Biochemical and immunological comparison of lipopolysaccharides from *Bordetella* species

KEN-ICHI AMANO,^{1*}† KAZUE FUKUSHI¹ and MITSURU WATANABE²

¹ Department of Bacteriology, Hirosaki University School of Medicine, Hirosaki, Aomori 036, Japan

² The Kitasato Institute, Shirokane, Minato-ku, Tokyo 108, Japan

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Lipopolysaccharides (LPS) isolated from *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* were analysed for their chemical composition, molecular heterogeneity and immunological properties. All the LPS preparations contained heptose, 3-deoxy-D-manno-2-octulosonic acid, glucosamine, uronic acid, phosphate and fatty acids. The fatty acids C14:0, C16:0 and β OHC14:0 were common to all the LPS preparations. LPS from *B. pertussis* strains additionally contained isoC16:0, those from *B. parapertussis* contained isoC14:0 and isoC16:0, and those from *B. bronchiseptica* contained C16:1. By SDS-PAGE, LPS from *B. pertussis* had two bands of low molecular mass, and the LPS from *B. parapertussis* and *B. bronchiseptica* showed low molecular mass bands together with a ladder arrangement of high molecular mass bands. Immunodiffusion, quantitative agglutination and ELISA demonstrated that the LPS from *B. pertussis* strains reacted with antisera prepared against whole cells of *B. pertussis* and *B. bronchiseptica*; LPS from *B. parapertussis* reacted with antisera to *B. parapertussis* and *B. bronchiseptica*, and LPS from *B. bronchiseptica* reacted with anti-whole cell serum raised against any of the three species. From these results, it is concluded that LPS from *B. bronchiseptica* has structures in common with LPS from *B. pertussis* and *B. parapertussis*, while the LPS from *B. pertussis* and *B. parapertussis* are serologically entirely different from each other.

Introduction

Lipopolysaccharide (LPS) of *Bordetella pertussis* has toxic activities similar to those of LPS from other Gram-negative bacteria (Finger *et al.*, 1976; MacLennan, 1960; Nakase *et al.*, 1970). In addition, LPS from *B. pertussis* sensitizes mice to histamine (Bergman & Munoz, 1977) and affects the lymphocytosis induced by pertussis toxin (Kurokawa *et al.*, 1978).

LPS from *B. pertussis* contain lipid A and an oligosaccharide core having 3-deoxy-D-manno-2-octulosonic acid (KDO) (Moreau *et al.*, 1984), but they do not contain the long-chain polysaccharide characteristic of the LPS of enterobacteria (Hitchcock *et al.*, 1986). Pepler (1984) reported that SDS-PAGE profiles of LPS from *B. pertussis* showed two bands by silver staining

which were similar to those given by R-type LPS from enterobacteria. LeDur *et al.* (1980) also described the presence of two chemically and immunologically distinct LPS in *B. pertussis* of different serological type. Very little is known about the LPS of *B. parapertussis* and *B. bronchiseptica*.

In the present work, we compared the chemical composition and SDS-PAGE profiles of LPS extracted from different phases of *B. pertussis*, and from the other two species of *Bordetella*, and compared the antigenicity of these LPS preparations by quantitative agglutination, immunodiffusion and ELISA.

Methods

Organisms and growth conditions. The following strains were used: *B. pertussis* Tohama [phase I, BpI(TOHAMA)], 18-323 [phase I, BpI(18-323)], Sakairi [phase III, BpIII(SAKAIRI)] and UV-1570 [phase III, BpIII(UV-1570)]; one strain of *B. parapertussis* [Bpp(21815)]; and two strains of *B. bronchiseptica* [Bbs(L3) and Bbs(H-214)]. All of these cultures were obtained from our laboratory stock. *B. pertussis* strain AK-168 [BpI(AK-168)] and *B. parapertussis* strain AK-167 [Bpp(AK-167)] were isolated from the same patient (Watanabe *et al.*, 1987).

† Present address: Central Research Laboratory, Akita University School of Medicine, Akita 010, Japan.

Abbreviations: LPS, lipopolysaccharide(s); KDO, 3-deoxy-D-manno-2-octulosonic acid; GlcN, glucosamine; GlcN-P, phosphoryl glucosamine.

All strains were grown in the synthetic liquid medium of Stainer & Scholte (1971), with shaking for 24–48 h at 36 °C. The cells were collected by centrifugation at 6000 *g* for 20 min, killed with 0.5% formaldehyde, and lyophilized.

Preparation of LPS. LPS was extracted from formaldehyde-treated whole cells by the hot phenol/water method of Westphal & Jann (1965). The extract was centrifuged at 100000 *g* for 2 h, and the sediment was washed twice with distilled water and lyophilized (designated crude LPS). This crude LPS (20 mg) was treated with 1 mg DNAase I (Sigma) and 1 mg of RNAase A (Sigma) overnight at 37 °C. Trypsin (Sigma, 2 mg) was then added and the mixture incubated for 1 d at 37 °C. The mixture was also treated with 2 mg proteinase K (Merck) at 37 °C for an additional 1 d. Finally, the mixture was centrifuged at 100000 *g* for 2 h or at 150000 *g* for 4 h, then the sediment was washed twice, and lyophilized. The product was called 'purified LPS'. Purified LPS from each strain was used in this work, except for the preparation from BpI(TOHAMA), which was crude LPS.

Analytical methods. The following assay methods were used: neutral sugars were assayed by the phenol/sulphuric acid method (Dubois *et al.*, 1956) with glucose as a reference standard; heptose was analysed by the cysteine/sulphuric acid method (Osborn, 1963) with glycerol-D-glucosamine (Sigma) as a reference; KDO was determined by the thiobarbituric acid method of Karkhanis *et al.* (1978) with a small modification (2 M-HCl hydrolysis for 90 min instead of 0.05 M-H₂SO₄ hydrolysis for 30 min) with KDO (Sigma) as a reference; protein was assayed by the Lowry method; total phosphorus was determined by the method of Lowry *et al.* (1954); amino acids and amino compounds, including amino sugars, were analysed in a K202 amino acid autoanalyser (Kyowa Seimitsu Co.) after hydrolysis for 4 h in 4 M-HCl at 100 °C in sealed ampoules (Amano *et al.*, 1987); fatty acids were analysed as methyl esters in a Hitachi 163 gas chromatograph on a Chromosorb WAW column containing 15% ethylene glycol succinate (Gasukuro Kogyo Co.) as described previously (Amano *et al.*, 1984). Identification and quantitative analysis of neutral sugars were done in a Hitachi 163 gas chromatograph with the sugars as alditol acetates on a 3% ECNSS-M Gas-Chrom Q glass column (Gasukuro Kogyo Co.) as described previously (Amano & Williams, 1984), and as trimethylsilyl derivatives on a 2.5% SE-30 Chromosorb WAW column (Gasukuro Kogyo Co.).

SDS-PAGE. The technique of Laemmli (1970) was used, with the following modifications. The 1.0 mm thick slab gel contained 17.5% (w/v) polyacrylamide, and the spacer gel 5%. Each sample (2–8 µg) was boiled for 5 min in Laemmli sample buffer and applied to each slot. The gels were stained with silver as described previously (Amano *et al.*, 1987).

Immunization. New Zealand White rabbits weighing 1.9–2.2 kg were pre-bled and given weekly intravenous doses of 1 mg formaldehyde-treated, lyophilized whole cells suspended in 0.4 ml distilled water. A total of four injections was given and the rabbits were bled 7 d after the fourth injection. The sera were separated by centrifugation.

Serological methods. Immunodiffusion assays were done in 1% (w/v) agarose gels according to the method of Ouchterlony (1958). A tube agglutination test (Nichols & Nakamura, 1980) and ELISA (Kasai *et al.*, 1986) were performed to determine serogroup antigen specificity.

Results

Isolation and chemical analysis of *Bordetella* LPS

The LPS preparations of the *Bordetella* species, except that of BpI(TOHAMA), were strongly associated with

Table 1. Chemical composition of LPS preparations from *Bordetella* species

Except for protein content, the results are expressed as molar ratios relative to heptose (taken as 1.0). The analyses were repeated 3 times for each strain; mean values are shown.

LPS from	Total GlcN	Neutral sugar*	Uronic acid†	KDO	Total phosphorus	Protein (% w/w)
BpI(TOHAMA)	2.2	1.0	0.5	0.6	1.9	trace
BpI(AK-168)	2.9	1.9	0.6	0.5	5.6	7.5
BpI(18-323)	1.5	0.5	0.7	0.7	2.9	trace
BpIII(SAKAIRI)	2.5	1.5	0.7	0.5	5.0	7.1
BpIII(UV-1570)	2.8	0.6	0.6	0.7	2.3	6.4
Bpp(21815)	4.2	0.5	0.7	0.7	4.0	1.8
Bpp(AK-167)	4.0	0.6	0.7	0.6	3.8	2.1
Bbs(L3)	3.5	1.0	0.1	0.7	3.7	2.4
Bbs(H-214)	4.6	1.7	0.9	0.6	3.6	2.4

* Neutral sugar minus heptose and uronic acid.

† Uronic acid minus heptose.

outer-membrane proteins; after ultracentrifugation they still consisted of 7.3–59.9% (w/w) protein as determined by the Lowry method (data not shown). After treatment with proteolytic enzymes (trypsin and proteinase K), the protein content decreased (trace to 7.5%) but was not completely removed (Table 1). Only the LPS from BpI(TOHAMA) was freed from the proteins by ultracentrifugation. Since after the enzyme treatments the LPS of *B. parapertussis* and *B. bronchiseptica* could not be precipitated by ultracentrifugation at 100000 *g* for 2 h, these preparations were sedimented at 150000 *g* for 4 h.

The molar ratios, relative to heptose, of total glucosamine [GlcN, including free GlcN and phosphorylated GlcN (GlcN-P), both detected by the amino acid analyser after acid hydrolysis], neutral sugar, uronic acid, KDO and total phosphorus in five LPS preparations from *B. pertussis* strains were different (Table 1). The molar ratios of the components described above in two preparations of LPS from *B. parapertussis* were very similar [4.2:0.5:0.7:0.7:4.0 for LPS from Bpp(21815) and 4.0:0.6:0.7:0.6:3.8 for LPS Bpp(AK-167)], while those in the two *B. bronchiseptica* preparations were different from each other [3.5:1.0:0.1:0.7:3.7 for LPS from Bbs(L3) and 4.6:1.7:0.9:0.6:3.6 for LPS from Bbs(H-214)]. The molar ratios of total GlcN to heptose in LPS from *B. parapertussis* and *B. bronchiseptica* were slightly higher than those from *B. pertussis*. The other ratios were not significantly different in the various LPS preparations.

Table 2 shows the fatty acid composition of the LPS preparations. Five LPS preparations from *B. pertussis* and two from *B. parapertussis* contained isoC16:0 in addition to C14:0, C16:0 and β OH C14:0 (β -hydroxy-

Table 2. Fatty acid composition of LPS preparations from *Bordetella* species

The results are expressed as percentages (w/w) of total fatty acids. The analyses were repeated 3 times for each strain; average values are shown.

Fatty acid	BpI (TOHAMA)	BpI (AK-168)	BpI (18-323)	BpIII (SAKAIRI)	BpIII (UV-1570)	Bpp (21815)	Bpp (AK-167)	Bbs (L3)	Bbs (H-214)
C12:0	—	—	—	—	—	—	—	—	4.6
isoC14:0	—	—	—	—	—	2.8	3.4	—	3.1
C14:0	28.5	29.3	27.6	21.0	29.7	27.3	20.3	18.8	32.9
isoC16:0	20.6	6.0	23.1	7.2	17.2	12.7	13.4	—	—
C16:0	7.2	14.2	13.5	7.4	7.2	26.8	37.2	21.8	10.1
C16:1	—	—	7.0	—	—	—	—	12.8	12.0
isoC18:0	—	—	7.3	—	9.9	—	—	—	—
C18:0	—	4.5	3.3	—	1.2	2.4	—	2.4	—
β OHC14:0	42.9	46.2	15.0	64.5	34.7	28.1	25.8	42.6	37.8

—, Not detected.

 Table 3. Amino acid and amino sugar composition of LPS preparations from *Bordetella* species

The results are expressed as molar ratios relative to heptose (taken as 1.0). The analyses were repeated 3 times for each strain; average values are shown.

Amino acid or amino sugar	BpI (TOHAMA)	BpI (AK-168)	BpI (18-323)	BpIII (SAKAIRI)	BpIII (UV-1570)	Bpp (21815)	Bpp (AK-167)	Bbs (L3)	Bbs (H-214)
Asp	—	0.1	—	0.1	0.2	0.1	0.1	—	0.1
Ser	—	0.4	—	0.3	0.2	—	0.1	—	0.1
Glu	—	0.6	—	0.5	0.4	—	0.1	0.1	0.1
Gly	—	0.2	—	0.1	0.3	0.1	0.1	—	0.1
Ala	—	1.1	—	0.9	0.8	0.1	0.2	0.1	0.2
Leu	—	0.6	—	0.5	0.4	—	0.1	0.1	0.1
Phe	—	0.4	—	0.3	0.2	—	—	—	0.1
Arg	—	0.1	—	0.1	0.1	—	0.1	—	0.1
GlcN	1.4	2.2	0.9	1.9	1.7	2.3	2.2	2.1	3.4
GlcN-P	0.8	0.7	0.6	0.6	1.1	1.9	1.8	1.3	1.1

—, Not detected.

myristic acid) as major fatty acid components, while two LPS preparations from *B. bronchiseptica* contained C14:0, C16:0, C16:1 and β OHC14:0, without isoC16:0. The data show that isoC16:0 is specific to *B. pertussis* and *B. parapertussis* LPS, and C16:1 is specific to *B. bronchiseptica* LPS.

Mannoheptose was the only neutral sugar detected in LPS from *B. pertussis* and *B. bronchiseptica* by gas chromatographic analysis of alditol acetate derivatives; it was not detected in LPS from *B. parapertussis* (data not shown). The same results were obtained when LPS acid hydrolysates were trimethylsilylated and analysed by gas chromatography (data not shown).

Amino acid and amino compound compositions of LPS

All the LPS preparations contained GlcN and GlcN-P, which are constituents of the lipid A of LPS from

enterobacteria (Table 3). Further, LPS from the *B. pertussis* strains, except BpI(TOHAMA) and BpI(18-323), contained all the amino acids listed in Table 3, and the LPS from *B. parapertussis* and *B. bronchiseptica* contained mainly aspartic acid, serine, glutamic acid, glycine, alanine, leucine and arginine, probably originating from the hydrophobic outer-membrane proteins.

Comparison of LPS by SDS-PAGE

The molecular size and microheterogeneity of LPS from *Bordetella* were compared by SDS-PAGE (Fig. 1). The patterns of five preparations from *B. pertussis* were composed of two low molecular mass bands in different ratios. The apparent molecular masses of the slow- and fast-migrating bands were 4.5 and 3.4 kDa, respectively. The 3.4 kDa band was present in low concentrations in LPS from *B. pertussis* (Fig. 1, lanes 1, 2, 4 and 5) with the

Fig. 1. Comparison of LPS preparations from *Bordetella* species and *Salmonella minnesota* by SDS-PAGE using 17.5% polyacrylamide gel. After electrophoresis the gel was stained with silver. Lanes: 1, BpI(THAMA); 2, BpI(AK-168); 3, BpI(18-323); 4, BpIII(SAKAIRI); 5, BpIII(UV-1570); 6, Bpp(21815); 7, Bpp(AK-167); 8, Bbs(L3); 9, Bbs(H-214); 10, *S. minnesota* wild-type LPS; 11, *S. minnesota* R-mutant LPS.

Table 4. ELISA titres against LPS employing rabbit antisera against whole cells of *Bordetella*
The tests were repeated twice and representative results are shown.

LPS from*	ELISA titre using polyclonal antibody against whole cells of:								
	BpI (TOHAMA)	BpI (AK-168)	BpI (18-323)	BpIII (SAKAIRI)	BpIII (UV-1570)	Bpp (21815)	Bpp (AK-167)	Bbs (L3)	Bbs (H-214)
BpI(TOHAMA)	75010	7540	3970	11350	58260	<100	<100	6080	49050
BpI(AK-168)	24460	3490	220	5600	52680	<100	<100	2600	17070
BpI(18-323)	8700	1400	200	3950	11030	<100	<100	2040	5460
BpIII(SAKAIRI)	43170	4250	900	8416	44630	<100	<100	3820	18190
BpIII(UV-1570)	14230	2840	140	33800	16570	<100	<100	2700	5900
Bpp(21815)	<100	<100	<100	<100	<100	6110	3890	1180	6270
Bpp(AK-167)	<100	<100	<100	<100	<100	4940	2930	1180	5040
Bbs(L3)	8330	3040	<100	4030	8840	1790	1050	2890	16180
Bbs(H-214)	6680	2400	130	1150	8650	2200	1950	3290	274390
<i>E. coli</i>	<100	<100	<100	<100	<100	<100	<100	<100	<100

* Antigens were used at $1 \mu\text{g ml}^{-1}$ (0.1 μg per well).

exception of BpI(18-323) (Fig. 1, lane 3). The gel patterns of the two LPS preparations from *B. parapertussis* showed a ladder-type arrangement of the bands of high molecular mass, and a single fast-migrating band of low molecular mass (Fig. 1, lanes 6 and 7). The latter band was similar in molecular mass to the 3.4 kDa band of LPS from *B. pertussis* strains. Two LPS preparations from *B. bronchiseptica*, however, showed different gel patterns from each other. The LPS from Bbs(L3) showed two bands, of 5.1 and 3.5 kDa, respectively (Amano *et*

al., 1988) (Fig. 1, lane 8), whereas that from Bbs(H-214) was composed of faintly stained ladder bands and a single major band of low molecular mass (Fig. 1, lane 9).

Antigenicity of LPS of *Bordetella*

The antigenicity of LPS of *Bordetella* species was examined by agglutination assay and the ELISA, using hyperimmune antisera against whole cells of *Bordetella* species. Table 4 shows the antigenicity of LPS prepara-

Fig. 2. Double immunodiffusion patterns of LPS preparations from *Bordetella* species. The centre wells contained sera against (a) whole cells of BpI(TOHAMA), (b) whole cells of BpIII(SAKAIRI), (c) whole cells of Bpp(21815), and (d) whole cells of Bbs(H-214), versus LPS from (1) BpI(TOHAMA), (2) BpI(AK-168), (3) BpIII(SAKAIRI), (4) BpIII(UV-1570), (5) Bpp(21815), (6) Bpp(AK-167), (7) Bbs(L3), and (8) Bbs(H-214).

tions determined by ELISA. The antisera against *B. pertussis* phase I reacted with all the LPS preparations from *Bordetella*, except those from *B. paraptussis*; the anti-BpIII sera also reacted with the same LPS preparations. Anti-Bpp sera reacted with LPS from *B. paraptussis* and *B. bronchiseptica*, but the reactivity with *B. bronchiseptica* LPS was weak. The anti-Bbs sera reacted with all the *Bordetella* LPS preparations. These results indicate that the LPS of *B. pertussis* is similar in all strains regardless of the phase, and that it has a structure in common with LPS from *B. bronchiseptica*. The LPS from *B. paraptussis* were also similar to those from *B. bronchiseptica*, but different from those of *B. pertussis*. The LPS from *B. bronchiseptica* contained antigenic structures in common with the LPS from the other *Bordetella* species.

In an Ouchterlony immunodiffusion test (Fig. 2), anti-BpI(TOHAMA) and anti-BpIII(SAKAIRI) sera reacted strongly with all the LPS preparations from *B. pertussis* and weakly with *B. bronchiseptica*, whereas the anti-Bpp(21815) serum reacted with LPS from *B. paraptussis* and *B. bronchiseptica*. The anti-Bbs(H-214) serum reacted with all the *Bordetella* LPS preparations. These

results confirmed the ELISA data. In the immunodiffusion test, multiple precipitin lines were formed between LPS and anti-*Bordetella* sera, especially with LPS from BpI(AK-168) and serum against whole cells of BpI(SAKAIRI) (Fig. 2b), and with LPS from *B. bronchiseptica* and serum against whole cells of Bbs(H-214) (Fig. 2d). These multiple lines were possibly due to protein contamination.

Discussion

Szabo and his colleagues reported that *B. pertussis* LPS has two chemically different fractions (LeDur *et al.*, 1980), containing KDO-P (Caroff *et al.*, 1987; Chaby & Szabo, 1975). They determined that the polysaccharide moiety of these LPS preparations was made up of a heptasaccharide, composed of glucose, *N*-acetylglucosamine, glucuronic acid, *N*-acetylgalactosaminuronic acid, heptose and KDO in a molar ratio of 1:2:1:1:1:1 (Moreau *et al.*, 1984). Peppler (1984) reported the presence of LPS in *B. bronchiseptica* by SDS-PAGE using proteinase-K-treated whole cells, and Li *et al.* (1988)

demonstrated LPS in *B. parapertussis* and *B. bronchiseptica* by SDS-PAGE using whole cells treated with pronase. In neither report was LPS isolated or the structure of LPS determined.

The chemical analysis in this study indicated that the various LPS preparations had components in common (GlcN, GlcN-P, heptose, neutral sugar, uronic acid, KDO, phosphate and fatty acids) but in different molar ratios. By gas chromatography, the only neutral sugar found in LPS preparations from *B. pertussis* and *B. bronchiseptica* was heptose, while no neutral sugar was found in LPS from *B. parapertussis* strains. Contrary to the report of Moreau *et al.* (1984), we did not find glucose as a component of the polysaccharide moiety in *B. pertussis* LPS. The preparation of Moreau *et al.* (1984) may have been different from ours and this may account for the discrepancy. Chaby & Szabo (1975) reported that the LPS of *B. pertussis* contained phosphorylated KDO (KDO-P) and this component did not give a positive thiobarbiturate reaction. Caroff *et al.* (1987) demonstrated that LPS from *B. parapertussis* and *B. bronchiseptica* were also thiobarbiturate negative, but after dephosphorylation these LPS preparations gave a positive reaction. Based on these reports, we used strong acid hydrolysis (2 M-HCl for 90 min) to release KDO from LPS preparations for the quantitative analysis of KDO (Brade *et al.*, 1983).

Peppler (1984) and Li *et al.* (1988) described the presence of two different LPS components in *B. pertussis* by immunoblotting using polyclonal and monoclonal antibodies, respectively. We also showed by SDS-PAGE two different molecular mass components in five LPS preparations from *B. pertussis*. These preparations contained smaller amounts of the fast-migrating band than of the slower-migrating band. Although we do not know the structural relationship between the two components, Li *et al.* (1988) suggested that the two components may be useful for the establishment of serogroups for *Bordetella* species. The chemical analyses, SDS-PAGE profile and immunological antigenicity indicated that the chemical structures of LPS from *B. pertussis* phases I and III are similar; the LPS from *B. pertussis* and *B. bronchiseptica* contain partial similarities and the LPS from *B. parapertussis* and *B. bronchiseptica* also contain partial common structures. The chemical and immunological structures of the LPS preparations from *B. pertussis* and *B. parapertussis* were completely different. Our data obtained by using polyclonal antibodies against whole cells of *Bordetella* confirmed the result of Li *et al.* (1988) that monoclonal antibody against *B. pertussis* cells reacted with LPS from *B. pertussis* and *B. bronchiseptica* but not with LPS from *B. parapertussis*. Thus, the LPS from *B. bronchiseptica* possess two different epitopes, one in common with the LPS from *B.*

pertussis and the other in common with the LPS from *B. parapertussis*.

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