Enterobacterial common antigen and O-specific polysaccharide coexist in the lipopolysaccharide of *Yersinia enterocolitica* serotype O : 3

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*Yersinia enterocolitica* serotype O : 3 produces two types of lipopolysaccharide (LPS) molecules to its surface. In both types the lipid A (LA) structure is substituted by inner core (IC) octasaccharide to which either outer core (OC) hexasaccharide or homopolymeric O-polysaccharide (OPS) is linked. In addition, enterobacterial common antigen (ECA) can be covalently linked to LPS, however, via an unknown linkage. To elucidate the relationship between ECA and LPS in *Y. enterocolitica* O : 3 and the effect of temperature on their expression, LPS was isolated from bacteria grown at 22 °C and 37 °C by consequent hot phenol/water and phenol-chloroform-light petroleum extractions to obtain LPS preparations free of ECA linked to glycerophospholipid. In immunoblotting, monoclonal antibodies TomA6 and 898, specific for OPS and ECA, respectively, reacted both with ladder-like bands and with a slower-migrating smear suggesting that the ECA and OPS epitopes coexist on the same molecules. These results were supported by immunoblotting with a monovalent *Y. enterocolitica* O : 3 ECA-specific rabbit antiserum. Also, two or three 898-positive (and monovalent-positive) TomA6-negative bands migrated at the level of the LA–IC band in LPS samples from certain OC mutants, most likely representing LA–IC molecules carrying 1–3 ECA repeat units but no OPS. These bands were also present in *Y. enterocolitica* O : 9 OC mutants; however, coexistence of ECA and OPS in the same molecules could not be detected. Finally, the LA–IC–ECA bands were missing from LPS of bacteria grown at 37 °C and also the general reduction in wild-type bacteria of ECA-specific monovalent-reactive material at 37 °C suggested that temperature regulates the expression of ECA. Indeed, RNA-sequencing analysis showed significant downregulation of the ECA biosynthetic gene cluster at 37 °C.

INTRODUCTION

Lipopolysaccharide (LPS) and enterobacterial common antigen (ECA) are major components of the outer leaflet in the outer membrane of Gram-negative, enteric bacteria (Acker et al., 1981; Seltmann & Holst, 2001). In general, a complete or S-form LPS consists of three regions that are distinguished by their structures, genetics, biosynthesis and biological functions (Raetz & Whitfield, 2002): the O-specific polysaccharide (OPS or O-antigen) (Jansson, 1999; Knirel & Kochetkov, 1994), the core region (Holst, 1999, 2002, 2011) and the lipid A (LA) (Zähringer et al., 1999, 1999); the latter in endotoxic active LPS represents the toxic moiety. The so-called rough (R) form LPS comprises
core region and LA only. The enterobacterial LPS core region may be further divided into an inner core (IC) close to the LA and a more remote outer core (OC). ECA that is uniquely present in Enterobacteriaceae is a polysaccharide built of \(-\rightarrow 3\)-\(\alpha\)-D-Fucp4NAc\(-\rightarrow 4\)-\(\beta\)-D-ManpNACA\(-\rightarrow 4\)-\(\alpha\)-D-GlccpNAC\(-\rightarrow 1\) repeat units. This polymer can either covalently anchor to the outer membrane via its own \(l\)-glycerolphospholipid (ECA\(_{PC}\)) or utilize in some bacteria the core oligosaccharide as an attachment point (ECA\(_{LPS}\)). The third form of ECA, found in the periplasmic space, is a cyclic form and has no lipid anchor (ECA\(_{CYC}\)) (Kajimura et al., 2005; Kuhn et al., 1988; Lugowski et al., 1983). Of these three ECA types, only ECA\(_{LPS}\) is naturally immunogenic and it has been detected from Proteus \(\beta\) mirabilis and \(P\). \(\alpha\) stagnation, \(P\). \(\alpha\) mirabilis, \(P\). \(\alpha\) rettgeri, \(P\). \(\alpha\) monacensis, \(S\). \(\alpha\) enterica, \(Y\). \(\alpha\) enteroxocolitica, \(Y\). \(\alpha\) pseudotuberculosis, \(Y\). \(\alpha\) frederiksenii and \(E\). coli. Although the role of ECA in Enterobacteriaceae is not fully understood, there is growing evidence for its protective function against environmental stress. Studies on enteric pathogens, Salmonella enterica and \(E\). coli, revealed its importance in virulence and resistance to e.g. low pH, bile salts, and defense mechanisms from the eukaryotic host (Barua et al., 2009; Danese et al., 1998; Ramos-Morales et al., 2003). It was also proposed that ECA may be required for proper formation of flagella and motility of Serratia marcescens (Castelli et al., 2008).

\(Y\). \(\alpha\) enterocolitica is a heterogenic species divided into pathogenic and non-pathogenic sero- and bio-types. The bacteria are able to grow under a wide spectrum of conditions: in the environment with temperatures ranging from below 0 °C to over 40 °C, in invertebrate and vertebrate warm-blooded animals and humans (Bottone, 1997; Wren, 2003). Human pathogenic \(Y\). \(\alpha\) enterocolitica strains belonging most commonly to serotypes \(O:3\), \(O:8\) and \(O:9\) cause diseases, ranging from acute enteritis (especially in children) and enterocolitis to mesenteric lymphadenitis, terminal ileitis and reactive arthritis (Bottone, 1997). Blood contaminated by \(Y\). \(\alpha\) enterocolitica has caused life-threatening situations after transfusion due to the bacterium’s ability to grow at 4 °C (Goverde et al., 1998; Skurnik, 1999).

The structure of \(Y\). \(\alpha\) enterocolitica \(O:3\) LPS has been determined (Hoffman et al., 1980; Holst, 2007; Pinta et al., 2009; Radziejewska-Lebrecht et al., 1994, 1998; Shashkov et al., 1995) and proved to be somewhat unorthodox (Fig. 1). The LA–IC structure is very similar to that of other \(Y\). pseudotuberculosis; however, the IC of individual \(Y\). \(\alpha\) enterocolitica \(O:3\) LPS moieties can be substituted by either OC or OPS but not by both and we suggested that this is due to the fact that both OC and OPS would be linked to the same Hep II residue of IC (Pinta et al., 2012). The OC is a hexasaccharide and the OPS is a homopolymer (Fig. 1). Both the OPS and the OC were shown to be necessary for the expression of full virulence of \(Y\). \(\alpha\) enterocolitica \(O:3\) (al-Hendy et al., 1992; Biedzka-Sarek et al., 2005, 2011; Skurnik et al., 1999). We previously demonstrated the presence of ECA\(_{LPS}\) in \(Y\). \(\alpha\) enterocolitica \(O:3\) wild-type strains and in R-mutants expressing full or truncated OC (Rabsztyn et al., 2011; Radziejewska-Lebrecht et al., 1998, 2003).

In the present study we wanted to elucidate further the structural aspects of \(Y\). \(\alpha\) enterocolitica ECA\(_{LPS}\) making use of its immunogenicity. We wanted to clarify the relationships of the different LA–IC substitutions (OC, OPS and ECA) in individual LPS molecules and whether growth temperature (22 °C or 37 °C) influenced this. The present work showed that OPS and ECA\(_{LPS}\) are simultaneously present in \(Y\). \(\alpha\) enterocolitica \(O:3\) LPS and that the cultivation temperature affects in addition to the expression of OPS also that of OC and ECA. Although we obtained immunological evidence that ECA\(_{LPS}\) is linked to the IC of LPS the exact location remained unresolved.

**METHODS**

**Bacterial strains, culture, and control LPS.** Bacterial strains used in this study are listed in Table 1 and all LPS preparations used for reference in Table 2. For large-scale extractions of LPS the bacteria were cultivated in a 15 l fermenter under aerobic conditions at 22 °C and 37 °C for approximately 24 h (OD\(_{600}\) ~15–20). The medium contained per litre: 10 g tryptone (Proneidase), 5 g yeast extract (Biokar), 5 g NaCl, 0.25 g CaCO\(_3\), 5.85 g H\(_2\)PO\(_4\), 1 mg biotin and 10 g glucose. Glucose was added during fermentation to a final amount of approximately 50–60 g l\(^{-1}\), and pH was maintained at ~6.9 by addition of NH\(_4\)OH. The bacteria were killed with 1 % (w/v) aqueous phenol, harvested by centrifugation, washed two times with 0.9 % (w/v) aqueous NaCl, then freeze-dried and stored at ~20 °C.

**Isolation of LPS.** The LPS was extracted from dry bacteria using the hot phenol/water (Ph/W) method (Westphal & Jann, 1965). Crude Ph/W LPS preparations were purified from remaining nucleic acids by digestion with Benzonase (EC 3.1.30.2, Merck, ≥125 U LPS mg\(^{-1}\)) and RNase (EC 3.1.27.5, Boehringer Mannheim, 16 μg LPS mg\(^{-1}\)) in Tris-HCl buffer (0.2 g MgCl\(_2\)-6H\(_2\)O and 6.05 g Tris-HCl 1 L\(^{-1}\), pH 8.0) at 37 °C for 6 h. Proteins were removed from samples by treatment with Proteinase K (EC 3.4.21.64, Merck, 20 μg LPS mg\(^{-1}\)) for 4.5 h at 50–60 °C, followed by dialysis against H\(_2\)O, concentration by rotary evaporation and lyophilization. The LPSs (1–3 %, w/v solution in ultrapure H\(_2\)O) were then ultracentrifuged (Beckmann L–80, 105 000 g, 4 °C, 20 h) and collected pellets were marked as clean Ph/W LPS. The purity with respect to nucleic acids and proteins of each LPS sample was checked using DU68 Beckman spectrophotometer and quartz cuvettes (1 cm) at A\(_{260-280}\) against 0.25 M NaOH blank. The protein content was additionally determined by the method of Lowry (Lowry et al., 1954). The clean Ph/W LPS preparations were subjected to phenol–chloroform–light petroleum (PCP) extraction to isolate LPS/PCP devoid of ECAPG (Galanos et al., 1969). For mutants YeO3-trs11, YeO3-trs22 grown at 22 °C, and YeO3-trs24 grown at 22 °C and 37 °C, precipitation of LPS with H\(_2\)O generated two forms of LPS/PCP; part of the LPS precipitated as a gel-like material (marked as LPS/PCP) while another LPS portion precipitated as flakes (LPS/PCP\(_{FL}\)). Both precipitates were collected and analysed separately. The LPS yields obtained from the \(Y\). \(\alpha\) enterocolitica \(O:3\) wild-type, YeO3-trs11 and YeO3-trs22 strains grown at 22 °C were higher than in strains grown at 37 °C and were (expressed as percentage of dry cell mass): 2.1 % vs 0.1 % (wild-type), 1.8 % vs 0.6 % (YeO3-trs11) and 2.1 % vs 1.3 % (YeO3-trs22). In contrast, in the...
**Fig. 1.** The heterogeneous structure of the LPS of *Y. enterocolitica* O:3. The LPS inner core (IC) is the acceptor for OC hexasaccharide or homopolymeric OPS (Pinta et al., 2012) and/or ECA LPS (Radziejewska-Lebrecht et al., 1998). The OC structure was recently revised (Pinta et al., 2009). The single asterisk (*) indicates the putative OPS-attachment residue of the IC. The double asterisk (**) indicates that ECA is covalently linked to IC at unknown position. All sugars were pyranoses. L, D -Hep, L -glycero -a -D -manno -heptopyranose; D , D -Hep, D -glycero -a -D -manno -heptopyranose; Kdo, 3-deoxy-a -D -manno -oct-2-ulopyranosonic acid; Glc, b -D -glucopyranose; Gal, a -D -galactopyranose; GalNAc, 2-acetamido-2-deoxy-a -D -galactopyranose; Sug, 2-acetamido-2,6-dideoxy-D -xylo -hex-4-ulopyranose; 6 d- L -Alt, 6-deoxy-b -L -altropyranose; a -D -Fuc4NAc, 4-acetamido-4,6-dideoxy-a -D -galactopyranose; b -D -ManNAcA, 2-acetamido-2-deoxy-b -D -mannopyranosuronic acid; a -D -GlcNAc, 2-acetamido-2-deoxy-a -D -glucopyranose. WaaL OS , OC-specific ligase; WaaL PS , OPS-specific ligase; ECA-specific ligase is unknown (Pinta et al., 2012).

**Table 1.** Bacterial strains used in this work

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype and comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6471/76</td>
<td>YeO3, serotype O:3, patient isolate, wild-type</td>
<td>Skurnik (1984)</td>
</tr>
<tr>
<td>6471/76-c</td>
<td>YeO3-c, virulence plasmid-cured derivative of YeO3</td>
<td>Skurnik (1984)</td>
</tr>
<tr>
<td>YeO3-trs11</td>
<td>Δwzx-wbcKL::Km-Gen Block, KmR</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-trs22</td>
<td>ΔwbcKL, deletion of a 706 bp Xbol fragment</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-trs24</td>
<td>Δwbp, 929 bp deletion of two internal XhoI fragments</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-R1</td>
<td>Spontaneous rough derivative of YeO3-c</td>
<td>al-Hendy et al. (1992)</td>
</tr>
<tr>
<td>YeO3-c-OCR-ECA</td>
<td>Δ(wzx-wbqC)Δ(wzee-wzyE), ECA-negative derivative of YeO3-c-OCR, KmR</td>
<td>Rabsztyn et al. (2011)</td>
</tr>
<tr>
<td>YeO3-c-OC</td>
<td>Δ(wzbqC), derivative of YeO3-c (OC neg)</td>
<td>Biedzka-Sarek et al. (2005)</td>
</tr>
<tr>
<td>YeO3-c-trs8-R</td>
<td>Δwzx-wbqKL::Km-GenBlock, spontaneous rough mutant, derivative of YeO3-c, KmR</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>Ruokola/71-c</td>
<td>Spontaneous virulence plasmid-cured derivative of serotype O:9 strain</td>
<td>Ruokola/71</td>
</tr>
<tr>
<td>YeO9-c-R3</td>
<td>Δper::KmGB; OPS-negative derivative of Ruokola/71-c, KmR</td>
<td>Skurnik et al. (2007)</td>
</tr>
<tr>
<td>YeO9-OCR</td>
<td>Δper::KmGB; phage 6R1-37-resistant spontaneous low-OC-expressing derivative of Ruokola/71</td>
<td>Skurnik et al. (2007)</td>
</tr>
<tr>
<td>YeO9-c-OC</td>
<td>(=Ruokola/71-c-6R1-37-R); spontaneous OC-negative derivative of Ruokola/71-c</td>
<td>Müller-Loennies et al. (1999)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> sv. Montevideo SH94</td>
<td>Wild-type</td>
<td>Männel et al. (1978)</td>
</tr>
</tbody>
</table>
YeO3-trs24 mutant grown at 22 °C, the yield of LPS was slightly lower than from 37 °C and was 2.2% and 2.7%, respectively.

**Antibodies and antisera.** Mouse monoclonal antibody (mAb) 898 specific for ECA (Meier-Dieter et al., 1989), recognizing ManNAcA was a kind gift of Professor D. Bitter-Suermann (Institute of Medical Microbiology, Medical School of Hannover, Germany). MAbs TomA6 and 2B5 which are specific for Y. enterocolitica O:3 OPS and OC, respectively, have been described previously (al-Hendy et al., 1991; Skurnik et al., 1995; Pinta et al., 2012). Horse-radish peroxidase (HRP) or alkaline phosphatase (AP) -conjugated secondary swine anti-rabbit or goat anti-mouse antibody (Dako A/S) was used as a secondary antibody in immunoblotting analysis. Monovalent rabbit anti-ECA antisera specific for ECA_{C_{m}} of Y. enterocolitica O:3 was obtained after adsorption of polyvalent antisera against YeO3-c-trs8-R with the ECA-negative strain YeO3-c-OCR-ECA as described elsewhere (Rabszyn et al., 2011). Polyclonal rabbit antisera against Y. enterocolitica serotype O:9 (used at 1 : 1000 dilution) was obtained after immunization with formalin-killed bacteria as described earlier for serotype O:3 (al-Hendy et al., 1991a).

**Polyacrylamide gel electrophoresis (PAGE), silver staining and immunoblotting.** Silver-stained SDS- or deoxycholate (DOC)-PAGE analyses were performed as described (Laemmli, 1970; Tsai & Frasch, 1982) using 5% stacking and 15% separating gel in SDS-PAGE and 5% stacking and 12% separating gel in DOC-PAGE. Immunoblotting of SDS-PAGE separated samples was performed as described (Towbin et al., 1979). Briefly, samples were transferred from SDS-PAGE gels to polyvinylidene difluoride membranes (Immobilon-P, pore size 0.45 μm, Millipore) by either liquid or semidry systems. Liquid transfers were carried out in AP buffer (8.4 g 1⁻¹ NaHCO₃ and 0.203 g 1⁻¹ MgCl₂·6H₂O dissolved in deionized water, pH 9.3) at 4 °C and 30 V for 18 h. The semidry transfer was carried out by semidy转让 blotter (Panther Semidy Transferblotter, Owl Separation Systems, Thermo Scientific) at 12 V, 22 °C, for 2 h. After blocking for 12–16 h at 4 °C in 5% skimmed milk/1 × TBS Tween 20 buffer (0.15 M NaCl, 10 mM Tris-HCl, 0.05% Tween 20, pH 7.5), the membrane was incubated for 1 h at 22 °C in a rolling tube with the primary antibody (rabbit antisera or monoclonal antibody) in 5% skimmed milk/1 × TBS Tween 20 buffer. To detect the bound antibodies, the membranes were incubated for 1 h at 22 °C in 1:2000-diluted HRP- or AP-conjugated secondary swine anti-rabbit or goat anti-mouse antibodies in 5% skimmed milk/1 × TBS Tween 20 buffer. After washings, the HRP-conjugated antibodies were detected with the enhanced chemiluminescence solution (0.1 M Tris-HCl, 12.5 mM luminol, 2 mM coumaric acid, 0.03% H₂O₂) exposed to X-ray film (Kodak BioMax MR) while the AP-conjugated antibodies were detected with 5-bromo-4-chloro-3-indolyl-phosphate and p-nitroblue tetrazoium chloride (Serva) in AP buffer.

**Analytical methods.** Neutral sugar analyses, determination of organic bound phosphate, and Kdo and 2-amino-2-deoxy-β-D-glucose (GkN) quantifications were performed as described, as was the determination of the absolute configurations of the sugars (Kaca et al., 1988; Vinogradov et al., 1992). For GLC of alditol acetates, a Shimadzu gas chromatograph (model GC-17A) was used equipped with an SPB-5 fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and a flame ionization detector (FID). The temperature programme was 150 °C for 3 min, then 3 °C min⁻¹ to 300 °C for 5 min.

**RNA-sequencing.** The total RNA of Y. enterocolitica O:3 strain grown under two different temperatures (22 °C and 37 °C) was isolated as described (Rivas et al., 2001). The RNA-sequencing and data analysis were performed at the FIMM Technology Centre Sequencing Unit (http://www.fimm.fi/en/technologycentre/). The ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit for Gram-negative Bacteria (Epicentre). Paired-end sequencing was performed on a HiSeq2000 sequencer (Illumina) with a read length of 90 nucleotides. The obtained sequencing reads were filtered for quality and aligned against the Y. enterocolitica strain Y11 genome (accession number FR729477) using the TopHat read aligner (Langmead et al., 2009). The Cufflinks program (Trapnell et al., 2013) was then used to obtain the FPKM (fragments per kilobase of gene per million aligned fragments) values for differential expression.

**RESULTS**

To elucidate the relationships between ECA and LPS in Y. enterocolitica O:3, LPS was isolated from the wild-type

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**Table 2. LPS preparations used for reference in this work**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Type of extraction*</th>
<th>Chemo-type**</th>
<th>Core region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. enterocolitica O:3 strains</td>
<td>PhW/PCP</td>
<td>S</td>
<td>Complete</td>
<td>Radziejewska-Lebrecht et al. (1998)</td>
</tr>
<tr>
<td>Ye75S</td>
<td>PhW/PCP</td>
<td>S</td>
<td>Complete</td>
<td>Radziejewska-Lebrecht et al. (1994)</td>
</tr>
<tr>
<td>Ye75R</td>
<td>PCP</td>
<td>Rc</td>
<td>No outer core</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-c-R1</td>
<td>PCP</td>
<td>Ra</td>
<td>Complete</td>
<td>Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-c-trs8-R</td>
<td>PhW then PCP</td>
<td>Rc</td>
<td>No outer core</td>
<td>Radziejewska-Lebrecht et al. (2003); Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-c-trs22-R</td>
<td>PhW then PCP</td>
<td>Rc-Ra</td>
<td>Truncated outer core</td>
<td>Radziejewska-Lebrecht et al. (2003); Skurnik et al. (1995)</td>
</tr>
<tr>
<td>YeO3-c-trs24-R</td>
<td>PhW then PCP</td>
<td>Rc</td>
<td>No outer core</td>
<td>Radziejewska-Lebrecht et al. (2003); Skurnik et al. (1995)</td>
</tr>
<tr>
<td>Salmonella enterica sv. Montevideo SH94</td>
<td>PCP</td>
<td>S</td>
<td>Complete</td>
<td>Männel et al. (1978)</td>
</tr>
</tbody>
</table>

*PhW/PCP as described (Männel et al., 1978), PhW (Westphal & Jann, 1965) and PCP (Galanos et al., 1969). All LPS preparations contain ECA_{LPS} except S. enterica sv. Montevideo SH94. **S, smooth with complete core and OPS; Ra, rough with complete core but without OPS; Rc, rough with only inner core without outer core and OPS.
strain 6471/76 and from three OPS-expressing (smooth) OC mutants (YeO3-trs11, YeO3-trs22 and YeO3-trs24, Table 1) bacteria cultivated at both 22 °C and 37 °C as described in Methods. These mutants were included in the study as they caused different humoral responses during experimental mouse infections (Skurnik et al., 1995, 1999). Based on chemical analyses, YeO3-trs11 and YeO3-trs24 lack OC completely (Rc type), while YeO3-trs22 produces a truncated OC tetrasaccharide (Muszyński, 2004). Initially we used SDS-PAGE combined with silver staining to characterize properties of LPS that was purified from ECAPG. Typically the wild-type Y. enterocolitica O:3 LPS resolves in PAGE into two major fractions: (i) at the top of gel is a broad smear due to high molecular mass (HMM) LPS molecules composed of LA, IC and OPS, and (ii) a faster-moving strong low molecular mass (LMM) band composed of LA, IC and OC. Sometimes also a very fast-migrating band composed of LA and IC could be seen.

In our study, LMM bands in the LPS/PCP samples of bacteria grown at 37 °C were moving slightly faster than those from bacteria grown at 22 °C and the LA–IC band was stronger. As expected the LA–IC–OC band was missing in the OC mutants (Fig. 2, compare lanes 1–2 to lanes 3–12). In addition, there was clear heterogeneity in the LMM region bands suggesting non-stoichiometric substitution of LA–IC with fatty acids, phosphates and/or 4-amino-4-deoxy-l-arabinose (Rebei et al., 2004; Reiners et al., 2012a, b). Interestingly, the strong fastest-moving LMM band was present in the LPS/PCP sample of YeO3-trs22 grown at 37 °C (see arrow in Fig. 2). This is likely due to disturbed IC biosynthesis in this strain at 37 °C, which was also detected as increased relative Kdo content in this LPS (Muszyński, 2004). It may be speculated that a hypothetical glycosyltransferase activity of the fusion protein WbcK’L’ (fusing N- and C-terminal fragments of two glucosyltransferases) expressed in YeO3-trs22 (Skurnik et al., 1999) would block the IC biosynthesis at 37 °C by erroneous glycosylation.

The LPS samples isolated from bacteria grown at 37 °C showed the clear downregulation of OPS in all strains except YeO3-trs24 (Fig. 2, lanes 11–12). These results were also confirmed by immunoblotting and chemical analyses. Immunoblotting with OPS-specific mAb TomA6 demonstrated significantly higher amounts of the OPS at 22 °C compared to 37 °C (Fig. 3b) except in the YeO3-trs24 samples (compare Fig. 3b, lanes 9–12 and compositions in inset table). Interestingly, the LPS/PCP2 preparations contained more 6-d-L-Amp than the LPS/PCP1 and this could explain the different nature of both precipitates. Possibly the increased amounts of HO2 in the last steps of the PCP procedure resulted in the precipitation of more hydrophilic LPS moieties abundant in OPS. In summary, except for strain YeO3-trs24, the wild-type and the OC mutants expressed higher amounts of OPS when grown at 22 °C and this is in good agreement with previous results (Acker et al., 1986; al-Hendy et al., 1991b; Bengoechea et al., 2002; Ogasawara et al., 1985; Wartenberg et al., 1983).

**OPS and ECA<sub>LPS</sub> coexist in Y. enterocolitica O:3 LPS**

The OPS-specific mAb TomA6 detected in addition to the homopolymeric OPS smear also ladder-like bands migrating in the front of the smear (Fig. 3b, lanes 3–4 and 6–12). A ladder pattern is an indication of the presence of a heteropolymeric component in the OPS-containing fraction of LPS molecules and ECA<sub>LPS</sub> would be the best candidate in the light of present knowledge. The substitution of the core moiety of OPS-carrying LPS molecules by ECA units would significantly change its molecular mass and would generate the ladder pattern with TomA6 immunoblotting (see Figs 1 and 3b). To further evaluate this hypothesis the LPS/PCP preparations were analysed by immunoblotting with ECA-specific mAb 898 (Fig. 3d). All the LPS/PCP samples were ECA-positive, except those from wild-type bacteria grown at 22 °C and YeO3-trs22 grown at 37 °C (Fig. 3d, lanes 1 and 8). The co-localization of the TomA6- and mAb 898-specific ladder-like bands suggested that they were due to ECA<sub>LPS</sub>. Noteworthy, mAb 898 stained also the top region of the HMM smear further supporting the coexistence of ECA<sub>LPS</sub> and OPS in the same LPS molecules [Fig. 3, compare (b) and (d)].

Interestingly, growth temperature affected the ECA expression such that in all OC mutants grown at 22 °C two to three strong mAb 898-positive (but TomA6-negative) bands were observed migrating above the LA–IC band (Fig. 3d, lanes 3, 4, 6, 7, 9 and 10). Most likely these represent LPS molecules carrying 1–3 ECA repeating units. These bands were not present in LPSs isolated from bacteria grown at 37 °C. This finding corroborates and...
complements our recent observations of a similar temperature-mediated effect on ECA expression in R-type mutants (Rabsztyn et al., 2011).

A novel *Y. enterocolitica*-specific ECA*LPS* epitope

Antisera from rabbits immunized with boiled YeO3-c-trs8-R bacteria were extensively adsorbed with the ECA-negative strain YeO3-c-OCR-ECA to obtain monovalent anti-YeO3 ECA antisera (Rabsztyn et al., 2011). Immunoblotting of whole cell lysates and purified LPS of YeO3-c-trs8-R suggested that the monovalent rabbit antibodies recognized an ECA-specific epitope not present in ECAPG and that this epitope was not recognized by mAb 898 (Rabsztyn et al., 2011). To elucidate this phenomenon further we analysed by immunoblotting several *Y. enterocolitica* O : 3 LPS mutants (Fig. 4a). The results supported the results presented above, that the ECA moiety is linked to the LPS molecules substituted by the OPS but not to those substituted by the OC. The adsorbed antiserum reacted with the HMM region

**Fig. 3.** Immunoblotting of LPS/PCP preparations of *Y. enterocolitica* O : 3 wild-type and OC mutant strains using OPS-specific mAb TomA6 and ECA-specific mAb 898. Loading: 10 µg LPS per lane in (a) and (b) and 15 µg LPS per lane in (c) and (d). (a, c) Silver-stained SDS-PAGE after transfer of the bands onto PVDF membrane. Note that ECA cannot be stained by silver. (b) Immunoblotting with mAb TomA6. The bracket in (b) indicates a zone with ladder pattern. Chemically determined content of 6-d-L-altrose (i.e., OPS) in LPS is given in the inset table. (d) Immunoblotting with mAb 898. Lanes: 1, wild-type (22 °C); 2, wild-type (37 °C); 3, YeO3-trs11 (LPS/PCP1, 22 °C); 4, YeO3-trs11 (LPS/PCP2, 22 °C); 5, YeO3-trs11 (37 °C); 6, YeO3-trs22 (LPS/PCP1, 22 °C); 7, YeO3-trs22 (LPS/PCP2, 22 °C); 8, YeO3-trs22 (37 °C); 9, YeO3-trs24 (LPS/PCP1, 22 °C); 10, YeO3-trs24 (LPS/PCP2, 22 °C); 11, YeO3-trs24 (LPS/PCP1, 37 °C); 12, YeO3-trs24 (LPS/PCP2, 37 °C). The bracket in (d) indicates the region of ladder-like bands clearly visible on lanes 3–5 but only faintly on lanes 2, 6, 7 and 9–12. Note also in (d) the 2–3 strong mAb 898-positive [mAb TomA6-negative, see (b)] bands above the lipid A + core region in LPSs from 22 °C-grown OC mutants.
corresponding to the OPS smear of all smooth YeO3 strains which is an important observation as the rabbit antiserum did not contain any OPS-specific antibodies (the strain used for immunization was rough). This suggested that the OPS smear region LPS molecules were detected by the adsorbed antiserum via the ECALPS epitope. The adsorbed antiserum also stained a fast-migrating band of the OC mutant strain YeO3-c-OC and also of the rough OC mutant YeO3-c-trs8-R (Fig. 4a) indicating that the ECA epitope is linked to IC very close to lipid A. The same lysates were also blotted with mAb 898, which detected well the ECA PG-specific ladder that migrated slightly faster than the OPS-specific smear (Fig. 4b).

Interestingly, the ECA reactivity was split between the OPS smear and the IC regions in the OC mutant strain (Fig. 4a, YeO3-c-OC). This strain produces both LA–IC–OPS and LA–IC-type LPS molecules; the latter are abundant apparently because they are not substituted by OC. This, on the other hand, would indicate that only a certain fraction of the LPS molecules can at any time be substituted by OPS even though there would be no competition with OC substitution. This is supported by the fractionation of PS obtained after mild hydrolysis (2–3 % HOAc, 3 h, 100 °C) of Ye75S LPS on a Sephadex G-50 column into OPS, core–OS and ‘Kdo’ fractions (data not shown). On the other hand, it seems apparent that the LA–IC molecules not substituted by OC could instead be substituted by ECA. Altogether, these results demonstrated that the bacteria have complicated machinery working in determining how LPS molecules are substituted by these three different oligo-/poly-saccharides.
In order to get insight into whether the growth phase influenced ECA_{LPS} expression, samples were withdrawn from logarithmic and stationary phase bacteria grown at both 22 °C and 37 °C and blotted with unadsorbed (Fig. 4c) and adsorbed (Fig. 4a, d) rabbit antisera as well as OC-specific mAb 2B5 (Fig. 4e). The results showed that the unadsorbed antiserum (Fig. 4c) reacted strongly with both the Y. enterocolitica O : 3 LA–IC- and LA–IC–OPS-containing LPS molecules, but only weakly with the lysate of S. enterica serovar Montevideo. The reactivity was slightly weaker with bacteria grown at 37 °C. The unadsorbed antiserum also detected the LA–IC band of the ECA-negative control strain YeO3-c-OCR-ECA (Fig. 4c). On the contrary, the adsorbed antiserum did not react at all with the latter strain demonstrating the efficiency of the adsorption, as it had lost the reactivity against the LA–IC bands and also showed a weaker reactivity in the region of the LA–IC–OPS molecules [Fig. 4, compare (c) and (d)]. The effect of temperature was very clear such that less smear reactivity was detected from 37 °C-grown bacteria. In Fig. 4(e), the reactivity of mAb 2B5 demonstrated that expression of OC is lower at 37 °C and this is well seen when the bacteria grow for extended time. Cell lysate of YeO3-c-OCR-ECA and LPS_{PCP} from YeO3-c-trs8-R showed no reactivity with mAb 2B5 as they were OC-deficient mutants. The effect of temperature on ECA expression appeared to be regulated at least in part at the transcriptional level as demonstrated by the transcriptional profiling of the ECA gene cluster in RNA-sequencing analysis (Fig. 5). The RNA-sequencing data shows that all the ECA gene cluster genes are repressed in bacteria grown at 37 °C when compared to bacteria grown at 22 °C and that this was most dramatic for the wzzE-wecE genes (Fig. 5).

**Presence of Y. enterocolitica-specific ECA_{LPS} epitope in serotype O : 9**

To find out whether the ECA_{LPS} epitope is present only in Y. enterocolitica serotype O : 3, we performed immunoblotting analysis with a set of serotype O : 3 and O : 9 strains (wild-type strain and OPS-and/or OC-deficient derivatives) using the adsorbed rabbit anti-ECA_{LPS} antiserum for the detection (Fig. 6). The immunoblotting demonstrated the presence of ECA_{LPS} also in serotype O : 9; however, clearly the situation was not identical to that of serotype O : 3 (Fig. 6b). While a distinct signal was detected in the O : 3 OPS region no such signal was present in the O : 9 OPS region; instead the strongest signal was seen in the O : 9 OC mutants where the LA–IC band reacted strongly with the adsorbed antiserum. To rule out the possibility that the O : 9 strains Ruokola/71-c and YeO9-c-OC would not express the OPS the membrane used in (b) was washed and reprobed with anti-YeO9 antiserum (Fig. 6c) and this clearly demonstrated that these two strains expressed the OPS and that the adsorbed anti-ECA_{LPS} antiserum failed to bind to the OPS region of the gel.

**DISCUSSION**

In the present work using chemical analysis and immunoblotting data we demonstrated that Y. enterocolitica O : 3 has a highly complex and heterogeneous LPS in which the IC can be substituted by homopolymeric OPS, heteropolymeric ECA or by the OC hexasaccharide. We also showed that the bacteria decrease the production of OPS, ECA and OC when grown at 37 °C further adding to this observed complexity. Finally, our results strongly support the conclusion that ECA and OPS coexist on the same LPS molecules. It is not clear, however, whether this coexistence is full or if it is only a fraction of OPS-substituted molecules that are also ECA_{LPS}-substituted. The simultaneous stepwise biosynthesis of LPS and turnover on the bacterial surface result in a heterogeneous population of molecules that makes the chemical and structural analysis of LPS challenging and, on top, isolation and fractionation methods may cause further alterations in LPS (Hitchcock & Brown, 1983; Jann et al., 1975; Munford et al., 1980; Nowotny, 1984; Tavío et al., 2000).

The temperature is known as a key player in the biology of Y. enterocolitica (Bottone, 1997; Cornielis et al., 1998). Both immunoblotting and chemical analyses demonstrated that the expression of the OPS at 22 °C was higher in the wild-type strain and OC mutants YeO3-trs11 and YeO3-trs22, and all these LPSs were recovered from cells in higher yields than from those cultivated at 37 °C. These data were in agreement with other reports on thermoregulation of OPS in Yersinia (Acker et al., 1986; al-Hendy et al., 1991b; Bengoechea et al., 2002; Ogasawara et al., 1985; Wartenberg et al., 1983). Conversely, OPS expression in YeO3-trs24 was temperature-independent suggesting that the strain has acquired a spontaneous mutation somewhere in the still...
uncharacterized temperature-regulation mechanism. While the nature of this mutation is still unknown, this aberrant behaviour might be the reason why YeO3-trs24-infected mice differed from wild-type- or other OC mutant-infected mice in humoral responses and in protective response against re-challenge by the wild-type strain (Skurnik et al., 1999). Since mAb TomA6 antibodies (specific for the OPS) were shown to be protective in passive immunizations (Barr et al., 1998, 2003) and that expression of ECA PG and ECA LPS in the S-type LPS of Ye75S and in R- mutants (Rabsztyn et al., 1998, 2003) and that expression of ECA PG and ECA LPS is decreased at 37 °C (Muszyński, 2004; Rabsztyn et al., 2011). In the current study we clearly show a downregulation of the expression of ECA LPS at 37 °C compared to ECA LPS of bacteria grown at 22 °C. This downregulation was observed in a series of immunoblottings with ECA-specific antibody and antisera (Figs 3 and 4) and further complemented by observed downregulation of the wec ECA gene cluster at the transcriptional level (Fig. 5). It was particularly dramatic in wzzE and the downstream wecB–wecA genes. These genes encode for ECA chain length regulatory protein and for enzymes required for the biosynthesis of ECA glycosyl precursors, respectively (Barr et al., 1999; Kajimura et al., 2005). The immunoblotting analyses of the present work suggested coexistence of OPS and ECA in the Y. enterocolitica O:3 LPS. Specifically the mAb 898-positive immunostaining of the HMM region of the gel was very likely due to LPS molecules co-substituted with OPS and ECA LPS.

The discovery of the 1–3 ECA bands in immunoblotting with mAb 898 (Fig. 3d) explains the presence of multiple LMM bands seen within the IC and OC bands in silver staining (Fig. 2). The molecular mass of the ECA subunit (approximately Mr. 608) is sufficient to generate these distinct ladder bands in the gels. In addition, these 1–3 ECA-positive bands can be detected in all the smooth OC mutants grown at 22 °C but not at 37 °C, yet the ECA-positive HMM smear can be observed at the higher temperature. It is worth noting that we could not observe any cross-dependence between OC and ECA LPS substitutions. In conclusion, our data strongly indicate that ECA LPS is covalently linked to IC. However, elucidation of the attachment point requires further structural work. Our preliminary data with deep rough Y. enterocolitica O:3 mutants suggest that the binding site must be at the Kdo region of LPS (data not shown).

The use of the monospecific rabbit antiserum against Y. enterocolitica O:3 ECA LPS revealed the presence of a novel epitope. It was apparent that the adsorbed antiserum did not recognize ECA PG (at least not well) indicating that the major epitope seemed to be the ECA LPS linkage region and that this epitope was not present in ECA PG. It is feasible that some antibodies may react weakly with ECA PG but that was not detected under the used blotting conditions. It must be noted that loading of the gels also makes a difference. With the loading of 10–15 μg of purified LPS
(Fig. 3) reaction with mAb 898 was observed, while with 1–2 μg loading no reaction was seen (data not shown) suggesting that only a small proportion of molecules carried the mAb 898 epitope. It was also apparent that the ECA_LPS-specific antibodies in the adsorbed serum did not need OPS for recognition as R-strains also reacted; however, for those strains only the LA–IC–ECA was detected (Fig. 4a). On the other hand, the adsorbed antisera did not react with LA–IC–OC which is logical as the immunizing bacteria expressed no OC (Rabsztyn et al., 2011).

Y. enterocolitica O:3 and O:9 share many similarities in that the LA–IC moiety in both serotypes can be substituted by either OC or homopolymeric OPS (Skurnik et al., 2007). It was interesting to know whether ECA and OPS coexistence is shared among structurally similar serotypes or whether it is serotype specific. Although we clearly demonstrate the presence of ECA_LPS in both serotypes, in serotype O:9 ECA_LPS is present most clearly in the OC mutants YeO9-c-OC and YeO9-OCR (Fig. 6) and only as LA–IC–ECA (Fig. 6b) but not LA–IC–OPS. As our analysis was based on immunoblotting using the O:3-specific monovalent anti-ECA antiserum we cannot rule out the possibility that the O:9 OPS (a homopolymer of N-formylperosamine) could present a steric hindrance or conformational change to the ECA-specific epitope in the O:9 LA–IC–OPS–ECA molecules, if present. Further studies are warranted to elucidate this possibility.

The increasing interest in the biological role of ECA justifies further studies that should be widened to different enteric bacteria. Our results indicating the coexistence of ECA and OPS in Y. enterocolitica O:3 LPS show that the bacterial leaflet forms a very complicated and flexible system that undergoes constant modulation to environmental factors like temperature. This novel finding of a family of LPS molecules implicates the future need to elucidate the biological role of ECA in Y. enterocolitica O:3 and other medically important serotypes.

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