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Functional Characterization of Bacterial Oligosaccharyltransferases Involved in O-Linked Protein Glycosylation▽

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Protein glycosylation is an important posttranslational modification that occurs in all domains of life. Pilins, the structural components of type IV pili, are O glycosylated in *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and some strains of *Pseudomonas aeruginosa*. In this work, we characterized the *P. aeruginosa* 1244 and *N. meningitidis* MC58 O glycosylation systems in *Escherichia coli*. In both cases, sugars are transferred en bloc by an oligosaccharyltransferase (OTase) named PglL in *N. meningitidis* and PilO in *P. aeruginosa*. We show that, like PilO, PglL has relaxed glycan specificity. Both OTases are sufficient for glycosylation, but they require translocation of the undecaprenol-pyrophosphate-linked oligosaccharide substrates into the periplasm for activity. Whereas PilO activity is restricted to short oligosaccharides, PglL is able to transfer diverse oligo- and polysaccharides. This functional characterization supports the concept that despite their low sequence similarity, PilO and PglL belong to a new family of "O-OTases" that transfer oligosaccharides from lipid carriers to hydroxylated amino acids in proteins. To date, such activity has not been identified for eukaryotes. To our knowledge, this is the first report describing recombinant O glycoproteins synthesized in *E. coli*.

Protein glycosylation is a common posttranslational modification in bacteria. Glycans are often used to decorate proteins at the bacterial surface or components of appendages, such as flagella and pili (38). Protein glycosylation has been described for both gram-negative and -positive bacteria. Although in most cases, the precise functions of the glycans attached to bacterial proteins have not been determined, glycoproteins may play roles in adhesion, stabilization of the proteins against proteolysis, and evasion of the host immune response (38). Glycans are generally attached to serine or threonine (O glycosylation) or to asparagine (N glycosylation) residues. Two different mechanisms for protein glycosylation can be distinguished by the mode in which the glycans are transferred to proteins. The first mechanism involves the transfer of carbohydrates directly from nucleotide-activated sugars to acceptor proteins. Examples of pathways using this mechanism are protein O glycosylation in the Golgi apparatus in eukaryotic cells (28) and flagellin O glycosylation in several bacterial species (24). In the second mechanism, an oligosaccharide is preassembled onto a lipid carrier before being transferred en bloc to protein acceptors by an oligosaccharyltransferase (OTase). This mechanism has been described for N glycosylation in the endoplasmic reticulum of eukaryotic cells (17) and in the general N glycosylation system of *Campylobacter jejuni* (45). To date, the *C. jejuni* system is the only well-characterized bacterial N glycosylation system. O glycosylation has been found in several bacterial species. In *Neisseria meningitidis* (commonly known as meningococcus [MC]), *Neisseria gonorrhoeae* (also known as gonnococcus [GC]), and some *Pseudomonas aeruginosa* strains, type IV pilins (structural components of type IV pili) are O glycosylated (6, 42). Pili are filamentous polymeric appendages that protrude from the bacterial surface and play a key role in bacterial virulence. Although the presence of glycosylated pilins has been known for many years, the mechanisms of glycosylation are just starting to be understood.

P. aeruginosa is an opportunistic pathogen that causes local infections in cystic fibrosis and immunocompromised patients. Pioneering work from Castric showed that the pilins are O glycosylated in *P. aeruginosa* 1244 (6). PilO is the enzyme responsible for the transfer of the glycan from its undecaprenol-pyrophosphate (Und-PP) carrier to the C-terminal serine 148 residue of *P. aeruginosa* 1244 pilin (6, 8). This O-linked glycan [α-5NβOHC(4)7NFmPse-(2→4)β-Xyl-(1→3)-β-FucNAc] is a product of the O antigen biosynthetic pathway and has the same structure as the O antigen of lipopolysaccharide (LPS) (7, 9). PilO exhibits relaxed glycan specificity, as different O antigens could be attached to pilin by heterologously expressing PilO in nonglycosylating *P. aeruginosa* strains (9). It has also been shown that PilO glycan recognition features lie within the reducing-end moiety of the carbohydrate chain (18). Recently, glycosylated pilins have also been found in other *P. aeruginosa* strains (21, 43). Interestingly, in *P. aeruginosa* 5196, the pilin glycan is not a by-product of O antigen biosynthesis; instead, it was determined to be a homo-oligomer of α-1,5-linked D-arabinofuranose (43). This structure has been found previously in the arabinogalactan and lipoarabinomannan of mycobacteria (5). The machinery responsible for pilin glycosylation in *P. aeruginosa* 5196 has not yet been identified. It has been suggested that pilin glycosylation may play a role in *P. aeruginosa* infection and colonization in cystic fibrosis patients (21, 35).

Pilus-mediated adhesion is essential for the virulence of both

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GC and MC (4). In GC, the causative agent of gonorrhea, pilin O glycosylation occurs at an internal serine residue (Ser63 of the mature protein). MC is a gram-negative bacterium best known for its role in meningitis. The exact glycosylation site for MC pilin remains to be identified, although it appears to be located at a serine or threonine between residues 50 and 73. The carbohydrate found attached to MC pilin is the trisaccharide β-D-Gal*p*(1→4)-α-D-Gal*p*(1→3)-DATDH, where DATDH is a 2,4-diacetimido-2,4,6-trideoxyhexopyranose (37). It has been proposed that this glycan may be important in evading the host immune system and in increasing pilin solubility (25, 30). The pathway leading to pilin glycosylation in *Neisseria* has not been fully characterized. Power et al. showed that mutagenesis of an open reading frame (ORF) named *pglL* in MC resulted in pilin with reduced electrophoretic mobility (31). More recently, Aas and coworkers characterized a set of GC mutants and defined the roles of several components of the glycosylation pathway (1). Mutagenesis of the *pglO* gene, which presents 95% identity to MC *pglL*, abolished pilin glycosylation in GC. Both studies proposed that PglL and PglO are the OTases responsible for pilin glycosylation in their respective systems. However, the OTase activity of these proteins has hitherto not been demonstrated.

The N glycosylation system of *C. jejuni* has been functionally transferred to *Escherichia coli*. The accessibility to powerful genetic tools and the panoply of mutants available in *E. coli* facilitated the characterization of the N glycosylation pathway and the enzymes involved, leading to the establishment of the basis for exploitation of the system for the synthesis of recombinant glycoproteins (3, 12, 20, 44, 45). In this work, we functionally characterized two bacterial OTases, PilO from *P. aeruginosa* 1244 and PglL from MC strain MC58, in *E. coli*. Both enzymes were expressed in the presence of their respective pilin substrates and diverse Und-PP-glycans as sugar donors. PglL-dependent pilin glycosylation was detected in *E. coli* cells, in the absence of any other neisserial protein, demonstrating that PglL possesses OTase activity. Furthermore, PglL has relaxed glycan specificity, as it was able to transfer diverse exogenous oligo- and polysaccharides to pilin. Additionally, the glycan acceptor site in MC pilin was determined. We also showed that translocation of the Und-PP-glycan to the periplasm is required for pilin glycosylation for PglL and PilO, indicating that the activity of both OTases is located at the bacterial periplasm.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* 1244 cells were grown on Luria-Bertani medium at 37°C. Trimethoprim (100 μg/ml), tetracycline (20 μg/ml), spectinomycin (80 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and ampicillin (100 μg/ml) were included in the medium when required.

Subcloning and expression of *P. aeruginosa* 1244 pilin and PilO. The *pilA* gene encoding the *P. aeruginosa* 1244 pilin protein was amplified by PCR with oligonucleotides PilAEcoRI (5′-AAGAATTCATGAAAGCTCAGAAGGGTTTTA C-3′) and PilABamHI (5′-AAGGATCCTTAGGATTTCGGGCAATTAGC-3′) by using *Pfu* DNA polymerase and pPAC46 (Table 1) as the template. The PCR product was cut with EcoRI and BamHI and inserted into the same sites of vector pEXT20 to create pAMF1. PilA expression was under the control of the P_{tac} promoter, inducible with IPTG (isopropyl-β-D-thiogalactopyranoside). PilA expression was detected by Western blotting as explained below. To obtain a plasmid expressing *pilO* under an arabinose-inducible promoter, pPAC46 was cut with PsuI and HindIII. The DNA fragment carrying the complete sequence of *pilO* and a 3′ fraction of *pilA* was ligated into the BamHI and HindIII sites of pSPORT1, resulting in the plasmid pDOM2. pDOM2 was cut with KpnI and HindIII, and the fragment was inserted into the same sites of vector pMLBAD to yield pDOM3 (Table 1).

Cloning and expression of MC pilin and PglL. The *pilE* gene encoding the pilin protein was amplified from the genomic DNA of MC strain MC58 by using *Pfu* DNA polymerase and oligonucleotides PilEEcoRI (5′-AAAGAATTCATG AACACCCTTCAAAAAGGTTTTACCCTTATCGAGC-3′) and PilEHindIII (5′-TTTAAGCTTTTAGCTGGCATCACTTGCGTCGCGGCAGGTTGACG-3′). The PCR product was cut with EcoRI and HindIII and cloned into the same sites of pEXT20 and pEXT21 to construct pAMF3 and pAMF6, respectively. The *pglL* gene was amplified by PCR with oligonucleotides PglLEcoRI (5′-AAAGAAT TCATGCCCGCTGAAACGACCGTATCCGGCGCGC) and PglLHindIII-His (5′-TTTAAGCTTTCAGTGGTGGTGGTGGTGGTGGTGGTGGTGTT TGCAGGGTTTTGCTTCCGGATGACCGGGC-3′) by using Vent DNA polymerase (New England Biolabs) with MC strain MC58 as the template. PglL carries a His_{10} tag at the C terminus. The PCR product was cut with EcoRI and HindIII and inserted into the same site of pSPORT1 to produce pAMF4. pAMF4 was cut with EcoRI and HindIII, and the fragment containing the *pglL* gene was ligated into the same site of pEXT22 to create pAMF5. pAMF14 was constructed as follows: C-His_6-tagged MC pilin was amplified with pAMF3 as the template by using *Pfu* DNA polymerase and oligonucleotides PilEEcoRI and PilESalI-His (5′-AATCCAGTCGACTTAGTGGTGGTGGTGGTGGTGGCTG GCATCACTTGCGTCGCGGCAGGTTGACG-3′). The PCR product was cut with EcoRI and SalI and inserted into the same sites of pEXT21 to construct pAMF14. All of the plasmids were IPTG inducible.

Western blot analysis. Western blot analyses were carried out as described previously (2). The presence of proteins on nitrocellulose membranes was detected with diverse antibodies and/or lectins. Polyclonal anti-*P. aeruginosa* pilin was kindly provided by Peter Castric (Duquesne University, Pittsburgh, PA). Monoclonal SM1 (41) antibody against MC pilin was provided by Momtaz Virji (University of Bristol, United Kingdom). R12 antiserum was provided by Markus Aebi (ETH, Zurich, Switzerland).

Site-directed mutagenesis. PilE mutants T62A and S63A were generated by using *Pfu* DNA polymerase and pAMF3 as a template according to the method described previously by Fisher and Pei (14). Mutagenesis was confirmed by DNA sequencing. Oligonucleotide sequences used for mutagenesis were 5′-CCCGG CAACAACGCCTCTGCCGGCG-3′ for T62A and T62A_S, 5′-CGCCGGCAG AGGCGTTGTTGCCGGG-3′ for T62A_As, 5′-CCCGGCAACAACACTGCC GCCGGCGTG-3′ for S63A and S63A_S, and (5′-CACGCCGGCGGCAGTG TTGTTGCCGGG-3′ for S63A_As. The mutated codons are underlined in the oligonucleotide sequences. The expression of both mutants was confirmed by Western blot analysis using SM1 monoclonal antibody.

LPS preparation and detection. Whole-cell lysates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer were digested overnight with proteinase K at 50°C. A protein-free sample was applied to 15% SDS-PAGE and electroblotted onto a nitrocellulose membrane. A Far Western blotting technique using STL3 lectin (recognizing rhamnose), anti-STL3 polyclonal antibody, and anti-rabbit antibody conjugated with peroxidase was employed to detect O7 LPS variants. STL3 lectin was kindly provided by Koji Muramoto (Tohoku University, Sendai, Japan) (39).

Purification of glycosylated pilin. Pilin from the MC58 strain (encoded by the *pilE* gene) glycosylated with the *C. jejuni* glycan was produced in *E. coli* SCM3 transformed with pAMF5, pAMF14 (expressing C-terminally His_6-tagged *pilE*, Table 1), and pACYC$pglB_{mut}$. IPTG (0.5 mM) was added to the cultures, and cells were harvested at stationary phase. Pellets were washed with 30 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl (buffer 1) and resuspended in the same buffer containing Complete EDTA-free protease inhibitor cocktail (Roche). Cells were disrupted by French press and centrifuged at 10,000 × *g* for 10 min to remove cell debris. Membranes were separated by ultracentrifugation (200,000 × *g* for 2 h) and resuspended in buffer 1 containing 2% *n*-dodecyl-β-D-maltoside (buffer 2). The suspension was centrifuged (200,000 × *g* for 1 h), and then imidazole was added to the supernatant at a final concentration of 20 mM. The solution was applied to a Ni-nitrilotriacetic acid agarose column (QIAGEN) equilibrated previously with buffer 2 (containing 20 mM imidazole) and washed with the same buffer to remove unbound proteins. The bound proteins were eluted from the column by using buffer 2 (containing 250 mM imidazole). The eluate was dialyzed overnight at 4°C in 50 mM Tris-HCl, pH 8.5, containing 10 mM NaCl, 1 mM dithiothreitol (DTT), and 0.8% *n*-dodecyl-β-D-maltoside (buffer 3). Protein solutions were applied to an soybean agglutinin (SBA)-agarose column (Vector Labs) equilibrated by buffer 3. Unbound proteins were removed by washing the column with buffer 3, and proteins were eluted with

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
E. coli		
W3110	IN(*rrnD-rrnE*)1 *rph-1*	12
DH5α	F$^-$ φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *gal phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
CLM24	W3110 lacking WaaL ligase	12
SΦ874	*lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL*	27
SCM3	SΦ874 Δ*waaL*	Valvano et al., unpublished data
SCM7	SΦ874 Δ*wec*	3
P. aeruginosa		
1244	Wild-type, serotype O7	6
1244 *pilO*	1244 lacking PilO	6
Plasmids		
pSPORT1	Cloning vector, Amp^r	Invitrogen
pMLBAD	Cloning and expression vector, arabinose inducible, Tp^r	22
pEXT20	Cloning and expression vector, IPTG inducible, Amp^r	10
pEXT21	Cloning and expression vector, IPTG inducible, Sp^r	10
pEXT22	Cloning and expression vector, IPTG inducible, Km^r	10
pPAC46	Encodes *P. aeruginosa* 1244 *pilA-pilO* operon, Amp^r	6
pACYC*pgl*	Carries the *C. jejuni* protein glycosylation locus (*pgl*) cluster, Cm^r	45
pACYC$pglB_{mut}$	Carries *C. jejuni pgl* containing mutations W458A and D459A in PglB, Cm^r	45
pACYC$pglK_{mut}$	Carries *C. jejuni pgl* containing a Km^r cassette in *pglK*, Cm^r and Km^r	3
pJHCV32	Encodes the O7 antigen cluster from *E. coli*, Tet^r	26
pJHCV32::Tn3HoHo1–134	Encodes the O7 antigen cluster from *E. coli* carrying a transposon in *wzy*, Tet^r Amp^r	26
pJHCV32::Tn3HoHo1–136	Encodes the O7 antigen cluster from *E. coli* carrying a transposon in *wzz*, Tet^r Amp^r	26
pCW27	*pglK* in pMLBAD/Myc-His_6, Tp^r	3
pDOM2	*pilO* subcloned in pSPORT1, Amp^r	This study
pDOM3	*pilO* subcloned in pMLBAD, Tp^r	This study
pAMF1	*pilA* subcloned in pEXT20, Amp^r	This study
pAMF3	*pilE* cloned in pEXT20, Amp^r	This study
pAMF4	His_{10}-tagged *pglL* cloned in pSPORT1, Amp^r	This study
pAMF5	His_{10}-tagged *pglL* cloned in pEXT22, Km^r	This study
pAMF6	*pilE* cloned in pEXT21, Sp^r	This study
pAMF14	His_6-tagged *pilE* cloned in pEXT21, Sp^r	This study

buffer 3 containing 0.5 M D-galactose. Protein fractions were collected and kept at −20°C. *P. aeruginosa* 1244 pilin (PilA) glycosylated with *C. jejuni* glycan was produced by *E. coli* CLM24 transformed with pPAC46 and pACYC$pglB_{mut}$. Glycosylated *P. aeruginosa* 1244 pilin was purified using SBA agarose as described above.

In-gel pilin digestion. In-gel digestion of pilins was carried out by following a protocol of Shevchenko et al. (34) with slight modifications. In brief, purified glycosylated pilins were run on a 15% SDS-PAGE gel and stained with Coomassie blue. Protein bands corresponding to glycosylated pilins were cut out. Gel pieces were washed with water and repetitively dehydrated and rehydrated by incubation with acetonitrile (100%) and acetonitrile-water 1:1 (vol/vol). Pilins were reduced by the addition of 10 mM DTT in 50 mM ammonium bicarbonate, and thiol groups were alkylated with 50 mM iodoacetamide in 50 mM ammonium bicarbonate. Subsequently, pilins were digested with different proteases, proteinase K (Roche), thermolysin (Calbiochem), or modified trypsin (Promega), as indicated. Peptides eluted from the gel pieces were desalted by ZipTip$_{C18}$ (Millipore) according to the protocol of the supplier. Desalted peptides were dried by SpeedVac, dissolved in 0.1% formic acid in water, and used for mass spectrometry (MS) analyses.

Nano-liquid chromatography-electrospray ionization MS and MS/MS analysis. Peptides obtained from protease hydrolysis were analyzed using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Q-TOF Premier (Waters), equipped with a nanoACQUITY Ultra performance liquid chromatography system (Waters). Briefly, 2 μl of the peptide solution was injected into a microprecolumn C_{18} cartridge that was connected to a 75-μm (inside diameter) by 150-mm Atlantis dC_{18} column (Waters). Solvent A consisted of 0.1% formic acid and 1% acetonitrile in water, and solvent B consisted of 0.1% formic acid and 1% water in acetonitrile. After a 2-min trap wash in the precolumn with solvent A at a flow rate of 10 μl/min, peptides were separated by using a solvent gradient and electrosprayed into the mass spectrometer at a flow rate of 300 nl/min. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide ions. The instrument was calibrated, and data acquisition was performed as described previously (46). The data analysis was carried out using MassLynx (Waters; MassLynx, version 4.1).

RESULTS

Functional expression of PilO in *E. coli*. To test the functionality of PilO from *P. aeruginosa* 1244 in *E. coli*, we expressed this enzyme in the presence of its cognate pilin and an Und-PP-glycan substrate in the CLM24 strain (Table 1). Like most of the *E. coli* K-12 strains, CLM24 does not synthesize its endogenous O antigen because of an inactivating insertion in a rhamnosyltransferase (23). Furthermore, the WaaL ligase responsible for the transfer of the O antigen to the lipid A-core has been inactivated in this strain (12). CLM24 is a convenient system for the assembly of foreign glycans because the absence of WaaL results in the accumulation of the Und-PP-linked

glycans (12). DiGiandomenico et al. have shown that PilO possesses relaxed sugar specificity, being able to transfer a variety of glycans to *P. aeruginosa* 1244 pilin (9). In this experiment, the heptasaccharide found commonly in *C. jejuni* N glycoproteins (GalNAc-α1,4-GalNAc-α1,4-[Glcβ1,3-]GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac where Bac is bacillosamine) was chosen because the enzymes required for its synthesis are all contained in a single genetic locus named *pgl* (45). Furthermore, the presence of the *C. jejuni* glycan attached to proteins can be detected by the well-characterized glycan-specific antiserum R12 (3, 19, 20, 45) and/or SBA lectin. The plasmid pACYC*pglB*$_{mut}$ carries the *pgl* locus, which carries a mutation inactivating PglB, the OTase involved in protein N glycosylation in *C. jejuni* (45). Whole-cell extracts from CLM24 transformed with pPAC46, which carries the *P. aeruginosa* pilin and the PilO enzyme, in the presence and absence of pACYC*pglB*$_{mut}$ were analyzed by Western blotting using anti-pilin antiserum (Fig. 1A). Extracts from *P. aeruginosa* 1244 and its *pilO*-deficient derivative were included as positive and negative controls, respectively (6). Because pilin and LPS contain the same sugars in *P. aeruginosa*, the anti-pilin antiserum also reacted with *P. aeruginosa* LPS (Fig. 1A, lanes 1 and 2). In *E. coli* cells carrying only *P. aeruginosa* pilin and PilO, two immunoreactive bands were detected with anti-pilin antiserum. The lower band showed the same mobility as mature pilin from the *pilO* mutant (Fig. 1A, lanes 2 and 3). Finlay et al. have shown that *P. aeruginosa* pilin expressed in *E. coli* is not processed completely and that the protein present is associated mainly to the inner membrane with and without its six-amino-acid leader sequence (13). Based on this finding, we assumed that the upper band corresponded to prepilin containing its signal peptide. Pilin from cells carrying *P. aeruginosa* pilin, PilO, and pACYC*pglB*$_{mut}$ showed an additional band of lower electrophoretic mobility (Fig. 1A, lane 4). The same band was recognized by the glycan-specific antiserum R12 (19, 45) and SBA lectin, which recognizes GalNAc, the main component of the *C. jejuni* heptasaccharide (Fig. 1B), suggesting that in *E. coli*, *P. aeruginosa* pilin could be glycosylated with the *C. jejuni* glycan.

To verify that glycosylation was PilO dependent, plasmids pDOM3 (expressing *pilO* under an arabinose-inducible promoter), pAMF1 (expressing *P. aeruginosa* pilin), and pACYC*pglB*$_{mut}$ (Table 1) were transformed into CLM24 cells and pilin glycosylation was analyzed by Western blotting. As a negative control, an extract of cells carrying the empty vector pMLBAD instead of pDOM3 was also analyzed. An arabinose-dependent band, also reacting with the *C. jejuni* glycan-specific antiserum R12, was detected only in the presence of PilO (Fig. 1C).

To define the glycan structure and glycosylation site of the *P. aeruginosa* pilin, we purified *P. aeruginosa* pilin from *E. coli* cells coexpressing PilO and carrying pACYC*pglB*$_{mut}$ by affinity chromatography using SBA agarose. The purified protein was reduced by incubation with DTT, alkylated with iodoacetamide, and digested in gel with proteinase K. The resulting peptides were analyzed by MS. Two peaks matching the predicted doubly charged peptide ^{144}NCPKS^{148} (modified with acrylamide) containing the glycan DATDH(HexNAc)$_5$Hex (m/z 1,012.8^{2+}) or (HexNAc)$_6$Hex (m/z 1,000.3^{2+}) were identified in the MS spectra (data not shown). These glycopeptides were subjected to collision-induced dissociation (MS/MS). The MS/MS data, presented in Fig. 2, confirmed the identity of these glycopeptides, which correspond to the *C. jejuni* glycan attached to the *P. aeruginosa* pilin peptide ^{144}NCPKS^{148} containing either DATDH or HexNAc at the reducing end. This

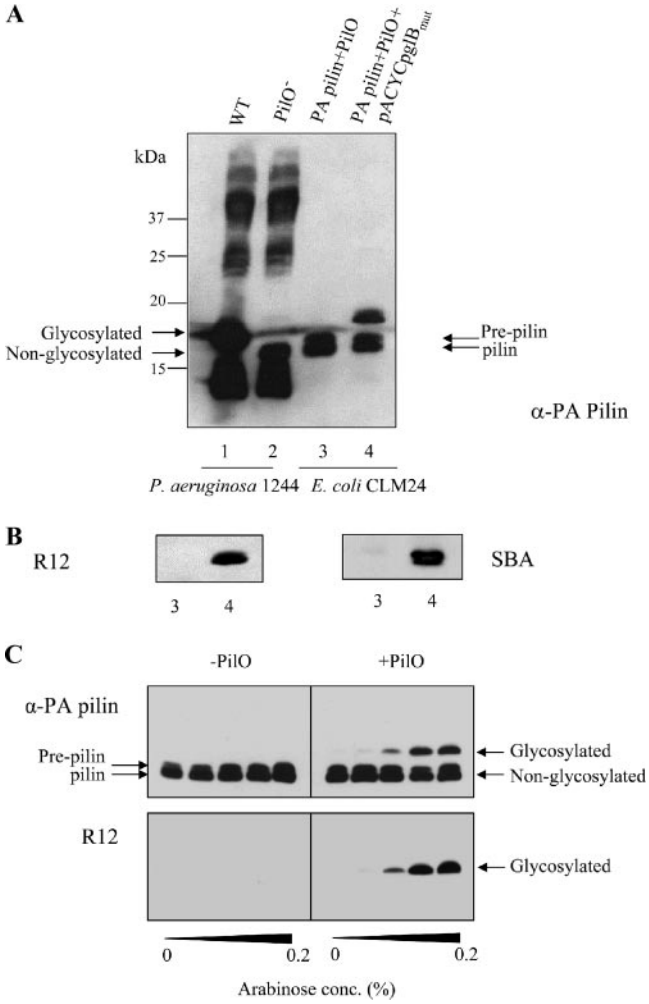

FIG. 1. Reconstitution of *P. aeruginosa* 1244 O glycosylation system in *E. coli*. (A) Western blot assay of whole-cell extracts containing unglycosylated and glycosylated *P. aeruginosa* (PA) 1244 pilin. Pilin was detected by using polyclonal anti-pilin antibody. Lanes: 1, *P. aeruginosa* 1244 expressing glycosylated pilin containing its endogenous trisaccharide; 2, *P. aeruginosa* 1244 *pilO* derivative expressing unglycosylated pilin; 3, *E. coli* CLM24 with pPAC46 (containing *pilA-pilO*); 4, *E. coli* CLM24 with pPAC46 and pACYC*pglB*$_{mut}$, which carries all the enzymes required for the assembly of the *C. jejuni* glycan except for PglB. WT, wild type. (B) Glycosylated *P. aeruginosa* pilin is recognized by the *C. jejuni* glycan-specific antiserum R12 (left panel) and the SBA lectin (right panel). SBA binds to GalNAc, the most abundant sugar of the *C. jejuni* glycan. Only lanes 3 and 4 of panel A show results of probes with antibody. (C) Whole-cell lysates of *E. coli* CLM24 transformed with pACYC*pglB*$_{mut}$, pAMF1 (expressing *P. aeruginosa* 1244 pilin), and pDOM3 (expressing PilO under an arabinose-dependent promoter) were analyzed by Western blotting (right panels). Extracts of cells carrying pMLBAD (empty vector) instead of pDOM3 were included as a negative control (left panels). PilA was detected by using anti-pilin polyclonal antibody (upper panels), and glycosylated pilin was detected by R12 antibody (lower panels). The arabinose concentrations (conc.) used were 0, 0.0002, 0.002, 0.02, and 0.2%. Plasmids are described in Table 1.

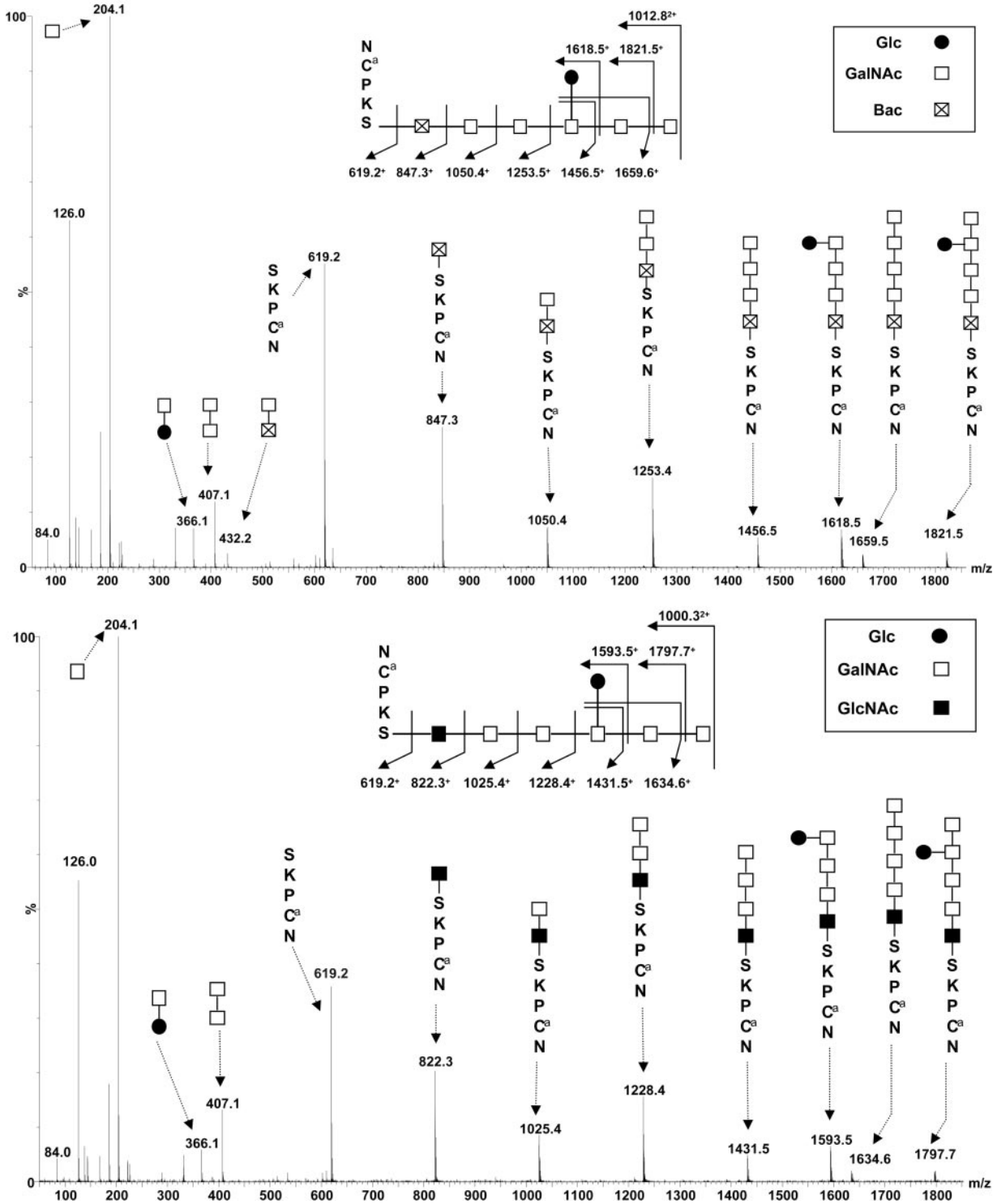

FIG. 2. MS/MS analysis shows glycosylation of recombinant *P. aeruginosa* pilin with the *C. jejuni* glycan at Ser148 in *E. coli*. MS/MS analysis of two glycopeptides matching the predicted doubly charged peptide ^{144}NCPKS^{148} modified with acrylamide and containing the glycan DATDH $(HexNAc)_5Hex$ (*m/z* $1{,}012.8^{2+}$) (upper panel) or $(HexNAc)_6Hex$ (*m/z* $1{,}000.3^{2+}$) (lower panel). The fragmentation patterns of the glycopeptides are shown in the insets. The assignment of the hexoses is based on the known structure of the *C. jejuni* glycan (47). DATDH is 2,4-diacetimido-2,4,6-trideoxyhexopyranose. C^a represents acrylamide attached to Cys.

variability in the glycan structure was reported previously for recombinant N glycoproteins expressing the *C. jejuni pgl* cluster in *E. coli* due to activity of chromosomal-encoded WecA (45). Ser148 is the only hydroxylated amino acid in this peptide, and therefore we concluded that this amino acid is the glycosylation site in our system. This was the only glycopeptide identified in our MS analysis. The glycosylation attachment site was therefore conserved when the *P. aeruginosa* system was transferred into *E. coli* (8).

These experiments demonstrated that the *C. jejuni* glycan was transferred to *P. aeruginosa* pilin by PilO, which was functionally expressed in *E. coli*. No other *P. aeruginosa* protein was required for glycosylation of *P. aeruginosa* pilin in *E. coli*, demonstrating that PilO is sufficient for glycosylation. In addition, these results confirm and extend previous observations regarding the relaxed sugar specificity of PilO (9, 18). The ability of PilO to transfer a glycan containing DATDH at the reducing end has not been reported previously.

Functional expression of PglL in *E. coli*. In a previous study, the mutagenesis of *pglL* in MC resulted in pilin with altered electrophoretic mobility (31). Although no further experimental data were shown, Power et al. speculated that PglL could be the enzyme responsible for the transfer of the glycan to pilin. Supporting this hypothesis, mutagenesis of PglO, the GC PglL homologue, resulted in unglycosylated pilin in GC (1). To determine the OTase activity of PglL, we expressed it in *E. coli* in the absence of any other neisserial protein and analyzed the glycosylation of MC pilin encoded by the *pilE* gene. CLM24 cells were transformed with plasmids pAMF3, which expresses the MC pilin, and pACYC$pglB_{mut}$. Two bands, corresponding to prepilin and mature pilin, were detected in whole-cell extracts by Western blot analysis using SM1, a monoclonal antibody directed against MC pilin (Fig. 3A, lane 1). When these cells were additionally transformed with pAMF5 encoding PglL, an extra band of lower electrophoretic mobility was detected with both SM1 (41) and the *C. jejuni* glycan-specific antiserum R12 (Fig. 3A and B, lanes 3), indicating that pilin was glycosylated. Unglycosylated pilin was still detected in the presence of PglL, indicating that either the process is not as efficient as it is in its native host or an excess of pilin was produced under our experimental conditions. Previous work identified the MC pilin glycosylation site as being contained within the tryptic peptide ^{45}SAVTEYYLNHGEWPGNNTSA GVATSSEIK^{73} (37). However, the exact site of glycosylation remained to be identified precisely (16, 25). Glycosylated MC pilin was purified by two successive affinity chromatography steps using Ni-nitrilotriacetic acid agarose and SBA agarose. The digestion of glycosylated pilin with trypsin and subsequent matrix-assisted laser desorption ionization MS analysis confirmed the previous result (data not shown). To identify the glycosylation site accurately, MC pilin was digested with thermolysin or proteinase K and the resulting peptides were analyzed by nano-liquid chromatography MS and MS/MS. The MS of thermolysin-digested MC pilin showed a doubly charged peak at *m/z* 1,430.0^{2+}, corresponding to the peptide ^{52}LNHG EWPGNNTSAG^{65} modified with the heptasaccharide DATD H(HexNAc)$_5$Hex. The MS/MS analysis of the glycopeptide ion at *m/z* 1,430^{2+} confirmed the presence of the *C. jejuni* glycan bound to the peptide ^{52}LNHGEWPGNNTSAG^{65} (Fig. 4A). The MS of proteinase K-digested MC pilin showed a doubly

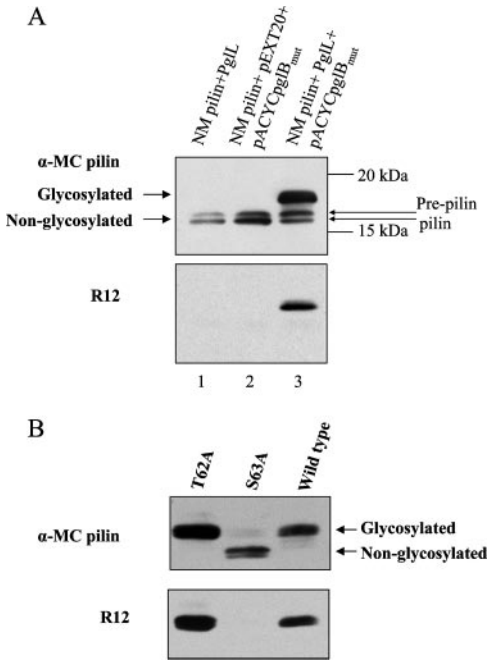

FIG. 3. Reconstitution of *N. meningitidis* (MC) MC58 O glycosylation system in *E. coli*. (A) Western blot assay of whole-cell *E. coli* CLM24 extracts producing unglycosylated and glycosylated MC pilin. Pilin was detected by the SM1 anti-pilin monoclonal antibody (upper panel) or the *C. jejuni* glycan antiserum R12 (lower panel). Lanes: 1, pAMF3 (expressing MC pilin) and pAMF5 (expressing PglL); 2, pAMF3, pACYC$pglB_{mut}$, and pEXT22 (cloning vector); 3, pAMF3, pACYC$pglB_{mut}$, and pAMF5. (B) Results of analysis similar to that for panel A, showing the effects of mutations S63A and T62A on pilin glycosylation. Plasmid details are presented in Table 1. NM, *N. meningitidis*.

charged glycopeptide peak at *m/z* 905.8^{2+}, corresponding to ^{63}SAGVA^{67} carrying the above-mentioned heptasaccharide. The MS/MS of this glycopeptide, presented in Fig. 4B, showed that the glycan was attached to the pentapeptide, in which Ser63 is the only amino acid that can be linked to a glycan. The presence of b and y fragment ions of the peptide is additional evidence to confirm the identity of the glycan carrier. From this set of experiments, we concluded that Ser63 is the site of O glycosylation in MC pilin. Supporting this conclusion, the replacement of Thr62 by Ala did not affect MC pilin glycosylation, whereas the mutagenesis of Ser63 impaired MC glycosylation severely (Fig. 3B). Interestingly, a band matching the molecular weight of glycosylated pilin was detectable in the S63A pilin mutant. This band was not detected with the antiserum R12. Whether this band corresponds to glycosylated pilin at another amino acid in this mutant is now under study. Our experiments show that the glycosylation site seems to be conserved when the MC system is transferred into *E. coli*.

The PglL-dependent pilin glycosylation occurred in *E. coli* cells in the absence of any other MC protein, demonstrating that PglL possesses OTase activity and is sufficient for glycosylation. The structure of the *C. jejuni* heptasaccharide transferred in this experiment by PglL is different from that of the trisaccharide found in MC pilin, indicating that like PilO, PglL has relaxed sugar specificity. This finding is also supported by

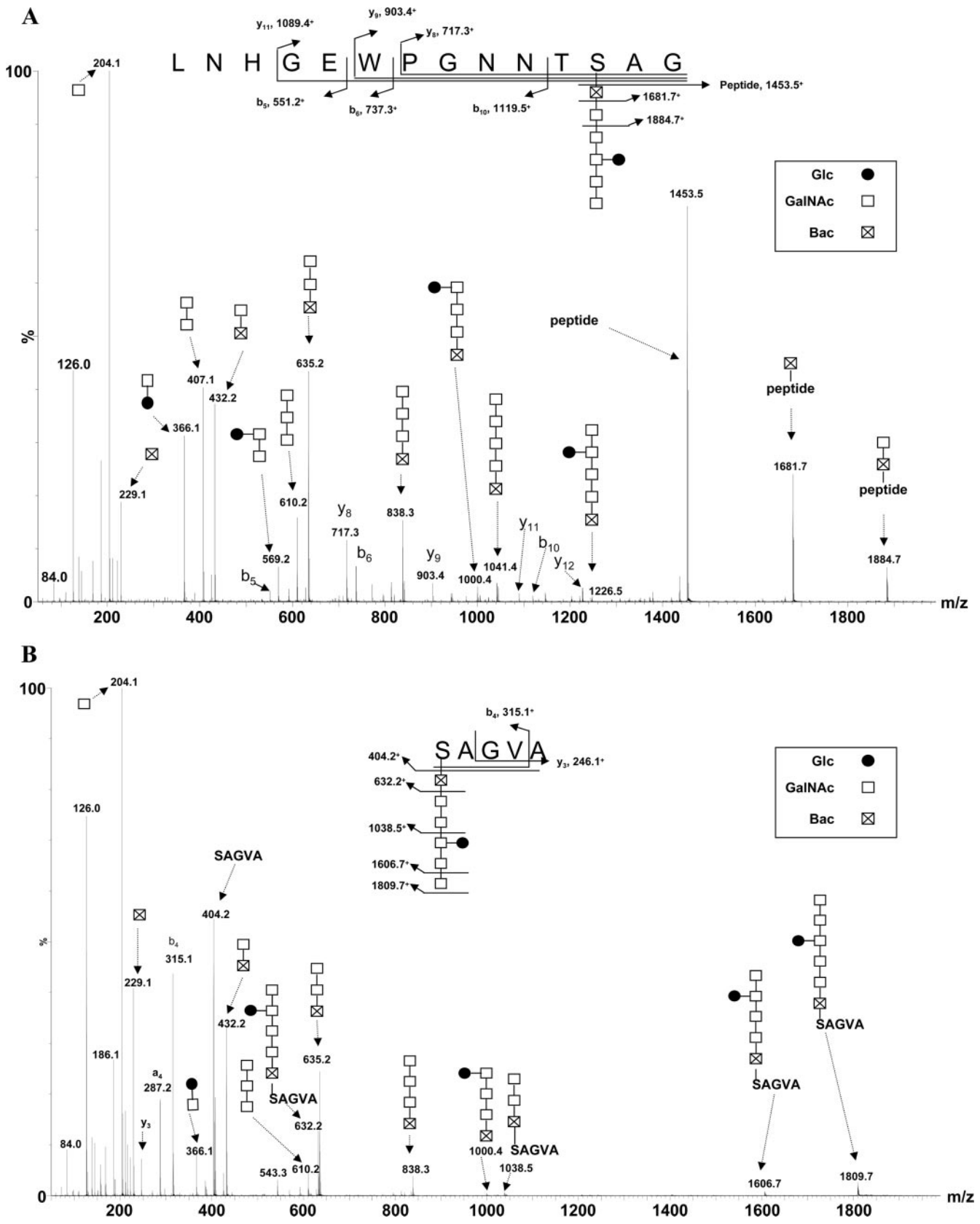

FIG. 4. MS/MS analysis shows glycosylation of recombinant MC pilin with the *C. jejuni* glycan in *E. coli*. (A) MS/MS spectrum of doubly charged ion at *m/z* 1,430.0^{2+}, corresponding to the glycopeptide ^{59}LNHGEWPGNNTSAG^{72} modified with DATDH(HexNAc)$_5$Hex resulted from thermolysin digestion. (B) MS/MS spectrum of a doublely charged glycopeptide ion at *m/z* 905.8^{2+}, corresponding to DATDH(HexNAc)5Hex attached to peptide ^{63}SAGVA^{67} resulted from proteinase K digestion of MC pilin. The fragmentation patterns of the glycopeptides are shown in the insets. As shown, the common peptide fragment ions (y and b) are observed in addition to the sugar fragments and the peptide with sugar fragments. The assignment of the hexoses is based on the known structure of the *C. jejuni* glycan (47).

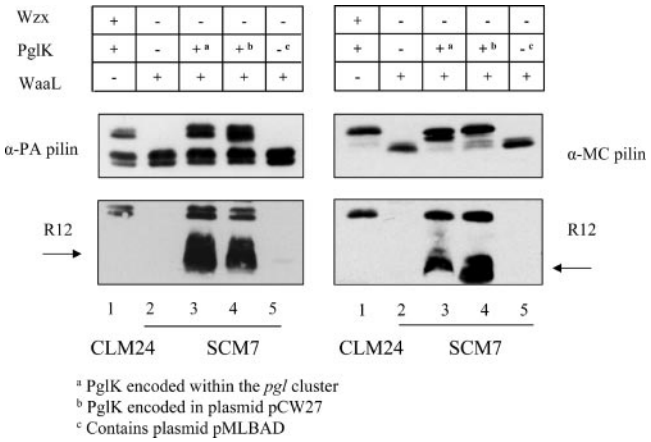

FIG. 5. Translocation of lipid-linked oligosaccharides to the periplasm is a prerequisite for glycosylation. Glycosylation of MC and *P. aeruginosa* pilins occurs only in the presence (+) of a functional flippase, either Wzx (lanes 1), a *pgl*-encoded PglK (lanes 3), or a PglK encoded in *trans* (lanes 4). Cell extracts were analyzed by Western blotting by using antibodies directed against *P. aeruginosa* and MC pilins (upper panels, left and right, respectively) and the glycan specific antiserum R12 (lower panels). Left panels, CLM24 containing pPAC46 and pACYC*pglB*$_{mut}$ (lanes 1); SCM7 transformed with plasmid pPAC46 and pACYC*pglK*$_{mut}$ (lanes 2); SCM7 containing pPAC46 and pACYCpgl (lanes 3); SCM7 transformed with pPAC46, pACYC*pglK*$_{mut}$, and pCW27, expressing PglK in *trans* (lanes 4), and SCM7 transformed with pACYC*pglK*$_{mut}$ and pMLBAD (cloning vector) (lanes 5). Right panels, same as left panels, except for plasmids pAMF5 and pAMF6 expressing MC pilin and PglL, respectively, instead of pPAC46. Details of the strains and plasmids are presented in Table 1. The arrows indicate the presence of LPS containing the *C. jejuni* oligosaccharide in the strains where a functional WaaL (ligase) and a flippase are present. −, absence of active protein.

the ability of PglL to transfer the *P. aeruginosa* O11 antigen (data not shown) and the *E. coli* O7 antigen (see below).

Translocation of Und-PP-glycan to the periplasm is required for PilO and PglL activity. In the synthesis of O antigen, peptidoglycan, exopolysaccharides, and capsules as well as in protein N glycosylation in *C. jejuni*, Und-PP-substrates are translocated or "flipped" into the periplasm by flippases (29). We investigated whether the translocation of Und-PP-glycans to the periplasm is also required for pilin glycosylation. The functional expression of PilO and PglL in *E. coli* allowed us to study pilin glycosylation in *E. coli* SCM7. The *E. coli* SCM7 strain lacks all known flippases, and it has been used recently to characterize PglK, the flippase of the *C. jejuni* glycosylation system (Table 1) (3).

pPAC46 (expressing *P. aeruginosa* pilin and *pilO*) and pACYC*pgl* or pACYC*pglK*$_{mut}$ (Table 1) were introduced into SCM7 cells (Fig. 5, left panels). *P. aeruginosa* pilin glycosylation was detected in cells carrying the intact *pgl* cluster, which carries a functional copy of the PglK flippase (Fig. 5, lane 3). *P. aeruginosa* pilin was not glycosylated in SCM7 cells carrying pACYC*pglK*$_{mut}$. pACYC*pglK*$_{mut}$ contains a nonpolar mutation in the *pglK* gene (3). In SCM7 cells carrying pACYC*pglK*$_{mut}$, the translocation of Und-PP-sugars into the periplasm is impeded due to the absence of flippases (Fig. 5, lane 2). The glycosylation was restored when the *pglK* mutation was complemented in *trans* (Fig. 5, lane 4). A similar analysis was carried out for PglL (Fig. 5, right panels). PglL activity was also dependent on the presence of a functional flippase in the cells. Thus, translocation of the Und-PP-linked oligosaccharide is required for PilO and PglL glycosylation, indicating that PilO and PglL activities are localized to the periplasm.

Carbohydrate chain length preferences of PglL and PilO. Although long polysaccharides are accessible to PilO at the periplasm of *P. aeruginosa* 1244, only a single O antigen-repeating unit was found attached to pilin (7). By exploiting the genetic tools available in *E. coli*, we tested whether PglL and PilO are able to transfer polymerized *E. coli* O7 antigen to their respective pilins. The SΦ874 strain (Table 1) carries a deletion encompassing the complete endogenous O antigen cluster. O7 antigen can be generated in this strain by using plasmids containing the gene cluster necessary for the synthesis of *E. coli* O7 antigen. Three O7 antigen variants were produced using different plasmids (Fig. 6A): wild-type O7 antigen, an O antigen polymerase (*wzy*) mutant that produces only a single O7 subunit, and an O chain length regulator (*wzz*) mutant that produces an O antigen with altered length distribution (26). In the *wzz* mutant, short O antigen chains containing only a few repeating O antigens are more abundant than in the wild-type O antigen (Fig. 6A).

We analyzed the ability of PglL to transfer these three variants of the O7 antigen in the SCM3 strain (Table 1), a derivative of strain SΦ874 lacking the *waaL* gene, encoding the O antigen ligase. SCM3 accumulates Und-P-P-linked oligosaccharides. A ladder of bands, exhibiting a pattern similar to that obtained for their respective O antigens was detected with the anti-pilin monoclonal SM1 antibody (Fig. 6B, lanes 1 to 4). This result suggested that PglL was able to transfer polymerized O antigen to MC pilin. T62A and S63A MC pilin mutants were tested as glycosylation acceptors for the Wzz O7 antigen variant. The high-molecular-weight forms of pilin disappeared only when Ser63, the glycan attachment site, was mutagenized (Fig. 6C), confirming that these bands represented MC pilin variants bound to the O7 antigen at Ser63.

A similar analysis was carried out for PilO. Up to two O7 antigen subunits were transferred to *P. aeruginosa* pilin in the *wzz* O7 mutant (Fig. 6B, lane 8). O7 antigen chains containing three or more repetitive subunits were not transferred to *P. aeruginosa* pilin, although the *wzz* mutant produces similar quantities of chains containing two, three, and four O7-repeating units (Fig. 4A, lane 3). Glycosylated *P. aeruginosa* pilin was not detected in the wild-type O7 strain because the formation of the short chain O antigen (i.e., one or two subunits) transferable by PilO are reduced by Wzz activity. Collectively, these experiments indicate that PglL can transfer long polysaccharides of a completely different structure from the native glycan, whereas PilO activity is intrinsically restricted to O antigen molecules containing up to two repetitive subunits.

DISCUSSION

In this study, we reconstituted *P. aeruginosa* 1244 and MC strain MC58 pilin glycosylation systems into *E. coli* cells. We detected some variability in the level of pilin glycosylation obtained (Fig. 3). This variation can be attributed to the diverse expression vectors used in different experiments and bearing different origins of replication. These vectors dictated the amounts of pilins and OTases produced rendering fully or

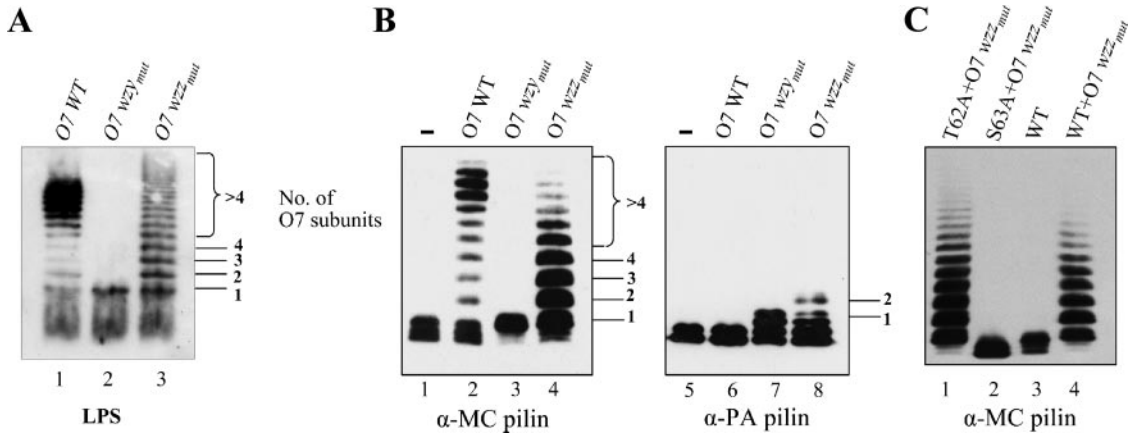

FIG. 6. PglL, but not PilO, transfers polysaccharides to pilin. (A) Lectin blot analysis of three different forms of *E. coli* O7 LPS produced in *E. coli* SΦ874. A lectin specific for rhamnose, one of the sugars of the O7 antigen, has been used: wild type (WT) (lane 1); *wzy* (polymerase) mutant (lane 2), and *wzz* (chain length regulator) mutant (lane 3). The numbers on the right indicate the numbers of O7-repeating units attached to lipid A-core. (B) The ability of PglL (left panel) and PilO (right panel) to transfer O7 antigen of different lengths to their respective pilins in the *E. coli* SCM3 strain (ligase-deficient derivative of SΦ874) was analyzed by Western blotting. MC pilins containing the three O antigen versions shown in panel A were detected by using the anti-MC pilin monoclonal antibody. PglL was able to transfer fully polymerized O7 antigen to MC pilin (lane 2). *P. aeruginosa* pilin containing O antigen of up to only two repeating units was detected in the *wzz* mutant strain (lane 8), despite the observation that O antigen containing two or more repeating units is equally abundant (panel A, lane 3). Lanes 1 to 4: pAMF5 (expressing PglL) and pAMF6 (expressing MC pilin). Additionally, lane 2 contains pJHCV32 (wild-type O7 antigen), lane 3 contains pJHCV32-134 (O7 *wzy* mutant), and lane 4 contains pJHCV32-136 (O7 *wzz* mutant). Lanes 5 to 8, pPAC46 (expressing *P. aeruginosa* pilin and PilO). Additionally, lane 6 contains pJHCV32, lane 7 contains pJHCV32-134, and lane 8 contains pJHCV32-136. (C) O7 antigen from the *wzz* mutant strain is not transferred to the S63A variant of MC pilin (lane 2). Glycosylation is not affected in the mutant T62A (lane 1). Unglycosylated (lane 3) and wild-type pilin glycosylated with the O7 antigen (lane 4) are included for comparison.

partially glycosylated pilin. Previous work has shown that the glycan attached to the C-terminal Ser residue of *P. aeruginosa* 1244 pilin is a product of the O antigen biosynthetic pathway and that transfer of the glycan is catalyzed by PilO (6, 7). *Neisseria* lacks the typical O antigen, and until recently, it was thought that the trisaccharide that decorates *Neisseria* pilins was formed by sequential transfer of individual sugars from the corresponding activated nucleotide sugars (38). In this work, we demonstrated that PglL can transfer diverse glycans preassembled onto the Und-PP carrier to pilin. The OTase activity of PglL was detected in *E. coli* cells in the absence of any other neisserial protein except for the pilin substrate. Our result is in agreement with the recent work by Aas et al., involving the PglO transferase in GC pilin glycosylation (1). By exploiting the availability of an *E. coli* strain lacking all the known Und-PP glycan flippases, we demonstrated that flipping of the Und-PP glycans is required for pilin glycosylation by both PilO and PglL, indicating that the activity of these enzymes is located at the bacterial periplasm. This finding is also supported by the ability of PglL to transfer polysaccharides that are polymerized in the bacterial periplasm.

Our work shows that PglL and PilO share several characteristics: they transfer their carbohydrates en bloc, use Und-PP-linked oligosaccharides as sugar donors, O glycosylate type IV pilin, have relaxed specificity, work in the periplasm, and are sufficient for glycosylation. Although PglL and PilO possess very limited sequence identity, our functional characterization of both enzymes in *E. coli* supports the concept that PilO and PglL belong to a new family of "O-OTases," i.e., OTases involved in O-linked protein glycosylation. A homologous domain of about 30 amino acids has been identified in these proteins, suggesting they are related evolutionarily (31). Interestingly, this domain is also present in enzymes involved in LPS biosynthesis, specifically in Wzy and WaaL. Wzy is responsible for the polymerization of the O antigen subunits, and WaaL is the ligase that transfers the polymerized O antigen from their Und-PP carrier to the lipid A-core (40). O-OTases, O antigen polymerases, and ligases catalyze different reactions, but they share the same type of Und-PP-linked sugar donor; therefore, we hypothesize that this common domain is involved in the recognition of the lipid-linked sugar substrate.

Similar to PglB, the "N-OTase" of the *C. jejuni* N glycosylation pathway, PglL and PilO can transfer a variety of carbohydrates other than their endogenous glycans to acceptor proteins (12). Notably, relaxed sugar specificity seems to be a common feature of enzymes that use Und-PP-linked oligosaccharides as the substrates. This characteristic is also shared by the WaaL ligase (32) and the Wzx flippase (11). Interestingly, we have shown here that the same heptasaccharide can be transferred by PglB forming an N linkage and by PilO and PglL, creating an O linkage. Furthermore, PglB and PglL can transfer polysaccharides, whereas PilO transfers only short glycan chains (Fig. 5). As PilO works at the periplasm, this observation cannot be attributed to a lack of access to the polymerized O antigen by PilO. An alternative hypothesis to explain the lack of polysaccharide transfer by PilO is that other proteins regulate PilO activity in *P. aeruginosa* 1244 to prevent the transfer of polysaccharides to pilin. As we worked with *E. coli*, where no other *P. aeruginosa* protein was present, this hypothesis seems unlikely. Our experiments suggest that PilO can discriminate between polymerized and unpolymerized O antigen by itself. We hypothesize that PilO has evolved to prevent the transfer of long O polysaccharides that would likely impede pilin transport and pilus assembly. On the con-

trary, *C. jejuni* PglB and MC PglL operate in bacteria lacking the typical LPS containing a long O antigen chain. PglL and PglB do not need to avoid polysaccharide recognition in their native environments and are able to transfer polysaccharides.

In addition to *Pseudomonas* and *Neisseria*, pilin glycosylation has been described recently for *Deinococcus geothermalis* (33) and it has been proposed that pilin is also glycosylated in *Francisella tularensis* (15). Although more experiments are required to confirm the presence of glycans attached to *F. tularensis* pilin, an ORF homologous to PilO has been identified in its genome, suggesting that the glycosylation process may occur by the same pathway described here. Additionally, ORFs with homology to *pilO* or *pglL* are found in the genomes of other bacteria from diverse genera, including *Burkholderia*, *Nitrosomonas*, *Shewanella*, and *Chromobacterium* (31). However, whether these genes code for O-OTases or O antigen ligases cannot be predicted currently from only their primary sequences. Another O-OTase may be involved in the glycosylation of pilin in *P. aeruginosa* 5196 (43). In any case, the identification of additional members of the O-OTase family is expected and it should not be a surprise to find additional O-OTases that evolved to O glycosylate other bacterial proteins. For example, there is evidence indicating that O-OTases could be involved in S-layer biosynthesis in gram-positive bacteria (36).

To our knowledge, this is the first report describing the synthesis of recombinant O glycoproteins with defined glycan structures in *E. coli.* The ability of PglL to transfer different polysaccharides in *E. coli* could have an application for the design and production of novel glycoprotein-based therapeutics and vaccines. The use of O-glycosylated pilin carrying different glycans as conjugated vaccines is being currently tested in our laboratory.

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