

Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: glycan substrate specificity

Antonio DiGiandomenico,^{1†} Mauricia J. Matewish,^{2†}
Amy Bisailon,² John R. Stehle,¹ Joseph S. Lam²
and Peter Castric^{1*}

¹Department of Biological Sciences, Duquesne University, Pittsburgh, PA 15282, USA. ²Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G2W1.

Summary

The structural similarity between the pilin glycan and the O-antigen of *Pseudomonas aeruginosa* 1244 suggested that they have a common metabolic origin. Mutants of this organism lacking functional *wbpM* or *wbpL* genes synthesized no O-antigen and produced only non-glycosylated pilin. Complementation with plasmids containing functional *wbpM* or *wbpL* genes fully restored the ability to produce both O-antigen and glycosylated pilin. Expression of a cosmid clone containing the O-antigen biosynthetic gene cluster from *P. aeruginosa* PA103 (LPS serotype O11) in *P. aeruginosa* 1244 (LPS serotype O7) resulted in the production of strain 1244 pilin that contained both O7 and O11 antigens. The presence of the O11 repeating unit was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Expression of the O-antigen biosynthesis cluster from *Escherichia coli* O157:H7 in strain 1244 resulted in the production of pilin that contained both the endogenous *Pseudomonas* as well as the *Escherichia* O157 O-antigens. A role for *pilO* in the glycosylation of pilin in *P. aeruginosa* is evident as the cloned *pilAO* operon produced glycosylated strain 1244 pilin in eight heterologous *P. aeruginosa* strains. Removal of the *pilO* gene resulted in the production of unmodified strain 1244 pilin. These results show that the pilin glycan of *P. aeruginosa* 1244 is a product of the O-antigen biosynthetic pathway. In addition, the structural diversity of the O-antigens used by the 1244 pilin glycosylation apparatus indicates that the glycan

substrate specificity of this reaction is extremely low.

Introduction

The pili of *Pseudomonas aeruginosa* are protein fibres that radiate from one or both cell poles (Paranchych and Frost, 1988; Strom and Lory, 1993). These structures are important in cell adhesion (Hahn, 1997) and are responsible for twitching motility (Bradley, 1980; Wall and Kaiser, 1999), a form of cell movement involved in the establishment and spreading of this opportunistic pathogen (Kang *et al.*, 1997; Comolli *et al.*, 1999). The pili of *P. aeruginosa* are made up of a single type of pilin subunit, which has the physical and chemical characteristics associated with those of the type IV pili (Paranchych and Frost, 1988; Strom and Lory, 1993). One of these characteristics is the N-methylation of the amino-terminal residue, phenylalanine. In addition to this modification, the pilin of *P. aeruginosa* 1244 is glycosylated (Castric, 1995). This process requires the presence of *pilO*, a gene that is a component of an operon containing *pilA*, the pilin structural gene.

Although once considered rare, glycosylation of prokaryotic protein is now known to occur in a variety of organisms (Messner and Schaffer, 2002). This modification is most commonly found in proteins associated with the cell surface, the best studied example being the archaeal and eubacterial S-layer glycoproteins (Messner and Schaffer, 2002). More recently, a number of examples of glycosylated surface proteins from pathogens have been demonstrated. Among Gram-positive organisms, proteins of *Streptococcus sanguis* and *Mycobacterium tuberculosis* have been shown to be glycosylated (Erickson and Herzberg, 1993; Dobos *et al.*, 1995). Examples of glycosylation of surface proteins among the Gram-negative pathogens include adhesins of *Escherichia coli* (Lindenthal and Elsinghorst, 1999; Benz and Schmidt, 2001), an outer membrane protein of *Chlamydia trachomatis* (Kuo *et al.*, 1996), the flagella of *Campylobacter* species (Doig *et al.*, 1996; Thibault *et al.*, 2001) and *P. aeruginosa* (Brimer and Montie, 1998; Arora *et al.*, 2001), and the pili of *Neisseria meningitidis* and *N. gonorrhoeae* (Parge *et al.*, 1995; Stimson *et al.*, 1995; Stimson *et al.*, 1996).

Accepted 23 July, 2002. *For correspondence. E-mail Castric@duq.edu; Tel. (+1) 412-396-6319; Fax (+1) 412-396-5907. †The first two authors contributed equally to this paper.

Examination of *P. aeruginosa* 1244 pili has shown that each subunit contains a single covalently bound glycan (Castric *et al.*, 2001) that is attached to serine 148 (Comer *et al.*, 2002), the carboxy-terminal pilin residue. Structural analysis has revealed the glycan to be a trisaccharide, α -5N β OHC₄7NFmPse-(2 \rightarrow 4)- β -Xyl-(1 \rightarrow 3)- β -FucNAc, which is attached to the serine β carbon through the C-1 of FucNAc (Castric *et al.*, 2001). These sugars, in the same order and linkage, are found in the serotype O7 (International Antigenic Typing System (IATS; Liu *et al.*, 1983) O-antigen, the serogroup to which strain 1244 belongs. These findings suggested that the pilin glycan and the O-antigen of this organism have a common metabolic origin.

The genes required for biosynthesis of the O-antigens of *P. aeruginosa* serotypes O5, O6 and O11 have been characterized (Burrows *et al.*, 1996; Belanger *et al.*, 1999; Dean *et al.*, 1999), and two genes, *wbpM* and *wbpL*, have been shown to be essential for the initial steps of O-antigen biosynthesis. *wbpM* encodes a bifunctional UDP-GlcNAc C6 dehydratase/C4 reductase that is essential for the formation of the nucleotide precursor UDP-QuiNAc (Burrows *et al.*, 1996; Cruzenet and Lam, 2001). This sugar nucleotide is converted into UDP-FucNAc by a subsequent epimerization step and is the precursor for the first sugar residue of the serotype O7 O-antigen unit. WbpL, a bifunctional glycosyltransferase, is required for the initiation of both A-band lipopolysaccharide (LPS; common antigen) and B-band LPS (O antigen) synthesis (Burrows *et al.*, 1996; Rocchetta *et al.*, 1998). This transferase initiates the assembly of individual O-antigen units by the transfer of FucNAc from UDP-FucNAc to undecaprenol phosphate. Consequently, mutations in *wbpM* in *P. aeruginosa* strains PAO1 (serotype O5) and PAK (serotype O6) result in the complete loss of O-antigen production, whereas null mutations in *wbpL* completely abolish the production of both A-band and O-antigen LPS. Therefore, *wbpL* and *wbpM* are ideal targets for constructing null mutants to investigate the role of O-antigen biosynthesis in the glycosylation of strain 1244 pilin.

We provide evidence in this paper that the pilin glycan is a product of the O-antigen biosynthetic pathway. This is performed using defined *wbpM* and *wbpL* mutants derived in the *P. aeruginosa* 1244 strain background. These results are confirmed by the finding that cloned biosynthesis gene clusters coding for heterologous O-antigen result in the production of pili containing heterologous O-antigen. Of particular significance is the evidence that the pilin glycosylation apparatus is non-selective with regard to O-antigen structure, showing no apparent specificity as to saccharide size, charge or structure. These results contribute to the understanding of pilus structure and function, and have important implications in pilus and LPS targeted vaccine design.

Results

Generation and analysis of 1244 *wbpM* and *wbpL* mutants

As *wbpL* and *wbpM* are involved in the initial steps of O-antigen biosynthesis, mutants of these genes in *P. aeruginosa* 1244 were generated to investigate whether the O7 O-antigen and the pilin glycan were products of this metabolic pathway. The nucleotide sequence for the *wbp* cluster in strain 1244 was unknown. However, based on the findings from previous studies (Burrows *et al.*, 1996; Belanger *et al.*, 1999), it was known that a highly conserved *wbpM* gene is located at the 3' end of the *wbp* cluster, and that *wbpL* is localized upstream of *wbpM* in a non-conserved serotype-specific region. The *wbpL* gene in strain 1244 (*wbpL*₁₂₄₄) could not be identified in Southern blot analysis even using low-stringency conditions. Therefore, taking advantage of the highly conserved nature of *wbpM* at the nucleotide level, a 1244 *wbpM* null mutant was first generated following an established method (Schweizer and Hoang, 1995) using an allelic replacement construct containing the *wbpM* gene from *P. aeruginosa* strain PAO1 (serotype O5) interrupted with a gentamicin resistance cassette (Burrows *et al.*, 1996). Southern blot analysis was performed on *XhoI*-digested chromosomal DNA from *P. aeruginosa* strain PAO1, 1244 wild type and the 1244 *wbpM* mutant using *wbpM*_{PAO1} as a gene probe (data not shown). The *wbpM* gene contains one *XhoI* site; therefore, the *wbpM*_{PAO1} probe hybridized to two DNA fragments from each strain. A *wbpM* probe-reactive fragment of 1.6 kb was observed in all three strains. Based on the available nucleotide sequence of the PAO1 *wbp* cluster (Burrows *et al.*, 1996; Stover *et al.*, 2000), it was deduced that the 1.6 kb fragment corresponds to the 3' end of the *wbpM* gene (Fig. 1). *wbpM* probe-reactive DNA fragments of sizes 4.3 kb, 2.2 kb and 3.1 kb were also observed from PAO1, 1244 wild type and the *wbpM* mutant respectively (Fig. 1). These DNA fragments correspond to the 5' end of *wbpM* and upstream DNA containing the less conserved serotype-specific region. Cloning and nucleotide sequencing analysis of the 3 kb *XhoI* fragment from the 1244 *wbpM* mutant revealed a complete open reading frame (ORF) at the 5' end composed of 1041 nucleotides, which encodes a 346-amino-acid protein with 58% identity and 74% similarity to WbpL from *P. aeruginosa* strain PAO1. In addition, the protein encoded by this ORF contained five conserved motifs characteristic of the WbpL-type family of glycosyltransferases (Rocchetta *et al.*, 1998), which belong to a larger family of UDP-GlcNAc/MurNAc:polyisoprenol-P GlcNAc/MurNAc-1-P transferases (Lehrman, 1994). This ORF was designated *wbpL*₁₂₄₄, and a *wbpL*₁₂₄₄ null mutant was successfully constructed using the gene replacement method described earlier (Schweizer and Hoang, 1995). Analysis

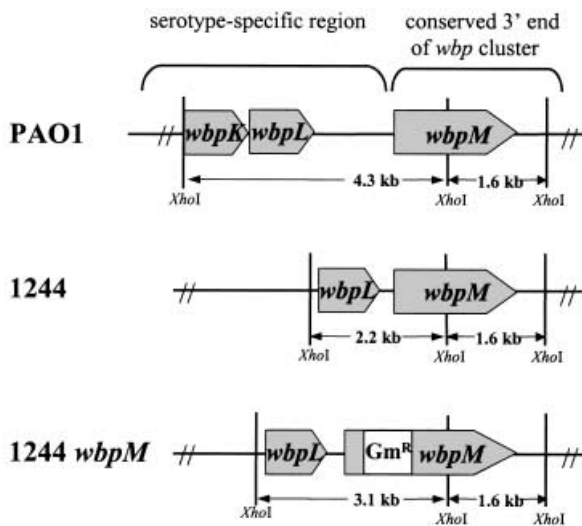


Fig. 1. Strategy for identifying *wbpM* and *wbpL* in strain 1244 based on comparison of location of these two genes in PAO1, 1244 wild type and 1244 *wbpM* mutant. The marked *XhoI* fragments represent the results from the Southern hybridization analysis.

of the LPS from 1244 *wbpM* and *wbpL* mutants showed that they are both deficient in B-band production, whereas the *wbpL* mutant is also deficient in A-band production (Fig. 2). Complementation experiments transforming *wbpM* (pFV163-26; *wbpM*_{PAO1}) and *wbpL* (pFVAB3-26; *wbpL*₁₂₄₄) into their respective mutants restored the phenotype to that of the parent (Fig. 2). The GenBank accession numbers for these genes are AY095101 for *wbpL*₁₂₄₄ and AF508154 for *wbpM*₁₂₄₄.

Pilin glycosylation state of 1244 wbpM and wbpL mutants

If the 1244 pilin glycan originates in the O-antigen path-

way, it would be expected that mutants defective in biosynthesis of this polysaccharide would produce altered pilin glycosylation. To test this, the *wbpM* and *wbpL*₁₂₄₄ mutants were analysed by Western immunoblotting to determine whether they produced pilin of a size and charge consistent with glycosylation. The pilin from each of these mutants was of an apparent molecular weight consistent with non-glycosylated pilin (Fig. 3A). Complementation of these mutants resulted in the production of pilin with the same molecular weight as authentic glycosylated 1244 pilin. To confirm these results, pilin produced by the mutants was separated by isoelectric focusing, blotted and probed with monoclonal antibodies, specific in one case for 1244 pilin protein and in the other for pilin glycan. It has been demonstrated previously that non-glycosylated 1244 pilin focuses at approximately pH 6.25, whereas glycosylated pilin has a pI of 4.75 (Castric, 1995). The pIs of the pilin from the *wbpM* and *wbpL* mutants were identical and matched that of non-glycosylated pilin, whereas the complemented mutants produced pilin with acidic isoelectric points indistinguishable from that of glycosylated pilin (Fig. 3B). In addition, the pilins from the complemented strains reacted with the glycan-specific monoclonal antibody 11.14, whereas the mutant pilins were non-reactive (Fig. 3C). These data showed that 1244 mutants defective in either the N-acetyl-fucosamine pathway (*wbpM*) or the initial transferase responsible for attachment of this sugar to the undecaprenol carrier lipid (*wbpL*) were unable to produce glycosylated pilin. Altogether, these results support the proposal (Castric *et al.*, 2001) that the O-antigen biosynthetic pathway is the metabolic source of the pilin glycan.

Heterologous pilin glycosylation by P. aeruginosa 1244

If the O-antigen biosynthetic pathway is the source of the

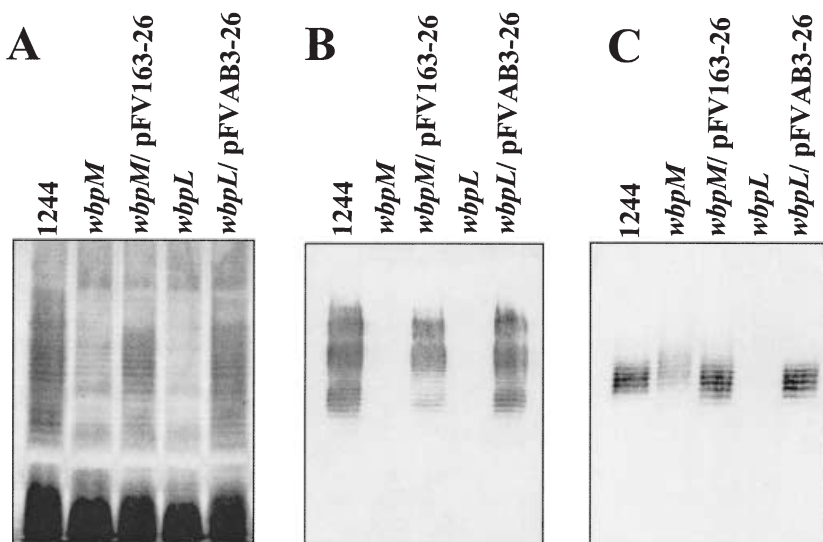


Fig. 2. Analysis of LPS from the parent 1244, *wbpM* and *wbpL* mutants. Silver-stained SDS-PAGE gel (A) and Western immunoblots using the O7 B-band specific mAb MF29-2 (B) and A-band specific mAb N1F10 (C). The 1244 *wbpM* and *wbpL* mutants are both deficient in B-band LPS, whereas the *wbpL* mutant is also deficient in A-band LPS. LPS of the *wbpM* and *wbpL* mutants is restored to that of the parent phenotype when complemented by pFV163-26 (*wbpM*_{PAO1}) and pFVAB3-26 (*wbpL*₁₂₄₄) respectively.

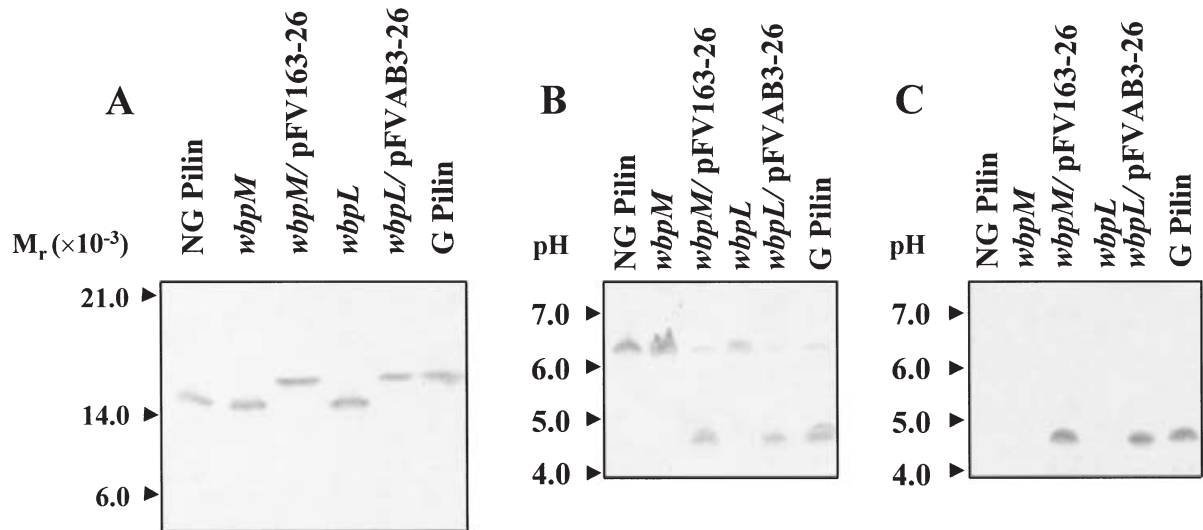


Fig. 3. Immunoblot analysis of pilin produced by *P. aeruginosa* 1244 *wbpM* and *wbpL* mutants. Western blot of cell extracts using anti-1244 pilin mAb 6.45 as probe (A). Immunoblot of pilin separated by electrofocusing using anti-1244 pilin mAb 6.45 (B) or anti-1244 pilin glycan mAb 11.14 (C) as probe. NG pilin is non-glycosylated 1244 pilin, and G is glycosylated pilin from the same strain. The 1244 *wbpM* and *wbpL* mutants both produce only non-glycosylated pilin. Complementation of these mutants with pFV163-26 (*wbpM*_{PAO1}) and pFVAB3-26 (*wbpL*₁₂₄₄), respectively, restored the ability to form glycosylated pilin.

glycan, and the glycan is identical to the O-antigen repeating unit, a question concerning pilin glycan substrate specificity arises. The high degree of variability in *P. aeruginosa* O-antigen structure (Knirel, 1990) suggests that there is either more than one glycosylating system available to this organism, depending on O-antigen, or one type of glycosylation machinery that is non-specific with regard to glycan structure.

To examine the glycan substrate specificity question, heterologous cloned O-antigen biosynthetic clusters were expressed in strain 1244, and the pilin produced was tested for glycosylation. To do this, the cloned O-antigen biosynthesis cluster from a serotype O11 strain of *P. aeruginosa*, contained in cosmid pLPS2, was mobilized into strain 1244 by triparental mating. Western immunoblotting analysis using O-specific antisera (Fig. 4A and B) showed that this strain produced O11 as well as host (O7) O-antigen. This indicated that the O-antigen transport and assembly apparatus of strain 1244 was compatible with the O-antigen repeating units of *P. aeruginosa* O11. However, these results were not conclusive to indicate that the pilin in the transformed strains is glycosylated with O11, O7 or both O-antigen sugars as their glycan constituents.

Pilin glycosylated with the cloned O-antigen repeating unit would be difficult to detect by Western immunoblotting using pilin-specific antibodies as this protein would have approximately the same molecular weight as native glycosylated pilin. In addition, the presence of LPS in the pilus preparations would preclude the use of LPS-specific sera in these blots. Therefore, pilin produced by *P. aeruginosa*

1244 (pLPS2) was subjected to isoelectric focusing to separate LPS from pilin, blotted to nitrocellulose and probed with pilin-specific and O-antigen-specific antibodies. Probing with a pilin-specific monoclonal antibody (Fig. 4C) showed that pilin from *P. aeruginosa* 1244 (pLPS2) was produced in both an acidic and a more neutral form. The pI of the acidic form matched that of wild-type glycosylated 1244 pilin, whereas the more neutral form co-migrated with non-glycosylated pilin. However, a Western immunoblot of this material, using a pilin protein-specific monoclonal antibody, indicated that no non-glycosylated pilin was present (results not shown). The O11 repeating unit, unlike the O7 repeating unit, is uncharged (Knirel, 1990) and, if attached to pilin, would produce a migration pattern identical to that of the non-glycosylated form. The presence of two pilin forms, differing by only glycan, was supported by probing identical blots with antisera specific for either the O11 antigen or the O7 antigen. Only the more neutral pilin form reacted with the O11-specific monoclonal antibody (Fig. 4D), whereas the acidic form reacted only with the O7 antibody (Fig. 4E). These results indicate that two populations of glycosylated pilins were produced. One contained bound O7 repeating unit, while the other had the O11 antigen subunit attached.

To substantiate the above findings further, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis was performed on purified pilin from the transconjugant strain *P. aeruginosa* 1244 (pLPS2). Non-glycosylated mature 1244 pilin

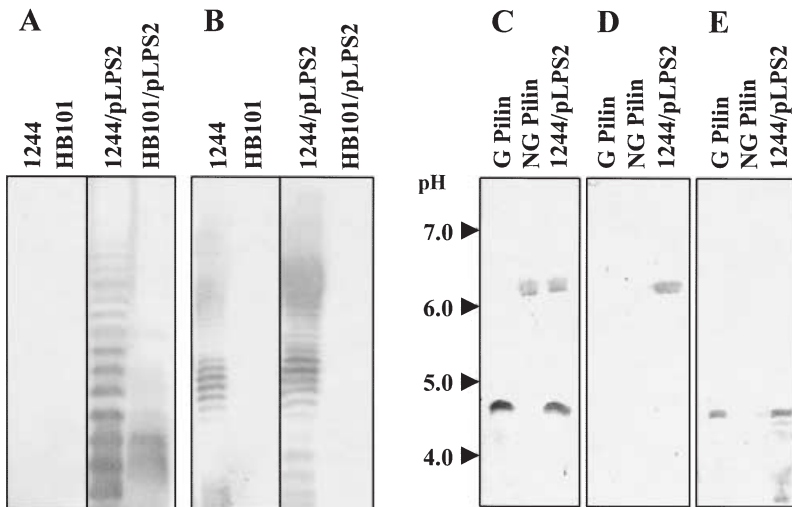


Fig. 4. Immunoblot analysis of LPS and pilin produced by *P. aeruginosa* 1244 (pLPS2). Western blot of LPS from *P. aeruginosa* 1244, *E. coli* HB101, *P. aeruginosa* 1244 (pLPS2) and *E. coli* HB101 (pLPS2) probed with anti-O11 mAb O11 (A) or anti-O7 mAb 11.14 (B). Immunoblot of pilin from *P. aeruginosa* 1244 (pLPS2) separated by electrofocusing and probed with anti-1244 pilin mAb 6.45 (C), anti-O11 mAb O11 (D) or anti-O7 mAb 11.14 (E). *P. aeruginosa* 1244 (pLPS2) produces LPS containing both O7 and O11 antigen and glycosylates its pilin with both these antigens.

is predicted to have a mass of 15 648 Da (Castric, 1995). Addition of the glycan mass, 666, would give 16 313, a value consistent with that determined previously (Castric *et al.*, 2001). Strain 1244 (pLPS2) pilin containing the O11 repeating unit, as defined by Knirel (1990), would be predicted to have a mass of 16 183. MALDI-TOF MS analysis of pure pilin produced by *P. aeruginosa* 1244 (pLPS2) showed two major peaks (results not shown). The base peak occurred at a mass of m/z 16 187 (± 10), a value that corresponded closely to pilin glycosylated with the O11 repeating unit. In contrast, analysis of glycosylated pilin produced from *P. aeruginosa* 1244 showed no peak at this mass. In addition, the putative heterologously glycosylated pilin produced a 30% base peak with a mass of m/z 16 316 (± 25), a size nearly identical to that predicted for glycosylated 1244 pilin.

To determine whether the O-antigen pathway from a source other than *Pseudomonas* could serve as the

glycan source for pilin glycosylation, the O-antigen biosynthetic cluster from *Escherichia coli* O157:H7 was expressed in *P. aeruginosa* 1244. Results from the Western immunoblotting analysis showed that the biosynthesis cluster from *E. coli* O157:H7, expressed from pDIG5, was able to support production of the *E. coli* O-antigen in *P. aeruginosa* 1244 (Fig. 5A and B). In addition, strain 1244 was able concomitantly to produce its own O-antigen. These results suggest that there is no incompatibility between the LPS assembly pathway of strain 1244 and the *E. coli* O157 O-antigen. The transconjugant strain *P. aeruginosa* 1244 (pDIG5) produced two electrophoretically distinct forms of 1244 pilin (Fig. 5C), one of which has a pI of ≈ 6.25 and corresponded to non-glycosylated 1244 pilin. No non-glycosylated pilin was detected in this preparation using Western blot with a pilin protein-specific monoclonal antibody (results not shown). The other pilin form has a pI of ≈ 4.75 and co-migrated with the glycosy-

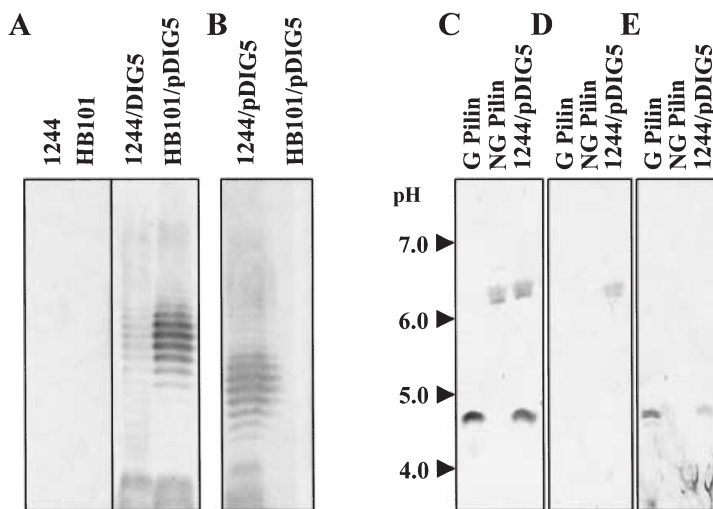


Fig. 5. Immunoblot analysis of LPS and pilin produced by *P. aeruginosa* 1244 (pDIG5). Western blot of LPS from *P. aeruginosa* 1244, *E. coli* HB101, *P. aeruginosa* 1244 (pDIG5) and *E. coli* HB101 (pDIG5) probed with an O157-specific polyclonal serum (A) or anti-O7 mAb 11.14 (B). Immunoblot of pilin from *P. aeruginosa* 1244 (pDIG5) separated by electrofocusing and probed with anti-1244 pilin mAb 6.45 (C), an O157-specific polyclonal serum (D) or anti-O7 mAb 11.14 (E). *P. aeruginosa* 1244 (pDIG5) produces LPS containing both *E. coli* O157 and *P. aeruginosa* O7 antigen and glycosylates its pilin with both these antigens.

lated form of 1244 pilin. Only the more neutral form of these pilins reacted with a polyclonal antiserum specific for the *E. coli* O157 O-antigen (Fig. 5D). The acidic pilin form reacted only with a monoclonal antibody specific for the *P. aeruginosa* O7 O-antigen (Fig. 5E). These results suggest that a fraction of the pilin subunits produced were glycosylated with the O157 repeating unit, whereas the remainder contains the O7 antigen.

Altogether, the results presented here provide concrete evidence that the O-antigen biosynthesis pathway serves as the metabolic source of the 1244 pilin glycan. In addition, they indicate that the glycosylation apparatus of strain 1244 has broad specificity. It is apparent that strain 1244 could use glycans that are significantly different in size, structure and charge from the homologous substrate.

Glycosylation of 1244 pilin by heterologous *P. aeruginosa* strains

Previous work has shown that PilO is an absolute requirement for pilin glycosylation in *P. aeruginosa* 1244 (Castric, 1995). As cloned heterologous O-antigen biosynthesis clusters can serve as a glycan source in strain 1244, it might be possible for PilO from this strain to be able to glycosylate 1244 pilin in heterologous strains if no other pilin-specific glycosylation elements were required. To investigate this, a cloned 1244 structural gene (*pilA*) was expressed in eight *P. aeruginosa* strains heterologous for O-antigen, in either the presence or the absence of *pilO*. As none of the strains tested produced pilin that reacted with the 1244 pilin-specific monoclonal antibody used in the assay, this strategy allowed for the unambiguous determination of heterologous pilin. Figure 6 shows the results of an experiment using one of these strains, *P. aeruginosa* PA103 (serotype O11), which lacks a *pilO* gene, as determined by Southern blot using 1244 *pilO* as

a probe (S. Bish and P. Castric, unpublished observations). This organism, containing only the vector control (pMMB66EH), produced no 1244 pilin, as determined by Western immunoblotting (Fig. 6A). The presence of strain 1244 *pilA* in the absence of *pilO* (pPAC24) resulted in the production of strain 1244 pilin with an apparent molecular weight equivalent to that of the non-glycosylated form. The inclusion of *pilO* (pPAC46) significantly increased the 1244 pilin molecular weight, suggesting that this protein had been post-translationally modified. The size difference between this protein and standard glycosylated pilin would be consistent with the presence of the O11 O-antigen repeating unit which, as described above, is smaller than the O7 structure (Fig. 6A).

To determine whether 1244 pilin produced by PA103 (pPAC46) contained bound O11 antigen, this protein was separated from contaminating LPS by isoelectric focusing, blotted and probed with antibodies specific for either 1244 pilin protein or the O11 antigen. The 1244 pilin produced by pPAC24 focuses at approximately pH 6.25 (Fig. 6B), which is equivalent to the pI of non-glycosylated 1244 pilin described in an earlier study (Castric, 1995). As the O11 polysaccharide repeating unit is uncharged, pilin containing this structure would be expected to have the same pI as non-glycosylated 1244 pilin, as described above. Only 1244 pilin produced in the presence of the *pilO* gene product reacted with the anti-O11 monoclonal (Fig. 6C), indicating that the protein contained bound O-antigen. These data suggest that PilO is capable of attaching the O11 repeating unit to 1244 pilin, indicating a low glycan specificity in the glycosylation reaction. These results were reproducible when the 1244 pilin expression was tested in seven other *P. aeruginosa* strains that produced structurally distinct O-antigens of differing serotypes: 1410 (O2), PAO1 (O5), PAKN1 (O6), 5870 (O13), 4239 (O16), 8433 (O19) and 7706 (non-typeable clinical isolate). In each case, 1244 pilin was produced only in the

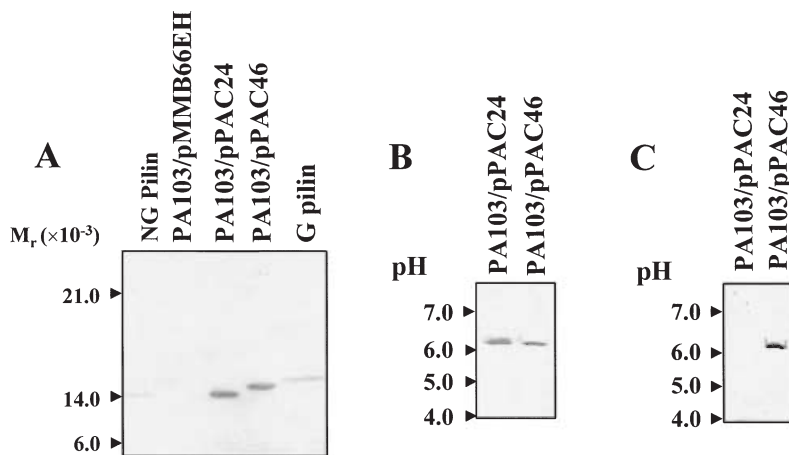


Fig. 6. Immunoblot analysis of pilin produced by *P. aeruginosa* 1244 (pPAC24) and *P. aeruginosa* 1244 (pPAC46). Western blot of cell extracts using anti-1244 pilin mAb 6.45 as probe (A). Immunoblot of pilin separated by electrofocusing using anti-1244 pilin mAb 6.45 (B) or anti-O11 mAb O11 (C) as probe. NG pilin is non-glycosylated 1244 pilin, and G is glycosylated pilin from the same strain. Strain 1244 pilin produced in strain PA103 in the presence of the *pilO* gene is glycosylated with O11 antigen.

presence of either pPAC24 or pPAC46. As with strain PA103, glycosylated pilin was detected only when the *pilO* gene was available (data not shown)

These results not only confirm the lack of glycan specificity in pilin glycosylation, but demonstrate the vital role that PilO plays in this process. In addition, these experiments suggest that PilO is the only requirement for pilin glycosylation that is not an integral part of another cell function.

Discussion

The structural similarity between the *P. aeruginosa* 1244 pilin glycan and the serotype O7 O-antigen repeating unit suggested that this saccharide was derived from the O-antigen biosynthetic pathway (Castric *et al.*, 2001). This was substantiated in the present paper by two lines of evidence. In the first, defined 1244 mutants defective in specific steps of the O-antigen biosynthetic pathway were unable to glycosylate pilin, whereas complemented mutants regained that ability. In the second, the expression of cloned O-antigen biosynthesis gene clusters in strain 1244 resulted in heterologous pilin glycosylation.

An important question arising from these results concerns how pilus and O-antigen biosynthesis intersect, in terms of both metabolism and cellular location, to produce pilin glycosylation. These pathways are multistep reactions that begin in the cytoplasm and culminate at the cell exterior (Mattick *et al.*, 1996; Darzins and Russell, 1997; Rochetta *et al.*, 1999). Evidence presented here indicates that a mutant (*wbpL*), lacking a transferase required for assembly of the O-antigen repeating unit, was unable to support pilin glycosylation. This suggests that pilin glycosylation does not occur through the sequential attachment of O-antigen sugars to pilin. This evidence, along with the relation of the glycan structure to the O-antigen (Castric *et al.*, 2001), also suggests that the glycosylation precursor may be the undecaprenol-bound repeating unit. If this is the case, pilin glycosylation could occur at the cell membrane in either the cytoplasm or, as a result of the Wzx-mediated transfer of the O-antigen repeating unit (Rochetta *et al.*, 1999), in the periplasm. In this situation, the pilin cytoplasmic membrane pool (Watts *et al.*, 1982) would provide the protein substrate. If pilin glycosylation occurs in the periplasm, it would be topologically similar to the dolichol pathway of protein glycosylation in eukaryotes (Burda and Aebi, 1999), with the periplasmic space functioning in a manner analogous to the lumen of the endoplasmic reticulum. This general strategy has been proposed previously for the attachment of the glycosaminoglycan and the sulphated oligosaccharides to the surface layer protein of *Halobacterium halobium* (Lechner and Wieland, 1989).

Wherever these pathways converge, it is clear that PilO is the only factor required for pilin glycosylation that is not a component of either O-antigen or pilus biosynthesis. It will be necessary to determine the mechanism of action, as well as specific details of glycan and pilin substrate specificity, to clarify the role of PilO in pilin glycosylation. Although much work remains to be done in PilO characterization, patterns of glycan substrate specificity are apparent from the work presented here, where it is shown that glycosylation could use eight different O-antigens, including *E. coli* O157:H7, as glycosylation substrate. The fact that the O-antigens used by saccharides in this study (Fig. 7) vary so greatly in structure, size and overall charge raises the intriguing question as to how PilO would recognize these molecules. Although common sugars and linkages are seen among certain related repeating units (for example, serotypes O2, O5 and O16), no major structures are shared by all groups. The results presented suggest that *P. aeruginosa* does not use A-band polysaccharide or peptidoglycan biosynthesis for pilin glycosylation, as no apparent pilin glycosylation occurs in the absence of O-antigen biosynthesis. Further, a single glycan isoform is produced by pilin glycosylation (Castric *et al.*, 2001), indicating that polymerized O-antigen repeating units do not serve as a glycosylation substrate. These findings suggest that recognition of the glycosylation substrate may be based on a combination of molecule size and the presence of some common structural elements. For example, if the glycan is transferred to pilin via undecaprenol phosphate, PilO may recognize portions of this molecule in addition to (or instead of) the O-antigen repeating unit.

As prokaryotes produce relatively few polysaccharides, and so have limited routes for biosynthesis of these molecules, it would not be surprising to find that these organisms must use pathways dedicated to saccharide production for protein glycosylation. This appears to be the case in examples such as pilin glycosylation by *N. meningitidis* (Jennings *et al.*, 1998; Powers *et al.*, 2000; Kahler *et al.*, 2001) and flagellin glycosylation by *P. aeruginosa* (Arora *et al.*, 2001). In *H. halobium*, a common pathway produces sulphated oligosaccharides used for glycosylation of the S-layer flagellin of this organism (Wieland *et al.*, 1985; Lechner and Wieland, 1989). An analogous situation may occur in the multiple glycosylation of *Campylobacter* proteins (Logan *et al.*, 1989; Guerry *et al.*, 1996). It is therefore unusual to find that pilin glycosylation in *P. aeruginosa* 1244 uses a major pathway (O-antigen biosynthesis) that normally operates to fulfil a separate physiological function. An obvious disadvantage to this arrangement is that the pilin-bound O-antigen repeating unit presents an additional antigenic target in which at least some of the antibodies directed against the lipopolysaccharide O-antigen, a major target of the

Serotype	Structure
<i>Pseudomonas aeruginosa</i>	
O2:	→4)-β-D-Man(2NAc3N)A-(1→4)-α-L-Gul(2NAc3NAc)A-(1→3)-β-D-FucNAc-(1→ 3 CH ₂ C=NH
O5:	→4)-β-D-Man(2NAc3N)A-(1→4)-β-D-Man(2NAc3NAc)A-(1→3)-α-D-FucNAc-(1→ 3 CH ₂ C=NH
O6:	→3)-α-L-Rha-(1→4)-α-D-GalNAcA-(1→4)-α-D-GalNFmA-(1→3)-α-D-QuiNAc-(1→ 3 6 OAc NH ₂
O7:	→4)-α-Pse(5N7NFm)-(2→4)-β-D-Xyl-(1→3)-β-D-FucNAc-(1→ 6 4 OCCH ₂ CH(OH)CH ₃ OAc
O11:	→2)-β-D-Glc-(1→3)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→
O13:	→2)-α-L-Rha-(1→3)-α-L-Rha-(1→4)-α-D-GalNAcA-(1→3)-β-D-QuiNAc-(1→ 3 OAc
O16:	→4)-β-D-Man(2NAc3N)A-(1→4)-β-D-Man(2NAc3NAc)A-(1→3)-β-D-FucNAc-(1→ 3 CH ₂ C=NH
<i>Escherichia coli</i>	
O157:	→3)-α-L-Fuc-(1→4)-β-D-Glc-(1→3)-α-D-GalNAc-(1→2)-α-D-PerNAc-(1→

Fig. 7. Chemical structures of the O-antigen repeating units from seven serotypes of *P. aeruginosa* (Knirel, 1990) and *E. coli* O157:H7 (Perry *et al.*, 1986).

immune response, would also be expected to recognize the pilus. This would be important as a monoclonal antibody reacting with the 1244 pilin glycan has been shown to block twitching motility by this organism (Castric *et al.*, 2001). On the other hand, the utilization of the O-antigen pathway for pilin glycosylation would represent a saving of energy expended for biosynthesis. A further advantage could be found in the low glycan substrate specificity of PilO. This property would guarantee that a *pilAO* operon entering the genome of a type IV pilin-producing species by reciprocal recombination would be able to code for pilin that had immediate access to the glycosylation substrate. Horizontal transfer of the ability to form glycosylated pili could then be carried out using small segments of information without the necessity for the mobilization of an entire pathogenicity island. This low specificity would also guarantee that variations in O-antigen biosynthesis (which could also be introduced by horizontal transfer), such as the addition or removal of functional groups, would not interfere with pilin glycosylation.

Previous work (Comer *et al.*, 2002) has shown that *P. aeruginosa* 1244 pili are able to stimulate glycan-specific antibodies. Further, the inhibition of twitching motility, a process important to *P. aeruginosa* virulence, by an anti-glycan monoclonal antibody (Castric *et al.*, 2001) indicates that this structure is a potential vaccine target. Of further significance is the finding that antibodies raised against the 1244 pilus glycan recognize LPS from this organism (Comer *et al.*, 2002). This suggests that pilus immunization could be used to raise a protective LPS-specific T-dependent B-cell response without the disadvantages of LPS toxicity and the requirement for LPS

purification. The ability of PilO to attach *E. coli* O157 O-antigen to 1244 pilin suggests that repeating units from a variety of Gram-negative pathogens could be used for pilin glycosylation. This raises the possibility of a pilus/O-antigen bioconjugate vaccine produced by the expression of a pathogen O-antigen cluster in a *P. aeruginosa* strain capable of glycosylating pilin. Such an effort will first require the determination of the range of glycan substrate specificity for PilO and the compatibility of pathogen O-antigen cluster expression with *P. aeruginosa*.

Experimental procedures

Culture conditions

All strains were grown aerobically on LB plates or broth cultures at 37°C. Broth cultures were grown on a rotatory shaker at 275 r.p.m. The following antibiotics (Sigma) were used in selective media at the indicated concentrations: ampicillin at 100 µg ml⁻¹ for *E. coli*; carbenicillin at 200 µg ml⁻¹ for *P. aeruginosa* 1244; gentamicin at 15 µg ml⁻¹ for *E. coli* and 300 µg ml⁻¹ for *P. aeruginosa* 1244; tetracycline at 15 µg ml⁻¹ and 50 µg ml⁻¹ for *E. coli* and *P. aeruginosa* respectively. IPTG (20 mM) and Xgal (40 µg ml⁻¹) (Gibco BRL) were added to solid media to determine loss of *lacZ* α-complementation in cloning experiments using the appropriate vector–host combinations. *Pseudomonas* isolation agar (PIA; Difco) was used for selecting *P. aeruginosa* transconjugants after the mating experiments.

Generation of 1244 wbpM null mutant

Pseudomonas aeruginosa 1244 *wbpM* mutants were generated by the gene replacement strategy described by Schweizer and Hoang (1995) using a previously generated

*wbpM*_{PAO1} knock-out construct (Burrows *et al.*, 1996). Briefly, this construct contains *wbpM* from *P. aeruginosa* strain PAO1 interrupted at the *NruI* site with a gentamicin resistance (*Gm*^R) cassette cloned into the gene replacement vector pEX100T. Homologous recombination of the *wbpM*_{PAO1::Gm}^R allele with *wbpM*₁₂₄₄ on the chromosome was confirmed by Southern blot analysis. Chromosomal DNA from 1244 and three *wbpM* mutants was isolated using DNAzol reagent (Gibco BRL) according to the manufacturer's instructions and digested with *XhoI*. Southern hybridizations were performed using the gentamicin resistance gene *aacC1* and *wbpM*_{PAO1} as separate DNA probes with a previously described protocol (Schweizer, 1993). These probes were labelled using a non-radioactive digoxigenin-dUTP labelling system (Boehringer Mannheim), and Southern blotting was performed under high stringency as described by the manufacturer.

Cloning of *wbpL* from strain 1244 and construction of *wbpL*₁₂₄₄ knock-out mutant

In Southern hybridization experiments described above for verifying the presence of the *Gm*^R in the 1244 *wbpM* knock-outs, a 3 kb *XhoI* fragment of 1244 DNA was found to hybridize to both the *wbpM*_{PAO1} and the *aacC1* probe in Southern blots. This fragment should contain *wbpL*, which is located 5'

to *wbpM* in strain PAO1. Thus, the following strategy was developed to clone *wbpL*₁₂₄₄. Genomic DNA from the 1244 *wbpM* mutant was digested with *XhoI* and electrophoresed on a 0.7% agarose gel. DNA bands containing *XhoI* fragments between 2.5 kb and 3.5 kb were isolated from the gel using GeneClean (MO BIO Laboratories), ligated to the cloning vector pBluescript-II SK and transformed into *E. coli* DH5 α by CaCl₂ transformation (Huff *et al.*, 1990). Transformants containing a plasmid with the 3 kb *wbpM*::*Gm*^R insert were selected for by growth on gentamicin–Luria agar plates. The presence of *wbpL* was verified by performing sequence analysis of the 3 kb insert of pFVAB1-SK. To generate the *wbpL* knock-out construct, a 1.5 kb *KpnI* fragment containing *wbpL*₁₂₄₄ was then cloned into the replacement vector pEX18Ap, and an 879 bp *Gm*^R cassette from pUCGM was inserted into the unique *NruI* site within *wbpL*₁₂₄₄; the new construct was designated pFVAB2-18Ap. The success of constructing 1244 *wbpL*::*Gm*^R mutants were confirmed by Southern blot analysis (Fig. 1).

DNA sequencing and analysis

The DNA sequence from the 3 kb insert of pFVAB1-SK obtained from strain 1244 *wbpM*::*Gm*^R was determined by the Guelph Molecular Super Centre (University of Guelph, Guelph, Ontario, Canada) using the d-Rhodamine terminator

Table 1. Strains and plasmids used in this study.

Strains/plasmids	Description	Source or reference
Strains		
<i>P. aeruginosa</i>		
1244	Wild type, IATS O7	Ramphal <i>et al.</i> (1984)
1244N3	<i>rpoN</i> , IATS O7	Ramphal <i>et al.</i> (1991)
PA103	Wild type, IATS O11	Liu (1973)
PAO1	Wild type, IATS O5	Holloway <i>et al.</i> (1979)
PAKN1	<i>rpoN</i> , IATS O6	Ishimoto and Lory (1989)
1410	Clinical isolate, IATS O2	Mercy Hospital, Pittsburgh, PA, USA
5870	Clinical isolate, IATS O13	Mercy Hospital, Pittsburgh, PA, USA
4239	Clinical isolate, IATS O16	Mercy Hospital, Pittsburgh, PA, USA
8433	Clinical isolate, IATS O19	Mercy Hospital, Pittsburgh, PA, USA
7709	Clinical isolate, IATS non-typeable	Mercy Hospital, Pittsburgh, PA, USA
<i>E. coli</i>		
SM10	<i>thi-1 thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu, Km ^r	Simon <i>et al.</i> (1993)
DH5 α	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco BRL
Plasmids		
pBluescript-II SK	2.9 kb cloning vector, Ap ^R	PDI Biosciences
pEX18Ap	5.8 kb gene replacement vector with multiple cloning sites from pUC18, <i>sacB</i> ⁺ , Ap ^R	Hoang <i>et al.</i> (1998)
pEX100T	5.8 kb gene replacement vector, <i>oriT</i> , <i>sacB</i> ⁺ , Ap ^R	Schweizer and Hoang (1995)
pUCGM	contains the 879 bp <i>Gm</i> ^R cassette, Ap ^R , <i>Gm</i> ^R	Schweizer (1993)
pUCP26	4.9 kb pUC18-derived broad-host-range cloning vector, Tc ^R	West <i>et al.</i> (1994)
pFVAB1-SK	3 kb <i>XhoI</i> fragment in pBluescript-II SK; contains <i>wbpL</i> ₁₂₄₄ and <i>wbpM</i> :: <i>Gm</i> ^R	This study
pFVAB2-18Ap	1.5 kb <i>KpnI</i> – <i>KpnI</i> insert in pEX18Ap containing <i>wbpL</i> ₁₂₄₄ , with a <i>Gm</i> ^R cassette cloned into <i>NruI</i> site	This study
pFVAB3-26	1.5 kb <i>KpnI</i> – <i>KpnI</i> fragment in pUCP26 for complementation; contains <i>wbpL</i> ₁₂₄₄	This study
pFV163-26	3.6 kb <i>XbaI</i> – <i>SaI</i> insert in pUCP26 for complementation; contains <i>wbpM</i> _{PAO1}	Burrows <i>et al.</i> (1996)
pDS300	Cosmid, <i>E. coli</i> O157 gene cluster	Maurer <i>et al.</i> (1999)
pLAFR1	Broad-host-range cosmid	Friedman <i>et al.</i> (1982)
pDIG5	pLAFR1 with O157 gene cluster	This study
pLPS2	pLAFR1 with <i>P. aeruginosa</i> O11 gene cluster	Goldberg <i>et al.</i> (1992)
pMMB66EH	Broad-host-range expression vector	Furste <i>et al.</i> (1986)
pPAC24	pEMMB66EH with strain 1244 <i>pilA</i>	Castric (1995)
pPAC46	pEMMB66EH with strain 1244 <i>pilAO</i>	Castric (1995)

cycle sequencing ready reaction kit (Perkin-Elmer). DNA sequence analysis was performed using the computer software program GENE RUNNER for Windows (Hastings Software), and nucleotide and protein alignments were performed by CLUSTALW (European Bioinformatics Institute; <http://www.ebi.ac.uk/clustalw/>). Nucleotide and amino acid homology searches were determined using the GenBank DNA and protein sequence databases through the National Center for Biotechnology Information BLAST network server (Altschul *et al.*, 1997).

Construction of pDIG5

As pDS300, a cosmid that contains the *E. coli* O157:H7 O-antigen gene cluster (Maurer *et al.*, 1999), was unable to replicate in *P. aeruginosa*, it was necessary to subclone the cluster region to a broad-host-range vector. An *EcoRI* partial digestion of pDS300 was ligated with pLAFR1 cut with the same enzyme. This DNA was packaged as lambda particles that were used to infect *E. coli*. Tetracycline-resistant colonies were tested for expression of O157 antigen by colony lift using a polyclonal antiserum (Difco Laboratories). Digestion of plasmid DNA with *EcoRI* followed by agarose electrophoresis revealed the presence of pLAFR1 vector and pDS300 insert DNA. Western immunoblotting analysis of positive clones using this same antiserum confirmed that the functional O-antigen cluster had been transferred to pLAFR1 producing pDIG5. This cosmid was mobilized to *P. aeruginosa* 1244 by triparental mating (Ruvkun and Ausubel, 1981), in which the expression of O157 antigen was confirmed by immunoblotting.

LPS and pilin preparation

LPS was prepared from whole-cell lysates of *P. aeruginosa* according to the method of Hitchcock and Brown (1983). Glycosylated and non-glycosylated pilin was prepared from *P. aeruginosa* 1244 as described previously (Castric *et al.*, 2001).

SDS-PAGE and isoelectric focusing

LPS was separated on 12.5% T Tris–glycine SDS-PAGE gels, whereas pilin samples were analysed using the same buffer system and 15% T resolving gels. LPS bands were visualized by the silver-staining method described by Dubray and Bezard (1982). Pili samples to be subject to isoelectric focusing were treated with 2% β -octylglucoside for 15 min at room temperature. This material was separated in a pH gradient from either 3.5 or 2.5 to 9.0 using a Pharmacia PhastSystem apparatus in which the gels had been equilibrated with 1.0% β -octylglucoside.

Immunoblot analysis

LPS or pilin separated by SDS-PAGE was transferred to nitrocellulose membrane by electroblotting, whereas diffusion blotting was used with pilin separated by isoelectric focusing. These blots were analysed in one of two ways. For LPS from the *P. aeruginosa* 1244 *wbpL* and *wbpM* mutants, the membrane was blocked with 3% skim milk and immersed in hybri-

doma culture supernatants containing monoclonal antibody (mAb) MF29-2 (specific for serotype O7 B-band LPS; Lam *et al.*, 1987) or mAb N1F10 (specific for the common antigen A-band LPS; Lam *et al.*, 1989). Detection of antibody reaction was carried out as described previously (Lam *et al.*, 1987). For all other blots, blocking was carried out as described previously (Castric *et al.*, 1989). LPS or pilin was detected using mAb 11.14 (specific for the 1244 pilin glycan and IATS serotype O7 O-antigen; Castric *et al.*, 2001), mAb O11 (specific for IATS serotype O11; ERFA, Westmount, Quebec, Canada), a polyclonal serum specific for *E. coli* O157 O-antigen (Difco, Detroit) or mAb 6.45 (specific for 1244 pilin protein; Castric and Deal, 1994). Detection of antibody reactions has been described previously (Castric *et al.*, 1989).

Pilin mass determination

Pilin mass analysis by MALDI-TOF mass spectrometry was performed by Dr M. Bier at the Mellon Institute Center for Molecular Analysis, Carnegie Mellon University, using a PerSeptive Biosystems Voyager STR with DE and a high m/z detector.

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Note added in proof

At the completion of our study, new information on the *P. aeruginosa* O-antigen gene cluster from an ATCC serotype O7 strain became available (Raymond, C. K., Sims, E. H., Kas, A., Spencer, D., Kutayavin, T., Ivey, R., Zhou, Y., Kaul, R., Clendenning, J. and Olson, M. V., direct submission accession number AC104737). Such information was useful to confirm the accuracy of sequencing results of the *wbpL* and *wbpM* genes of strain 1244.

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