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Review

Structures and Serology of the O-Specific Polysaccharides of Bacteria of the Genus *Citrobacter*

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Abstract. The review presents the structures of the O-specific polysaccharides (O-antigens) of the lipopolysaccharides isolated from over 25 *Citrobacter* strains, which represent different species and serogroups. The correlation between O-antigen structure and immunospecificity as well as numerous cross-reactions between *Citrobacter* and other enterobacterial species are discussed.

Key words: *Citrobacter*; O-antigen; lipopolysaccharide; structure; immunospecificity.

Introduction

Bacteria of the genus *Citrobacter* are Gram-negative bacilli of the family *Enterobacteriaceae.* These microorganisms are facultatively anaerobic and typically motile by means of flagellae; they can use citrate as the sole carbon source. The genus *Citrobacter* is taxonomically most closely related to *Salmonella* and *Escherichia coli*. *Citrobacter* strains are normal inhabitants of human and animal intestine, and are also commonly distributed in natural environment such as soil, water, sewage and food[71].

Citrobacter strains are opportunistic pathogens that are frequently isolated from wounds and cause gastrointestinal diseases[1, 22, 63], urinary tract infections and bacteremias[21], especially in immunocompromised patients. Some incidents of meningitis, brain abscesses and sepses in neonates have been also reported[2, 20, 37, 57, 76].

The genus *Citrobacter* was first described in 1928 by BRAAK[5] as *Bacterium freundii*. In 1932, WERKMAN and GILLEN[80] introduced the name *Citrobacter freundii* for lactose-fermenting coliform bacteria. This classification was not universally accepted and bacteria of the genus *Citrobacter* were subsequently described under various names, such as *Escherichia freundii, Colobactrum freundii, Paracolobactrum freundii, Padlevskia, Levinea, C. diversus*, the Ballerup group, the Bethesda group and the Bethesda-Ballerup group[20]. In 1958, the International Subcommittee of Taxonomy of *Enterobacteriaceae* adopted the term *C. freundii* for this highly heterogenous group of bacteria, which since the 1970s also comprises *C. amalonaticus* and *C. koserii.*

In 1993, the classification of *Citrobacter* was drastically revised and the species *C. freundii* redefined by BRENNER et al.[6] On the basis of DNA relatedness, 11 genomospecies were identified and separated by their

Abbreviations used: LPS – lipopolysaccharide, OPS – specific polysaccharide.

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biochemical profiles. The following homogenous species were distinguished: *C. koserii* (formerly *C. diversus*), *C. amalonaticus* and *C. farmerii* (formerly *C. amalonaticus* biogroup 1). According to the new classification, from the *C. freundii* complex (66 strains) only 9 strains belong to *C. freundii* and 57 strains formed the genomospecies 5, 6, 7 and 8, which were named as *C. youngae* (21 strains), *C. braakii* (15 strains), *C. werkmanii* (6 strains) and *C. sedlakii* (6 strains), respectively. The three unnamed genomospecies 9, 10 and 11 were later named as *C. rodentium*[70], *C. gillenii* and *C. murliniae*[7].

In 1954, WEST and EDWARDS[81] established for the Bethesda-Ballerup group an antigenic scheme comprising 32 O-serogroups, which was extended later by SEDLAK and SLAJSOVA[72, 73] to 43 O-serogroups. This scheme continues to be applied for the serotyping of *Citrobacter* strains even after the revision of the classification of the genus. Ninety reference strains listed in the antigenic scheme were phenotypically characterized and specified by MIKI et al.[56] based on the revised classification of *Citrobacter*. From these 90 strains, two strains were reclassified to *Hafnia alvei* and one strain was moved to *E. coli*. Among the remaining reference strains, *C. youngae* is the predominant species, followed by *C. braakii*, *C. werkmanii* and *C. freundii*. Some O-antigens are characteristic for more than one species.

From the chemical point of view, the O-antigen represents the polysaccharide chain of the S-form lipopolysaccharide (LPS), which is called O-specific polysaccharide (OPS). It is attached, via a core oligosaccharide, to lipid A, which is responsible for the endotoxic activities of the LPS. While lipid A is the most structurally conservative part of the LPS, the core region is less conservative, and the OPS is the most variable moiety.

Based on the sugar composition of the LPS, *Citrobacter* strains of different serogroups were classified by KELETI et al.[35] into 20 chemotypes, from which 11 chemotypes are identical to those occurring in the genera *Salmonella* and *Escherichia*. Strains of the three genera showed numerous serological cross-reactions. The serological specificity of the bacteria is defined by the fine structure rather than by the composition of the OPS. Therefore, in order to substantiate the antigenic heterogeneity and cross-reactivity on the molecular level, detailed structural studies of the OPS are performed, which help to improve the classification of *Citrobacter* strains. By now, structures of more than 25 *Citrobacter* OPS have been established and serological cross-reactions of a number of *Citrobacter* strains with *Salmonella*, *Escherichia*, *Hafnia*, *Klebsiella* and some other bacteria elucidated.

The present article is the first survey summarizing the structural data of *Citrobacter* O-antigens and their implication in the serological differences and similarities between different bacterial strains.

Typed Strains

C. youngae O1: 1, 2 (PCM 1506)

The branched OPS of *C. youngae* O1 (PCM 1506)[48] has a tetrasaccharide repeating unit containing D-mannose, D-rhamnose, D-ribose (Table 1). In chemotyping[35], ribose was not taken into account, being considered a constituent of nucleic acid rather than LPS. This and some other *Citrobacter* OPS include D-rhamnose, which is common to the O-antigens of many phytopathogenic bacteria, such as *Pseudomonas syringae* and *Xanthomonas campestris*, but uncommon to enterobacterial LPS apart from *Citrobacter*[28, 38] A peculiar feature of the serogroup O1 OPS is the presence of D-ribose in the α-furanose form, which occurs rarely in bacterial polysaccharides. In contrast to the more common β-ribofuranosyl group, the α-ribofuranosyl group is easily cleaved from the OPS under mild acidic conditions to give a linear backbone polysaccharide.

C. youngae O2: 5, 6 (PCM 1507), *C. werkmanii* O20: 40, 41, 43 (PCM 1553), *C. youngae* O25: 35, (36), 38 (PCM 1558)

The three strains produce the same OPS consisting of D-mannose, D-rhamnose and D-xylose that corresponds to chemotyping data of serogroup O25 but not serogroups O2 and O20[35]. The OPS has a branched tetrasaccharide repeating unit[23] (Table 1). Classification of strains *C. youngae* O2 (PCM 1507), *C. werkmanii* O20 (PCM 1553) and *C. youngae* O25 (PCM 1558) in different serogroups may result from a difference in the LPS core structure (compare data of *Citrobacter* O4, O27 and O36 given below) or a strong influence of H antigens.

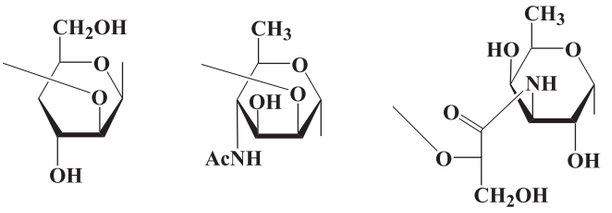
SDS/PAGE and immunoblotting analysis showed that from 5 strains formerly assigned to *Citrobacter* serogroup O2, only four strains, PCM 1507, PCM 1494, PCM 1495 and PCM 1496, possess an S-type LPS reactive with specific anti-O2 serum and, thus, do belong to serogroup O2. Strain PCM 1573 has an S-type LPS but does not react with anti-O2 serum and, hence, should be classified in another serogroup (authors' unpublished data).

C. youngae O3: 7, (8), 10 (PCM 1509)

The OPS of *C. youngae* O3 (PCM 1509) contains D-mannose and D-rhamnose, which is consistent with chemotyping data[35]. Computer-assisted analysis of the ^{13}C NMR spectrum showed that the OPS has a linear structure[39] (Table 1). Among the 18 strains (PCM 1497–1499, PCM 1508–1522) representing serogroup O3, strain PCM 1518 should be classified to another serogroup (authors' unpublished data).

C. youngae O4: 44, 45 (PCM 1525), *C. werkmanii* O27: 40, 41 (PCM 1560), *C. youngae* O36:? (PCM 1488)

Some *Citrobacter* O-antigens contain rarely occurring and even unique components, such as 4-deoxy-D--*arabino*-hexose, which is the monomer of the homopolymer OPS of *C. youngae* O4 (PCM 1525) and O36 (PCM 1488) as well as *C. werkmanii* O27 (PCM 1560)[67, 69] (Table 1, Fig. 1, structure **1**). So far, this sugar has not been found elsewhere in nature. The composition of the OPS is consistent with chemotyping data[35]. Variations in the LPS core structure are the reason for the classification of the strains having the same OPS in three different serogroups[66, 68]. Epitopes of two monoclonal antibodies to the serogroup O36 OPS were characterized, one of which, CB-2, reacted with the OPS, whereas the other, CB-8, recognized an O-acetylated oligosaccharide fragment intervening the OPS and the core region[75]. *Citrobacter* strains containing 4-deoxy-D-*arabino*-hexose do not cross react with any other enterobacterial organisms.



1 3 4

Fig. 1. The O-specific polysaccharides of *C. youngae* O4: 44, 45 (PCM 1525)[67] (**1**), *C. gillenii* O9: 48 (PCM 1537)[53] (**3**) and *C. youngae* O32: (23), 28 (PCM 1569)[43] (**4**) are homopolymers of rarely occurring monosaccharides

C. braakii O5: 73: Vi (PCM 1528), PCM 1487

4-Deoxy-D-*arabino*-hexose is also present as a terminal side-chain residue in the branched OPS of strains PCM 1487[24] and PCM 1528 (authors' unpublished data) of *C. braakii* O5 (Table 1). Strain PCM 1487 had not been originally classified, but the identity of its OPS structure to that of strain PCM 1528 from serogroup O5 enabled placing it in serogroup O5, too.

The OPS of several more *Citrobacter* strains, including *C. youngae* O15: 32, 34 (PCM 1571), *C. braakii* O17: 75 (PCM 2540) and *C. braakii* O18: 56 (PCM 1551), have the same main chain as *Citrobacter* PCM 1487 (authors' unpublished data), but their exact structures remain to be determined. *C. braakii* O5: 53, 54 (PCM 1527) is serologically identical to strains PCM 1528 and PCM 1487, whereas strain PCM 1529, which was formerly classified as *Citrobacter* O5, was moved to the *H. alvei* species[56]. An OPS-protein conjugate of *Citrobacter* PCM 1487, considered as a potential artificial vaccine, was characterized immunochemically[54].

C. braakii O6: 72 (PCM 1531)

The OPS of *C. braakii* O6 (PCM 1531) is another polysaccharide that contains a rarely occurring sugar, 4-deoxy-D-*arabino*-hexose, which is attached as a single side-chain monosaccharide unit (authors' unpublished data). The main chain is built up of L-rhamnose and D-fucose, the former being stoichiometrically O-acetylated at position 2 (elucidation of the exact OPS structure is in progress). In chemotyping[35], rhamnose and 4-deoxy-D-*arabino*-hexose were not determined as LPS constituents. The LPS that was recovered from the phenol phase of the phenol-water extract contained twice as much OPS as that obtained from the water phase, which could be accounted for by the hydrophobic nature of the OPS components.

C. braakii O7: 68 (PCM 1532)

According to MIKI et al.[56], serogroup O7 includes strains of two species, *C. braakii* PCM 1532 and *C. youngae* PCM 1503. The branched OPS of strain PCM 1532 has a D-mannan main chain and side chains of a single D-glucose residues[47] (Table 1). The D-mannan main chain has the same structure as the linear OPS of *E. coli* O9[64], *Klebsiella pneumoniae* O3[17] and *H. alvei* PCM 1223[32]. Despite the structural similarity of the O-antigens, no cross-reactivity was observed in Western immunoblotting between anti-*H. alvei* PCM 1223 O-serum and the LPS of *C. braakii* PCM 1532[32]. This could be accounted for by the masking of potential cross-reactive epitope(s) within the D-mannan chain by the lateral α-D-Glc*p* residue in the polysaccharide of strain PCM 1532.

Table 1. Structures of the O-specific polysaccharides of the lipopolysaccharides of *Citrobacter*

Serogroup (strain)	Structure of the repeating unit		Reference
O1 (PCM 1506)	→4)-α-D-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→ α-D-Rib*f*-(1→4)┘		48
O2 (PCM 1507) **O20** (PCM 1533) **O25** (PCM 1558)	→4)-α-D-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Rha*p*-(1→ α-D-Xyl*p*-(1→4)┘		23
O3 (PCM 1509)	→4)-α-D-Man*p*-(1→3)-β-D-Rha*p*-(1→4)-β-D-Rha*p*-(1→		39
O4 (PCM 1525) **O27** (PCM 1560) **O36** (PCM 1488)	→2)-β-D-ara4dHex*p*-(1→	**1**	67, 69
O5 (PCM 1487) (PCM1528)	→4)-α-D-Gal*p*NAc-(1→6)-α-D-Glc*p*NAc-(1→ β-D-ara4dHex*p*-(1→3)┘		24 a
O7 (PCM 1532)	→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→ α-D-Glc*p*-(1→3)┘		47
O7 (PCM 1503)	→4)-α-D-Man*p*-(1→3)-β-D-Rha*p*-(1→4)-β-D-Rha*p*-(1→ α-D-Glc*p*-(1→2)┘		a
O8 (PCM 1536, PCM 1572)	→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→ α-D-Xyl*f*-(1→2)┘		42
O9 (PCM 1537)	→3)-α-D-Rha*p*4NAc-(1→2)-α-D-Rha*p*4NAc-(1→2)-α-D-Rha*p*4NAc-(1→3)-α-D-Rha*p*4NAc2Ac-(1→ →2)-α-D-Rha*p*4NAc-(1→	**2** **3**	53
O12 (PCM 1544)	→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*-(1→ ~60% β-D-Glc*p*NAc-(1→3)┘		50
O12 (PCM 1542)	α-D-Glc*p*NAc-(1→4)┐ →6)-β-D-Glc*p*N*R*3HOBu-(1→3)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→ α-D-Glc*p*-(1→6)┘		34
O16 (PCM 1550)	D-Gro-1-*P*-(O→3)┐ α-D-Gal*p*-(1→6)┐ →6)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ α-D-Glc*p*-(1→2)┘		45
O21 (PCM 1554)	→6)-α-D-Man*p*3Ac-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→ α-D-Glc*p*-(1→3)┘		a

O23 (PCM 2352, PCM 1556)	→4)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Gal*p*NAc-(1→		30, 31
O24 (PCM 1557)	→3)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*3Ac-(→ α-L-Fuc*p*-(1→2)┘		a
O26 (PCM 1559)	→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→ α-D-Glc*p*-(1→2)┘		40
O28 (PCM 1561)	→2)-β-D-Rib*f*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→		41
O29 (PCM 1562) **O30** (PCM 1567)	→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→		40 a
O32 (PCM 1569)	→2)-L-GroA-(1→3)-α-D-Fuc*p*3N2Ac-(1→	**4**	43
O35 (PCM 1586)	→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Gal*p*-(1→	**5**	44
O38 (PCM 1489)	α-Abe*p*4Ac-(1→3)┐ →2)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→2)-α-D-Man*p*-(1→ α-D-Glc*p*-(1→2)┘		44
O41 (PCM 1444)	→2)-β-D-Glc*p*-(1→2)-β-D-Fuc*p*3N*R*3HOBu-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(→ α-D-Glc*p*-(1→2)┘		65
(396)	α-Abe*p*2Ac-(1→3)┐ →3)-α-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-β-D-Man*p*-(1→ α-D-Glc*p*-(1→3)┘		27
(NRCC 6070)	→4)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1→2)-α-D-Rha*p*4NAc-(1→2)-α-L-Fuc*p*-(1→		78
(NRCC 6052)	→2)-α-D-Rha*p*-(1→3)-β-D-Rha*p*-(1→4)-β-D-Glc*p*-(1→		78
(ATCC 51459)	→3)-α-D-Glc*p*NAc-1-*P*-(O→6)-α-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→ β-L-Rha*p*-(1→4)┘		55

Abbreviations: Abe – 3,6-dideoxy-D-*xylo*-hexose (abequose), Ac – acetyl, ara4dHex – 4-deoxy-D-*arabino*-hexose, Fuc3N – 3-amino-3-deoxyfucose, Gro – glycerol, GroA – glyceric acid, Rha4NAc – 4-acetamido-4-deoxy-D-rhamnose (N-acetylperosamine), *R*3HOBu – (*R*)-3-hydroxybutyryl.

[a] Authors' unpublished data.

C. youngae O7: (9), 13, 14 (PCM 1503)

The OPS of *C. youngae* O7 (PCM 1503) has a structure different from that of *C. braakii* O7 (strain PCM 1532) (authors' unpublished data). Its main chain is identical to the linear OPS of *C. youngae* O3 and similar to the main chains of the OPS of *C. youngae* O1 and *C. youngae* O2 (Table 1). They differ only in the interchange of some D-rhamnose residues with D-mannose residues, whereas the linkages of the monosaccharides, whether D-rhamnose or D-mannose, that occupy the same position in the repeating units are the same. In spite of the structural similarity of the OPS, O-antiserum against strain PCM 1503 did not cross-react with the majority of *Citrobacter* strains from serogroup O3.

A significant structural difference which is observed between the OPS of serogroup O7 strains belonging to two different species, *C. braakii* PCM 1532 and *C. youngae* PCM 1503, shows that their classification in one serogroup should be revised. Chemotyping data[35] are also consistent with the composition of none of these OPS.

C. braakii O8: 35, 37 (PCM 1536, PCM 1572)

The OPS of both *C. braakii* O8 strains studied, PCM 1536 and PCM 1572, are identical. In accordance with the chemotyping data[35], they contain D-rhamnose and furanosidic D-xylose, the latter, to the best of our knowledge, not being found in other bacterial polysaccharides. The OPS has a topology typical of many *Citrobacter* OPS which is characterized by a tetrasaccharide repeating unit with three monosaccharides in the main chain and one in the side chain[42] (Table 1).

C. gillenii O9: 48 (PCM 1537)

The O-antigen of *C. gillenii* O9 **(**PCM 1537) is represented by two structurally different polysaccharides, both composed of 4-acetamido-4-deoxy-D-rhamnose (*N*-acetyl-D-perosamine, D-Rha4NAc). One OPS has a tetrasaccharide repeating unit **2** and the other is an α1→2-linked homopolymer of Rha4NAc **3**[53] (Table 1, Fig. 1). This monosaccharide is difficult to detect in sugar analysis, and it was overlooked in the chemotyping of *Citrobacter* LPS[35]. One of the 3-substituted Rha4NAc residues in OPS **2** is partially O-acetylated at position 2 (the degree of O-acetylation is ~70%). OPS **2** and OPS **3** could be separated by gel-permeation chromatography on TSK HW-50S.

Based on matrix-assisted laser-desorption/ionization mass spectrometry data, it was suggested that the chain elongation in biosynthesis of both OPS proceeds by sequential transfers of single sugar units to the nonreducing end of the growing polysaccharide chain. This biosynthetic model requires the participation of several distinct transferases for the same monosaccharide. O-Acetylation in OPS **2** begins after the achievement of a certain chain length.

A polysaccharide with the same structure as OPS **3** has been previously reported as the O-antigen of *Vibrio cholerae* bio-serogroup Hakata[25] (serogroup O140[49]), whereas OPS **2** is unique for *Citrobacter*. An OPS with a pentasaccharide repeating unit of α1→2- and α1→3-linked residues of N-acetyl-D-perosamine or N-formyl-D-perosamine have been found in the LPS of *Brucella melitensis*[8, 9, 15] and *E. hermannii*[4], respectively. O-antigens of *B. abortus*[12, 14] and *Yersinia enterocolitica* O9[11, 13] are α1→2-linked homopolymers of N-formyl-D-perosamine, and a similar homopolymer of N-[(S)-2,4-dihydroxybutyryl]-D-perosamine was found in the LPS of *V. cholerae* O1[36].

Anti-O9 serum reacted with the homologous LPS in double immunodiffusion. In Western immunoblotting, it recognized mainly slowly migrating, high-molecular-mass LPS species. O-Deacylation of the LPS had no significant effect on its serological reactivity. From the separated OPS, only OPS **2** reacted in double immunodiffusion, whereas OPS **3** was inactive, probably because of a lower molecular mass. No significant cross-reactivity was observed between anti-O9 serum and *V. cholerae* O1 LPS, which can be accounted for by different N-acyl substituents at D-Rha4N (see above). The LPS from *E. coli* O157, which, among other monosaccharides, also contains D-Rha4NAc[62], also did not cross-react with anti-O9 serum in double immunodiffusion.

C. gillenii O12: 35, 36 (PCM 1544)

The OPS of *C. gillenii* O12 (PCM 1544) contains L-rhamnose, D-galactose and D-GlcNAc, which is in agreement with chemotyping data[35]. It is distinguished by non-stoichiometric substitution of the main chain with terminal GlcNAc residues (the degree of glycosylation is ~60%), whereas O-acetylation of the rhamnose residue at position 2 is stoichiometric[50] (Table 1). Smith degradation was efficiently applied for the determination of the structure of this OPS.

C. gillenii O12: 57 (PCM 1542)

In addition to D-galactose, D-GlcNAc and D-GalNAc, the OPS of *C. gillenii* O12 (PCM 1542) contains 2-deoxy-2-[(*R*)-3-hydroxybutyramido]-D-glucose

(D-GlcN*R*3HOBu), which is also a constituent of the OPS of *C. freundii* O41 (see below). Although strains PCM 1542 and PCM 1544 belong to the same serogroup O12, the structures of their OPS differ significantly[34] (Table 1). In a double immunodiffusion test with anti-O serum against strain PCM 1544, precipitation was observed only with the homologous LPS, but not with the LPS of strain PCM 1542. These findings taken together show that the O-antigens of *C. gillenii* PCM 1542 and PCM 1544 are related neither chemically nor serologically and, hence, should be reclassified in different O-serogroups.

C. youngae O16: 58 (PCM 1550)

The OPS of *C. youngae* O16 (PCM 1550) is distinguished by the presence of a phosphoglycerol group[45], which has the D configuration[26]. In chemotyping[35], phosphoglycerol was not considered as an LPS component. The OPS is acid-labile and is cleaved by the β1→6-linkage between residues of GalNAc and galactose in the course of dephosphorylation with 48% hydrofluoric acid to give a hexasaccharide that contains all sugar constituents of the OPS (D-glucose, D-galactose and D-GalNAc)[45] (Table 1). Other approaches used for the structural analysis of this OPS were Smith degradation and N-deacetylation followed by deamination with nitrous acid, which resulted in various smaller oligosaccharides studied by NMR spectroscopy and fast-atom bombardment collision-induced dissociation mass spectrometry.

The OPS of *H. alvei* PCM 1207 has been found to contain phospho-D-glycerol, too, which is attached at position 3 of a 4-substituted β-D-Gal*p*NAc residue in the main chain[26], i.e. to the same monosaccharide and at the same position as in the OPS of *C. youngae* PCM 1550. Immunoblotting and ELISA experiments demonstrated cross-reactivity between O-antiserum against *H. alvei* PCM 1207 and the LPS of strain PCM 1550 and indicated that a shared epitope resides on the O-antigen[26]. Both *H. alvei* PCM 1207 and *C. youngae* O16 OPS showed a high inhibitory activity, which decreased substantially upon dephosphorylation of the OPS. Based on these data, it was concluded that phospho-D-glycerol is an immunodominant group in the OPS responsible for the serological cross-reactivity of the two bacteria.

C. werkmanii O21: 60 (PCM 1554)

The OPS of *C. werkmanii* O21 (PCM 1554) includes D-glucose, D-mannose and D-GlcNAc, which is consistent with chemotyping data[35]. A pentasaccharide repeating unit of the OPS contains three mannose residues, one of which is partially O-acetylated (the degree of O-acetylation is ~30%) (authors' unpublished data) (Table 1). The OPS of strain PCM 1554 shares a branched trisaccharide fragment with the OPS of *C. braakii* PCM 1532 from serogroup O7 (Table 1), but it remains to be determined whether the two strains are serologically related.

C. freundii O23: 52
(PCM 2352, S-type; PCM 1556, SR-type)

In chemotyping[35], 4-deoxy-*arabino*-hexose was detected in the LPS of *C. freundii* O23, but later studies showed that the OPS contains only D-mannose and D-GalNAc[31]. The structure of the so-called biological repeating unit was determined for the OPS of *C. freundii* O23[31] (Table 1). This represents a preassembled oligosaccharide that is ligated to the LPS core with or without preceeding polymerisation in the course of biosynthesis to give S-type LPS (strain PCM 2352) or SR-type LPS (strain PCM 1556)[30], respectively. Remarkably, the GalNAc that links the repeating unit to the core has the β-configuration, whereas in the interior repeating units of the OPS, this sugar is α-linked (authors' unpublished data).

C. werkmanii O24: 49, 51 (PCM 1557)

In addition to the common monosaccharides and fucose detected in chemotyping the LPS[35], the OPS of *C. werkmanii* O24 (PCM 1557) was found to contain D-ribose and D-glucuronic acid (authors' unpublished data), the latter making it acidic. This OPS has the same structure, including the O-acetylation pattern, as that of *Salmonella enterica* ssp. *arizonae* O45[74].

C. werkmanii O26: 49, 50 (PCM 1559)

The OPS of *C. werkmanii* O26 (PCM 1559) contains D-glucose and D-ManNAc, which is not consistent with chemotyping data[35]. A branched structure was established for the trisaccharide repeating unit of this OPS (Table 1) (authors' unpublished data), which was originally published as that of *Citrobacter* O29b[40].

C. braakii O28: 70 (PCM 1561)

The sugar composition of the OPS of *C. braakii* O28 (PCM 1561)[41], which includes D-ribose and L-rhamnose, is inconsistent with chemotyping data[35]. A linear

trisaccharide repeating unit of this OPS[41] (Table 1) comprises a partial structure of the tetrasaccharide repeating unit of the OPS of *Klebsiella* O7 containing one additional 2-substituted α-L-Rha*p* residue[51].

C. braakii O29: 8, 10, 11 (PCM 1562)

The OPS of *C. braakii* O29 (PCM 1562, formerly O29a)[40] is a linear polysaccharide having the same structure as the main chain of the branched polysaccharide of *C. werkmanii* O26 (see above) (Table 1). ManNAc present in this OPS was overlooked in the chemotyping of the LPS[35].

C. braakii O30: 76 (PCM 1567)

The structure of OPS of *C. braakii* O30 (PCM 1567) is identical to that of *C. braakii* O29 (PCM 1562) (author's unpublished data). The strains O29 and O30 producing the same O-antigens may differ in LPS core structures, which should be studied.

C. youngae O32: (23), 28 (PCM 1569)

The OPS of *C. youngae* O32 (PCM 1569)[43] is a homopolymer of 3-amino-3,6-dideoxy-D-galactose (3-amino-3-deoxy-D-fucose, Fuc3N), which is N-acylated with the L-glyceroyl group and partially O-acetylated at position 2 (the degree of O-acetylation ~75%)[43] (Table 1, Fig. 1, structure **4**). The glyceroyl group, in turn, is glycosylated by the sugar component and, hence, this O-antigen is not a polysaccharide in the strict chemical sense since it contains not only glycosidic, but alternating glycosidic and amidic linkages. The composition of strain PCM 1569 is inconsistent with chemotyping data[35] of the LPS.

A different structure was established earlier for the OPS of serogroup O32[46], but later found to belong actually to a polysaccharide different from the O32 antigen. The same structure as PCM 1569 had been reported previously for the main chain of the cell-wall antigen of the Gram-positive microorganism *Eubacterium sabbureum* L13[29], which differs from the OPS of *C. youngae* O32 by the presence of lateral D-fructofuranose residues attached at position 3 of approximately every third Fuc3N residue.

C. braakii O35:? (PCM 1586)

The OPS of *C. braakii* O35 (PCM 1586)[44] contains D-galactose, 2-acetamido-2,6-dideoxy-L-galactose (2-acetamido-2-deoxy-L-fucose, FucNAc), which does not fit to chemotyping data[35] and, hence, the strain should be reclassified.

The linear structure **5** of the OPS of *C. braakii* O35[44] is identical to that of *S. enterica* ssp. *arizonae* O59[79]. Accordingly, the LPS of the two strains strongly cross-react with the corresponding anti-O sera[44]. Both anti-O sera also recognized the LPS of *Pseudomonas aeruginosa* O7 (former serogroup O11) in immunoblotting and ELISA, though the serological relatedness of the enteric bacteria to *P. aeruginosa* O7 is much weaker than to each other, and anti-*P. aeruginosa* O7 serum bound the LPS of neither *C. braakii* O35 nor *S. enterica* ssp. *arizonae* O59[44]. The observed one-way serological cross-reactivity is apparently based on structural similarities, but not identity, of the OPS, that of *P. aeruginosa* O7 having the structure **6** shown below[19]. Although different in sugar composition, both OPS have repeating units of the same size with the corresponding monosaccharide residues having the same absolute configurations and being connected by the same linkages. Conformational analysis confirmed the presence of structurally similar sites on the helix surfaces of each of the OPS, which may serve as a common epitope[44].

→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-
-(1→2)-β-D-Glc*p*-(1→ **5**

→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-
-(1→2)-β-D-Gal*p*-(1→ **6**

C. werkmanii O38:? (PCM 1489)

The OPS of *C. werkmanii* O38 (PCM 1489)[44] contains D-glucose, D-mannose, D-galactose, L-rhamnose and 3,6-dideoxy-D-*xylo*-hexose (abequose, Abe), which is in accordance with chemotyping data[35]. Abequose is an acid-labile monosaccharide, and mild acid delipidation of the LPS resulted in a significant loss of this sugar. Therefore, in order to determine the OPS structure, alkaline deacylation of the LPS was applied, which resulted in a regular polysaccharide without any loss of abequose[44].

The structure of the OPS of *C. werkmanii* O38[44] is similar to that of *S. enterica* sv. Kentucky, which differs only in the O-acetyl substitution[77]. Accordingly, a strong cross-reactivity of the two bacteria[73] and their LPS[44] has been demonstrated. The O-acetyl group on the abequose residue had little importance in manifesting the immunospecificity of *C. werkmanii* O38[44].

C. freundii O41:? (PCM 1444)

The OPS of *C. freundii* O41 (PCM 1444) contains D-glucose, D-galactose, D-GlcNAc, D-GalNAc and 3--amino-3,6-dideoxy-D-galactose amidated with (*R*)-3-hydroxybutyric acid (D-Fuc3N*R*3HOBu), and its biological branched hexasaccharide repeating unit has been established[65] (Table 1). The main chain of the OPS shows a similarity with that of *H. alvei* 1211[33]; particularly, the OPS share the trisaccharide fragment **7** in the main chain, which may be responsible for the weak cross-reactivity of the strains.

→2)-β-D-Glc*p*-(1→2)-β-D-Fuc*p*3N*R*3HOBu-
-(1→6)-α-D-Glc*p*NAc-(1→ **7**

Non-Typed Strains

Citrobacter sp. 396

The OPS of *Citrobacter* sp. 396 contains D-glucose, D-mannose, D-GlcNAc and abequose and has a branched heptasaccharide repeating unit[27], which is the largest one among the *Citrobacter* O-antigens (Table 1). It is closely related to the OPS of *S. enterica*, having the same side chain of 2-O-acetylabequose as *S. enterica* sv. Typhimurium (group B) and the main chain and the second side chain of glucose similar to those in *S. enterica* subgroups C_1 and C_4[38].

C. sedlakii NRCC 6070, *C. freundii* OCU158

Strains *C. sedlakii* NRCC 6070[78] and *C. freundii* OCU158[58] have similar OPS, which consists of D-glucose, L-fucose, D-GalNAc and Rha4NAc and has a linear tetrasaccharide repeating unit (Table 1). The same OPS as from *C. sedlakii* NRCC 6070 is produced by *E. coli* O157: H7[62], an enteric pathogen that can cause severe local and systemic diseases and is responsible for sporadic and for major outbreaks of hemorrhagic colitis and large numbers of hemolytic-uremic syndromes. Accordingly, *C. sedlakii* NRCC 6070[59] and its LPS[78] showed a strong serological cross-reactivity with anti-O serum against *E. coli* O157. The enzyme immunoassay using LPS derived either from *E. coli* O157 or from *C. freundii* OCU158 could equally detect high levels of serum antibodies against LPS in patients with enterohemorrhagic *E. coli* O157 infection[58]. A difference in the epitopes was demonstrated by the absorption of antibodies in patient serum by LPS from *E. coli* O157 or *C. freundii* and was attributable to the epitope specificity of the LPS core region and/or lipid A structure[58].

In addition to *C. sedlakii* and *C. freundii*, *E. coli* O157 is serologically related to *Brucella abortus*[12, 14], *B. melitensis*[8, 9], *S. enterica* O:30 (group N) [10, 61], *E. hermanii*[60], *P. maltophilia* 555[18], *Y. entercolitica* O9[11, 13], and *V. cholerae* O1[36]. The common epitope responsible for the serological cross-reactivity, 2-substituted D-perosamine residue N-acylated with various groups, is present in all cross-reactive bacteria, but only *Citrobacter* and *S. enterica* O:30 (group N) have the O-antigen that is structurally identical with that of *E. coli* O157. In a mouse infection model, *S. enterica* O:30 has shown promise as a live vaccine against *E. coli* O157: H7 gut colonization[16], and it is possible that the strains of *Citrobacter* can also function in a similar manner[78].

C. freundii NRCC 6052

A biochemically typical strain of *C. freundii* (NRCC 6052) produces a simple OPS that is composed of trisaccharide repeating units containing D-glucose and D-rhamnose[78] (Table 1).

Although this strain was reported to be another *Citrobacter* strain that shares an antigen with *E. coli* O157[3] and immunochemical studies showed the structural basis for the serological cross-reaction is underlying in the OPS moiety of the LPS[78], the OPS of *C. freundii* NRCC 6052 differs significantly from that of *E. coli* O157. Particularly, it lacks D-perosamine, implicated as the common epitope in the OPS of bacteria showing serological cross-reactivity with antisera against *E. coli* O157 (see the previous section). Therefore, it was suggested that the 2-substituted α-D-Rha*p* residue in the *C. freundii* NRCC 6052 OPS is structurally similar to the 2-substituted α-D-Rhap4NAc residue in the *E. coli* O157 OPS, thus presenting an example of mimicry within a bacterial polysaccharide antigen. However, modelling and oligosaccharide inhibition experiments will be required to confirm this point.

C. rodentium ATCC 51459

The OPS of a prototype transmissible murine colonic hyperplasia isolate of *C. rodentium* (ATCC 51459) contains D-glucose, D-GlcNAc and L-rhamnose and is built up of pentasaccharide repeating units linked through phosphate[55] (Table 1). The OPS is acid-labile owing to the presence of a glycosyl phosphate group in the main chain. It could be depolymerised to the pentasaccharide also by dephosphorylation with aqueous 48% hydrofluoric acid.

The OPS of *E. coli* O173[52] is remarkably similar to that of *C. rodentium* (ATCC 51459), differing only in the side chain sugar (α-L-fucose rather than β-L-rhamnose) and the glycosyl phosphate group (α-D-Glc*p*-1-*P* rather than α-D-Glc*p*NAc-1-*P*). It was suggested that the serological cross-reactions observed between *C. rodentium* strain and anti-O sera against *E. coli* O173 is attributed to a structural feature involving the common phosphorylated trisaccharide component **8** present in the main chain of both OPS[55].

-*P*-(O→6)-α-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→ **8**

Conclusions

Chemical studies performed by several research groups resulted in the elucidation of the composition and structures of 26 O-specific polysaccharides (O-antigens) of *Citrobacter* strains, which represent different species and different serogroups.

The O-antigens contain mainly neutral sugars, from which the most common are D-glucose, D-mannose and D-rhamnose. Two sugars, 4-deoxy-D-*arabino*-hexose and 3,6-dideoxy-D-*xylo*-hexose (abequose), are monosaccharides rarely occurring in nature. All monosaccharides except for pentoses are present in the pyranose form, D-xylose was found in both the pyranose and furanose forms and D-ribose only in the furanose form. Amino sugars represented by D-glucosamine, D-galactosamine, D-mannosamine, 2-amino-2-deoxy-L-fucose and 4-amino-4-deoxy-D-rhamnose (perosamine) are N-acetylated, and 3-amino-3-deoxy-D-fucose is acylated with either L-glyceroyl or (*R*)-3-hydroxybutyryl group. Two O-antigens are phosphorylated, one containing D-glycerol phosphate and the other a glycosyl phosphate group. In many serogroups the chemical composition of the O-antigens is not consistent with the original LPS chemotyping data.

The structures of *Citrobacter* O-antigens are highly diverse, the repeating units ranging from monosaccharide to heptasaccharide. There are three homopolymer O-antigens, consisting of 4-deoxy-D-*arabino*--hexose, 4-acetamido-4-deoxy-D-rhamnose (N-acetyl--D-perosamine) and 3-deoxy-3-(L-glyceroylamino)-D--fucose. The last monomer units are connected to each other by the glycosidic linkage between the sugar and glyceric acid moieties to build the structurally unusual O-antigen of serogroup O32.

The homopolymer of 4-deoxy-D-*arabino*-hexose was found in the serogroups O4, O27 and O36, whose classification in different serogroups could be accounted for by different LPS core structures (so far, six different *Citrobacter* LPS core types are known). Similarly, classification in different serogroups, O2, O20 and O25, of three strains that produce the same heteropolysaccharide O-antigen may result from differences in the LPS core structure. In contrast, some strains producing structurally different O-antigens are classified in the same serogroup (e.g. strains of serogroups O7 and O12), which could be due to the presence of common epitopes on the core region. Numerous *Citrobacter* strains are serologically related to other bacteria, mainly *Salmonella*, *Escherichia*, *Hafnia*, and *Klebsiella*, and some of these relationships could by substantiated on the level of the O-antigen structures.

The existing data show that the chemical and serological classification of *Citrobacter* strains requires revision. For this purpose, further immunochemical studies are needed to elucidate the O-antigen structures in serogroups not yet studied to reveal and substantiate on the molecular level serological relationships between strains of different *Citrobacter* serogroups and different enterobacterial genera and to find the position in the classification scheme of non-typed *Citrobacter* strains. It is also important to elucidate more precisely the serological and structural similarities and differences in the LPS core region in various serogroups and strains, which also contributes to the immunospecifity of *Citrobacter*.

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