



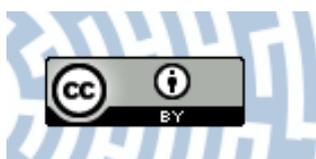
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Title: Serological characterization of the enterobacterial common antigen substitution of the lipopolysaccharide of "Yersinia enterocolitica" O:3

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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_{PG} and ECA_{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_{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_{PG} and ECA_{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_{LPS}-positive deepest-rough mutant contained only lipid A and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues of the inner core, suggesting that ECA_{LPS} was linked to the Kdo region of LPS in *Y. enterocolitica* O : 3.

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Abbreviations: CA, caprylic acid; DOC-PAGE, deoxycholate PAGE; ECA, enterobacterial common antigen; ECA_{CYC}, cyclic enterobacterial common antigen; ECA_{LPS}, LPS-bound enterobacterial common antigen; ECA_{PG}, phosphatidylglycerol-bound enterobacterial common antigen; ESI, electrospray ionization; Kdo, 3-deoxy-D-manno-oct-2-ulopyranosonic 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.

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

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-2-ulosonic 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 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 (Muszyński *et al.*, 2013). The ECA polysaccharide structure consists of linear trisaccharide repeating units composed of [→3)- α -D-Fucp4NAc-(1→4)- β -D-ManpNAc-(1→4)- α -D-GlcpNAc-(1→]. ECA can either be linked to phosphatidylglycerol (ECA_{PG}) 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 & Vécovi, 2011; Gilbreath *et al.*, 2012). Recently, the covalent linkage between ECA and LPS in *Shigella sonnei* phase II ECA_{LPS} was characterized (Goździewicz *et al.*, 2014). Interestingly, in certain Ra type *E. coli* strains only ECA_{LPS}, but neither ECA_{PG} 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 Muszyński *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_{PG} (Galanos *et al.*, 1969). LPS_{PCP} from Ra mutant YeO3-c-R1 and ECA_{PG} 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 μ m; Agilent) of polysilican 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 μ m; 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 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 μ l⁻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 μ l 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)

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

Bacterial strain	Description	Reference
YeO3-c-R1	Spontaneous rough (OPS-negative) derivative of serotype O:3 strain 6471/76-c; <i>Yersinia</i> virulence plasmid-cured	al-Hendy <i>et al.</i> (1992)
YeO3-c-R1-M196	<i>galU::</i> Cat-Mu. Deep-rough (Rd ₁ chemotype) Cat-Mu transposon insertion derivative of YeO3-c-R1, Clm ^R	This work
YeO3-c-R1-M164	<i>waaF::</i> Cat-Mu. Deep-rough (Rd ₂ chemotype), transposon insertion derivative of YeO3-c-R1, Clm ^R	This work
YeO3-c-R1-M205	<i>hldE::</i> Cat-Mu. Deep-rough (Re chemotype) transposon insertion derivative of YeO3-c-R1, Clm ^R	This work
YeO3-c-OCR-ECA	Δ(<i>wzx-wbcQ</i>) Δ(<i>wzzE-wzyE</i>). OPS-, outer core- and ECA-negative derivative of 6471/76-c, Km ^R	Rabsztyń <i>et al.</i> (2011)

were used as secondary antibodies in ELISA and Western blotting (WB), respectively.

Whole-cell lysates (WCLs). WCLs of bacteria were prepared as described by Rabsztyń *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 trypticase soy agar (TSA) medium at 22 and 37 °C, washed with sterile 0.85 % NaCl and collected by centrifugation (20 min, 1800 g, 4 °C), and the bacterial masses obtained were suspended in sterile 0.85 % NaCl, boiled at 100 °C for 2.5 h and centrifuged as above. The pellets were resuspended in sterile water and freeze-dried (Alpha; Martin Christ). Stock suspensions of 5 ml were prepared from the lyophilized bacterial masses (1 mg of dry bacterial mass per 1 ml of sterile 0.85 % NaCl). For immunization, 500 µl doses containing 50, 100, 200 and 500 µg of the lyophilized bacterial masses were prepared in sterile 0.85 % NaCl. Rabbits were immunized according to the '58 days' protocol (Swierzko *et al.*, 1993). The polyvalent antisera (approximately 15 ml per rabbit) obtained 7 days after the last injection were stored at -20 °C.

Caprylic acid (CA) extraction. Extraction of polyvalent antisera using CA was performed as described by Pupek (2009), with minor modifications. One millilitre of the polyvalent antiserum was mixed with 4 ml of 60 mM acetate buffer (pH 4.0) and the pH of the

mixture was adjusted to 4.5 with 1 M Tris. Thereafter, 125 µl CA was slowly added, stirring constantly for 30 min. After centrifugation (13000 g, 30 min, 4 °C), the supernatant was adjusted to pH 7.4 with 1 M Tris and filtered through a 0.22 µm Millipore filter. The sample was cooled to 4 °C and 1.53 g (final concentration approximately 1.9 M) ammonium sulfate was added, stirring constantly for 30 min at 4 °C. After centrifugation (10000 g, 15 min, 4 °C) the precipitate was suspended in 500 µl PBS and dialysed against PBS for 18 h. The buffer was changed and dialysis was continued for 4 h. After centrifugation (10000 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 Rabsztyń *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 antiserum, serially diluted in 0.1 % BSA, was applied to the wells, each in triplicate. After 1 h of incubation at

Table 2. LPS and ECA_{PG} control preparations used in this work

Preparation/strain	Description	Source/reference
LPS _{PCP} YeO3-c-R1	LPS _{PCP} containing ECA _{LPS} from Ra mutant YeO3-c-R1 cultivated at 22 or 37 °C, free of ECA _{PG}	DM* (Duda, 2007a)
LPS _{PCP} YeO3-c-RfbR7	LPS _{PCP} containing ECA _{LPS} from Rc mutant YeO3-c-RfbR7 cultivated at 22 or 37 °C, free of ECA _{PG}	DM* (Skurnik <i>et al.</i> , 1995)
LPS _{PCP} YeO3-c-R1-M196	LPS _{PCP} containing ECA _{LPS} from Rd ₁ mutant YeO3-c-R1-M196 cultivated at 22, 25 or 37 °C, free of ECA _{PG}	This work
LPS _{PCP} YeO3-c-R1-M164	LPS _{PCP} containing ECA _{LPS} from Rd ₂ mutant YeO3-c-R1-M164 cultivated at 22, 25 or 37 °C, free of ECA _{PG}	This work
LPS _{PCP} YeO3-c-R1-M205	LPS _{PCP} containing ECA _{LPS} from Re mutant YeO3-c-R1-M205 cultivated at 22, 25 or 37 °C, free of ECA _{PG}	This work
ECA _{PG} <i>S. Montevideo</i> SH94	ECA _{PG} was obtained from <i>S. Montevideo</i> SH94 cultivated at 37 °C by Ph/W and PCP extractions	DM* (Männel & Mayer, 1978)

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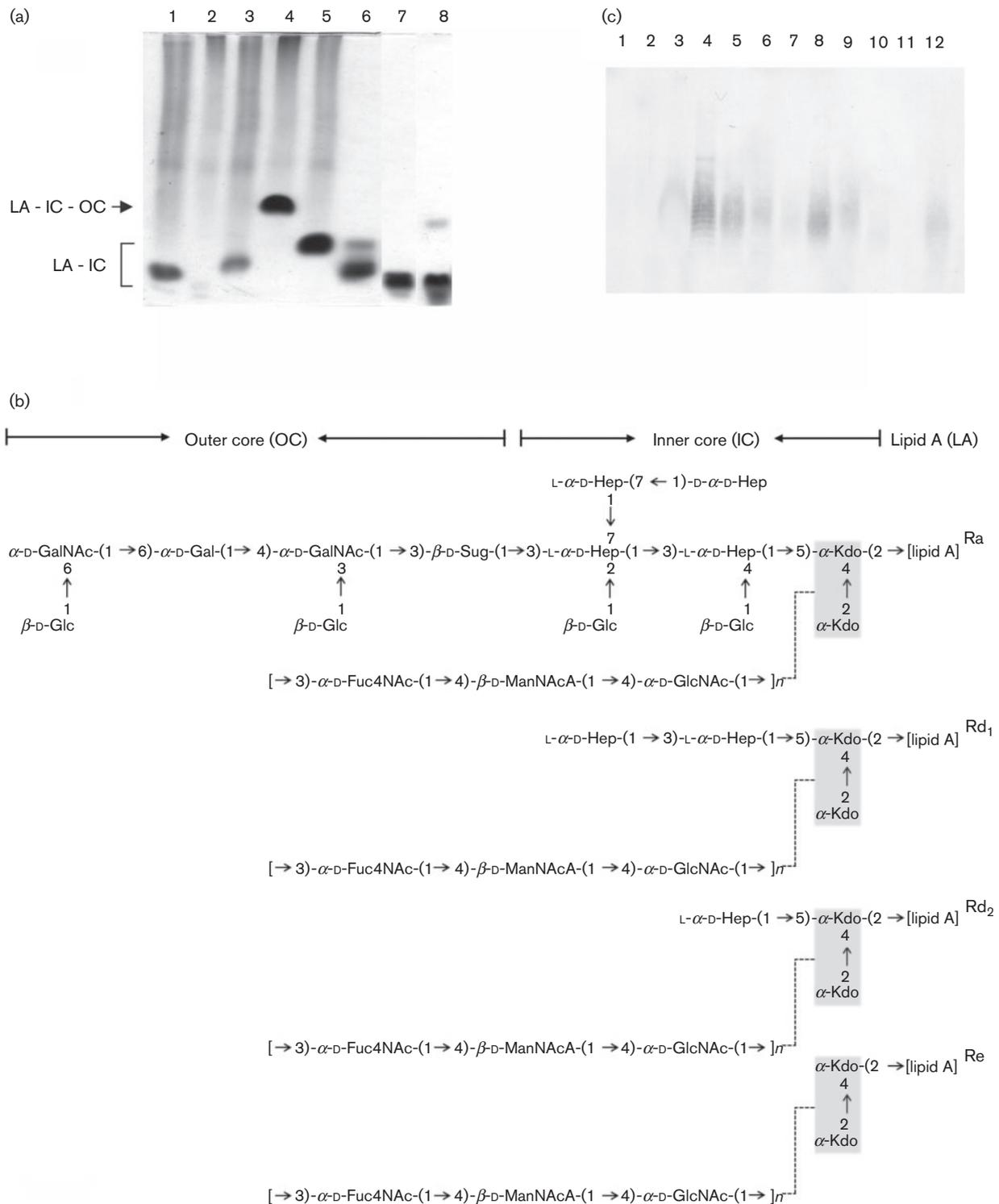


Fig. 1. Characterization of the LPS of YeO3 mutants. (a) Silver-stained DOC-PAGE profiles of LPS_{PCP} (0.25 μg) and WCL (2 μl) preparations of YeO3 mutants. Lane 1, WCL of M164; lane 2, WCL of M205; lane 3, WCL of M196; lane 4, LPS_{PCP} of YeO3-c-R1; lane 5, WCL of YeO3-c-OCR-ECA; lane 6, LPS_{PCP} 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-Hep, L-glycero-D-manno-heptopyranose; D,D-Hep, D-glycero-D-manno-heptopyranose; Kdo, 3-deoxy-α-D-manno-oct-2-ulonic acid; D-GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose, D-Glc, D-glucopyranose; D-GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; D-Gal, α-D-galactopyranose;

D-Sugp, 2-acetamido-2,6-dideoxy-D-xylo-hex-4-ulopyranose; D-Fucp4NAc, 4-acetamido-4,6-dideoxy-D-galactopyranose; D-ManpNAcA, 2-acetamido-2-deoxy-manno-pyranosuronic acid. (c) SDS-PAGE WB analysis of LPS_{PCP} preparations (3 µg per lane) of deep-rough mutants of YeO3 cultivated at 22 and 37 °C developed with monoclonal antibody mAb 898 specific for ECA at 1:200 dilution. Alkaline phosphatase-conjugated goat anti-mouse antibody was used at 1:2000 dilution. Lane 1, YeO3-c-R1 (22 °C); lane 2, YeO3-c-R1 (37 °C); lane 3, YeO3-c-RfbR7 (22 °C); lane 4, YeO3-c-RfbR7 (37 °C); lane 5, M196 (22 °C); lane 6, M196 (37 °C); lane 7, M164 (22 °C); lane 8, M164 (37 °C); lane 9, M205 (22 °C); lane 10, M205 (37 °C); lane 11, LPS_{Ph/W} YeO3-c-OCR-ECA (25 °C); lane 12, ECA_{PG}.

37 °C, wells were washed three times with TBS-Ca²⁺ 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 $\lambda_{405}=0.2$ was taken as a titre. The experiments were repeated three times.

SDS-PAGE and WB. ECA_{PG} of *S. Montevideo* SH94 and LPS_{PCP} of YeO3 strains were separated by SDS-PAGE (Mini-Protean; 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 uridylyltransferase, 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*-heptose (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 L,D-Hep and trace glucose, whereas LPS_{PCP} of M194 contained additional L-*glycero-D-manno*-heptopyranose (L,D-Hep) (1.7 times more than LPS_{PCP} of M164) and trace D-*glycero-D-manno*-heptopyranose (D,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 antisera against M196, M164 and M205 were first checked by ELISA for the presence of

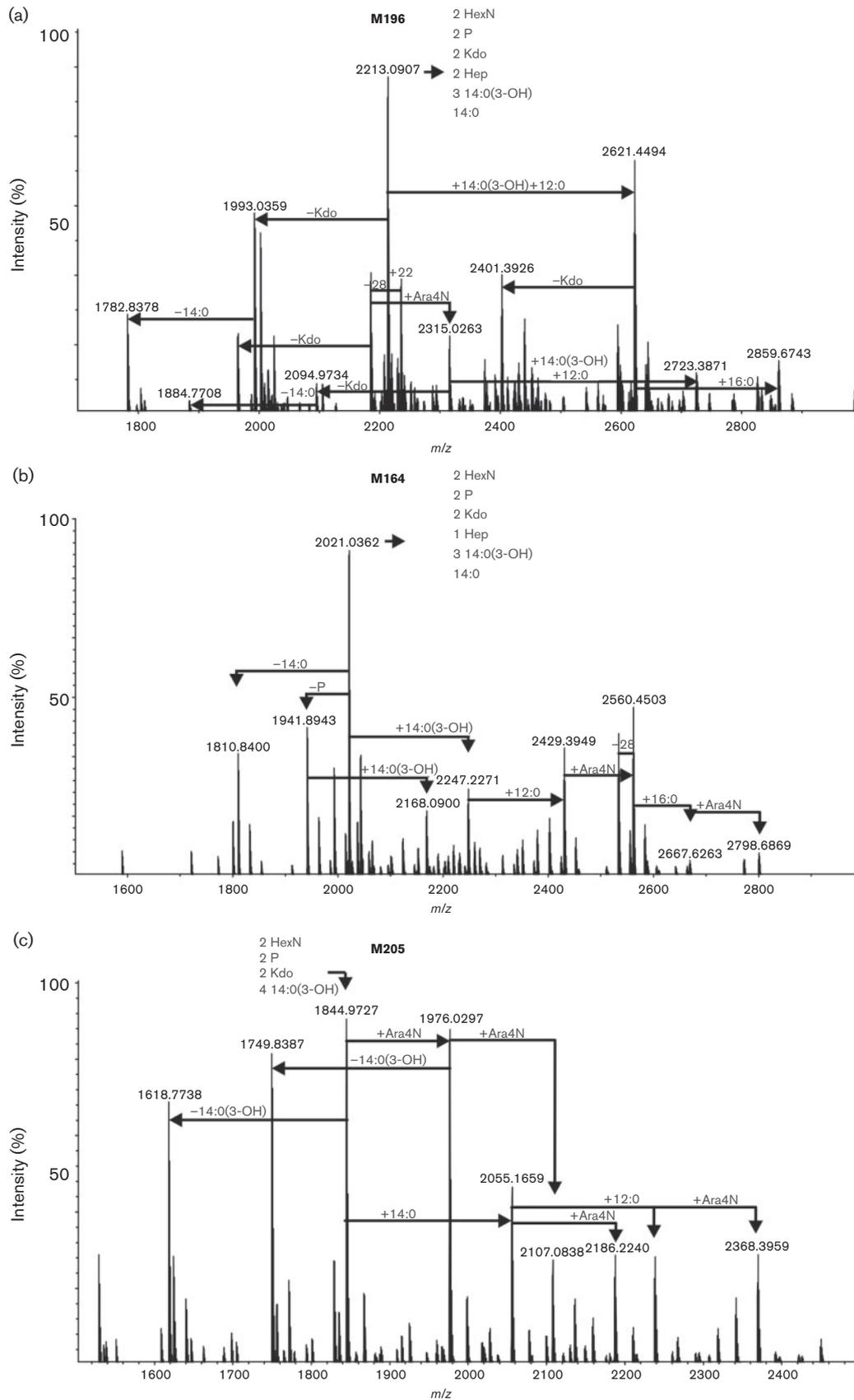


Fig. 2. The charge-deconvoluted ESI MS spectra of LPS_{PCP} samples from the deep-rough *Y. enterocolitica* strains. (a) YeO3-c-R1-M196, (b) YeO3-c-R1-M164 and (c) YeO3-c-R1-M205. The spectra were recorded in the negative ion mode. See text for details.

anti-LPS and anti-ECA antibodies. The untreated antisera reacted strongly with the ECA_{PG} 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 ECA_{PG} had dropped significantly and were 800 for all samples. When homologous LPS_{PCP} 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 LPS_{PCP} 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 LPS_{PCP} 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

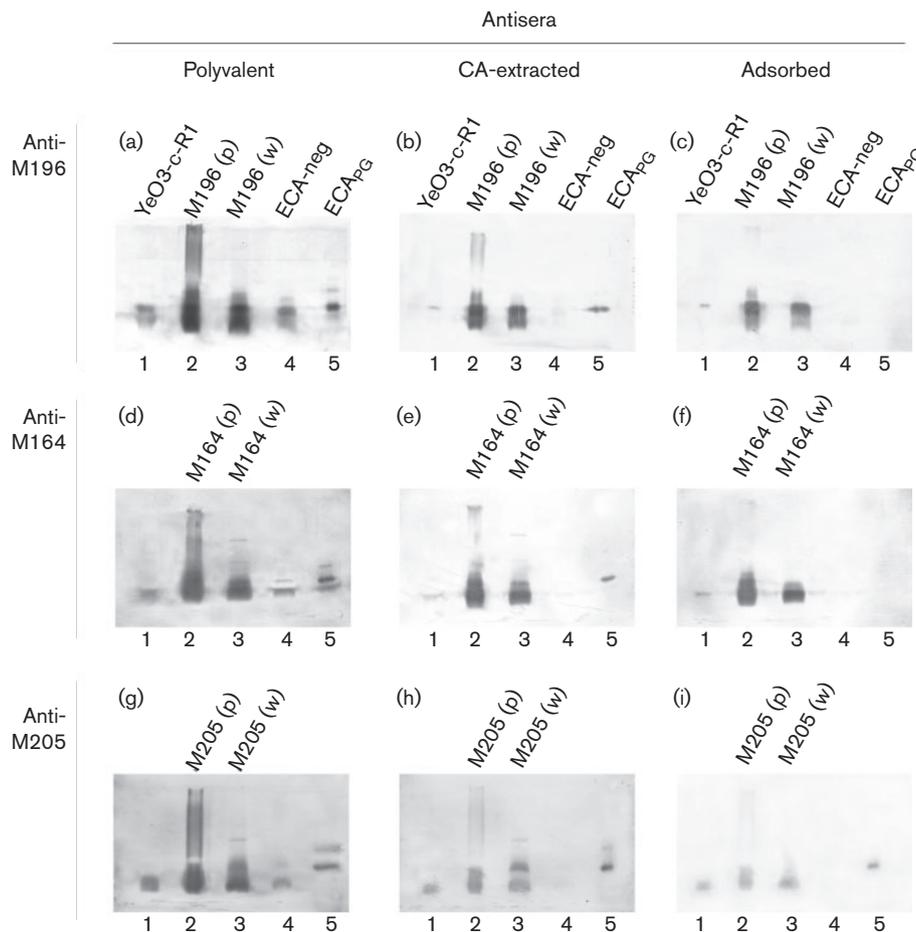


Fig. 3. WB analyses of rabbit anti-M196 (a–c), anti-M164 (d–f) and anti-M205 (g–i) antisera. (a, d, g) Untreated polyvalent antisera. (b, e, h) CA-extracted antisera. (c, f, i) Adsorbed (ECA-specific) antisera. The samples applied to the gels are indicated above the lanes and the lane numbers are indicated below. (p), LPS_{PCP} samples; (w), whole-cell lysate samples. Dilution of antisera: 1 : 1400. Loading: 2 µg for LPS_{PCP} preparations and ECA_{PG}; 2 µl for WCL.

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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