

Exploiting Bacterial Glycosylation Machineries for the Synthesis of a Lewis Antigen-containing Glycoprotein^{*[5]}

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Background: Bacterial glycosylation systems appear to be ideal for glycoengineering purposes.

Results: A Lewis x containing glycoprotein was synthesized using bacterial enzymes from four different species using *in vivo* and *in vitro* steps.

Conclusion: Glycosylation systems in bacteria open new avenues for the production of engineered glycoproteins.

Significance: Bacterial glycoengineering is a promising technology for the production of therapeutically valuable glycoproteins with expected high precision and low cost.

Glycoproteins constitute a class of compounds of increasing importance for pharmaceutical applications. The manipulation of bacterial protein glycosylation systems from Gram-negative bacteria for the synthesis of recombinant glycoproteins is a promising alternative to the current production methods. Proteins carrying Lewis antigens have been shown to have potential applications for the treatment of diverse autoimmune diseases. In this work, we developed a mixed approach consisting of *in vivo* and *in vitro* steps for the synthesis of glycoproteins containing the Lewis x antigen. Using glycosyltransferases from *Haemophilus influenzae*, we engineered *Escherichia coli* to assemble a tetrasaccharide on the lipid carrier undecaprenylphosphate. This glycan was transferred *in vivo* from the lipid to a carrier protein by the *Campylobacter jejuni* oligosaccharyltransferase PglB. The glycoprotein was then fucosylated *in vitro* by a truncated fucosyltransferase from *Helicobacter pylori*. Diverse mass spectrometry techniques were used to confirm the structure of the glycan. The strategy presented here could be adapted in the future for the synthesis of diverse glycoproteins. Our experiments demonstrate that bacterial enzymes can be exploited for the production of glycoproteins carrying glycans present in human cells for potential therapeutic applications.

The discovery of general protein glycosylation systems in Gram-negative bacteria inaugurated a new era in the production of recombinant glycoproteins (1, 2). Exploitation of these systems via glycoengineering has the potential to overcome many hurdles of the techniques currently available for the synthesis of glycoconjugates (3, 4). In contrast to protein glycosyl-

ation in eukaryotes, bacterial cells tolerate the manipulation of glycan moieties. In higher eukaryotes even minimal alterations in the glycan structure can be lethal, primarily because eukaryotic *N*-glycosylation is required for protein folding control (5). Although yeasts appear to be a promising system for production of humanized *N*-glycoproteins, it seems unlikely that these cells can be efficiently used for the production of glycoproteins containing glycans other than the high-mannose type (6). On the other hand, chemical synthesis of conjugates often requires harsh conditions for the attachment of the sugars, which usually affects the properties of the carriers (7). Furthermore, chemical cross-linking is difficult to control and therefore glycoconjugates produced via this method often present reproducibility and homogeneity issues (7). Utilization of living bacteria for the production of glycoproteins combines the specificities of enzymatic reactions for both, the assembly of the glycan and its conjugation to the polypeptide.

Oligosaccharyltransferases (OTases)³ are the key enzymes involved in such bacterial synthesis pathways. They are responsible for the transfer of glycans from the carrier lipid undecaprenyl pyrophosphate (UndPP) onto acceptor proteins (8). Importantly, some of the best characterized OTases display relaxed substrate specificity. Glycans attached to the carrier lipid can be transferred onto acceptor proteins with very limited or no requirements toward the structure of the oligo- or polysaccharide (9, 10). For instance, the presence of an acetamido group at C-2 of the reducing sugar residue and probably a linkage other than 1–4 between the two proximal sugars are the only known substrate requirements of the *Campylobacter jejuni* OTase PglB (1, 11). Therefore, if the desired glycan structure fulfills these requirements and can be assembled onto UndPP, it will be transferred onto acceptor proteins in the presence of PglB. No structural requirement for the glycan has been found for the *Neisseria* OTase PglL (9). Interestingly, UndPP serves as a carrier lipid for glycan assembly in many bacterial glycoconjugate pathways (12). One example is the lipopolysac-

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³ The abbreviations used are: OTase, oligosaccharyltransferases; UndPP, undecaprenyl pyrophosphate; Ni-NTA, nickel-nitrilotriacetic acid; FTICR, Fourier-transform ion cyclotron resonance.

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charide biosynthesis pathway, using UndPP for the assembly of its distal polysaccharide, named O antigen. The O antigen is then conjugated *en bloc* onto the lipid A-core by the O antigen ligase (13). Indeed, it was demonstrated that UndPP-linked, fully polymerized O antigens can also be transferred onto acceptor proteins by bacterial OTases (9, 10).

OTases recognize specific acceptor sites on acceptor proteins, making enzymatic glycan transfer superior to chemical conjugation in terms of precision and reproducibility. *C. jejuni* PglB attaches glycans in an *N*-linkage, recognizing the extended eukaryotic consensus sequence (D/E)XNX(S/T) (14). Any polypeptide engineered to contain this glyco-tag may serve as a *C. jejuni* PglB glycosylation target in the bacterial periplasm (14). The recognition determinants for other OTases are less clear. *Desulfovibrio desulfuricans* PglB does not recognize the same sequon as its *Campylobacter* counterpart (15) and it is unknown how *Neisseria* PglL recognizes its substrates (16).

Although the best characterized bacterial glycosylation systems originate from the pathogens *C. jejuni*, *Pseudomonas aeruginosa*, and *Neisseria* spp., these glycoprotein assembly machineries were functionally reconstituted in nonpathogenic and fast growing *Escherichia coli* laboratory strains (2, 17). This has opened up new avenues for the exploitation of these systems for the synthesis of engineered glycoproteins with potential therapeutic applications.

The flexibility of the bacterial glycosylation pathways prompted us to develop a strategy to exploit these systems for the synthesis of customized glycoproteins. The Lewis x (Le^x) antigen is a glycan structure (Gal β 1–4[Fuc α 1–3]GlcNAc-) that is abundantly expressed on a variety of human cells, as well as on some parasitic helminthes and the gastric pathogen *Helicobacter pylori* (18–20). Glycoconjugates containing Le^x have promising applications for the treatment of autoimmune diseases due to their immunosuppressant effect via interaction with the dendritic cell receptor DC-SIGN (21). Experiments in animal models have demonstrated that symptoms associated with autoimmune disorders are ameliorated upon treatment with Le^x -containing glycoproteins (22). The “old friend hypothesis” elaborates on this idea, proposing that reduced exposure to immune suppressive signals from microbes due to improved hygiene and generalized use of antibiotics has increased the prevalence of human immune disorders in modern times (23). The use of bacteria for the generation of immunosuppressant and other glycodrugs may be a valuable future technology. In this work, we describe a combined *in vivo* and *in vitro* approach for the production of a glycoprotein containing the Le^x antigen by exploiting bacterial enzymes.

EXPERIMENTAL PROCEDURES

DNA Manipulations and Plasmid Construction—*H. pylori* 11639 *fucT* full-length and truncated versions were excised from pGEM11639*fucT*fl and pGEM11639*fucT*tr (24), respectively, using SphI. The DNA ends were then blunted with a Klenow fragment and digested with PstI. The inserts were ligated into pEXT20, which was digested with SmaI and PstI. The resulting plasmids were pIH61, containing the full-length *fucT*, and pIH62, containing the truncated version.

Bacterial Strains and Growth Conditions—*E. coli* DH5 α or BL21 strains were grown in LB broth supplemented with the appropriate antibiotics to counterselect the eventual loss of plasmids (ampicillin = 100 μ g/ml, chloramphenicol = 10 μ g/ml in EtOH, trimethoprim = 100 μ g/ml).

Production and Detection of Glycoproteins in Living *E. coli*—*E. coli* DH5 α cells containing *Helicobacter influenzae* *lsg* genes on pGEMLOS5 (25), the *C. jejuni* *pglB* on pMAF10 or pWA1 containing an inactive *pglB* for control samples, and the *C. jejuni* *acrA*, encoding a periplasmic acceptor protein, on pMH5 (10), were induced with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside and 0.2% (w/v) arabinose at early log phase and grown overnight at 37 °C with shaking at 200 rpm. Periplasmic contents containing the glycoproteins were extracted using a lysozyme solution as described by Feldman *et al.* (10). Glycosylation of the acceptor protein AcrA was analyzed after separation of the periplasmic contents on SDS-PAGE by visualization with Coomassie Blue staining or Western blotting using a primary anti-AcrA antibody (Sacri antibody Services, University of Calgary) and a secondary goat anti-rabbit IRDye-680 antibody (Licor biosciences) with an Odyssey Infrared Imager (Licor Biosciences).

Purification of Glycoproteins before Fucosylation—Periplasmic fractions containing glycosylated histidine-tagged AcrA proteins were loaded onto 1-ml HisTrap FF columns (GE Healthcare Life Sciences) in 20 mM Tris-HCl, pH 8.0, containing 0.3 mM NaCl and 10 mM imidazole. The columns were washed with 50 ml of 20 mM Tris-HCl, pH 8.0, containing 20 mM imidazole and 0.3 M NaCl. The proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole and 0.3 M NaCl. Protein concentrations were measured using the Quick Start Bradford dye reagent and a BSA protein standard kit (Bio-Rad).

Expression of *H. pylori* Fucosyltransferases in *E. coli*—The *H. pylori* 11639 fucosyltransferase or its truncated version were either expressed in *E. coli* BL21 cells containing pGEM11639*fucT*fl or pGEM11639*fucT*tr (24) or in *E. coli* DH5 α cells containing pIH61 or pIH62. After induction with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside in early log phase, the cells were incubated for 6 h at 37 °C with shaking at 200 rpm. The cells were washed once with 20 mM HEPES, pH 7.0, buffer and disrupted by sonication in 20 mM HEPES containing a Complete EDTA-free proteinase inhibitor mixture (Roche Applied Science).

Addition of Radioactive Fucose in Vitro—Samples were prepared with 24 μ l of *E. coli* cell extract containing overexpressed fucosyltransferases, 20 μ g of purified AcrA, 160 μ g of BSA, and 1.2 units of Antarctic phosphatase (New England Biolabs) in 20 mM HEPES, pH 7.0, 50 mM NaCl, 35 mM MgCl₂, 20 mM MnCl₂, and adjusted to 38 μ l with H₂O. 2 μ l of a mixture of cold (0.2 μ M) and hot (80,000 dpm per sample) GDP-fucose was added. The samples were incubated at 37 °C for 3 h. For each sample, 10 μ l of Ni-NTA-agarose (Qiagen) was equilibrated with binding buffer (10 mM imidazole, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0) before the reaction samples were added. Samples were incubated for 1 h at room temperature while shaking, then washed with 20 mM imidazole, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0. The Ni-NTA-agarose together with the histidine-tagged

proteins were then transferred into 10 ml of Ecolite solution and radiation was measured with a scintillation counter.

Addition of Cold Fucose in Vitro—40- μ l samples were prepared as described for radioactive fucosylation, except using 100 μ g of cold GDP-fucose. Samples were incubated overnight at 37 °C, and then analyzed by Western blot and mass spectrometry.

In-gel Protein Digestion and Peptide Purification—After staining with Coomassie Blue, gel pieces containing glycosylated AcrA proteins were treated according to Shevchenko *et al.* (26) for trypsin digestion. Because AcrA does not contain disulfide bonds, reduction and alkylation steps were not required. Peptides were desalted using C18 Zip-Tips (Millipore).

LC ESI-Q-TOF—Dried desalted peptides were solubilized in 8 μ l of 0.1% (v/v) formic acid in H₂O. The Q-TOF Premier mass spectrometer (Waters Associates, Milford, MA) equipped with a nanoACQUITY ultraperformance liquid chromatography system (Waters) was operated as described elsewhere (17). Mass spectra were analyzed using MassLynx software (Waters).

LC-MALDI TOF-TOF—In gel trypsin-digested and desalted peptides were dried under vacuum centrifugation followed by resolubilization using 0.1% TFA in H₂O and injected onto an Easy nano-LC II system (Proxeon/Thermo Scientific). 10 μ l of sample was injected onto a BioSphere C18 (5 μ m) 100- μ m inner diameter \times 20-mm loading/de-salting column, washed with 0.1% TFA for 12 column volumes, and eluted onto a BioSphere C18 (5 μ m) 75- μ m inner diameter \times 100-mm analytical column. The mobile phases used for the elution of analytes from the analytical column were: 0.1% TFA in 98% H₂O and 2% acetonitrile as mobile phase A and 0.1% TFA in 95% acetonitrile and 5% H₂O as mobile phase B at a flow rate of 300 nl min⁻¹. Elution of the analytes was done by using a linear gradient from 0 B to 5% B over a period of 2 min, 5 B to 50% B over a period of 58 min, 50 B to 100% B over a period of 4 min and held at 100% B over a period of 11 min. The nanoLC separated peptides were directly spotted onto a Bruker Daltonics MTP AnchorChip™ 800/384 target and co-mixed with α -cyano-4-hydroxycinnamic acid matrix running at a flow rate of 1.26 μ l min⁻¹ using the Proteiner FcII sample spotting robot (Bruker Daltonics, Bremen, GmbH). HPLC fractions were collected with an 8-min delay using a spotting frequency of 20 s for a period of 64 min for a total of 192 spots. Mass spectra were obtained in the positive ion mode of ionization using a Bruker Daltonics (Bremen, GmbH) UltrafleXtreme MALDI TOF/TOF mass spectrometer. The FlexAnalysis software provided by the manufacturer was used for analysis of the mass spectra. The MS/MS spectra were obtained manually. The exact *m/z* used as the precursor *m/z* for MS/MS was determined first on the MALDI FTICR MS instrument (see below) and the MS/MS spectrum was automatically re-calibrated based upon this *m/z*.

Pronase E Digestion and Permethylolation of Glycan Moieties—Trypsinized peptides were incubated at 37 °C in 10 μ l of 0.1 M Tris-HCl, pH 7.5, containing 60 μ g of Pronase E. For glycan enrichment, the samples were applied three times onto active charcoal-containing MicroSpin columns (Harvard apparatus), which were previously hydrated for 10 min with H₂O and washed three times 80% acetonitrile, 20% H₂O containing 0.1%

TFA, followed by three washes with H₂O (27). The samples were washed twice with H₂O, eluted with 25% acetonitrile, and dried. Permethylolation was carried out in MicroTip columns (Harvard Apparatus), which were filled with NaOH beads (until <1 cm from top) and equilibrated with *N,N*-dimethylformamide (27). The dried glycans were resolubilized with 70% *N,N*-dimethylformamide, 25% methyl iodide, 5% H₂O, applied to the MicroTip columns and incubated for 15 min. After centrifugation at 950 \times *g*, 25 μ l of methyl iodide was added and the flow-through was reapplied for another 15 min. Flow-through and two washes with 100 μ l of acetonitrile were pooled. 300 μ l of dichloromethane were added and after mixing with 1 ml of 0.5 M NaCl for liquid-liquid extraction. The organic phase was dried after two washes with 0.5 M NaCl and three washes with H₂O together with a second extraction with 100 μ l of dichloromethane. Permethylated glycans were spotted on a Bruker Daltonics ground steel plate and 1 μ l of 2,5-dihydroxybenzoic acid (10 mg/ml in 30% H₂O, 70% CH₃CN containing 1 mM sodium acetate/ml) was spotted on top and allowed to dry. The permethylated glycans were then analyzed by MALDI-MS on a 9.4T Bruker Daltonics Apex Qe FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) to confirm the exact mass and elemental composition followed by MALDI-MS/MS analysis on a Bruker Daltonics Ultraflextreme mass spectrometer (Bremen, GmbH).

RESULTS

Synthesis of Glycoproteins with Terminal LacNAc Structures in Live Recombinant *E. coli*—A partial gene cluster from *H. influenzae*, containing lipooligosaccharide (LOS) synthesis genes (*lsg-f*) was previously expressed in *E. coli* by Phillips and colleagues (25). They analyzed the resulting chimeric lipopolysaccharide and found that the tetrasaccharide Gal β 1-4GlcNAc β 1-3Gal β 1-3GlcNAc-, derived from the *H. influenzae* LOS structure, was attached to *E. coli* lipid A-core. This tetrasaccharide contains a terminal LacNAc structure (Gal β 1-4GlcNAc-) and would only require a branching fucose residue attached via an α 1-3-linkage to the GlcNAc residue to constitute a Le^x antigen structure (Fig. 1). The synthesis of the identified chimeric glycolipid was shown to be dependent on the *E. coli* enzyme WecA (25). WecA is a glycosyltransferase, initiating the assembly of glycan subunits in the synthesis of the enterobacterial common antigen and the *E. coli* O antigen, generating UndPP-GlcNAc (13, 28). It can therefore be assumed that the *H. influenzae*-derived tetrasaccharide is assembled onto UndPP in *E. coli*. Because this glycan fulfills the substrate requirements of the *C. jejuni* OTase PglB and it is assembled on the carrier lipid UndPP, it is a potential substrate of PglB and other bacterial OTases for synthesis of glycoproteins.

We first tested if PglB was indeed able to generate glycoproteins containing the *H. influenzae*-derived tetrasaccharide as the glycan moiety. *E. coli* cells were co-transformed with plasmids containing the required *lsg* genes, the *C. jejuni* *pglB* OTase gene, and the *C. jejuni* *acrA* gene encoding an acceptor protein (2). After induction and overnight growth, periplasmic contents were extracted and the production of glycosylated proteins was analyzed by Western blotting (Fig. 2A). In the presence of both, the *lsg* cluster and *pglB*, three distinct bands were

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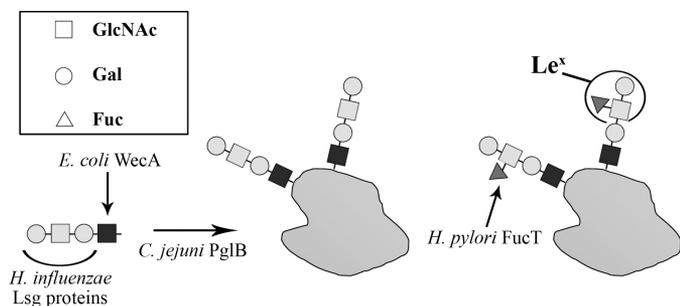


FIGURE 1. Strategy for the generation of Le^x-glycoproteins. The initiating glycosyltransferase WecA from *E. coli* was employed for the addition of the first GlcNAc residue onto the lipid carrier UndPP. Glycosyltransferase from the *H. influenzae* LOS biosynthesis cluster are introduced to the *E. coli* expression strain for the completion of the precursor glycolipid Gal β 1-4GlcNAc β 1-3Gal β 1-3GlcNAc-UndPP. The glycan is then conjugated in the same *E. coli* cells by the *C. jejuni* oligosaccharyltransferase PglB onto one of its protein acceptors, AcrA. The addition of the fucose residue in an α 1-3 linkage onto the exterior GlcNAc residue by *H. pylori* FucT *in vitro* completes the synthesis of the Le^x-glycoprotein. Squares indicate GlcNAc residues, circles the Gal residues, and triangles the Fuc residues. Sugar residues added by *E. coli* WecA are dark colored, by the *H. influenzae* Lsg proteins are light colored, and by the *H. pylori* FucT are gray colored.

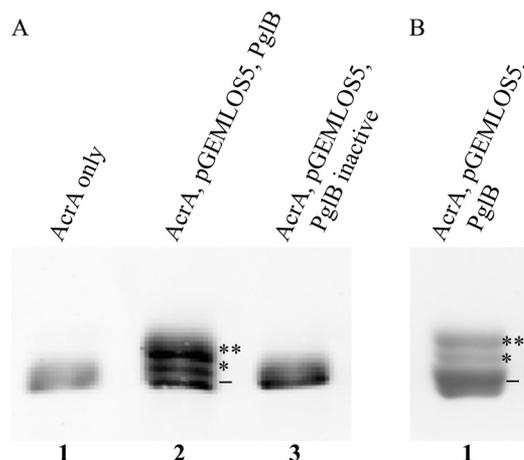


FIGURE 2. Protein glycosylation with the *H. influenzae*-derived glycan in living *E. coli*. Glycosylated AcrA acceptor proteins were analyzed by SDS-PAGE and Western blotting. A, periplasmic extracts containing AcrA proteins were analyzed by Western blotting using an anti-AcrA antibody. AcrA from cells containing only the *acrA* gene (lane 1) or an inactive *pglB* variant (lane 3) was observed as a single nonglycosylated moiety (–), whereas in the presence of all required functional genes, two additional protein species were produced (lane 2). The monoglycosylated AcrA band is marked with one asterisk, the diglycosylated band with two asterisks. B, glycosylated AcrA containing the *H. influenzae*-derived glycan was purified by nickel affinity chromatography and analyzed by SDS-PAGE and Coomassie staining.

detected using an anti-AcrA antibody. The fastest migrating band displayed the same electrophoretic mobility as nonglycosylated AcrA. Both slower migrating bands, however, suggested glycosylation and were designated as mono- and diglycosylated AcrA, respectively (Fig. 2A). The histidine-tagged AcrA protein was purified (Fig. 2B) and glycosylation was confirmed by mass spectrometry after digestion with trypsin. The two established AcrA acceptor peptides DFNR (551 Da) and AVFDN~~NN~~STLLPGAFATITSEGFQK (2755 Da) (the glycosylated asparagines are underlined) were analyzed for the presence of covalently attached glycans (2). The corresponding parental peaks of 1281 and 3485 Da and the fragmentation peaks showing the sequential loss of 162 Da (Hex) and 203 Da (HexNAc) were found in MS and MS/MS spectra, respectively (Fig. 3 shows the MS/MS

spectrum for the DFNR glycopeptide). This confirmed that the expected tetrasaccharide, with the sequence Hex-HexNAc-Hex-HexNAc, was transferred onto the AcrA acceptor protein in living *E. coli* cells. Interestingly, during analysis of the MS spectra, ion peaks compatible with the presence of elongated glycan moieties were occasionally found (supplemental Fig. S1).

In Vitro Fucosylation of an Engineered Bacterial Glycoprotein—Knowing that the *H. influenzae*-derived tetrasaccharide can be transferred onto protein acceptors in living *E. coli* cells, the next step was to fucosylate the terminal LacNAc structure to produce a glycoconjugate containing the Le^x antigen (Fig. 1). FucT from *H. pylori* is one of the most extensively characterized fucosyltransferases (24). It was shown that the fucosyltransferase activity of FucT from *H. pylori* 11639 readily modified LacNAc structures, whereas the similar type I disaccharide Gal β 1-3GlcNAc- was not a suitable acceptor (29). Therefore, it was expected that this fucosyltransferase would modify the *H. influenzae*-derived tetrasaccharide at the terminal LacNAc structure to generate a Le^x-epitope, and leaving the internal GlcNAc residue unchanged. A truncated version of this enzyme (FucT11639¹⁻⁴⁴¹) was previously demonstrated to have enhanced expression levels and improved solubility in *E. coli* (24). This recombinant enzyme seemed therefore ideal for this glycoengineering purpose. Due to the fact that *E. coli* cells do not synthesize GDP-fucose in normal lab growth conditions, and that stable coexpression of the OTase, the protein carrier, the Lsg and FucT glycosyltransferases, and the enzymes required for the synthesis of GDP-fucose is challenging and would require extensive genetic engineering of the *E. coli* cells, we decided to test the fucosylation *in vitro*.

Purified glycosylated AcrA containing the tetrasaccharide generated by the *H. influenzae* glycosyltransferases in *E. coli* as described previously was incubated with radioactively labeled GDP-fucose and crude extracts of *E. coli* cells expressing either the full-length or truncated *H. pylori* 11639 FucT (FucT_{FL} or FucT_{TR}). The glycoproteins were then purified once more using Ni-NTA-agarose. The rate of radioactive decays as a measure for the incorporated fucose residues was determined using a scintillation counter. Elevated counts in samples containing FucT_{FL}, and especially FucT_{TR}, suggested that fucose was transferred onto the glycoproteins *in vitro* (Fig. 4). Based on these results, we predicted that glycoproteins containing terminal Le^x-epitopes were produced via this combined *in vivo* and *in vitro* glycoengineering approach.

To further characterize the fucosylated glycoproteins, the *in vitro* assay was carried out using FucT_{TR} and unlabeled GDP-fucose. The resulting fucosylated glycoproteins were then purified, digested with trypsin, and the peptides were analyzed by mass spectrometry. Addition of a fucose residue increases the molecular mass of a molecule by 146.1 Da, leading to expected parental peaks at 1427 and 3631 Da for the fucosylated AcrA-glycopeptides. Indeed, these signals were detected with MALDI-MS and the peak corresponding to the nonfucosylated peptide, *m/z* = 3485 Da, decreased close to background intensity, suggesting nearly complete fucosylation of the acceptor glycoprotein (data not shown). MS/MS spectra of the 1427- and 3631-Da fragments contained a sequence of expected peaks

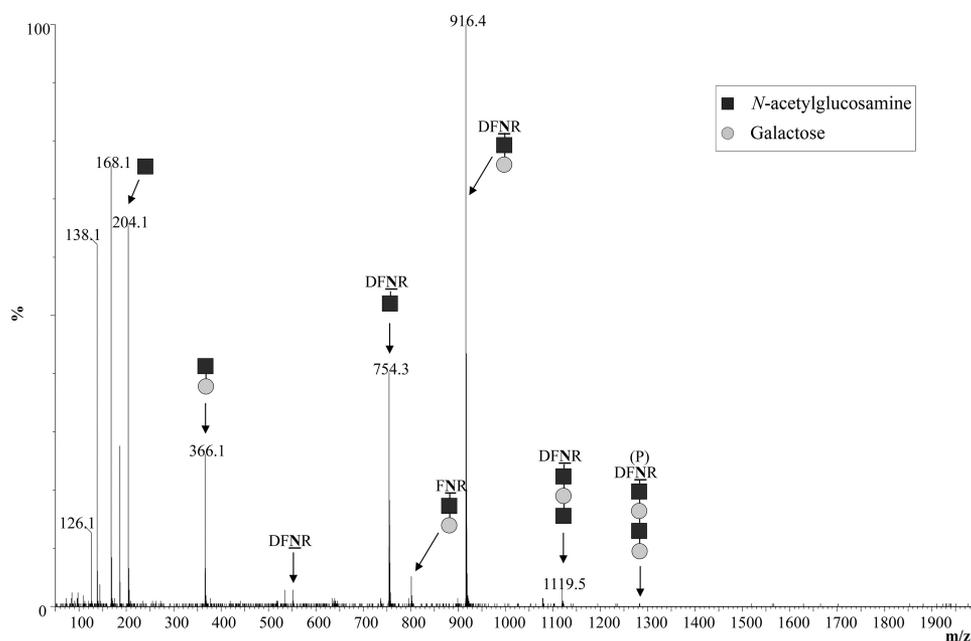


FIGURE 3. **MS/MS analysis of glycoproteins.** Glycosylated AcrA containing the *H. influenzae*-derived glycan added *in vivo* was analyzed by LC ESI-Q-TOF. Shown is the fragmentation spectrum of the Gal β 1-4GlcNAc β 1-3Gal β 1-3GlcNAc-DFNR glycopeptide. Dark squares indicate GlcNAc residues, light circles indicate Gal residues. The parental peak is marked with (P).

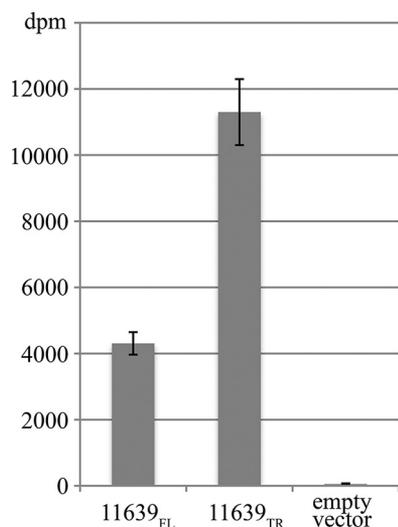


FIGURE 4. ***In vitro* activity of the *H. pylori* FucT on the *in vivo* generated precursor glycoprotein.** Fucosylation was determined after *in vitro* incubation of purified AcrA glycoproteins containing the *H. influenzae*-derived tetrasaccharide with extracts of *E. coli* cells expressing the *H. pylori* fucosyltransferases and radiolabeled GDP-fucose. The fucosyltransferases used were *H. pylori* 11639 FucT full-length (11639_{FL}) and truncated (11639_{TR}) versions or as negative control the empty vector pEXT20. The counts were collected from experiments conducted in triplicates.

confirming the successful incorporation of fucose residues (Fig. 5).

Further analysis of the fragmentation spectra of the DFNR-glycopeptide showed unexpected peaks at $m/z = 900$ and 1062 Da (marked with asterisks in Fig. 5A). Depending on the mass spectrometer used, these peaks were either prominent using ESI-Q-TOF (supplemental Fig. S2) or close to the background level in the MALDI TOF-TOF spectra (Fig. 5A). Both peaks matched with fragments still containing the DFNR peptide and a dHex (fucose) residue in the remaining glycan moiety. However, not more than one HexNAc residue appeared to be pres-

ent in either glycopeptide fragment. This suggested that the external GlcNAc residue, which was the predicted acceptor site for the fucose residue, was not required for fucosylation, and that the fucose was linked to another residue. Assuming that fucose was attached to the internal GlcNAc residue, the 900-Da fragment could correspond to Fuc α 1-4GlcNAc-DFNR and the 1062-Da fragment to Gal β 1-3[Fuc α 1-4]GlcNAc-DFNR. These MS data indicated therefore the presence of the unexpected glycan structure Gal β 1-4GlcNAc β 1-3Gal β 1-3[Fuc α 1-4]GlcNAc-(LacNAc-Le^a). Although the specificity of *H. pylori* 11639 FucT was previously determined to be restricted to type II acceptors and α 1-3 fucosyltransferase activity (29), it was a possibility that in the applied *in vitro* conditions, and offering the enzyme an unusual acceptor with an internal type I and an external type II structure, the enzyme specificity was altered. An alternative explanation was that these peaks were MS artifacts. It has been reported that fucose residues can rearrange during MS analysis, which can lead to misinterpretations concerning the sugar sequence of glycan moieties (30).

To conclusively resolve the structure of the fucosylated glycan moieties, the trypsinized peptides were Pronase E digested, leaving only the asparagine-linked glycans (31). These were then permethylated and subjected to MS/MS analysis (27). Permethylated overcomes the problem of fucose moieties re-arranging during MS/MS analysis. As free hydroxyl and amino groups are modified with methyl groups, adding a mass change of 14 Da at each permethylation site, this technique allows the determination of the number of linkages in which a sugar residue is involved. Analysis by MALDI-MS on the 9.4T FTICR MS was completed to determine the elemental composition of the permethylated glycan. The expected m/z for the sodium-cationized permethylated glycan was 1252.6045 and the observed m/z was 1252.6046 corresponding to an expected elemental composition of (C₅₅H₉₅N₃O₂₇+Na)⁺. The obtained MS/MS

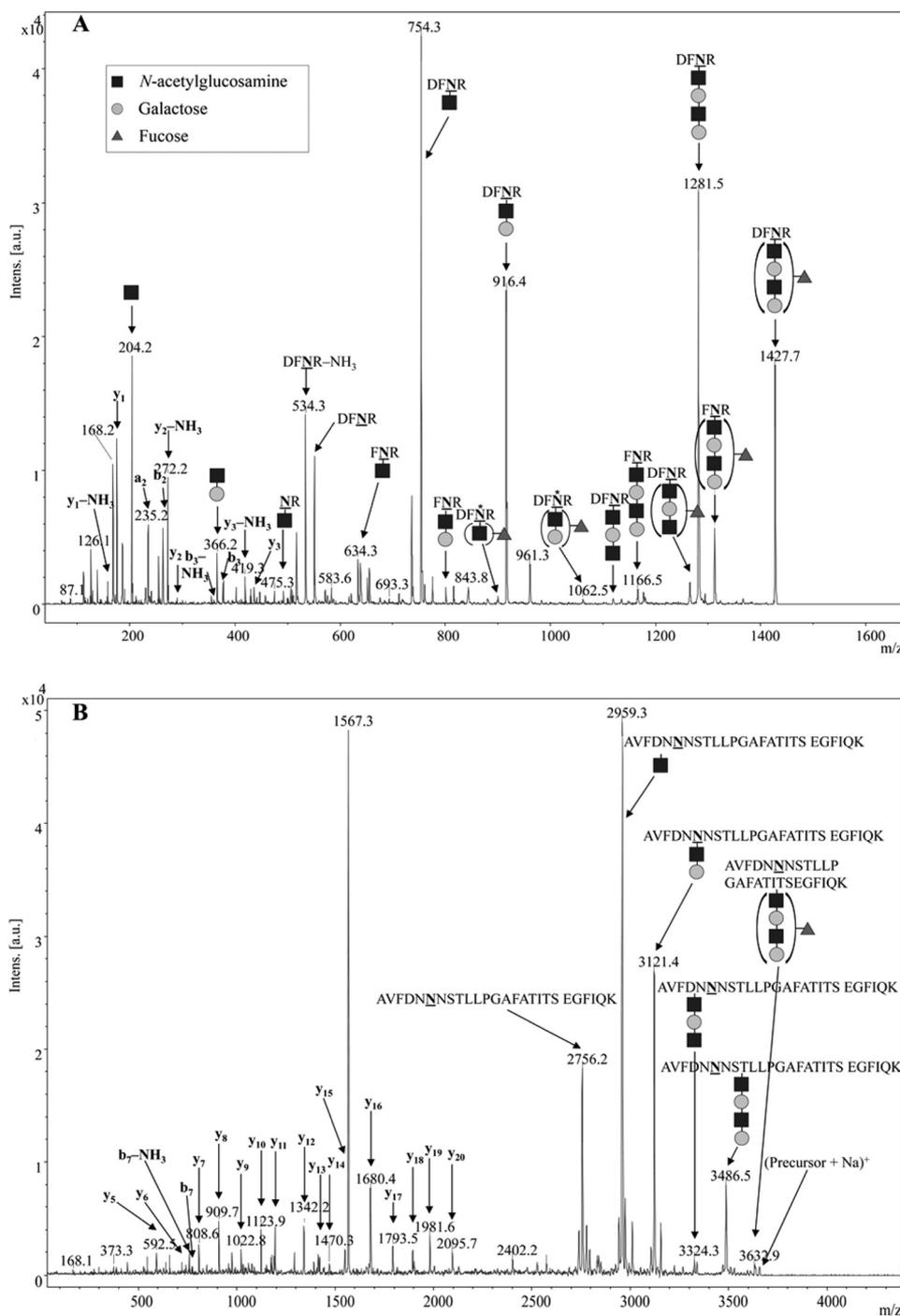


FIGURE 5. **MS/MS analysis of Le^x-glycoproteins.** Glycoproteins were analyzed by MS after *in vitro* fucosylation. The LC-MALDI TOF-TOF MS/MS fragmentation spectrum of the $(M + H)^+$ of the DFNR glycopeptide is shown in A, the MS/MS spectrum of the $(M + Na)^+$ peak of the larger AcrA glycopeptide is shown in B, with its corresponding MS spectrum shown under [supplemental Fig. S3](#). The mass difference between adjacent peaks in the glycan region of the MS/MS accurately corresponds to successive glycan loss although the absolute m/z shifts slightly higher than the expected mass as m/z increases. Note the MS spectrum ([supplemental Fig. S3](#)) accurately shows that the expected m/z was observed within 0.05 Da. Dark squares indicate GlcNAc residues, light circles indicate Gal residues, and gray triangles indicate Fuc residues. Asterisks mark unexpected peaks that misleadingly suggested fucosylation at the internal GlcNAc residue.

spectrum from the MALDI TOF/TOF instrument contained two major peaks at 615 and 660 Da (Fig. 6). Both masses corresponded to the expected fragments of the permethylated asparagine-glycan moiety containing a terminal Le^x-epitope. Consistent with what was observed by Liu *et al.* (31) the permethylated modified Asn shows a 143-Da loss in MS/MS observed as the mass difference between the precursor m/z

1252 and the first fragment ion at m/z 1109 (exact m/z by FTICR MS was 1109.5469 observed, 1109.5463 expected $(C_{49}H_{86}N_2O_{24} + Na)^+$ (31)). The peak at m/z 615 is consistent with a sodium-cationized y_2 ion containing the modified permethylated Asn plus HexNAc and hexose (by MALDI FTICR the exact m/z 615.2736, observed; and 615.2737, expected $(C_{26}H_{44}N_2O_{13} + Na)^+$). Similarly, the peak at m/z 660.35 corre-

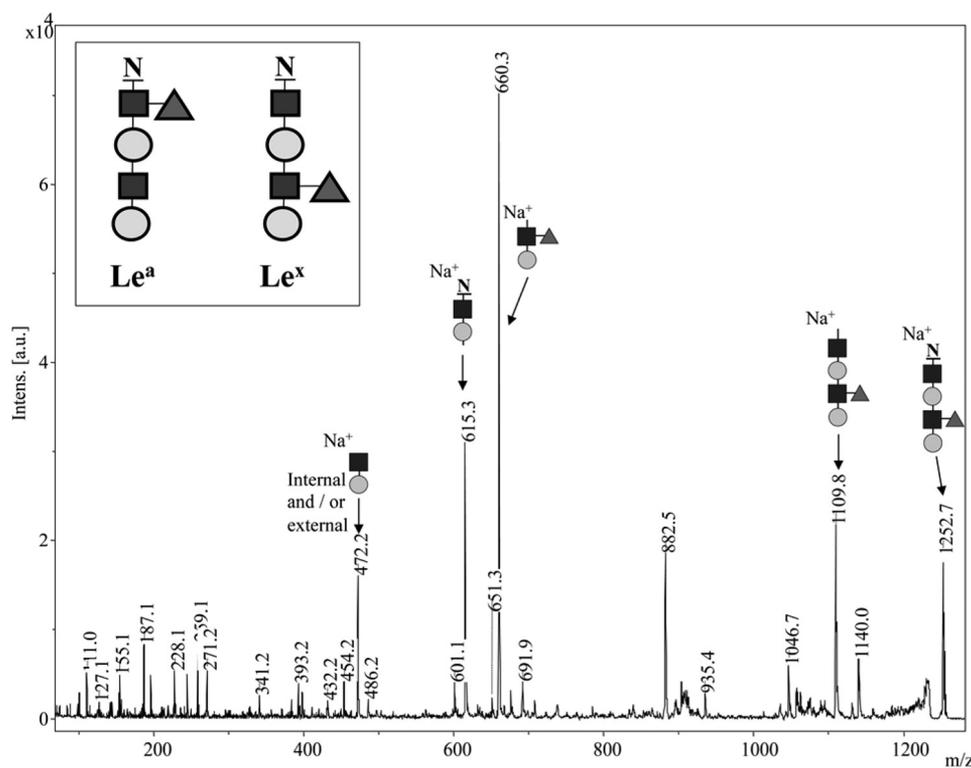


FIGURE 6. **MALDI TOF-TOF analysis of permethylated glycans to verify the Le^x structure.** Shown is the MALDI TOF-TOF fragmentation spectrum of the permethylated Asn-glycan, confirming the position of the fucose residue at the exterior GlcNAc residue (the Le^x structure). Peaks at 615.3 and 660.3 Da can only be derived from fragmentation of the expected Le^x-containing glycan. The peak at 472.2 Da may correspond to a Gal-GlcNAc fragment, which may be the internal or external disaccharide fragments, as they cannot be distinguished by the mass. The *inset* shows the correct glycan (containing the Le^x-epitope) in comparison to the alternative glycan containing the Fuc residue at the internal GlcNAc residue (Le^a-epitope). GlcNAc residues are indicated by *dark squares*, Gal residues by *light colored circles*, and Fuc residues by *gray triangles*.

sponded to a sodium-cationized b_2 ion (the remaining part of the permethylated molecule) consisting of a branched HexNAc (230 Da) and terminal Hex (219 Da) and terminal dHex (189 Da) (by MALDI FTICR the exact m/z 660.3202, observed; 660.3202, expected ($C_{29}H_{51}NO_{14} + Na$)⁺). This showed conclusively that fucose was located on the HexNAc residue remote from the Asn and not on the HexNAc moiety attached to the Asn. Therefore, a glycoprotein containing a Le^x-epitope was successfully produced with combined *in vivo* and *in vitro* techniques, using selected bacterial enzymes.

DISCUSSION

The synthesis of defined glycoconjugates is an important but challenging task for the pharmaceutical industry. In the future, the exploitation of glycoconjugate biosynthesis pathways functionally expressed in nonpathogenic bacteria may become a valuable technology for the production of such compounds. In this work, we have produced a glycoprotein containing an epitope commonly found in human tissues, using a combined approach consisting of *in vivo* and *in vitro* glycosylation steps. The strategy used is presented in Fig. 1.

Fucosylation of free oligosaccharides with LacNAc-epitopes for the assembly of the Le^x antigen was previously accomplished in live *E. coli* cells using *H. pylori* fucosyltransferases (32). The design of the glycoengineering approach followed here, however, imposes that the glycans must be built onto the UndPP carrier to allow *en bloc* transfer to acceptor proteins. This requires the participation of a distinct class of initiating

glycosyltransferases, capable of adding a sugar residue onto the lipid carrier (12). The substrate for the next glycosyltransferases is therefore fixed to the inner membrane. It is possible that due to sterical hindrance the fixed glycans might not be able to reach the active sites of some glycosyltransferases, which prevents the use of some enzymes for this second step. Based on previous work (25), it was correctly predicted that the set of *H. influenzae* glycosyltransferases selected for the *in vivo* glycosylation step was able to generate the required glycolipid anchored in the inner membrane. In the presence of the *C. jejuni* OTase PglB, the tetrasaccharide was transferred onto the AcrA acceptor protein. Some side products were generated, having slightly elongated glycans with an additional HexNAc residue or a Hex-HexNAc disaccharide. This was surprising and indicated a partial reduction in acceptor specificity of the responsible glycosyltransferases in the heterologous expression system.

Because of its terminal LacNAc structure, the tetrasaccharide assembled with the *H. influenzae* glycosyltransferases may be modified in various ways to generate humanized antigens. Addition of sialic acid, galactose, or GalNAc residues could result in epitopes mimicking gangliosides or blood group antigens. The goal of this work was the synthesis of a Le^x-containing glycoprotein by fucosylation of the LacNAc acceptor. The fucose incorporation was successfully accomplished by *in vitro* fucosylation of the *in vivo* assembled glycoprotein. Although MS/MS peaks at 900 and 1062 Da misleadingly indicated an unexpected site of fucosylation (LacNAc-Le^a-), the analysis of

the permethylated product confirmed that the expected Le^x-epitope was indeed produced. Curiously, fucose rearrangement in the gas phase was prominent using Q-TOF MS (supplemental Fig. S2), but only minor using MALDI-MS (Fig. 5A). This can be explained considering that the compounds spend several milliseconds in the collision cell of a Q-TOF but only microseconds of interaction time in a MALDI TOF/TOF.

The presented work demonstrates a step toward a novel technology for the production of glycoproteins. Our work was preceded by an analogous, although conceptually different approach for the synthesis of homogenous *N*-glycoproteins. The strategy, also consisting of an initial *in vivo* glycosylation step, and several modification steps *in vitro*, was recently reported by Schwarz *et al.* (33). In that work, AcrA was glycosylated *in vivo*, the glycan was trimmed by glycanases *in vitro*, and subsequently subjected to an enzymatic transglycosylation reaction to obtain *N*-glycoproteins carrying oligosaccharides of the high mannose type. Unlike the strategy presented by Schwarz *et al.* (33), our approach can be adapted to generate glycoproteins carrying human glycan epitopes of diverse nature. This includes glycans unlikely to be synthesized in engineered yeast or mammalian cells such as those present on glycolipids. The ultimate goal of the glycoengineering approach is to achieve complete synthesis *in vivo*, followed by simple purification of the customized glycoconjugates. Using an optimized set of glycosyltransferases, the all-enzymatic *in vivo* synthesis is expected to assure the highest product quality and low production costs. With this approach an almost limitless combination of designed glycan and protein acceptor structures appears possible. One limitation is that bacterial strains will have to be engineered to produce the activated sugar donors not available in *E. coli* under standard growth conditions, for example, GDP-fucose and CMP-sialic acid. However, strains producing these activated sugars have been constructed (34). A main focus of future work should be the thorough analysis and optimization of metabolic pathways and the expression and control of activity of glycosyltransferases originating from various species, in *E. coli*. These efforts are currently ongoing in our laboratory.

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Figure S1. Detection of elongated side-products in ESI-Q-TOF MS spectra

Glycosylated AcrA containing the *H. influenzae* derived glycan added *in vivo* was analyzed by LC ESI-Q-TOF. Shown is a MS spectrum containing the 1281.5Da peak for the single charged DFNR glycopeptide and peaks at 742.8Da and 823.7Da, being the double charged ions of elongated side products containing an additional HexNAc residue or an additional Hex-HexNAc disaccharide. Interestingly, some fragmentation was also already observed in the Q-TOF MS spectra, such as peaks corresponding to free sugar ions (204.1: HexNAc, 366.1: Hex-HexNAc). GlcNAc residues are indicated by dark squares, Gal residues by light circles.

Figure S2. ESI-Q-TOF MS/MS showing the fragmentation spectrum of the DFNR-glycopeptide

Shown is the fragmentation pattern for the DFNR-glycopeptide using ESI-Q-TOF MS/MS. Besides the expected peaks, additional peaks of 1062Da and 900Da (marked with asterisks) suggested fucosylation at the internal GlcNAc residue. The misleading 1062Da peak is more prominent in this spectrum compared to the same analysis performed using LC MALDI TOF/TOF (compare Figure 5A). Dark squares indicate GlcNAc residues, light circles Gal residues and gray triangles Fuc residues.

Figure S3. MS spectrum of larger AcrA glycopeptide

LC-MALDI TOF/TOF MS spectrum of the larger AcrA glycopeptide showing the (M+H)⁺ at m/z 3631.66 and (M+Na)⁺ at m/z 3653.62 peaks. The observed m/z is within 0.05 Da of the expected m/z for the glycosylated AcrA peptide.

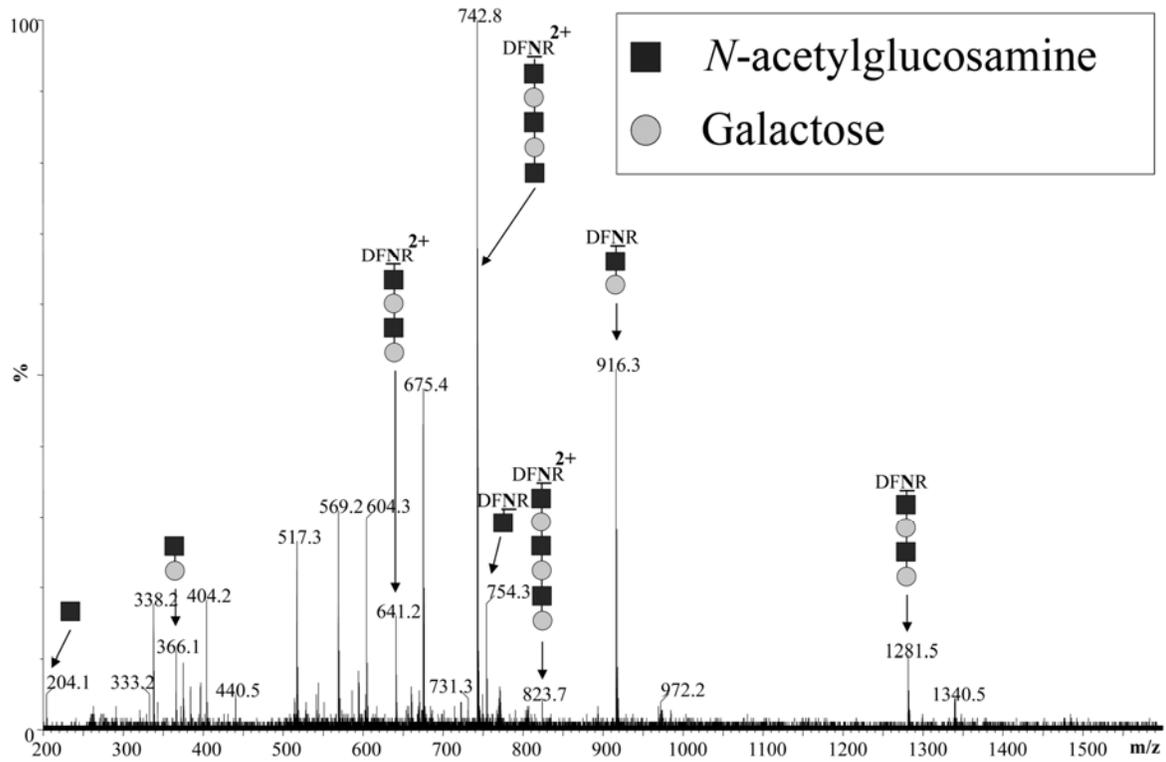


Fig S1

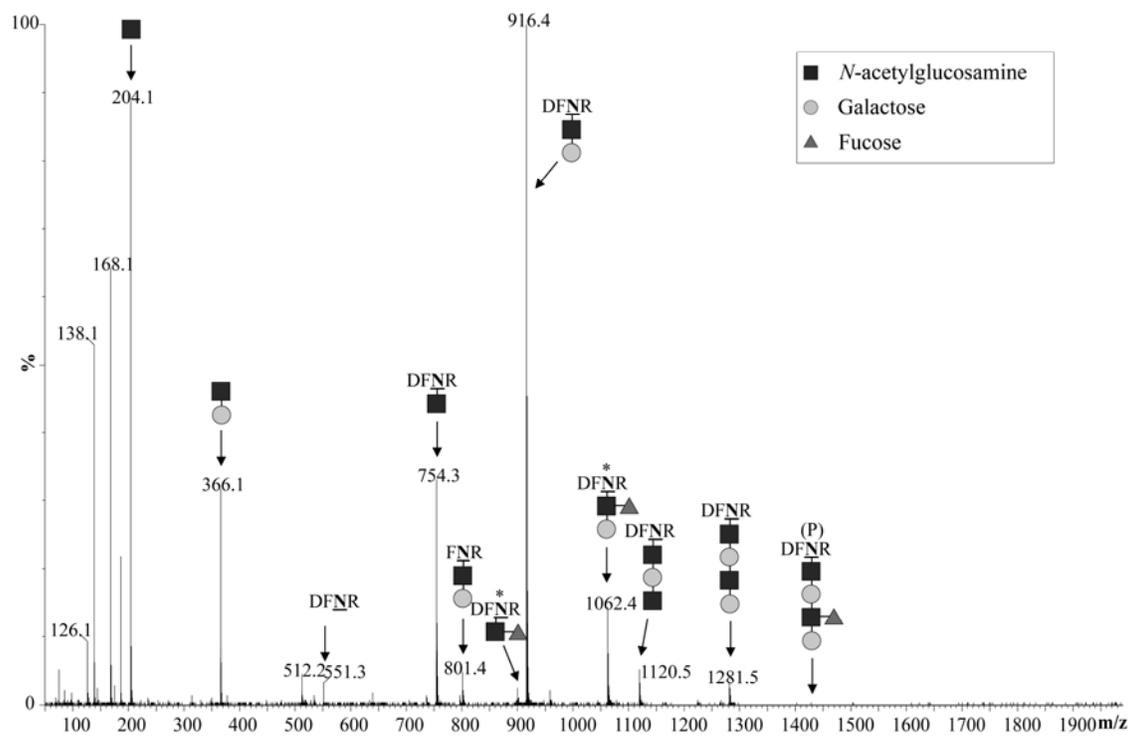


Figure S2

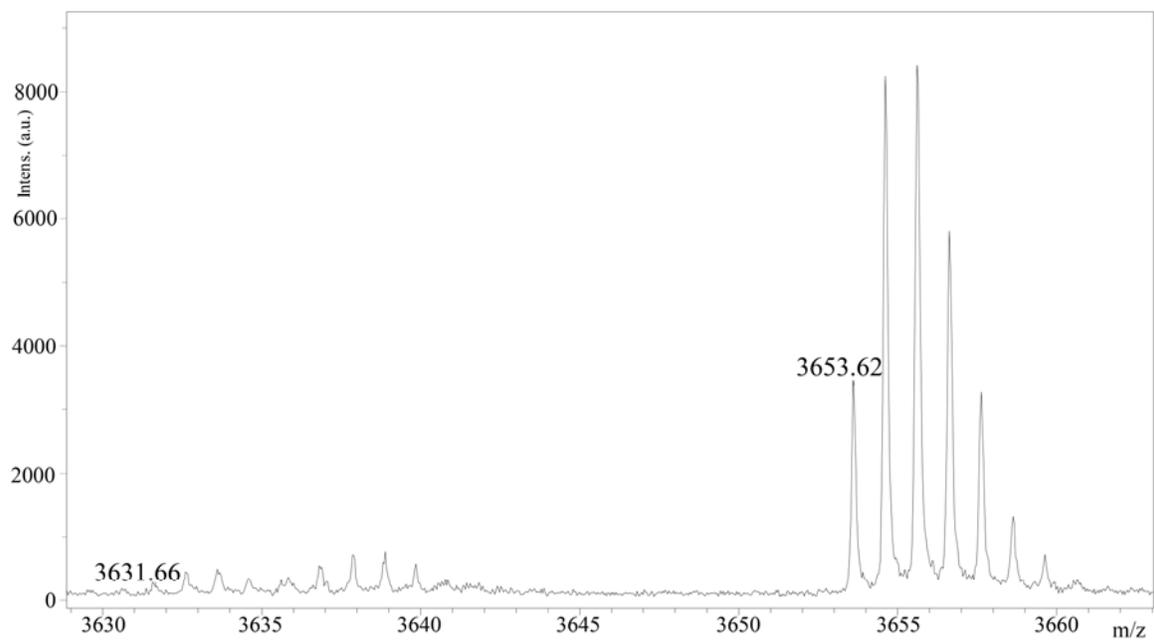


Figure S3

**Exploiting Bacterial Glycosylation Machineries for the Synthesis of a Lewis
Antigen-containing Glycoprotein**

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