Re-classification within the serogroups O3 and O8 of Citrobacter strains

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Abstract

Background: Citrobacter strains are opportunistic pathogens often responsible for serious enteric as well as extra-intestinal diseases, and therefore the O-antigenic scheme, still in use in diagnostic identification, should be set for proper serotyping. The structures of more than 30 different Citrobacter O-antigens (O-polysaccharide chains of the lipopolysaccharides) of 43 Citrobacter O-serogroups have been elucidated so far. However, relationships between strains in several heterogeneous serogroups still need to be clarified by immunochemical studies. These include complex serogroups O3 and O8, represented by 20 and 7 strains, respectively, which are the subject of the present work. Earlier, the O-polysaccharide structures have been determined for Citrobacter O3 strain Be35/57 (PCM 1508) and Citrobacter O8 strain Be64/57 (PCM 1536).

Results: Serological studies (immunoblotting) carried out on Citrobacter lipopolysaccharides from different strains ascribed to serogroups O3 and O8 showed that each of these serogroups should be divided into non-cross-reacting subgroups. Based on the results of chemical analyses and ¹H and ¹³C NMR spectroscopy the structure of Citrobacter O-antigens from strains PCM 1504 (O6) and PCM 1573 (O2) have been established. Chemical data combined with serological analyses showed that several Citrobacter strains should be reclassified into other serogroups.

Conclusions: Immunochemical studies carried out on Citrobacter LPS, described in this paper, showed the expediency of reclassification of: 1) strains PCM 1504 and PCM 1573 from serogroups O6 and O2 to serogroups O3 and O8, respectively, 2) strains PCM 1503 and PCM 1505 from serogroups O3 and O8 to new serogroups O3a and O8a, respectively.

Keywords: Citrobacter, Lipopolysaccharide, O-antigen structure, Serological specificity, Bacterial classification, Enterobacteria

Background

Bacteria of the genus Citrobacter of the family Enterobacteriaceae are normal inhabitants of animal and human intestinal tract. Some Citrobacter strains are often associated with extraintestinal disorders, among which the most significant are neonatal meningitis and brain abscesses [1, 2].

Serotyping and classification of these bacteria is important for diagnostic purposes. The genus Citrobacter is taxonomically most closely related to Salmonella and Escherichia coli. Currently, strains of the genus Citrobacter are classified into 11 genomospecies based on genetic studies [3]. Serological heterogeneity of Citrobacter strains is defined by the structure diversity of the O-antigen [4–6], which represents the O-specific polysaccharide chain (OPS) of the cell-surface lipopolysaccharide (LPS). Based on the LPS O-antigens, Citrobacter strains are divided into 43 O-serogroups [7] and 20 chemotypes [5].

Structural analysis of the OPS is crucial for unambiguous assignment of serotypes and their cross-reactivity. This is especially important in the case of Citrobacter where heterogeneity of strains within particular serogroup is observed and after re-classification some serotypes and O-antigens are overlapping in different species. Structures of the OPS and core domains of LPS are an efficient tool in classification of Gram-negative bacteria. Structural analysis of Citrobacter is an example for such approach enabling fine taxonomic classification.
of these bacteria. Elucidation of structures of over 30 different Citrobacter OPS improved the serological classification of strains and explained multiple cross-reactions between Citrobacter and other genera of the family Enterobacteriaceae, such as Hafnia, Escherichia, Klebsiella, and Salmonella [6, 8] (and refs cited herein). Although many Citrobacter OPS structures have been established, in several heterogeneous serogroups the O-antigens require further immunochemical studies.

The present work is devoted to serogroups O3 and O8, represented by 20 and 7 strains, respectively. The OPS structures have been determined for Citrobacter O3 strain Be35/57 (PCM 1508) [9] and Citrobacter O8 strain Be64/57 (PCM 1536) [10]. Serological studies of number of strains belonging to serogroups O3 and O8 indicated that each of these serogroups can be further divided into two non-cross-reacting subgroups. Moreover, it was shown that two strains from other serogroups, PCM 1504 (O6) and PCM 1573 (O2), should be reclassified into O3 and O8 serogroups, respectively. Two other strains, PCM 1503 and PCM 1505, should be reclassified from serogroups O3 and O8 into new serogroups. These studies prove that the existing O-antigenic scheme used for serotyping, classification and diagnostic purposes needs modifications to make it consistent with the recent taxonomic changes and serological and structural data on the Citrobacter O-antigens.

**Methods**

**Bacterial strains, cultivation, isolation of LPS and OPS**

Citrobacter strains listed in Table 1 were derived from the Polish Collection of Microorganisms (PCM) of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Analysis of bacteria performed by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) with standard procedure using Bruker Daltonics UltrafleXtreme spectrometer and Biotyper 3.1 software proved the classification of all strains to Citrobacter genus. Bacteria were cultivated in a Davis broth medium supplemented with casein hydrolysate and yeast extract (Difco), with aeration at 37 °C for 24 h, then harvested and freeze-dried.

The LPS were isolated from bacterial mass by the phenol-water procedure [11], recovered from water phase and purified as described [12]. The LPS (called LPS I) from two strains (Be35/57 and Be64/57) were isolated by dialysis of the phenol-water extract without separation of layers and purified with coldaq 50% trichloroacetic acid to precipitate proteins and nucleic acid [6]. The yield of the LPS was 1.5–2.5% of dry bacterial mass.

LPS was heated with 1% acetic acid at 100 °C for 1–2 h and the carbohydrate-containing supernatant was fractionated by gel filtration chromatography on a Sephadex G-50 Fine column (100 × 2.0 cm) in 0.05 M pyridinium acetate buffer pH 5.4 to obtain a high-molecular mass O-polysaccharide.

**Preparation of sera and immunochemical analysis**

Rabbit sera against whole cells of Citrobacter strains of serotype O3 (PCM 1497 and PCM 1508) and O8 (PCM 1533, 1536, 1572, 2539) were prepared as described earlier [13]. Sera against cells of strains PCM 1503, 1505 and PCM 1531 were from previous studies [8, 14, 15]. The animal studies were conducted in strict accordance with the ethical guidelines established by the National Ethics Committee and approved by the First Local Ethics Commission at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (LKE 53/2009).

SDS-PAGE of LPS was performed by the method of Laemmi [16]. The gels were stained with the silver reagent [17] or immunoblotted according to [18]. Briefly, after separation in SDS-PAGE, the LPS was transblotted from the gel onto a PVDF membrane (Immobilon P, Millipore). The air-dried membrane was incubated with anti-Citrobacter serum diluted in TBS-T (20 mM Tris-HCl, 50 mM NaCl, 0.05% Tween-20, pH 7) containing 1% BSA, washed with TBS-T and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase diluted in TBS-T. The immunoblot was visualised with the staining reagent (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.05 M Tris/HCl pH 9.5 containing 5 mM MgCl₂).

Passive hemagglutination assay was performed as described previously [13]. The LPS were at first heated (1 mg/ml PBS, 100 °C, 2 h) or alkali treated (0.25 M NaOH, 56 °C, 1 h) [19]. The sheep erythrocytes (0.2 ml of packed cells) were coated with a suspension of 1 mg LPS/ml PBS at 37 °C for 1 h. The hemagglutination assay was performed with 1% erythrocytes and ten-fold dilutions of serum, all in PBS at 37 °C for 2 h. Results were expressed as the reciprocal titres of the serum dilutions.

**Sugar and methylation analysis**

A sample of the OPS was hydrolyzed with 2 M TFA (120 °C, 2 h), monosaccharides were converted conventionally into the alditol acetates and analyzed by GLC-MS on a Hewlett-Packard 5971A instrument equipped with an HP-1 capillary glass column (12 m × 0.2 mm) using a temperature program of 150 °C (3 min) to 270 °C at 8 °C min⁻¹. Methylation of the OPS was performed by the method of Gunnarsson [20]. The partially methylated monosaccharides were derived by hydrolysis of the methylated polysaccharide as in sugar analysis or with 10 M HCl (80 °C, 30 min), converted into the alditol acetates and analyzed by GLC-MS as above.
NMR spectroscopy
Polysaccharide samples were freeze-dried twice from a 99.9% D₂O solution and dissolved in 99.95% D₂O. 
¹H- and 
¹³C–NMR spectra were recorded at 53 °C on a Bruker DRX-500 spectrometer (Germany) and chemical shifts are reported with internal acetone (δH 2.225, δC 31.45) as reference for calibration. The NMR spectra were recorded and data processed using standard Bruker software.

Results and discussion
Serological studies
The strains ascribed by several authors [4, 21, 22] to serogroups O3 and O8 are listed in Table 1. The names of species are used according to Miki et al. [21]. As the serological identity of several strains was ambiguous we have characterized them immunochemically. The passive hemagglutination test of Citrobacter O8 LPS with rabbit antisera against the whole bacteria revealed high degree

Table 1 Strains used in this work for studies of Citrobacter serogroups O3 and O8

| Collection number and name | Serotype | Collection | Serotype |
|----------------------------|----------|------------|----------|
| 1497 8/50 M.Wright Na12 | 3a,3b,1c:5,6 | (Lanyi, 1984)¹ | Na12 3:5,6 |
| 1508 35/57 P.R. Edwards | 3a,3b,1c:4,5 | (Miki et al., 1996)² | Na12 3:4,5 |
| 1498 9/50 M.Wright Na11 | 3a,3b,1c:8,9 | | Na11 3:8,9 |
| 1509 36/57 P.R. Edwards | 3a,3b,1c:7,8(10) | | 4439 3:7(8),10 |
| 1510 37/57 P.R. Edwards | 3a,3b,1c:8 | | Na22 3:8 |
| 1511 38/57 P.R. Edwards | 3a,3b,1c:8,9 | | Na11 3:8,9 |
| 1512 39/57 P.R. Edwards | 3a,3b,1c(9),13,14 | | 4203–54 3:9(9),13,14 |
| 1499 10/50 M.Wright Mich5 | 3:12 | | Mich 5 3:39 |
| 1513 40/57 P.R. Edwards | 3a,3b,1c:14,15,16 | | 1170–50 3:14,15,16 |
| 1514 41/57 P.R. Edwards | 3a,3b,1c:13,17 | | Mich 7 3:13(17) |
| 1515 42/57 P.R. Edwards | 3a,3b,1c:21,22 | | Fels 3:21,22 |
| 1516 43/57 P.R. Edwards | 3a,3b,1c:21,23 | | Va Ankers 3:21,22 |
| 1517 44/57 P.R. Edwards | 3a,3b,1c:21,24 | | 4850–50 3:21,24 |
| 1518 45/57 P.R. Edwards | 3a,3b,1c(21),25,27 | | 1109–51 3:21(21),25,27 |
| 1519 46/57 P.R. Edwards | 3a,3b,1c(9),29,30 | | 4045–50 3:9(9),29,30 |
| 1520 47/57 P.R. Edwards | 3a,3b,1c:32,33 | | 3241–50 3:32,33 |
| 1521 48/57 P.R. Edwards | 3a,3b,1c:39 | | Mich 5 3:39 |
| 1522 49/57 P.R. Edwards | 3a,3b,1c:47 | | Ga 97 3:47 |
| 1503 14/50 M.Wright Md 2 | 5:6,9 | | Md 2 7:9(9),13,14 |
| 1504 15/50 M.Wright Mich 10 | 6:15 | | Mich 10 8:21(21),25,26 |
| 1505 16/50 M.Wright Mich 11 | 6:16 | | Mich 11 8:32,33 |
| 1533 61/57 P.R. Edwards | 8a,1c:67 | | Va Singleton 8:67 |
| 1534 62/57 P.R. Edwards | 8a,8b:8,12 | | Fla 1608 8:8,12 |
| 1535 63/57 P.R. Edwards | 8a,8b(21),25,26 | | Mich10 8:21(21),25,26 |
| 1536 64/57 P.R. Edwards | 8a,8b:35,37 | | 3080–80 8:35,37 |
| 1572 106/59 P.R. Edwards | 8a,8c:35,37 | | 3080–50 8:35,37 |
| 2539 60/57 P.R. Edwards | 8a,1c | | (Bonn 5937 8:53,55) |
| 1573 107/59 P.R. Edwards | 2a,1b:21,22 | | Md 10 2:21,22 |
| 1496 5/50 M.Wright Mich 10 | 2:7 | | Md 10 2:21,22 |

¹Polish Collection of Microorganisms
²Institute of Hygiene and Epidemiology in Prague, Czech
³Center for Disease Control in Atlanta, USA
⁴acc. to [22]
⁵acc. to [21]
⁶Source strains of antigenic scheme of West and Edwards corresponding to numbers and names in other collections
⁷O-antigens of strains marked in italic were subjected to structural studies
of identity of strains PCM 1536 and PCM 1572, whereas the other LPS studied (PCM 2539) was different (Table 2). LPS of strain PCM 1533 showing low cross reactivity with LPS of PCM 1536 and PCM 1572 was considered as an R form, like LPS of strains PCM 1534 and PCM 1535 but their R character was not proved experimentally.

Within the entire serogroup O8 the OPS structure has been determined only for the *Citrobacter* Be64/57 (PCM 1536) [10], thus this strain is considered as a reference for the entire serogroup O8. In the immunoblotting assay (Fig. 1a, middle panel), the anti-1536 serum reacted with the homologous LPS as well as with LPS of strain PCM 1573, which has been originally ascribed to serogroup O2 [23]. This finding shows the expediency of transfer of *Citrobacter* PCM 1573 and PCM 1496 into serogroup O8 (Fig. 1a, Table 1). Data on the structure of the OPS of strain PCM 1573 presented below confirmed this conclusion.

Lipopolysaccharides from strains PCM 1504 and PCM 1505, putative members of serogroup O8 or O6 (Table 1), were not recognized either by anti-1536 (O8) (Fig. 1a and b, middle panels) or anti-1531 (O6) [15] sera. The lack of reactivity of the anti-1505 serum with LPS of strain PCM 1573 (O8) confirms that strain PCM 1505 should not be classified within the serogroup O8 (Fig. 1a, right panel). Moreover, the structural studies revealed the occurrence of D-galactofuranose in the OPS of strain PCM 1505 [14], a component that is not characteristic for serogroup O8. Therefore, *Citrobacter* PCM 1505 should be assigned to a new serogroup.

The other *Citrobacter* strain, namely PCM 2539, has been considered as a member of serogroup O8 (Table 1). The serum anti-2539 reacted with the homologous LPS (Table 2) as well as with LPS of strain PCM 1504 (Fig. 1b, right panel). In turn, the LPS of strain PCM 1504 was recognized by anti-1508 and anti-1497 sera (anti O3 sera; Fig. 2). These data allowed to classify both PCM 2539 and PCM 1504 strains as the members of serogroup O3.

| Table 2 | Titres of rabbit sera against whole bacteria in passive hemagglutination test with *Citrobacter* O8 LPS. Reciprocal titres in parentheses concern alkali treated LPS used for coating sheep erythrocytes |
|---------|--------------------------------------------------------------------------------|
|         | anti-2539 | anti-1533 | anti-1536 | anti-1572 |
| LPS 2539 | 2560      | 0         | 0         | 0         |
| (60/57)  | (2560)    | (0)       | (0)       | (0)       |
| LPS 1533 | 160       | 320       | 40        | 160       |
| (61/57)  | (0)       | (40)      | (0)       | (20)      |
| LPS 1536 | 0         | 160       | 1280      | 2560      |
| (64/57)  | (0)       | (0)       | (5120)    | (10240)   |
| LPS 1572 | 0         | 0         | 320       | 1280      |
| (106/59) | (80)      | (20)      | (7680)    | (20480)   |

The serogroup O3 also appeared to be complex. The OPS structure has been determined for *Citrobacter* Be35/57 (PCM 1508) [9] and is considered as a reference structure for the serogroup O3. SDS-PAGE and immunoblotting studies (Fig. 3) showed that the strains of serogroup O3 display diverse pattern and can be divided into several groups. The strains PCM 1497, 1498, 1499, 1511, 1512, 1514, 1516, 1519, 1521, and PCM 1522 were recognized by anti-1508 (O3) serum. Anti-1497 serum reacted additionally with LPS of strains PCM 1503 and PCM 1518 (Fig. 3c). However, anti-1503 serum recognized only LPS of strains PCM 1503 and PCM 1518, indicating that they are serologically different. LPS of the strains PCM 1508, 1509, 1510, 1513, 1517, and PCM 1520 contained low amount of OPS or were already in the R form. In addition, the anti-1503 (not shown) or anti-1508 sera did not recognize the strain PCM 1515 (Fig. 3). The other immunochemoical studies on the OPS of *Citrobacter* PCM 1503 [6, 8] and PCM 1505 [14] suggest that these two strains should be reclassified into new serogroups (Table 3). The structural and serological studies presented below indicated that serogroup O3 should be extended by adding strain PCM 1504, which has been ascribed previously to serogroup O6 or O8. In turn, strain PCM 1573 that has been classified
Fig. 2 SDS-PAGE and immunoblotting experiments for determination the serotype of *Citrobacter* PCM 1504. Silver-stained SDS-PAGE (a) and immunoblotting with anti-*C. youngae* PCM 1508 (b) and anti-*C. youngae* PCM 1497 (c) sera of LPS from *C. youngae* PCM 1504 (lane 1), *C. youngae* O3 PCM 1508 (lane 2 – LPS I [6]), *C. youngae* O3 PCM 1509 (lane 3)

Fig. 3 Cross-reactivity of LPS from *Citrobacter* strains classified in O3 serogroup. Silver-stained SDS-PAGE (a) and immunoblotting with anti-*C. youngae* PCM 1508 (b) and anti-*C. youngae* PCM 1497 (c) sera of the indicated *Citrobacter* LPS
previously to serogroup O2, should be transferred to serogroup O8 (Table 1).

Structural analysis of the O-polysaccharides from *Citrobacter* PCM 1573 and PCM 1504 strains

In order to confirm the above serological results, the structural studies were performed on the OPS of strains PCM 1509, 1573, and PCM 1504. The corresponding LPS of *Citrobacter* PCM 1509, 1573 and PCM 1504 strains were recovered in yields 4.6%, 0.4%, and 1.65% of a dry bacterial mass, respectively. In SDS-PAGE, the LPS 1504 and LPS 1573 preparations showed a ladder-like pattern characteristic for LPS of S-type. LPS 1509 preparation in SDS-PAGE experiment has lost its smooth (S) character, therefore it has not been subjected to structural analysis. The mild acid hydrolysis of the LPS followed by fractionation of the carbohydrate material (38%, 59%, and 57% of the LPS weight) on Sephadex G-50 afforded the main fraction P 1 (OPS), intermediate fraction P 2, core oligosaccharide fraction P 3 and Kdo-containing fraction P 4. The yields of the OPS and core fractions were 35% and 27.8% for PCM 1504, 53.6% and

### Table 3 Re-classification of *Citrobacter* strains in serogroups O3 and O8

| Structure                                                                 | Serotype | Citrobacter strain, Reference |
|---------------------------------------------------------------------------|----------|-------------------------------|
| →3): α−O−Rhap (1→3) α−O−Rhap (1→2) β−O−Rhap (1→) α−O−Xyl (1→4)           | O8       | Be64/67 [10], this study: PCM 1573 |
| →3): α−O−Rhap (1→3) α−O−Rhap (1→2) β−O−Rhap (1→) α−O−Gal (1→4)           | new O8a  | PCM 1505 [14] |
| →4): α−O−Manp(1→3) β−O−Rhap (1→4) β−O−Rhap (1→)                           | O3       | Be35/57 [9], this study: PCM 1504, 1497, 2539, 1509 |
| →4): α−O−Manp(1→3) β−O−Rhap (1→4) β−O−Rhap (1→) α−O−GlcP (1→2)           | new O3a  | PCM 1503 [8], this study: PCM 1518 |

![Fig. 4 NMR spectra of the isolated OPS. Shown are the $^1$H NMR spectra (a, c) and $^{13}$C NMR spectra (b, d) of the OPS of *Citrobacter* PCM 1573 (a, b) and PCM 1504 (c, d). Expanded regions of the $^1$H NMR spectra are shown in the insets.](image)
22.7% for PCM 1573, and 6.2% and 57.7% for PCM 1509, respectively, of the total material eluted from the column.

The sugar analysis of the OPS of strain PCM 1573 revealed the presence of two components, namely rhamnose and xylose in molar ratio 3.0:0.8 (hydrolysis with 2 M TFA). Methylation analysis showed the presence of terminal xylofuranosan, 2-substituted rhamnopyranose, 3-substituted rhamnopyranose and 2,3-disubstituted rhamnopyranose in molar ratios 0.5:0.9:1.0:1.0 (2 M TFA). The $^1$H NMR and $^{13}$C NMR spectra of the OPS of strain PCM 1573 (Fig. 4a, b respectively) were identical to those of OPS from strains PCM 1536 [10] and PCM 1572 (data not shown), representing serogroup O8. Therefore, NMR data confirm the results of the chemical analysis and the expediency of classifying strain PCM 1573 into serogroup O8.

Regarding the strain PCM 1504, sugar analysis of the OPS (hydrolysis with 2 M TFA) indicated the presence of rhamnose and mannose in molar ratio 1.9:1.0. Methylation analysis of the OPS revealed the presence of 4-substituted rhamnopyranose, 3-substituted rhamnopyranose, and 4-substituted mannopyranose residues in molar ratios 0.9:0.8:1.0, respectively. Moreover, the $^1$H NMR and $^{13}$C NMR spectra of the OPS of PCM 1504 strain (Fig. 4c, d, respectively) were identical to the spectra of Citrobacter strain Be35/57 [10] belonging to O3 serogroup. Thus, these data exclude the strain PCM 1504 from the O8 serogroup. The performed serological analysis indicated that the original smooth type LPS of strain PCM 1509 was identical to that of strain PCM 1508 (LPS I, O3) (Fig. 2).

Conclusions
In this paper we report that each of the complex serogroups O3 and O8 can be divided into two non-cross-reacting serogroups. The strains originally ascribed to the serogroups O2 (PCM 1573), O6 (PCM 1504, 1505) and O7 (PCM 1503) that O-antigenic structures are shown in Table 3, should be re-classified into serogroups O3 (PCM 1504) and O8 (PCM 1573). The strains PCM 1503 [8] and PCM 1505 [14] should be classified within new serogroups, namely O3a and O8a, respectively.

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors' contributions
EK, AG designed the study. EK, MS, AKK, SG, MM analyzed the data. EK, MS, AG, AKK wrote the manuscript. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate
Not applicable.

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Competing interests
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