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

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\textbf{Abstract.} In this paper we present the structure and describe serological properties of the O-specific polysaccharide of \textit{Proteus mirabilis} O13 lipopolysaccharide, which contains a unique component: an amide of D-galacturonic acid (D-GaLA) with an unusual amino acid, $N^\epsilon$-[($R$)-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 \textit{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 \textit{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 \textit{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 \textit{P. mirabilis} strains was observed and attributed to a common heat-stable antigen(s) different from the lipopolysaccharide.

\textbf{Key words:} \textit{Proteus mirabilis}; lipopolysaccharide; O-specific polysaccharide; serological reactivity; $N^\epsilon$-[($R$)-1-carboxyethyl]-L-lysine.

\textbf{Introduction}

\textit{Proteus mirabilis} rods are human opportunistic pathogens which make urinary tract, wound, burn, and respiratory tract infections difficult to treat. After \textit{E. coli}, they are the most common Gram-negative isolates from “urological” patients\textsuperscript{13}. 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 material\textsuperscript{7}. Fimbriae,
urease, immunoglobulin proteases, hemolysins, and outer membrane lipopolysaccharide (LPS) have been considered among the factors of virulence of these bacteria.  

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 amicridly 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^\*\{[(R)-1-carboxyethyl]-L-lysine. This unusual amino acid, called alaminolysine (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 acid and which was also involved in our serological studies. Preliminary data on the OPS structure of *P. mirabilis* O13 have been reported.

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 73K/49, came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). Growth of the bacteria was performed as described.

**Isolation and degradation of LPS and OPS.** Lipopolysaccharide of *P. mirabilis* O13 was isolated by extraction of dried bacterial cells with hot aqueous phenol. Alkali-treated LPS was prepared by treatment of the LPS with 0.25 M sodium methanolate in absolute methanol (37°C, 12 h). 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 previously. For degradation of the galacturonic acid residue, OPS was oxidized 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 CF₂COOH (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\*\{[(R)-1-carboxyethyl]-L-lysine (1) and N\*\{(S)-1-carboxyethyl]-L-lysine (2) were synthesised as described.

Solvolyisis 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 × 10⁸ 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 dose.

**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-
Sugars 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 $^1$H- and $^{13}$C-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 $\delta$ 47.1 and 58.9, compared with their positions at $\delta$ 40.6 and 51.6, respectively, in the corresponding free amino acids. Therefore, it was suggested that the unknown amino acid is N$^\text{a}$-1-carboxyethyl lysine (AlaLys).

To confirm this suggestion and to determine the absolute configuration, AlaLys was compared with the authentic samples of N$^\text{a}$-[(R)-1-carboxyethyl]-L-lysine (1) and N$^\text{a}$-[(S)-1-carboxyethyl]-L-lysine (2) (Fig. 1). Both isomers had the same retention time in amino acid analysis and the same $^1$H-NMR chemical shifts as the natural AlaLys. However, the $^{13}$C-NMR spectrum of natural AlaLys was indistinguishable from the spectrum of isomer 1 but different from that of isomer 2, the $^{13}$C-NMR chemical shifts being consistent with published data$^3$. 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 data$^{10}$: $[\alpha]_D + 9.7^\circ$ 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$^\text{a}$-[(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 linkages$^{15}$. The $^1$H- and $^{13}$C-NMR spectra of the resultant amide 3 showed signals for $\alpha$- and $\beta$-GalA and AlaLys. The signal for H-2 of the lysine residue
was shifted down-field to δ 4.4, compared with its position near δ 3.8 in the free amino acid, thus indicating acylation at N-2. Accordingly, C-6 of GalA resonated at δ 172.3, which is characteristic for amidites of uronic acids. These data are similar to those of an amidite of GlcA with AlaLys, isolated from the OPS of P. alcalifaciens O23, and showed that the OPS of P. mirabilis O13 contains N⁴-[a(R)-1-carboxyethyl]-N⁵-(D-galacturonoyl)-L-lysine (3) (Fig. 1).

The structure of the OPS was studied using two-dimensional NMR spectroscopy as described previously. 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 ¹³C-NMR spectrum of the OPS was assigned using a ¹H, ¹³C heteronuclear shift-correlated experiment (HMOC), and the sugar glycosylation pattern was determined by the ¹³C-NMR chemical shift data. 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 amidite of D-galacturonic acid with N⁴-[a(R)-1-carboxyethyl]-L-lysine, which has not been hitherto found in nature. An amidite of D-glucuronic acid with the same amino acid (4) has been previously reported as a component of the OPS of P. alcalifaciens O23; the full structure of this OPS is also shown in Fig. 2.

Serological studies

The following P. mirabilis O13 antigens and derived products were used in studies with rabbit polyclonal anti-O serum against P. mirabilis O13: LPS, alkali-treated (O-deacetylated) LPS, OPS, degraded by periodate oxidation followed by borohydride reduction O-specific polysaccharide, OPSSD – degraded by periodate oxidation followed by borohydride reduction O-specific polysaccharide, OPSSd – deaminated O-specific polysaccharide.

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, LPSON – alkali-treated lipopolysaccharide, OPS – O-specific polysaccharide, OPSSD – degraded by periodate oxidation followed by borohydride reduction O-specific polysaccharide, OPSSda – deaminated O-specific polysaccharide.

"Proteus mirabilis" O13

\[
\alpha-D-GalpA6AlaLys \\
\downarrow \\
\rightarrow 3)-\beta-D-GlcNAc-(1\rightarrow 3)-\alpha-D-Galp-(1\rightarrow
\]

"Proteus mirabilis" O13

\[
\rightarrow 6)-\beta-D-Galp-(1\rightarrow 6)-\beta-D-Glc-(1\rightarrow 3)-\beta-D-GlcNAc-(1\rightarrow 4)-\beta-D-GlcA6AlaLys-(1\rightarrow
\]

"Proteus mirabilis" O13

Fig. 2. Structures of the O-specific polysaccharides of Proteus mirabilis O13 and Providencia alcalifaciens O23. D-GalA6AlaLys and D-GlcA6AlaLys stand for N⁴-[a(R)-1-carboxyethyl]-N⁵-(D-galacturonoyl)-L-lysine and -D-glucuronoyl)-L-lysine, respectively.
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 inactive. 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. alcalafaciens* O23 LPS.
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. calcalificiens* 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 antigen\(^4\). 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* O3\(^{27}\) and O14\(^19\) (the structures of *P. mirabilis* O17 and O38 have not yet been established). It should be noted that in earlier studies by Penner and Hennessy\(^14\) 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)\(^5\), which might also reflect similarities between heat-stable antigens other than LPS.

### References


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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitory dose (ng)</th>
<th>Titer of antiserum after preincubation with inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. mirabilis</em> O13 LPS</td>
<td>9.5</td>
<td>&lt; 1 : 4000</td>
</tr>
<tr>
<td>Alkali-treated LPS</td>
<td>78</td>
<td>&lt; 1 : 4000</td>
</tr>
<tr>
<td>OPS</td>
<td>78</td>
<td>&lt; 1 : 4000</td>
</tr>
<tr>
<td>degraded by periodate oxidation followed by borohydride reduction OPS</td>
<td>&gt; 10 000</td>
<td>1 : 64 000</td>
</tr>
<tr>
<td>Deaminated OPS</td>
<td>10 000</td>
<td>1 : 32 000</td>
</tr>
<tr>
<td>Amino acid 1</td>
<td>&gt; 10 000</td>
<td>1 : 64 000</td>
</tr>
<tr>
<td>Amino acid 2</td>
<td>&gt; 10 000</td>
<td>1 : 64 000</td>
</tr>
<tr>
<td>Amide 3</td>
<td>&gt; 10 000</td>
<td>1 : 64 000</td>
</tr>
<tr>
<td><em>P. calcalificiens</em> O23 LPS</td>
<td>&gt; 10 000</td>
<td>1 : 64 000</td>
</tr>
</tbody>
</table>

The mean values of three independent experiments are shown.

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

<table>
<thead>
<tr>
<th>Strain/serogroup</th>
<th>Antiserum titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>not precipitated</td>
</tr>
<tr>
<td>PrK 26/57, O13</td>
<td>1 : 64 000</td>
</tr>
<tr>
<td>PrO 10/52, OKX, O3</td>
<td>1 : 16 000</td>
</tr>
<tr>
<td>S1959, O3</td>
<td>1 : 16 000</td>
</tr>
<tr>
<td>PrK 6/57, O3</td>
<td>1 : 8 000</td>
</tr>
<tr>
<td>PrK 11/57, O4</td>
<td>1 : 16 000</td>
</tr>
<tr>
<td>PrK 29/57, O14</td>
<td>1 : 8 000</td>
</tr>
<tr>
<td>PrK 32/57, O17</td>
<td>1 : 4 000</td>
</tr>
<tr>
<td>PrK 64/57, O38</td>
<td>1 : 8 000</td>
</tr>
</tbody>
</table>

* Serum after precipitation with O13 lipopolysaccharide.

The mean values of three independent experiments are shown.
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