A Single Nucleotide Exchange in the *wzy* Gene Is Responsible for the Semirough O6 Lipopolysaccharide Phenotype and Serum Sensitivity of *Escherichia coli* Strain Nissle 1917

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Structural analysis of lipopolysaccharide (LPS) isolated from semirough, serum-sensitive *Escherichia coli* strain Nissle 1917 (DSM 6601, serotype O6:K5:H1) revealed that this strain's LPS contains a bisphosphorylated hexaacyl lipid A and a tetradecasaccharide consisting of one *E. coli* O6 antigen repeating unit attached to the R1-type core. Configuration of the GlcNAc glycosidic linkage between O-antigen oligosaccharide and core (β) differs from that interlinking the repeating units in the *E. coli* O6 antigen polysaccharide (α). The *wa and *wb** gene clusters of strain Nissle 1917, required for LPS core and O6 repeating unit biosyntheses, were subcloned and sequenced. The DNA sequence of the *wa** determinant (11.8 kb) shows 97% identity to other R1 core type-specific *wa** gene clusters. The DNA sequence of the *wb** gene cluster (11 kb) exhibits no homology to known DNA sequences except *manC* and *manB*. Comparison of the genetic structures of the *wb**_{O6} (*wb** from serotype O6) determinants of strain Nissle 1917 and of smooth and serum-resistant uropathogenic *E. coli* O6 strain 536 demonstrated that the putative open reading frame encoding the O-antigen polymerase Wzy of strain Nissle 1917 was truncated due to a point mutation. Complementation with a functional *wzy* copy of *E. coli* strain 536 confirmed that the semirough phenotype of strain Nissle 1917 is due to the nonfunctional *wzy* gene. Expression of a functional *wzy* gene in *E. coli* strain Nissle 1917 increased its ability to withstand antibacterial defense mechanisms of blood serum. These results underline the importance of LPS for serum resistance or sensitivity of *E. coli*.**

Lipopolysaccharide (LPS) is a key component of the outer membrane of gram-negative bacteria. It is comprised of three distinct regions: lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide O antigen that causes a smooth phenotype. Lipid A is the most conserved part of LPS. It is connected to the core part, which links it to the O repeating units. In *Escherichia coli*, five different core structures (K-12 and R1 to R4) have been described (2, 18, 43). The O repeating units are highly polymorphic, and more than 190 serologically distinguished forms in *E. coli* are known today (35).

The LPS core-encoding genes are located at a conserved position on the *E. coli* K-12 chromosomal map (81 to 82 min) (5). The *wa** (formerly called *rfa*) gene clusters contain the genes which code for the enzymes required for the core assembly and consist of three operons (defined by the first genes in the operons; *gmhD*, *waaQ*, and *waaA*). Although the O-unit-encoding gene cluster is extremely polymorphic in the *E. coli*

species, it is localized at a conserved position on the *E. coli* K-12 chromosome between the *galF* and *gnd* genes (45.4 min) (5). These determinants consist of several sugar transferase-, epimerase-, and isomerase-encoding genes, the O-antigen flip-pase gene (*wzx*), the O-antigen polymerase gene (*wzy* [formerly called *rfc*]), as well as the genes coding for enzymes involved in carbohydrate biosynthesis pathways. Until now, several *E. coli* O-antigen-encoding gene clusters have been studied, e.g., those of serotypes O7, O111, O113, and O157 (32, 38, 54, 55). These gene clusters show no significant nucleotide homology with each other, with the exception of some common genes such as *manC* and *manB*. However, they contain a conserved range of predicted enzyme activities. The O6 antigen is widely distributed among pathogenic and nonpathogenic fecal *E. coli* isolates and is often found in uropathogenic *E. coli* strains. It is associated with R1-type core LPS and has not been investigated in detail so far.

E. coli strain Nissle 1917 (DSM 6601, serotype O6:K5:H1) is a nonpathogenic fecal isolate, which is used as a probiotic agent in medicine (7), mainly for treatment of various gastroenterological indications (23, 26, 30, 33, 41). This strain exhibits a serum-sensitive, semirough phenotype. Since colonies of this strain on agar plates show a special smooth-and-rough

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference
pGEM-T Easy	Cloning vector, Ap ^r	Promega
pBluescript II KS	Cloning vector, Ap ^r	Stratagene
pLDR8	<i>int</i> gene expression vector, Km ^r	9
pLDR9	Cloning vector for integration into the λ - <i>attB</i> , Km ^r	9
pLDR11	Cloning vector for integration into the λ - <i>attB</i> , Tc ^r	9
pGWB1917	<i>wb</i> [*] _{Nissle 1917} cloned into pGEM-T Easy, Ap ^r	This work
pGWB536	<i>wb</i> [*] ₅₃₆ cloned into pGEM-T Easy, Ap ^r	This work
pBWB536	<i>wb</i> [*] ₅₃₆ cloned into pBluescript II KS, Ap ^r	This work
pGLG2504	2,504 bp of the 5'-proximal region of <i>wb</i> [*] ₅₃₆ (including JUMPstart region, <i>wzx</i> ₅₃₆ , and the 986-bp fragment of <i>wzy</i> ₅₃₆) cloned into pGEM-T Easy, Ap ^r	This work
pBLG2504	2,504 bp of the 5'-proximal region of <i>wb</i> [*] ₅₃₆ (including JUMPstart region, <i>wzx</i> ₅₃₆ , and the 986-bp fragment of <i>wzy</i> ₅₃₆) cloned into pBluescript II KS, Ap ^r	This work
pGLG2849	2,849 bp of the 5'-proximal region of <i>wb</i> [*] ₅₃₆ (including JUMPstart region, <i>wzx</i> ₅₃₆ , and the 1,343-bp intact <i>wzy</i> ₅₃₆) cloned into pGEM-T Easy, Ap ^r	This work
pBLG2849	2,849 bp of the 5'-proximal region of <i>wb</i> [*] ₅₃₆ (including JUMPstart region, <i>wzx</i> ₅₃₆ , and the 1,343-bp intact <i>wzy</i> ₅₃₆) cloned into pBluescript II KS, Ap ^r	This work
pLBW1	<i>P_{bla}::wzy</i> ₅₃₆ cloned into pLDR11, Tc ^r	This work
pGPW1	<i>Pwb</i> [*] _{Nissle 1917::wzy₅₃₆ cloned into pGEM-T Easy, Ap^r}	This work
pLPW1	<i>Pwb</i> [*] _{Nissle 1917::wzy₅₃₆ cloned into pLDR9, Km^r}	This work

colonial morphology, it was speculated that this phenotypic appearance may be due to the presence of a modified LPS. Usually, the O-antigen side chain is synthesized by polymerization of identical O repeating units, assembled on a special phospholipid carrier (antigen carrier lipid) (42). The enzyme O-antigen ligase adds the O-antigen side chain to the core oligosaccharide, which is synthesized separately from the O-antigen side chain and linked to lipid A (29). As polymerization of the O subunits into a long-chain polysaccharide is catalyzed by the O-antigen polymerase Wzy, the question of whether the semirough phenotype of strain Nissle 1917 is the result of a nonfunctional O-antigen polymerase was raised.

Since LPS is located on the outer surface of bacterial cells, its expression is known to be responsible for many features of the cell surface of the gram-negative bacteria, such as resistance to detergents, hydrophobic antibiotics, serum complement factors, etc. (13, 14, 28, 49). It has been suggested that some of these characteristics, especially resistance to the bactericidal effect of serum complement system, are dependent on the length of the O-antigen side chain (39). LPS is believed to significantly contribute to virulence by protecting bacteria from the bactericidal effect of serum complement (20, 40, 51). Moreover, in contrast to the earlier view, it has recently been reported that the K5 capsule does not contribute as much to serum resistance of *E. coli* strains as the O antigen does (9). Therefore, it is important to study the effect of impaired O6 antigen synthesis of *E. coli* strain Nissle 1917 on its serum sensitivity, which together with the absence of virulence factors contributes to its safety and probiotic effect. Here we report the complete structure of the tetradecasaccharide of the LPS core region with one O6 antigen repeating unit attached, and the mode of linkage between the O antigen and core and the structure of lipid A were elucidated. The structure of the complete LPS molecule identified further confirms the results of genetic analysis of the semirough phenotype of *E. coli* strain Nissle 1917. The results of our chemical analysis completed and confirmed the nucleotide sequence and structural analyses of the O6 antigen gene cluster, the genetic basis for the semi-

rough phenotype of *E. coli* Nissle 1917, and its importance for this strain's serum sensitivity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *E. coli* strain Nissle 1917 (Mutaflor) (DSM 6601, serotype O6:K5:H1) was kindly supplied by Ardeypharm GmbH (Herdecke, Germany), and the uropathogenic *E. coli* strain 536 (O6:K15:H31) used had been isolated from a patient with acute pyelonephritis (4). The plasmids constructed in this study are listed in Table 1. The *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (44) with or without 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Where appropriate, ampicillin was added to the growth medium at a concentration of 50 µg/ml.

Isolation of LPS. Lyophilized cells (67.59 g) were extracted with phenol-water by the procedure of Westphal and Jann (58), and the crude LPS preparation was digested sequentially, first with RNase or DNase and proteinase K and then with phenol-chloroform-petrol ether by the method of Galanos et al. (12). The yield of the purified LPS was 2.77 g (4.1%).

Isolation and purification of lipid A and oligosaccharide moiety. A suspension of LPS (258.8 mg) in 0.1 M sodium acetate-hydrogen acetate (pH 4.4) (25 ml) was heated at 100°C for 1 h, a lipid precipitate was extracted with chloroform (three times with 25 ml of chloroform), the crude lipid A from the extract (23.2 mg) was fractionated on a 2-mm-diameter silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany) in chloroform-methanol-water (100:75:15, vol/vol/vol). Bands were visualized by dipping the plate in distilled water, marking the bands, scraping them off the plate, and elution from the silica gel by shaking the gels overnight with chloroform-methanol-water 100:100:30 (vol/vol/vol). Six fractions were obtained, including hexaacyl lipid A (*LA*_{hexa}) (2.06 mg) (*R_f* of 0.4) as the main fraction. For matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis, lipid A was dissolved in chloroform-methanol (8:2, vol/vol) and treated with Amberlite IRA-120 (H⁺) cation-exchange resin.

After extraction of lipids, the water phase was lyophilized and the product (272 mg) was fractionated on a column (3.5 by 90 cm) of TSK HW-40 (S) gel (Merck) in pyridine-hydrogen acetate-water (4:10:1,000, vol/vol/vol) with monitoring using a differential refractometer (Knauer, Berlin, Germany) to give an oligosaccharide (49.05 mg), together with monosaccharides. The oligosaccharide (42 mg) was further purified by high-performance anion-exchange chromatography by two runs on a semipreparative CarboPac PA1 column (9 by 250 mm) in a linear gradient of 0 (5 min) to 0.5 M sodium acetate at 4 ml/min using a Dionex system with pulse amperometric detection. Fractions were collected every minute and analyzed using the same system on an analytical CarboPac PA1 column (4.6 by 250 mm) using the same eluent at 1 ml/min. Two major products having retention times of 21.17 and 26.23 min by analytical chromatography (~12 and ~15 min by preparative chromatography) were obtained and desalted on a column (2.5 by 120 cm) of Sephadex G-10 (Pharmacia, Uppsala, Sweden) in water to give

oligosaccharides OSI (oligosaccharide I) (4.68 mg) and OSII (4.39 mg), respectively. Two minor products with retention times of 10.70 and 33.43 min were not studied.

Chemical analyses. The total amounts of amino sugars were determined by the modified Morgan-Elson test (48). Separate quantification of GlcN, GalN, and ethanolamine (Etn), as well as phosphorylated amino components (GlcN-*P* and Etn-*P*), was performed by high-pressure liquid chromatography using a PICO-TAG instrument (Waters) after hydrolysis with 4 M HCl (100°C, 16 h). The total amount of phosphate was determined by the method of Lowry et al. (27). The total amount of 3-deoxy-*D*-manno-octulosonic acid was determined by the thio-barbiturate test (57). Neutral sugars (Glc, Gal, Man, and *L*-glycero-*D*-manno-heptose) were analyzed by gas-liquid chromatography (GLC) and GLC-MS as the alditol acetates (45), prepared by hydrolysis with aqueous 2 M CF₃CO₂H (100°C, 4 h) followed by reduction with NaBH₄ in water and peracetylation with acetic anhydride in pyridine (1:1.5 [vol/vol], 85°C, 20 min). Fatty acids were determined by GLC as the methyl esters prepared by methanolysis (2 M HCl-methanol, 120°C, 16 h) followed by extraction with chloroform (59).

GLC, GLC-MS, and MALDI-TOF MS. GLC was performed on Varian 3700 GC or Hewlett-Packard HP 5890 Series II chromatographs equipped with a 30-m-long fused silica SPB-5 column (Supelco) using a temperature gradient from 150°C (3 min) to 320°C at 5°/min. GLC-MS was performed on a Hewlett-Packard HP 5989A instrument equipped with a 30-m-long HP-1 column (Hewlett-Packard) under the same chromatographic conditions as used for GLC.

MALDI-TOF MS was performed on a Bruker-Reflex II instrument (Bruker-Franzen Analytik, Bremen, Germany) in a linear configuration in the negative mode at an acceleration voltage of 20 kV and with delayed ion extraction. Samples were dissolved in chloroform (lipid A) or distilled water (oligosaccharides) at a concentration of 10 µg/µl, and 2 µl of solution was mixed with 2 µl of 0.5 M 2,4,6-trihydroxyacetophenone (Aldrich) in methanol as the matrix solution. Aliquots (0.5 µl) were deposited on a metallic sample holder and analyzed immediately after drying in a stream of air.

NMR spectroscopy. Prior to the measurements, the oligosaccharide samples were lyophilized twice from ²H₂O. The nuclear magnetic resonance (NMR) spectra were recorded at 300 K in 99.96% ²H₂O. Chemical shifts were referenced to internal sodium 3-trimethylsilylpropanoate-*d*₄ (δ_H 0), internal acetone (δ_C 31.45), or external 85% aqueous H₃PO₄ (δ_P 0.0). One-dimensional ¹H and ³¹P NMR and two-dimensional spectra were obtained with a Bruker DRX-600 spectrometer (Rheinstetten, Germany) at 600 and 243 MHz, respectively, and ¹³C NMR spectra were obtained with a Bruker AMX-360 spectrometer at 90 MHz. Bruker software XWINNMR version 2.6 was used to acquire and process the data. Mixing times of 100 and 500 ms were used in two-dimensional total correlated spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments, respectively.

SDS-PAGE, Western blotting, and immunization. Three types of antibodies were used in Western blot analysis: (i) polyclonal rabbit anti-*E. coli* O6 serum from J. Bockemühl (Hygiene Institut, Hamburg, Germany) obtained against *E. coli* Nissle 1917 (DSM 6601, serotype O6:K5:H1); (ii) polyclonal anti-*E. coli* R1 serum obtained against an *E. coli* R1 mutant; and (iii) monoclonal antibody WN1-222-5 cross-reactive with all *E. coli* LPS core types having a minimal structure (>Rd).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed as described previously (52). For Western blotting, gels were electrotransferred overnight onto polyvinylidene difluoride membranes (Qiagen) by Hank blotting (Bio-Rad) as described previously (37). Rabbits were immunized with heat-inactivated *E. coli* R1 bacteria as described previously (52).

Serum resistance assay. Serum resistance of *E. coli* strains was usually analyzed by incubating the bacteria in 90% human serum. A bacterial culture that had been allowed to grow overnight was diluted 1:100 in LB and grown to 90 Klett units. The bacteria were diluted 1:10 in human serum and incubated at 37°C. After 0, 1, 3, and 24 h, survival of the strains was tested by plating an aliquot on LB agar plates containing the appropriate antibiotic (19). In addition, to determine the serum resistance of strain Nissle 1917 after complementation with a single chromosomal copy of the *wzy*₅₃₆ gene (*wzy* from strain 536) or with an full-length, plasmid-encoded *wb**₅₃₆ gene cluster, growth of bacterial strains in 50% human serum was also measured. Serum sensitivity and efficiency were routinely checked by incubation of the strains in heat-inactivated serum (56°C, 30 min). After heat inactivation, serum-sensitive strains multiplied in serum but not in nontreated serum.

DNA technology. Isolation of DNA and recombinant DNA techniques were performed as described previously (44). Restriction enzymes were obtained from Amersham-Pharmacia Biotech (Freiburg, Germany) and used as recommended by the supplier. DNA primers were purchased from MWG Biotech AG (Eber-

sberg, Germany). The *wzy*₅₃₆ gene was integrated into the bacteriophage λ-specific chromosomal attachment site of *E. coli* strain Nissle 1917 as described previously (11).

Screening of an established cosmid genomic library of *E. coli* strain Nissle 1917. To identify a cosmid clone containing the R1-type encoding *waa* gene cluster of *E. coli* Nissle 1917, an established genomic library (Gigapack III Gold Packaging Extract; Stratagene) of strain Nissle 1917 was screened by PCR using primers R1C3 and R1K15 (2).

Amplification and subcloning of the O6 antigen-encoding gene cluster of *E. coli*. The primers used in this study are listed in Table 2. To amplify the *E. coli* O6-specific *wb** gene cluster, the already published oligonucleotides 482 and 412 were used. These primers are complementary to the JUMPstart region of the O6-specific *wb** determinant and the *gnd* gene (which is located downstream of *wb**), respectively (10). Long-distance PCR was performed by using the Expand Long Template PCR system (Roche Molecular Biochemicals, Mannheim, Germany). PCR was performed in an Eppendorf Thermocycler (Mastercycler personal) as follows. First, an initial denaturation step (94°C for 2 min) was performed. This step was followed by 10 cycles of PCR, with 1 cycle consisting of denaturation (10 s at 94°C), annealing (30 s at 64°C), and extension (12 min at 68°C). For the next 20 cycles, the extension step was increased by 20 s each time. A final elongation step (68°C for 12 min) was performed. The amplified PCR products were verified on a 0.8% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer. Partial amplification of the O6-specific *wb** gene cluster and amplification of PCR products used for cloning and complementation analysis were performed as described above, using appropriate annealing temperatures and elongation times.

Amplified PCR fragments were subsequently purified by phenol-chloroform (1:1, vol/vol) extraction and precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of 100% ethanol. The pellet was washed twice with 70% ethanol, dried, and resuspended in an appropriate amount of water. The PCR product was cloned into the vector pGEM-T Easy (Promega, Mannheim, Germany) by the protocol of the manufacturer.

Construction of chromosomally integrated *P*_{bla::wzy₅₃₆} and *Pwb_{Nissle 1917::wzy₅₃₆} fusions for complementation of *E. coli* strain Nissle 1917.** To avoid gene dosage effects and to create stably complemented strains that can be grown in the absence of antibiotics, strain Nissle 1917 was chromosomally complemented by integration of different promoter-*wzy*₅₃₆ fusions into the chromosomal attachment site of bacteriophage λ. Promoters P1 and P2 of the β-lactamase-encoding gene (*bla*) of pBR322 (8) together with a Shine-Dalgarno sequence were fused with the *wzy*₅₃₆ gene by PCR using primers LG7 and LG10, cloned into plasmid pLDR11. The resulting plasmid pLBW1 (Table 1) was subsequently used for the integration of the *P*_{bla::wzy₅₃₆} fusion into the bacteriophage λ attachment site of the *E. coli* Nissle 1917 chromosome as described by Diederich and coworkers (11). By using primers LG15 and LG16, the upstream region of the *wb** gene cluster which is expected to contain the promoter(s) of the *wb** determinant of strain Nissle 1917 (*Pwb**_{Nissle 1917}) was amplified (~450 bp). In parallel, the *wzy*₅₃₆ gene was amplified using primers LG8 and LG10. After digestion with *Xba*I, the two fragments were ligated, and PCR was performed with primers LG15 and LG10 using the ligation mixture as a template. The obtained PCR product represents the *wzy*₅₃₆ gene under transcriptional control of the *wb**_{Nissle 1917}-specific promoter. The fragment was then cloned into vector pLDR9, thus resulting in pLPW1, which was used for integration of the strain Nissle 1917-specific *Pwb**_{Nissle 1917::wzy₅₃₆} fusion into the bacteriophage λ attachment site of the *E. coli* Nissle 1917 chromosome as described previously (11).

DNA sequence analysis and sequence annotation. A small insert library (2 to 2.5 kb) was generated by mechanical shearing of DNA of the *waa** gene cluster-containing cosmid clone 8Y1 as well as of the cloned *wb** determinant of strain Nissle 1917 (34). After end repair with T4 polymerase, the fragments were ligated into the prepared pTZ19R vector. Isolated plasmids were sequenced from both ends using the dye terminator chemistry and analyzed on ABI337 sequencers (Applied Biosystems, Munich, Germany). The Phrap software implemented in the Staden software package was used for assembly and editing of the sequence data (47).

Homology searches and searches for conserved protein domains were performed with the BLASTN, BLASTX, and PSI- and PHI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) programs of the National Center for Biotechnology Information (NCBI) (1). The identification of the putative open reading frames (ORFs) was done using Vector NTI (InforMax, Oxford, United Kingdom) and the NCBI ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Prediction of membrane-spanning regions of proteins was done by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html).

Isolation and analysis of LPS side chain pattern. To isolate LPS from the *E. coli* strains used in this study, 2 ml of a culture grown overnight was collected by

TABLE 2. Primers used in this study

Primer	Position in <i>wa</i> * _{O6} and <i>wb</i> * _{O6} determinants	Sequence (5'→3')	Comment and/or reference
R1C3	9954–9972	GGG ATG CGA ACA GAA TTA GT	Located in <i>wa</i> * _{O6} (2)
R1K15	9422–9441	TTC CTG GCA AGA GAG ATA AG	Located in <i>wa</i> * _{O6} (2)
482	11283–11301	CAC TGC CAT ACC GAC GCC GAT CTG TTG CTT GG	8
412	267–296	ATT GGT AGC TGT AAG CCA AGG GCG GTA GCG T	8
M13-uni		TGT AAA ACG ACG GCC AGT	Promega
LG1	1553–1573	GTT TCT TGT ATT CAG TAT GCT	Located in <i>wb</i> * _{O6}
LG8	1553–1573	GCT CTA GAG CGT TTC TTG TAT TCA GTAT GCT	Located in <i>wb</i> * _{O6}
LG2	2648–2668	TGG GTT TGC TGT GTA TGA GGC	Located in <i>wb</i> * _{O6}
LG3	2990–3013	TAT GAG CCC TGT TAT AAC TTG GGA	Located in <i>wb</i> * _{O6}
LG4	3841–3864	CAC CTT GCC CTC CTG AAC CAT TAT	Located in <i>wb</i> * _{O6}
LG5	4963–4989	GAA TAG TTT ACC TGA GGA TTT TTT ATC	Located in <i>wb</i> * _{O6}
LG6	6166–6189	GTC TTC CTA CAC CCA CCA TCT CCA	Located in <i>wb</i> * _{O6}
LG7	8241–8262	CCA GCC ATA ATG ATA GGT GTA A	Located in <i>wb</i> * _{O6}
LG8		AAC CTG AAA GAA GGG GCG AAG	Located in <i>galF</i> (positions 841–861), amplification of <i>wb</i> * _{O6} upstream region
LG9	337–356	GCT CTA GAG CTT AGG TGT AAT TAT ATT ATT	Located in <i>wb</i> * _{O6} , amplification of <i>wb</i> * _{O6} upstream region
LG15	1553–1573	CCA TCG ATG GGT GCC TGA CTG CGT TAG CAA TTT AAC TGT GAT AAA CTA CCG CAT TAA AGC TTA TCG ATG ATA AGA GAG GTT TCT TGT ATT CAG TAT TGC T	Located in <i>wb</i> * _{O6} , <i>P_{bla}</i> fused to primer LG6
LG16	1553–1573	GCT CTA GAG CGT TTC TTG TAT TCA GTA TGC T	Located in <i>wb</i> * _{O6} , <i>Xba</i> I restriction site fused to primer LG6

centrifugation, washed with 1 ml of TNE (10 mM Tris [pH 8], 10 mM NaCl, 10 mM EDTA), and resuspended in 540 μ l of TNEX (TNE buffer with 1% [vol/vol] Triton X-100). Sixty microliters of lysozyme (5 mg/ml) (Sigma-Aldrich, Taufkirchen, Germany) was added, and the mixture was incubated for 20 min at 37°C. Prior to phenol extraction, 30 μ l of proteinase K (20 μ g/ml) (Sigma-Aldrich) was added, and the mixture was incubated for 2 h at 65°C. The aqueous phase was divided into two halves: one half was used for preparation of chromosomal DNA, and 20 μ l of the other half were used for analysis of the LPS by SDS-PAGE (24) using a 15% slab polyacrylamide gel. After electrophoresis, the gels were silver stained (50).

Nucleotide sequence accession numbers. The sequences of the *wa**_{O6} and *wb**_{O6} gene clusters of the *E. coli* strain Nissle 1917 (accession numbers AJ426044 and AJ426045, respectively) as well as that of the *wzy* gene of the *E. coli* O6 strain 536 (accession number AJ426423) were submitted to the EMBL nucleotide sequence database.

RESULTS

Chemical and serological characterization and degradation of LPS. The purified LPS was isolated from cells of *E. coli* Nissle 1917 (DSM 6601, serotype O6:K5:H1) by combination of the phenol-water (58) and phenol-chloroform-petroleum ether (12) procedures. SDS-PAGE of the LPS (Fig. 1) showed the predominance of a semirough-type species with one O-antigen repeating unit attached to the core-lipid A moiety.

On Western blots, the LPS reacted with homologous polyclonal anti-O6 serum, which recognized almost exclusively the semirough-type LPS species but not the rough form which, according to the silver staining procedure, was also present to a significant lower extent (data not shown). Polyclonal anti-*E. coli* R1 serum reacted also with the homologous LPS studied, and from these data, we concluded that LPS of *E. coli* Nissle 1917 has an R1-type core and the predominant LPS species has one O6 antigen repeating unit (semirough-type LPS).

Composition analysis data of the LPS are given in Table 3. Fatty acids present in the lipid A part are typical of the *E. coli*

LPS (60). In addition to GlcN and the so-called basal sugars (3-deoxy-D-manno-octulosonic acid [Kdo], L-glycero-D-manno-heptose [Hep], Glc, and Gal), which are obligatory components of the lipid A backbone (60) and the full-core oligosaccharide of *E. coli* (18), respectively, two additional sugar constituents, GalN and Man, were found. These can be attributed to the O6 antigen repeating unit, which is a pentasaccharide containing two residues of Man, one residue of GalNAc, and, depending on the strain, either one Glc residue and one

FIG. 1. Silver-stained SDS-PAGE of LPS from *E. coli* Nissle 1917 (lane 1) and *E. coli* rough mutants of core types R1 (lane 2), R2 (lane 3), R3 (lane 4), R4 (lane 5), and K-12 (lane 6).

TABLE 3. Composition of LPS of *E. coli* Nissle 1917 (DSM 6601)

Component	Content	
	nmol/mg	mol/mol of LPS ^a
Sugars		
Total hexosamine	591	3.8 (4)
GlcN ^b	283	1.8 (3)
GalN	139	0.9 (1)
Glc	1,069	6.9 (4)
Gal	474	3.0 (2)
Man	321	2.1 (2)
Hep	566	3.6 (3)
Kdo	248	1.6 (2)
Phosphate substituents		
<i>P</i>	1,163	7.4 (5)
Etn- <i>P</i> ^c	86	0.5 (0.5)
Fatty acids		
Dodecanoic acid	130	0.8 (1)
Tetradecanoic acid	156	1.0 (1)
3-Hydroxytetradecanoic acid	460	3.0 (4)
Hexadecanoic acid	Trace	

^a Values calculated from the LPS structure (Fig. 4 and 7) are given in parentheses.

^b Total of GlcN and GlcN-*P* (217 and 66 nmol/mg, respectively).

^c Total of Etn and Etn-*P* (60 and 26 nmol/mg, respectively).

GlcNAc residue or two residues of GlcNAc (21). Phosphate and ethanolamine phosphate (Etn-*P*) were found as well; the former has been reported to be a substituent of both lipid A and core, whereas the latter, most likely, originated from the core moiety (18).

For structural studies, the lipid A and carbohydrate parts were separated chemically by mild acid hydrolysis. Lipid A was fractionated by preparative thin-layer chromatography to give the representative hexaacyl *E. coli*-type lipid A (LA_{hexa}), which was studied by MALDI-TOF MS. The carbohydrate portion was fractionated by gel permeation chromatography on TSK HW-40 (S) followed by high-performance anion-exchange chromatography under neutral conditions to give two major oligosaccharides (OSI and OSII), which were studied using MALDI-TOF MS and NMR spectroscopy.

MALDI-TOF MS of lipid A. The negative-mode MALDI-TOF mass spectrum of the purified LA_{hexa} showed an intense peak of a pseudomolecular ion [M_{BH-LA} - H]⁻ at *m/z* 1797.4. This corresponds to the molecular formula C₉₄H₁₇₈O₂₅N₂P₂ and molecular mass 1798.4 Da, which are characteristic of a lipid A molecule with a bisphosphorylated lipid A backbone and the four 3-hydroxytetradecanoyl groups, one dodecanoyl group, and one tetradecanoyl group typical for the hexaacyl *E. coli*-type lipid A (LA_{hexa}). The absence of other peaks indicates the homogeneity of the isolated LA_{hexa}. The mass spectrum was essentially identical to that of the reference sample of *E. coli* LA_{hexa} from our laboratory (Forschungszentrum Borsstel), and no significant difference was observed between the LA_{hexa} studied and that from *E. coli* Re mutant F515 with respect to the capacity to induce production of cytokines in human mononuclear cells (data not shown).

In summary, these data show that LA_{hexa} from *E. coli* Nissle 1917 (DSM 6601) has the characteristic hexaacyl *E. coli*-type lipid A as shown in the LPS structure (see Fig. 4).

TABLE 4. ¹H and ¹³C NMR data of anomeric atoms of OSI and OSII from LPS of *E. coli* Nissle 1917 (DSM 6601)

Oligosaccharide and sugar residue	Chemical shift (ppm)				$J_{1,2}$ coupling constant of OSI (Hz) ^a
	δ_{HI}		δ_{CI}		
	OSI	OSII	OSI	OSII	
O6 antigen					
β -Glc _p	4.65	4.65	105.0	104.9	8.0
α -Gal _p NAc	5.26	5.26	100.2	100.2	4.0
$\rightarrow$ 2,3)- β -Man _p	4.80	4.81	101.9	101.9	nr
$\rightarrow$ 4)- β -Man _p	4.73	4.74	101.8	101.9	nr
$\rightarrow$ 3)- β -Glc _p NAc	4.86	4.86	102.6	102.6	8.4
R1-type core					
α -Gal _p	5.36	5.35	96.9	96.8	3.7
$\rightarrow$ 2)- α -Gal _p	5.61	5.60	92.7	92.8	3.3
$\rightarrow$ 3)- β -Glc _p	4.75	4.76	103.8	103.6	~7
$\rightarrow$ 2,3)- α -Glc _p	5.86	5.85	95.4	95.5	3.3
$\rightarrow$ 3)- α -Glc _p	5.27	5.30	102.6	101.9	~4
α -Hepp ^{III}	4.94	4.95	101.2	101.4	nr
$\rightarrow$ 3,7)- α -Hepp ^{II}	5.09 ^b	5.16 ^b	103.5	103.1	nr
$\rightarrow$ 3)- α -Hepp ^I	5.17 ^b	5.13 ^b	101.1	101.4	nr

^a nr, not resolved.

^b Chemical shift of the major signal.

MALDI-TOF MS of the O-antigen core oligosaccharides.

The negative-ion mode MALDI-TOF mass spectrum of OSI showed the major peak of a pseudomolecular ion [M_{OSI} - H]⁻ at *m/z* 2800.4, together with smaller peaks belonging to the corresponding sodium and potassium adduct ions. These data are compatible with a tetradecasaccharide containing eight hexoses, three heptoses, and two 2-acetamido-2-deoxyhexose residues, together with one Kdo, one ethanolamine, and three phosphate groups (Hex₈Hep₃HexNAc₂KdoP₃Etn; calculated molecular mass of 2799.8 Da for C₉₅H₁₆₃O₈₅N₃P₃).

The MALDI-TOF mass spectrum of OSII contained a [M_{OSII} - H]⁻ pseudomolecular ion peak at *m/z* 2677.9, which demonstrates that the molecular mass of OSII was 123 Da less than that of OSI. This difference corresponds to the mass of a phosphoethanolamine group, and therefore, we conclude that OSII is a tetradecasaccharide bisphosphate or pyrophosphate (Hex₈Hep₃HexNAc₂KdoP₂; calculated molecular mass of 2676.8 Da for C₉₃H₁₅₈O₈₂N₂P₂). The spectrum of OSII showed also less intense peaks at *m/z* 2515, 2242, and 1785, which, most likely, belong to fragment ions.

NMR spectroscopy of the O-antigen core oligosaccharides.

The ¹H and ¹³C NMR spectra of OSI and OSII were similar and contained signals of anomeric protons for 13 sugar residues at δ_{H} 4.65 to 5.86 and δ_{C} 92.7 to 105.0 (Table 4). This finding is in accordance with a tetradecasaccharide consisting of 13 aldose residues and Kdo at the reducing end. The spectra also contained signals for a methylene group of various Kdo forms [the major one was at δ_{H} 1.82 (d, *J*_{3,4} = 4.4 Hz, H3) and δ_{C} 35.2 (C3)], as well as signals for two N-acetyl groups at δ_{H} 2.04 and 2.06 (both s) and δ_{C} 23.3 and 23.5. A significant difference between the spectra of OSI and OSII is the presence of signals for a CH₂N group of Etn-*P* at δ_{H} 3.3 (t, *J* = 4.8 Hz) and δ_{C} 41.3 (*J*_{C,P} = 8.8 Hz) in the OSI spectrum; these signals are absent from the OSII spectrum. These data are in agreement with the MALDI-TOF MS data above.

Correspondingly, the ¹H,¹³C heteronuclear multiple quan-

FIG. 2. Anomeric region of a ^1H , ^{13}C HMQC spectrum of OSI from *E. coli* Nissle 1917. The corresponding part of the ^1H NMR spectrum is displayed to the left of the left vertical axis. For signal assignment, see Table 4.

tum correlation (HMQC) spectrum of OSI (Fig. 2) and OSII showed 13 major cross-peaks for the anomeric resonances and some additional minor cross-peaks for two Hep residues (Hep^I and Hep^{II}). The splitting of the Hep signals in the *F2* (vertical) dimension (^1H), but not in the *F1* (horizontal) dimension (^{13}C), was evidently caused by the influence of Kdo present in multiple forms. The anomeric ^1H and ^{13}C resonances were essentially the same in OSI and OSII (Table 4).

A set of two-dimensional experiments, including correlated spectroscopy (COSY), TOCSY, NOESY, and ^1H , ^{13}C HMQC, enabled assignment of signals essential for identification of the spin systems of sugar residues in OSI (Table 5). All residues were found to be in the pyranose form, and the configurations of their glycosidic linkages were determined on the basis of the ^1H and ^{13}C NMR chemical shifts and $J_{1,2}$ coupling constant values (Table 4). The configurations of β -Manp and α -Hepp were confirmed by NOE correlations between H1 and H3, H5 of the former or between H1 and H2 of the latter. The NOESY experiment also revealed the modes of glycosylation and the sequence of the sugar residues. The glycosylation pattern was confirmed by the ^{13}C NMR chemical shift data of the linkage carbons, which were similar for OSI and OSII (data not shown). The core carbohydrate backbone structure thus established is identical to that reported for the *E. coli* R1-type core (53).

The ^{31}P NMR spectrum of OSI (Fig. 3, shown along the *F1*

axis) contained signals for one phosphate group (P , δ 5.09) and one pyrophosphate group (P_α and P_β at δ -11.26 and -10.37, respectively, both $d, J_{P,P} = 20$ Hz). A ^1H , ^{31}P HMQC experiment (Fig. 3) correlated the signals of P with H4 of the 3,7-disubstituted Hep residue (Hep^{II}) at δ_P/δ_H 5.09/4.32, P_α with H4 of the 3-substituted Hep residue (Hep^I) at δ_P/δ_H -11.26/4.60, and P_β with Etn at δ_P/δ_H -10.37/4.20 (CH_2O , major) and -10.37/3.29 (CH_2N , minor). Therefore, position 4 of Hep^I and Hep^{II} in OSI is phosphorylated by Etn pyrophosphate and phosphate groups, respectively (Fig. 3).

The ^{31}P NMR spectrum of OSII showed signals for two phosphate groups at δ 4.66 and 4.92 and no signals for pyrophosphate groups. In the ^1H , ^{31}P HMQC spectrum, the phosphate signals gave cross-peaks with the H4 signals of Hep^I and Hep^{II} at δ_P/δ_H 4.92/4.35 and 4.66/4.32, respectively. Therefore, OSII has the same phosphorylation sites as OSI, but OSII contains two phosphate groups rather than one phosphate group and one ethanolamine pyrophosphate group and, thus, represents a partial structure of OSI.

It is noteworthy that the change in the phosphate substituent at Hep^I caused a marked shift of the H4 signal from δ 4.60 ($J_{4,P} = \sim 9$ Hz) in OSI to δ 4.36 in OSII. Some other significant discrepancies were observed between published data of *E. coli* R-mutant F470 LPS with R1-type core (52) and *E. coli* O6 antigen polysaccharide (18) on the one hand and those of OSI and OSII from *E. coli* Nissle 1917 on the other. Compared to

TABLE 5. ¹H NMR data of OSI from LPS of *E. coli* Nissle 1917 (DSM 6601)

Oligosaccharide and sugar residue	Chemical shift (ppm) ^a						
	H1	H2	H3	H4	H5	H6a,6b	H7a,7b
O6 antigen							
β-Glcp-(1→	4.65	3.38	3.50	3.43	3.42	3.72	
α-GalpNAc-(1→	5.26	4.19	3.95	4.00			
→2,3)-β-Manp-(1→	4.80	4.37	3.87	3.80	3.45		
→4)-β-Manp-(1→	4.73	3.95	3.87	3.80	3.54		
→3)-β-GlcpNAc-(1→	4.86	3.88	3.81	3.59	3.52		
R1-type core							
α-Galp-(1→	5.36	3.86	3.96	3.99			
→2)-α-Galp-(1→	5.61	3.98	4.22	4.00			
→3)-β-Glcp-(1→	4.75	3.40	3.74	3.52	3.46	3.73; 3.93	
→2,3)-α-Glcp-(1→	5.86	3.88	4.24	3.41	4.28	3.60; 3.74	
→3)-α-Glcp-(1→	5.27	3.54	4.18	3.77			
α-Hep ^{III} -(1→	4.94	3.99	3.90				
→3,7)-α-Hep ^{II} -(1→ ^b	5.09	4.31	4.05	4.32	3.81	4.28	3.67; 3.74
→3)-α-Hep ^I -(1→ ^b	5.17	4.06	4.15	4.60	4.18	4.11	
→5)-Kdo ^b			1.82	4.08	4.16		

^a Chemical shifts for NAc are δ 2.04 and 2.06, for Etn 3.29 (CH₂N) and 4.20 (CH₂O).
^b Chemical shifts of the major signals are given.

the R1 core, chemical shifts are different for β-Glc, which is terminal in the core of the R mutant but 3-substituted in OSI and OSII. As expected, the signals for C3 and H3 shifted most (from δ_C/δ_H 76.9/3.52 in the nonsubstituted core to δ_C/δ_H 85.0/

3.74 in OSI). Compared to the O6 polysaccharide, chemical shifts of two monosaccharides differ dramatically. One is α-GalNAc, which is 4-substituted in the O6 polysaccharide but terminal in OSI and OSII, the change resulting in a significant

FIG. 3. ¹H,³¹P HMQC spectrum of OSI from *E. coli* Nissle 1917. The ³¹P NMR spectrum and the corresponding part of the ¹H NMR spectrum are displayed above the horizontal axis and to the left of the left vertical axis, respectively.

FIG. 4. Structure of the complete semirough LPS from *E. coli* Nissle 1917 containing O-antigen core oligosaccharide and lipid A. Incomplete substitution with Etn-P is shown by broken lines.

upfield shift of the C4 signal from δ 78.6 to $\sim$ 70, respectively. The other is GlcNAc, which is α -linked in the O6 polysaccharide but β -linked in OSI and OSII (e.g., compare δ_{C3}/δ_{H3} 81.8/4.01 and 84.1/3.81 or δ_{C5}/δ_{H5} 73.1/4.17 and 76.7/3.52, respectively). Taken together, the results of chemical, MS, and NMR analyses of the LPS revealed the structure of LPS from *E. coli* type Nissle, which is shown in Fig. 4.

Sequence analysis of the *E. coli* strain Nissle 1917 *wa gene cluster encoding LPS R1 core type and of the *wb** determinant encoding the O6 repeating unit.** The cosmid clone 8Y1 containing the *wa** gene cluster from *E. coli* Nissle 1917 was identified using specific primers for screening of an established cosmid genomic library of this strain (2). The entire cosmid was sequenced and analyzed. The *wa**_{O6} gene cluster of *E. coli* strain Nissle 1917 showed 97% homology on the DNA level to the published sequence of the *E. coli wa** gene cluster encoding R1 core type (17) and a conserved location on the *E. coli* chromosomal map upstream of *kdtB* located at 81 min on the *E. coli* K-12 chromosome.

Amplification of the full-length *wb**_{O6} gene cluster from *E. coli* strains Nissle 1917 and 536 with published primers binding in the *wb** flanking regions (10) resulted in a DNA fragment of $\sim$ 11 kb in both *E. coli* strains. The PCR products of both strains exhibited an identical restriction pattern (data not shown) and were cloned into pGEM-T Easy, resulting in plas-

mids pGWB1917 and pGWB536, respectively. The *E. coli* strain Nissle 1917-specific DNA fragment was sequenced, and the genetic structure was analyzed in detail (Fig. 5). The *wb**_{O6} gene cluster of strain Nissle 1917 is 11,037 bp long and exhibits an overall G+C content of 36.4%, suggesting an acquisition of the *wb** determinant by horizontal gene transfer. Nine tightly linked, sometimes overlapping putative ORFs were identified (Fig. 5 and Table 6). The G+C content does not vary markedly between the different ORFs with the exception of the genes *manC* and *manB*, which show an even higher G+C content than the overall *E. coli* chromosome (50.8%). The identical genetic structure of the *wb** gene clusters of *E. coli* O6 strains Nissle 1917 and 536 was verified by PCR (data not shown) using different primer combinations as depicted in Fig. 5.

The deduced amino acid sequences of these ORFs were analyzed with regard to the presence of conserved domains and similarity to other protein sequences. On the basis of the results obtained, the identified putative ORFs of the *wb**_{O6} determinant were predicted to encode putative glycosyl- or mannosyltransferases (ORFs 3, 4, 5, and 7), a putative O6 antigen flippase Wzx (ORF 1), a putative O-antigen polymerase Wzy (ORF 2), a putative UDP-N-acetylglucosamine-4-epimerase or UDP-glucose-4-epimerase (ORF 6), a mannose-1-phosphate guanosyltransferase (ORF 8), and a phosphomannomutase (ORF 9) (Table 6). The nucleotide se-

FIG. 5. Genetic structure of the O6 side chain-encoding determinant of *E. coli* strains Nissle 1917 and 536. The positions and directions of identified ORFs and the binding sites of the primers used are indicated by arrows. The point mutation in *E. coli* strain Nissle 1917-specific O6 antigen polymerase-encoding gene *wzy*_{Nissle 1917} is indicated at the top of the figure (boldface nucleotides).

quences of ORFs 1 to 7 showed no homology on the DNA level to sequences available from public databases.

Comparison of ORF 2 of *E. coli* strains Nissle 1917 and 536, encoding the O6-specific O-antigen polymerase Wzy. ORF 2 was predicted to be the *wzy* gene encoding the putative O6 antigen-specific polymerase because of its location downstream of the putative *wzx* gene of the *wb** determinant. This was corroborated by the fact that 12 transmembrane helices were predicted from the deduced amino acid sequence of ORF 2 using TMpred software (data not shown). To determine whether the putative *wzy* genes of the serum-sensitive strain Nissle 1917 and its serum-resistant counterpart strain 536 differ from each other, the corresponding DNA sequences were compared. Interestingly, comparison of the sequences of the *E. coli* Nissle 1917- and 536-specific putative *wzy* gene demonstrated that in *wzy*_{Nissle 1917} a point mutation (C to A transition at position +986 with respect to the translational start of *wzy*_{Nissle 1917}) resulted in an internal stop codon (TCA to TAA) and consequently in truncation of *wzy*_{Nissle 1917} (328 amino acids) compared to *wzy*₅₃₆ (447 amino acids) (Fig. 5). This internal stop codon causes premature translation termination of *wzy*_{Nissle 1917} transcripts, thus leading to a nonfunctional O6-specific O-antigen polymerase. Therefore, the point mutation within *wzy*_{Nissle 1917} is proposed to be the reason for the semirough phenotype of *E. coli* strain Nissle 1917. This supports the results of the biochemical analysis of this strain's LPS, namely, that it is comprised of only one O repeating unit linked to the R1-type core.

Complementation of the *wzy*_{Nissle 1917} allele. To verify the proposed function of the putative *wzy* gene and to prove that the identified point mutation within *wzy*_{Nissle 1917} is the reason for the semirough phenotype of strain Nissle 1917, complementation experiments were performed. Strain Nissle 1917 was transformed with the full-length *wb** gene cluster from *E. coli* strain 536 on a plasmid (pBWB536). In addition, two fragments of the *E. coli* 536-specific *wb** gene cluster were subcloned into pBluescript II KS and transferred into strain Nissle 1917. One of these plasmids contained the O-antigen flippase gene (*wzx*) and a fragment of *wzy*₅₃₆ with the size of the truncated ORF *wzy*_{Nissle 1917} (pBLG2504). The other one consisted of the complete *wzx* and *wzy* genes of *E. coli* strain 536 (pBLG2849). To avoid gene dosage effects, *E. coli* strain Nissle 1917 was also chromosomally complemented by integration of a single copy of *wzy*₅₃₆ fused with promoters of the β -lactamase-encoding gene of pBR322 (*P*_{bla::wzy}₅₃₆) or with the up-

stream region of the *wb**_{Nissle 1917} gene cluster (*Pwb**_{Nissle 1917::wzy}₅₃₆) into the chromosomal attachment site of the λ bacteriophage (for details, see Materials and Methods). The *wb**_{O6}-specific upstream region of strain Nissle 1917 is 97% identical to the previously studied *wb**_{O7} promoter (U23775). The resulting strains, in which a functional *wzy*₅₃₆ copy has been stably integrated, were designated *E. coli* Nissle 1917 λ P_{bla::wzy}₅₃₆ and *E. coli* Nissle 1917 λ P_{wb*}_{Nissle 1917::wzy}₅₃₆, respectively.

Expression of O6 side chains was studied by SDS-PAGE. With regard to LPS side chain expression, strain Nissle 1917 and its derivatives were grouped into three classes: semirough, smooth, and smooth with reduced amount of O antigen (Fig. 6A). Only transformation of *E. coli* strain Nissle 1917 with a construct containing the full-length *wb** determinant from strain 536 (pBWB536) resulted in a smooth phenotype. Introduction of the shortened *wzy*₅₃₆ fragment (pBLG2504) representing the size of the truncated gene *wzy*_{Nissle 1917}, had no complementing effect (Fig. 6A). Derivatives of strain Nissle 1917 complemented with plasmid-encoded *wzy*₅₃₆ (pBLG2849) alone as well as the chromosomally complemented strains *E. coli* Nissle 1917 λ P_{bla::wzy}₅₃₆ and *E. coli* Nissle 1917 λ P_{wb*}_{Nissle 1917::wzy}₅₃₆ showed a smooth phenotype with reduced amounts of O antigen. The level of the O6 LPS side chain synthesized in strain Nissle 1917 (pBLG2849) was markedly lower than in the smooth strains Nissle 1917(pBWB536) and 536 but higher than that of the chromosomally complemented strains (Fig. 6A). Therefore, we conclude that the *E. coli* strain 536-specific *wzy* gene encodes the functional O6 antigen polymerase.

With one representative of these three groups of strains (semirough, smooth, and smooth but with reduced amounts of O antigen), serum resistance assays were performed to analyze whether the presence and amount of longer LPS side chains may contribute to serum resistance in *E. coli* strain Nissle 1917 (Fig. 6B). The smooth strain *E. coli* Nissle 1917(pBWB536) showed a markedly increased resistance to 50 and 90% human serum compared to that of the semirough wild-type strain *E. coli* Nissle 1917, which was not detected after 1 h of incubation in 50 and 90% human serum. In comparison to the wild-type strain, the smooth strain with a reduced amount of O antigen (strain Nissle 1917 λ P_{wb*}_{Nissle 1917::wzy}₅₃₆) survived better and was still detected after 24 h of incubation (4.07% $\pm$ 0.66% and 0.007% $\pm$ 0.001% survival of the inoculum in 90 and 50% serum, respectively) (Fig. 6B). Generally, serum resistance was in accordance with the number of O6 repeating units produced by the different strains, i.e., serum resistance was higher in

TABLE 6. Characteristics of the ORFs located in the O6-specific *wb** gene cluster of *E. coli* strain Nissle 1917

Putative ORF	Length (bp)	G + C content (%)	No. of aa ^a	Conserved domain(s)	Similar protein(s) (EMBL accession no.)	% Identical aa/% similar aa (total no. of aa)	Putative function of O6 protein
ORF 1	1,256	31.3	418	Polysaccharide biosynthesis proteins	Putative O-antigen transporter RfbX protein, <i>Shigella dysenteriae</i> (S34963) O-antigen transporter <i>E. coli</i> K-12 (169652)	24/45 (396) 23/44 (415)	O-antigen flippase Wzx
ORF 2	986, 1,343 ^c	30.5	328, 447 ^c		Hypothetical protein, <i>Streptococcus pneumoniae</i> R6 (AAL00026) β-1,3-glucan synthase GSC-1, <i>Pneumocystis carinii</i> (AAG02216)	25/48 (447) 24/40 (1944)	O-antigen polymerase Wzy
ORF 3	860	31.2	286	Glycosyltransferase family 2	Glycosyltransferase, <i>Bacillus halodurans</i> (BAB07432) putative β-1,3-glucosyltransferase Waav, <i>E. coli</i> F470 (AAC69672)	38/58 (303)	Glycosyltransferase
ORF 4	1,032	33.6	343	Glycosyltransferase group 1	Predicted glycosyltransferases, <i>Thermotoga bacteriophage</i> <i>tergocongensis</i> (AAM23571) glycosyltransferase, <i>Pyrococcus furiosus</i> DSM 3638 (AAL80431)	31/50 (327) 28/45 (404)	Glycosyltransferase
ORF 5	1,145	28	381		Glycosyltransferase, <i>Clostridium acetobutylicum</i> (AAK80991) putative mannosyltransferase, <i>Yersinia pestis</i> (CAC92344)	27/45 (336) 28/43 (393)	Glycosyltransferase
ORF 6	1,007	35.6	335	NAD-dependent epimerase/dehydratase family	UDP-glucose-4-epimerase, GalE, <i>Haemophilus influenzae</i> Rd (AAC22012)	25/39 (380) 54/71 (338)	UDP- <i>N</i> -acetylglucosamine-4-epimerase or UDP-glucose-4-epimerase
ORF 7	401	30.4	401	Glycosyltransferase group 1	UDP-galactose-4-epimerase, <i>H. influenzae</i> (CAA40568) UDP- <i>N</i> -acetylglucosamine-4-epimerase, <i>E. coli</i> O55 (AF461121)	54/71 (338)	
ORF 8	483	38.1	483	Mannose-6-phosphate isomerase family 2	WbaD (ORF7.17) function unknown, <i>Salmonella enterica</i> (AAB49389) glycosyltransferase, <i>Clostridium acetobutylicum</i> (AAK80997)	23/39 (331) 51/67 (399) 26/47 (420)	Glycosyltransferase
ORF 9	456	55.1	456	Phosphoglucosyltransferase/phosphomannomutase, α/β domain I and α/β domain II	Mannose-1-phosphate guanylyltransferase, <i>E. coli</i> O157:H7, (BAB36277) mannose-1-phosphate guanylyltransferase, <i>E. coli</i> K-12 (AAC75110) Phosphomannomutase <i>E. coli</i> O6 (AACG41759) Phosphomannomutase <i>E. coli</i> O41 (AACG41754)	71/84 (478) 99/99 (456) 98/98 (456)	Mannose-1-phosphate guanosyltransferase Phosphomannomutase

^a aa, amino acids.^b Length of *wzy* or *Wzy* in semirough *E. coli* strain Nissle 1917.^c Length of *wzy* *Wzy* in smooth *E. coli* strain 536.

FIG. 6. Influence of O6 LPS side chain expression on serum resistance. (A) SDS-PAGE analysis of the O6-specific LPS side chain length of *E. coli* strains Nissle 1917 and 536 and derivatives. Lane 1, *E. coli* 536; lane 2, *E. coli* Nissle 1917; lane 3, *E. coli* Nissle 1917(pBWB536); lane 4, *E. coli* Nissle 1917(pBLG2504); lane 5, *E. coli* Nissle 1917(pBLG2849); lane 6, *E. coli* Nissle 1917ΔPwb^{Nissle 1917::wzy₅₃₆}; lane 7, *E. coli* Nissle 1917ΔPwb^{Nissle 1917::wzy₅₃₆}. (B) Serum resistance of *E. coli* strains Nissle 1917 and 536 and derivatives. Serum resistance assays were performed in 90% (black symbols) and 50% human serum (white symbols). The percentage of surviving cells were plotted against incubation time in human serum. Cell numbers within the different inocula ($t = 0$) were set at 100%.

strains with larger numbers of O6-specific repeating units. This is further confirmation that *wzy₅₃₆* encodes the O6-specific antigen polymerase and that the C-to-A transition at position +986 in *wzy_{Nissle 1917}*, which results in a nonfunctional O-antigen polymerase, is responsible for the semirough phenotype of *E. coli* strain Nissle 1917. Therefore, we identified for the first time the gene encoding O6 antigen polymerase (*wzy*), proved its function, and demonstrated that the reason for the semirough phenotype of *E. coli* strain Nissle 1917 is a point mutation, which probably causes premature translation termination of *wzy*.

DISCUSSION

Our studies on LPS from *E. coli* Nissle 1917 (DSM 6601) resulted in elucidation of the structures of both lipid A and the carbohydrate moiety (Fig. 4). The representative fraction of lipid A is characterized by a bisphosphorylated glucosamine disaccharide backbone and six asymmetrically (four plus two) distributed fatty acid residues. This lipid A structure has also been reported for other *E. coli* strains (60).

The presence of the R1-type core in *E. coli* Nissle 1917 was first proposed in serological studies using polyclonal rabbit anti-*E. coli* O6 serum obtained against *E. coli* Nissle 1917 (DSM 6601, serotype O6:K5:H1) and polyclonal anti-*E. coli* R1 serum obtained against an *E. coli* R1 mutant. Also, serological studies indicated that LPS of *E. coli* Nissle 1917 has one O6 antigen repeating unit, thus allowing classification of the LPS as a semirough-type LPS, which was further confirmed by chemical analysis.

The structure of the carbohydrate portion was established

unequivocally by structural studies after LPS delipidation whereby the bisphosphorylated carbohydrate backbone of *E. coli* R1 core had been identified (53). However, although the position of the Etn pyrophosphate group has been suggested (18), it has not yet been demonstrated, and the present work shows, for the first time, direct evidence supporting its attachment to Hep^I. Substitution with this group seems to be incomplete, and as a result, in a comparable number of the LPS molecules, Hep^I carries either an Etn pyrophosphate group or a monophosphate group at position 4. The possibility of partial cleavage of the Etn pyrophosphate group during mild acid degradation of LPS cannot be excluded as well. Position 3 of the terminal β -Glc residue was found to be the site of attachment of the O-antigen polysaccharide chain to the R1-type core.

The *E. coli* O6 antigen is heterogeneous, and two structurally related types of the repeating unit which differ in the nature of a lateral sugar substituent have been recognized. One type is associated with K2, K13, and K15 antigens and characterized by the presence of a lateral β -Glc residue (21, 22). The other type was found in an *E. coli* strain with K54 antigen and has a lateral β -GlcNAc residue (21). As shown in Fig. 6, the O6 antigen of *E. coli* Nissle 1917 belongs to the first type. The O6 antigen oligosaccharide present in this strain corresponds to the so-called biological repeating unit, which is assembled on a lipid carrier and then polymerized in the O-antigen biosynthesis pathway characteristic of bacterial heteropolysaccharides (31). A defect in the O-antigen polymerase gene may result in the inability of the enzyme to produce a polysaccharide and thus give rise to an semirough-type LPS, like LPS of *E. coli*

Nissle 1917. Remarkably, the configuration of the glycosidic linkage of GlcNAc at the reducing end of the biological repeating unit depends on whether it connects the repeating unit to the core or to the neighboring repeat in the polysaccharide chain. This finding indicates different specificities, not only with respect to the substrate but also to the stereochemistry of the glycosidic linkage formation, of two enzymes involved in the transfer of the O-antigen to the core (ligase) and in the polymerization process (O-antigen polymerase).

Together with *E. coli* O1, O4, and O18 strains, those with the O6 antigen belong to the most frequent extraintestinal *E. coli* isolates (36). However, *E. coli* strains of serotype O6 are also commonly detected among intestinal isolates (6, 16, 25, 46). In the case of uropathogenic O6 clones, the gut may serve as a reservoir of infectious microorganisms for recurrent urinary tract infections (3). Also, the R1-type core is the most frequently occurring core type in *E. coli* clinical isolates (2, 3).

The full-length *wa** and *wb** gene clusters of *E. coli* strain Nissle 1917, which are required for biosynthesis of the *E. coli* R1 LPS core type and O6 antigen, respectively, have been cloned, sequenced, and analyzed. The nucleotide sequence of the *wa**_{O6} determinant is 97% identical to already known sequences of other R1 core type-specific *wa** gene clusters, e.g., *E. coli* strain F470. The *wb**_{O6} gene cluster has not been sequenced so far. In *E. coli* Nissle 1917, this determinant is located on the chromosome between the *galF* and *gnd* genes, as reported for other *wb** clusters (10). As also described for other O-antigen gene clusters, all putative ORFs of this strain's *wb** gene cluster, with the exception of *manC* and *manB*, have a relatively low G+C content, suggesting that it may have been acquired by horizontal transfer from other species. According to the corresponding deduced amino acid sequences, we have identified five putative ORFs specific for the O6 LPS serotype: one putative ORF coding for the O6 antigen flippase (*wzx*), the O6 antigen polymerase-encoding gene *wzy*, four putative glycosyltransferase-encoding genes, and a putative epimerase-encoding gene (Fig. 5). Although ORF 2 shows no marked similarity to other *wzy* genes, it was considered the putative O-antigen polymerase-encoding gene, as it is located downstream of the putative *wzx* gene. In addition, 12 transmembrane helices have been predicted from the deduced amino acid sequence. This is also the case for the putative Wzy proteins of an *E. coli* O113 and O8:K40 strain (EMBL accession numbers AF172324 and AF013583, respectively). Generally, the number of transmembrane helices of Wzy proteins of other *E. coli* serogroups is variable, ranging from 8 (*E. coli* O157:H7 strains EDL933 and Sakai and an *E. coli* O7 strain [EMBL accession numbers AAG57099, BAB36267, and AF125322, respectively]) to 10 in isolates of serogroups O55, O104, and O111 (EMBL accession numbers AAL67557, AAK64372, and AAD46730, respectively) or 11 in serogroup O4 (EMBL accession number U39042) and in *E. coli* K-12 (EMBL accession number AAB88404). The function of ORF 6 could not clearly be defined according to sequence similarity. As *N*-acetylglucosamine was identified to be present in the O6 O-unit structure, an UDP-*N*-acetylglucosamine-4-epimerase should be encoded within the *wb**_{O6} gene cluster. However, the deduced amino acid sequence of ORF 6 shows a higher similarity to UDP-glucose-4- and UDP-galactose-4-epimerases than to the

UDP-*N*-acetylglucosamine-4-epimerase of *E. coli* O55 (EMBL accession number AF461121) (56) (Table 6).

To correlate our genetic and structural analyses, the cloned O6 antigen gene cluster of the smooth *E. coli* strain 536 was compared with that of the semirough strain Nissle 1917. The full-length *wzy*_{O6} gene cluster of strain 536 was shown to be able to restore full-length O6 side chain synthesis and to complement the semirough phenotype of *E. coli* strain Nissle 1917. Therefore, it was concluded that the predicted mutation is located in this strain's O6 antigen gene cluster. A C-to-A transition within *wzy*_{Nissle 1917} which results in a premature stop codon (TAA) was identified. Since the rest of the nucleotide sequence of *wzy* in *E. coli* strains Nissle 1917 and 536 was identical, we complemented strain Nissle 1917 with the full-length *wzy*₅₃₆ gene and with the shorter form representing the size of the strain Nissle 1917-specific *wzy* gene and proved that only the intact *wzy*₅₃₆ gene is functional and able to restore the O6 side chain synthesis in *E. coli* Nissle 1917. Although chromosomally complemented derivatives of strain Nissle 1917 which carry one copy of *wzy*₅₃₆ in the chromosomal attachment site of the bacteriophage λ expressed lower amounts of LPS side chains than strain 536, our results demonstrate that expression of a functional O6-specific *wzy* gene results in polymerization of multiple repeating units within the O6 side chains of strain Nissle 1917 (Fig. 6A). Weaker expression of repeating units in strains complemented with *wzy*₅₃₆ alone compared to strains complemented with the plasmid-encoded *wb** determinant of strain 536 may be indicative of other factors which impair proper *wb** expression in strain Nissle 1917. Additionally, the different promoter-*wzy*₅₃₆ fusions may not be in accordance with the optimal arrangement of genes and the optimal distance between promoter and *wzy* translational start as they are in the *wb** operon.

Since the O6 serotype is widely distributed among wild-type (pathogenic and nonpathogenic) *E. coli* strains, we studied the importance of full-length O6 LPS side chains for serum resistance of different derivatives of *E. coli* strain Nissle 1917 (Fig. 6B), as this trait represents an important biological feature of this therapeutically used *E. coli* strain with respect to its biosafety. The results showed that the O6 antigen expression contributes to serum resistance of these strains. Serum resistance of *E. coli* Nissle 1917 was quantitatively related to the amounts of O6 antigen. This example underlines the impact of point mutations, in addition to DNA rearrangement as well as acquisition and deletion of large genetic determinants, for evolution of members of the family *Enterobacteriaceae* (15).

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