

# Glycomimicry: Display of the GM3 sugar epitope on *Escherichia coli* and *Salmonella enterica* sv Typhimurium

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Oligosaccharides present on the surface of pathogenic bacteria play an important role in their interaction with their host. Bacteria with altered cell surface structures can be used to study these interactions, and glycoengineering represents a tool to display a glycoepitope on a different bacterium. Here, we present non-pathogenic *Escherichia coli* and *Salmonella enterica* serovar Typhimurium expressing the sialyllactose oligosaccharide epitope of the ganglioside GM3. By expression of the galactosyltransferase LgtE and the sialic acid transferase Lst as well as the CMP-sialic acid synthetase SiaB from *Neisseria gonorrhoeae* and *Neisseria meningitidis* in engineered strains devoid of the sialic acid catabolism, the GM3 sugar epitope was displayed on these bacteria as demonstrated by live cell immunostaining and a detailed analysis of their lipooligosaccharides. These strains offer the possibility to investigate the role of sialic acid in the recognition of bacteria by the immune system in a non-pathogenic background.

**Keywords:** bacterial glycosylation/chimeric LPS/gangliosides/glycoengineering

## Introduction

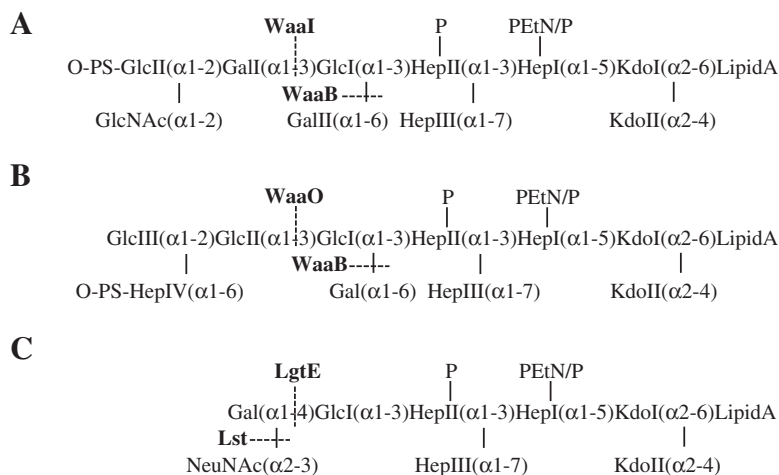
Bacteria produce many oligo- and polysaccharide structures. A high percentage of these is found in structures of the cell wall, e.g., in peptidoglycan, lipopolysaccharides (LPS) and lipooligosaccharides (LOS). Some human enteropathogens, like *Campylobacter jejuni* and *Neisseria meningitidis*, produce saccharide structures on their surface that resemble human carbohydrate structures (Tsai 2001; Yuki et al. 2004). This mechanism is termed molecular mimicry and might help the bacteria to avoid recognition by the human immune system. *C. jejuni* is one of the few bacteria able to synthesize sialic acid

(*N*-acetyl neuraminic acid; NeuNAc), and many strains include this sugar in their LOS, resulting in human ganglioside sugar mimicry (Aspinall, Fujimoto et al. 1994; Aspinall, McDonald et al. 1994; Ang et al. 2004). Gangliosides are glycosphingolipids containing at least one sialic acid residue. They are present in all mammalian cells but enriched in nerve cells and in brain tissue. The molecular mimicry of ganglioside sugar epitopes can lead to the autoimmune paralysis Guillain–Barré Syndrome (GBS) in approximately 1 in 1000 humans infected with *C. jejuni* (Ang et al. 2004; Yuki et al. 2004). Patients produce anti-ganglioside antibodies that do not only bind the ganglioside sugar epitopes on the surface of the pathogen but also the host's own gangliosides, mostly in the nodes of Ranvier and the motor nerve termini (Yuki et al. 1990; Hafer-Macko et al. 1996).

Recent studies used genetically engineered *Escherichia coli* to produce pure oligosaccharides in large quantities. These *E. coli* strains heterologously express glycosyltransferases from other bacteria, e.g., *N. meningitidis*, *Neisseria gonorrhoeae* or *C. jejuni* or even eukaryotes. The oligosaccharides produced include the carbohydrate moiety of nonsulfated HNK-1, Lewis X tetrasaccharides, globotriose and globotetraose, human milk oligosaccharides and the carbohydrate moieties of the gangliosides GM1 and GM2 (Priem et al. 2002; Antoine et al. 2003, 2005; Dumon et al. 2006; Yavuz et al. 2008). The assembly of these oligosaccharide structures takes advantage of the internalization of lactose by the genetically engineered bacteria. After internalization, lactose is used in the cytoplasm as a platform for the addition of other saccharides by heterologously expressed glycosyltransferases. Most of the oligosaccharides produced are secreted into the medium and can be purified easily (Bettler et al. 1999; Priem et al. 2002). This approach of using *E. coli* as a living factory replaces the time-consuming and laborious chemical or chemo-enzymatic synthesis and also allows the large-scale production of oligosaccharides (Bettler et al. 1999).

The synthesis of oligosaccharides in vivo can be combined with an approach utilizing the bacterial cell surface as a scaffold for different saccharide structures. In Gram-negative bacteria, the outer membrane is an asymmetric bilayer with glycerophospholipids in the inner leaflet and LPS in the outer leaflet. LPS consists of the lipid A moiety, an inner and an outer core to which in some organisms a polymeric O-antigen region is attached (Raetz and Whitfield 2002). The lipid A moiety comprises two glucosamine residues connected through a  $\beta$ 1,6 linkage. Acyl chains attached to these glucosamines anchor the molecule in the outer membrane. Proximal to the lipid A is

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**Fig. 1.** Lipopolysaccharide structures in *S. Typhimurium* (A), *E. coli* K-12 (B) and the engineered strains for GM3 display (C). Glc, glucose; Gal, galactose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-mannoheptose; GlcNAc, N-acetylglucosamine; NeuNAc, N-acetyl neuraminic acid; P, phosphate; PEtN, phosphoethanolamine. Adapted from Yethon, Gunn et al. (2000), Yethon, Vinogradov et al. (2000), Frirdich et al. (2003) and Kaniuk et al. (2004).

the inner core region. In most Gram-negative bacteria, this region is composed of two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and several L-glycero-D-mannoheptose (Hep) residues which can be substituted by phosphoethanolamine or hexoses. The distal outer core consists of hexoses and N-acetylhexoses (see Figure 1) (Schnaitman et al. 1991; Parker et al. 1992; Pradel et al. 1992; Schnaitman and Klena 1993; Heinrichs et al. 1998). To date, five core types have been found for *E. coli* (R1–R4 and K-12), whereas for *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) only two have been described (Lüderitz et al. 1982; Heinrichs et al. 1998; Olsthoorn et al. 1998). In *S. Typhimurium*, the polymeric O-antigen region is attached to the last glucose residue of the outer core (Hellerqvist et al. 1969). The outer core structure in *E. coli* K-12 strains terminates on the heptose residue HepIV to which the O-antigen region is attached (Holst et al. 1991; Heinrichs et al. 1998). *E. coli* K-12 strains used in the laboratory, however, do not produce the polymeric O16 O-antigen due to an insertion in *wbbL*. This gene encodes the rhamnosyltransferase that is involved in the addition of rhamnose to the second position of the O16 backbone (Liu and Reeves 1994; Stevenson et al. 1994; Yao and Valvano 1994).

Bacterial cell surfaces can be used to display engineered oligosaccharide structures in their lipopolysaccharide layer by two different pathways. On the one hand, the O-antigen ligase WaaL dependent transfer of oligo- and polysaccharides to *E. coli* or *S. Typhimurium* lipid A core can be exploited. The structures transferred include the *Haemophilus influenzae* lipooligosaccharide structure, the O-antigens from *Shigella dysenteriae* or *E. coli* O111 as well as the *C. jejuni* heptasaccharide normally linked to protein (Spinola et al. 1990; Falt et al. 1996; Szymanski et al. 1999; Wang et al. 1999; Phillips et al. 2000; Xu de et al. 2007). This approach requires the assembly of the engineered oligosaccharides on the lipid carrier undecaprenylpyrophosphate. On the other hand, truncated lipid A core structures as acceptors of foreign saccharide structures can be used. This approach has been employed by Paton and co-workers for the production of probiotics (Paton et al. 2001, 2005). The *E. coli* R1 strain CWG308 with a truncation of the lipid A core at the glucose I residue is used in these

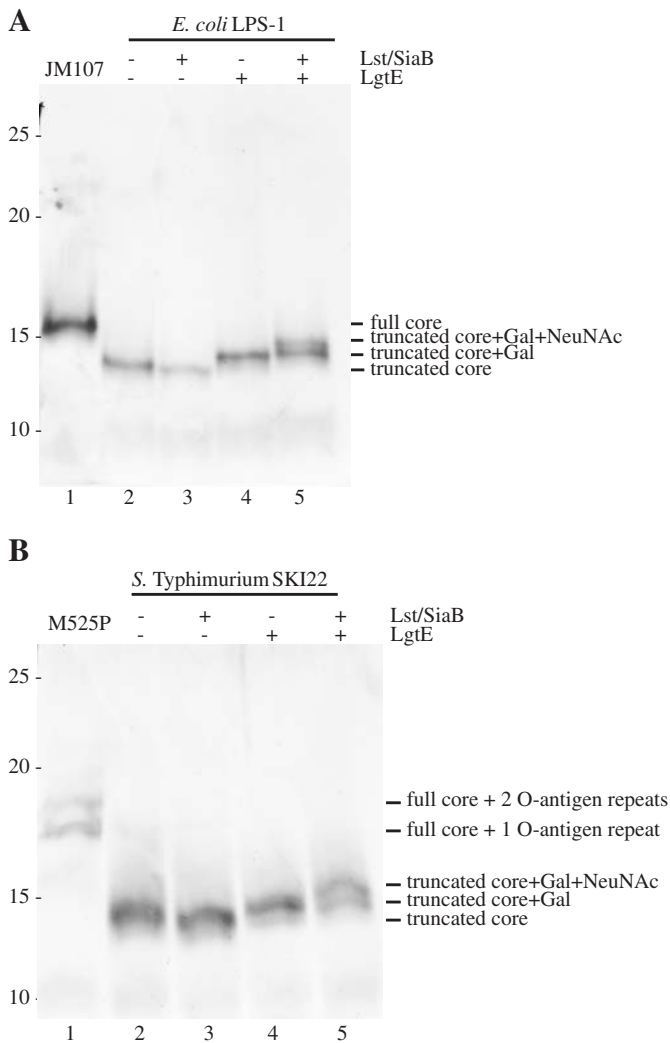
studies to heterologously express glycosyltransferases from *C. jejuni* and *N. meningitidis*. These then modify the truncated lipid A core to produce a chimeric LOS structure on *E. coli*. This system is similar to the “living factory” described above that employs the glucose moiety of internalized lactose as a platform for the addition of other saccharides.

We extended the approach by Paton and co-workers from *E. coli* R1 to *E. coli* K-12 and *S. Typhimurium* by constructing strains that lack the gene encoding the sialic acid aldolase NanA. This allowed the incorporation of sialic acid into oligosaccharides within the cell (Priem et al. 2002). Additionally, the strains were deleted in glycosyltransferase genes which led to the truncation of the lipid A core terminating with the glucose I residue. This was then used to attach the GM3 epitope by the heterologous expression of glycosyltransferases from *N. meningitidis* and *N. gonorrhoeae*.

## Results

### Truncation of lipid A core in *S. Typhimurium* and *E. coli* K-12 and display of the GM3 sugar on the truncated lipid A core

To produce a terminal Glc in *E. coli* K-12 lipid A core, the genes encoding the glycosyltransferases WaaO and WaaB were deleted. The resulting strain *E. coli* K-12  $\Delta nanA \Delta waaO \Delta waaB$  was termed LPS-1. In *S. Typhimurium*, the same truncation of the lipid A core was achieved by deleting the genes encoding the galactosyltransferases WaaI and WaaB, and the resulting strain was termed SKI22. Both strains used were also deleted in the gene encoding the sialic acid aldolase NanA to prevent the degradation of sialic acid added externally (see below). In order to produce *E. coli* and *S. Typhimurium* with a GM3 sugar epitope on the cell surface, the strains with the truncated lipid A core, LPS-1 and SKI22, were transformed with a plasmid encoding the  $\beta$ -1,4-galactosyltransferase LgtE from *N. gonorrhoeae* (pBBRLgtE) and with a plasmid encoding the CMP-sialic acid synthetase SiaB as well as the sialic acid transferase Lst from *N. meningitidis* (pKI3\*). The strains containing the corresponding vectors served as negative control. Strains were grown in Luria–Bertani (LB) medium supplemented with 2% sialic acid, and proteinase-K-treated whole cell extracts were



**Fig. 2.** The truncated lipid A core in *E. coli* LPS-1 (A) and *S. Typhimurium* SKI22 (B) can be modified with a GM3 epitope. Tris-Tricine PAGE of whole cell extracts from cells which were grown in the presence of external sialic acid and which expressed the indicated genes from plasmids followed by silver staining. Whole cell extracts from the parental strains were used as controls.

prepared. The display of the GM3 structure was judged by silver staining of a Tris-Tricine polyacrylamide gel electrophoresis (PAGE). To first demonstrate the truncation of the lipid A core, proteinase-K-treated whole cell extracts of the parental strains can be seen in lane 1 of Figure 2A for the LPS-1 parent JM107 and in lane 1 of Figure 2B for M525P, the parental strain of SKI22. These strains show bands of higher apparent molecular mass than the strains with the truncated core, corresponding to the full core for JM107 or the full core with additional one or two O-antigen subunits for M525P. In lane 4 of Figure 2A and B, the addition of the galactose residue by LgtE was detected for LPS-1 and SKI22, respectively, by a shift to a higher apparent molecular mass (see Figure 2). The expression of SiaB and Lst led to the transfer of a sialic acid moiety and a further shift to a higher apparent molecular mass. This demonstrated that the GM3 epitope was produced in an *E. coli* K-12 derivative and a *S. Typhimurium* strain and that it was transferred to the trun-

cated lipid A core in both strains. In the strains containing the plasmids expressing LgtE and Lst/SiaB (lane 5 in Figure 2A and B), it was observed that the transfer of the sialic acid residue by Lst was not complete and that some truncated lipid A core with only galactose added was still present in the preparation.

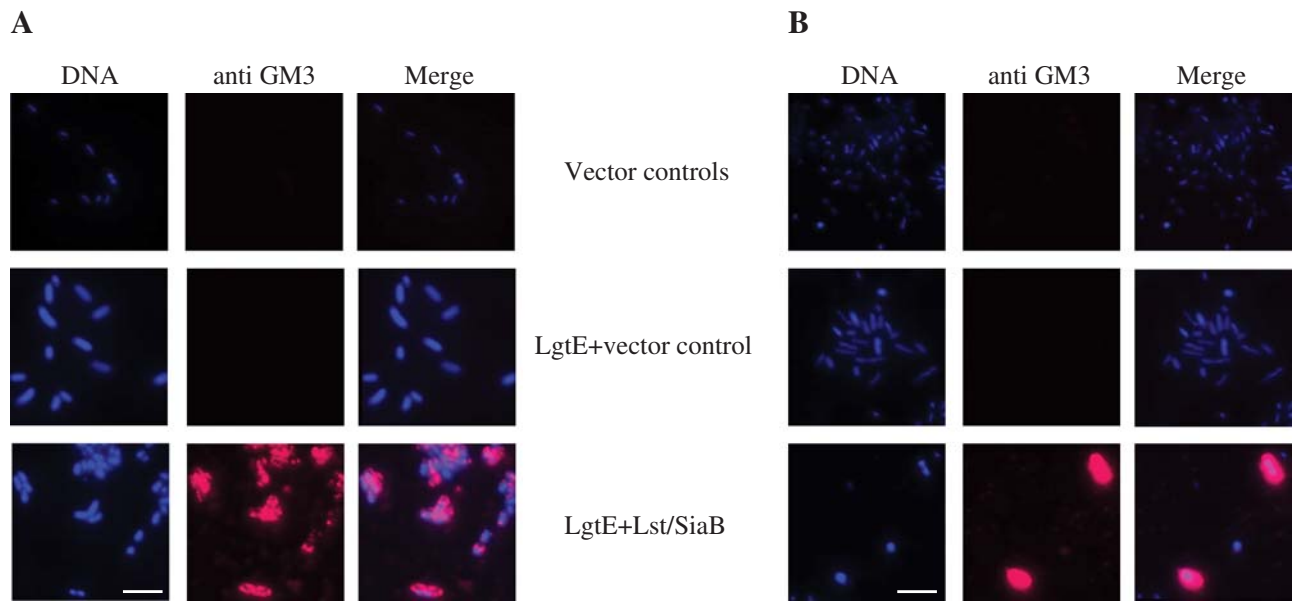
#### *Live cell microscopy demonstrated surface localization of GM3 sugar epitope on LPS-1 and SKI22*

For immunological studies, it was important that the GM3 sugar epitope was present on the cell surface. As cell surface localization cannot be judged with whole cell extracts, immunofluorescence analysis of live cells was performed. Cells containing the plasmids encoding the galactosyltransferase LgtE, the CMP-sialic acid synthetase SiaB and the sialic acid transferase Lst grown in medium containing sialic acid (see above) were incubated with a mouse monoclonal anti-GM3 antibody, stained with 4, 6-diamino-2-phenylindole (DAPI) and an anti-mouse-IgM-Alexa647 conjugate. It is evident from Figure 3 that the antibody did not bind to cells containing the truncated core (upper panel) or the truncated core with the galactose residue attached by LgtE (middle panel). When the genes encoding Lst and SiaB were expressed, cell surface staining was visible for *E. coli* LPS-1 and *S. Typhimurium* SKI22 (lower panel in Figure 3A and B). This demonstrated that the GM3 sugar epitope was present on the surface of the bacterial cells, although it was perceivable again that not all cells carry this epitope.

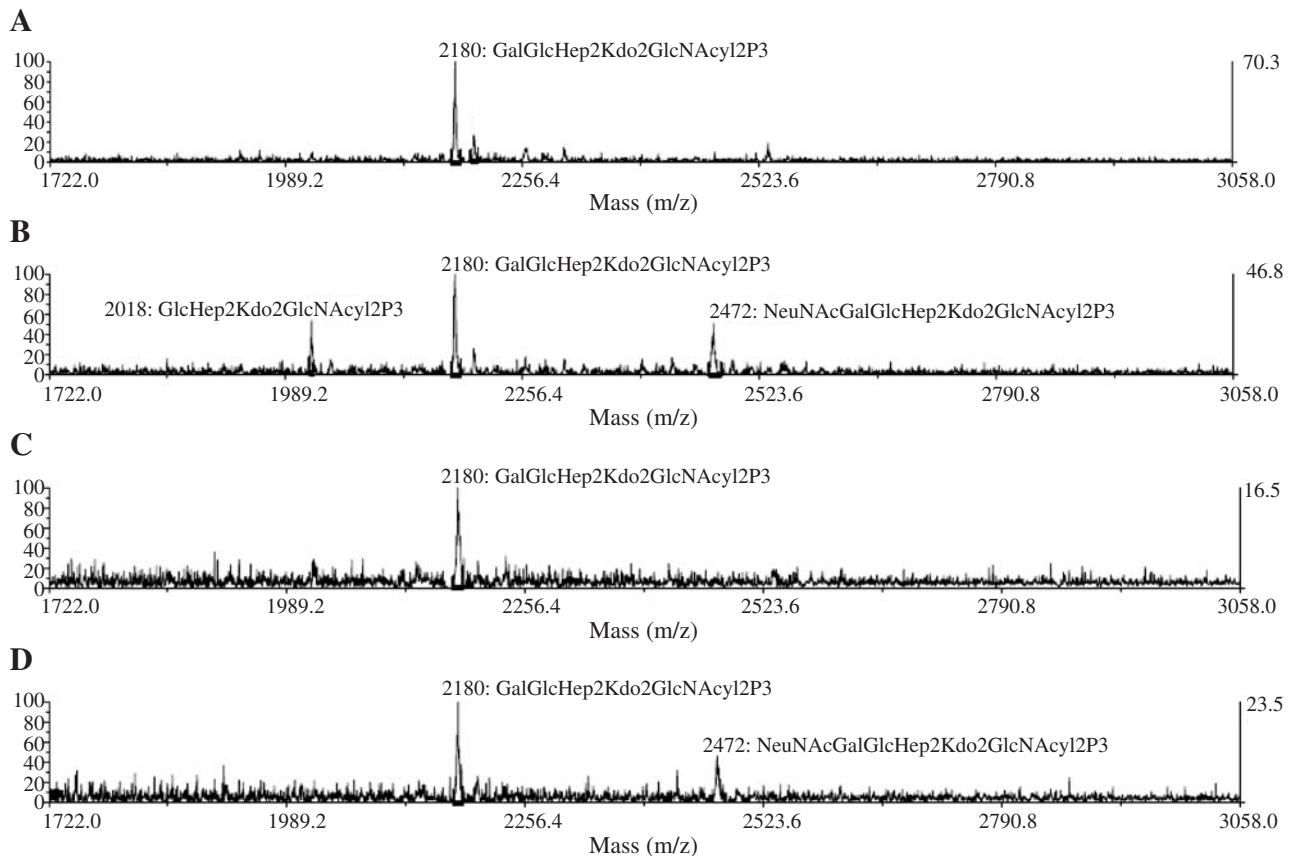
#### *Matrix-assisted laser desorption/ionization time-of-flight analysis revealed presence but low abundance of GM3 sugar epitope on truncated lipid A core*

Mass spectrometry was performed for further analysis of the structures present on the truncated lipid A core of *E. coli* LPS-1 and *S. Typhimurium* SKI22. LOS of the strains displaying the GM3 epitope and grown in medium containing sialic acid were isolated by the method developed by Galanos et al. (1969). The acyl chains attached to the hydroxyl groups of the glucosamine residues of the lipid A were cleaved by mild hydrazine treatment (Haishima et al. 1992). As can be seen in Figure 4B, the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis demonstrated that neither the addition of galactose nor of sialic acid to the lipid A core was complete in the *E. coli* LPS-1 strain. In *S. Typhimurium* SKI22, the galactose residue was added to nearly all lipid A core molecules, but sialic acid was not transferred to all galactosylated lipid A core molecules, similar to the situation in the *E. coli* strain (Figure 4D and B). As indicated in Figure 4, the molecular composition of the truncated lipid A core structure deduced from the detected mass is a P<sub>3</sub>-Glc-Hep<sub>2</sub>-Kdo<sub>2</sub>-lipid A structure instead of the reported P<sub>4</sub>-Glc-Hep<sub>3</sub>-Kdo<sub>2</sub>-lipid A structure (Müller-Loennies et al. 2003). This is most probably due to the substrate specificity of the lipid A core glycosyl- and phosphotransferases and will be discussed in more detail below.

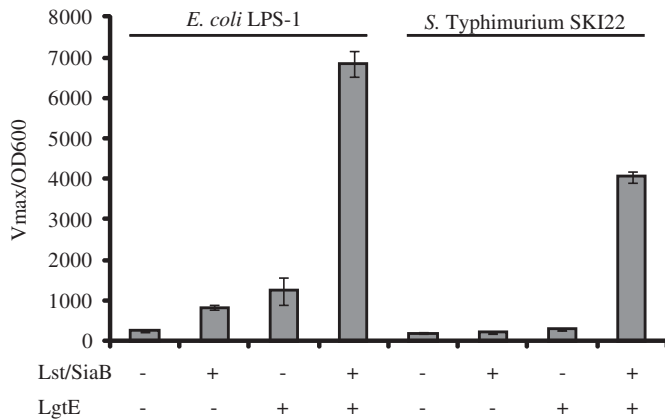
*Quantification of display showed higher display levels in E. coli*  
 As means of quantification of the display of the GM3 epitope on the *E. coli* LPS-1 and the *S. Typhimurium* SKI22 strains, we carried out a colorimetric antibody binding assay. For this, live



**Fig. 3.** Surface localization of the GM3 epitope on *E. coli* LPS-1 (**A**) and *S. Typhimurium* SKI22 (**B**). Immunofluorescence analysis of live cells that were grown in the presence of external sialic acid and which expressed the indicated genes from plasmids. Scale bar 5  $\mu\text{m}$ .



**Fig. 4.** MALDI-TOF analysis of purified, *O*-deacylated LOS from *E. coli* LPS-1 and *S. Typhimurium* SKI22 demonstrates the addition of NeuNAc in the presence of Lst/SiaB. (**A**) *E. coli* LPS-1 expressing the gene encoding for LgtE and containing the vector control for the sialyltransferase. (**B**) *E. coli* LPS-1 expressing the genes encoding for LgtE and Lst/SiaB. (**C**) *S. Typhimurium* SKI22 expressing the gene encoding for LgtE and containing the vector control for the sialyltransferase. (**D**) *S. Typhimurium* SKI22 expressing the genes encoding for LgtE and Lst/SiaB.



**Fig. 5.** Quantification of anti-GM3 binding to whole cells demonstrates higher display levels in *E. coli* LPS-1 when compared to *S. Typhimurium* SKI22. Live cells expressing the indicated genes from plasmids were incubated with the anti-GM3 antibody, and the amounts of bound antibody were quantified by the colorimetric assay described in the Materials and methods section.

cells grown in medium containing sialic acid were incubated with the anti-GM3 antibody EM5 followed by an incubation with an anti-mouse-IgM-HRP conjugate. The assay was developed with the colorimetric substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (see Materials and methods), and the kinetics of the color development were recorded. This assay demonstrated a 27-fold increase in signal intensity in the *E. coli* LPS-1 strain containing LgtE and Lst/SiaB as compared to the LPS-1 strain harboring the vector controls (Figure 5, left). In *S. Typhimurium* SKI22, the increase in signal intensity was 20-fold between SKI22 strain containing LgtE and Lst/SiaB in comparison to the SKI22 strain with the vector controls (Figure 5, right). This showed that *E. coli* LPS-1 cells bound more anti-GM3 antibody EM5 than the *S. Typhimurium* SKI22 cells and thereby indicated higher display levels of the GM3 sugar epitope on the *E. coli* strain.

## Discussion

The connection between the autoimmune disease GBS and sialylated structures in bacterial LOS is still not clear. The usual infectious agents associated with subsequent GBS include Epstein-Barr virus, *Mycoplasma pneumoniae*, *C. jejuni* and cytomegalovirus (Hughes and Cornblath 2005). Of these, *C. jejuni* is known to express sialylated LOS that mimics human gangliosides (Aspinall, Fujimoto et al. 1994; Ang et al. 2004; Yuki et al. 2004). However, not every structure mimicking gangliosides leads to GBS. *N. meningitidis* and *H. influenzae* strains have been demonstrated to produce LOS with a sialyl-LacNAc structure, which differs from the GM3 epitope only in the *N*-acetyl group present at C2 of the Gal residue (Mandrell et al. 1992; Yamasaki et al. 1993). Nevertheless, none of these strains has been reported to be a statistically significant antecedent infection in GBS patients.

With the aim of providing well-defined model organisms in order to study the connection between ganglioside mimicry in the LOS and evocation of an autoimmune disease, we constructed *E. coli* K-12 and *S. Typhimurium* that display the

GM3 sugar epitope on the cell surface as demonstrated by various methods. Mass spectrometry analysis of the *O*-deacylated isolated LOS proved the addition of galactose and sialic acid residues to the lipid A core. The molecular mass of the truncated lipid A core in the *E. coli* LPS-1 strain (2018 Da) was increased by 162 and 292 Da, respectively (Figure 5). However, the molecular mass of 2018 Da for the truncated lipid A core did not fit with the calculated mass of 2291.95 Da for the chemical composition lipid A-Kdo<sub>2</sub>-Hep<sub>3</sub>-Hex-P<sub>4</sub> of the three main glycoforms I-II-III present in *E. coli* K-12 (Müller-Loennies et al. 2003). The difference between the calculated and the observed molecular mass could be accounted for by the disappearance of one heptose residue and one phosphate group. In *E. coli* R1, it has been shown that the mutation of *waaY* yielded a strain with a core oligosaccharide devoid of phosphate on the HepII residue. Furthermore, a mutation of *waaQ* resulted in the loss of the branched HepIII residue on HepII, and it impeded the activity of *waaY* (Yethon et al. 1998). It is also known that a mutation of *waaG* which encodes the glucosyltransferase acting on HepI not only leads to the truncation of the lipid A core immediately behind the inner core heptose residues but also to a lack of phosphate groups on HepII (Yethon, Vinogradov et al. 2000). It is likely that a similar co-dependency between the monosaccharides present in the lipid A core and the phosphorylation of saccharides exists in *E. coli* K-12. Most probably, the deletion of *waaO* and *waaB* yields a suboptimal substrate for the gene product of *waaQ*, thereby inhibiting the addition of the HepIII residues. This then finally results in a lipid A-Kdo<sub>2</sub>-Hep<sub>2</sub>-Hex-P<sub>3</sub> structure because the gene product of *waaY* relies on the presence of the HepIII residue (Yethon et al. 1998). Yethon and co-workers also demonstrated that the genetic determinants for modifications of the lipid A core are conserved between *E. coli* and *S. Typhimurium* (Yethon, Gunn et al. 2000). Therefore, the structure lipid A-Kdo<sub>2</sub>-Hep<sub>2</sub>-Hex<sub>2</sub>-P<sub>3</sub> that we observed for the galactosylated truncated lipid A core in SKI22 can also be explained to stem from the same substrate specificities as in *E. coli* K-12. In a *S. Typhimurium* SKI22 strain containing the vector controls for LgtE and Lst/SiaB, a product at 2018 Da was visible, which demonstrates that in *S. Typhimurium* SKI22 the truncated lipid A core also possesses the structure lipid A-Kdo<sub>2</sub>-Hep<sub>2</sub>-Hex-P<sub>3</sub> (data not shown).

While mass spectrometry analysis and lipooligosaccharide separation by Tris-Tricine polyacrylamide gels demonstrated the presence of sialic acid on the truncated lipid A core structures of *E. coli* LPS-1 and *S. Typhimurium* SKI22, antibody binding proved the  $\alpha$ -2,3 linkage of the attached sialic acid residue. A competition assay with purified sialyllactose, but not with lactose, demonstrated the accuracy of the antibody binding (data not shown).

The *E. coli* and *S. Typhimurium* strain displaying the GM3 sugar epitope will be helpful tools when the role of sialic acid containing LOS structures in the recognition and the interaction of these structures with the immune system is being examined.

## Materials and methods

### Bacterial strains and growth conditions

A summary of bacterial strains used in this study can be found in Table I. Bacteria were grown in LB medium (10 g/L Bacto

**Table I.** Bacterial strains, plasmids and oligonucleotides used in this study

Bacterial strains	Genotype and phenotype	Source or reference
Strain		
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>SupE44 <math>\Delta</math>lacU169 (<math>\Phi</math>80lacZ<math>\Delta</math>M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	(Hanahan 1983)
JM107	<i>endA1 gyrA96 thi hsdR17 SupE44 relA1</i> $\lambda^{-}$ $\Delta$ ( <i>lac-proAB</i> ) [F' <i>traD36 proAB+ lacI<sup>f</sup> lacZ</i> $\Delta$ M15]	DSM; (Yanisch-Perron et al. 1985)
TA01	JM 107 $\Delta$ <i>nanA</i>	(Antoine et al. 2003)
LPS-1	TA01 $\Delta$ <i>nanA</i> $\Delta$ <i>wcaJ</i> $\Delta$ <i>waaO</i> $\Delta$ <i>waaB</i>	This study
<i>Salmonella enterica</i> sv. Typhimurium		
M525P	Wild type	(Mastroeni et al. 2000)
SK12	M525P $\Delta$ <i>nanA</i>	This study
SK114	M525P $\Delta$ <i>nanA</i> $\Delta$ <i>waaI</i>	This study
SK122	M525P $\Delta$ <i>nanA</i> $\Delta$ <i>waaI</i> $\Delta$ <i>waaB</i>	This study
<b>Plasmids</b>		
Plasmid	Genotype	Source or reference
pBBR1MCS-3	<i>Tet<sup>R</sup>, bhr ori</i>	(Kovach et al. 1995)
pBBR1gtE	<i>Tet<sup>R</sup>, bhr ori, N. gonorrhoeae lgtE</i> cloned XbaI/SacI into pBBR1MCS-3	This study
pACT3	<i>Cam<sup>R</sup>, p15A ori</i>	(Dykxhoorn et al. 1996)
pK13*	<i>Cam<sup>R</sup>, p15A ori, N. meningitidis lst</i> cloned XbaI/PstI with a C-terminal FLAG-tag downstream of <i>SiaB</i> from <i>N. meningitidis</i> cloned SacI/XbaI with a C-terminal c-myc-tag into pACT3	This study
pKD3	<i>bla FRT cat FRT PS1 PS2 oriR6K</i>	(Datsenko and Wanner 2000)
pKD4	<i>bla FRT aph FRT PS1 PS2 oriR6K</i>	(Datsenko and Wanner 2000)
pKD46	<i>bla P<sub>BAD</sub> gam bet exo pSC101 oriTS</i>	(Datsenko and Wanner 2000)
pCP20	<i>bla cat cI857 <math>\lambda</math>P<sub>R</sub> flp pSC101 oriTS</i>	(Datsenko and Wanner 2000)
<b>Oligonucleotides (5'→3')</b>		
LT2 NanA H1P1	CAGGTATAAAGGTAGGTAGTAAATATATTCATCATCCGTAGAGGTAGGTGTGTAGGCTGGAGCTGCTTC	
LT2 NanA H2P2	CACACTGTTGTAGGCCGGGCAAGCGTAGCGCCCCGGCATTATGCTG CATATGAATATCCTCCTTAGTTCCTATTCC	
LT2 I+B H1P1	TATTTCTATCTCAGGAAATGAATCCATTACATCACCTATGGGTGTGTAGGCTG GAGCTGCTTC	
LT2 waaI H2P2	CTATATTTTAAAAATTTTAAATAATGCAATATTCTCGAAATTACAAAAGTGA CATATGAATATCCTCCTTAGTTCCTATTCC	
LT2 I+B H2P2	GCTATTTAATATCTAAAGTCTTTAGATTAAGGTTTAACTCTGGGGTATAT CATATGAATATCCTCCTTAGTTCCTATTCC	
LT2 waaB H1P1	GTTATAATCTAAAGTCTGTTGAATGACTTCTTCTTCAAAAATATTTCTGCTCATGTGTAGGCTGGAGCTGCTTC	
LGTE1	GGATCCAGGAGTAAATAATGCAAAAACCACGTATCAGCTTGGC	
LGTE2	GAGCTCTTTTAATCCCTATATTTTACAC	
Lst Fw/XbaI new	AGTCTAGAAATTTGTCGGAATGGAGTTTATG	
Lst Rev/PstI FLAG new	AACCTGCAGTCACTTGTCTGTCGTCCTTGTAGTCAATTTTATCGTCAAATGTCAAATTC	
SiaB Fw/SacI	AGAGCTCCTGCTCAAAAACGTTTTATCGATTG	
SiaB Rev/XbaI c-myc new	AATCTAGATCACAGATCCTTCTGAGATGAGTTTTGTTCGCTTTCCTTGTGATTAAGAATGTTTTTC	
l	CCCAGGTCGTGATAAAGGGCGTCTTC	
m	TTAGGATCCACCTTCCAGCCATCCCTAT	
n	GGATCCAAAGCACTCGCGGCCGCAAAAGCTTGCAGAGCAAAAACC	
o	GTCGACCATCTTCCAGGGGAATTT	

tryptone, 5 g/L Bacto yeast extracts, 5 g/L NaCl). For sialylation experiments, LB was supplemented with 2% sialic acid (Jülich chiral solutions, Jülich, Germany). The pH of the LB with sialic acid was adjusted to 7.5 by addition of NaOH and sterilized by filtering. LB agar plates were supplemented with 1.5% (w/v) agar. Antibiotics were used at the following final concentrations: tetracyclin (tet) 10  $\mu$ g/mL, ampicillin (amp) 100  $\mu$ g/mL, kanamycin 50  $\mu$ g/mL, chloramphenicol (cam) 25  $\mu$ g/mL.

#### DNA manipulations

*N. meningitidis siaB* and *lst* were amplified from *N. meningitidis* MC58 (ATCC# BAA 335 D5) genomic DNA, while *H. influenzae lst* was amplified from *H. influenzae* RM118 genomic DNA (Derek Hood, Oxford). *N. gonorrhoeae lgtE* was amplified from *N. gonorrhoeae* genomic DNA. Restriction enzymes were purchased from MBI/Fermentas, whereas amplification of DNA was carried out with *Pfu* DNA polymerase isolated in the Institute of Microbiology, ETH Zurich, by standard protocols. All cloning steps were carried

out in *E. coli* DH5 $\alpha$ , and all constructs were confirmed by sequencing (Synergene Biotech, Schlieren, Switzerland).

#### Construction of the *E. coli* K-12 $\Delta$ *nanA* $\Delta$ *wcaJ* $\Delta$ *waaO* $\Delta$ *waaB* strain LPS-1

Inactivation of *waaB* and *waaO* was carried out by homologous recombination using the suicide plasmid pKO3. Primers (l) and (m) were used for polymerase chain reaction (PCR) amplification of 0.67 kb of DNA flanking the 5' end of *waaB* from JM107, whereas primers (n) and (o) were used for PCR amplification of 0.69 kb of DNA flanking the 3' end of *waaO*. The 665-bp fragment was digested with *Bam*HI and *Sal*I, while the 688-bp fragment was digested with *Bam*HI and *Sma*I. Both digested fragments were mixed and cloned into the suicide plasmid pKO3 digested with *Sma*I and *Sal*I. The resulting recombinant integrative plasmid pKO3-BO was transformed into TA1 $\Delta$ *nanA* cells; positive clones were screened for correct integration of the plasmid DNA by PCR with primers (l) and (o) (for the sequence, see Table I). The truncation of *wcaJ* is described elsewhere (Priem et al.).

*Construction of the M525P $\Delta$ nanA $\Delta$ waal $\Delta$ waab strain SKI22*

For the step-by-step deletion of the *nanA*, *waal* and *waab* genes, the method introduced by Datsenko and Wanner was used (Datsenko and Wanner 2000). Primers and plasmids employed in this approach are listed in Table I. First, the gene encoding the sialic acid aldolase NanA was deleted. DNA sequences from template plasmid pKD4 which carries a kanamycin resistance cassette flanked by FLP recognition target (FRT) sites were amplified using primers LT2 NanA H1P1 and LT2 NanA H2P2. The PCR product obtained was electroporated into strain M525P carrying the  $\lambda$  red recombinase encoding plasmid pKD46. Transformants in which the *nanA* gene had been exchanged with the kanamycin resistance cassette were selected on LB plates containing 50  $\mu$ g/mL final concentration kanamycin. Integration of the antibiotic cassette into the correct genomic region was verified by PCR. Loss of the antibiotic cassette from the chromosome was achieved by using pCP20 which encodes the FLP recombinase and recognizes the FRT sites. The resulting  $\Delta$ *nanA* strain was termed SKI2 after verification of the genomic deletion of *nanA* by PCR. In SKI2, the *waal* gene was deleted using the same approach and primers LT2 I+B H1P1 and LT2 *waal* H2P2. The resulting  $\Delta$ *nanA* $\Delta$ *waal* strain was termed SKI14 after verification of the genomic deletion of *waal* by PCR. In SKI14, the *waab* gene was deleted using the same approach again and primers LT2 *waab* H1P1 and LT2 I+B H2P2. The resulting  $\Delta$ *nanA* $\Delta$ *waal* $\Delta$ *waab* strain was termed SKI22 after verification of the genomic deletion of *waab* by PCR.

*Analysis of lipid A core*

Detection of lipid A core and its alteration was performed by silver staining. To achieve this, proteinase-K-treated whole cell extracts were prepared as follows: The equivalent of 1 OD<sub>600</sub> of induced overnight LPS-1 or SKI22 cells harboring pBBR1gtE and pKI3\* (or the corresponding vector controls) or the parental strains JM107 or M525P were resuspended in 500  $\mu$ L 0.065 M Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (*w/v*), 5%  $\beta$ -mercaptoethanol (*v/v*), 10% glycerin (*v/v*) and 0.05% bromophenol blue (*w/v*) and lysed for 5 min at 95°C. Proteins were digested by the addition of proteinase K (Roche, final concentration 0.4 mg/mL) for 1 h at 60°C. Equal volumes of each sample were then separated on a 17% Tris-Tricine-PAGE and stained with silver as described (Tsai and Frasch 1982).

*Live cell microscopy*

For live cell microscopy, the equivalent of 0.2 OD<sub>600</sub> of induced overnight LPS-1 or SKI22 cells harboring pBBR1gtE and pKI3\* (or the corresponding vector controls) were pelleted by centrifugation (8 min, 4°C, 8000  $\times$  g) and washed once with 1 mL phosphate-buffered saline (PBS). The samples were then treated with 5% bovine serum albumin (BSA) in PBS on a rotary wheel for 30 min at 4°C. Incubation with the primary GM3 recognizing antibody EM5 (Hugh Willison, Glasgow) in PBS/5% BSA followed for 45 min at 4°C on a rotary wheel. Unbound antibody was washed away in two washing steps using 1 mL PBS. Cells were then stained with DAPI (10  $\mu$ g/mL final concentration), and bound antibody was visualized with a goat-anti-mouse-IgM-AlexaFluor647 conjugate (Invitrogen) in a 45-min incubation step on ice in the dark. Unbound DAPI and

secondary antibody were removed by washing the cells three times with PBS. Finally, cells were resuspended in 60  $\mu$ L PBS for analysis under the microscope (Zeiss Axioplan 2), embedded in low melting agarose. Data acquisition and analysis for microscopy was carried out with Zeiss Axiovision 4.7, while images were combined using Adobe Photoshop CS3.

*Colorimetric antibody binding quantification to whole cells*

The procedure used for quantification of anti-GM3 antibody EM5 binding to whole cells was the same as for live cell microscopy up to the incubation step with the secondary antibody. As secondary antibody, a goat-anti-mouse-IgM-horse radish peroxidase conjugate (Santa Cruz) was used in PBS/5% BSA in an incubation step of 45 min at 4°C on a rotary wheel. Unbound horse radish peroxidase conjugate was washed away in two washing steps; cells were resuspended in 500  $\mu$ L 70 mM phosphocitrate buffer pH 4.2 and distributed in triplicates in a 96-well plate. A first measurement for OD<sub>600</sub> was carried out before adding the substrate ABTS (final concentration 1 mM in 70 mM phosphocitrate buffer pH 4.2 with addition of 0.03% H<sub>2</sub>O<sub>2</sub>) to the wells and recording the color development at 405 nm for 5 min in a SpectraMaxPlus (Molecular Devices). For analysis,  $v_{\max}$  was normalized to OD<sub>600</sub>.

*Isolation of lipooligosaccharides*

LOS of overnight induced LPS-1 or SKI22 cells harboring pBBR1gtE and pKI3\* (or the corresponding vector controls) was extracted using the phenol-chloroform-light petroleum method (Galanos et al. 1969). In brief, cells equivalent to approximately 4000 OD<sub>600</sub> were harvested and washed with 1  $\times$  PBS. The bacteria were then resuspended in phenol:chloroform:light petroleum (1:2.5:4) at a final concentration of 2 g per 50 mL and disrupted in a potter homogenizer. Samples were spun for 10 min at 4°C at 3800  $\times$  g, and the supernatant was incubated at 55°C in a water bath until complete evaporation of the chloroform-ether phase had taken place. LPS was precipitated by the addition of 1 volume of deionized H<sub>2</sub>O and pelleted for 10 min at 4°C at 3800  $\times$  g. At this stage, three separate layers were visible in the suspension, H<sub>2</sub>O in the upper phase, LOS in the middle phase and phenol in the lower phase. The phenol phase was removed with a Pasteur pipette, and a second precipitation of LOS from the phenol phase was performed by the addition of 1 volume of deionized H<sub>2</sub>O, and the centrifugation step was repeated. Both LOS phases were combined and spun at 4°C for 15 min at 11,000  $\times$  g. Residual phenol was removed with a Pasteur pipette, and the centrifugation step was repeated. The pellet containing the LOS was washed with 20 mL methanol, spun for 20 min at 17,500  $\times$  g, and the supernatant was discarded. Residual methanol was removed by heating the sample in a water bath to 55°C. The LOS pellet was resuspended in 10 mL of 0.1 mM MgCl<sub>2</sub> and centrifuged at 15°C, 100,000  $\times$  g for 16 h. The final pellet was resuspended in 2 mL of ddH<sub>2</sub>O and dried down in a vacuum.

*O-deacylation of isolated lipooligosaccharides*

O-deacylation of LPS was performed by mild hydrazine treatment (Haishima et al. 1992). LPS was dissolved in hydrazine hydrate to a final concentration of 20 mg/mL and incubated for 2 h at 37°C with constant shaking. To precipitate LPS after the

cleavage of the O-linked acyl chains, 15 volumes of ice-cold acetone were added. The sample was spun for 15 min at room temperature at  $16,000 \times g$ , and the pellet containing the O-deacylated LPS was washed with acetone and subjected to another centrifugation step for 15 min at room temperature at  $10,000 \times g$ . The supernatant was removed and the pellet air dried.

#### Mass spectrometry analysis

For MALDI-mass spectrometry profiling, samples dissolved in water at a final concentration of 5–10 mg/mL were mixed 1:1 with the 6-aza-2-thio-thymine (ATT) matrix (20 mg/mL in 70% MeOH with 10 mM ammonium citrate). Data acquisition was performed on 4800 Peptide Analyzer, (Applied Biosystem, Framingham, MA) using linear negative ion mode, with a total of 20 sub-spectra of 125 laser shots and each with laser energy set at 7000.

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#### Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); BSA, bovine serum albumin; DAPI, 4, 6-diamino-2-phenylindole; FRT, FLP recognition target; GBS, Guillain-Barré Syndrome; Hep, L-glycero-D-mannoheptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NeuNAc, N-acetyl neuraminic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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