First Evidence for a Covalent Linkage between Enterobacterial Common Antigen and Lipopolysaccharide in Shigella sonnei Phase II ECA_{LPS}\textsuperscript{*}

Received for publication, August 23, 2013, and in revised form, December 5, 2013 Published, JBC Papers in Press, December 9, 2013, DOI 10.1074/jbc.M113.512749

Tomasz K. Gozdziewicz\textsuperscript{1,2}, Czeslaw Lugowski\textsuperscript{1,3}, and Jolanta Lukasiewicz\textsuperscript{1,4}

From the \textsuperscript{1}Department of Immunochemistry, Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, PL-53-114 Wroclaw, Poland and the \textsuperscript{2}Department of Biotechnology and Molecular Biology, University of Opole, kard. B. Kominka 6A, PL-45-035 Opole, Poland

Background: Enterobacterial common antigen (ECA) is a surface antigen of all enteric bacteria.

Results: ECA polysaccharide substitutes the outer core region of \textit{Shigella sonnei} lipopolysaccharide (LPS).

Conclusion: First structural evidence for the existence of ECA covalently associated with LPS (ECA_{LPS}).

Significance: ECA_{LPS} is the only immunogenic form of ECA and could be a target for therapeutic strategies against nosocomial infections.

Enterobacterial common antigen (ECA)\textsuperscript{2} is a surface antigen present in Gram-negative bacteria belonging to the Enterobacteriaceae family, including \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, and \textit{Proteus} spp. Recent studies have indicated the importance of ECA for cell envelope integrity, flagellum expression, and resistance of enteric bacteria to acetic acid and bile salts. ECA, a heteropolysaccharide built from the trisaccharide repeating unit, \(\rightarrow 3\)-\(\alpha\)-\(d\)-Fucp\(4\)\(\text{NAc}-(1\rightarrow 4)\)-\(\beta\)-\(d\)-Manp\(N\text{AcA}-(1\rightarrow 4)\)-\(\alpha\)-\(d\)-Glc\(p\)N\(\text{Ac}-(1\rightarrow\) occurs as a cyclic form (ECA\textsubscript{Cyc}), a phosphatidylylglycerol (PG)-linked form (ECA\textsubscript{PG}), and an endotoxin/lipopolysaccharide (LPS)-associated form (ECA\textsubscript{LPS}). Since the discovery of ECA in 1962, the structures of ECA\textsubscript{PG} and ECA\textsubscript{Cyc} have been completely elucidated. However, no direct evidence has been presented to support a covalent linkage between ECA and LPS; only serological indications of co-association have been reported. This is paradoxical, given that ECA was first identified based on the capacity of immunogenic ECA\textsubscript{LPS} to elicit antibodies cross-reactive with enterobacteria. Using a simple isolation protocol supported by serological tracking of ECA epitopes and NMR spectroscopy and mass spectrometry, we have succeeded in the first detection, isolation, and complete structural analysis of poly- and oligosaccharides of \textit{Shigella sonnei} phase II ECA\textsubscript{LPS}. ECA\textsubscript{LPS} consists of the core oligosaccharide substituted with one to four repeating units of ECA at the position occupied by the O-antigen in the case of smooth \textit{S. sonnei} phase I. These data represent the first structural evidence for the existence of ECA\textsubscript{LPS} in the half-century since it was first discovered and provide insights that could prove helpful in further structural analyses and screening of ECA\textsubscript{LPS} among Enterobacteriaceae species.

Enterobacterial common antigen (ECA)\textsuperscript{2} is a surface antigen expressed by Gram-negative bacteria belonging to Enterobacteriaceae, including emerging drug-resistant pathogens such as \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, \textit{Proteus} spp., \textit{Citrobacter} spp., \textit{Serratia} spp., \textit{Proteus} spp., \textit{Yersinia} spp., and \textit{Plesiomonas shigelloides}. These bacteria are responsible for healthcare-associated infections, such as intestinal infections and nosocomial infections (e.g. sepsis). Drug resistance in a few members of this family, notably \textit{E. coli}, \textit{Klebsiella} spp. and \textit{Proteus} spp., is an increasing global problem. This stresses the need for new protective vaccines or therapeutic strategies against Gram-negative bacteria. Some of these could be based on ECA.

ECA is a heteropolysaccharide built from the trisaccharide repeating unit, \(\rightarrow 3\)-\(\alpha\)-\(d\)-Fucp\(4\)\(\text{NAc}-(1\rightarrow 4)\)-\(\beta\)-\(d\)-Manp\(\text{NAcA}-(1\rightarrow 4)\)-\(\alpha\)-\(d\)-Glc\(p\)N\(\text{Ac}-(1\rightleftharpoons 2\rightarrow 4)\), modified with O-acetyl groups. The biological significance of ECA is not fully understood and requires further study. However, Barua et al.\textsuperscript{5} showed the importance of ECA together with the O-specific polysaccharide of endotoxin (lipopolysaccharide (LPS)) for the resistance of Shiga toxinogenic \textit{E. coli} O157:H7 and \textit{Salmonella} to acetic acid and bile salts.\textsuperscript{6} In the case of \textit{Serratia marcescens}, mutations that disrupt the integrity of the ECA biosynthetic pathway severely diminish bacterial motility by influencing the transcription of genes involved in envelope integrity and flagellar expression (7, 8).

ECA occurs in three forms. The first two forms, a free, cyclic polysaccharide (ECA\textsubscript{Cyc}) localized to the periplasm (2, 3) and a linear, phosphatidylylglycerol (PG)-linked polysaccharide localized on the cell surface (ECA\textsubscript{PG}) (4, 9–12), are completely

\textsuperscript{*} This work was supported by Wroclaw Research Centre EIT + within the project “Biotechnologies and Advanced Medical Technologies”—BioMed (POIG.01.01.02-02-003/08) co-financed by the European Regional Development Fund (Operational Programme Innovative Economy, 1.1.2).

\textsuperscript{1} To whom correspondence should be addressed. Tel.: 48-713709927; E-mail: czaja@iitd.pan.wroc.pl.

\textsuperscript{2} The abbreviations used are: ECA, enterobacterial common antigen; ECA\textsubscript{Cyc}, cyclic form of enterobacterial common antigen; ECA\textsubscript{PG}, enterobacterial common antigen linked to phosphatidylglycerol; ECA\textsubscript{LPS}, enterobacterial common antigen linked to lipopolysaccharide or lipooligosaccharide; OS, oligosaccharide; PS, polysaccharide; LOS, lipooligosaccharide; dLOS, LOS devoid of the lipid A; ESI, electrospray ionization; IT, ion trap; TOCSY, total correlation spectroscopy; HMB, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; DEPT, distortionless enhancement by polarization transfer; Hep, l-glycero-d-manno-heptose; Kdo, 3-deoxy-d-manno-oct-2-ulosonic acid; P, phosphate; PETn, phosphoethanolamine; PPETn, pyrophosphorylethanolamine.

The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 5, pp. 2745–2754, January 31, 2014 © 2014 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
characterized and are nonimmunogenic. The third form is poorly understood, but is thought to be composed of ECA and LPS. LPS is the main surface antigen of Gram-negative bacteria and is a potent virulence factor. It consists of three main regions: the O-specific polysaccharide (smooth strains only), the core oligosaccharide (OS), and lipid A. To date, the nature of the association between ECA and LPS has not been elucidated and remains the last missing link to understanding ECA diversity. Additionally, ECA<sub>LPS</sub> is the only immunogenic form of ECA capable of eliciting cross-reactive anti-ECA antibodies (13, 14).

Paradoxically, given this structural uncertainty, the history of ECA began with the discovery of ECA<sub>LPS</sub>. The presence of ECA was originally inferred from the extent of serological cross-reactions in hemagglutination assays between the sera of patients and various E. coli O-serotypes during studies of urinary tract E. coli infections in 1962 by Kunin et al. (15). A few E. coli strains (serotype O14, O54, O124, and O144) elicited highly cross-reactive antibodies in rabbits (13, 14) that could be removed from anti-O14 serum by absorption with extracts of any strain of E. coli, while retaining the homologous reactivity of the serum. Thus, it was concluded that anti-O14 serum contained antibodies against an antigen that is common for all Enterobacteriaceae. This suggested that, unlike most E. coli strains, a few, especially O14, express this antigen in an immunogenic form, the presumptive ECA<sub>LPS</sub>, capable of eliciting cross-reactive anti-ECA antibodies (1, 13–15). Following this initial discovery of ECA, ECA<sub>PG</sub> was identified (4, 9, 10), and its chemical structure and covalent linkage to PG were ultimately elucidated for E. coli and Salmonella typhimurium (11, 12). The identification of ECA<sub>CYC</sub> and its structural analysis were completed even earlier for Shigella sonnei phase I (2, 3). Therefore, ECA<sub>PG</sub> and ECA<sub>CYC</sub> seem to be well characterized with respect to their occurrence (11), structural variability (16), and the genetic basis for their biosynthesis (17–19).

To date, no direct evidence for the existence of a covalent linkage between ECA and LPS has been reported. Since its initial discovery, ECA<sub>LPS</sub> in nature has usually been identified indirectly using serological approaches similar to those used to establish the immunogenic properties of E. coli O14 ECA<sub>LPS</sub> (1, 13–15). The only indication for the existence of ECA<sub>LPS</sub>, apart from its immunogenicity, has been co-migration of LPS/LOS in SDS-PAGE, as detected by specific anti-ECA antibodies/sera. In subsequent studies, Kunin et al. (9) and Mayer et al. (20) identified the presence of such an immunogenic form of ECA in rough mutants of E. coli Ra, R1, R4, and K-12 that express a complete core OS region of LOS. More recent and extensive serological studies have addressed the occurrence of ECA<sub>LPS</sub> in Yersinia enterocolitica (21–24) and Proteus mirabilis. ECA immunogenicity has also been demonstrated for several wild-type strains and mutants of Y. enterocolitica O-3 and O-9 as well as P. mirabilis (25). The authors of these latter studies utilized polyclonal and monoclonal antibodies against different forms of ECA to screen SDS-PAGE-separated LPS/LOS isolated from a broad range of wild-type and mutant bacteria to show the coexistence of ECA and LPS epitopes. Recent serological studies of Y. enterocolitica LPS pointed to the core OS of LPS as the probable location of the ECA and its coexistence with O-specific polysaccharide (21). The only attempted structural analyses of ECA<sub>LPS</sub> were reported for E. coli strain F470 (R1 core type) (26) and S. sonnei phase II (27). These analytical strategies utilized chromatography to separate different fractions of degraded LPS and characterize them for the presence of ECA constituents. The detection of ECA constituents was qualitative and was based only on sugar and methylation analyses and isotope labeling of ECA constituents (26) or NMR spectroscopy (27). Both studies reported the coexistence of the core OS and ECA constituents, but lacked direct evidence for a covalent linkage between them.

To resolve the ECA<sub>LPS</sub> structure, we sought to isolate ECA<sub>LPS</sub> and perform a detailed structural analysis using the sensitive techniques of NMR spectroscopy and mass spectrometry. For this investigation, we used S. sonnei phase II, a species that is the causative agent of dysentery. This mutant is well characterized with respect to the structure of the core OS and ECA<sub>CYC</sub>, expressing LOS with the core OS of E. coli R1 type and devoid of O-specific polysaccharide (PS) (2, 28, 29). The data presented here, coming a half-century after the initial discovery of ECA, represent the first structural evidence for the existence of ECA<sub>LPS</sub> and provide insights that could prove helpful in further structural analyses and screening of ECA<sub>LPS</sub> among Enterobacteriaceae species.

**EXPERIMENTAL PROCEDURES**

**Bacteria and Sera—**S. sonnei phase II was obtained from the Polish Collection of Microorganisms at the Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). The bacteria were grown to logarithmic phase in liquid Davis medium enriched with glucose, yeast extract, and casein hydrolysate; killed with 0.5% phenol; and centrifuged using a CEPA laboratory flow centrifuge. Rabbit polyclonal anti-ECA<sub>CYC</sub> and anti-E. coli R1 core OS sera were prepared as part of a previously published study (30, 31).

**Preparation of LOS and Oligosaccharides—**LOS was extracted from bacterial cells using a hot phenol/water method (32). Polyan-and oligosaccharides were obtained by mild acid hydrolysis of LOS (1.5% acetic acid, 100 °C, 30 min). The resulting mixture was centrifuged (40,000 × g), and the supernatant was collected, lyophilized, and fractionated on a Bio-Gel P-10 column (1.6 × 100 cm; Bio-Rad) equilibrated with 0.05 m pyridine/acetate acid buffer (pH 5.6). ECA was detected by dot-blotting of eluted fractions with anti-ECA<sub>CYC</sub> serum (30).

**Dot-blotting—**Fractions eluted from Bio-Gel P-10 were spotted onto a dried nitrocellulose membrane. The membrane was air-dried, and sample spotting was repeated two times. The membrane was blocked with 2% casein in TBS (10 mM Tris pH 7.4, 150 mM NaCl) for 1 h at 37 °C, and then incubated overnight with a solution of polyclonal rabbit anti-ECA<sub>CYC</sub> serum in TBS (200-fold dilution). The membrane was washed three times with TBS prior to incubation with 2000-fold diluted goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad). After washing with TBS, the membrane was stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.05 m Tris-HCl buffer (pH 9.6) containing 5 mM MgCl₂. The reaction was stopped by washing with an ethanol/water/acetic acid mixture.
Identification of the Covalent Linkage between ECA and LPS

RESULTS

Isolation and Purification of S. sonnei Phase II ECA<sub>LPS</sub>—The LOS of S. sonnei phase II was extracted from bacterial cells by a hot phenol/water method and was obtained with a yield of 2.11% of dry bacterial mass. Poly- and oligosaccharides (dLOS) were released from lipid A by mild acid hydrolysis of LOS (250 mg) in 1.5% acetic acid and separated by gel filtration on Bio-Gel P-10 (Fig. 1). Five fractions were eluted and analyzed for the presence of ECA epitopes by dot-blotting using polyclonal rabbit R1 core OS sera. Positive reactions with anti-ECA<sub>CYC</sub> serum were observed for fractions I and IV, which consisted of [ECA]<sub>2–4</sub>-dLOS containing two to four repeating units of ECA.

Acid solution (4.5:4.5:1) followed by washing with water and air drying.

Electrospray Mass spectrometry—MS and MS<sup>2</sup> experiments were carried out on an amaZon SL ion trap (IT) mass spectrometer (Bruker Daltonics) in both positive-ion and negative-ion modes. The samples were dissolved in acetonitrile/water/formic acid solution (50:50:0.5; 50 µg/ml). Source parameters were as follows: sample flow, 3 µl/min; ion source temperature, 200 °C; nitrogen flow, 5 liters/min at a pressure of 8 psi. Spectra were scanned in the 200–2000 m/z range. The system was calibrated in positive-ion mode using ESI-L tuning mix (Agilent Technologies) before acquisitions. MS<sup>2</sup> experiments were performed using an isolation width of 4 m/z, an amplitude value of 0.35, and a SmartFrag mode of 60–80%. The fragment ions structures were determined using GlycoWorkbench software (33).

NMR Spectroscopy—All NMR spectra were obtained using an Avance III 600 MHz (Bruker BioSpin) spectrometer equipped with a QCI-P cryoprobe. NMR spectra of isolated oligosaccharides were obtained in 2H<sub>2</sub>O using acetone as an internal reference (δ<sub>H</sub> 2.225 ppm; δ<sub>C</sub> 31.05 ppm). Oligosaccharides (1.8 mg) were repeatedly exchanged with 2H<sub>2</sub>O (99.95%) with intermediate lyophilization. The data were acquired and processed with standard Bruker software (TopSpin 3.1) and assigned using SPARKY (34). The signals were assigned based on two-dimensional experiments using correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum coherence-distortionless enhancement by polarization transfer (HSQC-DEPT), and HSQC-TOCSY. The mixing times in clean-TOCSY experiments were 30, 60, and 100 ms. The delay time in HMBC was 60 ms, and the mixing time for NOESY was 200 ms.

Compositional Analysis—Methylation analyses were performed according to the method described by Ciucanu and Kerek (35). Partially methylated alditol acetates were analyzed by gas chromatography (GC)-MS using a Thermo Scientific TSQ system with an RX5 fused silica capillary column (0.2 mm × 30 cm) and a temperature program of 150–270 °C at 12 °C min<sup>−1</sup>.

MS<sup>3</sup>). Electrospray ionization-ion trap mass spectrometry (ESI-IT-MS<sup>3</sup>) analyses confirmed dot-blotting results and the presence of different forms of ECA. Fraction I was identified as linear polymer of ECA subunit derived from ECA<sub>PG</sub> and devoid of PG moiety (data not shown). Fraction II was identified as ECA<sub>CYC</sub> (data not shown) as a result of co-purification of this form together with LPS. Fractions reacted with both sera (III, IV) were analyzed by ESI-IT-MS<sup>3</sup>. Analyses confirmed dot-blotting results and the presence of poly- and oligosaccharides derived from ECA<sub>LPS</sub> built of the core OS of LOS (dLOS) substituted with repeating units of ECA ([ECA]<sub>p</sub>-dLOS; n ≥ 1). Fraction III consisted of [ECA]<sub>2–4</sub>-dLOS containing two to four ECA repeating units (Fig. 2A), and fraction IV consisted of [ECA]<sub>1</sub>-dLOS containing one repeating unit of ECA (Fig. 2B) were identified. According to previously published data (28), fraction V consisted of different unsubstituted core OS glycoforms of E. coli R1 type. The final yield of [ECA]<sub>p</sub>-dLOS was 3% of released poly- and oligosaccharides (fractions III and IV).

Under standard conditions, a basic methylation analysis of [ECA]-dLOS indicated the presence of partially methylated alditol acetate of one constituent of ECA, 3-substituted-Glc<sub>NAc; </sub>as expected based on the susceptibility of →3)-α-D-Fucp4NAc1→ to acidic degradation and the nonvolatile character of →4)-β-D-ManpNACA-1→ derivative. The following derivatives of constituents of the R1 core OS, in addition to nonvolatile residues substituted with a phosphate (P) or phosphoethanolamine (PEtn) group, were also identified: terminal Glcp, terminal Galp, 3-substituted Glcp, 2-substituted Galp, 2,3-disubstituted Glcp, and terminal Hepp. In comparison with unsubstituted core OS, molar ratios of terminal Glc, terminal Gal, →3)-Glc, and →2)-Gal were 0.4:1.1:4.1:2.2, showing decreasing amounts of terminal Glc and increasing amounts of →3)-Glc. These data provide an initial indication of the position of substitution of the core OS by ECA at →3)-Glc that would prove to be in agreement with subsequent studies.

Mass Spectrometry Analysis of ECA<sub>LPS</sub>—A detailed analysis of the molecular mass of [ECA]<sub>p</sub>-dLOS fractions indicated the presence of a covalent linkage between ECA and different glycoforms of the core OS (Fig. 2, A and B). Glycoforms substituted with two, three, and four repeating units of ECA were identified in fraction III (Fig. 2A) and were represented by pre-
Identification of the Covalent Linkage between ECA and LPS by NMR Spectroscopy—Fraction IV containing [ECA]-dLOS was further investigated using one-dimensional and two-dimensional 1H, 13C, 31P NMR spectroscopy. The 1H (Fig. 3), 1H, 13C HSQC-DEPT, and HMBC NMR spectra (Fig. 4) contained mainly signals for 11 anomeric protons and carbons and the Kdo spin system confirming the dodecasaccharide structure of [ECA]-dLOS (Fig. 3); signals for three acetamide groups, the carboxylic group of ManNAc and the methyl group of FucpNAc, characteristic ECA residues, were also identified. Capital letters shown in the structure are used throughout the text and in tables (Tables 2 and 3) and figures (Figs. 3, 4, and 5) to refer to the corresponding sugars. Because the ECA repeating unit and R1 core OS structures were previously described, the chemical shift values were compared with published NMR data (37, 38).

Resides A–I were identified as constituents of the core OS, and residues J–L were identified as constituents of ECA (Fig. 3, inset structure). Residue A, with characteristic deoxy proton signals at δ 1.90 ppm (H-3ax) and 2.25 ppm (H-3eq) together with a high chemical shift of the C-5 signal (δ 73.3 ppm), was identified as 5-substituted 3-deoxy-α-D-manno-2-ulosesonic acid [→5]-α-Kdo[β]. Residue B, with a JH1,C1 of ~174 Hz, small vicinal couplings between H-1, H-2, and H-3, and a high C-3 signal (δ 78.5 ppm), was identified as 3-substituted 1-glycero-D-manno-HeppP-Etn. 1H, 31P HMBC experiments revealed connectivity between the pyrophosphate monoester signal (δp 11.6 ppm) and H-4 (δ 4.61 ppm) of residue B, suggesting a P-Etn substitution. Residue C, with a JH1,C1 of ~171 Hz, was assigned as 3,7-disubstituted 1-glycero-D-manno-HeppP-Etn based on chemical shifts reported previously (38), small vicinal couplings of H-1 and H-2, and relatively high chemical shifts of C-3 (δ 79.8 ppm) and C-7 (δ 68.4 ppm) signals. 1H, 31P HMBC
spectra showed connectivity between P (δ 0.8 ppm) and H-4 (δ 4.40 ppm) of residue C. Residue D, with a JH1,C1 of ~172 Hz, was assigned as terminal α-manno-hepp based on 1H,13C chemical shift values and small vicinal couplings between H-1, H-2, and the C-6 signal at δ 69.5, as previously shown for the monosaccharide α-manno-hepp (38). Residue E was recognized as 3-substituted α-D-Glcp based on the relatively large chemical shift of C-3 signal (δ 76.7 ppm) and chemical shifts similar to those reported previously (38). Residue F, with a JH1,C1 of ~175 Hz, was assigned as 2,3-disubstituted α-D-GlcP based on relatively high chemical shifts of C-2 (δ 73.3 ppm) and C-3 (δ 78.7 ppm) signals. Residue F’ was assigned as a residue F variant present in unsubstituted core OS (Table 2). Residue G, with a JH1,C1 of ~172 Hz, was identified as 2-substituted α-D-Galp based on the relatively large chemical shift in C-2 (δ 73.2 ppm). Residue H, with a JH1,C1 of ~178 Hz, was assigned as terminal α-D-Galp based on the large couplings between H-1, H-2, and H-3; small vicinal couplings among H-3, H-4, and H-5; and chemical shifts similar to those reported previously (38). Residue I was recognized as 3-substituted β-D-GlcP based on...
TABLE 2

| Residue | Description | H-1/C-1 | H-2/C-2 | H-3/[(H2ax,eq)/C-3] | H-4/C-4 | H-5/C-5 | H-6a, H-6b/C-6 | H-7a, H-7b/C-7 (NHAc) | H-8a, H-8b/C-8 [C(O)] |
|---------|-------------|---------|---------|---------------------|---------|---------|----------------|------------------------|----------------------|
| A       | =5)-a-Kdo   | NDa     | 196.3   | (1.29, 2.23)/34.1   | 4.11/66.3 | 4.17/73.3 | 3.69/69.7      | 3.80/72.6               | 3.47, 3.93/64.7      |
| B       | =3)-a-a-Hepp4PPEtn-(1→ | 5.20/100.1 | 4.01/71.6 | 4.08/78.5          | 4.61/72.3 | 4.22/72.0 | 4.10/69.3      | 3.72, 3.72/63.8         |                     |
| C       | =3,7)-a-a-Hepp4P4-1→ | 5.10/103.5 | 3.48/70.6 | 4.12/79.8          | 4.40/69.4 | 3.83/70.2 | 4.23/68.5      | 3.58, 3.75/68.4         |                     |
| D       | a-a-Hepp-(1→ | 5.20/100.2 | 3.93/70.7 | 3.87/71.4          | 3.84/66.9 | 3.61/71.9 | 4.04/69.5      | 3.65, 3.72/63.7         |                     |
| E       | =3)-a-a-GlcP-(1→ | 5.20/102.0 | 3.66/71.0 | 4.07/76.7          | 3.77/71.2 | 3.91/73.1 | 3.78, 3.92/60.5 |                     |                     |
| F       | =2,3)-a-a-GlcP-(1→ | 5.80/95.3 | 3.87/73.3 | 4.17/78.2          | 3.56/68.7 | 4.10/71.9 | 3.78, 3.95/61.0 |                     |                     |
| F'      | =2,3)-a-a-GlcP-(1→ | 5.81/95.1 | 3.88/73.3 | 4.19/78.8          | 3.57/68.7 | 4.11/72.0 | 3.78, 3.95/61.0 |                     |                     |
| G       | a-a-Gal-(1→ | 5.61/92.1 | 3.98/73.2 | 4.19/68.9          | 3.98/70.7 | 4.13/72.0 | 3.74, 3.74/61.9 |                     |                     |
| H       | a-a-Gal-(1→ | 5.31/96.6 | 3.85/69.0 | 3.95/70.1          | 3.99/70.1 | 4.13/72.0 | 3.75, 3.75/61.9 |                     |                     |
| I       | =3)-b-b-GlcP-(1→ | 4.73/103.3 | 3.39/73.6 | 3.68/85.8          | 3.49/68.9 | 3.44/76.3 | 3.73, 3.89/61.4 |                     |                     |
| I'      | b-b-GlcP-(1→ | 4.75/103.1 | 3.33/73.9 | 3.51/76.6          | 3.40/70.4 | 3.45/76.6 | 3.73, 3.91/61.4 |                     |                     |
| J       | =4)-a-a-GlcNAC-(1→ | 4.78/102.3 | 3.75/56.3 | 3.74/72.7          | 3.68/79.5 | 3.54/75.2 | 3.86, 3.70/60.9 |                     |                     |
| K       | =4)-b-b-ManNAC-(1→ | 4.93/99.7 | 4.49/54.2 | 4.07/73.2          | 3.82/74.8 | 3.86/77.2 | -1.175.1       |                     |                     |
| L       | a-a-FucPNAC-(1→ | 5.35/99.5 | 3.64/69.3 | 4.00/69.1          | 4.20/54.6 | 4.18/66.5 | 1.06/16.2       |                     |                     |

* ND, not determined.
* Residue F’ is a variant of residue F present in the core OS that is devoid of ECA trisaccharide.
* Residue I’ is a terminal residue I present in the core OS that is devoid of ECA trisaccharide.

TABLE 3

Selected inter-residue NOE and 3JH,H connectivities from the anomeric atoms of [ECA]-dLOS dodecasaccharide isolated from S. sonnei phase II LOS

Data indicating the covalent linkage between ECA and LOS are shown in bold.

| Residue | Description | Atom δH,C | δC | δH | Inter-residue atom/residue |
|---------|-------------|-----------|----|----|---------------------------|
| B       | =3)-a-a-Hepp4PPEtn-(1→ | 5.20/100.1 | 78.5 | 4.179 | H-5 of A                  |
| C       | =3,7)-a-a-Hepp4P4-1→ | 5.10/103.5 | 78.5 | 4.089 | C-3, H-3 of B              |
| D       | a-a-Hepp-(1→ | 4.98/100.2 | 68.5 | 3.59/3.74 | C-7, H-7a, H-7b of C        |
| E       | =3)-a-a-GlcP-(1→ | 5.20/102.0 | 68.5 | 4.120 | H-3 of C                  |
| F       | =2,3)-a-a-GlcP-(1→ | 5.80/95.3 | 4.070 | 4.070 | H-3 of E                  |
| F'      | =2,3)-a-a-GlcP-(1→ | 5.81/95.1 | 4.070 | 4.070 | H-3 of E                  |
| G       | =2)-a-a-Gal-(1→ | 5.61/92.1 | 3.870 | 3.970 | H-2 of F                  |
| H       | a-a-Gal-(1→ | 5.31/96.6 | 78.7 | 4.170 | H-2 of F                  |
| I       | =3)-b-b-GlcP-(1→ | 4.73/103.1 | 78.8 | 4.190 | H-3 of F'                 |
| I'      | b-b-GlcP-(1→ | 4.75/103.1 | 85.3 | 3.680 | H-3 of I                  |
| J       | =4)-a-a-GlcNAC-(1→ | 4.78/102.3 | 79.4 | 3.699 | H-4 of J                  |
| K       | =4)-b-b-ManNAC-(1→ | 4.93/99.7 | 74.7 | 3.810 | H-4 of K                  |
| L       | a-a-FucPNAC-(1→ | 5.35/99.5 | 74.7 | 3.810 | H-4 of K                  |

* Value represents NOE connectivities only.
* Residue F’ is a variant of residue F present in the core OS that is devoid of ECA trisaccharide.

FIGURE 5. A selected portion of the NOESY spectrum containing the anomeric protons region of [ECA]-dLOS dodecasaccharide isolated from S. sonnei phase II LOS. The cross-peaks are labeled as explained in the legend for Fig. 2. The signal indicating the linkage between ECA and dLOS is shown in bold.

1H,13C chemical shifts and the very high chemical shift of its C-3 signal (δ 85.4 ppm) as compared with that of residue I’ (δ 76.6 ppm), identified as terminal β-D-GlcP, which was present as an unsubstituted variant of residue I in the core OS devoid of ECA. This latter finding affirms results obtained by ESI-MS analysis (above), which detected a small amount of unsubstituted core oligosaccharides (dLOS). Residue J was assigned as 4-substituted α-D-GlcNAc based on its characteristic C-2 signal (δ 56.3 ppm) and the downfield chemical shift of its C-4 signal (δ 79.5 ppm). Residue K was assigned as 4-substituted β-D-ManNAcA based on its characteristic C-2 (δ 54.2 ppm) and C-6 (δ 175.1 ppm) signals and the relatively high chemical shift of C-4 (δ 74.8 ppm). Residue L was recognized as terminal α-D-FucNAc based on the characteristic exocyclic methyl group signals (δ 1.06/16.2 ppm) and chemical shift of the C-4 signal (δ 54.6 ppm).

Each disaccharide element was identified by HMBC (Table 3, Fig. 4) and NOESY experiments (Table 3, Fig. 5), providing a complete sequence of dodecasaccharide [ECA]-dLOS (Fig. 3, inset structure). The HMBC spectra showed cross-peak signals between the anomeric proton and the carbon at the linkage position and between the anomeric carbon and proton at the linkage position (Fig. 4). Thus, the combined results suggest a dodecasaccharide structure of S. sonnei phase II [ECA]-dLOS containing one repeating unit of ECA linked to the LOS via an α(1→3) linkage between →4)-α-D-GlcNAc-(1→ of ECA (res-
Identification of the Covalent Linkage between ECA and LPS

...idue I) and \(\rightarrow 3\)-\(\beta\)-D-Glcp (residue I) of the outer core OS (Fig. 3, inset structure). Additionally, biological repeating units of ECA with \(\rightarrow 4\)-\(\alpha\)-D-GlcNAc(1→, as a first residue, and glycoforms with two, three, and four repeating units together with some O-acetyl group modifications, were also identified.

DISCUSSION

Lipopolysaccharides, the main surface antigens of Gram-negative bacteria, are virulence factors belonging to pathogen-associated molecular patterns recognized by the innate immune system that are involved in the development of sepsis and septic shock. The structures of lipopolysaccharides are able to undergo modifications to evade bactericidal factors of innate and adaptive immunity pathways, yet retain some common epitopes. Among these common epitopes are inner core motifs built of Hep and Kdo, which are considered to be potential candidates for therapeutic strategies against infections caused by enteric bacteria (39). In the case of Enterobacteriaceae, it is thought that a small part of LPS is decorated with ECA, a population referred to as ECA\(_{LPS}\). ECA is shared among all species of Enterobacteriaceae and may be considered a potential target for the design of cross-reactive and bactericidal antibodies. Because of its coexistence with LPS, ECALPS is the only form of Enterobacteriaceae and may be considered a potential target for therapeutic strategies against infections caused by enteric bacteria (39).

In this study, the authors used high-resolution SDS-PAGE to separate LPS and LOS preparations for subsequent immunoblotting with the above described detection antibodies and concluded that both low molecular mass (one to three repeating units) and high molecular mass forms of ECA\(_{LPS}\) were present. However, further structural analyses are necessary to confirm serological results obtained for \(Y.\) enterocolitica.

It should be emphasized that there are obvious inherent difficulties in the structural analysis of ECA\(_{LPS}\), including the very low amounts of such glycoforms and their contamination with other forms of ECA (i.e., ECA\(_{PG}\) and ECA\(_{APG}\)). Cross-reactivity of absorbed anti-ECA sera provided preliminary evidence for the presence of low molecular mass forms of ECA (ECA\(_{PS}\)) in \(Y.\) enterocolitica O:3 ECA\(_{LPS}\). This finding was supported by more recent, extensive serological studies on \(Y.\) enterocolitica smooth and rough mutants (21). In this latter study, the authors used high-resolution SDS-PAGE to separate LPS and LOS preparations for subsequent immunoblotting with the above described detection antibodies and concluded that both low molecular mass (one to three repeating units) and high molecular mass forms of ECA\(_{LPS}\) were present. However, further structural analyses are necessary to confirm serological results obtained for \(Y.\) enterocolitica.

Moreover, we are going to use the delipidated form of this antigen (ECA\(_{LPS}\)-dLOS) to prepare a neoglycoconjugate with tetanus toxoid to elicit antienterobacterial antibodies broadly cross-reactive with all forms of ECA and core OS epitopes. We hope to find them to be a protective and bactericidal solution against nosocomial infections of Gram-negative bacteria such as \(Klebsiella, E.\) coli, \(Enterobacter, Proteus\) spp., or \(Serratia\) spp. Because a small part of the identified antigens were O-acetylated, we are going to remove all O-acetyl groups from the ECA\(_{LPS}\)-dLOS preparation to produce a glycoconjugate vaccine that elicits the production of antibodies aimed at epitopes present in enterobacterial isolates with and without O-acetyl groups in their ECA, thus generating a wider ranging response and higher serum bactericidal antibody titers. It is known that O-acetyl groups influence antigenicity and immunogenicity of poly- and oligosaccharide antigens (30, 42–46).

REFERENCES

1. Mäkelä, P. H., and Mayer, H. (1976) Enterobacterial common antigen. \(Bacteriol. Rev.\) 40, 591–632
Identification of the Covalent Linkage between ECA and LPS

2. Dell, A., Oates, J., Lugowski, C., Romanowska, E., Kenne, L., and Lindberg, B. (1984) The enterobacterial common-antigen, a cyclic polysaccharide. Carbohydr. Res. 133, 95–104.

3. Lugowski, C., Romanowska, E., Kenne, L., and Lindberg, B. (1983) Identification of a trisaccharide repeating-unit in the enterobacterial common-antigen. Carbohydr. Res. 118, 173–181.

4. Männel, D., and Mayer, H. (1978) Isolation and chemical characterization of the enterobacterial common antigen. Eur. J. Biochem. 86, 361–370.

5. Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K., and Ohta, M. (2002) Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing Escherichia coli O157:H7. Mol. Microbiol. 43, 629–640.

6. Domon, B., and Costello, C. E. (1988) A systematic nomenclature for carbohydrate fragmentations in FABMS/MS of glycoconjugates. Carbohydr. Res. 173, 215–218.

7. Radziejewska-Lebrecht, J., Skurnik, M., Shashkov, A. S., Brade, L., Rózálski, A., Bartodziejka, B., and Mayer, H. (1998) Immunochemical studies on R mutants of Yersinia enterocolitica O:3. Acta Biochim. Pol. 45, 1011–1019.

8. Lugowski, C., J. Bacteriol. 185, 5328–5332.

9. Kuhn, H. M., Meier-Dieter, U., and Mayer, H. (1988) ECA, the enterobacterial common antigen. FEMS Microbiol. Rev. 4, 195–222.

10. Kuhn, H. M., Neter, E., and Mayer, H. (1983) Modification of the lipid moiety of the enterobacterial common antigen by the "Pseudomonas factor". Infect. Immun. 40, 696–700.

11. Rick, P. D., Hubbard, G. L., Kitaoka, M., Nagaki, H., Kinoshita, T., Dowd, S., Simpleanu, V., and Ho, C. (1998) Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of Salmonella typhimurium defective in ECA synthesis. Glyobiology 8, 557–567.

12. Rick, P. D., Mayer, H., Neumeyer, B. A., Wolski, S., and Bitter-Suermann, D. (1985) Biosynthesis of enterobacterial common antigen. J. Bacteriol. 162, 494–503.

13. Kunin, C. M., and Beard, M. V. (1963) Serological studies of O antigens of Escherichia coli by means of the hemagglutination test. J. Bacteriol. 85, 541–548.

14. Ceroni, A., Maass, K., Geyer, H., Geyer, R., Dell, A., and Haslam, S. M. (2008) GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. J. Proteome Res. 7, 1650–1659.

15. Goddard, T. D., and Kneller, D. G. (2001) SPARKY version 3.5.13. SPARKY, San Francisco, California.

16. Erbel, P. J., Barr, K., Gao, N., Gerwig, G. J., Rick, P. D., and Gardner, K. H. (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in Escherichia coli. J. Bacteriol. 185, 1995–2004.

17. Lehrer, I., Vigeant, K. A., Tatar, L. D., and Valvano, M. A. (2007) Functional characterization and membrane topology of Escherichia coli WecA, a sugar-phosphate transferase initiating the biosynthesis of enterobacterial common antigen and O-antigen lipopolysaccharide. J. Bacteriol. 189, 2618–2628.

18. Mayer, H., Schmidt, G., Whang, H. Y., and Neter, E. (1972) Biochemical basis of the immunogenicity of the common enterobacterial antigen. Infect. Immun. 6, 540–544.

19. Muszyszyński, A., Rabszyn, K., Knapka, K., Duda, K. A., Duda-Grychtoł, K. T., Kasperkiewicz, K., Radziejewska-Lebrecht, J., Holst, O., and Skurnik, M. (2013) Enterobacterial common antigen and O-specific polysaccharide coexist in the lipopolysaccharide of Yersinia enterocolitica serotype O:3. Microbiology 159, 1782–1993.

20. Lugowski, C., J. Biol. Chem. 265, 13490–13497.

21. Vaguine, A. V., Knirel, Y. A., Thomas-Oates, J. E., Shashkov, A. S., and L’vov, V. L. (1994) The structure of the cyclic enterobacterial common antigen (ECA) from Yersinia pestis. Carbohydr. Res. 258, 223–232.

22. Lugowski, C., and Romanowska, E. (1991) Characterization of an enterobacterial common antigen (ECA) epitope recognized by anti-ECA-tetanus toxoid conjugate serum. FEMS Microbiol. Lett. 61, 315–318.
Identification of the Covalent Linkage between ECA and LPS

43. Richmond, P., Borrow, R., Findlow, J., Martin, S., Thornton, C., Cartwright, K., and Miller, E. (2001) Evaluation of de-O-acetylated meningococcal C polysaccharide-tetanus toxoid conjugate vaccine in infancy: reactogenicity, immunogenicity, immunologic priming, and bactericidal activity against \( O \)-acylated and de-\( O \)-acylated serogroup C strains. *Infect. Immun.* 69, 2378–2382

44. Richmond, P., Borrow, R., Goldblatt, D., Findlow, J., Martin, S., Morris, R., Cartwright, K., and Miller, E. (2001) Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. *J. Infect. Dis.* 183, 160–163

45. Richmond, P., Goldblatt, D., Fusco, P. C., Fusco, J. D., Heron, I., Clark, S., Borrow, R., and Michon, F. (1999) Safety and immunogenicity of a new *Neisseria meningitidis* serogroup C-tetanus toxoid conjugate vaccine in healthy adults. *Vaccine* 18, 641–646

46. Snape, M. D., and Pollard, A. J. (2005) Meningococcal polysaccharide-protein conjugate vaccines. *Lancet Infect. Dis.* 5, 21–30