Serological characterization of the enterobacterial common antigen substitution of the lipopolysaccharide of *Yersinia enterocolitica O:3*

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Enterobacterial common antigen (ECA) is a polysaccharide present in all members of Enterobacteriaceae anchored either via phosphatidylglycerol (PG) or LPS to the outer leaflet of the outer membrane (ECA\textsubscript{PG} and ECA\textsubscript{LPS}, respectively). Only the latter form is ECA-immunogenic. We previously demonstrated that *Yersinia enterocolitica O:3* and its rough (O-specific polysaccharide-negative) mutants were ECA-immunogenic, suggesting that they contained ECA\textsubscript{LPS}; however, it was not known which part of the LPS core region was involved in ECA binding. To address this, we used a set of three deep-rough LPS mutants for rabbit immunization. The polyvalent antisera obtained were: (i) analysed for the presence of anti-LPS and anti-ECA antibodies; (ii) treated with caprylic acid (CA) to precipitate IgM antibodies and protein aggregates; and (iii) adsorbed with live ECA-negative bacteria to obtain specific anti-ECA antisera. We demonstrated the presence of antibodies specific for both ECA\textsubscript{PG} and ECA\textsubscript{LPS} in all antisera obtained. Both CA treatment and adsorption with ECA-negative bacteria efficiently removed anti-LPS antibodies, resulting in specific anti-ECA sera. The LPS of the ECA\textsubscript{LPS}-positive deepest-rough mutant contained only lipid A and 3-deoxy-D-manno-oct-2ulosonic acid (Kdo) residues of the inner core, suggesting that ECA\textsubscript{LPS} was linked to the Kdo region of LPS in *Y. enterocolitica O:3*.

INTRODUCTION

LPS is an important component of most Gram-negative bacterial cell envelopes and can be involved in a broad range of physiological and pathological activities associated with the human host immune system (Meredith et al., 2006). In wild-type strains the smooth (S) form of LPS molecule consists of lipid A (LA) to which is covalently linked a heteropolysaccharide chain made up of a core oligosaccharide (OS) and an O-specific polysaccharide (OPS). Rough-type bacteria (R) produce LPS lacking OPS (Alexander & Rietschel, 2001; Chung & Raetz, 2010). In *Salmonella enterica* serovar Minnesota, inactivation of

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**Abbreviations:** CA, caprylic acid; DOC-PAGE, deoxycholate PAGE; ECA, enterobacterial common antigen; ECA\textsubscript{CYC}, cyclic enterobacterial common antigen; ECA\textsubscript{LPS}, LPS-bound enterobacterial common antigen; ECA\textsubscript{PG}, phosphatidylglycerol-bound enterobacterial common antigen; ESI, electrospray ionization; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; LA, lipid A; M164, strain YeO3-c-R1-M164; M196, strain YeO3-c-R1-M196; M205, strain YeO3-c-R1-M205; OPS, O-specific polysaccharide; OS, core oligosaccharide; PCP, phenol/chloroform–light petroleum; PG, phosphatidylglycerol; WB, Western blotting; WCL, whole-cell lysate; YeO3, *Yersinia enterocolitica* O:3.
different core biosynthetic genes resulted in gradually truncated core structures known as the Ra, Rb, Rc, Rd and Re chemotypes. Ra mutants synthesize full-core OS, while Rb to Rd mutants contain cores with a decreasing number of monosaccharide residues, down to Re mutants which contain only two 3-deoxy-d-manno-oct-2ulosonic acid (Kdo) residues and LA in their LPS. Other members of Enterobacteriaceae form similar but not identical rough core structures (Seltmann & Holst, 2001; Focà et al., 2012).

Bacilli from the genus Yersinia were earlier classified within the genus Pasteurella (Pasteurellaceae). The identification of enterobacterial common antigen (ECA) in their cell envelope resulted in their reclassification to Enterobacteriaceae (Kuhn et al., 1988). Yersinia enterocolitica O:3 (YeO3) is a rod-shaped, Gram-negative, non-sporulating, facultatively anaerobic and oxidase-negative human pathogen (Bottone, 1999; Fredriksson-Ahomaa & Korkeala, 2003). It causes yersiniosis, which is usually a mild diarrhoeal disease. Yersinia are able to grow over a wide range of temperatures (0–42 °C) which may result in life-threatening problems, for example when YeO3-contaminated blood was stored at 4 °C was later infused to patients (Goverde et al., 1998; Skurnik et al., 1999). The LPS of YeO3 has some unique features, which were thoroughly analysed at the compositional (Hoffman et al., 1980; Wartenberg et al., 1983), structural (Radziejewska-Lebrecht et al., 1994, 1998; Shashkov et al., 1995; Pinta et al., 2009) and genetic levels (Skurnik et al., 1995, 1999). A single LPS molecule is substituted at heptose II of the core octasaccharide either by a homopolymeric OPS or a so-called outer core hexasaccharide (Pinta et al., 2012) which actually represents one remaining repeat of an ancient but no longer expressed OPS. Another extraordinary feature of YeO3 LPS is that the core serves as acceptor for ECA (Radziejewska-Lebrecht et al., 1998).

It was also shown that OPS and ECA coexist in the same LPS molecules from YeO3 wild-type strains possessing either full or truncated or no outer core (Muszynski et al., 2013). The ECA polysaccharide structure consists of linear trisaccharide repeating units composed of \( [\text{a}-\text{D-Fucp} \text{NpNAC-(1→4)-}\beta\text{-D-ManpNAC-(1→4)-}\text{a}-\text{D-GlcpNAC-(1→4)}] \). ECA can either be linked to phosphatidylglycerol (ECAPG) or to LPS (ECA_{LPS}), or is present as a cyclical molecule without a lipid anchor (ECA_{CYC}) that is not surface-expressed (Kuhn et al., 1988; Castelli & Vescovi, 2011; Gilbreath et al., 2012). Recently, the covalent linkage between ECA and LPS in Shigella sonnei phase II ECA_{LPS} was characterized (Godzdiewicz et al., 2014). Interestingly, in certain Ra type E. coli strains only ECA_{LPS}, but neither ECAPG nor ECA_{CYC} were ECA-immunogenic (Kuhn et al., 1988). By contrast, YeO3 Rc mutants were shown to be ECA-immunogenic, suggesting that ECA was linked to the core region (Radziejewska-Lebrecht et al., 1998, 2003; Kasperkiewicz, 2002; Duda, 2007b).

To elucidate whether the complete core was needed to express ECA_{LPS} in YeO3, we used different deep-rough mutants with successively truncated core structures. Rabbit antisera raised against these mutants were shown to possess anti-ECA antibodies, indicating that ECA was linked to the Kdo region of YeO3 LPS.

**METHODS**

**Bacterial strains.** The bacterial strains used in this study are described in Table 1. Isolation of the deep-rough LPS mutants used in this work from the Cat-Mu transposon insertion library constructed to Y. enterocolitica strain YeO3-c-R1 was described by Pinta et al. (2012).

**Recombinant DNA methods.** The galU, waaF and hldE genes of YeO3 were amplified by PCR and cloned into plasmid pTM100 (Michiels & Cornelis, 1991). The plasmids obtained were mobilized into the respective mutants and the LPS profiles of the correct transconjugants were analysed by deoxycholate PAGE (DOC-PAGE) analysis and silver staining as described by Skurnik et al. (1999).

**LPS and ECA preparations (Table 2).** LPS_{PCP} preparations of the deep-rough mutants were prepared as described by Muszynski et al. (2013) with minor modifications. Briefly, crude LPS obtained from hot phenol/water extractions was purified by digestion with DNase and RNase at 37 °C for 10 h, followed by proteinase K treatment for 16 h at 56 °C. After ultracentrifugation (180 000 g, 4 °C, 20 h) and subsequent phenol/chloroform-light petroleum (PCP) extraction, pure LPS_{PCP} samples were obtained which were devoid of ECA_{PCP} (Galanos et al., 1969). LPS_{PCP} from Ra mutant YeO3-c-R1 and ECA_{PCP} from S. enterica serovar Montevideo SH94 were used as controls.

**Analytical methods.** Chemical composition analyses of LPS_{PCP} included Kdo and glucosamine determination (Brade et al., 1983; Kaca et al., 1988), and neutral sugar (Duda et al., 2011) and fatty acid (Wollenweber & Rietschel, 1990) analyses. GC analysis of alditol acetates was performed utilizing an HP 5890 (series II) gas chromatograph with a flame-ionization detector and a column (30 m × 2.5 mm × 0.25 μm; Agilent) of polysilicic SPD-5. Helium was used as carrier gas (70 kPa). The temperature programme in GLC was 3 min/150 °C/3 °C min⁻¹/320 °C. Data were integrated and stored using ChemStation software. GC analysis of methyl esters of fatty acids was performed in an HP 6890N gas chromatograph with a flame-ionization detector and a column (30 m × 0.32 mm × 0.25 μm; Agilent) of phenyl methyl siloxane HP-5. Helium was used as carrier gas (70 kPa). Separations were recorded using ChemStation software. The temperature programme in GC was 3 min/120 °C/5 °C/3 °C min⁻¹/320 °C/10 min⁻¹.

**Mass spectrometry.** Electrospray Fourier-transformed ion cyclotron resonance MS was performed in the negative ion mode using an APEX Qe-Instrument (Bruker Daltonics) equipped with a 7 T magnet and a dual Apollo ion source. Mass spectra were acquired in broad band modes. The samples (~ 10 ng μl⁻¹) were dissolved in a 50:50:0.001 (by vol.) mixture of 2-propanol, water and triethylamine. The samples were sprayed at a flow rate of 2 μl min⁻¹. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 150 °C. Mass spectra were calibrated externally by lipids with known structure and charged-deconvoluted and the given mass numbers were referred to the mono-isotopic masses of neutral molecules.

**Antibodies.** Diluted (1:2000) horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (P0448; Dako) and alkaline phosphatase-conjugated goat anti-rabbit antibodies (D0487; Dako)
were used as secondary antibodies in ELISA and Western blotting (WB), respectively.

Whole-cell lysates (WCLs). WCLs of bacteria were prepared as described by Rabsztyn et al. (2011).

Polyvalent antisera. New Zealand white rabbits with no detectable antibodies against ECA or LPS were immunized with boiled YeO3 deep-rough mutant bacteria. Briefly, the strains were cultivated in triplicate soy agar (TSA) medium at 22 and 37 °C, washed with sterile 0.85 % NaCl and collected by centrifugation (20 min, 1800 g), and the bacterial masses obtained were suspended in sterile 0.85 % NaCl, boiled at 100 °C, and the bacterial masses were obtained by centrifugation (10 000 g, 15 min, 4 °C). The supernatant was washed twice with PBS and dialysed against PBS for 18 h. The buffer was changed and dialysis was continued for 4 h. After centrifugation (10 000 g, 15 min, 4 °C), supernatant was collected and stored at −20 °C.

Adsorption of antisera. The polyvalent rabbit antisera were extensively adsorbed with live YeO3 ECA-negative strain YeO3-c-OCR-ECA (Table 1) as described by Rabsztyn et al. (2011).

ELISA. The anti-ECA and anti-LPS antibodies were measured using the ELISA protocol described by Swierzko et al. (1994), with minor modifications. Briefly, NUNC MaxiSorp 96-microtitre plates were coated with S. Montevideo SH94 ECA_PG (1 μg per well in 50 μl PBS) or with the YeO3 LPS_PCP preparations (5 μg per well in 50 μl PBS) and incubated at 4 °C for 18 h. The wells were then rinsed twice with Tris-buffered saline (TBS)+ TWEEN 20 (10 mM Tris, 120 mM NaCl, 0.05 % TWEEN 20, pH 7.4) containing 1 mM CaCl2 (TBS-Ca2+) and incubated for 1 h at 37 °C with 200 μl 0.1 % BSA in TBS-Ca2+. After washing as above, rabbit antisera, serially diluted in 0.1 % BSA, was applied to the wells, each in triplicate. After 1 h of incubation at 4 °C, the wells were rinsed and the bound antibodies were revealed using the anti-rabbit IgG peroxidase conjugate and the TMB solution.

Table 1. YeO3 strains used in this work, originating from strain 6471/76 (Skurnik, 1984)

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>YeO3-c-R1</td>
<td>Spontaneous rough (OPS-negative) derivative of serotype O:3 strain 6471/76-c; Yersinia virulence plasmid-cured</td>
<td>al-Hendy et al. (1992)</td>
</tr>
<tr>
<td>YeO3-c-R1-M196</td>
<td>galU::Cat-Mu. Deep-rough (Rd1 chemotype) Cat-Mu transposon insertion derivative of YeO3-c-R1, ClmR</td>
<td>This work</td>
</tr>
<tr>
<td>YeO3-c-R1-M164</td>
<td>waaF::Cat-Mu. Deep-rough (Rd2 chemotype), transposon insertion derivative of YeO3-c-R1, ClmR</td>
<td>This work</td>
</tr>
<tr>
<td>YeO3-c-R1-M205</td>
<td>hldE::Cat-Mu. Deep-rough (Re chemotype) transposon insertion derivative of YeO3-c-R1, ClmR</td>
<td>This work</td>
</tr>
<tr>
<td>YeO3-c-OCR-ECA</td>
<td>Δ(wzx-wzyE) Δ(wzzE–wzyE). OPS-, outer core- and ECA-negative derivative of 6471/76-c, KmR</td>
<td>Rabsztyn et al. (2011)</td>
</tr>
</tbody>
</table>

Table 2. LPS and ECA_PG control preparations used in this work

<table>
<thead>
<tr>
<th>Preparation/strain</th>
<th>Description</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>LPS_PCP YeO3-c-R1</td>
<td>LPS_PCP containing ECA_LPS from Ra mutant YeO3-c-R1 cultivated at 22 or 37 °C, free of ECA_PG</td>
<td>DM* (Duda, 2007a)</td>
</tr>
<tr>
<td>LPS_PCP YeO3-c-RhR7</td>
<td>LPS_PCP containing ECA_LPS from Rc mutant YeO3-c-RhR7 cultivated at 22 or 37 °C, free of ECA_PG</td>
<td>DM* (Skurnik et al., 1995)</td>
</tr>
<tr>
<td>LPS_PCP YeO3-c-R1-M196</td>
<td>LPS_PCP containing ECA_LPS from Rd1 mutant YeO3-c-R1-M196 cultivated at 22, 25 or 37 °C, free of ECA_PG</td>
<td>This work</td>
</tr>
<tr>
<td>LPS_PCP YeO3-c-R1-M164</td>
<td>LPS_PCP containing ECA_LPS from Rd2 mutant YeO3-c-R1-M164 cultivated at 22, 25 or 37 °C, free of ECA_PG</td>
<td>This work</td>
</tr>
<tr>
<td>LPS_PCP YeO3-c-R1-M205</td>
<td>LPS_PCP containing ECA_LPS from Re mutant YeO3-c-R1-M205 cultivated at 22, 25 or 37 °C, free of ECA_PG</td>
<td>This work</td>
</tr>
<tr>
<td>ECA_PG S. Montevideo SH94</td>
<td>ECA_PG was obtained from S. Montevideo SH94 cultivated at 37 °C by Ph/W and PCP extractions</td>
<td>DM* (Männel &amp; Mayer, 1978)</td>
</tr>
</tbody>
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Fig. 1. Characterization of the LPS of YeO3 mutants. (a) Silver-stained DOC-PAGE profiles of LPS_{PCP} (0.25 μg) and WCL (2 μl) preparations of YeO3 mutants. Lane 1, WCL of M164; lane 2, WCL of M205; lane 3, WCL of M196; lane 4, LPS_{PCP} of YeO3-c-R1; lane 5, WCL of YeO3-c-OCR-ECA; lane 6, LPS_{PCP} of M196; lane 7, LPS_{PCP} of M164; lane 8, LPS_{PCP} of M205. LA, lipid A; IC, inner core; OC, outer core. (b) Structures of LPS from deep-rough mutants: Rd1, M196; Rd2, M164; Re, M205, and LPS_{PCP} of their parental strain YeO3-c-R1, mutant Ra (Pinta et al., 2012). The dotted line indicates the non-stoichiometric substitution by ECA of one of two Kdo residues in the shaded rectangle. L-D-Hepp, L-glycero-D-manno-heptopyranose; D-D-Hepp, D-glycero-D-manno-heptopyranose; Kdo, 3-deoxy-D-manno-oct-2ulosonic acid; D-GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; D-Glc, D-glucopyranose; D-GalpNAc, 2-acetamido-2-deoxy-D-galactopyranose; D-Gal, α-D-galactopyranose;
37 °C, wells were washed three times with TBS-CaCl$_2$ buffer and incubated for 1 h at 37 °C with horseradish peroxidase-conjugated goat anti-rabbit antibodies, diluted 1:2000 in 0.1% BSA. The wells were then rinsed as above and the bound secondary antibodies were detected with 2,2’-azino-di(3-ethylbenzthiazolinsulfonate) (Sigma) as a substrate for peroxidase. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2% oxalic acid. The plate was read by using a Benchmark microplate reader (Bio-Rad) at 405 nm. The last antibody dilution showing extinction of $A_{405}$=0.2 was taken as a titre. The experiments were repeated three times.

**SDS-PAGE and WB.** ECA$_{PCP}$ of S. Montevideo SH94 and LPS$_{PCP}$ of YeO3 strains were separated by SDS-PAGE (Mini-Protein; Bio-Rad) (Laemmli, 1970). The gels were transferred to PVDF membrane (Biotrans blotting) and the membrane was processed for WB as previously described (Towbin et al., 1979; Radziejewska-Lebrecht et al., 2003).

**RESULTS AND DISCUSSION**

**Characteristics of YeO3 deep-rough Cat-Mu mutants**

The mutant strains YeO3-c-R1-M196, YeO3-c-R1-M164 and YeO3-c-R1-M205 (hereafter M196, M164 and M205, respectively) were isolated from the Cat-Mu transposon insertion library based on the lack of an outer core (Pinta et al., 2012). Identification of the Cat-Mu transposon insertion sites of the mutants revealed that certain genes involved in core biosynthesis had been inactivated. M196 carried the Cat-Mu insertion in the galU gene, M164 in the waaF gene and M205 in the hldE gene. GalU is glucose-1-P uridylytransferase, also called UDP-glucose pyrophosphorylase, involved in biosynthesis of UDP-glucose. WaaF is heptosyltransferase II (Gronow et al., 2000) and HldE is a kinase and ADP-transferase involved in the biosynthesis of ADP-glycero-manno-heptopyranose (McArthur et al., 2005). In DOC-PAGE analysis the strains were found to express deep-rough LPS with different degrees of core truncation, as shown by their WCL and LPS$_{PCP}$ profiles (Fig. 1a). The mutants were complemented in trans by the respective wild-type genes from a plasmid (data not shown). While the galU::Cat-Mu and hldE::Cat-Mu mutants were fully complemented, the waaF::Cat-Mu mutant was only partially so, perhaps reflecting the fact that waaF is located upstream of the waaC gene encoding heptosyltransferase I, and thus the mutant might have had some defect in the latter activity.

**Chemical composition of M196, M164 and M205 LPS**

Pure LPS$_{PCP}$ preparations from M196, M164 and M205 were obtained at yields of 0.8, 1 and 0.44%, respectively. Compositional analysis of these preparations identified fatty acids [predominantly 14:0(3-OH), 12:0 and 14:0, with trace 16:0], glucosamine and Kdo in all three mutants. LPS$_{PCP}$ of M164 contained additional 1,3-D-Hep and trace glucose, whereas LPS$_{PCP}$ of M194 contained additional 1-glycerol-3-manno-heptopyranose (1,3-D-Hep) (1.7 times more than LPS$_{PCP}$ of M164) and trace 1,4-glycerol-3-manno-heptopyranose (1,4-D-Hep) and glucose. These results were confirmed by high-resolution electrospray Fourier-transformed ion cyclotron resonance MS of the LPS$_{PCP}$ (Fig. 2), recorded in the negative ion mode. The major molecular mass ion (m/z 2213.0907) on the electrospray ionization (ESI) spectrum of LPS$_{PCP}$ of M196 (Fig. 2a) corresponded to a molecule composed of 2 hexosamine (HexN), 2 phosphate (P) residues, 2 Kdo, 2 heptose (Hep) residues, 3 14:0(3-OH) and 1 14:0 (calculated mass 2213.0991 u). The ion at m/z 2021.0362 of the spectrum of LPS$_{PCP}$ of M164 (Fig. 2b) was consistent with a molecule containing 2 HexN, 2 P, 2 Kdo, 1 Hep, 3 14:0(3-OH) and 1 14:0 (calculated mass 2021.0391 u). LPS$_{PCP}$ of M205 was deprived of Hep and composed only of 2 HexN, 2 P, 2 Kdo and 4 14:0(3-OH) with or without 1 Ara4N, as deduced from the presence of two major molecular mass ions at m/z 1976.0297 and 1844.9727, respectively (calculated masses 1976.0332 and 1844.9740 u, respectively) (Fig. 2c). The mass spectra possessed additional heterogeneity in the fatty acids, Kdo, P and Ara4N substitution patterns. In summary, the compositional and MS analyses of the respective LPS preparations of deep-rough YeO3 mutants confirmed the truncations of their core regions (Fig. 1b).

**Presence of ECA$_{LPS}$ in M196, M164 and M205**

We observed a positive reaction of specific anti-ECA monoclonal antibody 898 (Mab 898) with LPS$_{PCP}$ of all deep-rough mutants in WB analysis (Fig. 1c). As those LPS$_{PCP}$ were deprived of ECA$_{PG}$ we assumed that the reaction was due to the presence of ECA$_{LPS}$, which was confirmed in further studies (see below).

**Detection of anti-ECA- and anti-LPS-specific antibodies in ELISA**

The untreated polyvalent antiserum against M196, M164 and M205 were first checked by ELISA for the presence of
anti-LPS and anti-ECA antibodies. The untreated antisera reacted strongly with the ECAPG control with titres >256 000 (anti-M164), 6400 (anti-M196) and 6400 (anti-M205). The titres of the CA-treated and ECA-specific antisera against ECAPG had dropped significantly and were 800 for all samples. When homologous LPSPCP preparations were used as antigen, the untreated polyvalent antisera showed the same titre, >256 000. The titres of CA-treated and ECA-specific antisera against M196 and M205 were 8000 while that for M164 was 800. In conclusion, the data demonstrated that all antisera contained both anti-LPS and anti-ECA antibodies, suggesting that the deep-rough mutants were not only LPS-but also ECA-immunogenic. CA treatment as well as adsorption with an ECA-negative strain significantly reduced the antibody titres against both antigens.

Detection of anti-ECA- and anti-LPS-specific antibodies in immunoblotting

WB gave more detailed information than ELISA, demonstrating the presence of both anti-LPS and anti-ECA_LPS antibodies in antisera. To allow the identification of antigens migrating in the low (1–5 kDa) and high (10–50 kDa) molecular mass regions of the gel, 2 µg LPSPCP corresponding to 2 µL WCL was applied. The WB experiments were performed repeatedly with different dilutions of antisera, leading to the following representative results.

Antibodies of the untreated polyvalent antisera recognized antigens in the low-molecular mass regions of YeO3-c-R1 LPSPCP and in the 5–10 kDa region of the WCL of YeO3- c-OCR-ECA (Fig. 3a, d, g, lanes 1 and 4). The untreated
anti-M196 antiserum reacted strongly with low-molecular mass molecules of M196 LPS PCP and WCL (Fig. 3a, lanes 2 and 3), demonstrating the presence of anti-core antibodies in the serum. The reaction with high-molecular mass compounds of LPS PCP (Fig. 3a, lane 2) was due to the presence of ECA LPS molecules bound to LPS. Reaction with ECA PG (Fig. 3a, lane 5) indicated the presence of anti-ECA antibodies in the serum. The untreated anti-M164 (Fig. 3d, lanes 2 and 3) and anti-M205 (Fig. 3g, lanes 2 and 3) antisera gave comparable results to those of anti-M196.

The corresponding CA-treated and ECA-specific antisera produced similar but weaker patterns with the LPS PCP and WCL samples (Fig. 3b, c, e, f, h, i, lanes 2 and 3) and reacted mainly with low-molecular mass molecules. Moreover, these antisera had completely lost reactivity with the WCL of YeO3-c-OCR-ECA (Fig. 3b, c, e, f, h, i, lane 4), suggesting that the CA treatment had effectively removed the IgM antibodies that had apparently made up the majority of the anti-LPS, but also some of the anti-ECA antibodies (Fig. 3b, e, h, lanes 1–3). The results also demonstrated that the adsorption with live bacteria was successful as no anti-LPS antibodies were detected in the anti-ECA-specific sera (Fig. 3c, f, i, lane 4), although some anti-ECA PG antibodies were also removed (Fig. 3c, f, i, lane 5). We speculate that the anti-ECA antibodies in adsorbed antisera were responsible for the relatively weaker immunostaining of the LPS PCP and WCL preparations (Fig. 3c, f, i, lanes 2 and 3) than was the corresponding staining of the polyvalent antisera (Fig. 3a, d, g, lanes 2 and 3).

WB using ECA PG as antigen showed that all the polyvalent antisera contained anti-ECA PG antibodies, although the staining did not reveal the typical ladder-like banding profile. Instead, the antibodies recognized a molecule located in the low-molecular mass region and/or one or two sharp bands higher up in the gel (Fig. 3a, d, g, lane 5).

The CA-treated anti-M196 and anti-M164 antisera resulted in one sharp band in the low-molecular mass region (Fig. 3b, e, lane 5), while CA-treated anti-M205 recognized in addition a faster-migrating compound (Fig. 3h, lane 5), indicating that CA extraction led to partial loss of anti-ECA antibodies.

Interestingly, anti-M205 antiserum also reacted with ECA PG (Fig. 3i, lane 5), demonstrating that the Kdo region of LPS is probably an acceptor for ECA. This conclusion is further supported by the presence of ECA LPS in LPS PCP of M205 and by the finding that free LA of YeO3 was not ECA-positive (data not shown).

WB analysis of all the anti-LPS PCP antisera revealed some positive reactions with the adsorbed sera as a smear in the high-molecular mass region. As the bacteria lack OPS, the smear was probably a tailing artefact, especially as some tailing could also be seen in the WCL preparations. The very weak reactivity of the anti-ECA antibodies against ECA PG again demonstrated that the ECA epitope(s) present in ECA LPS are distinct from the ECA epitope(s) in ECA PG, in accordance with the results of Rabsztyn and co-workers (Rabsztyn et al., 2011).

In summary, we clearly demonstrated that anti-M196, anti-M164 and anti-M205 antisera contained anti-ECA antibodies, showing that these deep-rough strains are ECA-immunogenic. The WB results furthermore suggested that the ECA substitution in YeO3 LPS is located in the Kdo region, although structural studies will be required to confirm this.

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