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Characterization of Anti-ECA Antibodies in Rabbit Antiserum Against Rough *Yersinia enterocolitica* O:3*

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Abstract—Enterobacterial common antigen (ECA) is a characteristic surface component in bacteria belonging to the Enterobacteriaceae family. It is generally integrated in the outer membrane via a linkage to phosphatidylglycerol (ECA_{PG}) and at the same time in some special cases via a linkage to lipopolysaccharide (ECA_{LPS}); the latter form is immunogenic. *Yersinia enterocolitica* O:3 expresses both ECA_{PG} and ECA_{LPS}. To study whether ECA-immunogenicity of *Y. enterocolitica* O:3 is temperature-regulated, rabbits were immunized with ECA-expressing *Y. enterocolitica* O:3 bacteria grown at 22 and 37°C. To induce minimal amount of anti-LPS antibodies, immunization was performed with YeO3-c-trs8-R, an LPS mutant missing both O-polysaccharide and the outer core hexasaccharide. However, abundant antibodies specific for LPS core were still present in the obtained antisera such that the reactivity of ECA-specific antibodies could not be detected. To obtain "monovalent" anti-ECA antisera, the sera were absorbed with ECA-negative bacteria. Absorption with live bacteria removed efficiently the anti-LPS antibodies, whereas this was not the case with boiled bacteria. Western blotting revealed that the specificity of the monovalent anti-ECA antiserum was different from that of a monoclonal anti-ECA antibody (mAb 898) as it did not react with ECA_{PG}, and this suggested that in *Y. enterocolitica* O:3 ECA_{LPS} only one or two ECA repeat unit(s) is/are linked to LPS. Both ECA_{PG} and ECA_{LPS} expression were found to be regulated by temperature and repressed at 37°C.

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Key words: *Yersinia enterocolitica* O:3, enterobacterial common antigen, lipopolysaccharide, monovalent antiserum

Like other members of the Enterobacteriaceae family, the *Yersiniae* possess in the outer membrane two major surface components, i.e., lipopolysaccharide (LPS) and enterobacterial common antigen (ECA). LPS is much more abundant in the outer leaflet of Gram-negative bacteria than ECA [1, 2]. In wild type strains (smooth forms, S) LPS is composed of the O-specific polysaccharide, core oligosaccharide, and lipid A. Strains that are deprived of the O-polysaccharide are called rough (R) [1]. ECA is a polymer built up of trisaccharide →3)-α-D-Fuc*p*4NAc-(1→4)-β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→ repeating units and is anchored to the outer membrane either via a phosphatidylglycerol (ECA_{PG}) or lipid A–core of LPS (ECA_{LPS}) [3]. The ECA_{LPS} is immunogenic in rabbits, while ECA_{PG} is not unless combined with some proteins. ECA_{LPS} has been demonstrated in *Escherichia coli* possessing a complete core region but not in deep rough mutants originating from the R1, R2, R3, R4, and K-12 LPS core types [3, 4]. Similar to *E. coli*, in *Proteus mirabilis* strains only the complete LPS core serves as the

Abbreviations: ECA, enterobacterial common antigen; ECA_{LPS}, ECA linked to LPS; ECA_{PG}, ECA linked to phosphatidylglycerol; Fuc4NAc, 4-acetamido-4,6-dideoxygalactose; LPS, lipopolysaccharide; LPS/PCP, LPS obtained by phenol/chloroform/light petroleum extraction; ManNAcA, 2-acetamido-2-deoxymannuronic acid.

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acceptor of ECA [5]. Contrary to this, it was reported that rough mutants of *Yersinia enterocolitica* O:3 strains possessing a complete LPS core as well as those with a truncated core or no outer core were ECA-immunogenic, suggesting that ECA was linked to the inner core region of their LPS [6-10]. In addition, the *Y. enterocolitica* O:3 wild type strain Ye75S possessing O-specific polysaccharide and a full core as well as its mutant Ye75R were ECA-immunogenic in rabbits [6].

The biological function of ECA is not well understood. It was suggested that ECA is responsible for the expression of the full pathogenic capacity of bacteria as ECA-negative mutants of *Salmonella enterica* serovar Typhimurium were 10-times less virulent than ECA-positive strains [11]. A protective function of the surface form of ECA in survival of bacteria against environmental stress was also indicated [12]. Expression of most virulence factors in *Y. enterocolitica* O:3 including that of LPS is temperature-dependent [13]. To study whether this hold also true for ECA, ECA_{LPS} was isolated from rough mutants of *Y. enterocolitica* O:3 cultivated at 22 and 37°C, and rabbits were immunized with the rough mutants cultivated at 22 and 37°C to analyze the presence of anti-ECA antibodies in the resulting rabbit antisera [8-10]. Immunoblotting data of the isolated ECA_{LPS} samples suggested that more ECA might be expressed at 37°C than at 22°C [10]; however, the data were not conclusive as the ECA_{LPS} isolations were not suitable for comparative analysis. Furthermore, quantification of the anti-ECA antibodies in the polyvalent antisera by Western blotting was impossible owing to the presence of abundant anti-LPS antibodies [9].

The aim of this study was to obtain antiserum enriched in antibodies specific for ECA (a so-called "monovalent" serum) to be used as a tool to study ECA-immunogenicity of *Y. enterocolitica* grown at different temperatures. Therefore, anti-LPS antibodies were removed from the polyvalent rabbit antiserum by using ECA-negative bacteria for absorption.

MATERIALS AND METHODS

Bacterial strains, antisera, and LPS and ECA preparations. The bacterial strains and plasmids are described in Table 1, LPS and ECA preparations in Table 2, antisera in Table 3, and primers in Table 4. When required, appropriate antibiotics were added to growth media as follows: kanamycin (Km, 100 μg/ml), chloramphenicol (Clm, 20 μg/ml), and ampicillin (Amp, 50 μg/ml).

Construction of the ECA-negative strain. The ECA gene cluster sequence of *Y. enterocolitica* O:3 was obtained from the genomic sequence project. The gene cluster is

Table 1. Bacterial strains and plasmids used in this work

Bacterial strain or plasmid	Description	Source or reference
Y. enterocolitica		
YeO3-c-trs8-R	Δ(*wzx-wbcL*)::Km-GenBlock (LPS outer core negative). Spontaneous rough LPS mutant. Derivative of serotype O:3 strain 6471/76-c. Km^R	[21, 22]
YeO3-c-OCR	Δ(*wzx-wbcQ*) (LPS outer core negative). Spontaneous rough mutant. Derivative of serotype O:3 strain 6471/76-c	[23]
YeO3-c-OCR-ECA	Δ(*wzx-wbcQ*) Δ(*wzzE-wzyE*). ECA-negative derivative of YeO3-c-OCR. Km^R	this work
S. enterica		
SH94	Serovar Montevideo, wild type	[24]
E. coli		
S17-1λ_{pir}	Strain used as host for suicide vectors	[25]
Plasmids		
pRV1-AKG	Suicide vector for ECA gene cluster deletion. Clm^R Km^R	this work
pUC4K	Source of KmGB cassette. Amp^R Km^R	Pharmacia, USA
pRV1	Suicide vector. Clm^R	[21]
pGEM-T	Cloning vector. Amp^R	Promega

Table 2. LPS and ECA preparations used in this work

Preparation	Description	Reference
LPS/PCP Ye75S (22°C)	LPS/PCP preparation from Ye75S strain (S type LPS) cultivated at 22°C obtained by phenol/water and PCP extraction [26]	MPI [26]
LPS/PCP Ye75R (37°C)	LPS/PCP preparation from Ye75R strain (Rc type LPS) cultivated at 37°C obtained by PCP extraction [27]	MPI [28]
LPS/PCP YeO3-c-trs8-R (22°C)	LPS/PCP preparation of strain YeO3-c-trs8-R cultivated at 22°C obtained by phenol/water [29] followed by PCP extraction [27]	[6, 9, 10]
LPS/PCP YeO3-c-trs8-R (37°C)	LPS/PCP preparation of strain YeO3-c-trs8-R cultivated at 37°C obtained by phenol/water [29] followed by PCP extraction [27]	US [6, 7, 9, 10]
ECA_{PG}	ECA_{PG} extracted from *S.* Montevideo SH94 by phenol/water and PCP extraction as described [26]	MPI [26]

Note: MPI, Max Planck Institute for Immunobiology, Freiburg, Germany; US, Department of Microbiology, University of Silesia, Katowice, Poland.

Table 3. Antisera and antibodies used in this work

Antiserum or antibody	Description	Reference
Rabbit anti-YeO3-c-trs8-R (22°C) antiserum	Polyvalent rabbit antiserum against YeO3-c-trs8-R strain cultivated at 22°C. Immunization with boiled bacteria according to the "long" protocol [30]	[9, 10]
Rabbit anti-YeO3-c-trs8-R (37°C) antiserum	Polyvalent rabbit antiserum against YeO3-c-trs8-R strain cultivated at 37°C. Immunization with boiled bacteria according to the "long" protocol [30]	[9, 10]
mAb 898	Monoclonal antibody specific for ECA	[31]
D0486	AP-conjugated goat anti-mouse immunoglobulins	DAKO, Glostrup, Denmark
D0487	AP-conjugated goat anti-rabbit immunoglobulins	–"–
P0447	HRP-conjugated goat anti-mouse immunoglobulins	–"–
P0217	HRP-conjugated swine anti-rabbit immunoglobulins	–"–

organized similarly to that of *E. coli* (*wecA*, *wzzE*, *wecB*, *wecC*, *rmlB*, *rmlA*, *wecD*, *wecE*, *wzxE*, *wecF*, *wzyE*, *wecG*) [14]. The allelic exchange strategy was designed to delete the region between the genes *wecA* and *wecG*, i.e., to retain the WecA activity required also in LPS biosynthesis. To replace the *wzzE-wzyE* fragment with kanamycin resistance GenBlock (KmGB), the suicide vector pRV1-AKG was constructed as follows. In the first-step PCRs, the *wecA*-specific 525-bp DNA fragment of *Y. enterocolitica* O:3 was amplified with primer pair wecA-f/wecA-R-GB, the *wecG*-specific 518-bp fragment with primer pair wecG-F-GB/wecG-R, and the 1156-bp KmGB-fragment of pUC4K with primer pair km-GB66-f/km-GB66-r (Table 4). In the second round of PCRs, the purified *wecA*, *wecG*, and KmGB fragments were joined together using primers wecA-f/wecG-R to obtain the final 2.2-kb AKG fragment. The purified AKG fragment was ligated to plasmid pGEM-T. The resulting plasmid was used as a template to amplify the cloned AKG-fragment, and the fragment was phosphorylated and cloned to the EcoRV site of suicide vector pRV1 (Table 1). The ligation mixture was transformed into *E. coli* S17-1λ_{pir}, and the resulting suicide vector was named pRV1-AKG. To replace by allelic exchange the ECA gene cluster to KmGB, pRV1-AKG was introduced into *Y. enterocolitica* strain YeO3-c-OCR by conjugation and the transconjugants were screened for Km-resistant (Km^R) Clm-sensitive (Clm^S) clones. The loss of the ECA gene cluster from the obtained transconjugates was confirmed by PCR using primers WecA-F2/WzzE-R1 (Table 4) that produce a 902-bp fragment with wild type bacteria and no products with the ECA-mutant. One resulting ECA-negative strain was named YeO3-c-OCR-ECA.

Absorption with boiled ECA-negative bacteria. The YeO3-c-OCR-ECA bacteria were cultured on three tryp-

Table 4. Primers used in this work

Primer	Sequence	Description	PCR product
wecA-F	TATTAGGCCGCCGCTATAAA	*wecA* forward primer	525 bp, left flank of AKG-fragment
wecA-R-GB66	tttgagacacaacgtggctttccCACTGC CTAAAAGCCTCTGG	*wecA* reverse primer, 5′-end with KmGB sequence (lower case)	
wecG-F-GB66	cctcactttctggctggatgatgTGGCGA AATTGTTGTATTGG	*wecG* forward primer, 5′-end with KmGB sequence (lower case)	518 bp, right flank of AKG-fragment
wecG-R	GGTTTGCCACCGACTAAAAA	*wecG* reverse primer	
Km-GB66-f	ggaaagccacgttgtgtctcaaa	KmGB forward primer	1156 bp, middle part of AKG-fragment
Km-GB66-r	catcatccagccagaaagtgagg	KmGB reverse primer	
wecA-F2	CGGCGATAAATGCTTTCAAT	*wecA* forward primer upstream of primer wecA-F	902 bp, primers located outside the 525 bp *wecA* fragment
wzzE-R1	TATTGACTGTTGGCCGGTCT	*wzzE* reverse primer	

ticase soya agar slants. After 24-h incubation at 22°C, the bacteria were collected and washed with sterile 0.85% NaCl (3 ml per slant) in order to prepare the inoculum. Next, 9 ml was used to inoculate a Roux bottle with 300 ml of trypticase soya agar medium and incubated at 22°C for 48 h. The collected bacteria were washed with 10 ml NaCl, the resulting bacterial suspension was centrifuged (4300*g*, 4°C, 30 min), and the sediment was washed three times with 0.85% NaCl. The bacterial mass was further suspended in 10 ml of 0.85% NaCl, incubated at 100°C for 2.5 h, centrifuged as above, and the resulting wet bacterial mass was used for absorptions.

The rabbit antiserum against 22°C-grown YeO3-c-trs8-R bacteria was diluted 1 : 20 with sterile 0.85% NaCl. A 5 ml-sample was mixed with 4 g wet bacterial mass and incubated at 37°C for 2 h with shaking. After centrifugation (4300*g*, 22°C, 1 h), a 1 ml aliquot of the absorbed antiserum was removed for later analysis, and the remaining 4 ml was absorbed with a new portion of wet bacterial mass (2 g) at 4°C for 18 h with shaking. After centrifugation as above, the absorbed and twice absorbed antisera were passed through 0.22-μm sterile filter (Millipore, USA) and stored at −20°C [15].

Absorption with live ECA-negative bacteria. The ECA-negative strain YeO3-c-OCR-ECA was grown in 100 ml Luria–Bertani broth [16] supplemented with 50 μg/ml kanamycin. After 24-h incubation at 37°C with shaking (200 rpm), the bacteria were centrifuged (2600*g*, 22°C, 15 min). The resulting bacterial sediment was washed with sterile phosphate buffered saline and centrifuged as above. The wet bacterial mass was used for absorption.

A 2-ml sample of 1 : 20 diluted rabbit antiserum against 37°C-grown YeO3-c-trs8-R bacteria was gently mixed with 0.5 g of wet bacterial mass and incubated on ice for 30 min. After centrifugation (14,000*g*, 22°C, 8 min) a new 0.5 g portion of wet bacterial mass was mixed with the supernatant and incubated on ice for 30 min. After centrifugation as above, the twice absorbed antiserum was passed through a 0.22-μm sterile Millipore filter and stored at −20°C [17].

Whole cell lysates. To prepare whole cell lysates, YeO3-c-trs8-R (22 and 37°C), YeO3-c-OCR-ECA, and *S. enterica* sv. Montevideo (*S.* Montevideo) SH94 strains were grown on Luria–Bertani broth for 18 h. After incubation, the OD_{600} of the culture was determined. Then 1 ml culture was centrifuged (14,000*g*, 22°C, 5 min) and the supernatant was removed. The pellet was resuspended into 2× SDS-sample buffer in ratio 100 μl/OD_{600} = 1. Next, the sample was incubated at 100°C for 10 min, cooled to ~22°C, and 2 μl proteinase K (20 mg/ml) was added. After 18-h incubation at 60°C, the lysates were stored at −20°C. The whole cell lysates were used as antigens for testing antiserum absorbed with live ECA-negative bacteria.

SDS-PAGE and Western blotting. The non-absorbed and twice absorbed anti-YeO3-c-trs8-R antisera were analyzed by immunoblotting using as antigens the ECA_{PG} standard preparation from *S.* Montevideo SH94 and LPS/PCP preparations from *Y. enterocolitica* strains YeO3-c-trs8-R (22 and 37°C), Ye75S (22°C), and Ye75R (37°C) as well as whole cell lysates of YeO3-c-trs8-R (22 and 37°C), YeO3-c-OCR-ECA, and *S.* Montevideo SH94. Electrophoretic separation of the samples was performed in 15% SDS-PAGE as described [18].

Western blotting was performed by two different protocols. (i) The ECA_{PG} and LPS/PCP samples were transferred after SDS-PAGE from the gel onto a PVDF membrane (polyvinylidene difluoride, Immobilon™-P, pore size 0.45 μm; Millipore) using a Biotrans blotting apparatus (Kucharczyk, Poland) at 30 V, 4°C, for 18 h [19]. After blocking for 2 h (22°C) in 10% skimmed milk/dot blot buffer (50 mM Tris-HCl, 0.2 M NaCl, pH 7.4), the membrane was incubated 12-16 h at 22°C with gentle shaking with the primary antibody (antiserum or monoclonal antibody) in 10% skimmed milk/dot blot buffer. For detection of the bound antibodies, the membrane was incubated for 2 h at 22°C in (1 : 2000)-diluted alkaline phosphatase (AP)-conjugated secondary goat anti-rabbit or anti-mouse antibodies (DAKO, Denmark) in 10% skimmed milk/dot blot buffer and stained with 5-bromo-4-chloro-3-indolyl-phosphate (Serva, Germany) and *p*-nitroblue tetrazolium chloride (Serva) in AP-buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.3).

(ii) The samples were transferred after SDS-PAGE from the gel onto a PVDF membrane by semidry blotter (Panther™ Semidry Electroblotter, Owl Separation Systems; Thermo Scientific, USA) at 12 V, 22°C, for 2 h. After blocking 12-16 h at 4°C in 5% skimmed milk/1× Tris-buffered saline (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 (antiserum or monoclonal antibody) in 5% skimmed milk/1× TBS Tween 20 buffer. To detect the bound antibodies, the membrane was incubated for 1 h at 22°C in (1 : 2000)-diluted horseradish peroxidase (HRP)-conjugated secondary swine anti-rabbit or goat anti-mouse antibodies (DAKO) in 5% skimmed milk/1× TBS Tween 20 buffer. After washing, the secondary antibodies were detected with the enhanced chemiluminescence solution (0.1 M Tris-HCl, 12.5 mM luminol, 2 mM coumaric acid, 0.03% H_2O_2) exposed to X-ray film (Kodak BioMax MR, USA).

RESULTS AND DISCUSSION

Construction and characterization of the ECA-negative mutant. The strain YeO3-c-OCR-ECA was constructed as described in "Materials and Methods". The *wzzE-wzyE* region of the ECA gene cluster was replaced by KmGB using allelic exchange. The strain did not express ECA as confirmed by immunoblotting (data not shown).

Absorption with boiled ECA-negative bacteria. SDS-PAGE and Western blotting of the LPS/PCP preparations from *Y. enterocolitica* Ye75S, Ye75R, and YeO3-c-trs8-R strains as antigens revealed strong immunostaining in the high (10-50 kDa) and low (1-5 kDa) molecular mass regions of the gel with the non-absorbed anti-YeO3-c-trs8-R antiserum (Fig. 1a, lanes *1-5*). After absorption

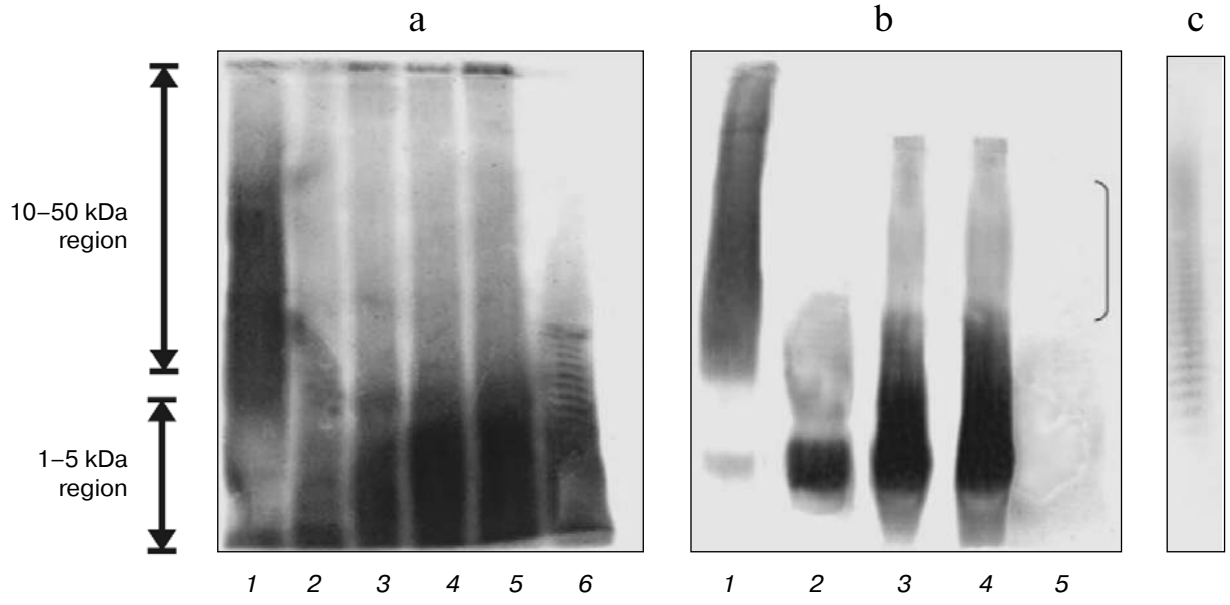

Fig. 1. Western blotting analysis of rabbit anti-YeO3-c-trs8-R (22°C) antiserum before and after absorption with boiled YeO3-c-OCR-ECA bacteria: a) non-absorbed antiserum (1 : 600). Lanes: *1*) Ye75S (22°C), 15 μg; *2*) Ye75R (37°C), 15 μg; *3*) YeO3-c-trs8-R (22°C), 15 μg; *4*) YeO3-c-trs8-R (22°C), 25 μg; *5*) YeO3-c-trs8-R (22°C), 40 μg; *6*) ECA_{PG}, 4 μg; b) antiserum (1 : 600) absorbed with boiled ECA-negative bacteria. Lanes: *1*) Ye75S (22°C), 15 μg; *2*) Ye75R (37°C), 15 μg; *3*) YeO3-c-trs8-R (22°C), 15 μg; *4*) YeO3-c-trs8-R (22°C), 25 μg; *5*) ECA_{PG}, 5 μg. The faint ECA bands are indicated by a bracket; c) control blotting with ECA-specific monoclonal antibody (1 : 200) and ECA_{PG} (2 μg) as antigen.

with boiled ECA-negative bacteria, the absorbed antiserum still contained antibodies against all these antigens (Fig. 1b, lanes *1-4*) but the reaction with LPS/PCP of Ye75R in the 10-50 kDa region was weaker than for the non-absorbed antiserum and occurred mainly in the lower part of the region (Fig. 1b, lane *2*).

Immunoblotting with the ECA_{PG} standard preparation as antigen using the non-absorbed antiserum (Fig. 1a, lane *6*) and the ECA-specific monoclonal antibody as control (Fig. 1c) showed a strong immunostaining with a characteristic ladder-like banding profile. The ECA_{PG} preparation reacted with the absorbed antiserum, but the staining was faint and detected as a large spot in the 1-5 kDa region and a lower part of the 10-50 kDa region (Fig. 1b, lane *5*).

In conclusion, absorption with boiled bacteria did not remove effectively the anti-LPS antibodies.

Absorption with live ECA-negative bacteria. Western blotting with the LPS/PCP preparation from YeO3-c-trs8-R and whole cell lysates from both YeO3-c-trs8-R and the ECA-negative strain YeO3-c-OCR-ECA as antigens demonstrated that the non-absorbed antiserum contained specific anti-core LPS and anti-ECA antibodies (Figs. 2a and 3a). After absorption twice with live ECA-negative bacteria the absorbed antiserum did not react with any antigens present in the whole cell lysate of the YeO3-c-OCR-ECA (Figs. 2b (lane *3*) and 3b (lane *1*)). Therefore, in this case the anti-LPS antibodies were efficiently removed from the antiserum. The remaining antibodies immunostained a relatively narrow band in the ECA-positive YeO3-c-trs8-R preparation (Figs. 2b (lanes *1* and *2*) and 3b (lane *2*)), in contrast to a broad band stained by the non-absorbed antiserum (Figs. 2a and 3a). This suggested that the narrow band carried the ECA-specific antigen (provided that the absorbed antiserum contained antibodies only against ECA). The absorbed antiserum showed a weaker reaction with the YeO3-c-trs8-R antigens than the non-absorbed antiserum (compare Figs. 2a and 2b). Interestingly, the narrow band immunostained by the absorbed antiserum was located in the low molecular mass region without any sign of a ladder-like pattern, indicating the presence of one (or two) ECA repeat unit only (Fig. 3b, lane *2*). Control immunoblotting with the ECA-specific monoclonal antibody mAb 898 revealed a positive reaction in the high (10-50 kDa) molecular mass region with both LPS/PCP and whole cell lysate from the Rc mutant YeO3-c-trs8-R and no reaction with the ECA-negative strain (Figs. 2c and 3c).

The whole cell lysate from YeO3-c-trs8-R strain cultivated at 22°C (Figs. 2b and 2c, lanes *1*) showed a stronger reaction with the ECA-specific monoclonal antibody and the absorbed antiserum than the lysate from the same strain cultivated at 37°C (Figs. 2b and 2c, lanes *2*). This suggests that both ECA_{PG} and ECA_{LPS} expression is regulated by temperature and is repressed at 37°C.

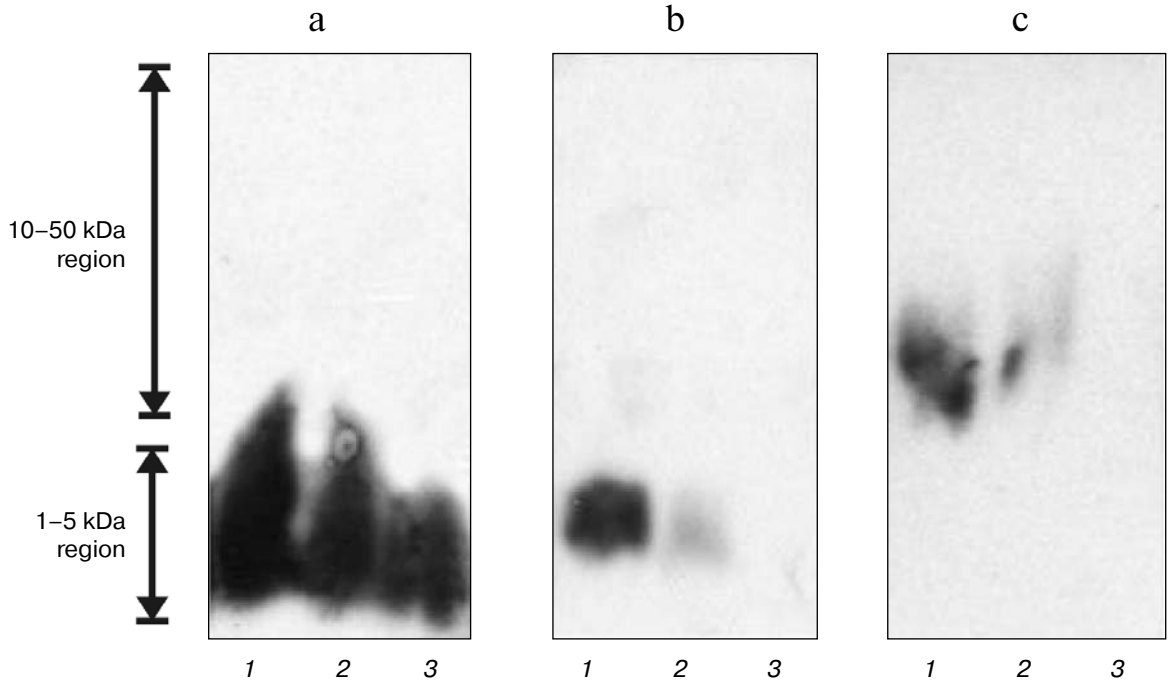

Fig. 2. Western blotting analysis of rabbit anti-YeO3-c-trs8-R (37°C) antiserum before and after absorption with live YeO3-c-OCR-ECA bacteria: a) non-absorbed antiserum (1 : 600); b) absorbed antiserum (1 : 600); c) ECA-specific mAb 898 (1 : 3000). Lanes: *1*) YeO3-c-trs8-R (22°C); *2*) YeO3-c-trs8-R (37°C); *3*) YeO3-c-OCR-ECA. 2 µl of whole cell lysate was loaded to each lane.

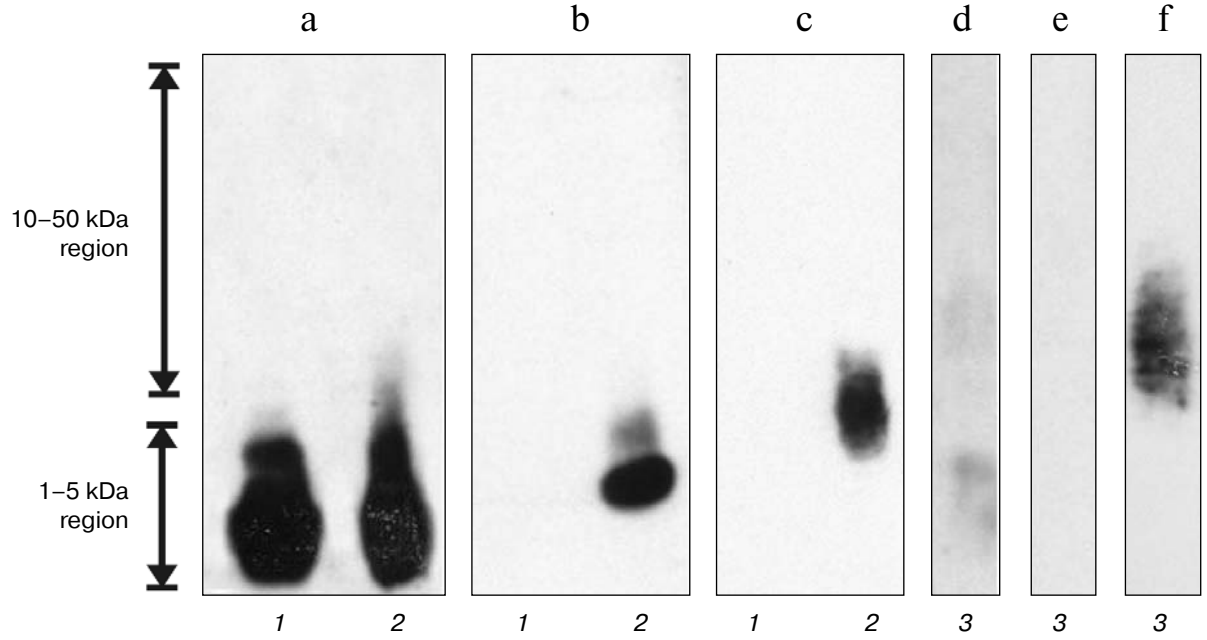

Fig. 3. The absorbed rabbit anti-YeO3-c-trs8-R (37°C) antiserum and the monoclonal antibody react with different epitopes: a, d) non-absorbed antiserum (1 : 100); b, e) absorbed antiserum (1 : 100); c, f) ECA-specific mAb 898 (1 : 3000). Lanes: *1*) whole cell lysate of YeO3-c-OCR-ECA, 4 µl; *2*) LPS/PCP preparation of YeO3-c-trs8-R (37°C), 1 µg; *3*) whole cell lysate of *S.* Montevideo SH94, 4 µl.

Western blotting with the whole cell lysate from *S.* Montevideo SH94 as antigen revealed a faint immunostaining with the non-absorbed antiserum (Fig. 3d), no reaction with the absorbed antiserum (Fig. 3e), and a strong immunostaining with the monoclonal antibody specific for ECA with the characteristic ladder-like banding profile (Fig. 3f). A comparison of the specificities of the absorbed antiserum (Figs. 2b (lanes *1* and *2*) and 3b (lane *2*)) and the ECA-specific monoclonal antibody (Figs. 2c (lanes *1* and *2*) and 3c (lane *2*)) demonstrated that the absorbed antiserum and the monoclonal antibody reacted with different epitopes.

In summary, absorption with live ECA-negative bacteria removed the anti-LPS antibodies from the polyvalent antiserum against strain YeO3-c-trs8-R more efficiently than absorption with boiled bacteria. Although LPS is a heat-stable antigen, boiling might have denatured its epitopes or, more likely, caused epitope blocking, e.g. by denatured surface structures thus resulting in inferior absorption efficiency of the boiled bacteria.

Our data also indicated that the ECA-specific antibodies in the polyvalent anti-YeO3-c-trs8-R antiserum were mainly against an ECA_{LPS}-specific epitope that is not present in ECA_{PG}. Additionally, the data suggested that only single ECA-units are ligated to LPS molecules, raising a question why they were not detected in earlier structural analyses of *Y. enterocolitica* O:3 LPS [6, 20]. A likely explanation is that ECA substitution of LPS is non-stoichiometric and that a small fraction of ECA-substituted LPS molecules could be detected by immunoblotting while at best traces of the ECA-specific sugars were seen in structural and compositional analyses. In addition, two of the constituents of the ECA repeating unit, e.g. ManNAcA and Fuc4NAc, are relatively labile monosaccharides. The modern analytical mass spectrometry methods, which are sensitive enough for detection of non-stoichiometric substitutions, could be a useful tool in establishing the linkage of ECA to LPS molecule. Further work is needed to elucidate this question.

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REFERENCES

1. Alexander, C., and Rietschel, E. T. (2001) *J. Endotoxin Res.*, **7**, 167-202.
2. Vaara, M. (1999) in *Endotoxin in Health and Disease* (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds.) Marcel Dekker, New York, pp. 31-38.
3. Kuhn, H.-M., Meier-Dieter, U., and Mayer, H. (1988) *FEMS Microbiol. Rev.*, **54**, 195-222.
4. Kasperkiewicz, K., Skurnik, M., Brade, L., Muszynski, A., and Radziejewska-Lebrecht, J. (2004) *Abstr. Book 3rd German-Polish-Russian Meeting on Bacterial Carbohydrates*, Wroclaw, Poland, Abstract P10.

5. Duda, K. A., Duda, K. T., Beczala, A., Kasperkiewicz, K., Radziejewska-Lebrecht, J., and Skurnik, M. (2009) *Arch. Immunol. Ther. Exp.*, **57**, 147-151.
6. Radziejewska-Lebrecht, J., Skurnik, M., Shashkov, A. S., Brade, L., Rozalski, A., Bartodziejska, B., and Mayer, H. (1998) *Acta Biochim. Pol.*, **45**, 1011-1019.
7. Kasperkiewicz, K. (2002) *ECA-Immunogenicity of Yersinia enterocolitica Rough Mutants*: Ph. D. Thesis [in Polish], Department of Microbiology, University of Silesia, Katowice, Poland.
8. Radziejewska-Lebrecht, J., Kasperkiewicz, K., Skurnik, M., Brade, L., Steinmetz, I., Swierzko, A. S., and Muszynski, A. (2003) *Adv. Exp. Med. Biol.*, **529**, 215-218.
9. Duda, K. T. (2007) *Reactivity of Polyclonal Antisera against R Mutants of Yersinia enterocolitica O:3*: Ph. D. Thesis [in English], Department of Microbiology, University of Silesia, Katowice, Poland.
10. Duda, K. A. (2007) *Immunochemical Studies on Lipopolysaccharides from R Mutants Yersinia enterocolitica O:3*: Ph. D. Thesis [in English], Department of Microbiology, University of Silesia, Katowice, Poland.
11. Valtonen, M. V., Larinkari, U. M., Plosila, M., Valtonen, V. V., and Makela, P. H. (1976) *Infect. Immun.*, **13**, 1601-1605.
12. Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K., and Ohta, M. (2002) *Mol. Microbiol.*, **43**, 629-640.
13. Bottone, E. J. (1997) *Clin. Microbiol. Rev.*, **10**, 257-276.
14. Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) *Science*, **277**, 1453-1462.
15. Paziak-Domanska, B., Chmiela, M., Jarosinska, A., Majeed, F. A., Czkwianianc, E., Planeta-Malecka, I., and Rudnicka, W. (2000) *Pediatria Wspolczesna. Gastroenterologia, Hepatologia i Zywienie Dziecka*, **2**, 283-286.
16. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, New York.
17. Skurnik, M. (1985) *Infect. Immun.*, **47**, 183-190.
18. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
20. Pinta, E., Duda, K. A., Hanuszkiewicz, A., Kaczynski, Z., Lindner, B., Miller, W. L., Hyytiainen, H., Vogel, C., Borowski, S., Kasperkiewicz, K., Lam, J. S., Radziejewska-Lebrecht, J., Skurnik, M., and Holst, O. (2009) *Chemistry*, **15**, 9747-9754.
21. Skurnik, M., Venho, R., Toivanen, P., and Al-Hendy, A. (1995) *Mol. Microbiol.*, **17**, 575-594.
22. Skurnik, M., Venho, R., Bengoechea, J.-A., and Moriyon, I. (1999) *Mol. Microbiol.*, **31**, 1443-1462.
23. Biedzka-Sarek, M., Venho, R., and Skurnik, M. (2005) *Infect. Immun.*, **73**, 2232-2244.
24. Mayer, H., and Schmidt, G. (1979) *Curr. Top. Microbiol. Immunol.*, **85**, 99-153.
25. De Lorenzo, V., and Timmis, K. N. (1994) in *Bacterial Pathogenesis*, Pt. A (Clark, V. L., and Bavoil, P. M., eds.) Academic Press, USA, pp. 386-405.
26. Mannel, D., and Mayer, H. (1978) *Eur. J. Biochem.*, **86**, 361-370.
27. Galanos, C., Luderitz, O., and Westphal, O. (1969) *Eur. J. Biochem.*, **9**, 245-249.
28. Radziejewska-Lebrecht, J., Shashkov, A. S., Stroobant, V., Wartenberg, K., Warth, C., and Mayer, H. (1994) *Eur. J. Biochem.*, **221**, 343-351.
29. Westphal, O., and Jann, K. (1965) *Methods Carbohydr. Chem.*, **5**, 83-91.
30. Swierzko, A., Brade, L., Paulsen, H., and Brade, H. (1993) *Infect. Immun.*, **61**, 3216-3221.
31. Peters, H., Jurs, M., Jann, B., Jann, K., Timmis, K. N., and Bitter-Suermann, D. (1985) *Infect. Immun.*, **50**, 459-466.