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Structure Elucidation of the O-Antigen of *Salmonella enterica* O51 and Its Structural and Genetic Relation to the O-Antigen of *Escherichia coli* O23*

A. V. Perepelov[1#], Bin Liu[2,3#], Dan Guo[2,3], S. N. Senchenkova[1], A. S. Shahskov[1], Lu Feng[2,3], Lei Wang[2,3], and Y. A. Knirel[1]**

[1]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 119991 Moscow, Russia; fax: (499) 137-6148; E-mail: perepel@ioc.ac.ru*
[2]*TEDA School of Biological Sciences and Biotechnology, Nankai University, TEDA, Tianjin 300457, P. R. China*
[3]*Tianjin Key Laboratory for Microbial Functional Genomics, TEDA College, Nankai University, TEDA, Tianjin 300457, P. R. China; E-mail: striker198126@yahoo.com.cn*

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Abstract—The O-polysaccharide (O-antigen) of *Salmonella enterica* O51 was isolated by mild acid degradation of the lipopolysaccharide and its structure was established using sugar analysis and NMR spectroscopy. The O-antigen of *Escherichia coli* O23, whose structure was elucidated earlier, possesses a similar structure and differs only in the presence of an additional lateral α-D-Glc*p* residue at position 6 of the GlcNAc residue in the main chain. Sequencing of the O-antigen gene clusters of *S. enterica* O51 and *E. coli* O23 revealed the same genes with a high-level similarity. By comparison with opened gene databases, all genes expected for the synthesis of the common structure of the two O-antigens were assigned functions. It is suggested that the gene clusters of both bacteria originated from a common ancestor, whereas the O-antigen modification in *E. coli* O23, which, most probably, is induced by prophage genes outside the gene cluster, could be introduced after the species divergence.

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Key words: *Salmonella enterica*, *Escherichia coli*, lipopolysaccharide, bacterial polysaccharide, structure, gene cluster, O-antigen

Clones of *Escherichia coli* include both commensal and pathogenic strains. *Salmonella enterica* is recognized as one of the important pathogens of animals and humans. Serotyping of these bacteria is based on their surface antigens, including O-antigens, and about 174 O-serogroups in *E. coli* and 46 O-serogroups in *S. enterica* are recognized [1, 2].

The O-antigen (O-polysaccharide, OPS) is a part of the lipopolysaccharide of Gram-negative bacteria. It consists of oligosaccharide repeats (O-units), usually containing two to eight sugar residues. The OPS is one of the most variable constituents on the cell surface due to variations in the types of sugars present, their arrangement and linkages, and is subject to intense selection by the host immune system and bacteriophages. The OPS is also an important virulence factor, and its loss makes many pathogens serum sensitive or otherwise seriously impaired in virulence [3-5].

In *E. coli*, *S. enterica*, and *Shigella* spp., genes for the O-antigen biosynthesis are normally clustered between *galF* and *gnd* and belong to three different groups: i) genes for synthesis of nucleotide precursors of sugars used as specific O-antigen constituents; ii) genes encoding glycosyl transferases; iii) O-unit processing genes, including genes for O-unit flippase (*wzx*) and O-antigen polymerase (*wzy*) in the Wzy-dependent synthesis pathway [6]. The

Abbreviations: COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; OPS, O-polysaccharide; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; TSP, sodium trimethylsilyltetradeuteropropionate.

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[#] These authors contributed equally to this work.

** To whom correspondence should be addressed.

genes in the last two groups are specific to a unique O-antigen structure [7]. Genetic variations in the O-antigen gene clusters contribute major differences between the diverse O-antigen forms.

In this study, the structure of the O-antigen of *S. enterica* O51 was determined and found to be closely related to that of *E. coli* O23 reported earlier [8]. The O-antigen gene clusters of *S. enterica* O51 and *E. coli* O23 were sequenced and found to possess a high-level similarity to each other. An evolutionary relationship between the two bacteria studied is discussed.

MATERIALS AND METHODS

Salmonella enterica O51 and *E. coli* O23 strains were obtained from the Institute of Medical and Veterinary Sciences (Adelaide, Australia). *Salmonella enterica* O51 type strain G1456 was grown to late log phase in 8 liters of Luria–Bertani broth (1% tryptone, 0.5% yeast extract, 1% NaCl) using a 10-liter fermentor (BIOSTAT C-10; B. Braun Biotech International, Germany) under constant aeration at 37°C and pH 7.0. Bacterial cells were washed and dried as described [9]. The lipopolysaccharide (250 mg) was isolated from dried cells (7 g) by the phenol–water method [10] and purified by precipitation of nucleic acids and proteins with aqueous 50% trichloroacetic acid at 4°C followed by dialysis.

The lipopolysaccharide of *S. enterica* O51 (~90 mg) were delipidated with aqueous 2% acetic acid (6 ml) at 100°C until lipid precipitation (~1 h). The precipitate was removed by centrifugation (13,000*g*, 20 min) and the supernatant fractionated on a column (56 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 0.05 M pyridinium acetate buffer, pH 4.5, with monitoring using a differential refractometer (Knauer, Germany). The OPS was isolated in a yield 30% of the lipopolysaccharide mass.

The OPS of *S. enterica* O51 was hydrolyzed with 2 M trifluoroacetic acid (120°C, 2 h), and the monosaccharides were analyzed by GLC of the alditol acetates on a Hewlett-Packard 5890 chromatograph (USA) equipped with an Ultra-2 column (Supelco, USA) using a temperature gradient of 160 to 290°C at 3°C/min. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides as described [11].

Prior to NMR measurements, samples were deuterium-exchanged by freeze-drying from D_2O and then examined as solutions in 99.96% D_2O at 30°C. Spectra were recorded on a Bruker Avance 600 spectrometer (Germany) using TSP (δ_H 0) and acetone (δ_C 31.45) as internal reference. 2D NMR spectra were obtained using standard Bruker software, and the Bruker TopSpin program was used to acquire and process the NMR data. Mixing times of 100 and 150 msec were used in TOCSY and ROESY experiments, respectively.

Chromosomal DNA was prepared as described [12]. Primers WL_1098 (5′-ATTGGTAGCTGTAAGCCAAGGGCGGTAGCGT-3′) and WL_2211 (5′-CACTGCCATACCGACGACGCCGATCTGTTGCTTGG-3′) based on JUMPStart and *gnd* genes, respectively, were used to amplify the DNA of *S. enterica* O51 and *E. coli* O23 O-antigen gene clusters, using the Expand Long Template PCR system (Roche, USA). The PCR cycles used were as follows: denaturation at 94°C for 10 sec, annealing at 60°C for 30 sec, and extension at 68°C for 15 min. The PCR products were digested with DNase I (11284932001; Roche Diagnostics, USA), and the resulting DNA fragments were cloned into a pGEM-T Easy vector system to produce a gene bank. Sequencing was carried out by the Tianjin Biochip Corporation (China), using an ABI 3730 automated DNA sequencer (Applied Biosystems, USA). Sequences of the O-antigen gene clusters were analyzed as described [13].

RESULTS AND DISCUSSION

Structure elucidation of the O-antigen of *S. enterica* O51. The lipopolysaccharide was isolated from dried bacterial cells of *S. enterica* O51 by the Westphal procedure [10] and degraded with mild acid. A polysaccharide fraction was isolated by gel-permeation chromatography on Sephadex G-50. Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the polysaccharide revealed Glc, Gal, GlcNAc, and GalNAc in the ratio of ~1 : 1 : 2 : 1. GLC of the acetylated (*S*)-2-octyl glycosides showed that all monosaccharides have the D configuration.

The ^{13}C NMR spectrum of the O-polysaccharide (Fig. 1) showed signals for five anomeric carbons at δ 98.7-106.4, three nitrogen-bearing carbons (C2 of amino sugars) at δ 49.9-57.1, five CH_2O groups (C6 of hexoses) at δ 61.1-62.4 (non-substituted) and 69.1 (C6 of a glycosylated hexose residue; data of DEPT test), other sugar ring carbons in the region δ 70.0-82.4, and three N-acetyl groups at δ 23.6-23.9 (CH_3), 175.9-176.0 (CO). The ^1H NMR spectrum of the O-polysaccharide contained, *inter alia*, signals for five anomeric protons at δ 4.48-5.45 and three N-acetyl groups at δ 2.01-2.08.

The ^1H and ^{13}C NMR spectra of the OPS were assigned (Table 1) using two-dimensional correlation spectroscopy, including $^1H,^1H$ COSY, TOCSY, $^1H,^{13}C$ HSQC, and HMBC experiments. Five sugar spin systems were revealed, which, based on characteristic coupling constants, were assigned to Glc (**A**), Gal (**B**), GalNAc (**C**), $GlcNAc^I$ (**D**), and $GlcNAc^{II}$ (**E**). The $J_{1,2}$ coupling constant values of ~3 Hz indicated that units **A** and **C** are α-linked, whereas a relatively large $J_{1,2}$ value of 7-8 Hz showed that units **B**, **D**, and **E** are β-linked.

The signals for C6 of unit **A**, C3 and C4 of unit **B**, C3 of units **C** and **D** were shifted significantly downfield to δ

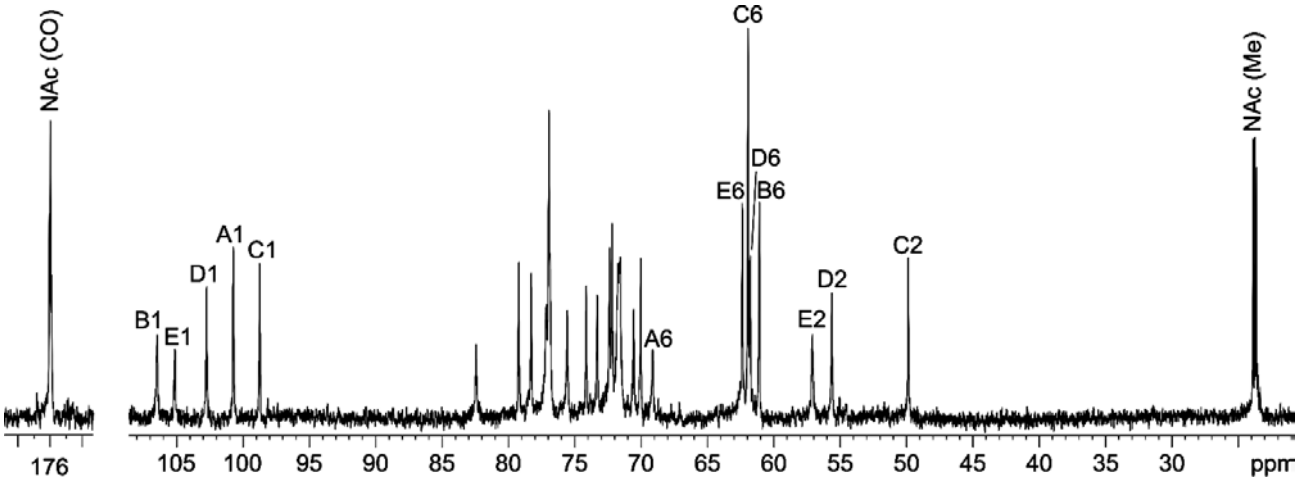

Fig. 1. ^{13}C NMR spectrum of the O-polysaccharide of *S. enterica* O51. Numerals refer to carbons in sugar residues denoted by letters as shown in Table 1.

69.1, 82.4, 76.9, 78.3, and 79.2, respectively, as compared with their positions in the corresponding non-substituted monosaccharides [14, 15]. These displacements are due to α-glycosylation effects and define the glycosylation pattern of the monosaccharides in the repeating unit of the O-polysaccharide.

The sequence of the monosaccharide residues was determined by a two-dimensional ROESY experiment. The spectrum showed cross-peaks at δ 4.88/4.22, 4.48/3.91, 5.45/3.73, 4.57/3.95, and 4.64/3.74, which were assigned to interresidue correlations between the following anomeric protons and protons at the linkage carbons: **A** H1/**B** H4; **B** H1/**C** H3; **C** H1/**D** H3, **D** H1/**A** H6a, and **E** H1/**B** H3, respectively. These data were confirmed by a ^{1}H,^{13}C HMBC experiment, which showed cross-peaks between the following anomeric protons and linkage carbons: **A** H1/**B** C4, **B** H1/**C** C3, **C** H1/**D** C3, **D** H1/**A** C6, and **E** H1/**B** C3 at δ 4.88/76.9, 4.48/78.3, 5.45/79.2, 4.57/69.1, and 4.64/82.4, respectively.

Therefore, the O-polysaccharide of *S. enterica* O51 has the structure **1** showed in Fig. 2.

Characterization of the O-antigen gene cluster of *S. enterica* O51. A sequence of 9091 base pairs (bp) between JUMPStart and *gnd* was examined, and seven open reading frames (*orfs*) having the same transcriptional direction were identified (Fig. 3). All *orfs* were assigned functions based on sequence similarity to previously described gene sequences available from the GenBank database (Table 2).

Genes for synthesis of the nucleotide precursors of common sugars (Glc, Gal, and GlcNAc in case of *S. enterica* O51) are located outside of the O-antigen gene

Table 1. ^{1}H and ^{13}C NMR chemical shifts of the O-polysaccharide of *S. enterica* O51 (δ, ppm)

Sugar residue	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 (6a, 6b) C6
→6)-α-D-Glc*p*-(1→ **A**	4.88 100.7	3.43 73.8	3.71 74.1	3.42 70.6	4.33 71.6	3.95, 4.19 69.1
→3,4)-β-D-Gal*p*-(1→ **B**	4.48 106.4	3.59 71.7	3.74 82.4	4.22 76.9	3.73 76.8	3.75, 3.79 61.1
→3)-α-D-Gal*p*NAc-(1→ **C**	5.45 98.7	4.37 49.9	3.91 78.3	4.17 70.0	3.89 72.2	3.75, 3.75 61.9
→3)-β-D-Glc*p*NAc-(1→ **D**	4.57 102.7	3.84 55.6	3.73 79.2	3.74 72.4	3.44 76.9	3.78, 3.89 61.8
β-D-Glc*p*NAc-(1→ **E**	4.64 105.1	3.59 57.1	3.53 75.6	3.41 71.7	3.41 77.1	3.75, 3.93 62.4

Note: Signals for the N-acetyl groups are at δ_H 2.01-2.08; δ_C 23.6-23.9 (Me), and 175.9-176.0 (CO).

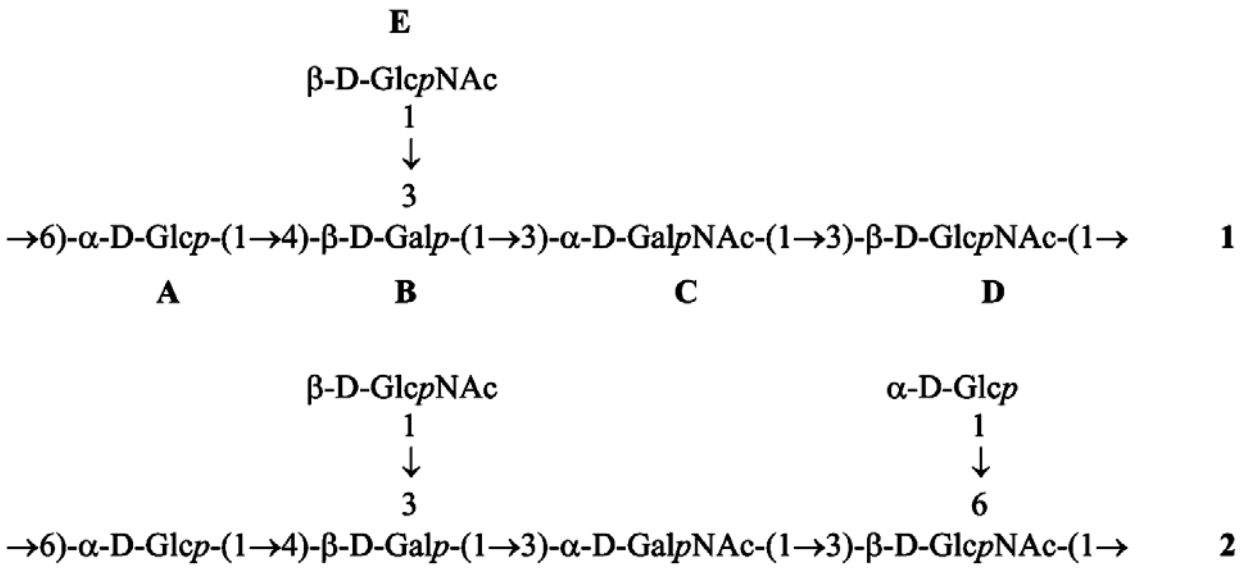

Fig. 2. Structures of the O-polysaccharides of *S. enterica* O51 (**1**) and *E. coli* O23 (**2**).

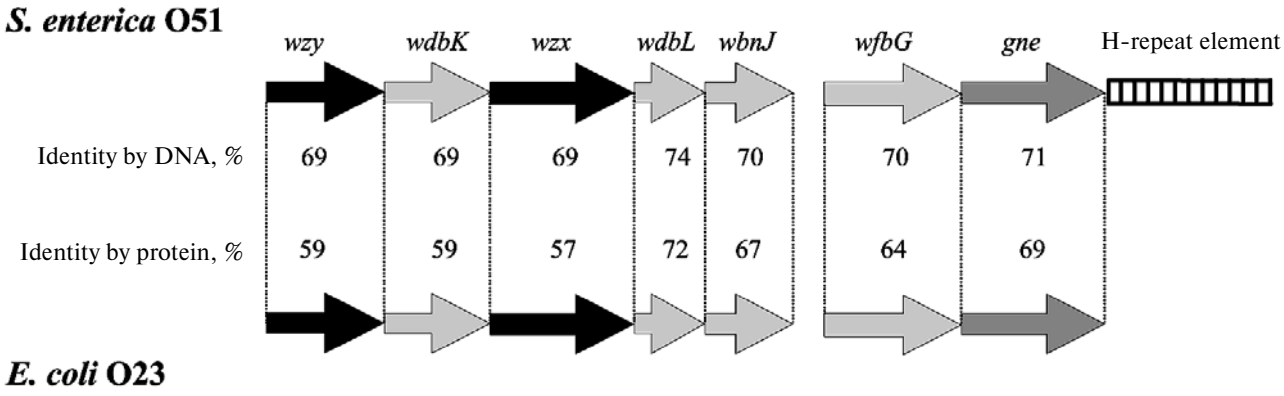

Fig. 3. Comparison of the O-antigen gene clusters of *S. enterica* O51 and *E. coli* O23.

cluster [16]. Therefore, a gene for synthesis of UDP-GalNAc only was expected in the *S. enterica* O51 gene cluster. Orf7 shares 61% identity with UDP-GlcNAc C4-epimerase (Gne) of *Yersinia enterocolitica* 0:8, which converts UDP-GlcNAc to UDP-GalNAc. It was suggested that Orf7 has the same function, and *orf7* was named accordingly.

Orf1 and Orf3 are the only two proteins with predicted transmembrane segments. Orf1 has nine well-proportioned transmembrane segments. It shares 46% similarity to the putative Wzy protein (O-antigen polymerase) of *S. enterica* group C1. Orf3 has 10 predicted transmembrane segments. It shares 46% similarity to the putative Wzx protein (O-unit flippase) of *Shigella boydii* type 9. Therefore, *orf1* and *orf3* were proposed to be the genes for O-antigen polymerase (*wzy*) and O-unit flippase (*wzx*), respectively.

Transfer of GlcNAc-1-P or GalNAc-1-P to an undecaprenol phosphate carrier catalyzed by WecA initiates the repeating unit synthesis in many *E. coli*, *S. enterica*, and *Shigella* spp. strains. Gene *wecA* is located outside of the O-antigen gene cluster [17], and, therefore, only four glycosyl transferase genes were expected in the O-antigen gene cluster of *S. enterica* O51.

Orf5 shares 46% identity with glycosyltransferase WbnJ of *E. coli* O86, which is responsible for the synthesis of the β-Gal-(1→3)-GalNAc linkage [18]. It was proposed that Orf5 has the same function as WbnJ. Orf6 shares 66% identity with glycosyltransferase WfbG of *S. enterica* O55 [19], and it was proposed that Orf6 catalyzes the formation of the α-GalNAc-(1→3)-GlcNAc linkage as this is the only linkage shared by the O-antigens of *S. enterica* O51 and O55. It can be suggested also that D-GlcNAc **D** rather than D-GalNAc **C** is the first sugar of

Table 2. Characteristics of the ORFs in the O-antigen gene cluster of *S. enterica* O51

No. orf	Gene	Position of gene	G + C (%)	Conserved domain(s)	Similar protein(s), strain(s) (GenBank accession No.)	% identical/% similar (number of a.a. overlap)	Putative function of protein
1	*wzy*	196..1221	26.9		O-antigen polymerase, *S. enterica* group C1 (AAB49386)	24/46 (327)	O-antigen-polymerase
2	*wdbK*	1218..2084	28.6	glycosyl transferase group 2 (PF00535), $E_{value} = 8.8\ e^{-31}$	glycosyltransferase, *Streptococcus thermophilus* (CAI34545)	32/48 (285)	glycosyl-transferase
3	*wzx*	2057..3259	27.3		O-antigen flippase, *S. boydii* type 9 (AAL27353)	27/46 (156)	O-antigen-flippase
4	*wdbL*	3256..3912	30.0	glycosyltransferase sugar-binding region (PF04488), $E_{value} = 4.8\ e^{-18}$	glycosyltransferase, *Prevotella oris* C735 (EFI49643)	38/58 (226)	glycosyl-transferase
5	*wbnJ*	3939..4673	31.2	glycosyl transferase group 2 (PF00535), $E_{value} = 1.3\ e^{-24}$	WbnJ, *E. coli* O86 (AAV80758)	46/66 (242)	–"–
6	*wfbG*	4900..6027	32.7	glycosyl transferase group 1 (PF00534), $E_{value} = 6\ e^{-36}$	WfbG, *S. enterica* O55 (ADI39346)	66/83 (372)	–"–
7	*gne*	6054..7082	34.9	epimerase (PF01370), $E_{value} = 5.9\ e^{-54}$	Gne, *Y. enterocolitica* (type 0:8) (AAC60777)	61/76 (336)	UDP-GlcNAc C4-epi-merase

the repeating unit of *S. enterica* O51. Orf2 and Orf4 belong to the glycosyl transferase group 2 family (PF00535, *E* value = $8.8\times e^{-31}$) and glycosyl transferase sugar-binding region family (PF04488, *E* value = $4.8\times e^{-18}$), respectively. Therefore, *orf2*, *orf4*, *orf5*, and *orf6* were proposed to be putative glycosyl transferase genes, and were named *wdbK*, *wdbL*, *wbnJ*, and *wfbG*, respectively.

Downstream of *gne*, an H-repeat element (positions 7329 to 8621) was found, which shares 78% DNA sequence identity with the H-repeat element of *E. coli* K12 [20] (Fig. 3). H-repeats have been suggested to mediate gene transfers and to play a role in formation of new O-antigen gene clusters [21]. However, current information is not enough to speculate on a role of the H-repeat in the formation of the *S. enterica* O51 O-antigen gene cluster.

Relatedness of the O-antigen structures and gene clusters of *S. enterica* O51 and *E. coli* O23. For a long time, only three common O-antigens have been known in *E. coli* and *S. enterica* (O55/O50, O111/O35, and O157/O30, respectively) [22]. Recently, more identical or closely related O-antigens have been found between the two species, including O71/O28, O85/O17, O103/O55, O123/O58, O145/O48, and O166/O66 pairs [23-27], as well as O17, O44, O73, O77/O6,14 and O118, O151/O47 groups [28, 29].

The structure **1** established in this work for the O-antigen of *S. enterica* O51 is closely related to the structure **2** of the O-antigen of *E. coli* O23 (Fig. 2), which has been elucidated earlier [8]. The latter differs only in the presence of a lateral α-D-Glc*p* residue attached at position 6 of the 3-substituted GlcNAc residue. Therefore, a new pair, *S. enterica* O51/*E. coli* O23, is added to the list of related O-serogroups of the two bacteria.

To substantiate genetically the structural similarity between the O-antigens of *S. enterica* O51 and *E. coli* O23, the O-antigen gene cluster of the latter was sequenced too. As expected, the O23 cluster was found to contain the same set of genes in the same order as in *S. enterica* O51 (Fig. 3). Therefore, the genes were assigned the same functions and given the same names. The corresponding genes from the two gene clusters share DNA identity from 69 to 74% and protein identity from 57 to

72% (Fig. 3). The only major difference between the two O-antigen gene clusters is that the H-repeat element is absent from the cluster of *E. coli* O23.

The similarity of the O-antigen gene clusters is consistent with the structural similarity of the O-antigens of *S. enterica* O51 and *E. coli* O23. As for the additional lateral glucosylation in *E. coli* O23, this is presumably encoded by prophage genes located elsewhere in the chromosome, as, e.g. serotype-converting genes involved in the synthesis of *S. flexneri* O-antigens [30]. It can be suggested that the O-antigen gene clusters of *S. enterica* O51 and *E. coli* O23 diverged from a common ancestor, as found in the other related *E. coli*/*S. enterica* gene clusters encoding the same or similar O-antigen structures. The prophage-induced O-antigen modification in *E. coli* O23 could be introduced after the species divergence.

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