Pseudomonas aeruginosa D-Arabinofuranose Biosynthetic Pathway and Its Role in Type IV Pilus Assembly*§

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Pseudomonas aeruginosa strains PA7 and Pa5196 glycosylate their type IVa pilins with α1,5-linked D-arabinofuranose (D-Araf), a rare sugar configuration identical to that found in cell wall polymers of the Corynebacterineae. Despite this chemical identity, the pathway for biosynthesis of α1,5-D-Araf in gram-negative bacteria is unknown. Bioinformatics analyses pointed to a cluster of seven P. aeruginosa genes, including homologues of the Mycobacterium tuberculosis genes Rv3806c, Rv3790, and Rv3791, required for synthesis of a polyenyl-linked D-ribose precursor and its epimerization to D-Araf. Pa5196 mutants lacking the orthologues of those genes had non-arabinosylated pilins, poor twitching motility, and significantly fewer surface pili than the wild type even in a retraction-deficient (pilT) background. The Pa5196 pilus system assembled heterologous non-glycosylated pilins efficiently, demonstrating that it does not require post-translationally modified subunits. Together the data suggest that pilins of group IV strains need to be glycosylated for productive subunit-subunit interactions. A recombinant P. aeruginosa PA01 strain co-expressing the genes for D-Araf biosynthesis, the pilin modification enzyme TfpW, and the acceptor PilAIV produced arabinosylated pili, confirming that the Pa5196 genes identified are both necessary and sufficient. A P. aeruginosa epimerase knock-out could be complemented with the corresponding Mycobacterium smegmatis gene, demonstrating conservation between the systems of the Corynebacterineae and Pseudomonas. This work describes a novel Gram-negative pathway for biosynthesis of D-Araf, a key therapeutic target in Corynebacterineae.

The Gram-negative opportunistic pathogen Pseudomonas aeruginosa can post-translationally modify its flagellins (the major subunits of flagella) and pilins (the major subunits of type IV pilis (T4P) via O-glycosylation with strain-specific sugars (1–3). The post-translationally modifications are thought to modulate interactions with eukaryotic hosts because both flagella and T4P are exposed on the cell surface and are involved in colonization. In the case of T4P, loss of pilin glycosylation has been demonstrated to decrease fitness in a mouse model of acute infection (4). Each strain of P. aeruginosa expresses one of five alleles of type IVa pilin (5), and those of groups I (PilA) and IV (PilAIV) have been experimentally demonstrated to be glycosylated by distinct mechanisms (3, 6, 7). Group I pilins are modified on a conserved C-terminal Ser residue with a single lipopolysaccharide (LPS) O-antigen unit by the TfpO (also called PilO) O-oligosaccharyltransferase (6). Strains with different LPS serotypes express group I pilins modified with glycans matching that of O-antigen of the background strain (8). Inactivation of tfpO prevents pilin glycosylation but does not block expression of surface pili or pilus-mediated “twitching” motility (1).

In contrast, PilAIV is modified on multiple Ser and Thr residues in the predicted αβ-loop and β-sheet regions with D-arabinofuranose (D-Araf) residues arranged as monomers or α1,5-linked dimers, trimers, and potentially longer polymers (3). D-Araf is an uncommon sugar in prokaryotes. The α1,5-linked configuration is found mainly in the cell wall polymers lipoarabinomannan (LAM) and arabinogalactan of Corynebacterineae, a group including the major human pathogens Mycobacterium tuberculosis, Mycobacterium leprae, and Mycobacterium avium (9). We showed previously (7) that antibodies raised against LAM recognize glycosylated PilAIV and vice versa. The TfpW protein encoded immediately downstream of the pilin gene was implicated as a glycosyltransferase C family pilin O-oligosaccharyltransferase because tfpW knock-out and putative active site point mutants express non-glycosylated pilins (7). The loss of pilin arabinosylation markedly decreased the amount of surface pili expressed by the tfpW mutant, implying that glycosylation may be necessary for normal pilus assembly (7). This idea was supported by the observation that overexpression of PilAIV in a non-piliated mutant of P. aeruginosa lacking the glycosylation system did not restore motility or pilation (10).

In addition to the Corynebacterineae, D-Araf has been identified as a component of nodulation factors in some strains of rhizobia and of some O-antigens (11). The pathway for its biosynthesis in Gram-negative bacteria, including P. aeruginosa, is unknown. Synthesis could proceed via a nucleotide sugar precursor as is common for the majority of Gram-negative cell surface glycans (12, 13) or by a lipid-linked precursor as described for the Corynebacterineae (11). Here we describe the
Identification of seven *P. aeruginosa* genes potentially involved in D-Ara biosynthesis and show that three are essential for pilin arabinosylation, normal pilus assembly, and twitching motility. The pilin arabinosylation system was reconstituted in a laboratory strain of *P. aeruginosa* that does not normally express glycosylated pilin, confirming that the genes identified were both necessary and sufficient. The D-ribose to D-Ara epimerization step of arabinian biosynthesis was recently hailed as a “magic drug target” as compounds targeting this aspect of the pathway effectively kill both intracellular and extensively drug-resistant *M. tuberculosis* (14–16). The *P. aeruginosa* pilin arabinosylation system will be useful for the study of D-Ara biosynthesis and the identification of new inhibitors of the pathway.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains used in this study are listed in Table 1. Bacteria were maintained at −80 °C as glycerol stocks and routinely grown in Luria-Bertani (LB) broth or on LB agar plates (1.5% agar) with antibiotics where indicated at the following concentrations: for *E. coli*, 15 μg/ml gentamicin or 100 μg/ml ampicillin; and for *P. aeruginosa*, 30 μg/ml gentamicin or 200 μg/ml carbenicillin. 1- Arabinose was included at specific concentrations where indicated to induce expression from the pBADGr ara promoter. For complementation of Pa5196, 0.01% l-arabinose was used, whereas 0.05% l-arabinose was used for complementation of PAO1.

**Recombinant DNA Techniques**—Standard PCR and cloning techniques were used to generate knock-out and complementation constructs as listed in Table 1 using the primers listed in supplemental Table S1. *Escherichia coli* DH5α or the dam−/dcm− strain C2925 (New England Biolabs) were used for cloning, whereas *E. coli* SM10 was used to introduce knock-out constructs into *P. aeruginosa* by biparental mating. All restriction and DNA polymerase enzymes were from Fermentas and used according to the manufacturer’s recommendations.

**Twitching Motility Assays**—Twitching motility was measured as described previously (3) with modifications. Briefly, bacterial strains were stab-inoculated to the bottom of 1% LB agar plates containing antibiotics and l-arabinose. After a 48-h incubation at 37 °C in a humidified container, the agar was carefully removed, and the twitching zones on the plastic surface were stained for 15 min with 1% (w/v) crystal violet in 3.0 ml of PBS depending on the size of the pellet, and dialyzed in 50 mM NH₄HCO₃ using a dialysis cassette (Slide-A-Lyzer, 3500 molecular weight cutoff, Thermo Scientific).

**Mass Spectrometry Analyses**—The intact mass of pilins was determined as described previously (7). Briefly, pilin solutions were desalted by centrifugal filtration (Millipore 0.5-ml Amicon Ultra filter unit, 3000 molecular weight cutoff membrane), evaporated to dryness on a Savant centrifugal evaporator, and resuspended in 10 μl of concentrated formic acid. The proteins were solubilized by the addition of 90 μl of hexafluoroisopropanol. For some of the pilin samples, the quality of the electrospray ionization-MS spectra was significantly improved by the addition of 200–300 μl of deionized water. Mass spectra were acquired on a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Waters). Pilin solutions were infused at 1 μl/min into the nanoelectrospray interface, and spectra were recorded in the m/z range 800 to 2000 (one acquisition per s). MaxEnt (Waters) was used to derive protein molecular weight profiles from the spectra.

**RESULTS**

**Identification of Putative D-Araf Biosynthetic Pathway in *P. aeruginosa***—Strains Pa5196 and PA7 express pilins (PilAIV) modified with D-Araf (3, 7). Using the publicly available PA7 genome sequence (17), we searched for genes that could encode the biosynthesis of D-Araf. Our previous work (7) showed that only group IV strains produced pilins modified with D-Araf, implying that only they have the biosynthetic machinery to make the sugar. Examination of available *P. aeruginosa* genomes revealed a number of open reading frames (ORFs)
## TABLE 1

### Strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant characteristics | Source or Ref. |
|----------------------------|--------------------------|----------------|

#### E. coli strains
- **DH5α**
  - F* endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR supE44 thi-1 hsdR17(rk−,mrk−), λ− ara-14 leuB6 thiA1 lacY1 tsx78 galU44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) T6*<br>  - New England Biolabs
- **C2925**
  - Km, thi-1, brl, leu, tonA, lacY1, supE44, recA1, RFP-2′-Te-Mu, pir<br>  - Invitrogen

#### M. smegmatis mc2155

#### M. tuberculosis H37Rv

#### P. aeruginosa strains
- **PAO1**
  - Group II T4P<br>  - Laboratory stock
- **PA196**
  - Group IV T4P; rectal isolate<br>  - This study
- **Pa5196 tfpW/FRT**
  - EZ::Tn FRT insertion in tfpW<br>  - This study
- **Pa5196 6246:FRT**
  - FRT insertion at position 309 (EcoRV) in PsPA7_6246<br>  - This study
- **Pa5196 6247:FRT**
  - FRT insertion at position 173 (AfeI) in PsPA7_6247<br>  - This study
- **Pa5196 6248:FRT**
  - FRT insertion at position 569 (XhoI) in PsPA7_6248<br>  - This study
- **Pa5196 6249:FRT**
  - FRT insertion at position 105 (NruI) in PsPA7_6249<br>  - This study
- **Pa5196 6250:FRT**
  - FRT insertion at position 336 (BclI) in PsPA7_6250<br>  - This study
- **Pa5196 6251:FRT**
  - FRT insertion at position 422 (EcoRI) in PsPA7_6251<br>  - This study
- **Pa5196 6252:FRT-6251**
  - FRT insertion at position 540 (NruI) on the pilT background<br>  - This study
- **Pa5196 tfpW pilT**
- **Pa5196 PfP7_6248 pilT**
  - FRT insertion at position 540 (NruI) in pilT on the tfpW background<br>  - This study
- **PAO1 NP + AWX**
  - PAO1 pilA mutant complemented with pilA*, tfpW, tfpX from Pa5196 in pBADGr<br>  - This study

#### Plasmids
- **pEX18Ap**
  - Carbenicillin-resistant suicide vector used for gene replacement<br>  - 34
- **pLPL2**
  - Suicide vector encoding Fp recombines<br>  - 34
- **pFS856**
  - Source of FRT-flanked gentamicin resistance cassette<br>  - 34
- **pUCP20**
  - Broad host range arabinose inducible vector used for complementation; gentamicin resistance marker<br>  - 35
- **pBADGr**
  - Carbenicillin-resistant vector used for complementation; TEM-1 resistance marker<br>  - 34
- **pEX18Ap + 6246:GmFRT**
  - PsPA7_6246 knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 309 (EcoRV) in PsPA7_6246<br>  - This study
- **pEX18Ap + 6247:GmFRT**
  - PsPA7_6247 knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 173 (AfeI) in PsPA7_6247<br>  - This study
- **pEX18Ap + 6248:GmFRT**
  - PsPA7_6248 knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 569 (XhoI) in PsPA7_6248<br>  - This study
- **pEX18Ap + 6249:GmFRT**
  - PsPA7_6249 knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 105 (NruI) in PsPA7_6249<br>  - This study
- **pEX18Ap + 6251:GmFRT**
  - PsPA7_6251 knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 336 (BclI) in PsPA7_6251<br>  - This study
- **pEX18Ap + pilT:GmFRT**
  - pilT knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position (NruI) in pilT<br>  - 10
- **pBADGr + AWX**
  - Complementation construct carrying the Pa5196 pilA, tfpW, and tfpX genes<br>  - 3
- **pBADGr + PsPA7_6248**
  - Complementation construct carrying the Pa5196 PsPA7_6248 gene<br>  - This study
- **pBADGr + Pa5196**
  - Complementation construct carrying the Pa5196 PsPA7_6246 gene<br>  - This study
- **pBADGr + MSMEG6382**
  - Complementation construct carrying the M. smegmatis MSMEG6382 gene<br>  - This study
- **pBADGr + Rv3790**
  - Complementation construct carrying the M. tuberculosis Rv3790 gene<br>  - This study
- **pUCP20 + 6245–6251**
  - Complementation construct carrying the Pa5196 PsPA7_6245–6251 genes, lac promoter<br>  - This study
- **pUCP20 + 6247–6251**
  - Complementation construct carrying the Pa5196 PsPA7_6247–6251 genes, lac promoter<br>  - This study
- **pUCP20 + 6245–6249**
  - Complementation construct carrying the Pa5196 PsPA7_6245–6249 genes, lac promoter<br>  - This study
- **pUCP20 + 6246-6249, 6247+GmFRT**
  - Complementation construct carrying the Pa5196 PsPA7_6246-6249, 6247+GmFRT genes, lac promoter<br>  - This study
- **pUCP20 + 6246–6249**
  - Complementation construct carrying the Pa5196 PsPA7_6246–6249 genes, lac promoter<br>  - This study
- **pUCP20 + 6246–6249, 6247+GmFRT**
  - Complementation construct carrying the Pa5196 PsPA7_6246–6249, 6247+GmFRT genes, lac promoter<br>  - This study
- **pUCP20 + 6246–6249, 6247+GmFRT**
  - Complementation construct carrying the Pa5196 PsPA7_6246–6249, 6247+GmFRT genes, lac promoter<br>  - This study
- **pUCP20 + 6246–6249, 6247+GmFRT**
  - Complementation construct carrying the Pa5196 PsPA7_6246–6249, 6247+GmFRT genes, lac promoter<br>  - This study
unique to PA7, many of which were annotated as phage-related genes or insertion sequences (data not shown), but there were no candidate arabinose biosynthetic genes among them. We next searched for *P. aeruginosa* orthologues of the *M. tuberculosis* ORFs and those of strain PA7 encoding the oxidoreductase DprE1 and the short-chain dehydrogenase/reductase DprE2, respectively, which together catalyze the two-step 2′-epimerization of decaprenyl-P-D-ribose to the essential precursor, decaprenyl-P-D-arabinofuranose (Fig. 1A) (18). BLASTP searches of available *P. aeruginosa* genomes (19) using Rv3790 and Rv3791 as query sequences revealed potential homologues in all genomes examined, although the overall similarities were higher than those of strain PA7 (33 and 30% identity, respectively). The genes were located in one of two adjacent but divergently oriented clusters containing a total of seven genes (Fig. 1B). The five-gene cluster encodes a hypothetical protein (6247; the prefix PsPA7 is omitted from this point forward for brevity) with weak similarity to GtRA-like glycosyltransferases involved in LPS and teichoic acid synthesis, the aforementioned homologues of *M. tuberculosis* Rv3790 and Rv3791 (6248 and 6249), a hypothetical protein (6250) with limited similarity to glycosyltransferases, and a hypothetical protein (6251) with similarity to 3′-acyltransferases (Fig. 1B). Examination of the potential operon structure using the MicrobesOnline Operon Prediction algorithm (20) suggested that only the first three genes in the cluster were likely to be co-transcribed. Two *P. syringae* strains (*P. syringae* sv. *phaeolicola* 1448A and *P. syringae* pv. *syringae* B728A) also have contiguous homologues of Rv3790 and Rv3791 (Fig. 1B). A search of the NCBI databases with the PA7 genes 6248 and 6249 revealed that the closest hits outside of *Pseudomonas* were in genera found in the environment such as *Chlorobium*, *Syntrophobacter*, *Sulfurovum*, *Rhodobacter*, and *Rhizobium* where they are also arranged as a contiguous pair although not in syntenic clusters (data not shown). Hits in other *P. aeruginosa* genomes had less similarity to the *Mycobacterium* genes and were not contiguous; therefore, they were unlikely to be genuine orthologues, a hypothesis supported by functional analyses (below).

The two genes in the divergently oriented cluster (6246 and 6245; Fig. 1B) are homologues of the *M. tuberculosis* genes Rv3806c and Rv3807c, respectively, encoding the decaprenyl-P-ribose-5-P synthetase (21) and a putative decaprenyl-phosphoryl-5-phosphoribose phosphatase required to form decaprenyl-P-D-ribose, the precursor of decaprenyl-P-D-arabinofuranose (Fig. 1A). Homologues of these genes were absent from other *Pseudomonas* genomes with the exception of the above *P. syringae* strains in which the homologues of Rv3806c, Rv3790, and Rv3791 are contiguous (Fig. 1B). In PA7, the putative D-arabinose biosynthetic genes are adjacent to ORFs 6244 through 6237, required for the biosynthesis, polymerization, and export of the nucleotide sugar GDP-D-rihamnose to form the A-band O-antigen common to all *P. aeruginosa* strains (22). This genetic organization differs from that of other *P. aeruginosa* strains (supplemental Fig. S1) where the A-band O-antigen biosynthetic genes are adjacent to an unrelated gene cluster potentially involved in the biosynthesis of an unknown polysaccharide as it includes putative glycosyltransferase genes. Related genes are located downstream of the D-Ara cluster in
P. aeruginosa D-Araf Biosynthesis

**A.**

![Image](49x428 to 299x733)

**B.**

![Image](1x1 to 2x2)

**FIGURE 2. Phenotypes of mutants lacking 6246 to 6251.** A, twitching motility of the Pa5196 wild type, the tfpW mutant, and the 6246 through 6251 mutants. The gene map from Fig. 18 is shown at the top for reference. B, pilus preparations of each of the strains in A separated with SDS-PAGE and stained with Coomassie Brilliant Blue or probed with antibodies to PilAIV (anti-pilin) or to M. tuberculosis lipomannan (anti-LAM). M, molecular mass markers in kDa. Mutations that disrupt pilin glycosylation as shown in the bottom panel also reduce the amount of recoverable surface Pil and twitching motility.

PA7 but in the opposite orientation, suggesting that its 6245–51 cluster was acquired via horizontal gene transfer. Evidence of homologous recombination within the first gene of the A-band LPS cluster is apparent upon comparison of the RmD sequences of PA7 with those of other P. aeruginosa strains. The N terminus of PA7 RmD is divergent, whereas the C terminus is conserved (supplemental Fig. S1). In contrast, Gmd, which is encoded immediately downstream of RmD, is completely conserved among P. aeruginosa strains (data not shown).

**Validation of Gene Assignment by Mutagenesis and Complementation**—To test for the potential involvement of the 6245–51 genes in D-Araf biosynthesis, we generated single knock-outs of 6246 through 6251 in strain Pa5196 as well as a double knock-out of 6250 and 6251. The requirement for 6245 in D-Araf/biosynthesis was tested by reconstitution experiments (below). Pa5196 was used as the parent strain for mutagenesis because PA7 is multidrug-resistant and poorly piliated (7, 17), making it unsuitable. PCR and DNA sequencing were used to verify the presence of the genes of interest in Pa5196 (data not shown); in the absence of a Pa5196 genome sequence, we use the PA7 gene numbering. Fig. 2 shows the phenotypes of the resulting Pa5196 single mutants with respect to twitching motility (Fig. 2A) and surface pilation and pilin modification (Fig. 2B). The phenotypes of the 6250–51 double mutant were indistinguishable from those of the 6250 and 6251 single mutants (data not shown).

Inactivation of 6246, 6248, or 6249 resulted in a marked decrease in twitching motility compared with the wild type similar to that of the previously characterized tfpW mutant (7) (Fig. 2A). Western blot analyses of sheared surface proteins showed that each of these mutants expressed pilins of reduced mass that failed to react with anti-LAM serum (Fig. 2B). In contrast, disruption of the other genes (6247, 6250, or 6251; Fig. 2) did not affect twitching motility, pilus modification, or pilus assembly, suggesting that they are dispensable for biosynthesis of D-Araf. Because of the proximity of the A-band O-antigen cluster to the genes of interest (supplemental Fig. S1), we also generated a mutant in 6243 (gmd), which encodes the first committed step of D-rhamnose biosynthesis (23). The potential participation of the A-band pathway in D-Araf biosynthesis was ruled out as the gmd mutation had no effect on motility or pilation (data not shown).

The inability of P. aeruginosa strains other than PA7 or Pa5196 to glycosylate PilAIV, when it is expressed in trans suggests that they lack the ability to synthesize D-Araf even though they have potential Rv3790 and Rv3791 orthologues. When the RV3790 homologue PA1416 from the group II strain PAO1 was expressed in the Pa5196 6248 mutant, it did not complement motility (Fig. 3A), pilation, or glycosylation (Fig. 3B), suggesting that, despite having modest sequence similarity (27% identity), PA1416 is not an Rv3790 orthologue. To test for conservation of the pathway between Pseudomonas and Mycobacterium, the 6248 mutant was complemented with the corresponding ORFs from Mycobacterium smegmatis (MSMEG _6382) and _M. tuberculosis_ (Rv3790) (Fig. 3). Despite the high level of sequence identity (83%) between the Mycobacterium gene products, only the _M. smegmatis_ gene complemented the Pa5196 mutant.

Reconstitution of D-Araf Biosynthetic Pathway in P. aeruginosa PAO1—Having determined that 6246, 6248, and 6249 were necessary for biosynthesis of D-Araf in Pa5196, we tested whether they were sufficient. We showed previously that a recombinant _P. aeruginosa_ PAO1 strain expressing the Pa5196 pilin and TfpW had poor twitching motility and expressed low levels of non-glycosylated pilin, implying pilus assembly defects in the absence of glycosylation (10). Transformation of that strain with the 6245–6251 genes increased twitching motility to levels commensurate with the native PAO1 pilin (Fig. 4A), and the pilin subunits were post-translationally modified with D-Araf (Fig. 4B). A shorter 6247–6251 construct did not complement glycosylation and instead suppressed motility relative to the control, suggesting that one or both of the divergently oriented genes 6245 and 6246 are required for D-Araf biosynthesis in the PAO1 background and that the absence of the necessary gene(s) had a detrimental effect. A construct expressing 6246–49 was sufficient for complementation, showing that the putative phosphatase gene 6245 was not essential and confirming that the 6250–51 genes were dispensable. To define the minimal number of genes required for the production of D-Araf in PAO1, we further disrupted the 6247 gene, which gave no change in phenotype when inactivated in Pa5196 (Fig. 2), in the 6246–49 construct. The resulting three-gene construct com-
implemented the recombinant strain (Fig. 4), showing that 6246, 6248, and 6249 are sufficient for synthesis of arabinosylated pili.

Mass Spectrometry Analysis of Pilins from Mutant and Recombinant Strains—The pilins of Pa5196 are variably modified at Thr-64 and Thr-66 with at least trisaccharides of \( 1,5-D\text{-Ara}f \) and at positions Ser-81, Ser-82, Ser-85, and Ser-89 with at least mono- or disaccharides (7). Although TfpW was implicated as the sole enzyme involved in transfer of \( D\text{-Ara}f \) to the pilins, it was unclear whether it was also responsible for translocating the sugars from the cytoplasm to the periplasm for attachment to the pilins (flippase activity) and/or forming the \( 1,5 \) linkage between \( D\text{-Ara}f \) residues (glycosyltransferase activity). The latter hypothesis is based on the limited sequence similarity of TfpW to the EmbA, EmbB, and EmbC glycosyltransferases (Fig. 1B) involved in formation of \( 1,2-, 1,3-, \) and \( 1,5\)-linked arabinans in mycobacteria (7). The only potential glycosyltransferase encoded within the \( D\text{-Ara}f \) biosynthetic cluster, 6250, was dispensable for synthesis and addition of \( D\text{-Ara}f \) residues to the pilins (Figs. 2 and 4), but we could not rule out a role for its product in formation of the \( 1,5 \) linkage. Therefore, mass spectrometry was used to determine whether the patterns of glycosylation on pilins recovered from the Pa5196 6250–51 mutant and the recombinant PAO1 strains were similar to those reported previously (7) for pilins from Pa5196. Fig. 5A shows that pili isolated from the Pa5196 6250–51 mutant had a pattern of glycosylation similar to the wild type (7) with up to 18 sugars attached to the protein. Because only five potential sites of modification on PilAIV were identified previously (7), the data indicate that polymers of \( D\text{-Ara}f \) residues seen on the Pa5196 pilin, although the distribution of masses was broader with increased representation of pilins modified with seven (mass, 16,053 Da) or fewer sugar...
residues and some with up to 21 (mass, 17,893 Da) residues. Interestingly, whereas elimination of 6250 and 6251 had little effect on the glycosylation pattern (Fig. 5C), consistent with the mutant phenotypes, loss of the putative phosphatase gene 6245 increased the abundance of less heavily glycosylated species and decreased the abundance of highly glycosylated peaks (Fig. 5D). Disruption of 6247 with a GmR cassette on the 6245–6249 or 6246–6249 plasmids had subtle effects on the pattern of glycosylation (Fig. 5, E and F) that were likely due to increased 6248–6249 expression from the constitutive promoter of the resistance marker. Together with the knock-out phenotypes in Fig. 4, the data show that 6246, 6248, and 6249 are the minimum number of genes required for D-Araf biosynthesis.

FIGURE 5. Mass spectrometry analysis of intact pilins. Pili were isolated and subjected to electrospray ionization-MS as described under “Experimental Procedures” to determine the extent and pattern of pilin glycosylation. Presented here are the reconstructed molecular mass profiles. A, Pa5196 6250–51 double mutant. B, PAO1 NP + AWX + 6245–6251. C, PAO1 NP + AWX + 6245–6249. D, PAO1 NP + AWX + 6246–6249. E, PAO1 NP + AWX + 6245–6249, 6247::GmFRT. F, PAO1 NP + AWX + 6246–6249, 6247::GmFRT. As previously observed for the Pa5196 wild type (3), a characteristic pattern of evenly spaced peaks, each separated by 132 Da (the mass of a single arabinofuranose unit), is observed in the reconstructed molecular mass profiles of all the strains analyzed. Sodium adduct peaks are prominent in the profiles of some of the isolates. The mass of the unmodified protein is 15,132 Da; the peaks are labeled with the number of d-Araf residues present on the pilin.

role of pilin glycosylation in assembly of surface pili—The reduced motility and surface piliation that resulted from loss of any of tfpW, 6246, 6248, or 6249 in Pa5196 or upon expression of PilAIVTfpWX in PAO1 NP without the d-Araf biosynthetic genes suggested a defect in pilus assembly in the absence of glycosylation. To address this idea, we inactivated the PilT retraction ATPase in Pa5196, its tfpW mutant, and the d-Araf-deficient 6248 mutant. Blocking pilus retraction traps polymerized pili on the bacterial surface and therefore reports on the maximum level of assembly possible for a particular strain.

Fig. 6 shows that although the pilT mutant of Pa5196 has a typical hyperpiliated phenotype double mutants lacking pilT and tfpW or pilT and 6248 express levels of surface pili that are similar to those of the non-glycosylated single mutants. Therefore, although pilin arabinosylation is not essential for pilus assembly (because there remains a small amount of surface pili
DISCUSSION

The emergence of multidrug- and extensively drug-resistant strains of *M. tuberculosis*, one of the world’s most prevalent human pathogens, means there is an urgent need for new antimycobacterials (15). The unique cell envelope of the Corynebacterineae is a prime target as many of the enzymes involved in its biosynthesis, including those involved in synthesis of D-Ara, the essential precursor of LAM and arabinogalactan, are essential for viability and lack human homologues. Several key players in the LAM and arabinogalactan biosynthetic pathways have recently been identified, including the three essential enzymes decaprenyl-P-ribose-5-P synthetase (*Rv3806c*) and the decaprenyl-P-ribose epimerase composed of DprE1 (*Rv3790*) and DprE2 (*Rv3791*) (18, 21, 25).

Although D-Ara is an integral cell envelope component in the Corynebacterineae, it is rare in other species, and therefore little is known about its biosynthesis in those backgrounds. Some plant-associated bacteria have D-Ara as part of their host-specific nodulation factors, and mutagenesis studies of *Azorhizobium caulinodans* led to the identification of the noe gene cluster potentially involved in D-Ara biosynthesis (26). However, the functions of most of the Noe proteins have not been determined. Here we have identified the minimal set of genes required to synthesize D-Ara in *P. aeruginosa* and showed that they encode orthologues of *Mycobacterium decaprenyl-P-ribose-5-P synthetase*, DprE1 and DprE2 (Fig. 1).

The 6246 protein is an orthologue of *Rv3806c* (decaprenyl-P-ribose-5-P synthetase), an essential protein in *M. tuberculosis* (27). The requirement for such a protein in *P. aeruginosa* pilin arabinosylation suggests that the D-Ara precursor is synthesized as a lipid-linked phosphosugar intermediate rather than the undecaprenyl pyrophosphate-linked intermediates derived from sugar nucleotide precursors that are more typical of LPS and capsule biosynthesis (12, 28). Because Gram-negative bacteria have not been shown to synthesize decaprenyl (C55P) phosphate, the *P. aeruginosa* enzyme may use undecaprenyl (C55) phosphate as the carrier lipid. A recent study of *Rv3806c* function showed that it could use a variety of polyprenyl lipids, including C55 as substrates (21), supporting the idea that the *P. aeruginosa* enzyme could do so as well. Therefore, we suggest that such enzymes, including 6246, should be referred to more generally as polyprenyl-P-ribose-5-P synthases. The same researchers (21) performed site-directed mutagenesis of *Rv3806c* to confirm their identification of potential polyprenyl and phospho-P-ribose pyrophosphate binding sites. Comparison of the sequences of *Rv3806c* with *A. cauliformis* NocC, 6246, and the *P. syringae* orthologues *Psyr_2303* showed that all of the key functional residues identified by mutagenesis are conserved (supplemental Fig. S2).

Whether a dedicated phosphatase is required for formation of polyprenyl-P-ribose is an unresolved question in the field (11). The necessity for a dedicated enzyme is not supported by our data as constructs lacking the 6245 gene support D-Ara biosynthesis (for example, see Fig. 4B, last lane). It is possible that the dephosphorylation of polyprenyl-P-ribose-5-P can occur nonspecifically via the action of other phosphatases in the cell but that the process is more efficient if the dedicated enzyme is present. This hypothesis is supported by the data in Fig. 5, C and D, which show that there is a marked increase in more heavily glycosylated species when 6245 is provided. However, enhancing expression of 6248–6249 by insertion of a resistance marker with a constitutive promoter within 6247 increased the levels of pilin glycosylation even in the absence of 6245 (Fig. 5F). In *M. tuberculosis*, *Rv3807c* (the ORF upstream of *Rv3806c*; Fig. 1) is proposed to encode the relevant phosphatase (11), but there is currently no evidence for its involvement in cell wall biosynthesis. Unlike *Rv3806c* mutants, those lacking *Rv3807c* are viable, suggesting that cell wall synthesis continues in its absence (27).

The DprE1 enzyme encoded by *Rv3790* was recently demonstrated to be the target of exciting new classes of drugs: the benzothiazinones that kill multidrug-resistant *M. tuberculosis* and the dinitrobenzamides that kill both extracellular and intracellular bacteria (14–16, 29). However, variants of DprE1 with point mutations at a crucial Cys-387 residue are resistant...
to both families of compounds (14). Resistant forms of the M. smegmatis enzyme have a C387G substitution, whereas the P. aeruginosa orthologue has an Ala at the corresponding position (supplemental Fig. S3) and would therefore be predicted to be resistant. It is interesting to note that the M. smegmatis gene, but not that of M. tuberculosis, was able to complement the P. aeruginosa mutant (Fig. 3). Because they catalyze a two-step reaction, the DprE1 and DprE2 enzymes likely function as a complex, and it is possible that only M. smegmatis DprE1 is compatible with P. aeruginosa DprE2.

Because of the orientation of pilin subunits with their N-terminal domains embedded in the inner membrane and C-terminal domains exposed in the periplasm, we speculate that the glycans are transferred to the pilons on the outer face of the inner membrane. For this step to occur, the glycans must be assembled in the cytoplasm and translocated to the periplasm via a flipase reaction. Alternatively, they could be assembled and translocated by a single protein as has been proposed for WbbF of Salmonella borreze involved in synthesis of its O:54 O-antigen, a homopolymer of N-acetylmannosamine, or for the hyaluronic acid synthesize of Streptococcus pyogenes that synthesizes a GlcNAc homopolymer (30, 31). No putative flipase enzymes were identified in this work, and provision of the Pa5196 PilAIV-TfpWX proteins with the three polypropenyl-P-d-Araf/biosynthetic genes was sufficient to reconstitute pilin glycosylation in PAO1 (Figs. 4 and 5). Therefore, TfpW could potentially be a multifunctional enzyme responsible for translocation, polymerization, and oligosaccharyl transfer of the pilin arabinans.

The requirement that PilAIV proteins be O-glycosylated for efficient assembly is unusual for type IV pilins. The small amount of pilin recovered may under-represent the total amount of assembled fibers if they are shorter than normal as such fibers would not be recovered by shearing. However, examination of the cells by electron microscopy does not provide evidence for short fibers (not shown). TfpW does not modify heterologous pilins with d-Araf when they are expressed in Pa5196 NP, although such pilins are readily assembled by the Pa5196 pilus machinery (Fig. 7), suggesting that glycosylation is not required for recognition. Instead, the most likely explanation is that the native conformation of the unmodified PilAIV protein is atypical in some way. Glycosylation of PilAIV would generate a conformation that is readily assembled even in heterologous strains (Figs. 4 and 5). Furthermore, it is possible that the atypical structure hypothesized for unmodified PilAIV is necessary for substrate recognition by TfpW, explaining why heterologous pilins are not modified in the Pa5196 background despite having available Ser and Thr residues in positions corresponding to those modified in PilAIV (7).

In conclusion, we have now defined the components that are necessary and sufficient for P. aeruginosa to synthesize α1,5-linked d-Araf and to attach the glycans to PilAIV. We showed that this unusual post-translational modification is important for pilus assembly and function. The similarity of the pathways between P. aeruginosa and Mycobacterium is interesting from the standpoint of bacterial evolution and provides an opportunity to use P. aeruginosa for further investigation of d-Araf biosynthesis.

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