of the lipid A portion of gram-negative bacterial LPS, was found in all *C. burnetii* LPSs. After acetic acid hydrolysis, the majority of this sugar was released from the aggregated hydrophobic precipitate into the aqueous supernatant (Table 6). This fractionation is not characteristic of the lipid A glucosamine of gram-negative bacterial LPS. Moreover, ethanolamine in *C. burnetii* LPS was fractionated into the precipitate by acetic acid hydrolysis. Usually, ethanolamine in gram-negative bacterial LPS is released into aqueous solution (supernatant) after mild acid hydrolysis. Therefore, the putative lipid A moiety of *C. burnetii* LPS

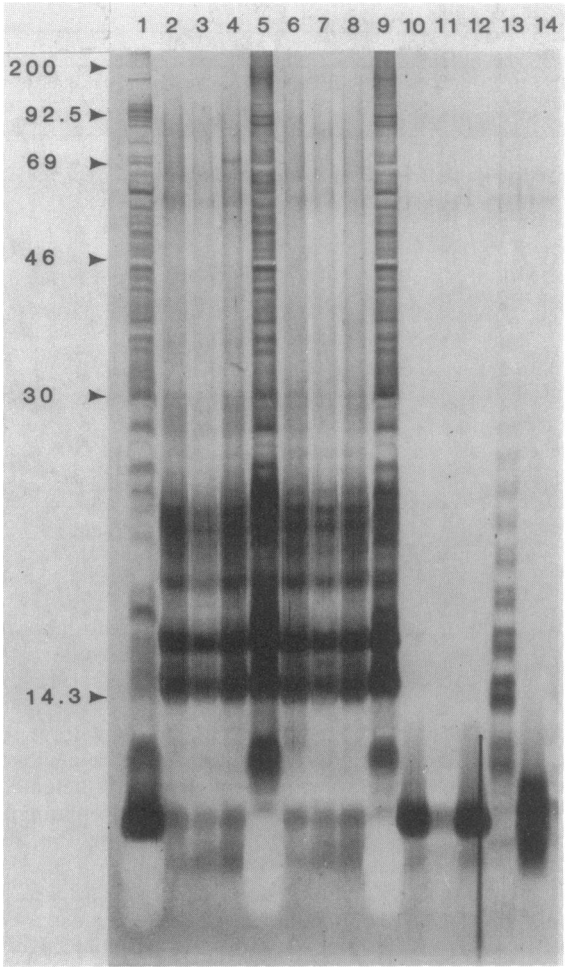

FIG. 2. SDS-PAGE of whole cells and LPS fractions from *C. burnetii* and *S. typhimurium*. Samples were boiled for 5 min with Laemmli sample buffer (17), and 20-μl samples (10 μg) were applied to each slot. After electrophoresis, the slab gel was fixed and silver stained after periodate oxidation. Lanes: 1, CB9MII whole cells; 2, CB9MI CPWE; 3, CB9MI CPWE supernatant; 4, CB9MI LPS; 5, CB9MI whole cells; 6, CBOI CPWE; 7, CBOI CPWE supernatant; 8, CBOI LPS; 9, CBOI whole cells; 10, CB9MII CPWE; 11, CB9MII CPWE supernatant; 12, CB9MII LPS; 13, LPS from *S. typhimurium* wild strain LT2; 14, LPS from *S. typhimurium* Re mutant strain G30/C21. Molecular weight markers: myosin 200K; phosphorylase b, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; lysozyme, 14.5K.

appears to be different in chemical composition and structure from the gram-negative bacterial lipid A.

A unique ninhydrin-positive compound, which was present in LPS I but not LPS II, was tentatively identified as a disaccharide galactosaminuronyl glucosamine. The final structure and molecular weight of this unique LPS I component are currently under investigation. Similar hexosaminuronic acid-containing disaccharides, mannosaminuronyl fucosamine, galactosaminuronyl fucosamine, and mannosaminuronyl glucosamine, have also been isolated from acid hydrolysates of polysaccharides from gram-positive bacteria, *Staphylococcus aureus* T (43), *S. aureus* M (18), and *Bacillus* species (44). The disaccharide described here may be an important antigenic determinant of LPS I since this compound was found in the supernatant fraction after acetic acid hydrolysis.

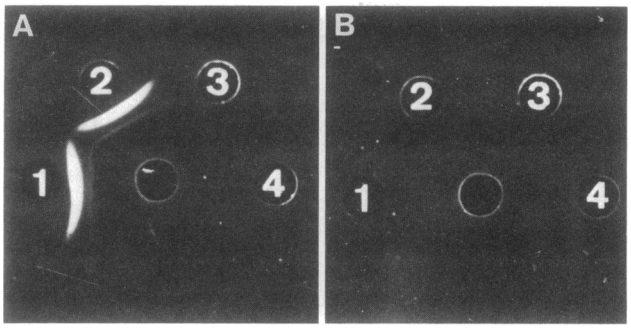

FIG. 3. Ouchterlony immunodiffusion patterns of LPS I (strains 9MI and CBOI) and LPS II (strain CB9MII). The center wells contained (A) hyperimmune rabbit antiserum against phase I whole cells (phase I and II antibodies) and (B) hyperimmune rabbit antiserum against phase II whole cells versus LPS from (1) CB9MI CMR, (2) CBOI CMR, (3) CB9MII whole cells, and (4) CB9MII CMR.

LPS I concentrations of the KDO-like compound and fatty acids (41 to 54 nmol/mg and 0.6 to 2.2%, respectively) were very low as compared with those of the LPSs of gram-negative *Enterobacteriaceae* wild strains (at least 200 nmol/mg and over 10%, respectively). Differences in KDO and fatty acid contents were also noted between LPSs from *S. typhimurium* Re mutants, which contain KDO (ca. 700 nmol/mg) and fatty acids (about 40%). Thus, the chemical composition of LPS from *C. burnetii* was significantly different from that of the LPSs of gram-negative *Enterobacteriaceae*. In addition, the different molar ratios of chemical components of LPS I and LPS II suggest that the structure of LPS I may correspond to smooth LPS, whereas LPS II may correspond to rough LPS. The significance of the minor chemical differences detected between the LPS I from strain CB9MI and that from strain CBOI remains to be shown.

The fatty acid profiles of *C. burnetii* whole cells (37), cell walls (2a), and LPS I (6) show a high percentage of branched-chain fatty acids. Our studies corroborate previous studies (6, 37) and show minor differences in the fatty acid contents of LPS I and LPS II. Although $C_{18:1}$, $C_{10:1}$, and C_{15} (anteiso) compounds were not detected in LPS II and strain CBOI LPS I, the significance of this observation is not readily apparent. In addition, the amino acids associated with these purified LPSs were very similar. The chemical association of these proteins or peptides with the LPSs of *C. burnetii* has not been resolved.

LPS II contained only one silver-stainable band (apparent molecular weight, 10.5K) by SDS-PAGE. This band had a similar migration to that of the major band of the LPS from the *S. typhimurium* Re mutant, which has a molecular weight of 2.8K. Thus, the molecular weight of LPS II might be ca. 3K. Generally, SDS-PAGE of LPS from gram-negative bacteria shows many bands at regular intervals, referred to as laddering (11, 25, 35). However, LPS I is composed of at least six major bands with the silver-staining method, and these bands are not generally represented as doublets. Our data on LPS profiles in SDS-PAGE may be interpreted as mixtures of various unrelated LPSs or a mixture of six or more LPSs which may correlate with variation in saccharide chain length. Based on the chemical compositions and the banding patterns, it seems that the phase variation of *C. burnetii* cells may be similar to the smooth-to-rough mutational variation found in gram-negative *Enterobacteriaceae*.

The phase variation of *C. burnetii* was originally described

as a serological phenomenon distinguishing two variants, phase I and phase II. The mechanism of this phenomenon is, so far, unknown. However, many investigators have suggested that the phase variation occurring in *C. burnetii* is similar to the smooth-to-rough mutational variation observed in gram-negative *Enterobacteriaceae* (3, 5, 13–15, 28, 29). Recently, Baca et al. (3) presented serological evidence of identity of both LPS materials in complement fixation and immunodiffusion assays. The results of the immunological experiments with polyclonal anti-*C. burnetii* sera indicate significant differences between LPS I and LPS II antigenicities. Thus, LPS I from strains CB9MI and CBOI had specific antigenic characters distinctly different from those of LPS II. However, the putative lipid A moieties of LPS I and LPS II share serological cross-reactivity with each other and with *S. typhimurium* lipid A (30). The question of similarities and differences in antigenicity and epitope structures of LPSs require further study with highly specific polyclonal and monoclonal antibodies.

The data of others and our data are not adequate to allow us to define the structural features of LPS I and LPS II. The isolation of LPS I and LPS II by phenol-water extraction and ultracentrifugation followed by acetic acid cleavage of acid-labile linkages revealed a putative lipid A and saccharide O-specific moieties. More importantly, we identified a unique disaccharide galactosaminuronyl glucosamine, which was specific for smooth LPS I and was not detected in the rough LPS II preparations. In addition, the SDS-PAGE profiles of *C. burnetii* LPSs may be indicative of variation of molecular weight species generated by smooth-to-rough mutational variation of saccharide chain length. We are currently using high-pressure liquid chromatography, gas chromatography, and mass spectrometry to identify the unknown compounds, the structures of the putative saccharide O-specific chain, and putative lipid A and monoclonal antibodies to probe the immunological specificies of the *C. burnetii* LPSs.

ACKNOWLEDGMENTS

We thank Marius Peacock and Scott Stewart for technical assistance. The Assistance provided by Robert Evans and Charles Taylor for photography and illustration and review of the manuscript by the Laboratory of Microbial Structure and Function staff are gratefully appreciated.

LITERATURE CITED

1. **Amano, K., H. Hayashi, Y. Araki, and E. Ito.** 1977. The action of lysozyme on peptidoglycan with *N*-unsubstituted glucosamine residues. Isolation of glycan fragments and their susceptibility to lysozyme. Eur. J. Biochem. **76**:299–307.
2. **Amano, K., and J. C. Williams.** 1983. Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysozyme hydrolysis correlated with a high degree of peptide cross-linking. J. Bacteriol. **153**:520–526.

2a. **Amano, K., and J. C. Williams.** 1984. Biochemical and immunological properties of *Coxiella burnetii* cell wall and peptidoglycan-protein complex fractions. J. Bacteriol. **160**:982-988.

3. **Baca, O. G., I. L. Martinez, A. S. Aragon, and D. Klassen.** 1980. Isolation and partial characterization of a lipopolysaccharide from phase II *Coxiella burnetii*. Can. J. Microbiol. **26**:819–826.
4. **Baca, O. G., and D. Paretsky.** 1974. Some physiological and biochemical effects of a *Coxiella burnetii* lipopolysaccharide preparation on guinea pigs. Infect. Immun. **9**:939–945.
5. **Brezina, R.** 1958. Contribution to the study of phase variation in *Coxiella burnetii*. Acta Virol. **2**:91–102.
6. **Chan, M. L., J. McChesney, and D. Paretsky.** 1976. Further characterization of a lipopolysaccharide from *Coxiella burnetii*. Infect. Immun. **13**:1721–1727.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
8. **Fiset, P.** 1957. Phase variation of *Rickettsia* (*Coxiella*) *burneti*. Study of the antibody response in guinea pigs and rabbits. Can. J. Microbiol. **3**:435–445.
9. **Fiset, P.** 1964. Serologic techniques, p. 225–255. *In* R. J. C. Harris (ed.), Techniques in experimental virology. Academic Press, Inc., London.
10. **Galanos, C., O. Luderitz, E. T. Rietschel, and O. Westphal.** 1977. Newer aspects of the chemistry and biology of bacterial lipopolysaccharides, with special reference in their lipid A component, p. 239–334. *In* T. W. Goodwin (ed.), The biochemistry of lipids II, vol. 14. University Park Press, Baltimore.
11. **Goldman, R. C., and L. Leive.** 1980. Heterogeneity of antigenic-side chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. Eur. J. Biochem. **107**:145–153.
12. **Hoyer, B. H., R. A. Ormsbee, P. Fiset, and D. B. Lackman.** 1963. Differentiation of phase I and phase II *Coxiella burnetii* by equilibrium density gradient sedimentation. Nature (London) **197**:573–574.
13. **Kazar, J., R. Brezina, S. Schramek, J. Urvolgyi, V. Pospisil, and E. Kovacova.** 1974. Virulence, antigenic properties and physicochemical characteristics of *Coxiella burnetii* strains with different chick embryo yolk sac passage history. Acta Virol. **18**:434–442.
14. **Kazar, J., E. Skultetyova, and R. Brezina.** 1975. Phagocytosis of *Coxiella burnetii* by macrophages. Acta Virol. **19**:426–431.
15. **Kishimoto, R. A., and J. S. Walker.** 1976. Interaction between *Coxiella burnetii* and guinea pig peritoneal macrophages. Infect. Immun. **14**:416–421.
16. **Krauss, H., H. Schiefer, and H.-D. Schmatz.** 1977. Ultrastructural investigations on surface structures involved in *Coxiella burnetii* phase variation. Infect. Immun. **15**:890–896.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Liau, D.-F., M. A. Melly, and J. M. Hash.** 1974. Surface polysaccharide from *Staphylococcus aureus* M that contains taurine, D-aminogalacturonic acid, and D-fucosamine. J. Bacteriol. **119**:913–922.
19. **Lowry, O. H., N. R. Roberts, K. Y. Leiner, M.-L. Wu, and A. L. Farr.** 1954. The quantitative histochemistriy of brain. I. Chemical methods. J. Biol. Chem. **207**:1–17.
20. **Luderitz, O., A. M. Staub, and O. Westphal.** 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**:192–255.
21. **Muhlradt, P., V. Wray, and V. Lehman.** 1977. A ^{31}P-nuclear-magnetic-resonance study of the phosphate groups in lipopolysaccharide and lipid A from *Salmonella*. Eur. J. Biochem. **81**:193–203.
22. **Ormsbee, R. A., and M. G. Peacock.** 1976. Rickettsial plaques assay and cloning procedures. Tissue Culture Assoc. **2**:475–478.
23. **Osborn, M. J.** 1963. Studies on the gram-negative cell walls. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.
24. **Ouchterlony, O.** 1958. Diffusion-in-gel methods for immunological analysis. Prog. Allergy **5**:1–78.
25. **Palva, E. T., and P. H. Makela.** 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Eur. J. Biochem. **107**:137–143.
26. **Park, J. T., and M. J. Johnson.** 1949. A submicrodetermination of glucose. J. Biol. Chem. **181**:149–151.
27. **Schramek, S., and R. Brezina.** 1976. Characterization of an endotoxic lipopolysaccharide from *Coxiella burnetii*. Acta Virol. **20**:152–158.
28. **Schramek, S., and R. Brezina.** 1979. Isolation of endotoxic lipopolysaccharide from phase II *Coxiella burnetii*. Acta Virol. **23**:349.
29. **Schramek, S., R. Brezina, J. Kazar, and T. N. Khavkin.** 1978. Attempts at demonstration of lipopolysaccharide in phase II

Coxiella burnetii. Acta Virol. **22**:509–511.

30. **Schramek, S., and C. Galanos.** 1981. Lipid A component of lipopolysaccharides from *Coxiella burnetii*. Acta Virol. **25**:230–234.
31. **Schramek, S., and H. Meyer.** 1982. Different sugar compositions of lipopolysaccharides isolated from phase I and phase II cells of *Coxiella burnetii*. Infect. Immun. **38**:53–57.
32. **Stoker, M. G. P., and P. Fiset.** 1956. Phase variation of the Nine Mile and other strains of *Rickettsia burnetii*. Can. J. Microbiol. **2**:310–321.
33. **Taylor, R. L., and H. E. Conrad.** 1972. Stoichiometric deployerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. Biochemistry **11**:1383–1388.
34. **Trevelyan, W. D., D. F. Procter, and J. S. Harrison.** 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
35. **Tsai, C. H., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
36. **Tsuji, A., T. Kinoshita, and M. Hoshino.** 1969. Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-methyl-2-benzothiazolone hydrazone hydrochloride. Chem. Pharm. Bull. **17**:1505–1510.
37. **Tzianabos, T., C. W. Moss, and J. E. McDade.** 1981. Fatty acid composition of rickettsiae. J. Clin. Microbiol. **13**:603–605.
38. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedures. Methods Carbohydr. Chem. **5**:83–91.
39. **Williams, J. C., and J. L. Cantrell.** 1982. Biological and immunological properties of *Coxiella burnetii* vaccines in C57BL/10 ScN endotoxin-nonresponder mice. Infect. Immun. **35**:1091–1102.
40. **Williams, J. C., M. R. Johnston, M. G. Peacock, L. A. Thomas, S. Stewart, and J. L. Portis.** 1984. Monoclonal antibodies distinguish phase variants of *Coxiella burnetii*. Infect. Immun. **43**:421–428.
41. **Williams, J. C., M. G. Peacock, and T. F. McCaul.** 1981. Immunological and biological characterizations of *Coxiella burnetii*, phases I and II, separated from host components. Infect. Immun. **32**:840–851.
42. **Williams, J. C., and S. Stewart.** 1984. Identification of immunogenic proteins of *Coxiella burnetii* phase variants. *In* L. Leive and D. Schlessinger (ed.), Microbiology—1984. American Society for Microbiology, Washington, D.C.
43. **Wu, T. C. M., and J. T. Park.** 1971. Chemical characterization of a new surface antigenic polysaccharide from a mutant of *Staphylococcus aureus*. J. Bacteriol. **108**:874–884.
44. **Yoneyama, T., Y. Koike, H. Arakawa, K. Yokoyama, Y. Sasaki, T. Kawamura, Y. Araki, E. Ito, and S. Takao.** 1982. Distribution of mannosamine and mannosaminuronic acid among cell walls of *Bacillus* species. J. Bacteriol. **149**:15–21.