



Structure and Serological Characterization of an N^{ϵ} -[(R)-1-Carboxyethyl]-L-Lysine-Containing O-Chain of the Lipopolysaccharide of *Proteus mirabilis* O13

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Abstract. In this paper we present the structure and describe serological properties of the O-specific polysaccharide of *Proteus mirabilis* O13 lipopolysaccharide, which contains a unique component: an amide of D-galacturonic acid (D-GalA) with an unusual amino acid, N^{ϵ} -[(R)-1-carboxyethyl]-L-lysine (alaninolysine, AlaLys). Selective chemical degradations of either GalA or AlaLys resulted in the loss of the serological reactivity of the polysaccharide with anti-O serum against *P. mirabilis* O13. Neither synthetic stereoisomers of AlaLys nor the isolated amide of GalA with AlaLys inhibited the reaction of the O-antiserum with the homologous lipopolysaccharide. The O-antiserum did not cross-react with the lipopolysaccharide of *Providencia alcalifaciens* O23 containing an amide of D-glucuronic acid with AlaLys. These data showed that both uronic acid and amino acid components of the amide play an important role in manifesting the *P. mirabilis* O13-specificity, but the full specific epitope also includes another O-specific polysaccharide component(s). A cross-reactivity of anti-O13 serum with some other *P. mirabilis* strains was observed and attributed to a common heat-stable antigen(s) different from the lipopolysaccharide.

Key words: *Proteus mirabilis*; lipopolysaccharide; O-specific polysaccharide; serological reactivity; N^{ϵ} -[(R)-1-carboxyethyl]-L-lysine.

Introduction

Proteus mirabilis rods are human opportunistic pathogens which make urinary tract, wound, burn, and respiratory tract infections difficult to treat. After *Es-*

cherichia coli, they are the most common Gram-negative isolates from “urological” patients¹³. Strains that are classified into serogroups O3, O6, O10, O13, O23 and O26–O30 of the Kauffman-Perch scheme are most commonly found in the clinical material⁹. Fimbriae,

Author's fees were financed by the Association for the Joint Administration of Copyright KOPIPOL (Kielce, Poland) from funds collected on the basis of the Law on Author's Rights.

Abbreviations used: LPS – lipopolysaccharide, LPSOH – alkali-treated lipopolysaccharide, OPS – O-specific polysaccharide, OPSda – deaminated O-specific polysaccharide, OPSSD – degraded by periodate oxidation followed by borohydride reduction O-specific polysaccharide.

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urease, immunoglobulin proteases, hemolysins, and outer membrane lipopolysaccharide (LPS) have been considered among the factors of virulence of these bacteria^{12, 18}.

A peculiar feature of *Proteus* LPS is the presence of uronic acids (usually D-GlcA or D-GalA) in the O-specific polysaccharide chain (OPS, O-chain), which in some strains are amidically substituted with amino acids (L-Ala, L-Ser, L-Thr, L-Lys). These constituents have been shown to play a crucial role in manifesting the O-specificity of *P. mirabilis* O3, O27, O28, and *P. penneri* 12 (ATCC 33519)^{1, 4, 17, 21, 24}. In this paper we present the full structure and describe serological properties of *P. mirabilis* O13 OPS, which contains a novel amide of D-galacturonic acid with *N*^ε-[(*R*)-1-carboxyethyl]-L-lysine. This unusual amino acid, called alaninolysine (AlaLys), has been only once reported in bacterial polysaccharides, namely in the LPS of *Providencia alcalifaciens* O23, in which it forms an amide with D-glucuronic acid⁵ and which was also involved in our serological studies. Preliminary data on the OPS structure of *P. mirabilis* O13 have been reported²⁰.

Materials and Methods

Bacterial strain and growth. *P. mirabilis* strain S1959 was from the collection of the Institute of Microbiology and Immunology (University of Łódź, Poland). *P. mirabilis* O13, strain PrK 26/57, and other *P. mirabilis* strains were derived from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *P. alcalifaciens* O23, strain 738/49, came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). Growth of the bacteria was performed as described²².

Isolation and degradations of LPS and OPS. Lipopolysaccharide of *P. mirabilis* O13 was isolated by extraction of dried bacterial cells with hot aqueous phenol²⁵. Alkali-treated LPS was prepared by treatment of the LPS with 0.25 M sodium methanolate in absolute methanol (37°C, 12 h)²⁶. The sediment was washed with cold methanol, dissolved in deionized water, and purified by gel-permeation chromatography on Sephadex G50. The LPS was degraded with dilute acetic acid at 100°C, and the OPS was isolated on Sephadex G50 as described previously²⁰. For degradation of the galacturonic acid residue, OPS was oxidated with 0.1 M sodium periodate at ambient temperature for 48 h in the dark and the product was next reduced with an excess

of sodium borohydride for 2 h. Deamination of the OPS was performed with 5% sodium nitrite in aqueous 20% acetic acid at ambient temperature for 1 h. The modified polysaccharides were isolated by gel chromatography on Sephadex G50 or TSK HW-40 (S).

Chemical analyses. Acid hydrolysis of the OPS was performed with 2 M CF₃COOH (120°C, 2 h). Neutral sugars and uronic acids were identified using a Biotronik LC-2000 analyser (Germany) on a Dionex A × 9–11 resin in 0.5 M sodium borate, pH 8.0, at 65°C and 0.04 M sodium phosphate, pH 2.4, at 70°C, respectively. Amino components were identified using a Biotronik LC-2000 analyzer on an Ostion LG AN B resin in 0.35 M sodium citrate, pH 5.28, at 80°C.

For isolation of AlaLys, the OPS hydrolysate was fractioned on TSK HW-40 (S) in water, and AlaLys was converted into ammonium salt using a column of an IRA-120 (H⁺-form) resin and elution with aqueous 5% ammonia. The authentic samples of *N*^ε-[(*R*)-1-carboxyethyl]-L-lysine (**1**) and *N*^ε-[(*S*)-1-carboxyethyl]-L-lysine (**2**) were synthesised as described^{3, 5}.

Solvolysis of the OPS with anhydrous trifluoromethanesulfonic acid was performed at -4°C for 2 h. After neutralisation with aqueous 25% ammonia at 4°C, amide of GalA with AlaLys (**3**) was isolated by gel chromatography on TSK HW-40 (S).

NMR spectroscopy. NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D₂O at 70, 57 and 27°C for the OPS, AlaLys, and amide **3**, respectively, using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process the NMR data. A mixing time of 230 ms was used in a rotating-frame nuclear Overhauser effect (ROESY) experiment.

Rabbit anti-O13 serum. New Zealand rabbits were immunized with a suspension (1.5 × 10¹⁰ CFU ml⁻¹) of *P. mirabilis* O13 heat-killed (2 h, 100°C) cells in doses of 250, 500 and 1000 µl over three weeks via the marginal ear vein injection. Blood was drawn from the animals on day 58 after receiving the first immunization dose¹⁹.

ELISA and inhibition assay. The MaxiSorp (Nunc) immunoplates were coated with autoclaved bacterial cells (5 µg per well) or LPS (0.5 µg per well) in phosphate buffered saline (PBS), pH 7.2. After a 3 h incubation at 37°C with gentle shaking and 16 h at 4°C, the plates were washed 6 times with PBS and blocked with 10% skimmed milk in PBS. After a 1 h incubation at 37°C with diluted rabbit antiserum and then with peroxidase-conjugated goat anti-rabbit IgG (Sigma), the color reaction was developed using 2,2'-azino-bis(3-

-ethylbenz-thiazoline-6-sulfonic acid) as substrate for peroxidase. The last serum dilution, giving $OD \geq 0.2$ at 405 nm, measured with an EL 312e Reader (Bio-Tek Instruments), was taken as the serum titer.

In the inhibition test, the dilution of antiserum giving $OD = 1.00$ in ELISA with the homologous LPS was used. The last dose of inhibitors causing a decrease in the OD value to 0.50 was taken as the inhibitory dose.

Other serological techniques. In the absorption test, 1 ml prediluted antiserum (1:250 in PBS) was incubated with 200 μ g autoclaved bacteria for 2 h at ambient temperature and 16 h at 4°C with gentle shaking. For ELISA, the absorbed serum was centrifuged (4 min, $12\,000 \times g$) and diluted to 1:500.

The quantitative precipitation test was performed essentially as described²³. The amount of protein in the precipitate was determined by the Lowry procedure¹¹. In some experiments, after precipitation of LPS-specific antibodies with a dose of antigen giving the maximal level of the reaction, the antiserum was used in ELISA.

Passive immunohemolysis and passive hemagglutination tests were performed according to published procedures⁷.

Results and Discussion

Composition and structure of the OPS of *P. mirabilis* O13

Sugar analysis of the OPS, including determination of the absolute configuration of the monosaccharides, revealed D-galactose (D-Gal), 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), and D-galacturonic acid. Amino acid analysis showed the presence in the OPS hydrolysate of a neutral amino acid, which was separated from the monosaccharides by gel chromatography. The ¹H- and ¹³C-NMR spectra of the amino acid showed signals for lysine and alanine, but the signals for C-6 of lysine and C-2 of alanine were shifted down-field to δ 47.1 and 58.9, compared with their positions at δ 40.6 and 51.6, respectively, in the corresponding free amino acids. Therefore, it was suggested that the unknown amino acid is *N*^ε-(1-carboxyethyl) lysine (AlaLys).

To confirm this suggestion and to determine the absolute configuration, AlaLys was compared with the authentic samples of *N*^ε-[(*R*)-1-carboxyethyl]-L-lysine (**1**) and *N*^ε-[(*S*)-1-carboxyethyl]-L-lysine (**2**) (Fig. 1). Both isomers had the same retention time in amino acid analysis and the same ¹H-NMR chemical shifts as the natural AlaLys. However, the ¹³C-NMR spectrum of

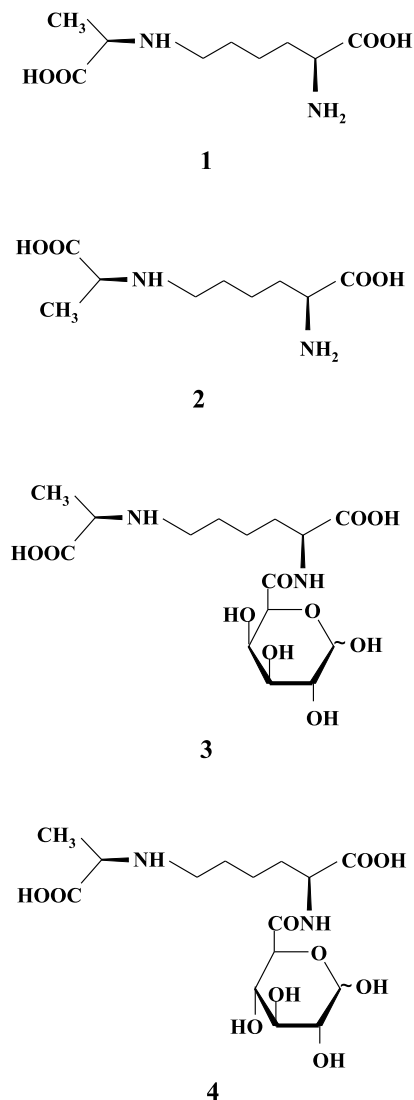


Fig. 1. Structures of *N*^ε-[(*R*)-1-carboxyethyl]-L-lysine (**1**), *N*^ε-[(*S*)-1-carboxyethyl]-L-lysine (**2**), *N*^ε-[(*R*)-1-carboxyethyl]-*N*^α-(D-galacturonoyl)-L-lysine (**3**) and *N*^ε-[(*R*)-1-carboxyethyl]-*N*^α-(D-glucuronoyl)-L-lysine (**4**)

natural AlaLys was indistinguishable from the spectrum of isomer **1** but different from that of isomer **2**, the ¹³C-NMR chemical shifts being consistent with published data⁵. A positive optical rotation value of the natural AlaLys, $[\alpha]_D + 6.5^\circ$ (*c* 0.4, water), showed that the lysine residue had the L configuration (compare published data¹⁰: $[\alpha]_D + 9.7^\circ$ and $+11.6^\circ$ (water) for compounds **1** and **2**, respectively). Therefore, the unusual amino acid present in the OPS of *P. mirabilis* O13 was *N*^ε-[(*R*)-1-carboxyethyl]-L-lysine.

To determine the location of AlaLys, the OPS was subjected to solvolysis with anhydrous trifluoromethanesulfonic acid, which cleaves glycosidic but not amidic linkages¹⁵. The ¹H- and ¹³C-NMR spectra of the resultant amide **3** showed signals for α - and β -GalA and AlaLys. The signal for H-2 of the lysine residue

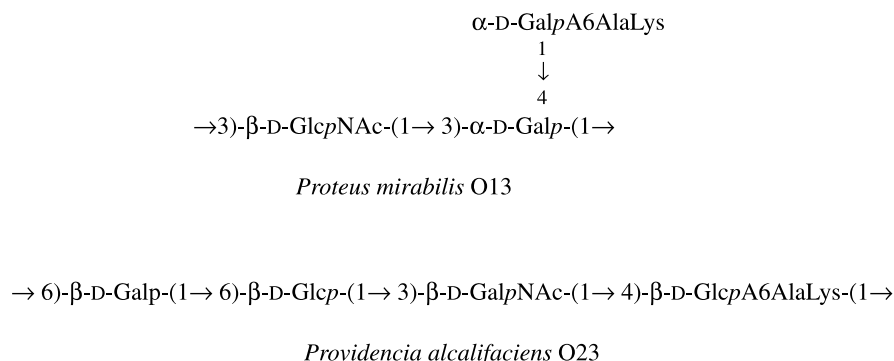


Fig. 2. Structures of the O-specific polysaccharides of *Proteus mirabilis* O13 and *Providencia alcalifaciens* O23. D-GalA6AlaLys and D-GlcA6AlaLys stand for N^{ϵ} -[(R)-1-carboxyethyl]- N^{α} -(D-galacturonoyl)-L-lysine and -(D-glucuronoyl)-L-lysine, respectively

was shifted down-field to δ 4.4, compared with its position near δ 3.8 in the free amino acid⁵, thus indicating acylation at N-2. Accordingly, C-6 of GalA resonated at δ 172.3, which is characteristic for amides of uronic acids². These data are similar to those of an amide of GlcA with AlaLys, isolated from the OPS of *P. alcalifaciens* O23⁵, and showed that the OPS of *P. mirabilis* O13 contains N^{ϵ} -[(R)-1-carboxyethyl]- N^{α} -(D-galacturonoyl)-L-lysine (3) (Fig. 1).

The structure of the OPS was studied using two-dimensional NMR spectroscopy as described previously²⁰. Briefly, the ^1H -NMR spectrum of the OPS was assigned using ^1H , ^1H shift-correlated experiments (COSY and TOCSY) and spin systems of α -Galp, β -GlcNAc, and α -GalpA were identified. The ^{13}C -NMR spectrum of the OPS was assigned using a ^1H , ^{13}C heteronuclear shift-correlated experiment (HMQC), and the sugar glycosylation pattern was determined by the ^{13}C -NMR chemical shift data¹⁰. Finally, a nuclear Overhauser effect experiment (ROESY) revealed inter-residue connectivities between the transglycosidic protons and, thus, demonstrated the sequence of the monosaccharide residues.

On the basis of the data obtained, it was concluded that the OPS of *P. mirabilis* O13 has the structure shown in Fig. 2. The OPS contains a unique component: an amide of D-galacturonic acid with N^{ϵ} -[(R)-1-carboxyethyl]-L-lysine, which has not been hitherto found in nature. An amide of D-glucuronic acid with the same amino acid (4) has been previously reported as a component of the OPS of *P. alcalifaciens* O23⁵; the full structure of this OPS⁶ is also shown in Fig. 2.

Serological studies

The following *P. mirabilis* O13 antigens and derived products were used in studies with rabbit poly-

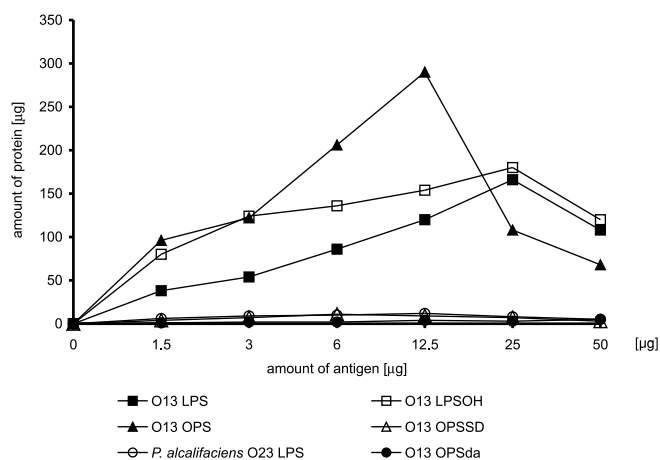


Fig. 3. Quantitative precipitation of anti-O serum against *P. mirabilis* O13 with homologous and heterologous antigens. The mean values of three independent experiments are shown. LPS – lipopolysaccharide, LPSOH – alkali-treated lipopolysaccharide, OPS – O-specific polysaccharide, OPSSD – degraded by periodate oxidation followed by borohydride reduction O-specific polysaccharide, OPSda – deaminated O-specific polysaccharide

clonal anti-O serum against *P. mirabilis* O13: LPS, alkali-treated (O-deacylated) LPS, OPS, degraded by periodate oxidation followed by borohydride reduction OPS (with GalA selectively destroyed), deaminated OPS (with AlaLys selectively destroyed), and amide of D-GalA with AlaLys 3. In addition, synthetic amino acids 1 and 2 and LPS of *P. alcalifaciens* O23 containing amide of D-GlcA with AlaLys 4 (Fig. 1, 2) were involved.

Anti-O13 serum reacted with the homologous LPS or alkali-treated LPS in ELISA, passive hemolysis, and passive hemagglutination tests to the titers 1 : 64 000, 1 : 51 200 and 1 : 40 960, respectively. In quantitative precipitation assay, both LPS and alkali-treated LPS bound comparable amounts of antibodies with the

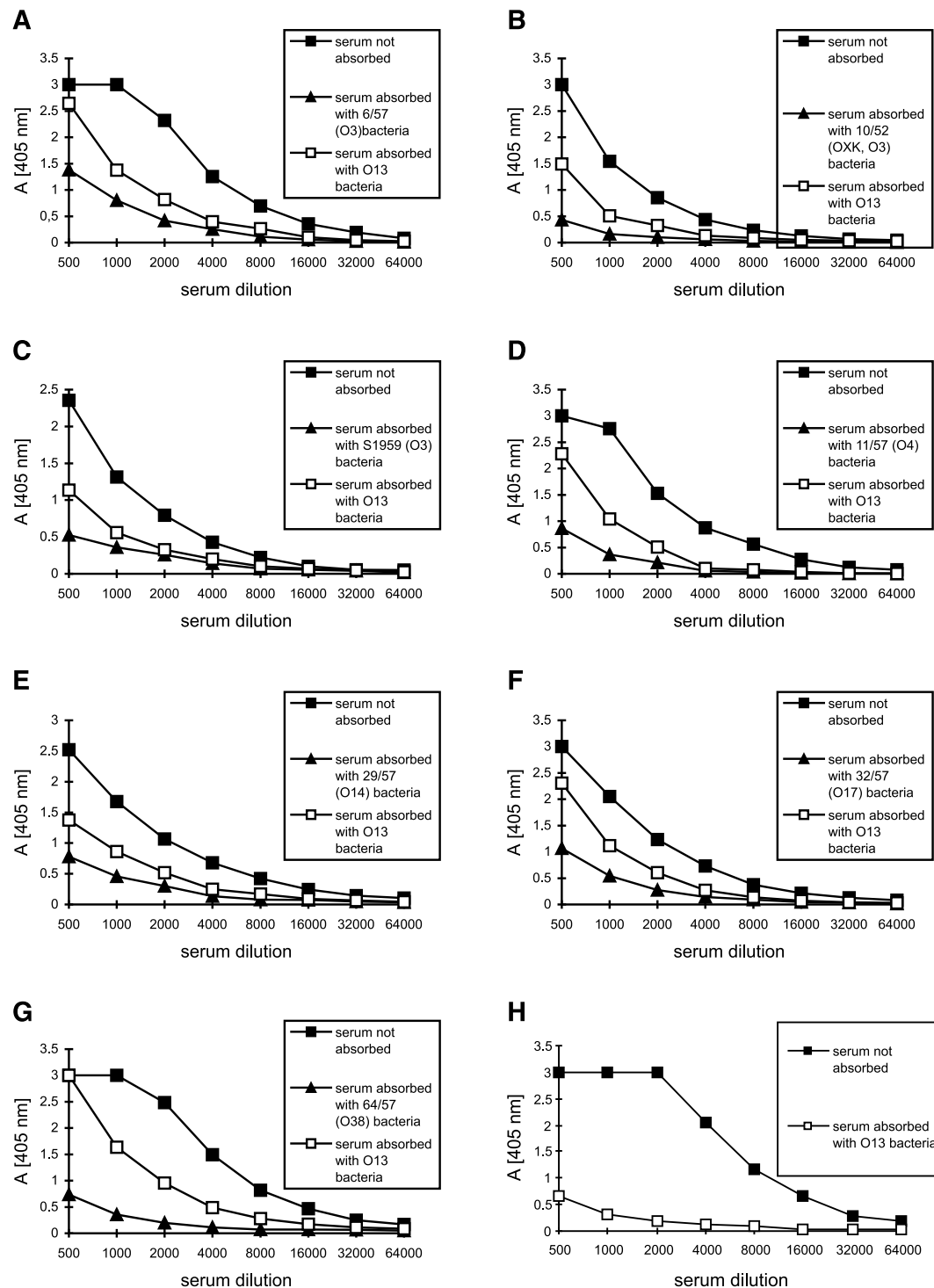


Fig. 4. Reactions of absorbed and non-absorbed anti-O13 serum with homologous and heterologous antigens in ELISA. The mean values of three independent experiments are shown. As coating antigens were used dry cells of strains: A – *P. mirabilis* PrK 6/57 (O3), B – *P. mirabilis* PrO 10/52 (OXK, O3), C – *P. mirabilis* S1959 (O3), D – *P. mirabilis* 11/57 (O4), E – *P. mirabilis* PrK 29/57 (O14), F – *P. mirabilis* PrK 32/57 (O17), G – *P. mirabilis* PrK 64/57 (O38), H – *P. mirabilis* PrK 26/57 (O13)

maximum at an antigen dose of 25 μg (Fig. 3). The purified OPS precipitated nearly twice as many antibodies at a dose of 12.5 μg compared with LPS. The degraded by periodate oxidation followed by borohydride reduction OPS and deaminated OPS were inac-

tive. Therefore, destruction of either GalA or AlaLys affected the major antigenic determinant and, hence, both components are necessary for binding *P. mirabilis* O13-specific antibodies. No reaction was observed between anti-O13 serum and *P. alcalifaciens* O23 LPS.

Table 1. Inhibition of the reaction of anti-O serum against *P. mirabilis* O13 with the homologous LPS in ELISA

Inhibitor	Inhibitory dose (ng)	Titer of antiserum after preincubation with inhibitor
<i>P. mirabilis</i> O13 LPS	9.5	< 1 : 4 000
Alkali-treated LPS	78	< 1 : 4 000
OPS	78	< 1 : 4 000
degraded by periodate oxidation followed by borohydride reduction OPS	>10 000	1 : 64 000
Deaminated OPS	10 000	1 : 32 000
Amino acid 1	>10 000	1 : 64 000
Amino acid 2	>10 000	1 : 64 000
Amide 3	>10 000	1 : 64 000
<i>P. alcalifaciens</i> O23 LPS	>10 000	1 : 64 000

The mean values of three independent experiments are shown.

Table 2. Reactions of anti-O13 serum with series of *P. mirabilis* antigens in ELISA

Strain/serogroup	Antiserum titer	
	not precipitated	precipitated ^a
PrK 26/57, O13	1 : 64 000	1 : 8 000
PrO 10/52, OXK, O3	1 : 16 000	1 : 8 000
S1959, O3	1 : 16 000	1 : 8 000
PrK 6/57, O3	1 : 8 000	1 : 8 000
PrK 11/57, O4	1 : 16 000	1 : 16 000
PrK 29/57, O14	1 : 8 000	1 : 4 000
PrK 32/57, O17	1 : 4 000	1 : 4 000
PrK 64/57, O38	1 : 8 000	1 : 4 000

^a Serum after precipitation with O13 lipopolysaccharide.

The mean values of three independent experiments are shown.

This finding confirmed that AlaLys alone is not sufficient for binding *P. mirabilis* O13-specific antibodies.

P. mirabilis O13 LPS, alkali-treated LPS, and OPS strongly inhibited the reaction of anti-O13 serum with the homologous LPS in ELISA. Preincubation of anti-O13 serum with all these antigens decreased the titer of the reaction to < 1: 4000. In contrast, the degraded by periodate oxidation followed by borohydride reduction OPS and deaminated OPS, as well as amino acids 1 and 2, amide 3, and LPS of *P. alcalifaciens* O23, showed no or almost no inhibitory activity (Table 1). Similar results were obtained with these products in inhibition of passive hemolysis (data not shown). A weak inhibition observed with the deaminated OPS may be due to incomplete deamination or the presence of a minor antibody fraction not detectable in precipitation test which does not require AlaLys for binding. Therefore, although amide 3, which is the lateral sugar

in OPS, plays a crucial role in manifesting the O13-specificity, the full specific epitope also includes another OPS component(s) present in the main chain.

Anti-O13 serum was found to react in ELISA with whole cells of strains of four other *P. mirabilis* O-serogroups from 23 O-serogroups of the Kauffman-Perch scheme tested, namely *P. mirabilis* O3, O14, O17, and O38 (Table 2). Absorption from anti-O13 serum of O13-LPS specific antibodies (by *P. mirabilis* O13 LPS at a dose of 25 µg) decreased the titer of the cross-reactions only insignificantly (Table 2).

Moreover, absorption of the investigated serum with cross-reacting bacterial cells did not change the level of its reaction with the homologous cells in ELISA, whereas the cross-reactions of absorbed serum with the corresponding antigens were strongly inhibited. The level of inhibition was markedly lower when serum pretreated with *P. mirabilis* O13 cells was tested (Fig. 4).

These results suggest that the cross-reactivity observed is due to the presence of a common heat-stable antigen(s) different from LPS, like, e.g., the enterobacterial common antigen⁸. In accordance with this conclusion, no marked similarity is observed between the structure of the O-specific polysaccharide of *P. mirabilis* O13 (this work) and those of *P. mirabilis* O3²⁷ and O14¹⁶ (the structures of *P. mirabilis* O17 and O38 have not yet been established). It should be noted that in earlier studies by PENNER and HENNESSY¹⁴ the cross-reactivity of anti-O13 serum shown by us were not observed. This discrepancy may be accounted for by different procedures employed by the two groups for rabbit immunization (with formalin-treated bacterial suspensions vs. heat-killed cells) and different serological assays (passive hemagglutination vs. ELISA). Interestingly, the same authors described, among “multiply agglutinating isolates of *P. mirabilis*”, a strain agglutinated by anti-O sera against *P. mirabilis* O3 (titer 1 : 80), *P. mirabilis* O13 (1 : 5120), *P. mirabilis* O18 (1 : 1280) and *P. mirabilis* O30 (> 1 : 40960)¹⁴, which might also reflect similarities between heat-stable antigens other than LPS.

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Received in March 2000

Accepted in September 2000