Studies on the antigenic S-type lipopolysaccharides of *Brucella abortus* strains 7 and Mustapha

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Antigenic phenol phase S-type lipopolysaccharides (LPS) isolated from Brucella abortus (B. abortus) *strains 7 and Mustapha were observed to have ^{13}C n.m.r. spectra which were almost identical to the one reported for the* Brucella abortus *1119-3. The glycosyl content of the lipid A obtained from the LPS of strain 7 was found to be 2-acetamido-2-deoxyglucose only while strain Mustapha was found to contain both 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose. The fatty acid present in the lipid A of both strains was mainly n-hexadecanoic acid. Octadecanoic acid, 3-hydroxytetradecanoic acid as well as small quantities of 3-hydroxydodecanoic acid were also identified. This contrasts with the earlier reports of the absence of 3-OH-14:0 in the LPS of* Brucella abortus.

Keywords: Brucella abortus; lipopolysaccharide; n.m.r. spectroscopy; fatty acids

Introduction

Brucella abortus is the main cause of contagious abortion in cattle. The disease brucellosis was first reported in Nigeria in 1928[1] and it has been shown to be a problem to all breeds of cattle in that country. The overwhelming majority of brucellosis outbreaks in Nigeria are caused by biotypes 1, 2 and 3[2]. The economic importance of brucellosis calls for extensive study of the causative organism. Earlier literature[3–7] contains some reports on the identification of glucose, galactose and mannose in the LPS of *Brucella abortus*. However Carroff *et al.*[8] carried out extensive studies on the antigenic S-type LPS of *B. abortus* 1119-3. This led to the identification of the *O*-chain polysaccharide of *B. abortus* S-type lipopolysaccharide as a linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units. Kreutzer *et al.*[9] observed that hexadecanoic acid and octadecanoic acid were the major fatty acid constituents in the lipid and also noted the absence of 3-hydroxytetradecanoic acid, a common marker of enteric LPS. However Caroff *et al.*[8] reported the presence of 3-hydroxy tetradecanoic acid in the LPS of *B. abortus*.

In this report, we present the results of the chemical studies on the LPS from the cell wall of *B. abortus* biotype 1, strains 7 and Mustapha which among others have been implicated in brucellosis in Nigeria.

Experimental

All the reagents used in this report were of analytical grade. The following strains of *B. abortus*: biotype 1 strain 7 and biotype 1 strain Mustapha typed at the Ministry of Agriculture and Veterinary Laboratory, New Haw, Weybridge, Surrey, England, were obtained from the collection of the National Veterinary Research Institute (NVRI) Vom. Strain 7 was isolated from the South Devon breed 27 at the Livestock Investment Breeding Centre in Kano while the strain Mustapha was isolated from Alhaji Mustapha's herd, a local milk collection centre in Maiduguri.

Culturing conditions

Each strain was supplied as a lyophilized culture and was grown on potato dextrose agar under CO_2 for 48 h at 37°C. The bacterial growth was harvested with sterile 1% saline. The cells were then centrifuged and killed with acetone to obtain the acetone dried cells which gave an average yield of 17 mg from 100 mg of wet cells.

Extraction of the crude LPS

The LPS was extracted by the hot water–phenol method[10]. Twenty g dry weight of each *B. abortus* strain gave an average yield of 1.7 g of the crude aqueous phase LPS and 2.0 g of the crude phenol-phase LPS (PPLPS). The latter was purified according to the method of Moreno *et al.*[1] and a yield of 32 mg was obtained from 100 mg of the crude.

Isolation of the polysaccharide (PS) and lipid A from the LPS

Delipidation was by mild acid hydrolysis with 0.1 M acetic acid as described in another report[12]. Fifty mg of the LPS gave the following yield of PS: 22 mg of strain 7 and 23 mg of strain Mustapha.

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^{13}C n.m.r.

To 20 mg of each PS was added 0.4 ml of 99% D_2O. The spectra were recorded on a GX-100 Jeol spectrometer at 70°C with full proton decoupling. Chemical shifts are reported using dioxane (^{13}C δ 67.4 ppm) as internal reference for strain 7 and acetone as internal reference (^{13}C δ 31.04 ppm) for strain Mustapha.

Sugar analysis of lipid A[12,13]

The method of Sloneker[13] was employed with some modifications[12]. The alditol acetates were analysed on a Hewlett Packard 5830A instrument fitted with a flame ionization detector and an SE-54 fused capillary column. Both injector and detector temperatures were 270°C while the column temperature was 185°C held for 8 min and increased to 230°C at 5°/min. In order to determine the percentage of the backbone sugars present in the lipid, the sugar analysis was repeated using mannose as internal standard.

Fatty acid analysis

This was carried out as previously described[12].

Results and discussion

The ^{13}C n.m.r. data of the phenol *O*-polysaccharides of the two strains are recorded in *Table 1*. The decoupled spectra of the strain 7 showed signals at 101.32, 77.93, 69.01, 52.93, 68.56, 17.79 and 165.60 ppm and are assigned to the carbon atoms of 1, 2-linked 4,6-dideoxy-4-acyl-α-hexopyranosyl residue according to the assignment of Caroff *et al.*[8]. The decoupled spectra of strain Mustapha showed signals at 101.43, 77.98, 69.11, 52.98, 68.53, 17.79 and 166.76 ppm and are assigned as for strain 7. *Table 1* also showed the values of (δ^C-δ) where δ^C are the chemical shift values reported for the 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residue present in the LPS of the *B. abortus* 1119-3[8]. The single signal present in the anomeric region indicates that the PS is a homopolymer.

Six of the seven signals in the proton decoupled ^{13}C-n.m.r. spectra are assigned to the ring carbon atoms and the seventh to the substituent on C-4. The signal at about 166 ppm indicates this seventh carbon atom to be a carbonyl. The substituent cannot be an acetamido group because this would give rise to an additional signal which would arise from the methyl group of the acetamido group. This then suggests that the substituent on C-4 is a formamido group and not an acetamido group.

The n.m.r. data strongly indicate the homopolymer in the LPS of strains 7 and Mustapha to be 1,2-linked-4,6-dideoxy-4-formamido-α-linked hexopyranosyl units. The very small values in the (δ^C-δ) columns lead us to conclude that the polysaccharides in the LPS of the strains 7 and Mustapha are identical with the corresponding one in the *B. abortus* 1119-3.

An attempt to hydrolyse the *O*-PS of the two strains by 4 M HCl resulted in a black precipitate indicating degradation of the polysaccharide. The complete procedure for the preparation of the alditol acetates was carried out but no sugar was identified on the g.l.c. This degradation can be attributed to the formamido group present on the carbon-4.

The quantity of the glucosamine and galactosamine indicated by the results of the sugar analysis of the lipid A was rather small to account for being the only backbone sugars for the fatty acid molecules present. For example, 1 mg of the lipid A of strain Mustapha was found to contain 5 μg of glucose, ~23 μg of glucosamine and ~7 μg of galactosamine, while 1 mg of the lipid A strain 7 gave a yield of only ~10 μg of glucose and ~23 μg of glucosamine. Various hydrolytic conditions: 4 M HCl for 4 h, 4 M HCl for 18 h, and 2 M TFA for 18 h were employed to find out if a higher recovery of the

Table 1 ^{13}C n.m.r. shifts of the native *O*-chain from the phenol-phase LPS of the *B. abortus* strain 7 (S-7) and strain Mustapha (S-M)

Carbon atom	Shifts *O*-PS (ppm)		(δ^C-δ) ppm	
	S-7	S-M	S-7	S-M
C-1	101.32	101.43	+0.49	+0.38
C-2	77.93	77.98	+0.36	+0.28
C-3	69.01	69.11	+0.41	+0.41
C-4	52.93	52.98	+0.22	+0.17
C-5	68.56	68.53	+0.28	+0.31
C-6	17.79	17.79	+0.28	+0.28
NHCHO	165.60	166.76	+0.56	−0.60

δ = chemical shift values of the carbon atoms in the native *O*-chain of S-7 and S-M

δ^C = chemical shift values of the carbon atoms in the native *O*-chain of *B. abortus* 1119-3[8]

Table 2 Characterization of the fatty acids present in the phenol phase LPS and the isolated lipid A of *B. abortus* strains 7 (S-7) and Mustapha (S-M)

Fatty acid methyl ester	G.l.c. relative retention time (t_R)[a]	% of fatty acids				Mass spectrometry	
		S-7		S-M		Base peak	Characteristic ion
		LPS	Lipid A	LPS	Lipid A		
14:0	1.00	—	—	3	4	[b]	
16:0	1.41	64	73	44	52	74	270 (M), 227 (M-43)
18:0	1.78	23	18	19	12	74	298 (M), 255 (M-43)
3-OH-12:0	0.88	—	3	—	7	[c]	
3-OH-14:0	1.33	13	6	34	25	[c]	

[a] Relative retention times are based on tetradecanoic acid methyl ester (14:0, t_R = 1.00)

[b] Signals were very weak on g.c.–m.s

[c] Although strong signals were observed on the g.l.c. the response under the operating conditions of the g.c.–m.s. was rather weak

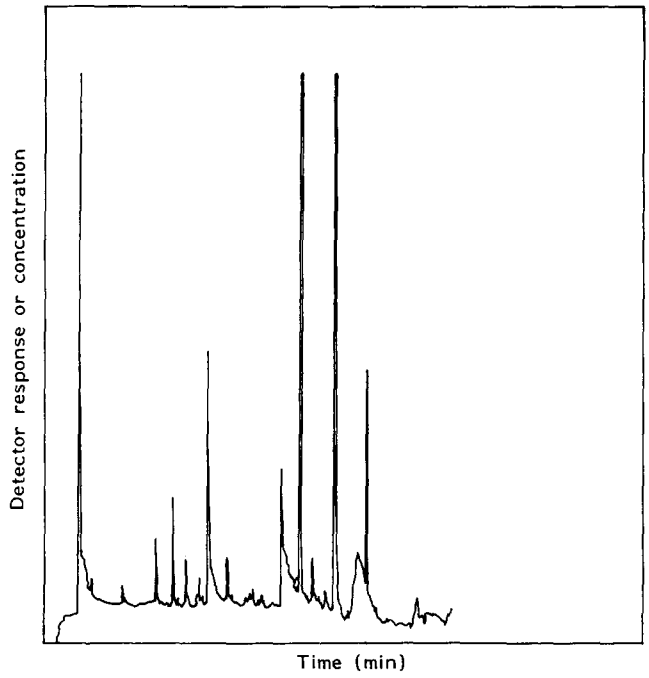

Figure 1 Gas–liquid chromatogram of the fatty acids of the LPS from strain 7

backbone sugars could be obtained. However essentially the same results were obtained.

The amount of individual fatty acids present in the phenol phase LPS and the isolated lipid A from strains 7 and Mustapha was calculated from the gas–liquid chromatograms. As shown in *Table 2*, the major fatty acid of the PPLPS was n-hexadecanoic acid. n-Octadecanoic acid was also found present and the presence of these two fatty acids was confirmed by g.c.-m.s.

3-Hydroxydecanoic acid was found present in the lipid A but not in the LPS of the two strains. 3-Hydroxytetradecanoic acid was identified in both the LPS and lipid A of the two strains. It was found present in large proportions in strain Mustapha: 34% in the LPS and 25% in the lipid A. It is present to a much smaller extent in the LPS and lipid A of strain 7. The presence of 3-hydroxytetradecanoic acid deserves some comments because it had earlier been reported absent in the LPS of *Brucella*[14,15] except for a contrary report by Caroff *et al.*[8]. In view of these earlier reports, a minimum of about 10 determinations was carried out on the LPS of each strain and this fatty acid was found present in all the determinations. Since the final proof of its presence rested on mass spectral analysis, the samples were injected on the g.c.-m.s. However, at the operating conditions of the instrument, the signal corresponding to 3-hydroxytetradecanoic acid on the g.l.c. was very weak on the g.c.-m.s. Because of this poor response, it was not possible to obtain the mass spectra of the fatty acid. The identification therefore rested on the results from the g.l.c. and a typical chromatogram is shown in *Figure 1*. It is quite possible that the use of capillary g.l.c. in this analysis has made this identification possible.

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