Pseudomonas aeruginosa 1244 Pilin Glycosylation: Glycan Substrate Recognition

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The pilin of Pseudomonas aeruginosa 1244 is glycosylated with an oligosaccharide that is structurally identical to the O-antigen repeating unit of this organism. Concordantly, the metabolic source of the pilin glycan is the O-antigen biosynthetic pathway. The present study was conducted to investigate glycan substrate recognition in the 1244 pilin glycosylation reaction. Comparative structural analysis of O subunits that had been previously shown to be compatible with the 1244 glycosylation machinery revealed similarities among sugars at the presumed reducing termini of these oligosaccharides. We therefore hypothesized that the glycosylation substrate was within the sugar at the reducing end of the glycan precursor. Since much is known of PA103 O-antigen genetics and because the sugars at the reducing termini of the O7 (strain 1244) and O11 (strain PA103) are identical (β-N-acetyl fucosamine), we utilized PA103 and strains that express lipopolysaccharide (LPS) with a truncated O-antigen subunit to test our hypothesis. LPS from a strain mutated in the wbjE gene produced an incomplete O subunit, consisting only of the monosaccharide at the reducing end (β-D-N-acetyl fucosamine), indicating that this moiety contained substrate recognition elements for WaaL. Expression of pilAO1244 in PA103 wbjE::aacC1, followed by Western blotting of extracts of these cells, indicated that pilin produced has been modified by the addition of material consistent with a single N-acetyl fucosamine. This was confirmed by analyzing endopeptidase-treated pilin by mass spectrometry. These data suggest that the pilin glycosylation substrate recognition features lie within the reducing-end moiety of the O repeat and that structures of the remaining sugars are irrelevant.

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that expresses type IV pili (33), fibrous surface appendages that protrude from the poles of the cell (20). Pili contribute to bacterial pathogenicity by initiating the colonization of host tissue through adhesion and mediating motility across surfaces (33). A single pilus fiber is a polymer of a proteinaceous subunit, referred to as pilin (33). Pilin of P. aeruginosa 1244 is modified by glycosylation, a process that requires the presence of the enzyme PilO (6).

Since the discovery of archaeal S-layer glycoproteins (35), numerous accounts of protein glycosylation in prokaryotes have been recorded, especially among surface proteins of pathogens (44, 57). Examples of gram-positive bacteria in which this posttranslational modification has been documented include Streptococcus mutans (9), S. parasanguis (53), Mycobacterium tuberculosis (15), and Bacillus anthracis (55). Among the gram-negative bacteria, much of the research attention has focused on the two glycosylation systems of Campylobacter jejuni (23, 56, 58), in addition to the flagellin glycosylation of P. aeruginosa 1244 (3) and Helicobacter pylori (43) and the pilin glycosylation of Neisseria spp. (26, 37, 54) and P. aeruginosa 1244 (6, 7, 51).

P. aeruginosa 1244 pilin contains a single covalently bound glycan (7) that is O linked to the β-carbon of Ser148 (10), the carboxy-terminal residue (8). The pilin glycan is a trisaccharide, structurally identical to the O-antigen repeating unit of the serotype O7 lipopolysaccharide (LPS) of strain 1244 (7), which suggested that the glycan might originate in the same metabolic pathway as O-antigen biosynthesis. Evidence supporting this was provided by the finding that the mutation of genes involved in initial steps of O-antigen biosynthesis (either wbpM or wbpL) abolished pilin glycosylation (14). In addition, expression of heterologous O-antigen gene clusters in P. aeruginosa 1244 allowed for pilin to be decorated with the heterologous saccharide, confirming that pilin glycosylation and O-antigen biosynthesis shared a common metabolic origin (14). Furthermore, the putative oligosaccharyltransferase, PilO, is the only protein required for glycosylation that is not involved in O-antigen or pilin synthesis (14).

P. aeruginosa O-antigen biosynthesis proceeds by the “Wzy-dependent” mechanism (13, 17), during which individual O-antigen repeating units are constructed on the cytoplasmic side of the cell membrane by the sequential addition of nucleotide-activated sugars to the carrier lipid, undecaprenyl phosphate (Und-P) (17, 39). In P. aeruginosa PA103, WbpL catalyzes the transfer of β-D-FucNAc to Und-P (13), followed by the addition of α-L-FucNAc by a previously uncharacterized transferase. Finally, the glucosyltransferase, WbjA, adds β-D-Glc to complete the O11 subunit (12). The putative flipase, Wzx, translocates the undecaprenyl pyrophosphate (Und-PP)-linked O repeat to the periplasmic face (31, 34, 36) of the cell membrane, where O-antigen polymerization (34) is mediated by Wzy (13, 39). O-antigen chain length is regulated by Wzz (41), and the O ligase, WaaL, transfers the entire O-antigen to the core polysaccharide (1, 36). A Wzy mutant cannot synthesize polymerized O-antigen; however, these cells are capable of

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producing a core that contains a single O-antigen repeating unit, a phenotype referred to as “core + 1” (13). Interestingly, mutation of \( \text{wby} \) yielded cells that produced a core containing an incomplete O subunit and possessed a core + 2/3 phenotype (12). These phenotypes suggest that O-antigen polymerization and complete assembly of the individual O subunit are not necessary for core-O-antigen ligation (12). A similar phenomenon was observed in a mutant of \textit{Escherichia coli} K-12 that could only synthesize the first sugar of its O subunit (17).

Results from a previous study that investigated pilin specificity in the \textit{P. aeruginosa} 1244 glycosylation reaction suggested that the positioning of Ser at the pilin C terminus is critical for recognition by the glycosylation machinery (25). Although no other specific recognition features are present, the pilin surface charge must be compatible with the glycosylation apparatus (25). Glycosylation requires an enzyme with a specificity for other specific recognition features present, the pilin surface that the positioning of Ser at the pilin C terminus is critical for.

### MATERIALS AND METHODS

#### Bacterial strains and media.

\textit{P. aeruginosa} strains and plasmids used in the present study are listed in Table 1. \textit{E. coli} DH5\textalpha{} (Invitrogen), SM10 (50), and HB101 (2) were used for genetic manipulations. Luria (L) broth or agar was used for routine growth of bacteria. Cetrimide agar base (Difco) was used for the isolation of \textit{P. aeruginosa} after triparental matings. For analysis of pilin, overnight cultures were used to inoculate petri plates containing CAYE solid medium, which consisted of 0.75% Casamino Acids, 0.15% yeast extract, and 2% agar. The media contained gentamicin (250 \( \mu \text{g/ml for } \text{P. aeruginosa} \)), kanamycin (30 \( \mu \text{g/ml for } \text{E. coli} \)), carbenicillin (250 \( \mu \text{g/ml for } \text{P. aeruginosa} \)), ampicillin (50 \( \mu \text{g/ml for } \text{E. coli} \)), and/or tetracycline (50 \( \mu \text{g/ml for } \text{P. aeruginosa} \)), as required. When necessary, the medium was supplemented with 5% sucrose or 5 mM IPTG (isopropyl-\( \beta \)-thiogalactosidase).

#### DNA manipulations.

Plasmid DNA was isolated with Qiagen Plasmid Miniprep Kit (Qiagen Sciences, Valencia, CA) or Wizard Plus Miniprep (Promega Corp., Madison, WI). Restriction endonucleases and modification enzymes (New England Biolabs, Beverly, MA; Boehringer Mannheim Corp., Indianapolis, IN) were used as specified by the manufacturer.

The \text{wby}E sequence was obtained from a PCR fragment amplified from the pLS2 plasmid. Primers for the amplification of the \text{wby}E gene were 83-30 (5'GCTGAAACCCCTCGGCTTTAAGG-3') and 6-95-E (5'-GCATCGCGATTCACCTCCG-5'). These primers were used as specified by the manufacturer.

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#### Table 1. \textit{P. aeruginosa} strains and plasmids used in this study

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<th>\textit{P. aeruginosa} strain or plasmid</th>
<th>Description*</th>
<th>Source or reference</th>
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<tr>
<td>PA103</td>
<td>Wild type; IATS O11</td>
<td>30</td>
</tr>
<tr>
<td>1244N3</td>
<td>( \text{rpm} ) (( \text{Te} )); IATS O7</td>
<td>40</td>
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<tr>
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<td>( \text{wzy} ) (( \text{Gm} ))</td>
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<tr>
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<tr>
<td>PA103 \text{wby};\text{Cl}</td>
<td>( \text{wby} ) (( \text{Gm} ))</td>
<td>30</td>
</tr>
<tr>
<td>PA103 \text{wby};\text{Cl}</td>
<td>This study</td>
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* \( \text{Ap} \), ampicillin resistance; \( \text{Cb} \), carbenicillin resistance; \( \text{Te} \), tetracycline resistance; \( \text{Gm} \), gentamicin resistance; \( \text{Km} \), kanamycin resistance.

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#### Plasmids

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<td>pRK2013</td>
<td>Km(^{\text{r}}); helper plasmid for triparental mating</td>
</tr>
<tr>
<td>pLS2</td>
<td>Broad-host-range cosmiding containing the \textit{P. aeruginosa} PA103 O11 gene cluster</td>
</tr>
<tr>
<td>pEX100T</td>
<td>5.8-kb gene replacement vector, ( \text{oriT; acb} \text{B}^{\text{r}} ); ( \text{Ap}^{\text{r}} )</td>
</tr>
<tr>
<td>pUCGm</td>
<td>Contains a ( \text{Gm} ) \text{r} \text{cassette (ac};\text{Cl}) \text{; ( \text{Ap}^{\text{r}} ), ( \text{Gm}^{\text{r}} )}</td>
</tr>
<tr>
<td>pEX100T-\text{wby};\text{ac};\text{Cl}</td>
<td>pEX100T with insertion of the ( \text{Gm} ) \text{r} \text{cassette (ac};\text{Cl}) \text{ in \text{wby}E}</td>
</tr>
<tr>
<td>pUC18</td>
<td>pUC18-derived broad-host-range shuttle vector; ( \text{Ap}^{\text{r}}/\text{Cb}^{\text{r}} )</td>
</tr>
<tr>
<td>pCD207</td>
<td>pUC18 with \textit{P. aeruginosa} PA103 \text{wby}E</td>
</tr>
<tr>
<td>pMMB66EH</td>
<td>8.8-kb broad host range expression vector; ( \text{Ap}^{\text{r}}/\text{Cb}^{\text{r}} )</td>
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<td>pPAC46</td>
<td>pMMB66EH with \textit{P. aeruginosa} 1244 \text{pIL4O}</td>
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<tr>
<td>pPAC24</td>
<td>pMMB66EH with \textit{P. aeruginosa} 1244 \text{pIL4}</td>
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were isolated with a Qiaquick gel extraction kit (QIAGEN) as specified by the manufacturer. Nucleotide sequence determination of both strands of the cloned DNA was carried out by the Biomolecular Research Facility (University of Virginia). Nucleotide sequence fragments were assembled and analyzed with the Gene Construction Kit (SciQuest, Research Triangle, NC) computer program.

In vitro mutagenesis and gene replacement. A wbjE mutation was made in the chromosome of P. aeruginosa PA103 as follows. A blunt-ended wbjE PCR product, as described above, was cloned into the Smal site in the PEX100T vector. A nonpolar mutation of wbjE was constructed in vitro by insertion of a gentamicin resistance gene (aacCI), recovered as an 854-bp SalI fragment from pUCGm, into the SalI site in wbjE on pEX100T. This construct (pEX100T-wbjE::aacCI) was introduced into the mobilizing E. coli strain SM10, and SM10(pEX100T-wbjE::
aacCI) was conjugated with P. aeruginosa PA103 by pelleting approximately equal numbers of donor (grown overnight at 37°C) and recipient (grown overnight without shaking at 42°C) strains in microcentrifuge tubes and spotting them onto the center of an L agar plate. After incubation for 12 to 18 h at 37°C, the cells were resuspended in 1 ml of L broth and plated on gentamicin-containing cetrimide plates. Colonies arising after 48 h were purified on the same medium and the swabbed onto L agar plates containing both gentamicin and sucrose. Since pEX100T contains the aacB gene, which renders gram-negative bacteria sensitive to sucrose, colonies arising on sucrose- and gentamicin-containing plates carried wbjE::aacCI on the chromosome and had lost the vector-associated aacB gene. These colonies were tested for sensitivity to carbencillin to confirm the loss of vector DNA. Gene replacement was confirmed by PCR amplification of chromosomal DNA with primers flanking the aacCI insertion site in wbjE.

Complementing plasmid. The same Vent-PCR product that was used for the gene replacement, as described above, was cloned in Smal-digested digested pUKU51. pUCGm. Restriction analysis indicated that the fragment was inserted in the orientation opposite that of the lacZ gene on the plasmid. The recombinant plasmid, referred to as pCD207, was transformed into P. aeruginosa PA103 wbjE::aacCI by the electroporation protocol of Enderle and Farwell (16). For pilin analysis, triparental mating was used to mobilize pMMB66EH-derivated plasmids into P. aeruginosa strains (42).

Isolation of LPS. P. aeruginosa LPS was isolated by the proteinase K digestion method of Hichens and Brown (24), followed by hot-phenol extraction with slight modifications. Cells grown overnight in L broth with appropriate antibiotics were equilibrated to an optical density of 0.5 at a wavelength of 600 nm. This volume of bacterial suspension was then transferred to a microcentrifuge tube and centrifuged at 13,100 x g for 5 min. The supernatant was removed, and the pellet was resuspended in 200 µl of lysis buffer (2% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1 M Tris [pH 6.8]), and boiled for 15 min. The lysed cells were treated with RNase and DNase for 30 min at 37°C. The cells were then digested for 3 h at 59°C with 120 µg of protease K. The digested lysates were then extracted with an equal volume of 90% phenol for 15 min at 65°C with periodic vortexing. After centrifugation at 16,000 g for 30 min at 4°C, the lipid A-containing aqueous layers from these extractions were removed, an equal volume of 90% phenol for 15 min at 65°C with periodic vortexing. After centrifugation at 16,000 g for 30 min at 4°C, the lipid A-containing aqueous layers from these extractions were removed, and the phenol-water layers were removed sequential extraction with diethyl ether. The aqueous layers from these extractions were removed, and the phenol-water layers were removed.

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Analysis of pilin glycosylation. The pilus glycosylation patterns were subjected to two overnight dialyses against 6 liters of deionized water (0.025% sodium azide) at 4°C. Dialyzed samples were analyzed via matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry by Mark Bier of the Mellon Institute Center for Molecular Analysis, Carnegie Mellon University, using a PerSeptive Biosystems Voyager STR with DE and a high mass detector. O-antigen repeating unit structures (28, 38) were drawn by using ChemDraw 9.0 for comparative analysis.

RESULTS

Structural comparison of O-antigen repeating units. In a previous study that investigated the glycan specificity of PilO, results indicated that nine P. aeruginosa serotypes (seven having known O-antigen structures) and E. coli O157, all possessed an O-subunit that could serve as the 1244 pilin glycan (Fig. 1). This suggested that common structures among these O subunits could be important for recognition by the 1244 glycosylation machinery. Comparative structural analysis of these sugars revealed drastic differences in charge, size, configuration, linkage, and number of sugars (Fig. 1). However, it is evident that the Und-PP/pilin-linked moieties (reducing end) share structural similarities (Fig. 1). Similarities include a C-1 linkage to the Und-PP carrier, a D configuration, a C-5 linkage to the adjacent sugar, and the presence of a 2-N-acetyl group (Fig. 1). Much of the variance between these O repeats lies within the remaining carbohydrate moieties (Fig. 1). For instance, the serotype O7 repeat of strain 1244 is a trisaccharide in which the sugar in the third position (counting the Und-PP/pilin-linked moiety at the reducing end as the first position), pseudaminic acid, is a 14-carbon sugar in the α configuration. This pseudaminic acid is linked (1→4) to the second sugar, xylose, a β-5-carbon sugar. In contrast, the O6 repeating unit is a tetrasaccharide, in which the second and third moieties are β-sugars (linked 1→3), consisting of seven and nine carbons, respectively, whereas the fourth moiety is in the α configuration and consists of six carbons. Structural comparison of the O repeats from Fig. 1 suggests that the moiety at the reducing terminus may possess the glycan recognition features necessary for catalysis by the pilin glycosylation machinery and that structural aspects of the other saccharides are nonessential.

Characterization of PA103 wbjE::aacCI. To experimentally determine whether the structural elements of the glycan necessary for glycosylation are within the reducing-end moiety of the O-antigen repeating unit, we required a mutant incapable of complete O-subunit assembly beyond the first sugar, β-N-acetyl fucosamine (Fig. 1). Because little is known of the O-antigen genetics of strain 1244 (serotype O7), we utilized a strain more commonly studied in O-antigen biosynthesis, PA103 (serotype O11), as a genetic background (12, 13). Notably, the first sugar moiety the O7 serotype O subunit is identical to that of the serotype O11 subunit (β-D-FucNAc; Fig. 1). Because wbjE encodes the only remaining hypothetical glycosyltransferase in the O11 biosynthetic gene cluster (12, 13),...
we hypothesized that WbjE mediated the attachment of α-L-FucNAc to undecaprenyl pyrophosphate-linked β-D-FucNAc during assembly of the O11 O-antigen repeating unit. If this was true, its inactivation would result in an incomplete O-antigen subunit consisting only of β-D-FucNAc. A mutation of wbjE was constructed in vitro by insertion of a gentamicin resistance gene (aacC1). LPS was isolated from PA103, PA103 wzy::aacC1 (13), PA103 wbjA::aacC1 (12), PA103 wbjE::aacC1, and PA103 wbjE::aacC1/pCD207 (Table 1). This LPS was subjected to SDS-PAGE, followed by silver staining, which revealed that PA103 makes a long side chain O-antigen (Fig. 2) that was not detectable in the LPS mutants. O antigen was noted in the PA103 wbjE::aacC1 mutant containing the complementing clone pCD207, indicating that the wbjE mutation was nonpolar (Fig. 2).

The size of the LPS core was compared in these strains (Fig. 2). LPS from all strains contained a band corresponding to a complete core (Fig. 2). As anticipated, LPS from PA103 and an O-antigen polymerase mutant, PA103 wzy::aacC1, contained core + 1 (13), and PA103 wbjA::aacC1 makes a core and a core + 2/3 (Fig. 2) (12). A small amount of LPS that migrated faster than the core + 2/3 was observed with PA103 wbjE::aacC1 (Fig. 2). Complementation with the plasmid pCD207 resulted in the expression of a complete core + 1 (Fig. 2). These data suggest a core + 1/3 phenotype for PA103 wbjE::aacC1, indicating that WbjE adds the α-L-FucNAc to undecaprenyl pyrophosphate-bound β-D-FucNAc (Fig. 3) and, as in PA103 wbjA::aacC1, an incomplete O subunit can be transferred to the core (12).

Pilin glycan substrate recognition. To determine whether the solitary reducing-end sugar in the O-antigen repeat could be transferred to P. aeruginosa 1244 pilin, we expressed pPAC46 (which contains pilAO1244) in PA103 wbjE::aacC1. To further examine the effect of incomplete synthesis of the O-subunit on glycosylation, pPAC46 was also expressed in PA103
Subsequently, plasmid-encoded pilin was assayed for glycosylation by subjecting extracts of these cells to Western blot analysis using a 1244 pilin-specific MAb as a probe (Fig. 4). Pilin produced by 1244N3/pPAC46 and 1244N3/pPAC24 were used as glycosylated and nonglycosylated standards (6), respectively. This blot revealed that pilin produced by PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46 had a slightly higher apparent molecular weight than the nonglycosylated pilin standard (Fig. 4). PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 synthesized pilin that had a slightly lower apparent molecular weight than both the glycosylated pilin standard and the heterologously glycosylated pilin of PA103/pPAC46 (Fig. 4) (14). However, the apparent molecular weight of PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 was higher than pilin produced by PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46 had a slightly higher apparent molecular weight than the nonglycosylated pilin standard (Fig. 4). PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 synthesized pilin that had a slightly lower apparent molecular weight than both the glycosylated pilin standard and the heterologously glycosylated pilin of PA103/pPAC46 (Fig. 4) (14). However, the apparent molecular weight of PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 was higher than pilin produced by PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46 (Fig. 4). This blot suggests that the incomplete O subunits synthesized by the \textit{wbjE} and \textit{wbjA} mutants are being utilized for glycosylation, since the difference in the apparent molecular weight of the pilin produced is consistent with the size differences we observed in the LPS (Fig. 2 and 4). It is therefore likely that in PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46, the pilin glycan is a single \textit{\beta}-FucNAc and a disaccharide in PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46, whereas PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 produces pilin glycosylated with the entire O11 subunit (14) (Fig. 1).

To conclusively determine the glycosylation status of pilin produced by PA103\textit{wbjA}\textsubscript{::}aacC1/pPAC46 and PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46, purified pilus preparations were treated with the endopeptidase, GluC (10), followed by MALDI-TOF analysis (Fig. 5). The largest pilin fragment produced by GluC digestion (Fig. 5A) consists of the C-terminal 51 residues, which contains the glycosylation site, with a molecular weight predicted from the amino acid sequence to be 5,331 (10). GluC-treated pilins produced by 1244N3/pPAC46 and 1244N3/pPAC24 were used as glycosylated and nonglycosylated controls (6), respectively (spectra not shown; Fig. 5C). The largest fragment mass from the GluC-treated glycosylated pilin control contained covalently bound material with a molecular weight of 670 (Fig. 5C), which is consistent with the value determined for the strain 1244 glycan structure as previously determined (7). The nonglycosylated pilin produced a GluC fragment within the range of the value predicted by the amino acid sequence (Fig. 5C). Endopeptidase treatment of pilin produced by PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46 produced a fragment with a molecular weight of 5,705 (±20) with no signal seen at the mass predicted by the pilin gene, indicating the addition of material with the molecular weight of ~374 had been covalently linked to the protein (Fig. 5B and C). Endopeptidase treatment of pilin produced by PA103\textit{wbjE}\textsubscript{::}aacC1/pPAC46 produced a fragment with a mass of 5,519 Da (±20), revealing the presence of additional covalently bound material with a molecular weight of ~188 (Fig. 5B and C). These data support the description of

\begin{figure}
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\includegraphics[width=0.8\textwidth]{pathway.png}
\caption{Proposed pathway for the assembly of \textit{P. aeruginosa} serogroup O11 O-antigen and pilin glycosylation. WbpL initiates O-antigen synthesis by the addition of nucleotide-activated \textit{\textit{\beta}}-acetyl fucosamine onto Und-P (41). We propose that WbjE adds the second \textit{\beta}-acetyl fucosamine, whereas WbjA adds the terminal glucose to complete the O11 subunit (12). Subsequent O-antigen biosynthesis steps involve the transportation to the periplasm (Wzx), polymerization of O-antigen units (Wzy), chain length determination (Wzz), and linkage of the O antigen to the LPS core (WaaL) (13, 41). We have previously noted that LPS from PA103\textit{wbjA}\textsubscript{::}aacC1 contains a core \textit{\textit{\beta}}/3, indicating that the O-antigen subunit need not be complete prior to transfer to the lipid A-core (12). In pilin glycosylation, PilO mediates the transfer of a single, complete O-subunit from Und-PP to pilin (14).}
\end{figure}
the LPS phenotypes (Fig. 2) and the pilin Western blot analysis (Fig. 4) in reference to the size of the incomplete O-antigen subunits. The additional value of \[\text{H}374\] for GluC-treated PA103 \(\text{wbjA}::\text{aacC1}/\text{pPAC46} \text{ pilin}\) is consistent with the molecular weight predicted for the first two carbohydrate moieties of the O11 subunit (12). This suggested that the pilin glycan produced here consists of the disaccharide, \[\text{H}9251\] -L-FucNAc-(1\(\rightarrow\)3)-\[\text{H}9252\] -D-FucNAc-(1\(\rightarrow\)Ser148). Pilin produced by PA103 \(\text{wbjE}::\text{aacC1}/\text{pPAC46}\) contained a modification with a molecular weight of 188, a value consistent with the first carbohydrate moiety of the O11 subunit, suggesting that this pilin glycan likely consists of \[\text{H}9252\] -D-FucNAc-(1\(\rightarrow\)Ser148). The unlabeled peak detected in both spectra (Fig. 5B) was likely generated due to the presence of one of the peptide fragments of the GluC-digested 1244 pilin that did not contain the glycan or was due to a contaminant of the pilus preparation. These results confirm that WbjE adds the second sugar, \(\alpha\)-L-N-acetyl fucosamine, to the O11 O-antigen subunit. Furthermore, the first sugar of this subunit, \(\beta\)-D-N-acetyl fucosamine, can be transferred to the core oligosaccharide, as well as pilin, indicating that this sugar contains the substrate recognition features necessary for catalysis by both WaaL and PilO.

**DISCUSSION**

The results of this study indicated that WbjE catalyzed the addition of the second sugar, \(\alpha\)-L-N-acetyl fucosamine, to the O11 subunit (Fig. 3). Because the first sugar of the O11 subunit is identical to the reducing-end moiety of the O7 subunit of strain 1244, and therefore the 1244 pilin glycan (7), \(\text{pilAO}_{1244}\) was expressed in the \(\text{wbjE}\) mutant and pilin was tested for glycosylation to analyze glycan substrate recognition. Pilin produced contained a modification that had a molecular weight consistent with a single \(\beta\)-D-FucNAc. This suggested that the reducing-end moiety of the O-antigen subunit possesses the necessary structures for glycosylation and that the remaining sugars of the glycan precursor are nonessential.

Previous studies have shown that incomplete O subunits can be ligated to the core oligosaccharide (12, 17, 27, 60). The data presented here indicated that a single \(\beta\)-D-FucNAc was transferred to the core oligosaccharide in the \(\text{wbjE}\) mutant. It is therefore likely that the structural information recognized by WaaL of \(P.\ aeruginosa\) PA103 was also in the reducing sugar of the O repeat. This interpretation is strengthened by previous data indicating that WaaL is capable of ligating a number of different polysaccharides to the core (19), since all of the O repeats tested possessed similar reducing sugars. In addition, gene replacement of \(\text{glf}\) in an \(E.\ coli\) K-12 O-antigen mutant strain produced an LPS phenotype like that of the PA103 \(\text{wbjE}::\text{aacC1}\), indicating a similarity between WaaL specificity of both systems (17). These results are consistent with an earlier study showing that Wzx from several different O-antigen systems seemed to have specificity for the sugar at the reducing terminus of Und-PP-linked O subunit (32). Collectively, these data suggest that among related groups of gram-negative bacteria the reducing sugar of the O repeat acts as a...
FIG. 5. GluC digestion of 1244 pilin. (A) Amino acid sequence of 1244 pilin in which vertical arrows indicate GluC cleavage sites. The largest GluC fragment (underlined) consists of residues 98 to 148 and is predicted to have a molecular mass of 5,331 Da. This fragment contains the 1244 pilin glycosylation site. Set148 (10). (B) MALDI-TOF spectra for GluC-treated pilin produced by PA103 wbjA::aacC1/pPAC46 and PA103 wbjA::aacC1/pPAC46 are shown. (C) The deduced glycan mass is listed for each of the pilins subjected to GluC digestion and MALDI-TOF.

universal foundation, in which enzymes involved in the construction of surface antigens are compatible. Therefore, a variety of sugars can be added to this foundation sugar, which is recognized by machinery critical for surface display, such as PilO and WaaL. If this is true, it appears as if evolution has provided an efficient means to ensure maximum surface diversity by providing congruity between products of horizontally transferred genes. For example, if a heterologous O-antigen biosynthetic operon was passed from one bacterium to another, the saccharide produced would likely be compatible with endogenous PilO and/or WaaL and therefore surface expressed, due to the similarities in O-subunit reducing-end sugars. Because waaL and pilO are not located in the same operon as the O-antigen biosynthetic genes (6, 41), there is little likelihood of cohorizontal transfer, underlying the necessity for cohesion with heterologous O antigens. Notably, WaaL mediates the addition of polymerized O antigen and also has specificity for the common antigen, since this enzyme mediates the ligation of both polysaccharides to the core-lipid A (1). However, PilO does not transfer polymerized O antigen or common antigen subunits onto pilin, since each pilin monomer contains a single glycan identical to the O-antigen repeating unit with no evidence of alternate glycoforms (7). Because PilO does not transfer polymerized O antigen to pilin, this suggests that structural attributes of the long side chain O antigen-Und-PP contribute to the loss of affinity for the pilin glycosylation machinery. This may be due to the size of the polymerized O antigen or to an increase of affinity for WaaL. Additional work will be needed for clarification.

To discern structural components of the reducing-end sugar of the O-repeat important for glycan substrate recognition, cloned heterologous O-antigen biosynthetic operons producing structurally distinct reducing end sugars (29) could be expressed in P. aeruginosa 1244, where the pilin produced may be assayed for presence of the heterologous glycan. For example, the O subunit of Burkholderia cepacia serotype B [β-D-Gal-(1→3)-α-D-Fuc] or those of various P. syringae strains (repeats of L- and/or D-Rha in various arrangements) contain sugars at their reducing termini dissimilar from those discussed here (29). The results of such studies may serve to define the glycan specificity of the 1244 pilin glycosylation reaction. Until these experiments are conducted, one cannot dismiss the possibility that the Und-PP carrier alone, or a minimal component of the bound carbohydrate, such as the C-1, or the ether oxygen, contains the substrate recognition properties for the 1244 pilin glycosylation machinery.

A plasmid encoding pilAO1244 was expressed in the wbjE and wbjA mutants and, subsequently, pilin produced were extracted and subjected to GluC digestion in which fragments were analyzed by MALDI-TOF. Due to the intrinsic properties of pilin, such as extracellular location, fibrous morphology, and solubility in water, pilin can be easily purified from cell cultures. In addition, GluC digestion separates pilin into pieces readily detectable by MALDI-TOF analyses, in which the fragment containing the glycan is the largest (10). This methodology not only represents an efficient means of analysis of the pilin glycan molecular weight but also an expedient technique for analysis of an organism’s O-antigen repeating unit. In this novel approach, pilin is used as an O-subunit “trap.” Therefore, one may analyze the repeating unit apart from the core and lipid A, a previously unattainable endeavor. The sugars of the glycan could be sequenced and further analyzed by using procedures previously described (7). Alternative means to analyze O antigen include acidic and alkaline degradation of extracted LPS, processes that do not disrupt the core-O-polysaccharide bond, and could potentially chemically modify the O antigen (28).

Previously, LPS biosynthesis and eukaryotic protein glycosylation have been hypothesized to have a common evolutionary origin (4, 5) based on similarities between the eukaryotic dolichol and prokaryotic Und-P carrier lipids, the metabolic assembly of oligosaccharides, in addition to sequence similarity between eukaryotic and prokaryotic glycosyltransferases. Feldman et al. suggested that the N-linked glycosylation systems of C. jejuni and eukaryotes are homologous processes (18). Similar to the 1244 pilin glycosylation system, the C. jejuni N-linked glycosylation system has shown the capability to utilize O antigen for protein glycosylation in an engineered E. coli strain. When pglB from C. jejuni was coexpressed with different LPS biosynthesis gene clusters in an E. coli LPS mutant (waaL and the rhamnosyl transferase wbbL), production of recombinant AcrA containing covalently bound, full-length O-antigen occurred (18). If the eukaryotic N-linked glycosylation system had evolved from a similar system to that of C. jejuni, it is plausible that 1244 pilin glycosylation represents an ancestral form. This conclusion is based on the intrinsic utilization of the O subunit as the 1244 pilin glycan and the above-mentioned
Because O antigen can be covalently linked to protein by N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in E. coli, Shigella sonnei serotype O157 (29). As previously mentioned, the E. coli serotype O157 subunit can be utilized as the 1244 pilin glycan, whereas S. sonnei and F. tularensis appear to produce O subunits with reducing-end moieties that are structurally compatible for glycosylation (Fig. 6B). Other than generating a vaccine using pilin as the glycosylated protein, the breadth of protection may be increased by mutation of nonpilin proteins, such as toxoids, to possess the 1244 pilin glycosylation substrate (25). For example, combining expression of the S. sonnei O-antigen gene cluster and mutated shiga toxin, allowing compatibility with the 1244 glycosylation machinery, may yield a biologically produced, bivalent antitoxin, anti-LPS vaccine.

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