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

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Pseudomonas aeruginosa **strains PA7 and Pa5196 glycosylate their type IVa pilins with α1,5-linked D-arabinofuranose (D-Ara*f*), 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-Ara*f* 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 polyprenyl-linked D-ribose precursor and its epimerization to D-Ara*f*. 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* PAO1 strain co-expressing the genes for D-Ara*f* biosynthesis, the pilin modification enzyme TfpW, and the acceptor $PilA_{IV}$ 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-Ara*f*, 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 pili (T4P)[2]) via *O*-glycosylation with strain-specific sugars (1–3). The post-translational 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 ($PilA_I$) and IV ($PilA_{IV}$) 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, $PilA_{IV}$ is modified on multiple Ser and Thr residues in the predicted αβ-loop and β-sheet regions with D-arabinofuranose (D-Ara*f*) residues arranged as monomers or α1,5-linked dimers, trimers, and potentially longer polymers (3). D-Ara*f* 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 $PilA_{IV}$ 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 $PilA_{IV}$ in a non-piliated mutant of *P. aeruginosa* lacking the glycosylation system did not restore motility or piliation (10).

In addition to the Corynebacterineae, D-Ara*f* 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

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[2] The abbreviations used are: T4P, type IV pili; D-Ara*f*, D-arabinofuranose; LAM, lipoarabinomannan; FRT, flippase recognition target; NP, non-piliated.

identification of seven *P. aeruginosa* genes potentially involved in D-Ara*f* 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 pili, confirming that the genes identified were both necessary and sufficient. The D-ribose to D-Ara*f* epimerization step of arabinan 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*f* 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. L-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 distilled H_2O. After decanting the crystal violet, the plates were gently rinsed with tap water to remove excess dye and air-dried. The areas of twitching zones were measured using NIH ImageJ software.

Surface Protein Isolation, SDS-PAGE, and Western Blot Analyses—Surface proteins were isolated by shearing as described previously (3). Briefly, strains were streaked in a grid-like pattern on LB plates containing antibiotics and L-arabinose. Two plates per sample were used. After overnight incubation at 37 °C, the cells were gently scraped from the agar surface using a coverslip and resuspended in 5 ml of PBS. After vigorous vortexing for 30 s to shear pili and flagella, the cells were removed by centrifugation, and supernatant proteins were precipitated overnight at 4 °C by the addition of 1 M $MgCl_2$ to give a final concentration of 100 mM. The precipitated proteins were harvested by centrifugation at 4 °C, resuspended in 50–100 μl of 1× sample buffer (80 mM Tris, pH 6.8, 5.3% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromphenol blue, 2% (w/v) SDS) depending on the size of the pellet. Samples were boiled for 10 min before separation on 15% SDS-polyacrylamide gels at 150 V with a prestained molecular weight marker (Fermentas).

After SDS-PAGE, proteins were transferred to nitrocellulose for 1 h at 220 mA. The membranes were blocked for 2 h at room temperature with 5% skim milk. The blots were incubated with primary antibodies for 12 h at 4 °C. For detection of the pilin, anti-$PilA_{IV}$ (1:5000 in PBS; rabbit number 286) was used. For detection of the pilin glycan, anti-*M. tuberculosis* LAM (1:1000 in PBS) obtained through NIAID, National Institutes of Health Contract HHSN266200400091C entitled "Tuberculosis Vaccine Testing and Research Materials," awarded to Colorado State University, was used. For detection, the blots were incubated for 1 h at room temperature with goat anti-rabbit-IgG-alkaline phosphatase conjugate (Bio-Rad), diluted 1:3000 in PBS, washed 4 × 5 min in PBS, and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer (Bio-Rad).

For mass spectrometry, the Pa5196 *PsPA76250–51* double mutant was streaked in a grid pattern on 20 LB agar plates that were incubated overnight at 37 °C. Recombinant PAO1 strains were similarly grown on LB plates containing 30 μg/ml gentamicin, 200 μg/ml carbenicillin, and 0.05% L-arabinose. Pili were isolated as described previously (7). Precipitated proteins were harvested by centrifugation, resuspended in a total of 0.5–3.0 ml of PBS depending on the size of the pellet, and dialyzed in 50 mM NH_4HCO_3 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. All 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 ($PilA_{IV}$) modified with D-Ara*f* (3, 7). Using the publicly available PA7 genome sequence (17), we searched for genes that could encode the biosynthesis of D-Ara*f*. Our previous work (7) showed that only group IV strains produced pilins modified with D-Ara*f*, 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 nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_{K^-} m_{K^+}), λ−	Invitrogen
C2925	*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)* Tet^S *endA1 rspL136* (Str^R) *dam13::Tn9* (Cam^R) *xylA-5 mtl-1 thi-1 mcrB1 hsdR2*	New England Biolabs
SM10	Km^R, *thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir*	32
M. smegmatis mc2155	Source of DNA for complementation of Pa5196 mutants	Jun Liu, University of Toronto
M. tuberculosis H37Rv	Source of DNA for complementation of Pa5196 mutants	Jun Liu, University of Toronto
***P. aeruginosa* strains**		
PAO1	Group II T4P	Laboratory stock
PAO1 NP	*Tn5-phoA* insertion at base 163 in *pilA*	33
Pa5196	Group IV T4P; rectal isolate	5
Pa5196 *tfpW*	EZ::Tn FRT insertion in *tfpW*	7
Pa5196 *6246::FRT*	FRT insertion at position 309 (EcoRV) in *PsPA7_6246*	This study
Pa5196 *6247::FRT*	FRT insertion at position 173 (AfeI) in *PsPA7_6247*	This study
Pa5196 *6248::FRT*	FRT insertion at position 569 (XhoI) in *PsPA7_6248*	This study
Pa5196 *6249::FRT*	FRT insertion at position 105 (NruI) in *PsPA7_6249*	This study
Pa5196 *6250::FRT*	FRT insertion at position 336 (BclI) in *PsPA7_6250*	This study
Pa5196 *6251::FRT*	FRT insertion at position 422 (EcoRI) in *PsPA7_6251*	This study
Pa5196 *6250::FRT-6251::FRT*	FRT insertions at position 336 of *PsPA7_6250* and position 422 of *PsPA7_6251*	This study
Pa5196 *pilT::FRT*	FRT insertion at position 540 (NruI) in *pilT*	This study
Pa5196 *tfpW pilT*	FRT insertion at position 540 (NruI) in *pilT* on the *tfpW* background	This study
Pa5196 *PsPA7_6248 pilT*	FRT insertion at position 540 (NruI) in *pilT* on the *PsPA7_6248::FRT* background	This study
PAO1 NP + AWX	PAO1 *pilA* mutant complemented with $pilA_{IV}$-*tfpW-tfpX* from Pa5196 in pBADGr	10
Plasmids		
pEX18Ap	Carbenicillin-resistant suicide vector used for gene replacement	34
pFLP2	Suicide vector encoding Flp recombinase	34
pPS856	Source of FRT-flanked gentamicin resistance cassette	34
pBADGr	Broad host range arabinose-inducible vector used for complementation; gentamicin resistance marker	35
pUCP20	Carbenicillin-resistant vector used for complementation; TEM-1 resistance marker	34
pEX18Ap + 6246::GmFRT	*PsPA7_6246* knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 309 (EcoRV) in *PsPA7_6246*	This study
pEX18Ap + 6247::GmFRT	*PsPA7_6247* knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 173 (AfeI) in *PsPA7_6247*	This study
pEX18Ap + 6248::GmFRT	*PsPA7_6248* knock-out construct with SstI-flanked Gm-FRT cassette inserted at position 569 (XhoI) in *PsPA7_6248*	This study
pEX18Ap + 6249::GmFRT	*PsPA7_6249* knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position 105 (NruI) in *PsPA7_6249*	This study
pEX18Ap + 6250::GmFRT	*PsPA7_6250* knock-out construct with BamHI-flanked Gm-FRT cassette inserted at position 336 (BclI) in *PsPA7_6250*	This study
pEX18Ap + 6251::GmFRT	*PsPA7_6251* knock-out construct with EcoRI-flanked Gm-FRT cassette inserted at position 422 (EcoRI) in *PsPA7_6251*	This study
pEX18Ap + *pilT*::GmFRT	*pilT* knock-out construct with SmaI-flanked Gm-FRT cassette inserted at position (NruI) in *pilT*	10
pBADGr + AWX	Complementation construct carrying the Pa5196 $pilA_{IV}$, *tfpW*, and *tfpX* genes	3
pBADGr + *PsPA7_6248*	Complementation construct carrying the Pa5196 *PsPA7_6248* gene	This study
pBADGr + PA1416	Complementation construct carrying the PAO1 *PA1416* gene	This study
pBADGr + MSMEG6382	Complementation construct carrying the *M. smegmatis MSMEG6392* gene	This study
pBADGr + Rv3790	Complementation construct carrying the *M. tuberculosis Rv3790* gene	This study
pUCP20 + 6245–6251	Complementation construct carrying the Pa5196 *PsPA7_6245-51* genes, *lac* promoter	This study
pUCP20 + 6247–6251	Complementation construct carrying the Pa5196 *PsPA7_6247-51* genes, *lac* promoter	This study
pUCP20 + 6245–6249	Complementation construct carrying the Pa5196 *PsPA7_6245-49* genes, *lac* promoter	This study
pUCP20 + 6245–6249, 6247::GmFRT	Complementation construct carrying the Pa5196 *PsPA7_6245-49* genes, with an insertion of the SmaI-flanked Gm-FRT cassette at position 173 (AfeI) in *PsPA7_6247*, *lac* promoter	This study
pUCP20 + 6246–6249	Complementation construct carrying the Pa5196 *PsPA7_6246-49* genes, *lac* promoter	This study
pUCP20 + 6246–6249, 6247::GmFRT	Complementation construct carrying the Pa5196 *PsPA7_6246-49* genes, with an insertion of the SmaI-flanked Gm-FRT cassette at position 173 (AfeI) in *PsPA7_6247*, *lac* promoter	This study

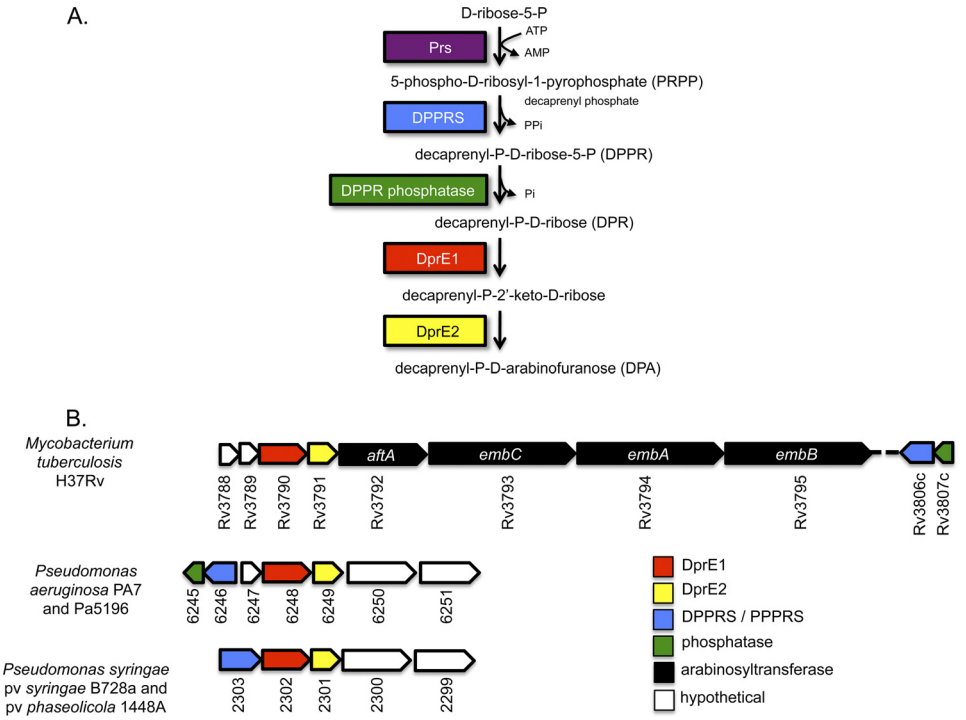

FIGURE 1. **D-Arabinofuranose biosynthesis in *P. aeruginosa*.** *A*, proposed pathway of D-Araf biosynthesis based on studies in the Corynebacterineae. Phospho-D-ribose pyrophosphate, the first intermediate of the pathway, is likely generated by Prs, a common enzyme involved in bacterial purine metabolism. In PA7, Prs is encoded by *PsPA7_5320*. *B*, genes involved in D-Araf biosynthesis in *Mycobacterium* and *Pseudomonas*. Open reading frames encoding the enzymes that are predicted to generate the intermediates shown in *A* are shown in matching colors. Genes are numbered according to the H37Rv, PA7, and B728a genome designations with the strain prefixes eliminated for clarity and are not drawn to scale. *DPPRS*, decaprenyl-P-ribose-5-P synthetase; *PPPRS*, polyprenyl-P-ribose-5-P synthetase.

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 Rv3790* and *Rv3791* genes 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. 1*A*) (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 highest between the *M. tuberculosis* ORFs and 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. 1*B*). 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 glucosyltransferases 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. 1*B*). 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. *phaesolicola* 1448A and *P. syringae* pv. *syringae* B728A) also have contiguous homologues of *Rv3790* and *Rv3791* (Fig. 1*B*). 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*, *Sulforovum*, *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. 1*B*) 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. 1*A*). 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. 1*B*). 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-rhamnose 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-Araf cluster in

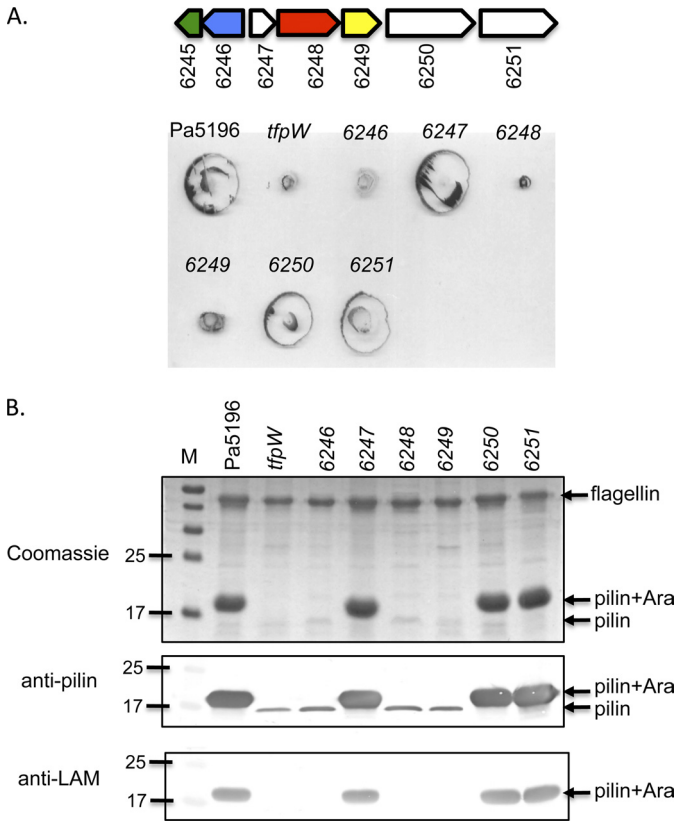

FIGURE 2. **Phenotypes of mutants lacking *6246* to *6251*.** *A*, twitching motility of the Pa5196 wild type, the *tfpW* mutant, and the *6246* through *6251* mutants. The gene map from Fig. 1*B* is shown at the *top* for reference. *B*, pilus preparations of each of the strains in *A* separated with SDS-PAGE and stained with Coomassie Brilliant Blue or probed with antibodies to $PilA_{IV}$ (anti-pilin) or to *M. tuberculosis* lipoarabinomannan (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 pili 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-Ara*f* 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-Ara*f* 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. 2*A*) and surface piliation and pilin modification (Fig. 2*B*). 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. 2*A*). 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. 2*B*). 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-Ara*f*. 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-Ara*f* synthesis was ruled out as the *gmd* mutation had no effect on motility or piliation (data not shown).

The inability of *P. aeruginosa* strains other than PA7 or Pa5196 to glycosylate $PilA_{IV}$ when it is expressed in *trans* suggests that they lack the ability to synthesize D-Ara*f* 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. 3*A*), piliation, or glycosylation (Fig. 3*B*), 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-Ara*f* 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 pili, 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. 4*A*), and the pilin subunits were post-translationally modified with D-Ara*f* (Fig. 4*B*). 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-Ara*f* 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-Ara*f* 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-

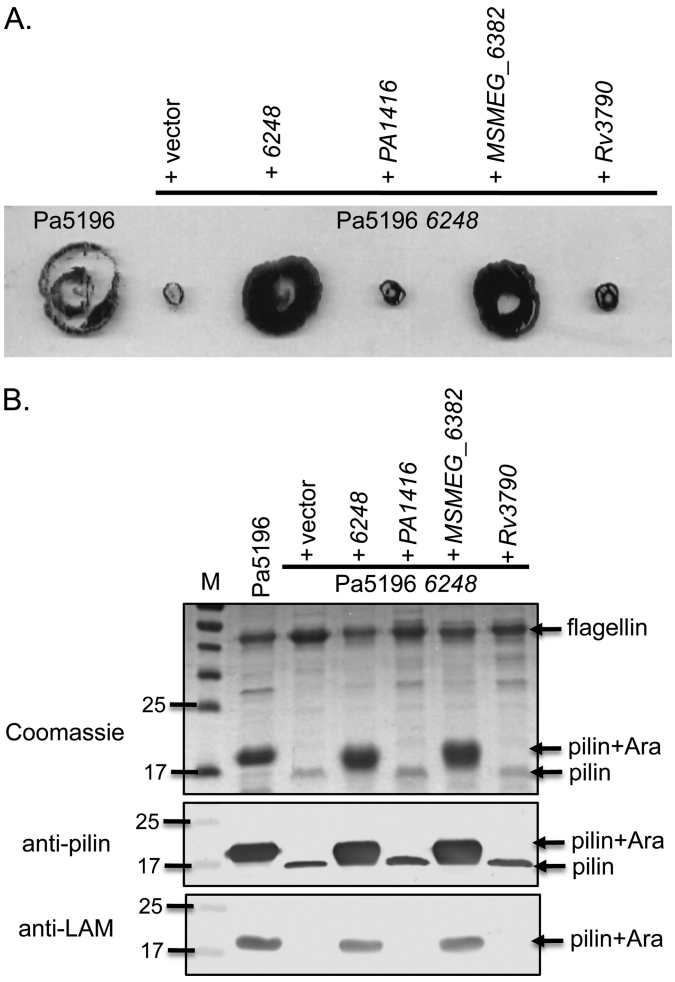

FIGURE 3. **Complementation of Pa5196 *6248* (*dprE1*) mutant.** *A*, twitching motility of the Pa5196 wild type and its *6248* mutant complemented with the pBADGr vector control, the cognate gene, or homologues from *P. aeruginosa* PAO1 (*PA1416*), *M. smegmatis* (*MSMEG_6382*), or *M. tuberculosis* (*Rv3790*). *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 $PilA_{IV}$ (anti-pilin) or to *M. tuberculosis* lipoarabinomannan (anti-LAM). *M*, molecular mass markers in kDa. Genes that restore twitching motility also restore pilin glycosylation and increase the amount of recoverable surface pili.

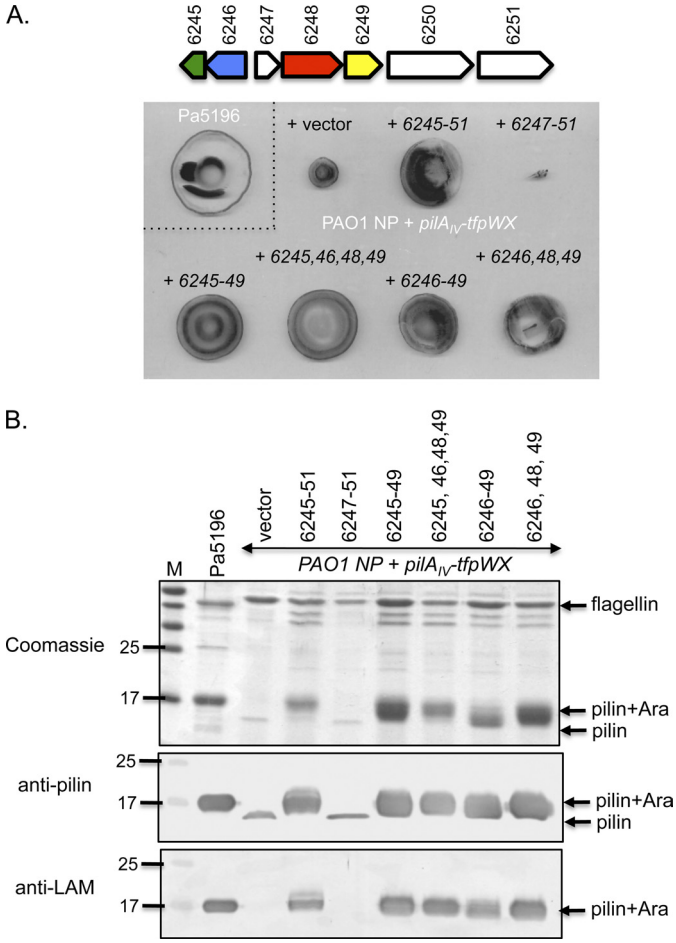

FIGURE 4. **Reconstitution of Pa5196 pilin arabinosylation system in PAO1.** *A*, twitching motility of the Pa5196 strain (*top left*) and an NP PAO1 mutant complemented with the Pa5196 $pilA_{IV}$, *tfpW*, and *tfpX* genes in pBADGr plus a vector control (pUCP20) or various combinations of the Pa5196 *6245–6251* genes in pUCP20. The gene map from Fig. 1*B* 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 $PilA_{IV}$ (anti-pilin) or to *M. tuberculosis* lipoarabinomannan (anti-LAM). *M*, molecular mass markers in kDa. The minimum set of genes required for modification of $PilA_{IV}$ includes *6246*, *6248*, and *6249* (*last lane*).

plemented 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 α1,5-D-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-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 α1,5 linkage between D-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. 1*B*) involved in formation of α1,2-, α1,3-, and α1,5-linked arabinans in mycobacteria (7). The only potential glycosyltransferase encoded within the D-Ara*f* biosynthetic cluster, *6250*, was dispensable for synthesis and addition of D-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 α1,5 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. 5*A* 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 $PilA_{IV}$ were identified previously (7), the data indicate that polymers of α1,5-D-Araf were present, and we could therefore eliminate 6250 as a potential α-1,5-D-Ara*f*-transferase. Mass spectrometry on pilins isolated from PAO1 non-piliated (NP) carrying the $pilA_{IV}$-*tfpWX* cassette and various combinations of genes from the *6245–6251* cluster showed that expression of the entire cluster gave pilins with the characteristic pattern of multiple D-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

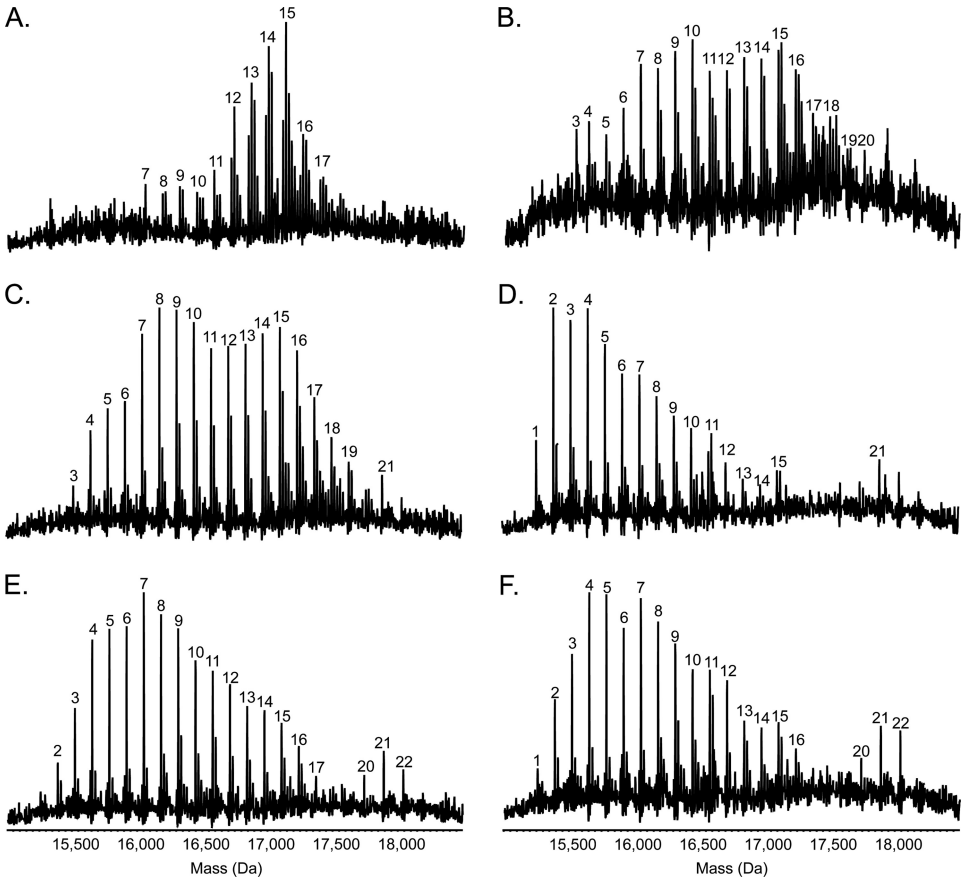

FIGURE 5. **Mass spectrometry analysis of intact pilins.** Pili were isolated and subjected to electrospray ionization-MS as described under "Experimental Procedures" to determine the extent and pattern of pilin glycosylation. Presented here are the reconstructed molecular mass profiles. *A*, Pa5196 *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-Ara*f* residues present on the pilin.

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. 5*C*), 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. 5*D*). Disruption of *6247* with a Gm^R 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-Ara*f* biosynthesis.

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 $PilA_{IV}$TfpWX in PAO1 NP without the D-Ara*f* 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-Ara*f*-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.

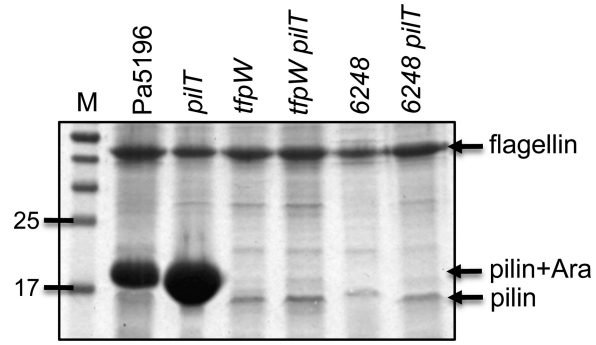

FIGURE 6. **Pilin glycosylation is important for pilus assembly.** Pilus preparations were separated with SDS-PAGE and stained with Coomassie Brilliant Blue. *M*, molecular mass markers in kDa. Each of the mutants was created in Pa5196 as described under "Experimental Procedures." Inactivation of either *tfpW* (7) or the *dprE1* homologue *6248* prevents pilin glycosylation. Blockade of pilus retraction via inactivation of *pilT* does not increase the level of recoverable surface pili, indicating a profound assembly defect.

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

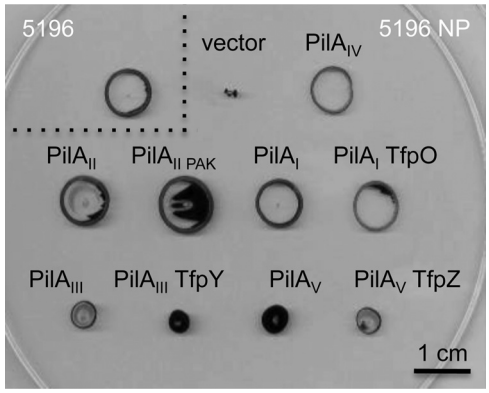

FIGURE 7. **Pa5196 assembly system is not specific for glycosylated pilins.** Twitching motility of the Pa5196 wild type (5196) and its non-piliated *pilA$_{IV}$* mutant (5196 NP) complemented with the pBADGr vector control, the native *pilA$_{IV}$* gene, or pilin genes from *P. aeruginosa* strains of other pilin groups: group II pilins from strains PAO1 (PilA$_{II}$) or PAK (PilA$_{IIPAK}$), group I pilin from strain 1244 alone or with the *tfpO* gene, group III pilin from strain PA14 alone or with the *tfpY* gene, and group V pilin from strain 8110594 alone or with the *tfpZ* gene (5, 10). The non-glycosylated group I and II pilins complement the Pa5196 NP mutant to the same extent as its native pilin, showing that the assembly system does not require that pilins be arabinosylated. The reduced twitching conferred by group III and group V pilins may arise from reduced compatibility with the minor pilins of Pa5196 that are most similar to those of group I and II strains (24).

and motility in strains incapable of pilin glycosylation), the process is markedly impaired in its absence. We next asked whether the decrease in piliation was caused by an inability of the Pa5196 pilus machinery to recognize and assemble pilins that lack D-Ara*f* modifications. Non-glycosylated pilins from other strains of *P. aeruginosa* were expressed in a *pilA$_{IV}$* mutant of Pa5196 (denoted Pa5196 NP). Fig. 7 shows that non-glycosylated group II pilins from strains PAO1 and PAK restore levels of motility to the Pa5196 NP mutant similar to that of its glycosylated native pilin; therefore, the assembly system is not specific for post-translationally modified pilins. Both glycosylated and non-glycosylated group I pilins from group I strain 1244 restore similar levels of motility in the Pa5196 NP background. As seen in previous studies with PAO1 NP, group III and V pilins were less efficient in restoring motility potentially due to reduced compatibility with the group I/II-like minor pilins of Pa5196 (10, 24).

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*f*, the essential precursor of LAM and arabinogalactan, are essential for viability and lack human orthologues. 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-D-ribose epimerase composed of DprE1 (Rv3790) and DprE2 (Rv3791) (18, 21, 25).

Although D-Ara*f* 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*f* 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*f* 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*f* 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*f* 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 (C_{50}) phosphate, the *P. aeruginosa* enzyme may use undecaprenyl (C_{55}) phosphate as the carrier lipid. A recent study of Rv3806c function showed that it could use a variety of polyprenyl lipids, including C_{55}, 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-D-ribose-5-P synthetases. The same researchers (21) performed site-directed mutagenesis of Rv3806c to confirm their identification of potential polyprenyl and phospho-D-ribose pyrophosphate binding sites. Comparison of the sequences of Rv3806c with *A. caulinodans* NoeC, 6246, and the *P. syringae* orthologue 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-D-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*f* biosynthesis (for example, see Fig. 4*B*, *last lane*). It is possible that the dephosphorylation of polyprenyl-P-D-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. 5*F*). 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 pilins 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 flippase 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 synthase of *Streptococcus pyogenes* that synthesizes a GlcNAc homopolymer (30, 31). No putative flippase enzymes were identified in this work, and provision of the Pa5196 $PilA_{IV}$-TfpWX proteins with the three polyprenyl-P-D-Ara*f* 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 $PilA_{IV}$ proteins be *O*-glycosylated for efficient assembly is unusual for type IV pilins. The small amount of pili 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-Ara*f* 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 $PilA_{IV}$ protein is atypical in some way. Glycosylation of $PilA_{IV}$ 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 $PilA_{IV}$ 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 $PilA_{IV}$ (7).

In conclusion, we have now defined the components that are necessary and sufficient for *P. aeruginosa* to synthesize α1,5-linked D-Ara*f* and to attach the glycans to $PilA_{IV}$. 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-Ara*f* biosynthesis.

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