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A comparative study of lipopolysaccharides from two *Coxiella burnetii* strains considered to be associated with acute and chronic Q fever

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Coxiella burnetii strains Nine Mile and Priscilla, considered to be associated with acute and chronic forms of Q fever, were investigated for variation in composition of their lipopolysaccharides. Though SDS-PAGE profiles of the lipopolysaccharides were distinct, chemical analyses showed only small differences in their overall composition. Further studies on lipid A-deprived O-polysaccharide fractions of both lipopolysaccharides, obtained by steric-exclusion chromatography, revealed noticeable differences in distribution and chemical composition of the O-polysaccharide chains. It is likely that *C. burnetii* strains are capable of synthesizing chemically distinct subclasses of O-specific polysaccharide molecules differing in their antigenic reactivities. The results provide suggestive evidence that virulence of *C. burnetii* may be modulated through lipopolysaccharide composition and structure. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Coxiella burnetii is an obligatory, intra-phagolysosomal parasitizing bacterium which is the causative agent of Q fever. In humans, infection with *C. burnetii* can be asymptomatic or result in acute (Marrie, 1990) or chronic disease (Raoult et al., 1990). Although the acute form of the disease is curable by a proper treatment with tetracyclines (Yeaman and Baca, 1990), there is a constant threat of valvular endocarditis, which is the most serious complication of chronic Q fever (Raoult et al., 1990). The pathogenesis of Q fever endocarditis is unknown, and mechanisms causing lesions in the cardiac valves are controversial. It should be clarified whether microbial factors or some predisposition of the host lead to the conditions of this disease. Some investigators suggest that there are specific strains causing acute or chronic disease (Samuel et al., 1985). Genomic DNA restriction fragment length polymorphism (Heinzen et al., 1990,Mallavia et al., 1991) and chemotype determination of lipopolysaccharides (LPS) (Hackstadt, 1986) were the basis for such differentiation. In the latter case, however, no detailed compositional analysis of the LPS was undertaken. Other investigators believe that there is no difference in pathogenicity among *C. burnetii* strains and occurrence of endocarditis is determined by host factors rather than by properties of the pathogen. Evidence for this hypothesis came from clinical observations (Raoult et al., 1990) and serotyping of *C. burnetii* isolates from acute and chronic Q fever patients (Yu and Raoult, 1994).

An LPS has been considered to be a major determinant of virulence expression and infection of *C. burnetii* (Baca and Paretsky, 1974,Williams, 1991). It is a predominant surface antigen, capable of inducing antibody response, and it is considered to be a protective immunogen (Gajdošová et al., 1994). Considering these facts, the investigations of composition and structure of the LPS isolated from the strains suggested to be associated with acute and chronic forms of Q fever could bring new data on the possible role of the LPS in the establishment of chronic or persistent infections by *C. burnetii*. In our initial studies, the LPS isolated from Nine Mile and Priscilla strains, considered (Samuel et al., 1985) to be associated with acute and chronic forms of Q fever, respectively, were analyzed for their chemical composition in considerable detail. The results are reported herein.

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MATERIALS AND METHODS

Cultivation and purification of C. burnetii cells

C. burnetii strains Nine Mile and Priscilla, serologically in phase I (yolk sac passage 3 in our laboratory), were propagated in chicken embryo yolk sacs. After cultivation, the *C. burnetii* cells were purified by a modified procedure of Ormsbee (1962). Briefly, yolk sacs with the rickettsial cells were suspended to 20% with aqueous 1 M NaCl containing 0.2% phenol and the suspension was allowed to stand at 5°C for 5 days. The mixture was then centrifuged at 14000 × *g*, 20°C for 40 min. The sediment was resuspended in aq. 0.85% NaCl and the cells were purified further by extraction with ether. Purified *C. burnetii* cells were centrifuged from aqueous phase at 10000 × *g*, 20°C for 30 min, and stored preferentially at 5°C in aq. 0.85% NaCl containing 0.1% phenol.

Isolation of the LPS

The cells (1 g each) from Nine Mile and Priscilla strains were extracted with chloroform–methanol (2:1, v/v) at 20°C overnight. The extraction was repeated with the fresh solvent mixture for 2 h. The cell suspension was centrifuged at 3000 × *g*, 20°C for 10 min, and the sediment was washed with acetone and ether. In a typical isolation procedure dried cells were suspended in preheated distilled water (60 ml, 68°C) and extracted with an equal volume of aq. 90% phenol as described (Westphal and Jann, 1965). The aqueous phase was collected after centrifugation. The phenol phase was re-extracted with water twice and the combined aqueous phases were evaporated to a low volume and dialyzed against distilled water for 4 days. The crude LPS (120 mg) was solubilized in 50 mM Tris–HCl buffer (20 ml, pH 7.5) and treated simultaneously with RNase (EC 3.1.27.5) and DNase I (EC 3.1.21.1), both from bovine pancreas (Boehringer Mannheim, Germany) for 4 h at 37°C, and then with proteinase K (EC 3.4.21.14, from *Tritirachium album*, Sigma, St. Louis, MO, USA) for 6 h at 37°C. After dialysis against distilled water and lyophilization, the LPS was purified by ultracentrifugation at 120000 × *g* for 4 h. The final yield was 105.2 mg of the LPS corresponding to 10.5% of the *C. burnetii* cells. In order to obtain higher amounts of the LPS from both *C. burnetii* strains, the above procedure was repeated several times.

Steric-exclusion chromatography

Prior to steric-exclusion chromatography, both the Nine Mile (510 mg) and Priscilla (580 mg) LPS were each partially hydrolyzed with aq. 1% (v/v) acetic acid (50 ml) for 2 h at 100°C, and the solutions were centrifuged at 14000 × *g* for 20 min to remove the lipid A in the pellets. The pellets were washed twice with distilled water followed by centrifugation at 14000 × *g* for 20 min to remove residual polysaccharides. Supernatants were pooled, concentrated by evaporation, and lyophilized. The yields of O-specific polysaccharides (PS) of Nine Mile and Priscilla LPS were 357 and 360 mg, respectively. The PS (350 mg each) were dissolved in redistilled water (4 ml) and fractionated on a column (2.5 × 87 cm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) using water as the eluant at a flow rate of 9.6 ml h^{-1}. The separated PS fractions were monitored with an RI detector (RIDK 102, Laboratorní prístroje, Praha, Czech Republic), and by the phenol–sulphuric acid reaction (Dubois et al., 1956).

Gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in slabs containing 12.5% of polyacrylamide and the gels were silver-stained for LPS as described (Škultéty and Toman, 1992). An SDS Molecular Weight Markers kit was purchased from Sigma (St. Louis, MO, USA).

Analytical methods

3-Deoxy-D-manno-2-octulosonic acid (Kdo), protein, phosphate, and hexosamine contents were determined as reported (Toman and Škultéty, 1996). The LPS and the individual PS fractions (300 μg each) were hydrolyzed with 2 M trifluoroacetic acid (0.1 ml) at 100°C for 2 h. After evaporation, each residue was dissolved in 1 M ammonium hydroxide solution (0.1 ml) containing sodium borodeuteride (1 mg) and kept at room temperature overnight. After usual work-up, the dried residues were acetylated with acetic anhydride (50 μl)–pyridine (50 μl) mixture at 100°C for 1 h. Water was then added and the solvents were removed by evaporation. The residues were extracted with chloroform–water (140 μl, 1:1, v/v) three times, the organic layers were pooled, dried (Na_2SO_4) and evaporated. The acetylated samples were finally dissolved in chloroform and injected directly onto a gas chromatography column.

Gas chromatography–mass spectrometry (GC–MS) of alditol acetates was performed on a Finnigan MAT SSQ 710 mass spectrometer using a fused-silica capillary column SP-2330 (30 m × 0.25 mm; Supelco, Bellefonte, USA). The initial oven temperature was 160°C, programmed to enhance for 10°C min^{-1} up to 240°C at a helium gas flow rate of 25 cm s^{-1}. Electron impact mass spectra were recorded at an electron energy of 70 eV, and an ion source temperature of 150°C.

RESULTS

The SDS–PAGE profiles of the LPS isolated from the *C. burnetii* strains Nine Mile and Priscilla are given in Fig. 1. At first glance, remarkable differences between the both profiles are evident. The LPS profile of the Nine Mile strain has two strong bands in the region of about 10–17 kDa and a group of unevenly distributed bands in the region of about 20–29 kDa. On the other hand, the LPS from Priscilla strain reveals one strong band at about

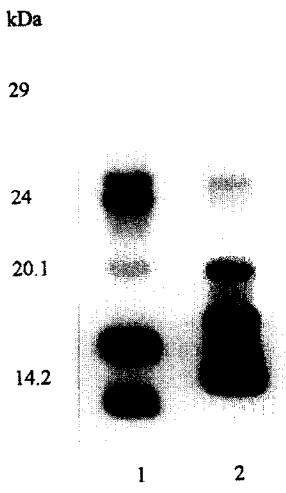

Fig. 1. SDS–PAGE silver stain of the *C. burnetii* LPS, strains Nine Mile and Priscilla (lanes 1 and 2). The amounts of LPS used per lane were 20 μg.

Table 1. Yields of PS fractions of *C. burnetii* LPS from Nine Mile and Priscilla strains obtained on Sephadex G-50 column chromatography

Strain	Nine Mile					Priscilla				
Fraction	A	B	C	D	E	A	B	C	D	E
Yield (%)	20.2	24.3	27.5	16.4	11.6	5.9	9.6	56.4	20.7	7.4

15 kDa and additional bands in the region of 17–29 kDa. Differences are also evident in the intensity of these minor bands. The bands are more intense with the Priscilla strain in the region of 17–20 kDa, whereas with the Nine Mile strain, the stronger bands are found at 25–29 kDa. When small amounts of LPS were applied to the gel, only the fastest-migrating bands were stained. Like Hackstadt (1986), we have also noticed that the slower-migrating LPS species extending above the 29 kDa region are stained poorly or not at all with the silver staining procedure. However, this region showed the greatest reactivity on immunoblots with rabbit polyclonal antisera raised against the *C. burnetii* whole cells of the strains Nine Mile and Priscilla.

After mild acid hydrolysis of both LPS and lipid A removal the remaining PS were fractionated on a column of Sephadex G-50. Five fractions **A–E** were obtained in each case. Yields of the individual fractions are given in Table 1. It is evident that fraction **C** represented the highest amount of the carbohydrate material recovered from both strains. The fractions **E** contained monosaccharides, mainly dihydrohydroxystreptose (Strep, 3-C-(hydroxymethyl)-L-lyxose) and virenose (Vir, 6-deoxy-3-C-methyl-D-gulose) (Toman et al., 1998), released from the respective LPS after mild acid treatment (see below). The amount of PS fractions recovered was quite regular in the case of the Nine Mile strain in contrast with that of the Priscilla strain in which the highest amount of PS recovered was found in the fraction **C** (Table 1).

Colorimetric estimations of the fractions (**A–D**) are presented in Table 2. In the Nine Mile strain, the phosphate content was low in fractions **A** and **B**, and increased substantially in fraction **C**, and mainly in fraction **D**. PS fractions **A** and **B** from the Priscilla strain had much lower phosphate contents than those found with the former strain and, in addition, a comparatively low value was also found in fraction **C**, which represented the main portion (56.4%) of the carbohydrate material recovered (Table 1). Thus, it is evident that most of the phosphate was concentrated in the low-molecular weight fractions **D**. In both strains, the Kdo content increased with the decreasing molecular weight of individual fractions. A slight deviation was observed with fraction **C** of the Nine Mile strain which had a higher Kdo content than that of the corresponding fraction **D**. Analysis of hexosamines revealed distinct features of their occurrence in the PS molecules of both strains. In the Priscilla strain, an increase of the hexosamine content towards the lower molecular weight fractions **C** and **D** is evident, whereas in the case of the Nine Mile strain this trend is not observed and the highest content of hexosamine is found in fraction **B**. The value obtained is almost twice as high as that found in fraction **C**, which represents the second highest hexosamine content.

It is well known that the phosphate and Kdo assays measure residues in the core region of LPS, whereas the assay for hexosamine detects residues in both the O-antigen and the core-lipid A regions. The data shown in Table 3 indicate

Table 2. Colorimetric estimations of the PS fractions of Nine Mile and Priscilla LPS obtained on Sephadex G-50

Strain	Fraction	Phosphate (nmol mg^{-1})	Kdo[a] (nmol mg^{-1})	Kdo[b] (nmol mg^{-1})	Hexosamine (nmol mg^{-1})
Nine Mile	LPS (native)	138.7	88.9	56.9	287.1
	A	25.4	54.5	36.8	63.2
	B	28.6	85.3	57.7	221.2
	C	136.6	109.6	74.0	116.9
	D	434.9	99.1	67.4	5.8
Priscilla	LPS (native)	123.1	94.9	63.7	288.3
	A	7.8	46.1	27.6	78.9
	B	4.6	54.2	35.3	72.8
	C	43.3	84.3	52.1	131.7
	D	411.2	160.5	90.6	144.1

[a,b]Estimated by the thiobarbituric acid assay after hydrolysis in 1 M HCl and in 0.1 M sodium acetate buffer, pH 4.4, respectively

Table 3. Data from chemical analyses of PS fractions of Nine Mile and Priscilla LPS

Strain	Fraction	% phosphate recovered[a]	Hexosamine/ Kdo[b]	Phosphate/ Kdo[b]
Nine Mile	A	4.2	1.2	0.5
	B	5.7	2.6	0.3
	C	31.1	1.1	1.3
	D	59.0	0.1	4.4
Priscilla	A	0.4	1.7	0.2
	B	0.4	1.3	0.1
	C	22.1	1.6	0.5
	D	77.1	0.9	2.6

[a]Percentage of total amounts of phosphate in each of the fractions
[b]Relative molar ratios. The total Kdo content was used in calculations

that with phosphate, as an indicator of molar amounts of the PS, the short-chain fractions **D** represented 59 and 77% of the total PS molecules, while the long-chain fractions **A** represented only 4 and 0.4% in the Nine Mile and Priscilla strains, respectively. Though the strains showed four different PS size populations their distribution differed remarkably in the higher molecular weight region. The molar ratios of hexosamine to Kdo indicate, inter alia, a relatively low content of hexosamines in the PS chains and reveal the possibility that some subclasses of PS molecules, e.g. fraction **D**, Nine Mile strain, may lack these sugar residues. The molar ratios of phosphate to Kdo give additional evidence that most of the phosphate is located in the lipid A proximal regions of the short-chain PS molecules, which represent the major size population in both strains.

Table 4 presents GC–MS analyses of the individual PS fractions of both Nine Mile and Priscilla strains; the corresponding alditol acetates are given. In each case, fraction **E** represents the monosaccharides released upon mild acid treatment of the parent LPS. Mainly Strep, Vir, and smaller amounts of D-mannose (D-Man), and D-glycero-D-manno-heptose (D,D-Hep) were released under these conditions. D-Man and D,D-Hep were present in appreciable amounts in all PS fractions (**A**–**D**) of both strains. Their contents reached maximum in fractions **C**, which were also shown (Table 1) to represent the highest portion of the PS material recovered. In contrast with this, the content of Vir and Strep was much more variable in the individual PS fractions. In the Nine Mile strain, the content of Vir was quite steady except for fraction **C**. The increased proportions of Strep were found in the higher molecular weight fractions **A** and **B**. The picture is quite different with the Priscilla strain. Vir is the dominant sugar in fraction **A**, and its content decreases towards the lower molecular weight fractions. A totally opposite trend is observed with Strep, the highest content of which was found in the lowest molecular weight fraction **D**. From the minor sugars detected, D-xylose (D-Xyl) was present in an appreciable amount. The highest content of the sugar was found in fractions **C** and **B** of the Nine Mile and Priscilla strains, respectively. At present, it is not known (Škultéty and Toman, 1994) with certainty whether D-Xyl and other minor sugars are constituents of the major smooth (S) LPS. The unknown compound (U) could not be characterized more deeply thus far. Nevertheless, it appears to be of sugar origin.

DISCUSSION

Based upon silver-stained SDS–PAGE profiles of the purified LPS from various strains of *C. burnetii* a differentiation of the strains suggested to be associated with acute and chronic forms of Q fever was attempted (Hackstadt, 1986). The LPS profiles of the former strains were characterized by two strong bands in the region of 10–17 kDa, whereas the latter showed one strong band in this region. The restriction endonuclease mapping of *C. burnetii* plasmids demonstrated (Heinzen et al., 1990; Mallavia et al., 1991) an association between plasmid type and disease. A clear correlation between the various groups established on the basis of plasmid profile and the groups based on LPS profile has been reported. Most recently, however, the

Table 4. Sugar composition of the fractions obtained from lipid A-deprived Nine Mile and Priscilla LPS after separation on Sephadex G-50

Strain	Fraction	Composition (peak area % of alditol acetates)								
		Ara	Vir[a]	Xyl	Man	Gal	Glc	Strep[a]	U[a]	Hep
Nine Mile	LPS (native)	1.3	22.4	4.1	31.4	0.6	1.2	14.5	1.4	23.1
	A	0.6	24.3	5.8	27.8	1.5	2.9	13.4	1.2	22.5
	B	0.3	26.4	0.9	33.3	0.4	0.8	10.2	1.8	25.9
	C	0.6	11.9	8.9	40.4	0.2	0.6	6.7	1.7	29.0
	D	5.7	22.8	1.7	33.9	0.9	1.6	7.6	1.4	24.4
	E	—	35.1	—	8.9	—	—	53.7	—	2.3
Priscilla	LPS (native)	1.6	13.4	4.9	32.3	1.1	1.6	15.9	0.6	28.6
	A	1.2	38.5	7.1	19.8	0.5	6.6	4.4	0.8	21.1
	B	1.5	23.9	11.2	32.9	0.4	2.5	5.3	0.3	22.0
	C	1.8	7.8	4.1	37.8	1.7	1.0	11.3	0.8	33.7
	D	1.7	10.1	5.5	27.7	0.3	2.1	24.8	0.4	27.4
	E	—	31.7	—	12.5	—	—	49.2	—	6.6

E, contains monosaccharides released from the respective LPS after mild acid treatment
[a]The abbreviations are described in the text

differentiation based on plasmid type has appeared ambiguous as a plasmid common to *C. burnetii* strains associated with acute and chronic Q fever was found (Válková and Kazár, 1995). Hackstadt (1986) made no attempts to chemically characterize the LPS of *C. burnetii* and, thus, to explain the migration patterns or antigenicity of the multitude of bands seen on SDS–PAGE. Therefore, in the present work, we focused on a detailed chemical characterization of the two LPS from the prototype *C. burnetii* strains, Nine Mile and Priscilla.

Although SDS–PAGE profiles (Fig. 1) of the LPS from Nine Mile and Priscilla strains differed considerably from one another, the colorimetric estimations (Table 2) and GC–MS analyses (Table 4) showed only small differences in their overall composition. However, analyses of their PS fractions, obtained by steric-exclusion chromatography, showed noticeable differences in phosphate, Kdo, and hexosamine contents (Tables 2 and 3), and in the composition of constituent sugars (Table 4). Moreover, differences in size and shape of PS molecules from both investigated strains could be anticipated. It is also of interest that there is a different content/distribution of Vir, and mainly Strep, in the individual PS fractions. These sugars have not been found in other LPS and can be considered as important chemotaxonomic markers. Moreover, their release from the parent LPS, under mild acidic conditions, led to a considerable decrease of the serological activity of the LPS in the passive hemolysis test (Schramek et al., 1985). Both sugars are located in the PS chain of the LPS, mostly in the terminal position (Mayer et al., 1988, Toman, 1991). As can be seen from Table 4, each PS fraction showed a high content of D,D-Hep. No L-glycero-D-mannoheptose (L,D-Hep) was detected. D,D-Hep is the biosynthetic precursor (Raetz, 1990) of L,D-Hep and is present in most enteric LPS only in addition to the latter sugar. We have shown (Toman and Škultéty, 1996) that D,D-Hep is a constituent sugar of the lipid A proximal core region of *C. burnetii* rough (R) LPS and, thus, its high content in fractions **A–D** indicates that the PS chains of both LPS are truncated. In addition, the data presented point to the fact that the PS chain subclasses could be chemically distinct.

It is also of interest to compare the binding of two monoclonal antibodies (MAbs) 1/4/H (Sekeyová et al., 1995) and 4/11 (a gift from Dr. D. Thiele, Giessen, Germany) with both the LPS antigens in immunoblot. The MAb 4/11 appeared to be highly specific as only one strong band was visible in the region of about 10–14 kDa (Toman, Sekeyová, Škultéty and Kováčová, unpublished results). This should indicate the presence of a similar structural epitope recognized by the MAb in both the LPS. In contrast with this, the MAb 1/4/H reacted strongly with the Nine Mile LPS in the region of about 17–28 kDa and reacted weakly with the Priscilla LPS at 29 kDa and strongly in the high-molecular mass range of 70–90 kDa (Sekeyová et al., 1995). Therefore, these findings may indicate differences in the structural features of both the LPS.

Interestingly, the short-chain population made up 59 and 77% of Nine Mile and Priscilla samples on a molar basis (Table 3). In addition, there were larger amounts of the intermediate-chain populations than of the long-chain populations, especially with the Priscilla strain. For comparison, members of the family Enterobacteriaceae show a distribution of 44–60% of the LPS molecules in the low-molecular weight population and 30–50% in the high-molecular weight population (Lüderitz et al., 1982, Peterson and McGroarty, 1985). Since the hydrophilic PS chains extend from the *C. burnetii* outer membrane into the aqueous environment, the observed heterogeneity of O-chain lengths indicates that the surface topography of *C. burnetii* is irregular, and that accessibility of the lipid A group of the LPS could vary in different regions on the surface of the bacterium. The presence of O-antigen-containing LPS influences a number of cell surface phenomena of *C. burnetii*, e.g. bacteriophage recognition (Kazár et al., 1975), infectivity and virulence (Baca and Paretsky, 1974, Williams, 1991), immunological properties and characterization (Schramek et al., 1983, Gajdošová et al., 1994, Sekeyová et al., 1995), and antibiotic susceptibility (Yeaman and Baca, 1990). The low level of LPS on *C. burnetii* that contains a long PS chain is probably sufficient to create a uniform cover over the cell, since the surface is inaccessible by antisera prepared against *C. burnetii* phase II cells (Schramek et al., 1978), which have the R-LPS located in the outer membrane (Toman and Škultéty, 1996). It has also been suggested (Williams and Waag, 1991) that *C. burnetii* is synthesizing PS chains of unusual sugar composition and structure with the aim to escape the immune system of the host, which might have difficulties in triggering effective defense mechanisms against the bacterium.

In conclusion, we have shown several pieces of evidence indicating that *C. burnetii* Nine Mile and Priscilla strains are capable of synthesizing chemically distinct subclasses of PS molecules differing in their antigenic reactivities. The study demonstrates that LPS variation in *C. burnetii* is more extensive than previously believed, and provides suggestive evidence that the virulence of the bacterium may be modulated through LPS composition and structure. In future, more detailed investigations will be undertaken in this respect.

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