On the Serological Specificity of the *Escherichia coli* O8 and O9 Antigens

By KLAUS JANN, BARBARA JANN, VERONIKA WINTER
AND CHRISTINE WOLF-ULLISCH

Max-Planck-Institut für Immunbiologie, Freiburg, West Germany

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The O8- and O9-specific lipopolysaccharides of *Escherichia coli* lost their serological activity during liberation of the polysaccharide moieties (α -mannans) by mild acid hydrolysis, as tested by passive haemagglutination and haemagglutination inhibition. The serological activities and specificities were restored by substitution of the polysaccharides with 1 to 2 stearyl groups per polysaccharide chain. The mannans obtained by biosynthesis *in vitro* were serologically active only when bound to the membrane-associated hydrophobic carrier molecule. Liberation of the polysaccharides from the carrier by treatment with aqueous phenol resulted in loss of the serological activity. The O8- and O9-specific mannans of *E. coli* are thus serologically active when they are part of an amphiphilic molecule and not as free polysaccharides.

INTRODUCTION

Among the many O groups of *Escherichia coli* (Ørskov *et al.*, 1967, 1977), groups 8 and 9 have several unique characteristics. Certain types of K antigens – those that are either acidic cell wall lipopolysaccharides or capsular acidic polysaccharides of very high molecular weight – occur only in these O groups, i.e. together with the O8 or O9 antigen (Ørskov *et al.*, 1977). These two O antigens are cell wall lipopolysaccharides. Their O-specific polysaccharide moieties are mannans, bound to core lipid A (Fig. 1). The formulations of their repeating units are (Reske & Jann, 1972; Prehm *et al.*, 1976):

Although the repeating oligosaccharide of the O9 mannan contains that of the O8 mannan, there is no serological relationship (cross-reaction) between the O8 and O9 antigens.

We have found that the serological specificity of both antigens is lost during the liberation of the respective polysaccharides from the lipid A moiety by treatment with mild acid (K. Jann & B. Jann, unpublished data, cited in Ørskov *et al.*, 1977). When studying the membrane-associated biosynthesis of the O9 antigen we found that the product of the *in vitro* synthesis (the O9 mannan), after liberation from the cytoplasmic membrane with aqueous phenol or dilute acid at elevated temperature, did not react with anti-O9 antibody (Kopmann & Jann, 1975).

These findings raised the question of the O8 and O9 specificities. Two alternatives would account for the findings: (i) the specificity is due to a special modifying group, easily split by acid and not added at all in the *in vitro* synthesis; or (ii) the specificity is related to a

Fig. 1. Schematic representation of the O8 and O9 lipopolysaccharides. The arrow indicates the site of degradation of the molecule into polysaccharide and lipid moieties. For further information, see Lüderitz *et al.* (1971) and Jann & Westphal (1975).

special spatial arrangement, maintained only in association with a hydrophobic entity such as lipid A or the carrier molecule of the biosynthesis. The evidence presented in this paper favours the latter interpretation.

METHODS

Bacteria. *Escherichia coli* r492 (O8:K27⁻:H⁻) and *E. coli* r397 (O9:K29⁻:H⁻) were used for the isolation of the lipopolysaccharides. *In vitro* syntheses of the O8 and O9 mannans were performed with the phosphomannoseisomerase-less mutants r860 (from r492; Kopmann & Jann, 1975) and r945 [from r860 by exchange of the *rfb* gene locus of *E. coli* O9 for that of *E. coli* O8 by conjugation with *E. coli* r549 (O8:K27⁻:H⁻, Hfr59; Schmidt & Westphal, 1968)].

Media. Strains r492 and r397 were grown on D1.5 agar (Schlecht & Westphal, 1966) and strains r860 and r945 in A3 medium (Kopmann & Jann, 1975), containing 0.5 % (w/v) glucose.

Chemicals. Stearoyl chloride, dimethylformamide and pyridine were obtained from Roth Co., Karlsruhe, West Germany; GDPmannose from Boehringer; and GDP[¹⁴C]mannose from Amersham-Buchler, Frankfurt, West Germany.

Abbreviations. LPS, Lipopolysaccharide; LPS-alk, alkali-treated lipopolysaccharide; PS-st, stearoylated polysaccharide; O8-TX, O9-TX, products of *in vitro* O8 or O9 mannan biosynthesis extracted with Triton X-100 in Tris buffer; O8-PW, O9-PW, products of *in vitro* O8 or O9 mannan biosynthesis extracted with 45 % (w/v) aqueous phenol.

Antisera. Anti-O8-LPS and anti-O9-LPS antisera were obtained by hyperimmunization of rabbits with heat-killed acapsular (K⁻) *E. coli* O8:K27⁻:H⁻ and *E. coli* O9:K29⁻:H⁻ (Mayer & Schmidt, 1973). Antisera against the polysaccharide moieties (mannans bound to core, Fig. 1) were raised by intravenous injection of rabbits with the acid-hydrolysed *Salmonella* r mutant r595 modified with stearoylated polysaccharides at a polysaccharide/bacteria ratio of 1:10 (w/w), according to Galanos *et al.* (1971); these antisera are termed anti-PS-st antisera. A goat anti-rabbit globulin antiserum was purchased from the Behringwerke, Marburg, West Germany.

Isolation of lipopolysaccharides and preparation of polysaccharide moieties. Agar-grown bacteria were extracted with 45 % phenol at 65 °C as described by Westphal & Jann (1965). The lipopolysaccharides were obtained from the aqueous phases by repeated centrifugation at 100 000 g. The final pellets were taken up in water and freeze-dried. The yields were about 2 % of the bacterial dry weight.

The lipopolysaccharides were degraded in 1 % (v/v) acetic acid at 100 °C for 90 min (Jann *et al.*, 1975; Müller-Seitz *et al.*, 1968; Schmidt *et al.*, 1969) and the flocculant lipid A was then removed by centrifugation in a bench top centrifuge. The polysaccharide which was obtained from the supernatant was purified by chromatography on Sephadex G-50. The material eluted in the void volume was freeze-dried.

Stearoylation of polysaccharides. The polysaccharides obtained from the O8- and O9-specific lipopolysaccharides (Fig. 1) were substituted with stearoyl groups as described by Hämmerling & Westphal (1967). In short, 50 mg polysaccharide, dried over P₂O₅ *in vacuo* at 45 °C, were dissolved in 5 ml anhydrous dimethylformamide; then 0.6 ml anhydrous pyridine and 10 mg distilled stearoyl chloride, dissolved in 0.6 ml dimethylformamide, were added and the mixture was stirred in a sealed ampoule at room temperature for 3 d. The reaction mixture was then diluted with 0.5 ml water and poured into 25 ml ethanol. The precipitate was washed with alcohol, dissolved in 5 ml water containing 1 % (v/v) 1-butanol and purified by chromatography on Sephadex G-50. It was obtained in 90 to 95 % yield. The stearoyl content of the reaction product was determined by gas chromatography of the methyl esters using a Varian Aerograph series 14000 and a Hewlett Packard integrator model 3380; the chromatographic runs were done at 170 °C on EGSSX.

***In vitro* synthesis of polysaccharides.** The incubation mixtures for the *in vitro* synthesis of the O8 and O9 mannans contained membranes (5 mg protein) obtained from *E. coli* r860 according to Osborn *et al.* (1972) or EDTA-bacteria (10⁹ cells) obtained from *E. coli* r945 according to Robbins *et al.* (1964) and GDP[¹⁴C]-

Table 1. *Percentage composition of the polysaccharide preparations before (PS) and after (PS-st) stearyl substitution*

Preparation	Man	Glc	Gal	Hep	KDO	Stearate
O8 PS	79.8	5.1	3.2	4.2	0.8	—
O9 PS	75.7	7.2	3.4	4.5	0.9	—
O8 PS-st	79.5	5.0	3.1	4.2	0.8	4.2
O9 PS-st	75.5	7.2	3.2	4.2	0.8	4.6

Man, mannose; Glc, glucose; Gal, galactose; Hep, L-manno-D-glycero-heptose; KDO, 2-keto-3-deoxy-octonate.

mannose (0.2 mM, final concentration; 10^6 c.p.m.) in 0.1 M-Tris/HCl buffer, pH 7.5 (final volume 1 ml). The mixtures were incubated for 30 min at 20 °C and the reaction was then stopped by rapid cooling in an ice bath, followed by centrifugation (105 000 g, 1 h, for membranes; 20 000 g, 20 min, for EDTA-bacteria). The pellets were washed once with the Tris buffer and centrifuged again.

The radioactive mannan was extracted from the bacteria and membranes with 45 % aqueous phenol at 68 °C. This extraction and the subsequent purification were described previously (Kopmann & Jann, 1975). To obtain the carrier-bound mannan, the above pellets were extracted with 10 mM-Tris/HCl, pH 7.5, containing 0.01 % (v/v) Triton X-100. Incubations were at room temperature for 30 min. After centrifugation (conditions as above) the supernatants were concentrated in an Amicon cell. To the resulting solutions, glycerol (30 %, v/v, final concentration) and ethanol (10 %, v/v, final concentration) were added and the concentration was continued. The solvents were then removed by ultrafiltration in 0.1 M-Tris buffer, pH 7.5. The final Triton X-100-free solutions contained the radioactive product.

Passive haemagglutination and inhibition. Human erythrocytes of blood group A were coated with the lipopolysaccharides treated with 0.25 M-NaOH at 56 °C for 1 h (LPS-alk) or with the stearyl-substituted polysaccharide moiety of the LPS (PS-st). Haemagglutination was tested (Jann *et al.*, 1965; Neter *et al.*, 1956) in antisera against the O8 antigen, the O9 antigen and the respective polysaccharide moieties.

To test inhibition of haemagglutination, the sera were preincubated with serial dilutions of the potential inhibitors in 0.9 % (w/v) saline (Jann *et al.*, 1965).

Antigen binding test. The test was performed according to Rude *et al.* (1971). To 50 µl of the radioactive product (10^3 to 1.5×10^4 c.p.m.) in phosphate-buffered saline (PBS) 50 µl of antiserum were added. In different experiments the antisera were used either undiluted or in dilutions of 1:2, 1:10, 1:40 and 1:80 in normal rabbit serum which had been diluted 1:10 with PBS. After 1 h at 37 °C and 2 d at 4 °C, 100 µl of a goat anti-rabbit globulin antiserum were added and the mixtures were incubated as above. They were then centrifuged at 30 000 rev. min⁻¹ for 30 min in a SW50.1 rotor. The supernatants were collected, and the pellets were washed once with PBS and suspended in 100 µl PBS. Radioactivity was determined in the first supernatants and the final pellet.

Measurement of radioactivity. This was measured in a Packard scintillation counter model 3390. The scintillation fluid contained 11.76 g diphenyloxazole and 0.24 g bis-*o*-methylstyrylbenzene in a mixture of 1 l Triton X-100 and 2 l toluene.

RESULTS

To characterize the molecular basis of the O8 and O9 specificities of *Escherichia coli*, we have studied the corresponding lipopolysaccharides and their polysaccharide moieties (Fig. 1). The latter were obtained in two ways. (i) Mild acid hydrolysis liberated the polysaccharide moiety from lipid A such that the mannan remained bound to the core oligosaccharide. (ii) *In vitro* biosynthesis using EDTA-bacteria or membrane preparations resulted in a mannan bound to a hydrophobic carrier molecule which is membrane-associated. The free mannan could be isolated from the membranes with aqueous phenol at 68 °C (Flemming & Jann, 1978).

The lipopolysaccharides and their polysaccharide moieties

Table 1 shows the composition of the polysaccharides obtained from the lipopolysaccharides by mild hydrolysis.

For our serological studies we required the polysaccharide moieties of the O8 and O9 lipopolysaccharides in a form which allowed them to attach to erythrocytes. We therefore

Table 2. *Reciprocal titres in the passive haemagglutination of alkali-treated LPS (LPS-alk) and stearyl-substituted polysaccharides (PS-st) in anti-O8 and anti-O9 antisera*

Antigen	Antiserum			
	Anti-O8-LPS	Anti-O8-PS-st	Anti-O9-LPS	Anti-O9-PS-st
O8 LPS-alk	10240	2560	10	10
O8 PS-st	10240	5120	10	10
O9 LPS-alk	10	10	1280	1280
O9 PS-st	10	10	640	320

substituted the polysaccharides with stearyl groups according to Hämmerling & Westphal (1967). The purified products contained about 1 to 3 stearyl groups per polysaccharide chain (Table 1) with a molecular weight of about 10000 to 12000 (Reske, 1970; Reske & Jann, 1972). In contrast to the unsubstituted polysaccharides, the stearylated polysaccharides gave rise to turbid aqueous solutions. In the analytical ultracentrifuge they sedimented rather quickly with *S* values ranging between 3 and 10, indicating a micellar state.

Table 2 shows the results of passive haemagglutination tests using the lipopolysaccharides (LPS) and the stearyl derivatives of the respective polysaccharides (PS-st) in the antisera directed against these antigens (anti-LPS and anti-PS-st). The free polysaccharide moieties of the lipopolysaccharides, i.e. the unsubstituted mannans linked to the core oligosaccharide, were not reactive in any of the antisera. In contrast, the stearyl derivatives had the same serological activity as the lipopolysaccharides from which the polysaccharide moieties were obtained. They also had the same specificity, with no cross-reaction between the stearylated polysaccharides obtained from the O8- and O9-specific lipopolysaccharides. The reactivity and specificity of the lipopolysaccharides and those of the stearyl derivatives from the corresponding polysaccharides were also comparable when tested in the sera obtained with the artificial antigens (anti-PS-st). This was corroborated by gel diffusion in which the homologous lipopolysaccharides and stearylated polysaccharides showed lines of identity when tested against the anti-polysaccharide antisera obtained with the artificial antigens (data not shown).

In the homologous serological systems, i.e. in the agglutination of erythrocytes coated with the O8 or O9 lipopolysaccharides in anti-O8 or anti-O9 antisera, the unsubstituted and stearyl-substituted polysaccharides were tested as inhibitors (Table 3). Clearly, the inability of the polysaccharides to inhibit the antigen-antibody reaction was overcome by stearyl substitution. As with the passive haemagglutination tests, the inhibition studies showed that there was no cross-reaction between the stearylated polysaccharides.

It is unlikely that the O8 and O9 specificities of the lipopolysaccharides, which are lost during liberation of the polysaccharides from lipid A and restored by stearylation, are due to some acid-labile determinant present in the lipopolysaccharides and absent in the polysaccharides.

The polysaccharides obtained by in vitro biosynthesis

The products of incubations of EDTA-bacteria or membranes with GDP-[¹⁴C]mannose were obtained in two ways. (i) Extraction with Triton X-100 in Tris buffer at pH 7.5 and room temperature yielded the mannans bound to the hydrophobic carrier molecule (designated O8-TX and O9-TX). (ii) Extraction with 45% aqueous phenol at 68 °C yielded the free mannans (designated O8-PW and O9-PW). The mobilities of these preparations in sodium dodecyl sulphate-polyacrylamide gel electrophoresis are shown in Fig. 2. O8-TX and O9-TX migrated into the gel, indicating that they were amphiphilic molecules able to interact with sodium dodecyl sulphate. Their electrophoretic mobility corresponded to that of the intermediate of the mannan biosynthesis as described previously (Kopmann & Jann, 1975). O8-PW and O9-PW did not migrate at all.

Table 3. *Inhibition of passive haemagglutination by LPS, the polysaccharide moieties (PS) and their stearyl derivatives (PS-st)*

In the haemagglutinating systems, erythrocytes coated with alkali-treated LPS were used in serum diluted one step less than the final dilutions in the passive haemagglutination experiments. Results are expressed as μg inhibitor ml^{-1} in PBS.

Inhibitor	Haemagglutinating system	
	O8/anti-O8	O9/anti-O9
O8 LPS	0.02	> 250
O8 PS	> 250	> 250
O8 PS-st	0.4	> 250
O9 LPS	> 250	0.02
O9 PS	> 250	> 250
O9 PS-st	> 250	1.6

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the radioactive products of *in vitro* incubations after extraction of the membranes with Triton X-100 (O8-TX, O9-TX; —) or with 45 % aqueous phenol (O8-PW, O9-PW; ---). TD, Tracking dye (pyronin G). The electrophoresis was run on 0.6×10 cm gels of 5.8 % polyacrylamide in the presence of 1 % sodium dodecyl sulphate. The gels were cut into 2 mm slices (fractions) which were hydrolysed in 2 M-HCl and counted as described previously (Kopmann & Jann, 1975).

The purified radioactive products were subjected to the antigen binding test (Rüde *et al.*, 1971). They were first incubated with anti-O8-LPS or anti-O9-LPS antisera or normal serum from rabbits. The antigen-antibody complexes were then precipitated with anti-rabbit Ig antiserum. As shown in Table 4, the free polysaccharides (O8-PW and O9-PW) did not bind anti-O8 or anti-O9 antibodies. However, the polysaccharides bound to the hydrophobic carrier (O8-TX and O9-TX) exhibited distinct and specific binding, although much lower than the theoretical 100 % binding. The binding curve obtained with O9-TX is shown in Fig. 3. The results obtained with O8-TX were very similar (data not shown). In both cases about 50 % binding of the added radioactive products to specific homologous antibody was observed.

The results of the antigen binding test agree with those from tests of passive haemagglutination and its inhibition. The polysaccharides are serologically active only when they are bound to the carrier and thus are part of an amphiphilic molecule.

Table 4. *Antigen binding test of carrier-bound (-TX) and free (-PW) products of the O8 and O9 in vitro synthesis*

Antisera were diluted 1:2 and the preparations contained 8000 c.p.m. incorporated from GDP[¹⁴C]mannose. Normal rabbit serum was used as a control.

<i>In vitro</i> product	Percentage of total radioactivity precipitated by:		
	Anti-O8 antiserum	Anti-O9 antiserum	Normal serum
O8-TX	18.5	1.5	1.5
O8-PW	1.5	0.8	1.5
O9-TX	0.5	20.5	1.0
O9-PW	1.0	2.0	1.7

Fig. 3. Binding of anti-O9-LPS antibody to the *in vitro* product (O9-TX) obtained with membranes from *E. coli* f860 by incubation with GDP[¹⁴C]mannose followed by extraction with Triton X-100.

DISCUSSION

The results indicate that the O8 and O9 mannans of *Escherichia coli* are serologically active only in association with a hydrophobic residue – either lipid A, stearyl residues or the carrier molecule of the biosynthesis. The role of the hydrophobic part of the molecule may be considered in two ways. It may serve to impose on the polysaccharide a secondary structure which is necessary for the O-antigenic expression, or it may form micelles in which the polysaccharide is presented to the antibody in a closely packed form, thus assuming the O8- or O9-specific state. The latter interpretation seems more likely. Although some acidic polysaccharides with very high molecular weights exhibit regions of helical superstructure (Rees, 1972; Moorhouse *et al.*, 1977; Wolf *et al.*, 1978), such molecular arrangements have not yet been implicated in serological specificity. Also, distinct superstructures are difficult to envisage with neutral polysaccharides having molecular weights of only 10000 to 12000 (Reske, 1970; Reske & Jann, 1972). We demonstrated by analytical ultracentrifugation that the stearylated O8 and O9 polysaccharides are highly aggregated (3 to 10 S) and the micellar nature of the lipopolysaccharides is well documented (see Jann & Westphal, 1975). Therefore it is much more likely that the serological specificities of the O8 and O9 mannans depend on their micellar presentation. This implies that the primary structure is important (no cross-reaction between both mannans) but not sufficient for the serological expression of the polysaccharides. The results of the antigen binding assay with the biosynthetic products can be discussed in this respect. Only 20 to 50% of the radioactive carrier-bound mannans were precipitated with the homologous antisera. We have recently found (Jann & Kanegasaki, unpublished results) that the sites of biosynthesis of the O9 antigen seem to be located in special areas of the cytoplasmic membrane. Thus the vesicles studied in the

antigen binding assay are likely to be a heterogeneous population with respect to the products of biosynthesis (mannans). Possibly, vesicles with the polysaccharide chains exposed in a dense array did bind the antibodies better than those which contained fewer chains. A similar conclusion was drawn from the interaction of coliphage $\Omega 8$ with its substrate, the O8 antigen of *E. coli* (Prehm *et al.*, 1976).

The O3 and O5 antigens of *Klebsiella* are lipopolysaccharides with α -mannans which are structurally (Curvall *et al.*, 1973; Lindberg *et al.*, 1972) and serologically closely related to the O8 and O9 antigens of *E. coli*. We have found that these *Klebsiella* O antigens also lose their O specificity when the polysaccharides are detached from lipid A by mild acid hydrolysis. Thus it is interesting to note that all the bacterial O antigens in which we assume a close apposition of polysaccharide chains to be essential for their immunological expression have α -mannans as their O-specific polysaccharides.

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