Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*

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***Campylobacter jejuni* has a general N-linked protein glycosylation system that can be functionally transferred to *Escherichia coli*. In this study, we engineered *E. coli* cells in a way that two different pathways, protein N-glycosylation and lipopolysaccharide (LPS) biosynthesis, converge at the step in which PglB, the key enzyme of the *C. jejuni* N-glycosylation system, transfers O polysaccharide from a lipid carrier (undecaprenyl pyrophosphate) to an acceptor protein. PglB was the only protein of the bacterial N-glycosylation machinery both necessary and sufficient for the transfer. The relaxed specificity of the PglB oligosaccharyltransferase toward the glycan structure was exploited to create novel *N*-glycan structures containing two distinct *E. coli* or *Pseudomonas aeruginosa* O antigens. PglB-mediated transfer of polysaccharides might be valuable for *in vivo* production of O polysaccharides-protein conjugates for use as antibacterial vaccines.**

conjugate vaccines | oligosaccharyltransferase | STT3 | glycoengineering

The increasing evidence that prokaryotes can glycosylate proteins has put an end to the doctrine that restricted glycoproteins to eukaryotes. Many bacteria, including several human pathogens, contain glycoproteins in their surface appendages such as flagella and pili (1). Recently, it has been discovered that the food-borne pathogen *Campylobacter jejuni* bears a general protein N-glycosylation system, responsible for the glycosylation of at least 30 different proteins (2–4). In *C. jejuni*, lack of N-glycosylation results in loss of immunogenicity of multiple proteins, decreased adherence and invasion *in vitro*, and loss of mouse colonization *in vivo* (5). The *C. jejuni* protein glycosylation machinery is encoded by a single locus named *pgl* (4). A heptasaccharide consisting of bacillosamine, glucose, and five *N*-acetylgalactosamine residues was found on proteins as the end-product of this biosynthetic pathway (2, 6).

A unique oligosaccharide structure covalently linked to Asn residues in different proteins is reminiscent of the N-linked glycosylation process in eukaryotic cells, where the $Glc_3Man_9GlcNAc_2$ oligosaccharide is assembled on a lipid carrier, dolichyl pyrophosphate, at the membrane of the endoplasmic reticulum (7). The preassembled oligosaccharide is then transferred to select Asn residues within the sequence Asn-X-Ser/Thr of nascent polypeptide chains (8). This process is catalyzed by the oligosaccharyltransferase (OST), a protein complex that is best characterized in yeast where it consists of nine different subunits (9). Stt3p is the most conserved protein in this complex and was suggested to be the catalytic subunit of the enzyme (7, 10–12). PglB, encoded in the *C. jejuni* N-glycosylation locus is similar in sequence and presumed overall structure to Stt3p and is essential for bacterial N-glycosylation (6). Based on these observations, it was postulated that bacterial and eukaryotic N-linked protein glycosylation are homologous processes (6). This hypothesis was supported by the finding that as in the eukaryotic system, the bacterial glycan acceptor sequence is Asn-X-Ser/Thr, where X can be any amino acid except proline (13). By analogy to the eukaryotic glycosylation system, and based on the homology of some *pgl*-encoded proteins with other bacterial proteins, it is proposed that the bacterial oligosaccharide is assembled on a lipid carrier (bactoprenyl pyrophosphate) at the cytoplasmic side of the inner membrane, flipped into the periplasm by the putative WlaB flippase, and transferred to Asn residues of periplasmic proteins by the PglB OST (Fig. 1*A*) (2, 6, 14, 15).

The proposed N-glycosylation pathway of *C. jejuni* has significant similarities to the "polymerase-dependent" pathway described for the synthesis of O antigen or O polysaccharide in many Gram-negative bacteria (Fig. 1*B*) (16). O antigen is the outer component of LPS and the major contributor to the antigenic variability of the bacterial cell surface (17). O antigen biosynthesis starts with the transfer of a sugar phosphate from a UDP-donor to an undecaprenyl phosphate carrier (Fig. 1*B*) (17). Different glycosyltransferases sequentially add the remaining monosaccharides from nucleotide-activated donors to complete the lipid-linked O antigen subunit that is then translocated to the periplasmic side of the inner membrane by the Wzx flippase (18). In the periplasm, Wzy catalyzes the polymerization of the O antigen subunits, and the polymerized O antigen is transferred to lipid A core to form LPS, in a step involving WaaL, the putative O antigen ligase (Fig. 1*B*) (17, 19). LPS is subsequently transported to the outer leaflet of the outer membrane. O antigen exhibits a preferred and strain-specific size distribution pattern, which is mediated by Wzz protein (20).

The similarity between the O polysaccharide and the proposed N-glycosylation biosynthetic pathways (Fig. 1 *A* and *B*) prompted us to investigate whether PglB was able to covalently link O antigen to an acceptor protein, as a way to test the hypotheses that (*i*) PglB alone is sufficient for OST activity and (*ii*) undecaprenyl pyrophosphate-linked oligosaccharides serve as substrates in this reaction (Fig. 1*C*). Achieving these objectives would open up possibilities for engineering novel glycoconjugates.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. *Escherichia coli* strains were grown on LB at 37°C. Trimethoprim at 100 μg/ml, tetracycline at 20 μg/ml, spectinomycin at 80 μg/ml, chloramphenicol at 20 μg/ml, and ampicillin at 100 μg/ml were added to the media for selection as needed. *E. coli* DH5α (Invitrogen) was the host for cloning experiments. Plasmids pACYC184 and pBR322 (NEB, Beverly, MA) were used as cloning vectors. Plasmids pACYC184(*pgl*), pACYC184(pgl_{mut}) (encoding the *C. jejuni pgl* cluster), pMF19 (encoding the rhamnosyl transferase *wbbL*), pJHCV32, and its *wzy* mutant derivative carrying the O7 antigen genes are described in refs. 18 and 21. Plasmid pLPS2 expressing

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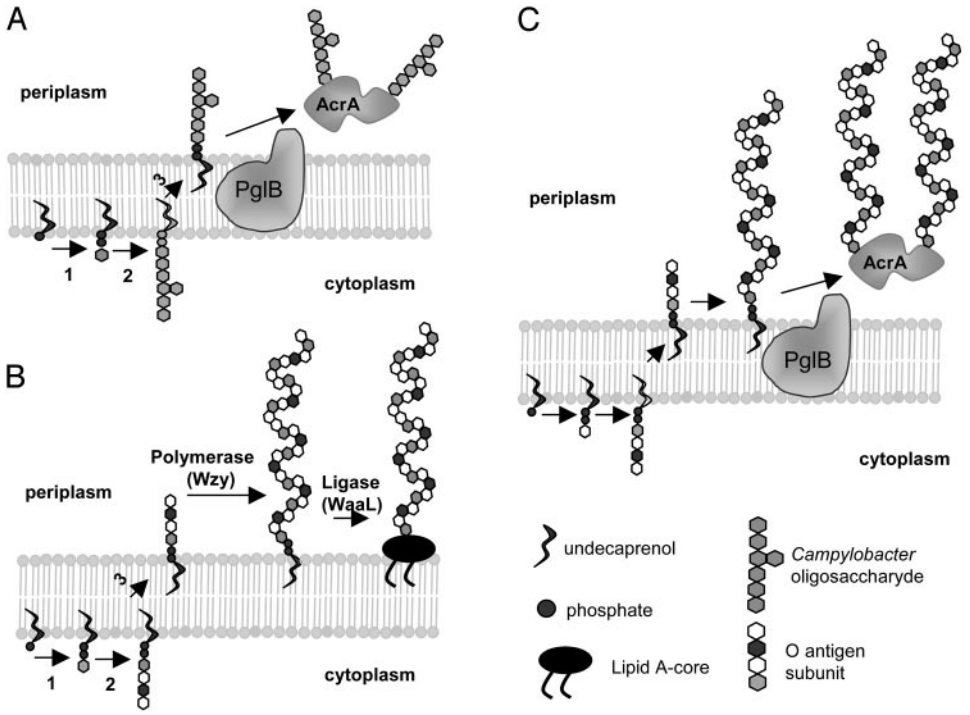

Fig. 1. Similarities between N-glycosylation and O antigen biosynthesis. (*A*) Hypothetical pathway for N-glycosylation in *C. jejuni*. Several glycosyltransferases sequentially add the sugars to a lipid carrier (1–2). The lipid-linked oligosaccharide is flipped by WlaB (3). PglB transfers the oligosaccharide to Asn residues of the acceptor protein. (*B*) LPS biosynthesis in *E. coli*. Several glycosyltransferases add sequentially the sugars to a lipid carrier, bactoprenyl pyrophosphate (1–2). The lipid-linked O antigen subunits are flipped by Wzx (3) and polymerized by the Wzy polymerase. The WaaL ligase transfers the O polysaccharide to the Lipid A-core, assembled by an independent pathway. (*C*) Combination of both pathways. In a Δ*waaL* ligase mutant, introduction of PglB in *E. coli* results in the transfer of the O polysaccharide to a protein acceptor.

Pseudomonas aeruginosa O11 (6, 22, 23) and the corresponding empty vector control pLAFR1 were kindly provided by Peter Castric (Duquesne University, Pittsburgh). The strains and plasmids used are listed in Table 1.

Construction of CLM24 Ligase Defective Strain. Deletion of the *waaL* chromosomal gene was performed as described by Datsenko and Wanner (24). We generated primers composed of 40–45 nucleotides corresponding to regions adjacent to the gene targeted for deletion. The primers (5′-GCAGTTTTGGAAAAGTTATCATCATTATAAAGGTAAAACAT*GTGTAGGCTGGAGCTGCTTCG* and 5′-AGTGAGTTTTAACTCACTTCTTAAACTTGTTTATTCTTAA*CATATGAATATCCTCCTTAG*) also contained 20 additional nucleotides that annealed to the template DNA from plasmid pKD4 (italics), which carries a kanamycin-resistance gene flanked by FRT (FLP recognition target) sites (24). Competent cells were prepared by growing *E. coli* W3110 carrying pKD46 (24) in LB containing 0.5% (wt/vol) arabinose, and the PCR products were introduced by electroporation. The plasmid pKD46 encodes the Red recombinase of the λ phage, which was placed under the control of the arabinose-inducible promoter P_{BAD}. Kanamycin-resistant colonies were screened by PCR with primers

Table 1. Strains and plasmids used in this study

Strain/plasmid	Characteristic/description	Source
DH5α	F− φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (rk−, mk+) *gal-pho*A *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1	Invitrogen
W3110	*rph-1* IN(*rrnD-rrnE*) 1	Laboratory stock
CLM24	W3110, Δ*waaL*	This work
pACYC(*pgl*)	Encodes the *C. jejuni pgl* cluster, Cm^R	6
pACYC(pgl_{mut})	Encodes the *C. jejuni pgl* containing mutations W458A and D459A in PglB, Cm^R	6
pMLBAD	Cloning vector, arabinose-inducible, Tmp^R	31
pMAF10	HA-tagged PglB cloned in pMLBAD, Tmp^R	This work
pWA1	HA-tagged $PglB_{mut}$ cloned in pMLBAD, Tmp^R	This work
pWA2	Soluble periplasmic hexa-His-tagged AcrA under control of Tet promoter, in pBR322, Amp^R	This work
pMH2	Soluble periplasmic hexa-His-tagged AcrA (N123L) under control of Tet promoter, in pBR322, Amp^R	This work
pMH3	Soluble periplasmic hexa-His-tagged AcrA (N273L) under control of Tet promoter, in pBR322, Amp^R	This work
pMH4	Soluble periplasmic hexa-His-tagged AcrA (N123L, N273L) under control of Tet promoter, in pBR322, Amp^R	This work
pMH5	Soluble periplasmic hexa-His-tagged AcrA under control of Tet promoter, in pACYC, Cm^R	This work
pJHCV32	Encodes the O7 antigen cluster from *E. coli,* Tet^R	21
pJHCV32::Tn*3*HoHo1–136	Encodes the O7 antigen cluster from *E. coli* carrying a transposon in *wzy,* Tet^R Ap^R	21
pLPS2	Encodes the O11 antigen from *P. aeruginosa,* Tet^R	23
pMF19	Expresses WbbL rhamnosyltransferase; restores O16 antigen biosynthesis, Sp^R	18
pCP20	*FLP*$^+$, λ cI857$^+$, λ p_R Rep^{ts}, Ap^R, Cm^R	24
pKD4	Template plasmid for mutagenesis, Ap^R, Kn^R	24
pKD46	γ, β, and *exo* from λ phage, *araC-*P_{araB}, Ap^R	24

annealing to regions outside of the mutated gene. Next, the antibiotic gene was excised by introducing the plasmid pCP20 (24) encoding the FLP recombinase. Plasmids pKD46 and pCP20 are both thermosensitive for replication and were cured at 42°C.

Cloning and Expression of PglB and $PglB_{mut}$. The *pglB* gene was amplified by PCR with oligonucleotides PglB*Eco*RI (AAGAATTCATGTTGAAAAAAGAGTATTTAAAAAACCC) and PglB*Nco*I-HA (AACCATGGTTAAGCGTAATCTGGAACATCGTATGGGTAAATTTTAAGTTTAAAAACCTTAGC), using *Pfu* polymerase with pACYC(*pgl*) as template. Oligonucleotide PglB*Nco*I-HA encodes an HA-tag to follow PglB expression by Western blot. The PCR product was digested with *Eco*RI and *Nco*I and cloned in the same sites of vector pMLBAD. The plasmid obtained was named pMAF10. Arabinose-dependent expression of PglB was confirmed by Western blot. pWA1, expressing $PglB_{mut}$, was constructed in an identical manner as pMAF10, but pACYC-(pgl_{mut}) was used as template for the PCR.

Cloning and Expression of Soluble and Mutant Forms of AcrA. To express soluble periplasmic AcrA, controlled by the constitutive tetracycline promoter, pET24(AcrA-per) (13) was cut with *Eco*130I. The Klenow fragment was used to generate blunt ends, and the linearized plasmid was cut with *Xba*I. The released 1.2-kbp fragment was ligated into pBR322, which was previously digested with *Nde*I and *Nru*I, resulting in plasmid pWA2. To express AcrA with point mutations in the two glycosylation sites (Asn-123 and Asn-273), different approaches were chosen. A 915-bp fragment was cut from pET24(AcrA-Asn-2) (13) with *Psi*I and *Xho*I and cloned into pWA2 cut with the same enzymes resulting in plasmid pMH2. In this plasmid, soluble AcrA with the point mutation N123L is expressed in the periplasm with a hexa-His-tag at the C terminus. For introduction of point mutations N273L and N123L, N273L, the QuikChange mutagenesis kit (Stratagene) was used with the primers as described in ref. 13 and plasmids pWA2 and pMH2 as template, respectively, resulting in plasmids pMH3 and pMH4. To construct a plasmid containing a different origin of replication, containing a different resistance, and expressing soluble AcrA with the hexa-His-tag under the control of the tet promoter, pWA2 was cut with *Eco*RV and *Ehe*I, and the resulting 1.5-kbp fragment was ligated to pACYC184 cut with the same enzymes, resulting in plasmid pMH5.

Production and Purification of Glycosylated AcrA. PglB was induced in *E. coli* cells expressing O antigen genes and *acrA* by the addition of arabinose to 0.2% (wt/vol). After induction at 37°C for 5 h, arabinose was added again to ensure PglB expression when the carbon source becomes limiting (as the cells metabolize the arabinose). Cells were harvested by centrifugation after a 20-h induction. Equivalent preparations of periplasmic extracts were carried out either by osmotic shock lysis or lysozyme treatment. For the former, cells were consecutively incubated first in 20% sucrose/30 mM Tris·Cl (pH 8.0)/1 mM EDTA and then in 5 mM $MgSO_4$ at 0°C for 2–4 h (20 OD_{600} units/ml). The latter consisted of a single incubation in 30 mM Tris·Cl (pH 8.5)/20% (wt/vol) sucrose/1 mM EDTA/1 mg/ml lysozyme (Sigma). A final centrifugation step in both methods yielded periplasmic proteins in the supernatant. For purification, the extracts were diluted with 1/9 vol of 10× buffer A (300 mM Tris·Cl, pH 8.0/3 M NaCl), sterile-filtered, loaded on a HisTrap HP column (Amersham Pharmacia Biosciences) at 1–5 ml/min, washed with at least 25 column volumes of buffer A containing 20 mM imidazole, and eluted into 1–3 ml of buffer A containing 0.25 M imidazole.

Western Blotting. Western blotting was performed as described in ref. 25. Anti-AcrA antibodies are described in ref. 6. Antiserum against *E. coli* O7 was obtained from Statens Serum Institut (Copenhagen). Antiserum against *E. coli* O16 was obtained from Laboratorio de Referencia de *E. coli* (Lugo, Spain). Antibodies against *P. aeruginosa* O11 were kindly provided by Joanna Goldberg (University of Virginia, Charlottesville).

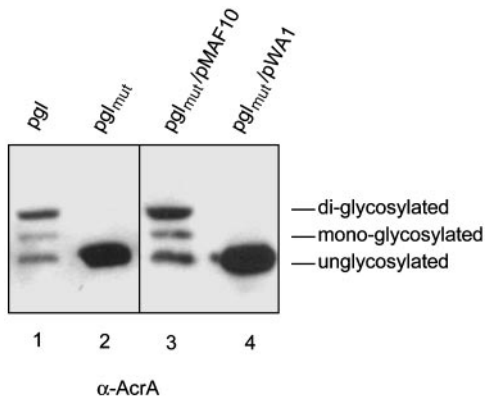

Fig. 2. Complementation of a *pglB* mutant by an HA-tagged, plasmid-encoded PglB. Whole-cell extracts of *E. coli* cells expressing AcrA and carrying the complete *C. jejuni pgl* cluster (Table 1) were analyzed by SDS/PAGE, transferred to nitrocellulose membranes, and detected with antibodies directed against AcrA. Three bands corresponding to unglycosylated, monoglycosylated, and diglycosylated AcrA were observed (lane 1). Bacteria transformed with mutated *pgl* cluster (pgl_{mut}, Table 1) that contains an inactive PglB ($PglB_{mut}$) produced only unglycosylated AcrA (lane 2). Expression of the functional PglB encoded in pMAF10 (lane 3), but not the $PglB_{mut}$ encoded in pWA1 (lane 4), restored glycosylation in cells expressing pgl_{mut}.

Characterization of Glycosylated Peptides. Purified AcrA samples were separated by SDS/PAGE and stained by using Novex colloidal blue reagent (Invitrogen), and the desired protein was excised, lyophilized, and digested with trypsin (EC 3.4.21.4, Promega) overnight. Peptides were extracted from gel pieces and purified by using a C-18 microtrap peptide cartridge (Jones Chromatography) in preparation for sequencing by mass spectrometry (MS) and tandem MS (MS/MS), using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass). The cyanogen bromide (CNBr) digest was performed in 50 μl of 70% aqueous formic acid (vol/vol) with the addition of one to two crystals of CNBr. The solution was incubated at room temperature for 5 h. The reaction was terminated by lyophilizing the sample after the addition of 4 vol of water. MS and MS/MS spectra were collected in the positive ion mode as described in ref. 6. Data were acquired and processed by using MASSLYNX software (Micromass). The instrument was precalibrated by using a 1 pmol/μl solution of [Glu-1]-fibrinopeptide B in acetonitrile/5% aqueous acetic acid (1:3, vol/vol).

Results

Functional Expression of PglB. In our earlier work, we showed that coexpression of the complete *C. jejuni pgl* locus and AcrA, a periplasmic component of a multidrug efflux pump, resulted in N-glycosylated AcrA in *E. coli*. Replacement of PglB by $PglB_{mut}$ (PglB with amino acid substitutions W458A and Y459A), abolished glycosylation (6). We have now constructed pMAF10, a plasmid that expresses an HA-tagged version of PglB, and pWA1, an analogous plasmid that expresses $PglB_{mut}$. In both cases, PglB expression was controlled by an arabinose-inducible promoter. Production of PglB was verified by Western blot (data not shown). To show that HA-tagged PglB was fully functional, we introduced pMAF10 or pWA1 in *E. coli* cells carrying the *pgl* cluster expressing $PglB_{mut}$, and a soluble form of a C-terminal His_6-tagged AcrA. Introduction of pMAF10, but not pWA1, resulted in production of glycosylated AcrA at comparable levels to those of cells expressing the wild-type *pgl* locus (Fig. 2).

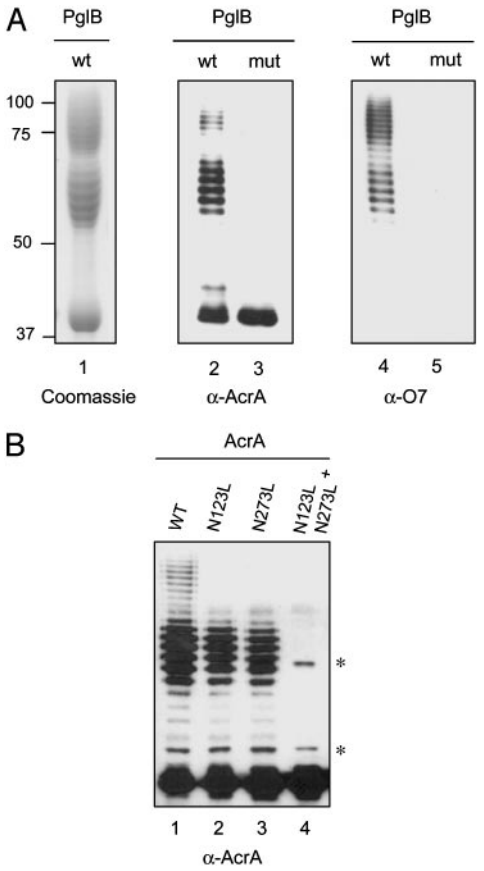

Fig. 3. Glycosylation of AcrA with O7 polysaccharide. (*A*) AcrA was purified from *E. coli* CLM24 strain expressing wild-type O7 polysaccharide and PglB from pMAF10 (wt) or $PglB_{mut}$ from pWA1 (mut). Purified proteins were analyzed by SDS/PAGE and visualized by Coomassie blue staining (lane 1) or transferred to nitrocellulose membranes and detected with antibodies directed against AcrA (lanes 2 and 3) or O7 antigen (lanes 4 and 5). (*B*) Periplasmic extracts of wild-type AcrA (lane 1) and AcrA carrying mutated glycosylation sites (Table 1) were also analyzed by using anti-AcrA antibodies (lanes 2–4). The bands visualized in lane 4 (*) correspond to unspecific binding of the anti-AcrA antibody to a protein present in the extract.

PglB Can Transfer O7-Polysacharide to AcrA. According to our hypothesis, prevention of O antigen transfer to lipid A-core by the O antigen ligase (WaaL) should favor the PglB-mediated transfer of O antigen from its carrier to the protein acceptor because of accumulation of bactoprenyl pyrophosphate-linked polysaccharide (Fig. 1). Therefore, we constructed the *E. coli*-K12 strain CLM24 that lacks the O antigen ligase. CLM24, as with most of *E. coli* K12 strains, does not synthesize O antigen because it carries an inactivating insertion in w*bbL*, the gene that encodes a rhamnosyl transferase necessary for the transfer of the second sugar of the O16 subunit (18, 26). To generate O polysaccharide, we introduced the gene cluster necessary for the synthesis of *E. coli* O7 antigen in CLM24 strain. In addition, we coexpressed in this strain *C. jejuni* PglB (encoded in pMAF10) and AcrA. As a negative control, we used cells expressing $PglB_{mut}$ (pWA1). After induction of PglB, AcrA was purified from periplasmic extracts by using affinity chromatography. Purified AcrA was analyzed by SDS/PAGE and visualized by Coomassie blue or immunodetection (Fig. 3*A*). By Coomassie blue staining, we detected a band of the same mass as that of unglycosylated AcrA (40 kDa) and a ladder of bands of higher molecular weight with two clusters centered at ≈60 and ≈80 kDa (Fig. 3*A*, lane 1). These bands reacted with anti-AcrA antibodies (Fig. 3*A*, lane 2), suggesting that they correspond to modified forms of AcrA. These larger polypeptides, but not the unmodified form of AcrA, were also detected by the anti-O7 antiserum (Fig. 3*A*, lane 4) and by a lectin that binds specifically to rhamnosyl residues (27) (data not shown). The high-molecular-weight bands were absent in the samples obtained from cells expressing $PglB_{mut}$ (Fig. 3*A*, lanes 3 and 5) or when the O7 antigen operon was omitted (data not shown). Thus, these results suggested that O7 polysaccharide was covalently linked to AcrA. The bands corresponding to O7 polysaccharide-linked AcrA showed extensive heterogeneity due to the typical variability of the chain length of the O polysaccharides generated by the Wzy polymerase (17).

PglB Transfers O7 Antigen or *C. jejuni* Glycan to the Same Selected Sites in AcrA. As shown previously, AcrA has five Asn-X-Ser/Thr sequons that could be potentially used as sites for glycan attachment, but only two of these sites, Asn-123 and Asn-273, are glycosylated in *E. coli* as well as in *C. jejuni* (6, 13). Therefore, the

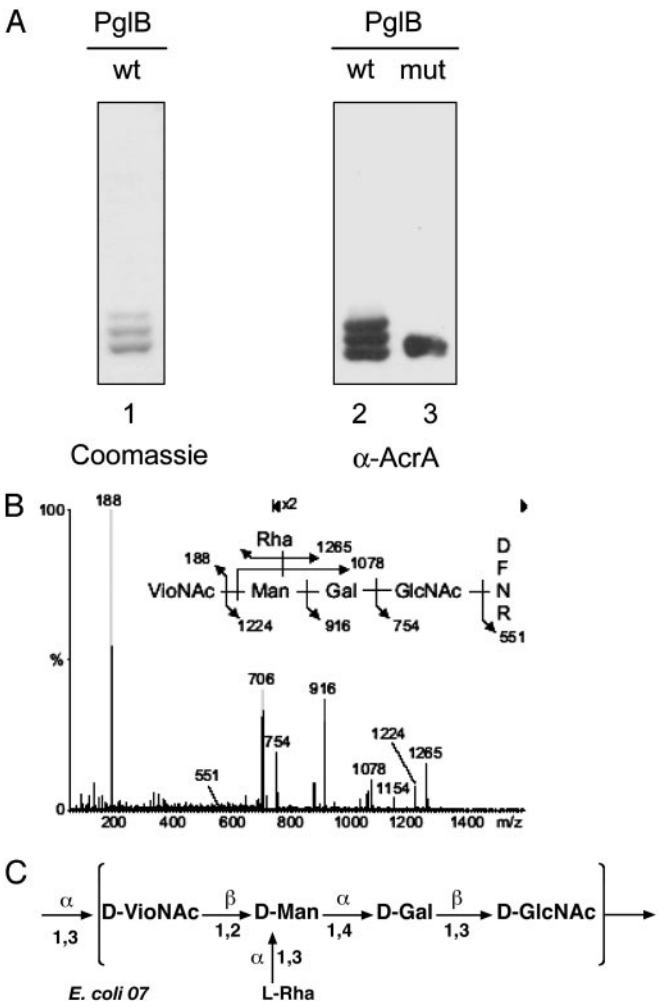

Fig. 4. Transfer of a single O7 antigen subunit to AcrA. (*A*) AcrA was purified from cells carrying the O7 antigen gene cluster with an inactive Wzy polymerase (Table 1) and expressing PglB or $PglB_{mut}$, separated by SDS/PAGE, and visualized by Coomassie blue staining (lane 1) or transferred to nitrocellulose membranes and detected with antibodies directed against AcrA (lanes 2 and 3). (*B*) MS/MS of glycosylated peptide derived from tryptic cleavage of AcrA purified from cells carrying PglB and the O7 antigen gene cluster with an inactive Wzy polymerase. CAD MS/MS spectrum of $[M + 2H]^{2+}$ m/z 706. Fragmentation patterns for the glycan are shown in the *Inset*. (*C*) Structure of the repetitive O7 subunit. GlcNAc, *N*-acetylglucosamine; Gal, galactose; Man, mannose; Rha, rhamnose; VioNAc; *N*-acetylviosamine.

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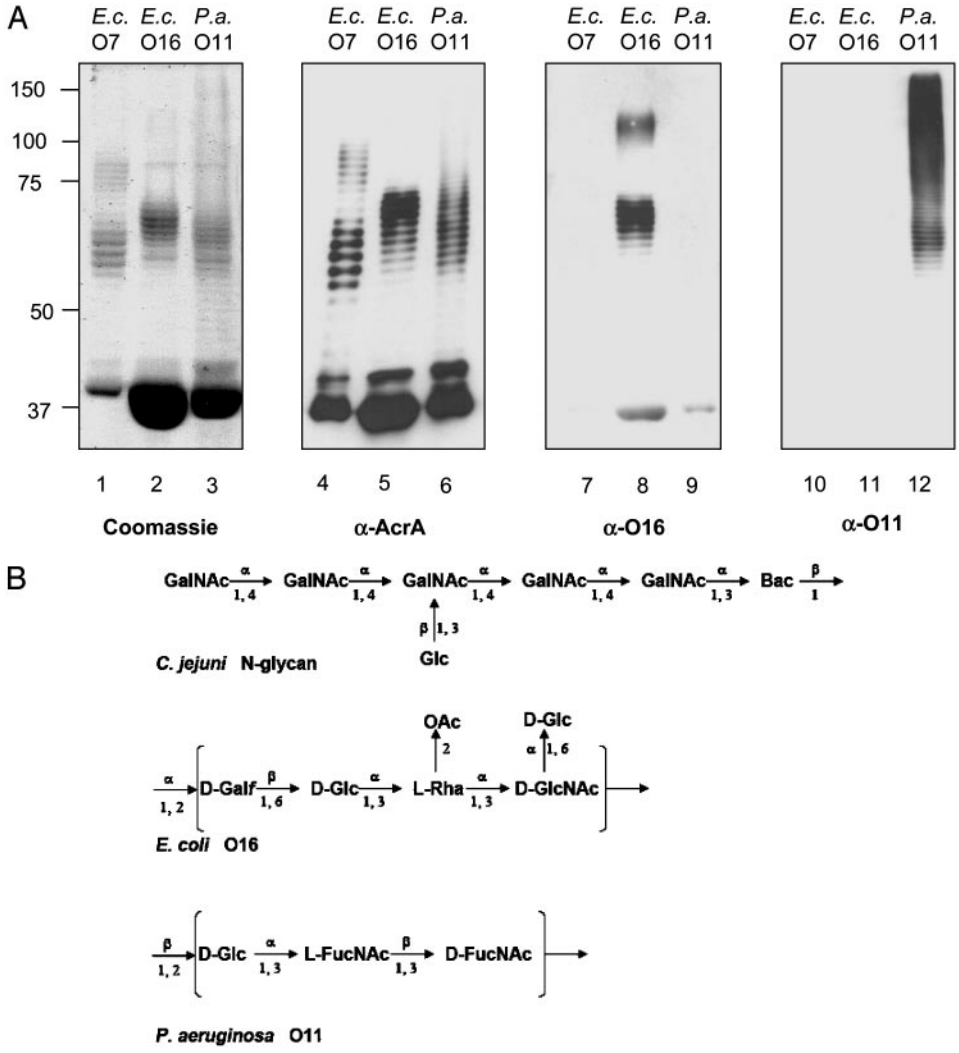

Fig. 5. Glycosylation of AcrA with *E. coli* O16 and O11 *P. aeruginosa* polysaccharides. (*A*) AcrA was purified from *E. coli* CLM24 transformed with pMAF10, expressing *E. coli* O16 or *P. aeruginosa* O11 polysaccharides (Table 1). Samples were analyzed by SDS/PAGE, followed by Coomassie blue staining (lanes 1–3) or transfer to a nitrocellulose membrane and immunodetection with antibodies recognizing AcrA (lanes 4–6), O16 (lanes 7–9), or O11 (lanes 10–12) polysaccharide. Note the different amount of unglycosylated AcrA detected by Coomassie blue present in lanes 1–3 that contain similar amounts of glycosylated protein. Large amounts of AcrA are also detected with the polyclonal anti-O16 antibody (lanes 8 and 9). (*B*) Structures of the *C. jejuni* oligosaccharide and the repetitive units of the *E. coli* O16 and *P. aeruginosa* O11 polysaccharides. The structure of the *E. coli O7* repetitive unit is shown in Fig. 4*C*.

two series of bands ≈60 and ≈80 kDa shown in Fig. 3*A* were assigned to mono- and diglycosylated AcrA, respectively. To show that the same N-glycosylation sites in AcrA were used for the covalent attachment of the O7 polysaccharides, we tested the glycosylation of AcrA mutants, in which one or both Asn residues were substituted by Leu (Fig. 3*B*, lanes 1–4). In periplasmic extracts of cells expressing either N123L or N273L AcrA mutants, the bands corresponding to AcrA with two polysaccharide chains were no longer detected, whereas AcrA containing one polysaccharide chain was still visualized (Fig. 3*B*, lanes 2 and 3). However, in the double mutant (N123L, N273L), AcrA was no longer glycosylated (Fig. 3*B*, lane 4). These results confirmed that PglB attaches O7 polysaccharide and *C. jejuni* oligosaccharide to the same Asn residues in AcrA.

Glycopeptide Analysis Confirms the Transfer of O7 Antigen to AcrA. MS analysis of glycopeptides resulting from trypsin digestion of purified samples failed to show O7 polysaccharide bound to peptides derived from AcrA, probably because of the high molecular weight of the sugar chains. Therefore, we purified AcrA from the CLM24 strain expressing PglB and the O7 antigen cluster with an inactivating transposon inserted into the *wzy* polymerase gene (21). In this strain, three bands were detected by Coomassie blue staining (Fig. 4*A*, lane 1) or Western blot analysis by using anti-AcrA antibodies (Fig. 4*A*, lane 2). We postulated that these bands represent unglycosylated, monoglycosylated, and diglycosylated forms of AcrA carrying a single O7 antigen subunit. Only the band corresponding to unglycosylated AcrA was detected in cells expressing the $PglB_{mut}$ (Fig. 4*A*, lane 3). AcrA produced in the *wzy* mutant strain was digested with trypsin, and the product mixture was analyzed by tandem electrospray MS. The predicted doubly charged glycosylated tryptic peptide (DFNR) spanning Asn-123 (m/z of 706^{2+}) was subjected to collisionally activated dissociation (CAD) MS/MS. Although some weak signals were observed indicative of a glycopeptide, the spectrum was dominated by fragment ions derived from an unmodified AcrA peptide (ASVDSAYGQALMAK) of the same doubly charged mass. To overcome this problem, the sample was treated with cyanogen bromide (CNBr), which cleaved this peptide at the methionine and shifted it to a lower mass. The data obtained from CAD MS/MS of m/z 706^{2+} after CNBr digestion contained abundant singly charged fragment ions from m/z 1,265 to m/z 551 (Fig. 4*B*), consistent with the predicted glycopeptide carrying one O7 antigen subunit (Fig. 4*C*).

PglB Can Transfer Diverse O Polysaccharides. To further analyze the specificity of PglB, we also tested whether the *E. coli* O16 and *P. aeruginosa* O11 polysaccharides can be transferred to AcrA. As mentioned before, CLM24 does not synthesize O antigen. However, biosynthesis of O16 antigen can be restored by complementation with a plasmid encoding *wbbL* gene (26). Thus, we expressed

the rhamnosyl transferase in CLM24 together with AcrA and PglB (18). After purification of AcrA and separation by SDS/PAGE, we showed by Coomassie blue staining (Fig. 5*A*, lane 2) and by Western blot analysis with anti-AcrA (Fig. 5*A*, lane 5) and O16 antigen-specific antibodies (Fig. 5*A*, lane 8) that PglB can also transfer *E. coli* O16 polysaccharide to AcrA. Similarly, we also purified AcrA from *E. coli* cells coexpressing PglB, AcrA, and the *P. aeruginosa* O11 antigen gene cluster (Fig. 5*A*). Glycosylated AcrA containing O11 polysaccharide was visualized by Coomassie blue staining (Fig. 5*A*, lane 3), anti-AcrA antibodies (lane 6), and *P. aeruginosa* O11 antigen-specific antibodies (lane 12). Only unglycosylated AcrA was detected in the presence of $PglB_{mut}$ (data not shown). Therefore AcrA was glycosylated with different O antigens, confirming that PglB has a relaxed specificity for its glycan substrate. Based on the relative abundance of modified AcrA, efficiency of glycosylation with the O16 and the O11 polysaccharides was judged to be lower than that with the O7 antigen (Fig. 5*A*, lanes 1–3). However, we cannot establish whether these differences in efficiency of glycosylation reflect PglB substrate specificity or availability of the lipid-linked sugar substrate.

Discussion

The evident similarities between the O polysaccharide and the proposed *C. jejuni* N-glycosylation biosynthetic pathways (Fig. 1 *A* and *B*) led us to examine the possibility of combining both pathways. In this study we have engineered *E. coli* cells in a way that those pathways converge at the step in which the PglB OST transfers O polysaccharide from its lipid carrier to Asn residues in an acceptor protein (Fig. 1*C*). We conclude that (*i*) PglB is sufficient for OST activity and (*ii*) PglB mediates the transfer of oligosaccharides and polysaccharides from the undecaprenyl pyrophosphate carrier to proteins.

Our experiments show that PglB can transfer diverse oligosaccharides and polysaccharides from *E. coli* and *P. aeruginosa* to proteins, in addition to the *C. jejuni* glycan. Therefore, PglB exhibits relaxed specificity toward the lipid-linked glycan substrate, because it can transfer structures differing in sugar composition and size (Figs. 4*C* and 5*B*). This relaxed specificity is in contrast to the typical eukaryotic OST, where a terminal α1-2-linked glucose residue is of central importance for efficient glycosylation (28). Interestingly, it has been reported that the sugars transferred during pilin *O* glycosylation in *P. aeruginosa* are products of the O antigen biosynthetic pathway and that the process occurs with low glycan substrate specificity (22). Because O antigen polymerization takes place at the periplasmic side of the inner membrane (17), the efficient transfer of O polysaccharide to proteins by PglB confirmed that N-glycosylation in bacteria takes place in the periplasm.

Our results strongly support the hypothesis that the *C. jejuni* and eukaryotic N-glycosylation are homologous processes, but in contrast to the multimeric eukaryotic OST (9), PglB alone is sufficient for OST activity. At present, the specific functions of the various subunits of the eukaryotic OST are unknown. In eukaryotic cells, translation, translocation into the endoplasmic reticulum, and glycosylation must be coordinated. It is probable that OST subunits function as chaperones to prevent the folding of the nascent polypeptide and to maintain the acceptor sequence in a "glycosylatable" conformation. It is likely that N-glycosylation in bacteria occurs posttranslocationally. Only those glycosylation sites on folded proteins showing the conformation required for recognition by PglB might be indeed glycosylated. The simplicity of the bacterial OST can be exploited to investigate aspects of N-glycosylation that are difficult to study in the eukaryotic system.

Bacterial polysaccharides conjugated to proteins have been developed as vaccines and in some cases effectively used during the last two decades. This is illustrated by the *Haemophilus influenzae* type b conjugate vaccine that has virtually eradicated invasive *H. influenzae* type b disease in vast parts of the world (29, 30). Current debate focuses on the efficacy of the conjugate vaccines and the cost-effectiveness of their use globally. The relaxed specificity of PglB could be exploited for the design and production of antibacterial conjugated vaccines by coexpressing PglB, an acceptor protein as a carrier, and the gene clusters encoding the synthesis of O antigens, exopolysaccharides, or capsules that are assembled via undecaprenyl pyrophosphate-linked sugars intermediates. This procedure is expected to be advantageous because glycoproteins could be purified in a few steps from *E. coli* fermentations, without the need for culturing pathogenic or slow-growing bacteria. Because no chemical treatments such as removal of endotoxin and crosslinking are necessary and the length of the O polysaccharide is controlled *in vivo*, the conjugates contain a defined and reproducible sugar pattern. Furthermore, the production of O antigen–protein conjugates by using this technology would allow the production of conjugates containing acid labile structures that cannot be produced by the traditional methods.

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