N-Linked glycosylation is a frequent protein modification that occurs in all three domains of life. This process involves the transfer of a preassembled oligosaccharide from a lipid donor to asparagine side chains of polypeptides and is catalyzed by the membrane-bound oligosaccharyltransferase (OST). We characterized an alternative bacterial pathway wherein a cytoplasmic N-glycosyltransferase uses nucleotide-activated monosaccharides as donors to modify asparagine residues of peptides and proteins. N-Glycosyltransferase is an inverting glycosyltransferase and recognizes the NX(S/T) consensus sequence. It therefore exhibits similar acceptor site specificity as eukaryotic OST, despite the unrelated predicted structural architecture and the apparently different catalytic mechanism. The identification of an enzyme that integrates some of the features of OST in a cytoplasmic pathway defines a novel class of N-linked protein glycosylation found in pathogenic bacteria.

N-Linked glycosylation is characterized by an N-glycosidic linkage between the side chain amide of an asparagine residue of proteins and an oligosaccharide. This type of glycosylation occurs in both prokaryotes and eukaryotes and requires the assembly of an oligosaccharide on a polisoprenoid lipid by sequential addition of monosaccharides, catalyzed by cytosolic glycosyltransferase. The resulting lipid-linked oligosaccharide is translocated to the luminal side of the endoplasmic reticulum membrane or the plasma membrane of prokaryotes, where it may be further elongated. The glycan is then transferred to the $\delta$-amino group of asparagine residues within the consensus sequence NX(S/T) of polypeptides. This reaction is catalyzed by oligosaccharyltransferase (OST), a membrane-bound enzyme that can be composed of several different subunits (1). As oligosaccharide transfer takes place in the endoplasmic reticulum or in the periplasm, N-glycosylation affects proteins trafficking along the secretory pathway.

N-Glycosylation exhibits important physiological functions. In the early secretory pathway of eukaryotes, N-glycans present on newly synthesized proteins orchestrate the folding of glycoproteins and act as a signal for directing misfolded polypeptides to degradation (2). After being processed in the Golgi organelle, N-linked glycans are relevant, among other processes, for the modulation of the immune system and for the control of immune cell homeostasis and inflammation (3, 4). The importance of this protein modification is supported by its incidence: more than half of all eukaryotic proteins are glycosylated (5).

About 8 years ago, a study uncovered that the extracellular HMW1A adhesin of the Gram-negative bacterium *Haemophilus influenzae* is modified with hexose monosaccharides on asparagine residues (6). The pioneering work of St Geme and co-workers (7, 8) subsequently showed that the modified asparagine residues are found within the same consensus sequence recognized by OST (i.e. NX(S/T)) and that the enzyme responsible for this modification is the HMW1C protein, capable of transferring Glc or Gal from nucleotide-activated substrates. Moreover, it has been proposed that HMW1C also forms hexose-hexose bonds. Modification of HMW1A adhesin prevents its premature degradation and promotes HMW1A to be displayed at the cell surface, a prerequisite for HMW1A-mediated adherence to human epithelial cells (8). Additionally, a recent report demonstrated that an HMW1C homolog from *Actinobacillus pleuropneumoniae* mediates N-linked glycosylation of the *H. influenzae* HMW1A protein (9).

To characterize accurately the mechanisms of this alternative N-glycosylation pathway and to gain insight into the specificity of the key enzyme N-glycosyltransferase (NGT), we established a platform suitable for *in vitro* glycosylation and undertook a detailed analysis of the reaction products. We adopted MS and gel electrophoresis for detection of glycosyltransferase activity using peptides and proteins as substrates and performed NMR studies to characterize the reaction adducts. We show that the HMW1C homolog from *A. pleuropneumoniae* is an inverting NGT that transfers a glucose or galactose moiety to asparagine, but it does not further elongate the N-linked monosaccharide. Instead, we found that another glucosyltransferase was able to elaborate the N-linked glucose. We compared the acceptor substrate range of NGT and OST and observed a highly similar specificity of the two different
enzymes. We concluded that NGT integrates polypeptide recognition common to the OST-based modification into a novel framework, resulting in a general N-glycosylation system that operates in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Fermentas. T4 DNA ligase was from PerkinElmer Life Sciences. UDP-Glc, UDP-GlcNAc, and UDP-GalNAc were from Sigma. UDP-Gal was obtained from VWR International. Synthetic peptides were purchased from JPT Peptide Technologies. UDP-Gal was obtained from VWR International. Synthetic peptides were purchased from JPT Peptide Technologies.

**Construction of Plasmids**—Escherichia coli DH5α was chosen as host for cloning. The NGT and α1,6-glucosyltransferase (α6GlcT) genes were amplified by PCR using genomic DNA from Yersinia enterocolitica strain 8081, A. pleuropneumoniae strain L20, or A. pleuropneumoniae strain AP76 as a template. Fragments containing the NGT gene were cut with Xhol and ligated into pEC(AcrA-cyt), previously digested with NdeI, blunted by treatment with Klenow fragment, and digested with Xhol. The α6GlcT gene was cloned into the pEXT21 plasmid. All open reading frames are in-frame with a hexahistidine tag at the N terminus. All plasmid constructs were verified by sequencing of relevant fragments (Microsynth AG).

**Protein Expression, Purification, and Analysis**—E. coli DH5α cells harboring a plasmid for expression of a relevant protein were grown in volumes of 1 liter at 37 °C in LB medium. Ampicillin (100 mg/liter) or chloramphenicol (25 mg/liter) was recommended by the provider. Purification of polypeptide with trypsin (Promega) overnight at 37 °C. Peptides were bound to a C18 cartridge for removal of salts, eluted with a solution of 70% acetonitrile and 0.1% formic acid, and subjected to MS analysis.

**Glycosylation Analysis of Proteins**—MALDI-MS and MS/MS analyses of synthetic peptides were performed on a model 4800 proteomics analyzer (Applied Biosystems) operated in the positive reflectron mode. The peptides eluted from the C18 cartridge or the ZipTipC18 pipette tip were mixed 1:1 with α-cyano-4-hydroxycinnamic acid (5 mg/ml in 70% acetonitrile and 0.1% trifluoroacetic acid) as a matrix for spotting onto the target plate. Peptide mixtures from the AcrA glycosylation reaction were analyzed with an Eksigent nano-HPLC system using an autosampler with a self-made reverse-phase tip column (75 μm × 80 mm) packed with C18 material (AQ, 3 μm, 200 Å; Bischoff GmbH, Germany). The gradient consisted of 10%–30% acetonitrile in 0.2% formic acid at a flow rate of 250 nl/min for 60 min and 30–50% acetonitrile in 0.2% formic acid for 5 min. High accuracy mass spectra were acquired with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in the mass range of m/z 300–1700 and a target value of 1 × 10⁶ ions. Up to 20 data-dependent MS/MS spectra of the most intense ions with a charge state of +2 or higher were recorded in parallel at the ion trap using collision-induced dissociation.

**NMR Analysis**—Unless indicated otherwise, samples were lyophilized and dissolved in D₂O at 1 mM. All samples were measured at 303 K on a 500-MHz AVANCE III spectrometer equipped with an inverse triple-resonance cryogenic probe (Bruker). Standard two-dimensional ¹³C-¹H heteronuclear single-quantum coherence (HSQC), ¹³C-¹H heteronuclear multiple-bond correlation, and ¹H-¹H total correlation spectroscopy (TOCSY) (τmix = 13 and 120 ms) spectra were recorded to assign the peptide and glycan chemical shifts. In addition, a two-dimensional ¹³C-¹H relayed HMBC-COSY spectrum (2D, 38) was measured to assist the assignment process. To detect through-bond correlations between glycan protons and the asparagine HN21, a two-dimensional ¹H-¹H TOCSY spectrum was recorded in 95% H₂O and 5% D₂O with a mixing time of 80 ms. All spectra are referenced to 2,2-dimethyl-2-silapentane sulfonic acid. ¹³C chemical shifts are indirectly referenced using a scaling factor (Δ) of 0.251449530 (13). All spectra were processed with Topspin 2.1 (Bruker) and analyzed by Sparky (39).

**RESULTS**

* A. pleuropneumoniae and Y. enterocolitica HMW1C Homologs Modify a DANYTK Peptide—To gain a comprehensive view of potential NGT proteins, we searched the genome data base for bacteria encoding HMW1C homologs and iden-
glycan being separated from those of the peptide (Fig. 2).

Interestingly, many of the microorganisms that encode a putative NGT are pathogenic and cluster in the OCTOBER 7, 2011•VOLUME 286 • NUMBER 40

FIGURE 1. Modification of TAMRA-labeled peptides by putative glycosyltransferases. Reaction products were separated by Tricine/SDS-PAGE, and fluorescence signals were acquired by an image analyzer. Lanes 1 and 2 are negative controls, where no UDP-Glc (lane 1) or no enzyme (lane 2) was added to the reaction. ApL20, A. pleuropneumoniae strain L20; Ye, Y. enterocolitica strain 8081; Ap76, A. pleuropneumoniae strain AP76.

The enzyme transferred glucose or galactose, but not GlcNAc, to the DANYTK peptide (Fig. 2). The enzyme transferred glucose or galactose, but not GlcNAc, to the DANYTK peptide (Fig. 2). The enzyme transferred glucose or galactose, but not GlcNAc, to the DANYTK peptide (Fig. 2). The enzyme transferred glucose or galactose, but not GlcNAc, to the DANYTK peptide (Fig. 2).

We expressed the HMW1C homologs from Y. enterocolitica strain 8081, A. pleuropneumoniae strain L20, A. pleuropneumoniae strain AP76, and X. campestris ATCC 33913 in E. coli and purified them. To test the proteins for glycosyltransferase activity, we adapted an in vitro assay developed for analysis of OST activity (17). We incubated the purified proteins with UDP-Glc and the hexapeptide DANYTK labeled at the N terminus with the fluorescent dye TAMRA. After separation of the reaction products by Tricine/SDS-PAGE and detection of fluorescence signals, we observed that Y. enterocolitica and the two A. pleuropneumoniae homologs modified the TAMRA-labeled peptide, visualized by a shift in electrophoretic mobility (Fig. 1). By contrast, X. campestris OGTT did not exhibit glycosyltransferase activity for this acceptor peptide in the presence of UDP-Glc, UDP-Gal, UDP-GlcNAc, or UDP-GalNAc under the experimental conditions tested (supplemental Fig. 2). In the following, we focused our study on the A. pleuropneumoniae strain AP76 enzyme.

A. pleuropneumoniae HMW1C Homolog Is an Inverting N-Glycosyltransferase—To prove glycosylation of the peptide directly and to characterize the site and chemical structure of the modification, we analyzed the glycosylation products using NMR spectroscopy. The assignment of the peptide and glycan was achieved using through-bond short- and long-range couplings. The $^{13}$C-$^1$H HSQC spectrum, a fingerprint of the glycopeptide, showed $^{13}$C-$^1$H correlations, with the signals of the glycan being separated from those of the peptide (Fig. 2A). As is typical for a hexose, seven carbohydrate-specific signals were observed. The anomeric C-1 $^1$C chemical shift of 82 ppm was a strong indication of a linkage to nitrogen. Similar anomeric C-1 shifts have been found in bacterial and eukaryotic N-glycans (18–20), in sharp contrast to chemical shifts around 100 ppm typical for O-glycosidic linkages. A comparison of all observed $^1$H and $^13$C chemical shifts of the glycan with those obtained for published model compounds revealed a striking coincidence with those of Glc-$\beta$Asn (supplemental Table 1) (21). With a perfect match of all six $^13$C chemical shift values and the single reported $^1$H chemical shift (22), we identified the Glc-$\beta$Asn structure. To confirm the linkage, we measured a $^1H$-$^1H$ coupling constant of 9.9 Hz typical for a $\beta$-configuration and recorded a two-dimensional TOCSY spectrum in H$_2$O (Fig. 2B). Through-bond correlations between the asparagine side chain NH and glucose protons were clearly visible, with the asparagine side chain NH chemical shift at 8.88 ppm being similar to those reported for bacterial and eukaryotic N-glycans of 8.62–8.73 ppm (18, 23). Thus, we established that the A. pleuropneumoniae HMW1C homolog is an inverting NGT.

Next, we analyzed the donor specificity of this NGT in vitro. The enzyme transferred glucose or galactose, but not GlcNAc or GalNAc, to the DANYTK peptide (Fig. 2C). In the presence of a 100-fold molar ratio of donor to acceptor, the conversion to glycopeptide was quantitative in the presence of UDP-Glc, whereas it was marginal in the presence of UDP-Gal. Importantly, NGT glycosylated the peptide in the presence of EDTA, proving that glycosyl transfer does not require metal ions. We also monitored the products of the reaction by MS (Fig. 2D). Analysis of unmodified TAMRA-DANYTK resulted in two major species, matching with TAMRA-DANYTK (calculated molecular mass, 1122.19 Da; observed, 1122.49 Da) and a by-product, TAMRA-DANYTK$_2$ (calculated molecular mass, 1814.50 Da; observed, 1813.78 Da). After incubation with NGT and UDP-Glc, we detected species corresponding to TAMRA-DANYTK-Glc (calculated molecular mass, 1284.35 Da; observed, 1284.58 Da) and TAMRA-DANYTK$_2$-Glc (calculated molecular mass, 1975.66 Da; observed, 1975.88 Da). A similar result was obtained after incubation with UDP-Gal. In all cases, we observed addition of a single hexasine moiety to the asparagine residue.

A. pleuropneumoniae Encodes a Polymerizing Glucosyltransferase That Elongates N-Linked Glucose—We inspected the NGT-encoding genomic region of A. pleuropneumoniae strain AP76 (supplemental Fig. 3). This region contains genes encoding putative proteins involved in the uptake of mannitol and its conversion to glucosamine 6-phosphate, two isomerases, a nucleosidase, and a methylthiotransferase. Interestingly, the ORF next to the NGT gene encodes a putative glucosyltransferase (APP7_1696). We expressed a C-terminally tagged protein in E. coli. When we incubated the purified protein with the TAMRA-labeled product of the NGT reaction, we detected a mobility shift upon Tricine/SDS-PAGE indicative of elaboration of the glycopeptide (Fig. 3A). This modification occurred in the presence of UDP-Glc, but not in the presence of UDP-Gal, UDP-GlcNAc, or UDP-GalNAc. The glucosyltransferase activity appeared to be cation-independent (supplemental Fig. 4).
**FIGURE 2.** *A. pleuropneumoniae* NGT is an inverting glycosyltransferase with specificity for UDP-glucose. A, $^{13}$C-$^1$H correlations in an HSQC spectrum of TAMRA-DANYTK-Glc. Signals originating from the covalently linked glucose are shown in purple. The asterisk denotes signals from an impurity of the original peptide. B, $^1$H-$^1$H TOCSY spectrum recorded with a mixing time of 80 ms and four transients. C, Tricine/SDS-PAGE analysis of glycosylation products. TAMRA-labeled peptides were incubated with NGT and different UDP-monosaccharide donors in the presence or absence of EDTA. D, MALDI-MS analysis of the TAMRA-labeled peptide (upper panel) in the presence of NGT and UDP-glucose (middle panel) or UDP-galactose (lower panel).
We analyzed the reaction product by MS and found an addition of two glucose moieties in the presence of a 1:100 acceptor/donor ratio (Fig. 3B). Notably, we observed addition of up to six glucose units in the presence of an excess of the donor and with increasing amounts of glucosyltransferase (supplemental Fig. 5). MS/MS analysis of the precursor ions corresponding to glucosylated peptides (m/z = 1446.61, 1608.68, 1770.74, 1932.80, 2094.86, and 2256.93) gave fragmentation compatible to modified TAMRA-DANYTK (data not shown).

To determine the chemical structure and stereochemistry of the reaction product, we analyzed the glycopeptide by NMR spectroscopy. The 13C-1H HSQC spectrum displayed signals of three different glucose units (Fig. 3C). Two new signals appeared in the anomeric region at ~100 ppm, in addition to the previously observed signal at ~82 ppm originating from the \( \alpha \)-linked glucose (supplemental Fig. 6). The signals were assigned with a two-dimensional TOCSY spectrum (Fig. 3D) and 13C-1H long-range correlations via J couplings. The first set of signals belonging to the \( \alpha \)-linked glucose displayed a C6 chemical shift of 68.3 ppm that differed from the initial glycopeptide harboring a single glucose unit (C6, 63.3 ppm). This was indicative of a carbohydrate attachment at O6. The signals of a second glucose unit originated from a terminal glucose (C6, 63.2 ppm), whose chemical shifts coincided with those of Glc-1,6Glc. The third set of signals displayed chemical shifts of a bridging glucose unit that is 1,6-linked on either side. Chemical shifts calculated

![Diagram](image-url)

**FIGURE 3.** An \( \alpha \)6GlcT elongates \( \alpha \)-linked glucose. **A**, glucosylated TAMRA-labeled peptides were incubated with \( \alpha \)6GlcT and different UDP-monosaccharide donors and then separated by Tricine/SDS-PAGE. **B**, MALDI-MS analysis of glucosylated products showed that the glucosyltransferase added two units of glucose to the \( \alpha \)-glucose. **C**, HSQC spectrum showing 13C-1H correlations of TAMRA-DANYTK-Glc. Signals of three different glucose species are visible. Glc\(_a\), terminal glucose; Glc\(_b\), bridging glucose; Glc\(_a\), \( \alpha \)-linked glucose. **D**, 1H-1H TOCSY spectrum (120-ms mixing time, D\(_2\)O) allows identification of the different glucose species within the sample. The signal labeled with an asterisk might come from a second signal set for a bridging glucose different from Glc\(_b\).
FIGURE 4. Overlapping substrate specificity of NGT and OST. A, MALDI-MS analysis of the glycosylation products using model peptides in the presence of UDP-glucose without (left panels) or with (right panels) NGT. Upper panels, spectrum of the SIVNPGGSDLTYTIER peptide (calculated molecular mass, 1720.89 Da); middle panels, spectrum of the acetyl-SIVNPGGSDLTYTIER-amide peptide (calculated molecular mass, 1745.87 Da); lower panels, spectrum of the acetyl-SIVNPGGSDLTYTIER-amide peptide (calculated molecular mass, 1775.97 Da). B, list of synthetic peptides that are glycosylated by NGT. The modified asparagine residues are highlighted in black. The amino acids in position +2 are shown in gray. The peptide sequences derive from the yeast glycoproteins identified by the UniProt entry names indicated on the right. C, LC-electrospray ionization-MS/MS analysis of the tryptic products of AcrA. Left panel, the spectrum from fragmentation of the doubly charged precursor ion at \( m/z \) 738.34 corresponds to the peptide EYDSSLATFNNSK. Right panel, the spectrum from fragmentation of the doubly charged ion at \( m/z \) 819.36 matches the glycopeptide EYDSSLATFN(Glc)NSK.
with the algorithm CASPER (26) further supported the assignment. We concluded that the APP7_1697 gene encodes a polymerizing α6GlcT that elaborates the product of the NGT reaction.

NGT Exhibits Acceptor Site Specificity Overlapping OST—We analyzed glycosylation of the SIVPGGSNLTYIER peptide present in the yeast glycoprotein lysophospholipase 2 (Pib2). After incubation with NGT and UDP-Glc, analysis of the reaction products by MS indicated that the peptide was modified (Fig. 4A, upper panels). The fragmentation spectrum of the ion at m/z 1882.93 was consistent with glucosylation of the asparagine within the NLT site (supplemental Fig. 7). Importantly, alteration of the NLT sequon to QLT or NPT abolished glycosylation (Fig. 4A), demonstrating a similar property of NGT as for OST with respect to acceptor sequon specificity (27).

We extended our analysis and examined glycosylation of a group of model peptides with the NX(S/T) consensus sequence in different positions (N terminus, central, C terminus). We chose sequences found to be glycosylated in yeast glycoproteins (Fig. 4B). Glycosylation at the NX(S/T) site was observed for all peptides (supplemental Fig. 8), indicating that the NX(S/T) consensus sequence was recognized by NGT. Although our analysis by MS/MS was not quantitative, the position of the consensus sequence within the peptide did not seem to affect glycosylation, and we did not observe a sequence preference for Ser or Thr at position +2. Importantly, we did not detect glycosylation of short peptides such as DQNAT and DFNV (data not shown), known substrates identified in vitro for bacterial OST from Campylobacter jejuni (28).

To further probe the acceptor site specificity of NGT, we tested glycosylation of the AcrA protein from C. jejuni, a substrate of bacterial and eukaryotic OSTs (29). MS analysis revealed that NGT was able to modify all four glycosites present in AcrA with a glucose moiety (Fig. 4C and supplemental Fig. 9). Altogether, these experiments proved that NGT and OST share the basis for recognition of acceptor substrates. Moreover, glycosylation of AcrA demonstrated that NGT operates on folded proteins.

**DISCUSSION**

In this work, we have provided direct proof that the soluble enzyme NGT glycosylates asparagine side chains of peptides and proteins. The modified asparagines are found within the NX(S/T) sequence. Proline is not tolerated in the X position, and asparagine cannot be replaced by glutamine. Any amino acid is allowed in positions next to the sequon. NGT shows preference for long polypeptides, indicating that binding of the substrate to the enzyme might require an extended contact surface. At the same time, modification of different NX(S/T) sequences on one protein suggested that NGT does not recognize a specific target but acts as a key component of a general protein glycosylation system.

NGT transfers one glucose unit from UDP-galactose to acceptor sites, forming β-glycosidic linkages in a metal ion-independent manner. UDP-galactose appears to be accepted as a substrate donor, albeit with low efficiency.

These features mark a clear distinction from the conventional N-glycosylation, wherein OST is localized in a membrane, uses a complex lipid-bound oligosaccharide as a donor, requires a metal ion for catalysis, and exclusively modifies proteins trafficking along the secretory pathway. Moreover, the two enzymes are structurally unrelated, as NGT is predicted to have a GT-B fold, whereas OST clusters in the GT-C group (30). Given these premises, it is remarkable that NGT and OST exhibit the same NX(S/T) acceptor sequence specificity. The amino acid at position +2 appears not be essential for catalysis by OST, but is rather involved in binding of the acceptor substrate by the enzyme (31). Indeed, both NGT and OST can glycosylate sites that do not contain Ser or Thr at position +2 (7, 32–34). It will be important to determine the substrate recognition mode of NGT, the biophysical properties of the consensus sequence, and the mechanisms that selected this sequon for general glycosylation systems in two convergent evolutionary processes. In this context, it is interesting to note that the glycoproteins produced by the cytoplasmic glycosylation system of H. influenzae are secreted proteins.

Cytoplasmic N-glycosylation does not appear to be a single-monosaccharide modification, as the N-glucose can be extended with α1,6-linked glucose in A. pleuropneumoniae. The enzyme responsible for glucose transfer is considered a distant relative of family GT4 glycosyltransferases based on sequence similarity. Remarkably, whereas we confirmed that the enzyme exhibits a retaining mechanism, its polymerase

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5 A. Nägeli, F. Schwarz, Y.-Y. Fan, and M. Aebi, unpublished data.
activity appears to be a novel feature of a member of this class of glycosyltransferases.

In summary, this study illustrates a cytoplasmic pathway of N-linked protein glycosylation (Fig. 5) with overlapping properties with the conventional N-glycosylation process. Future research will have to address the mechanism, extent, and physiological roles of this protein modification. Furthermore, as NGTs are encoded on the genome of relevant pathogens, it will be interesting to study how the immune system of the hosts reacts to this type of N-glycosylation. Finally, NGTs represent a promising tool that complements established platforms for production of glycoconjugates and for site-specific modification of proteins (35–37).

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