Electrophoretic and Immunochemical Study of the Lipopolysaccharides Produced by Chemostat-grown Escherichia coli O157

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Two chemically different O-polysaccharides, a low molecular mass form of LPS and core LPS produced by chemostat-grown E. coli O157, were analysed by SDS-PAGE, silver staining and immunoblotting. The reactivities of the different O-polysaccharides with antiserum prepared against E. coli O157 grown in batch culture, Salmonella O30 or Brucella abortus were very similar, showing that the O-polysaccharides share at least some antigenic determinants. The reactions of the low molecular mass LPS with the antiserum indicated it was semi-rough LPS having one repeat unit of the O-polysaccharide attached to core LPS.

INTRODUCTION

The serotype-specific antigens of smooth strains of Gram-negative bacteria are carried on the lipopolysaccharide (LPS) of their outer membrane, specifically on the O-polysaccharide portion of the molecule. Structural studies of LPS have helped to determine the molecular basis of serological classifications and reactivities. The serological cross-reactions which occur between Escherichia coli O157, Brucella abortus, Yersinia enterocolitica O9 and Vibrio cholerae strains have been related to the presence of 1-2-linked 4-amino-4,6-dideoxy-α-D-mannopyranose (D-perosamine) residues in the O-polysaccharide repeating unit of their LPSs (Perry et al., 1986).

E. coli O157, grown using chemostat culture, produces different forms of LPS depending on the growth rate and growth-limiting nutrient (Dodds et al., 1987). At a high growth rate (D = 0.8 h⁻¹, doubling time (td) approx. 52 min), E. coli O157 produced a very small amount of O-polysaccharide which was different from the O-polysaccharide previously characterized in that it lacked 2-aminohexose. Perry et al. (1986) identified the phenol-phase LPS from E. coli O157 grown in batch culture as a linear polysaccharide having the structure [→3)-α-D-GalNAcP-(1→2)-α-D-PerNAcP-(1→3)-α-L-FucP-(1→4)-β-D-GlcP-(1→)], which tests positively for 2-aminohexose. The exceedingly small amount of O-polysaccharide (1-2 mg purified from 100-150 mg of LPS) produced at the high growth rate has made chemical studies difficult. The predominant form of LPS produced by E. coli O157 at the high growth rate was of low molecular mass.

The immunoblotting technique has been used to study the interactions between antibodies and SDS-PAGE separated LPSs, improving the characterization of LPS (Chart et al., 1984; Dooley et al., 1985; de Jongh-Leuvenink et al., 1985). In this paper, we report results of studies on the different forms of LPS produced by E. coli O157 by using electrophoresis and immunoblotting with sera prepared against E. coli O157 (grown in batch culture), B. abortus and Salmonella O30.

METHODS

Bacteria and growth conditions. E. coli O157:H7 (LCDC 82-1933, NRCC 4125) was obtained from the Laboratory Centre for Disease Control, Health and Welfare Canada. Cells were grown in chemostats as continuous cultures in a glucose-mineral salts medium with either glucose (1 g l⁻¹) or Mg²⁺ (5 mg MgCl₂·6H₂O l⁻¹) as the limiting nutrient as described previously (Dodds et al., 1987). The dilution rates used were

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The extracts from the water and phenol phases were each taken up in ultracentrifugation (105,000 g for 1 h until the LPS preparations gave a single sharp peak at 460 nm in the carbocyanine dye assay (Johnson & Perry, 1976) and no measurable absorption peaks in the UV region (310–200 nm).

**LPS extraction.** The hot phenol-water procedure was used to extract LPS from cells (Westphal & Jann, 1965). The extracts from the water and phenol phases were each taken up in 1% NaCl and LPS was pelleted by ultracentrifugation (105,000 g, 4 °C, 16 h). The pellets were dissolved in water and lyophilized. Ultracentrifugation was repeated until LPS preparations gave a single sharp peak at 460 nm in the carbocyanine dye assay (Johnson & Perry, 1976) and no measurable absorption peaks in the UV region (310–200 nm).

**SDS-PAGE.** Purified LPS samples (10 μg) or the LPS of proteinase-K digested whole cells (Hitchcock & Brown, 1983) were analysed by electrophoresis in duplicate 1-5 mm, 14% (w/v) polyacrylamide gels using the system described by Laemmli (1970). Purified LPS from B. abortus 11193-3, Pasteurella hemolytica serotype 4 and Salmonella adelaidae, which have respectively, monosaccharide, disaccharide and pentasaccharide repeating units in their O-polsaccharide, were used as standards (Perry & Babiuk, 1984). The current was set at 15 mA per gel until the tracking dye was through the stacking gel, and then at 35 mA per gel until the dye was 10 cm into the separating gel. One gel was fixed and stained using the silver stain of Tsai & Frasch (1982), while the duplicate gel was used for immunoblotting.

**Antisera.** Antisera raised against E. coli O157 grown for 18 h at 37 °C were prepared by immunization of guinea pigs with formalin-killed E. coli O157 (Hitchcock & Brown, 1983) and was used as per the manufacturer’s instructions (Bio-Rad). The second antibodies used were affinity-purified goat anti-rabbit IgG HRP conjugate (Bio-Rad) and Affinitip Goat Anti-Rabbit IgG (H+L) (Dakopatts). Antisera raised against E. coli O157 grown for 18 h at 37 °C in Brain Heart Infusion broth (Difco) was kindly provided by D. W. Griffith, and was prepared as follows. Rabbits were inoculated intravenously with a freshly prepared suspension of formalin-killed E. coli O157 in 0.85% NaCl containing 2 × 109 cells ml−1. At 4 d intervals the rabbits were injected with increasing amounts (0.5, 1.0, 2.0 and 3.0 ml) of the antigen suspension. The antisera were collected 8 d after the last injection.

**Immunoblotting.** LPS from polyacrylamide slab gels was transferred to nitrocellulose sheets (Bio-Rad) using the transfer buffer of Towbin et al. (1979) and a Trans-Blot Electrophoretic Cell (Bio-Rad) by electroblotting overnight at 30 V and 0.1 A. Subsequent silver staining of the polyacrylamide gel showed that, while transfer of LPS was not complete, there was no visible difference in transfer of the various LPSs. Unreacted sites on the nitrocellulose sheets were blocked with 3% (w/v) gelatin in 20 mM-Tris/HCl pH 7.5 containing 0.5 M-NaCl (TBS) for 60 min. The nitrocellulose sheets were washed twice for 10 min with 0.05% (w/v) Tween 20 in TBS (TTBS) before overnight incubation with the species-specific first antibody diluted 1:1000 in 1% (w/v) gelatin–TTBS. Unbound antibodies were removed by washing the sheets three times for 10 min each with TTBS. The sheets were then incubated for 2 h with the appropriate horse-radish peroxidase (HRP) conjugated immunoglobulin diluted 1:3000 in 1% gelatin–TTBS, before being washed three times for 10 min each with TTBS and once for 10 min with TBS. Finally, sheets were incubated for 30 min with a colour development solution containing 4-chloro-1-naphthol prepared according to the manufacturer’s instructions (Bio-Rad).

**Absorption of antigen.** E. coli O157 was grown in L-broth for 18 h at 37 °C. Bacteria were harvested by centrifugation, washed and resuspended in TBS to an OD660 of 0.6. Cell suspension (5 ml) was added to anti-E. coli sera diluted 1:1000 in 50 ml of 1% gelatin–TBS. This mixture was incubated at room temperature for 1 h. Cells were removed by centrifugation at 12,000 g for 10 min. The efficiency of absorption of the antiseraum was monitored using proteinase-K digested cells which had been grown in L-broth for 18 h at 37 °C.

**RESULTS**

Analysis by SDS-PAGE (Fig. 1a) showed the various forms of LPS produced by E. coli O157 grown under different conditions as established previously (Dodds et al., 1987). Although LPS was present in both the aqueous and phenol phases after hot phenol-water extraction of cells, the phenol phase preferentially extracted high molecular mass LPS and, by comparison with the LPS of proteinase-K digested whole cells, was shown to be representative of the LPS produced by cells grown at low growth rates or in batch culture. The aqueous phase contained more low molecular mass LPS and was representative of the LPS produced by cells grown at high growth rates (Dodds et al., 1987). Phenol-phase LPS from C-limited or Mg2+-limited cells grown at D = 0.1 or 0.4 h−1 showed high molecular mass ‘ladder-like’ bands characteristic of smooth LPS
Fig. 1. SDS-PAGE analysis of purified LPS from *E. coli* O157. (a) Silver stained gel. (b) Immunoblot reacted with polyclonal rabbit antisera raised against phenol-killed *E. coli* O157. Lanes: 1, purified LPS of *B. abortus*; 2, 3, phenol-phase LPS from C-limited cells grown at $D = 0.1 \text{ h}^{-1}$, two different experiments; 4, phenol-phase LPS from C-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 5, phenol-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 6, phenol-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.1 \text{ h}^{-1}$; 7, aqueous-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.1 \text{ h}^{-1}$; 8, aqueous-phase LPS from C-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 9, phenol-phase LPS from C-limited cells grown at $D = 0.8 \text{ h}^{-1}$; 10, 11, 12, aqueous-phase LPS from C-limited cells grown at $D = 0.8 \text{ h}^{-1}$, three different experiments; 13, aqueous-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 14, purified LPS from *P. haemolytica*. 
containing O-polysaccharides of heterogeneous chain length, as well as lower molecular mass bands characteristic of rough LPS (lanes 2–6). Very little or no high molecular mass LPS was evident in the aqueous-phase LPS from C-limited cells grown at \( D = 0.8 \text{ h}^{-1} \) or from \( \text{Mg}^{2+} \)-limited cells grown at \( D = 0.4 \text{ h}^{-1} \) (lanes 10–13). The predominant form of LPS from these cells stained as distinct bands, apparently intermediate in molecular mass. Re-electrophoresis, in which most of this material migrated to the position of the second lowest molecular mass band in the first dimension, showed that the intermediate bands represented multimers or aggregates of low-molecular mass LPS (Dodds et al., 1987).

The antigenic reactivity between the LPSs produced by chemostat-grown \( E. \text{coli} \) O157 and polyclonal antiserum raised against \( E. \text{coli} \) O157 grown in batch culture was examined by immunoblotting (Fig. 1b). Purified LPS from \( B. \text{abortus} \) 119-3 and \( P. \text{hemolytica} \) serotype 4 (Fig. 1, lanes 1 and 14, respectively), used as standards for the silver stain, also served as negative controls for the immunoblot assay. The banding patterns shown by phenol-phase LPS from C-limited or \( \text{Mg}^{2+} \)-limited cells grown at \( D = 0.1 \) or \( 0.4 \text{ h}^{-1} \) were very similar in both the silver-stained gel and the immunoblot (Fig. 1, lanes 2–6) indicating the antiserum reacted with both the \( O \)-polysaccharide and the core regions of these LPSs. A slightly stronger reaction was shown with the core of these LPSs. An equally strong immunoblot reaction was obtained with the LPS produced by C-limited cells grown at \( D = 0.8 \text{ h}^{-1} \) and by \( \text{Mg}^{2+} \)-limited cells grown at \( D = 0.4 \text{ h}^{-1} \) which formed multimers on SDS-PAGE (Fig. 1b, lanes 9–13). Although high molecular mass \( O \)-polysaccharide bands could not be distinguished when the LPSs from these cells were silver stained, they were evident in the immunoblot, showing the increased sensitivity of the immunoblot technique (Fig. 1b, lanes 9–13). The core region of all aqueous-phase LPS preparations and the core region of phenol-phase LPS from C-limited cells grown at \( D = 0.8 \text{ h}^{-1} \) (Fig. 1b, lanes 7–13) reacted very weakly or not at all with the antiserum showing a clear difference to the silver-stained gel and to the reactions of phenol-phase LPS from C-limited or \( \text{Mg}^{2+} \)-limited cells grown at \( D = 0.1 \) or \( 0.4 \text{ h}^{-1} \) (Fig. 1b, lanes 2–6).

The \( O \)-polysaccharide of the LPS produced by \( S. \text{landau} \) O30 and that of \( E. \text{coli} \) O157 grown in batch culture are identical except that half the \( D \)-glucopyranosyl residues in the \( S. \text{landau} \) O30 LPS bear \( O \)-acetyl substituents at C-6 (Perry et al., 1986). Anti-Salmonella O30 serum was reacted with the \( E. \text{coli} \) O157 LPSs (Fig. 2). The two standard LPSs, \( S. \text{adelaide} \) O35 and \( P. \text{hemolytica} \) serotype 4, served as negative controls (Fig. 2b, lanes 1 and 14, respectively). The antiserum did not react with any of the core LPSs of \( E. \text{coli} \) O157 but did react with all other forms of LPS. The relative intensities of the bands other than core LPS in the immunoblot were approximately the same as in the silver-stained gel.

Serological cross-reactions which occur between \( E. \text{coli} \) O157 and \( B. \text{abortus} \) are due to the presence of perosamine in their \( O \)-polysaccharides (Perry et al., 1986). Since one of the differences between the LPS produced by chemostat-grown \( E. \text{coli} \) at high and low growth rates was in the amino sugar composition, the reactivity of the LPSs produced by \( E. \text{coli} \) O157 with \( B. \text{abortus} \) antiserum was examined (Fig. 3). In the silver-stained gel, \( B. \text{abortus} \) LPS showed a blurred band of high molecular mass and another of low molecular mass, while in the immunoblot a much larger region stained, again showing the increased sensitivity of this method. \( P. \text{hemolytica} \) serotype 4 LPS (Fig. 3b, lane 14) showed no reaction with the antiserum. Not all of the \( E. \text{coli} \) O157 LPSs reacted with the antiserum. The high molecular mass \( O \)-polysaccharide bands reacted very weakly or not at all (Fig. 3b, lanes 2–4, 10, 13). The multimeric LPS bands reacted much more strongly (Fig. 3b, lanes 5–7, 9, 11, 12). Again, the \( E. \text{coli} \) core LPSs did not react.

Anti-\( E. \text{coli} \) serum was absorbed with \( E. \text{coli} \) cells grown overnight in batch culture to remove antibodies specific for the \( O \)-polysaccharide containing 2-aminohexose. Cells grown in batch culture were previously found to produce smooth LPS with this \( O \)-polysaccharide (Perry et al., 1986). Absorbing the antiserum greatly reduced the reaction of the \( O \)-polysaccharide bands in the immunoblot (Fig. 4b, lanes 2–4, 8). This is most evident in the proteinase-K digested whole-cell lysates (Fig. 4b, lane 8) of cells grown overnight in batch culture. High molecular mass 'ladder-like' bands characteristic of smooth LPS containing \( O \)-polysaccharides of heterogeneous
Fig. 2. SDS-PAGE analysis of purified LPS from E. coli O157. (a) Silver stained gel. (b) Immunoblot reacted with polyclonal rabbit antisera raised against Salmonella O30, absorbed to remove other agglutinins. Lanes: 1, purified LPS of S. adelaide O35; 2, 3, phenol-phase LPS from C-limited cells grown at $D = 0\cdot 1$ h$^{-1}$; 4, phenol-phase LPS from C-limited cells grown at $D = 0\cdot 4$ h$^{-1}$; 5, aqueous-phase LPS from C-limited cells grown at $D = 0\cdot 4$ h$^{-1}$; 6, phenol-phase LPS from C-limited cells grown at $D = 0\cdot 8$ h$^{-1}$; 7, 8, 9, aqueous-phase LPS from C-limited cells grown at $D = 0\cdot 8$ h$^{-1}$, three different experiments; 10, phenol-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0\cdot 4$ h$^{-1}$; 11, aqueous-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0\cdot 4$ h$^{-1}$; 12, aqueous-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0\cdot 8$ h$^{-1}$; 13, phenol-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0\cdot 1$ h$^{-1}$; 14, purified LPS from P. haemolytica.

Chain length were evident in the silver-stained LPS profile of these cells but they were very weak in the immunoblot. The material that did react in the immunoblot was of lower molecular mass than smooth LPS and could have been due to a reaction of the antiserum with some cellular proteins. Reactions of the low molecular mass LPS were unaffected by absorption of the antiserum.
Fig. 3. SDS-PAGE analysis of purified LPS from *E. coli* O157. (a) Silver stained gel. (b) Immunoblot reacted with polyclonal bovine antisera raised against *B. abortus*. Lanes: 1, 14, purified LPS from *B. abortus*; 2, 3, phenol-phase LPS from C-limited cells grown at $D = 0.1\ h^{-1}$, two different experiments; 4, phenol-phase LPS from C-limited cells grown at $D = 0.4\ h^{-1}$; 5, aqueous-phase LPS from C-limited cells grown at $D = 0.8\ h^{-1}$; 6, phenol-phase LPS from C-limited cells grown at $D = 0.8\ h^{-1}$; 7, 8, 9,
Fig. 4. SDS-PAGE analysis of LPS from *E. coli* O157. (a) Silver stained gel. (b) Immunoblot reacted with polyclonal rabbit antiserum raised against *E. coli* O157. Lanes 1–8, reaction after absorption of antiserum with live cells of *E. coli* O157. Lanes: 1, 9, purified LPS from *S. adelaide* O35; 2, 10, phenol-phase LPS from C-limited cells grown at $D = 0.1 \text{ h}^{-1}$; 3, 11, phenol-phase LPS from C-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 4, 12, phenol-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 5, 6, 13, 14, aqueous-phase LPS from C-limited cells grown at $D = 0.8 \text{ h}^{-1}$, two different experiments; 7, 15, aqueous-phase LPS from Mg$^{2+}$-limited cells grown at $D = 0.4 \text{ h}^{-1}$; 8, proteinase-K digested whole cell lysates of *E. coli* O157.
**DISCUSSION**

In a previous report (Dodds et al., 1987) we showed that the structure of the LPS produced by chemostat-grown *E. coli* O157 depended on the rate at which the organism was grown and on the nutrient that was growth limiting. The LPS produced by C-limited cells grown at $D = 0.1$ or $0.4$ h$^{-1}$ and by Mg$^{2+}$-limited cells grown at $D = 0.1$ h$^{-1}$ was identical to that produced by batch-grown cells, the O-polysaccharide of which has been characterized as a linear polysaccharide having the structure $[-3)-\alpha-D-GalNAcp-(1\rightarrow2)-\alpha-D-PerNAcp-(1\rightarrow3)-\alpha-L-Fucp-(1\rightarrow4)-\beta-D-Glcp-(1\rightarrow)]_n$ (Perry et al., 1986). C-limited cells grown at $D = 0.8$ h$^{-1}$ and Mg$^{2+}$-limited cells grown at $D = 0.4$ h$^{-1}$ produced a very small amount of smooth LPS with an O-polysaccharide lacking 2-aminohexose, thus differing from the aforementioned O-polysaccharide, and a large proportion of low molecular mass LPS which tended to form multimers on SDS-PAGE. In this study, we have further characterized the LPSs produced by *E. coli* O157 using electrophoresis and immunoblotting.

The O-polysaccharide from cells of *E. coli* O157 grown under C-limitation at $D = 0.8$ h$^{-1}$ or under Mg$^{2+}$-limitation at $D = 0.4$ h$^{-1}$ differs chemically from, but appears to have some of the same antigenic determinants as, the O-polysaccharide from cells grown in batch culture or under C-limitation at $D = 0.1$ and $0.4$ h$^{-1}$ or under Mg$^{2+}$-limitation at $D = 0.1$ h$^{-1}$. Both O-polysaccharides reacted with the polyclonal serum prepared against *E. coli* O157 grown in batch culture (Fig. 1b), a reaction which demonstrated the sensitivity of the immunoblot assay since these bands were not evident in the LPS from cells grown under C-limitation at $D = 0.8$ h$^{-1}$ or under Mg$^{2+}$-limitation at $D = 0.4$ h$^{-1}$ after silver staining. Both O-polysaccharides also reacted with antiserum specific for the O-polysaccharide of *Salmonella* O30 (Fig. 2b). *E. coli* O157 grown in batch culture cross reacts with group N *Salmonella* (antigen O30) due to O-polysaccharides which are identical except that half of the D-glucopyranosyl residues in the group N *Salmonella* LPS are 6-O-acetylated (Perry et al., 1986). Antiserum raised against *B. abortus* gave very little or no reaction with the O-polysaccharides produced by *E. coli* O157 (Fig. 3b) even though the presence of perosamine in both O-polysaccharides can cause cross reactions (Perry et al., 1986). Because the reactivities of the different O-polysaccharides produced by *E. coli* O157 to the different antisera were so similar, we conclude that the differences between them are subtle.

The low molecular mass aqueous-phase LPS produced by C-limited cells grown at $D = 0.8$ h$^{-1}$ and by Mg$^{2+}$-limited cells grown at $D = 0.4$ h$^{-1}$ which formed multimers on SDS-PAGE also appears to share some antigenic determinants with the O-polysaccharide produced by *E. coli* O157 grown in batch culture or under C-limitation at $D = 0.1$ or $0.4$ h$^{-1}$ or under Mg$^{2+}$-limitation at $D = 0.1$ h$^{-1}$. It reacted just as strongly as O-polysaccharide with antisera raised against batch-grown *E. coli* O157 (Fig. 1b), but this antiserum may contain antibodies directed at determinants other than O-polysaccharide. The low molecular mass LPS also reacted just as strongly as O-polysaccharide with antisera specific for *Salmonella* O30 using electrophoresis and immunoblotting.

The fastest migrating band of *E. coli* O157 LPS, representing core LPS (Goldman & Leive, 1980; Munford et al., 1980; Palva & Makela, 1980), unexpectedly showed differences in reactivity to the antiserum raised against *E. coli* O157 (Fig. 1b). Core LPS from all phenol preparations except for that from C-limited cells grown at $D = 0.8$ h$^{-1}$, reacted with the
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antiserum whereas the core LPS from aqueous-phase preparations did not. Because the structure of the core is thought to be essentially conserved (Mayer et al., 1985; Luderitz et al., 1982) and because these bands all showed the same mobility on SDS-PAGE, the differences are probably the result of minor differences in substitutions.

In our previous study (Dodds et al., 1987), we found that a much higher proportion of rough LPS was produced by E. coli O157 at high growth rates. Such changes have been related by others to a decrease in the activity of R-core-O-polysaccharide ligase relative to the other enzymes involved in LPS biosynthesis (McConnell & Wright, 1979). The immunoblot assay has shown that the different O-polysaccharides produced by E. coli O157 have some antigenic determinants in common and the differences between the two are probably minor, possibly involving differences in modification of the polysaccharide after its assembly. The low molecular mass LPS produced by C-limited cells grown at a growth rate of 9×10⁻² h⁻¹ represents semi-rough LPS (core LPS plus only one repeat unit of O-polysaccharide) suggesting differences in the polymerization of repeating units. Thus, results from this study suggest that other aspects of the biosynthesis of LPS are also affected by growth conditions.

REFERENCES


