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QUARTERLY

Immunochemical studies on R mutants of *Yersinia enterocolitica* O:3°

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Three mutants of *Yersinia enterocolitica* O:3, namely: YeO3-R1, YeO3-RfbR7 and YeO3-c-trs8-R were classified on the basis of sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) profile of isolated lipopolysaccharides (LPS) as belonging to the Ra- (the first) and the Rc-type (the other two mutants). Methylation analysis, in addition to ^{13}C and ^{1}H NMR studies of purified core oligosaccharides revealed structures similar to those established previously for the full core of *Y. enterocolitica* O:3 in the case of the Ra mutant, and identical to that reported for the Rc mutant Ye75R, in the case of the two other mutants.

The O-specific sugar, 6d-L-altrose, which forms a homopolymeric O-chain, was present in small amounts in all three LPS preparations, as well as in the core oligosaccha-

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Abbreviations: LPS, lipopolysaccharide; DOC, deoxycholate; ECA, enterobacterial common antigen; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; Mab, monoclonal antibody; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid.

ride preparations along with the Ra and the Rc sugars, characteristic of the *Y. enterocolitica* O:3 core. This result is in line with genetic data, indicating that it is the *inner* core region which is the receptor for the O-specific chain in *Y. enterocolitica* O:3.

This region seems likewise to be the anchoring region for the enterobacterial common antigen (ECA), as shown by SDS/PAGE/Western blot analysis with monoclonal antibodies against ECA. In addition, we also demonstrated that the Ye75R mutant Rc and its parental strain Ye75S, both were ECA-immunogenic strains. So far, ECA-immunogenic strains, i.e. those with LPS-linked ECA, were only identified in *E. coli* mutants of the R1, R4 and K-12 serotype.

All Gram-negative bacteria with the exception of the genus *Sphingomonas* possess lipopolysaccharides as main constituents of their outer membrane [1]. Lipopolysaccharides are indispensable for bacterial survival and growth, one of their important function being maintaining of the proper arrangement of individual components in the outer membrane of the cell wall. In the case of *Sphingomonas paucimobilis* this function is performed by glycosphingolipid [2].

Generally, LPS of the wild type bacteria (S-forms) consist of long polysaccharide chains (O-specific chains), which are linked to the lipid A moiety (the hydrophobic anchor to the outer membrane) *via* the core oligosaccharide. Lipopolysaccharides of the rough type strains consist only of lipid A and core oligosaccharide – complete or truncated [1, 3]. The precise location, however, where the O-chains are attached to the core region was elucidated so far only for a few species of *Salmonella* (see Nikaido, 1969; 1970, and Hämmerling *et al.*, 1970, as cited by Lüderitz *et al.* [3]), *Shigella* [4], *Citrobacter* [5] and *Pseudomonas* [6] and it was always the complete core which was needed for a proper attachment of these O-chains. In addition, the full core can also accept in some cases additional surface constituents, e.g. K-antigens, T-antigens and the enterobacterial common antigen (ECA) [7]. The latter is composed of chains, built of a trisaccharide repeating unit ...[→3) αFuc4NAc-1.4β ManNAcA 1.4 αGlcNAc 1]$_n$ → with its reducing end (in the non-immunogenic form) linked to an L-glycerophosphatide, or it can occur as a cyclic form [8]. ECA is present in all members of the *Enterobacteriaceae* family, but the LPS-linked immunogenic form has been observed only in R-mutants having complete core-oligosaccharides of the R1, R4 or the K-12 core types [7].

Species of the genus *Yersinia* also possess ECA, and before phylogenetic studies based on 16S rRNA homology had been performed, this fact served as an argument for excluding these bacterial species from the *Pasteurellaceae* family and classified them as *Enterobacteriaceae* [9].

The genus *Yersinia* comprises such important human pathogens as *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. The presence of ECA in *Y. enterocolitica* cells was revealed by immunocytochemical studies [10]. There are about 70 serotypes of *Y. enterocolitica* as determined by variability of chemical composition of the O side chains of their lipopolysaccharides [11, 12].

Previous studies on *Y. enterocolitica* of the O:3 serotype revealed the presence of a homopolymeric O-chain, composed of β-(1→2) linked units of 6d-L-altrose. This strain is of importance as it is a causative agent of reactive arthritis in humans. It is noteworthy that LPS is so far the only component of bacterial origin identified within the inflamed joints of patients suffering from reactive arthritis triggered by *Salmonella*, *Shigella* or *Yersinia* [11].

In the present immunochemical studies on three *Y. enterocolitica* O:3 R mutants, we found that a truncated core oligosaccharide of the Rc type, is the place of ECA binding, similarly to the binding place of O-specific chain as it was revealed recently [13].

MATERIAL AND METHODS

Cultivation of bacteria and isolation of lipopolysaccharides. The mutants YeO3-R1, YeO3-RfbR7 and YeO3-c-trs8-R of *Y. enterocolitica* O:3 were obtained from the strain collection of Mikael Skurnik at the Turku Centre for Biotechnology, University of Turku (Finland).

Mass cultivation of these strains was carried out under aerobic conditions in Tryptic Soy Broth medium (Difco) at 28°C in a fermentor. Bacteria were killed with 1% aqueous phenol, washed three times with water, then twice with ethanol and finally dried with acetone and diethylether. Lipopolysaccharide in each case was obtained by the hot phenol/water extraction, according to Westphal *et al.* [14], and purified by repeated runs (105000 × ***g***, 3 times, 4 h each) in a preparative ultracentrifuge.

Compositional analysis. Neutral sugars were determined as alditol acetates after hydrolysis with 0.1 M HCl for 48 h at 100°C, using a Varian gas chromatograph (model 1520B), equipped with a fused silica wall-coated open tubular (WCOT) capillary column HP-5 (30 m × 0.25 mm inner diameter) with a temperature program of 150°C for 5 min, then 5°C min^{-1} ramp rate to 310°C, using D-xylose as an internal standard [15]. The absolute configuration of sugars were identified by a modification of the Gerwig *et al.* method [16], i.e. by GLC of their acetylated but-2-yl glycosides, following butanolysis in (+)-butan-2-ol (0.5 ml) and acetyl chloride (35.5 μl) at 85°C for 24 h and peracetylation. Phosphorus was determined by the method of Lowry *et al.* [17]. The content of Kdo, galactosamine and fucosamine was not quantified, but their presence was observed by NMR analysis.

Preparative isolation of the core oligosaccharide. The core oligosaccharides were obtained by very mild hydrolysis (0.1 M sodium acetate buffer, pH 4.4, 3 h, 100°C) of lipopolysaccharides of the respective mutants [18]. The hydrolysate was centrifuged, and the supernatant after evaporation was fractionated on a Sephadex G-50 column (120 cm × 2.5 cm) at room temperature, and eluted with water. Sugar-containing fractions were pooled, lyophilized and subjected to chemical and structural analyses.

The sediment containing lipid A and not completely hydrolyzed LPS was further treated with 1% acetic acid, for 1.5 h at 100°C. The hydrolysate was treated as described above, the fractions containing sugars were only subjected to the chemical analysis (not shown).

Analysis by methylation, NMR spectroscopy, mass spectrometry. These techniques were performed as described in detail in [15] (and will be not again given here). Since the results for two of the examined mutants revealed their structural identity with that of the core oligosaccharide of Ye75R [15], they will be presented only briefly in "Results and Discussion"

It should be added that, the structural analysis of the core oligosaccharide of the third mutant, Ra YeO3-R1, is still under investigation by NMR, thus the present publication contains only preliminary data. Later, its structure will be published together with the structure of the core oligosaccharide of Ye75S, isolated from the LPS of a wild type *Y. enterocolitica* O:3 strain, which was presented in part at the Eurocarb VIII[1].

SDS/PAGE analysis and serological studies. Lipopolysacharides of the three examined *Y. enterocolitica* O:3 mutants were subjected to SDS/PAGE and SDS/PAGE/Western blot analyses. LPS used as standards originated from our previous studies: those of *Y. enterocolitica* O:3, from the wild type strain Ye75S and its Rc mutant Ye75R were extracted by the method of Westphal *et al.* [14], whereas LPS of the Re mutant of *Sal-*

[1]8th European Carbohydrate Symposium, Sevilla, Spain, 1995, Abstracts, p. B O-17.

monella minnesota R595 was a kind gift from Dr. S. Schlecht (Max-Planck Institut, Freiburg, Germany).

Anti-O and anti-R sera were obtained by immunization of New Zealand White rabbits with heat-killed *Y. enterocolitica* O:3 S form (Ye75S) and Rc mutant (Ye75R), respectively. Immunization was carried out with a bacterial suspension (1.5×10^{10} CFU ml^{-1}) in doses of 0.25, 0.5 and 1 ml over a period of three weeks. The sera obtained five days after the last injection were stored at -20°C.

SDS/PAGE was carried out in the buffer system of Laemmli [19] with 13% separating gel and 5% stacking gel and the plate was stained after periodate oxidation with silver nitrate [20]. In Western blot analysis performed according to Towbin *et al.* [21] LPS was electrotransferred from the gel onto a nitrocellulose sheet and developed with a rabbit polyclonal antiserum, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova, Germany), the latter requiring 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine and *p*-nitroblue tetrazolium chloride (Bio-Rad, Munich, Germany) as substrates. Polyclonal rabbit antisera were used in a dilution of 1:100, whereas anti-rabbit IgG was diluted 1:1000.

Mab6, against O-polysaccharide chains of *Y. enterocolitica* O:3, were from the collection of Departments of Medical Microbiology and Medicine, Turku University, Turku (Finland) and was used at a dilution of 1:16.

MabECA – against the enterobacterial common antigen, was a kind gift from Prof. D. Bitter-Suermann (Institute of Medical Microbiology, Hannover, Germany) and was used at a dilution of 1:100.

Purified LPS were used in SDS/PAGE/Western blot analysis performed in the above described system, and proteinase K (EC 3.4.21.14; Boehringer, Mannheim, Germany) digested (12 h, 37°C) prior to use of monoclonal antibody Mab6 or MabECA.

RESULTS AND DISCUSSION

Isolation of lipopolysaccharides, products derived thereof and gel electrophoresis

LPS of Ra mutant YeO3-R1 and LPS of R mutant YeO3-RfbR7, as well as LPS of R mutant YeO3-c-trs8-R isolated by the phenol/water extraction [14], were obtained from all strains at high yields of about 3.5%, based on bacterial dry mass.

The SDS/PAGE, known to be a powerful tool for visualizing the natural heterogeneity of S-type LPS specimens, as well as – in the case of R mutants – their presumable R chemotype [22], was used prior to detailed chemical analysis. Thus, the three LPSs mentioned above were compared prior to their structural analysis, in SDS/PAGE with a well characterized Ye75R-Rc LPS sample of *Y. enterocolitica* mutant Rc [15] and with a Ye75S-LPS originating from *Y. enterocolitica* O:3 wild type strain, as well as with LPS of Re-type of *S. minnesota* R595 mutant (Fig. 1).

Lipopolysaccharide of YeO3-R1 migrated like the full core moiety of Ye75S-LPS, which confirmed its Ra character as well as the pattern observed when DOC/PAGE was performed [13]. LPS YeO3-RfbR7 and LPS YeO3-c-trs8-R migrated like LPS Ye75R which showed their Rc character, recognized for the last specimen [13, 15]. All lipopolysaccharides examined showed some heterogeneity, confirmed also by chemical and structural analyses.

Lipopolysaccharide samples of YeO3-R1 (420 mg), YeO3-RfbR7 (900 mg) and YeO3-c-trs8-R (514 mg), when hydrolysed with acetate buffer, pH 4.4, afforded the carbohydrate material and lipid A-moiety. Carbohydrate material was fractionated on a Sephadex G-50 column, giving in each case a small quantity of the high molecular mass fraction (Fr. I): 5 mg (1.2%), 15 mg (1.6%) and 4 mg (0.8%), respectively, and the core oligosaccharide frac-

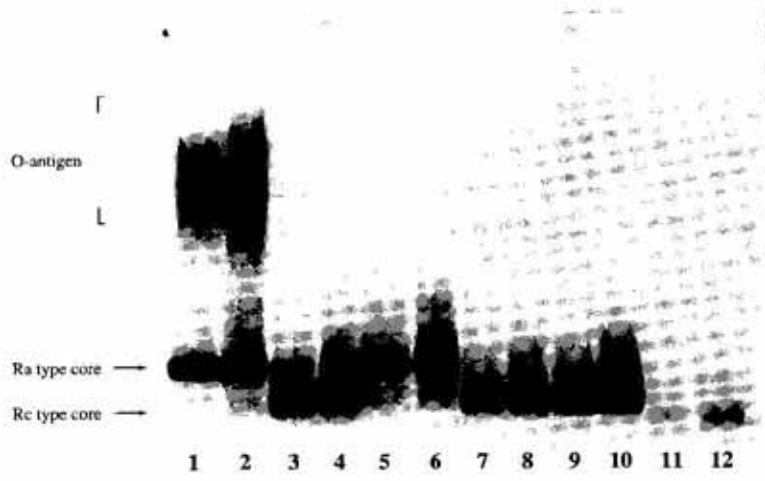

Figure 1. SDS/PAGE of isolated lipopolysaccharide samples from *Yersinia enterocolitica* O:3 S and R strains and *Salmonella* Re strain.

Purified LPS samples, 3 μg and 5 μg of each, were loaded and separated in a 13% separating and 5% stacking SDS/polyacrylamide gel. Staining was with periodate/Ag according to Tsai & Frasch [20]. Lanes 1 and 2 – *Y. enterocolitica* O:3, Ye75S; lanes 3 and 4 – *Y. enterocolitica* O:3, Ye75R; lanes 5 and 6 – *Y. enterocolitica* O:3, YeO3-R1; lanes 7 and 8 – *Y. enterocolitica* O:3, YeO3-RfbR7; lanes 9 and 10 – *Y. enterocolitica* O:3, YeO3-c-trs8-R; lanes 11 and 12 – *S. minnesota* R595.

tion: 76 mg (18%), 93 mg (10.3%), 42 mg (8.2%), respectively. From "lipid A" samples, obtained in suspiciously large amounts (about 80% of LPS used) another portion of polysaccharide could be released, namely: 36 mg (8.6% of LPS YeO3-R1), 136 mg (15.2% of LPS YeO3-RfbR7) and 81 mg (15.8% of LPS YeO3-c-trs8-R) after subjecting them to a stronger hydrolysis (1% acetic acid, 2 h, 100°C). Since we expected the presence of a labile-linked deoxy*amino* sugar (FucNAc), only those products liberated by mild acetate buffer hydrolysis at pH 4.4 were further examined.

Chemical and structural studies

The chemical composition of lipopolysaccharides and products derived thereof is shown in Table 1. Glucose, DD heptose, LD heptose, Kdo, that is those constituents, which are characteristic of a Rc-type core of Ye75R, in the molar ratios of 2:1:3:1 [15], were representative also for core oligosaccharides, as well as for LPS of YeO3-RfbR7 and YeO3-c-trs8-R mutants pointing to their Rc chemotype.

Mutant YeO3-R1 contained, in addition to Rc core type sugars, also galactose and two

Table 1. ***Chemical composition of lipopolysaccharides and derived products from R mutants of Yersinia enterocolitica* O:3.**

LPS was obtained from dry bacterial mass by phenol/water extraction and purified by ultracentrifugation. Mild hydrolysis of LPS released its carbohydrate part which fractionated on Sephadex G-50 yielded a high molecular mass Fr. I and core oligosaccharide fraction (core OS). Sugars were determined as alditol acetates [15], their absolute configuration identified by a modifed Gerwig *et al.* method [16], phosphorus by the Lowry method [17].

Mutant	YeO3 - R1			YeO3 - RfbR7			YeO3 - c - trs8-R		
	Amount in								
Sugar	LPS	Fr. I	core OS	LPS	Fr. I	core OS	LPS	Fr. I	core OS
	% (by mass)								
6-d-LAltrose	0.3	8.3	trace	trace	trace	trace	trace	trace	trace
D-Arabinose	0.3	2.0	0	0.2	11.5	0.8	0.3	10.1	0.9
D-Mannose	0.2	10.3	0	0.3	1.4	0.1	0.4	1.1	0
D-Galactose	3.6	0.9	7.2	0.2	1.0	0.4	0.2	0.2	0
D-Glucose	19.2	21.6	25.9	6.7	11.6	24.6	8.3	6.5	19.1
D-Galactosamine	+	+	+	0	0	0	0	0	0
D-Fucosamine	+	+	+	0	0	0	0	0	0
DD-Heptose	5.1	0.5	9.8	4.7	5.9	20.8	5.6	3.3	15.5
LD-Heptose	15.4	1.9	32.2	14.7	18.2	63.0	17.9	9.8	46.8
Kdo	+	+	+	+	+	+	+	+	+
P	n.t.	0.09	0.03	n.t.	0.09	0.07	n.t.	n.t.	n.t.

Abbreviations: trace, below 0.03%; +, component observed by NMR analysis, not quantified; n.t. - not tested.

aminosugars, namely galactosamine and fucosamine. Its chemical composition was the same as those previously established by analysis of the core oligosaccharide Ye75S isolated from LPS of the wild type strain *Y. enterocolitica* O:3. The structure of Ye75S core oligosaccharide[1] consisted of a hexasaccharide built of two terminal D-glucose residues *beta* 1.6 and *beta* 1.3 linked, one D-galactose residue *alpha* 1.4 linked, two D-galactosamine residues *alpha* 1.6 and *alpha* 1.3 linked and one D-fucosamine residue *beta* 1.3 linked. This hexasaccharide was attached to C3 of the branching LD heptose of the inner core with the same structure as for Ye75R core oligosaccharide [15], as depicted in Fig. 2.

Results of the structural studies (comprising methylation and NMR analyses) on YeO3-RfbR7 and YeO3-c-trs8-R core oligosaccharides revealed their structural identity with that presented in Fig. 2 for the Ye75R mutant Rc, whereas these carried on YeO3-R1 core oligosaccharide were showing its similarity to the structure characteristic for the full core oligosaccharide Ye75S. Some differences were observed in a phosphate substitution pattern, what needs to be checked by 600 MHz analysis.

Recently, genetic studies revealed that a truncated core oligosaccharide of the Rc type of *Y. enterocolitica* O:3 mutants trs (temperature responding, [13]) is the acceptor for the O-specific chain. This chain is a homopolymer, and thus giving a smear on the PAGE pattern, Fig. 1, built of 35–70 residues of β6-deoxy-L-altrose [13]. This deoxy sugar was present in small amounts in LPS preparations from all three mutants studied, as well as in the high molecular fractions (Fr. I) of two Rc mutants, and in a substantial amount in Fr. I of a Ra type mutant YeO3-R1 (Table 1). Fr. I contained in addition to 6d-L-altrose the proper core oligosaccharide sugars as well as arabinose and mannose, most probably due to a contamination, originating from some glucan.

Serological studies

SDS/PAGE/Western blot analysis, performed with LPS of all *Y. enterocolitica* O:3

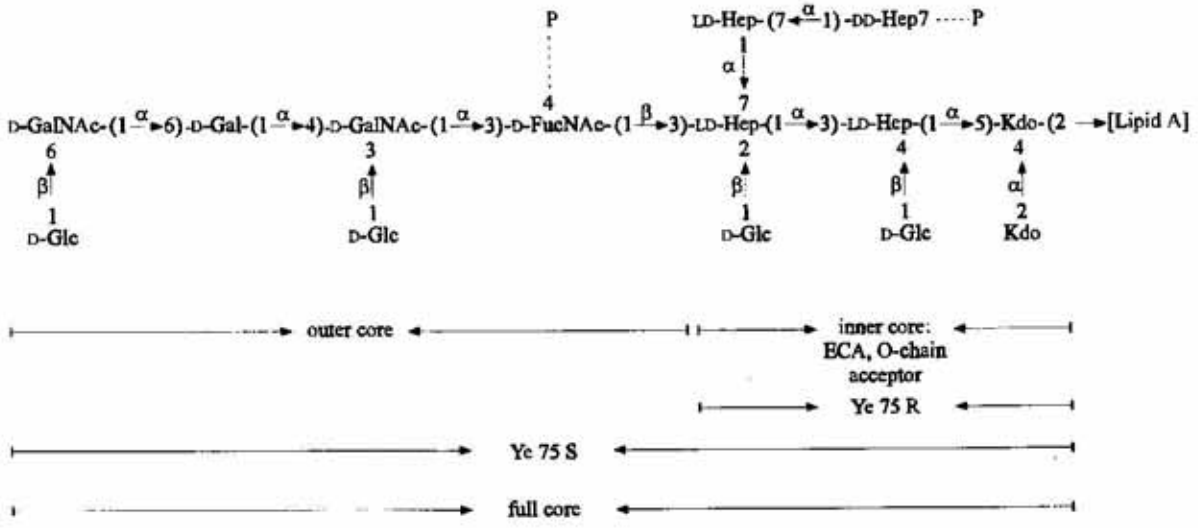

Figure 2. Structure of *Y. enterocolitica* O:3 core.

Ye ..., names of strains used in the present and earlier studies[1] [15]. →, Not equimolar substitution; D-Glc, D-glucose; D-Gal, D-galactose; D-GalNAc, 2-acetamido-2-deoxy-D-galactose; D-FucNAc, 2-acetamido-2,6-dideoxy-D-galactose; DD-Hep 7···P, D-*glycero*-D-*manno*-heptose 7-phosphate; LD-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; P, phosphate.

mutants, LPS of Ye75S and LPS of Re mutant *S. minnesota* using anti O-serum against the Ye75S wild type strain revealed not only a positive reaction with LPS Ye75S and LPS of all *Yersinia* R mutants, but also with Re mutant *S. minnesota* R595. In addition, and very unexpectedly, a ladder-like pattern, not visulaized by direct silver staining (Fig. 1), was observed (see Fig. 3). These results did not allow to answer unequivocally the question concerning the presence of 6d-L-altrose in the core oligosaccharides of Ye O3 mutants. No additional information became available (not shown) when Mab6 against 1.2 linked 6d-L-altrose was used in the system, instead of Ye75S antiserum. Here the positive reaction was observed only for LPS Ye75S, although the Dot test was positive also for Ra mutant YeO3-R1. The possible explanation of these results is that Mab6 as well as antibodies in the antiserum against the Ye75S strain recognize mainly 1.2 linked β-6d-L-altrose residues, and that only one terminal residue of this deoxysugar is present in the core oligosaccharide of Ye O3-R mutants.

A very intriguing, mentioned above, ladder-like banding pattern (Fig. 3), seen for LPS of all Ye O3 mutants was also observed when R-antiserum against the Ye75R strain was used in Western blot analysis, as it is shown in Fig. 4. With the help of Mab against ECA used in a parallel Western blot analysis (not shown) we could establish that the observed banding was due to the presence of the en-

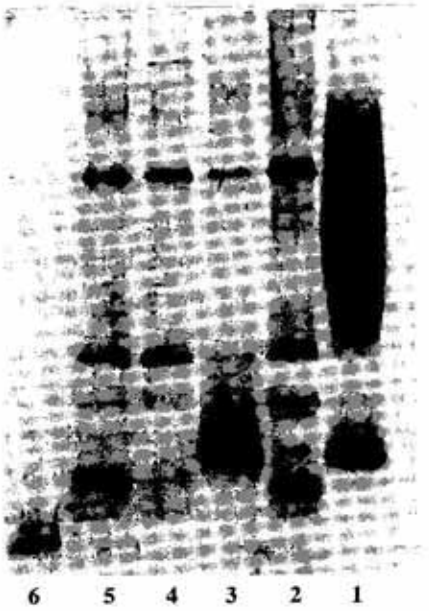

Figure 3. SDS/PAGE/Western blot analysis of lipopolysaccharide samples from *Y. enterocolitica* O:3 S and R strains and *Salmonella* Re strain with antiserum against Ye75S bacteria and goat IgG.

Analysis was performed with proteinase K digested LPS samples (5 μg each) from *Y. enterocolitica* O:3 S and R strains, and rabbit antiserum against *Y. enterocolitica* 75S heat killed bacteria and alkaline phosphatase-conjugated goat anti-rabbit IgG as the second antibody. For SDS/PAGE details see legend to Fig. 1. Lane 1 – *Yersinia enterocolitica* O:3, Ye75S; lane 2 – *Yersinia enterocolitica* O:3, Ye75R; lane 3 – *Y. enterocolitica* O:3, YeO3-R1; lane 4 – *Y. enterocolitica* O:3, YeO3-RfbR7; lane 5 – *Y. enterocolitica* O:3, YeO3-c-trs8-R; lane 6 – *S. minnesota* R595.

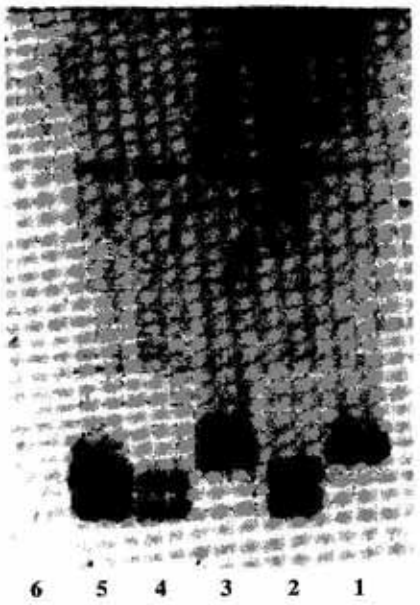

Figure 4. SDS/PAGE/Western blot analysis of lipopolysaccharide samples from *Y. enterocolitica* O:3 S and R strains and *Salmonella* Re strain with antiserum against Ye75R bacteria and goat IgG.

Analysis was performed as described in the legend to Fig. 3, except that only rabbit antiserum against *Y. enterocolitica* 75R (with Rc-type LPS) heat killed bacteria was used. Lane 1 – *Y. enterocolitica* O:3, Ye75S; lane 2 – *Y. enterocolitica* O:3, Ye75R; lane 3 – *Y. enterocolitica* O:3, YeO3-R1; lane 4 – *Y. enterocolitica* O:3, YeO3-RfbR7; lane 5 – *Y. enterocolitica* O:3, YeO3-c-trs8-R; lane 6 – *S. minnesota* R595.

terobacterial common antigen in all lipopolysaccharide preparations of *Yersinia* O3 R mutants. Since in this experiment we used proteinase K digested lipopolysaccharides, we were able to rule out the possibility that the banding was due to contamination of LPS with some proteins. The polysaccharide part of ECA does not stain directly with silver, since it has no vicinal OH-groups which could be split by periodate oxidation. When ECA is linked to LPS it should become visible due to the presence of the core region, unless it carries very long ECA-chains, which seems to be the case with the examined mutants. Usually the amounts of core-linked ECA, although very important for its immunogenicity, are very small [7].

From the fact that rabbit antisera obtained with the S and the Rc mutant of *Y. enterocolitica* O:3 contain a considerable amount of anti-ECA antibodies and from our previous knowledge that only LPS-linked ECA, as it has been established for some *E. coli* mutants [7], is immunogenic, one can assume that ECA is also here attached, like the O-chain [13], to the inner part of the *Y. enterocolitica* O:3 core. Both, the fact that ECA is co-extracted with LPS by the phenol/water method and that mutant *S. minnesota* R595 is an ECA-negative Re mutant are well documented [7, 23]. From the fact that ECA seems to be immunogenic in S and R forms of *Y. enterocolitica* O:3 one could speculate that infections with *Y. enterocolitica* should be paralleled by a considerable rise in the ECA-titer in the patients blood, which can easily be followed by passive hemagglutination. Further study with other serotypes are needed to prove whether this is also relevant for other serotypes of *Y. enterocolitica*.

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