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Coexistence of Two Distinct Versions of O-Antigen Polymerase, Wzy-Alpha and Wzy-Beta, in *Pseudomonas aeruginosa* Serogroup O2 and Their Contributions to Cell Surface Diversity[▽]

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Assembly of B-band lipopolysaccharide (LPS) in *Pseudomonas aeruginosa* follows a Wzy-dependent pathway, requiring the O-antigen polymerase Wzy and other proteins. The peptide sequences of the wzy_{α} product from strains of serotypes O2, O5, and O16 are identical, but the O units in O5 are α-glycosidically linked, while those in O2 and O16 are β-linked. We hypothesized that a derivative of the D3 bacteriophage wzy_{β} is present in the chromosomes of O2 and O16 and that this gene is responsible for the β-linkage. By a combination of PCR and primer walking, wzy_{β} genes of both serotypes have been amplified and cloned. They are identical but share only 87.42% sequence identity with their xenolog in D3. A chromosomal knockout mutant of O16 wzy_{β} was made, and it produces semirough LPS devoid of B-band O antigen. The cloned wzy_{β} is capable of complementing the O16 wzy_{β} mutant, as well as cross-complementing a wzy_{α} knockout mutant. However, in the latter case, the restored O antigen was β-linked. Using reverse transcription-PCR, we showed that wzy_{α} was transcribed in O2 and O16 strains and was functional, since both of these genes could complement the wzy_{α} mutant of O5. With the coexistence of wzy_{α} and wzy_{β} in O2 and O16 and the B-band O polysaccharides in these being β-linked, we hypothesized that *iap*, an inhibitor of the alpha-polymerase gene, must be present in these serotypes. Indeed, through PCR, TOPO-cloning, and nucleotide-sequencing results, we verified the presence of *iap* in both O2 and O16 serotypes.

Lipopolysaccharide (LPS) is an important virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa*. This glycolipid is found in the outer leaflet of the outer membrane and is composed of three main parts: lipid A, core, and O antigen. *P. aeruginosa* produces two distinct forms of LPS, called A band and B band. The O antigen of A band is composed of a homopolymeric repeating chain of D-rhamnose. B-band LPS, on the other hand, consists of heteropolymeric O-antigen units (28). Differences in the B-band O antigen divide *P. aeruginosa* into 20 distinct serotypes, as defined by the International Antigenic Typing Scheme (33, 34). The gene cluster for the biosynthesis of B-band LPS has been designated *wbp* (32). Based on results from Southern hybridization, Burrows et al. (9) found that the *wbp* cluster is highly conserved among serotypes O2, O5, O16, O18, and O20, which form the O2 serogroup (46). The cloning and sequencing of the LPS biosynthetic clusters from each of these serotypes by Raymond et al. (40) further substantiated the conserved nature of the LPS genes from members of this serogroup. The O antigens of these serotypes show immunochemical cross-reactivity when typed by slide agglutination using polyclonal rabbit antisera (29), but they are distinct from each other based on the type of glycosidic linkage or isomers/epimers of monosaccharides present and the presence or absence of acetyl substituent in their O-antigen sugars (23, 24) (Fig. 1).

wzy_{α} (formerly called *rfc*) has been shown to encode the B-band O-antigen polymerase and was responsible for the formation of α-linked O-antigen repeat units in serotype O5, strain PAO1 (14). Its nucleotide sequence differed from that of wzy_{α} of serotype O2 by one nucleotide, but the amino acid encoded within the same codon remained unchanged. Since the O antigens of serotype O5 are α-linked while those of serotype O2 are β-linked, it was puzzling how a single protein could catalyze glycosidic linkages of opposite stereochemistry (41). By examining the D3 phage genome sequence, which was annotated in 2000 (25), our group had identified a 3.6-kb fragment in the D3 genome capable of mediating serotypic conversion identical to that observed in a D3 lysogen strain (Fig. 2) (39). Three open reading frames (ORFs) were identified in this DNA fragment, and they encode a putative α-polymerase inhibitor (Iap), an O acetylase (Oac), and a β-polymerase (Wzy_{β}). The Wzy_{β} protein has a size of 42.6 kDa and contains nine predicted transmembrane segments, with a large periplasmic loop near the C terminus (39). It showed no primary amino acid sequence homology to other proteins, a characteristic that is consistent with Wzy/Rfc proteins from many gram-negative bacteria. wzy_{β} from D3 was used to complement a PAO1-derived (serotype O5) wzy_{α} null mutant that produced semirough LPS. Interestingly, the complemented strain exhibited an LPS banding pattern resembling that of serotype O16. In Western immunoblotting, the LPS bands reacted with O16-specific monoclonal antibody (MAb) (MF47-4), but not with O5-specific MAb (MF15-4), indicating that the restored O-antigen repeat units were β-linked. These data suggested that the D3 wzy_{β} encodes a functional O-antigen polymerase capable of catalyzing β-glycosidic linkages.

Two other ORFs in the 3.6-kb D3 DNA fragment, *iap* and

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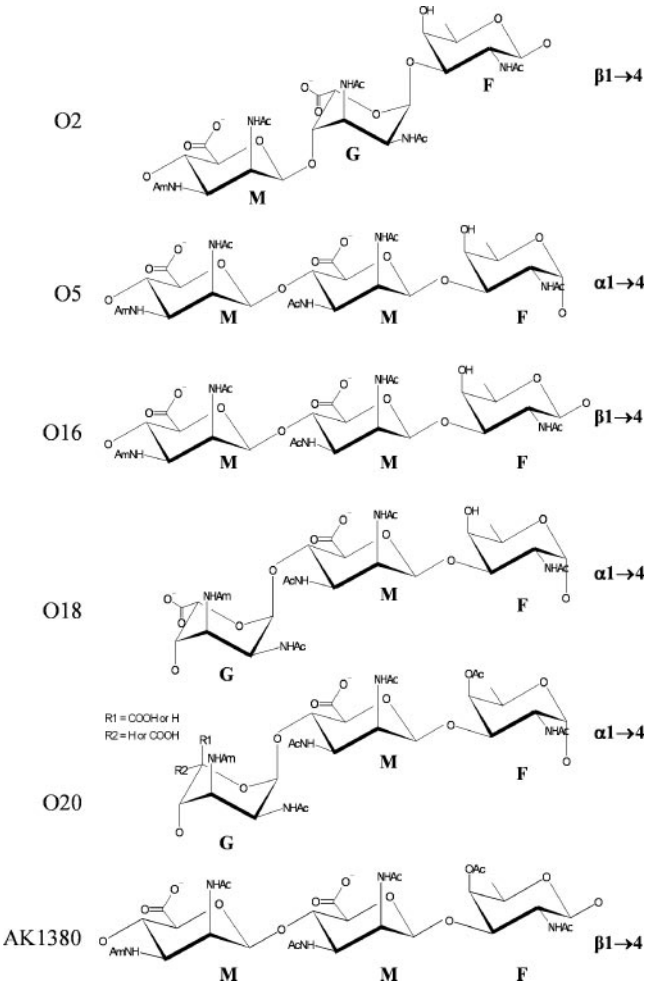

FIG. 1. Chemical structures of the *P. aeruginosa* serogroup O2 O-antigen repeat units. Serotypes O2, O5, O16, O18, and O20, which make up the O2 serogroup, as well as the D3 lysogen (AK1380), have highly similar O-antigenic sugar structures. Differences among them were due to the type of glycosidic linkage (α-D- versus β-D-*N*-acetylfucosamine) or isomer (β-D-mannuronic versus α-L-guluronic) or the presence/absence of *O*-acetyl groups at the C-4 of the fucosamine moiety (23, 26, 39). G, guluronic acid; M, mannuronic acid; F, fucosamine; OAc, O-acetyl group; NHAc, acetamido group; NHAm, acetamidino group.

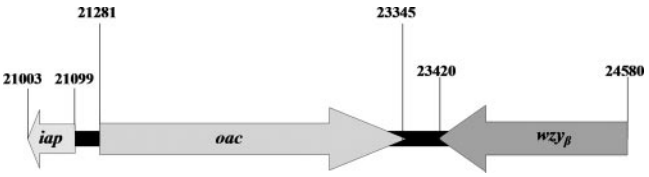

FIG. 2. The 3.6-kb seroconverting cassette of the D3 bacteriophage genome. The three genes involved in serotype conversion encode the Wzy_{α} inhibitor (*iap*; 96 bp), O-acetylase (*oac*; 2,064 bp), and O-antigen β-polymerase (wzy_{β}; 1,160 bp). The *iap*, *oac*, and wzy_{β} genes correspond to nucleotides 21099 to 21003, 21281 to 23345, and 24580 to 23420 of the D3 genome, respectively (25, 39).

oac, were found to encode proteins that also contribute to the mechanism of serotype conversion in *P. aeruginosa* strain PAO1. A plasmid containing the 96-bp *iap* ORF was capable of inhibiting long-chain B-band LPS production when transformed into serotypes O5 and O18 (39). The tranformants were still capable of producing A-band LPS. Transformation of the *iap* plasmid construct into serotype O16, which has β-linked units, showed no effect. These data indicate that Iap is an inhibitor of O-antigen polymerase with specificity for serotypes possessing α-linked O-antigen repeat units (39).

The *oac* ORF was the only one of the three genes in the 3.6-kb D3 DNA fragment that showed sequence similarity to other proteins. This 2,064-bp ORF was homologous to known *O*-acetyltransferase proteins (11). When PAO1 was transformed with a plasmid containing all three seroconverting genes, the resulting LPS produced by the transformant showed strong reaction with polyclonal anti-O20 antiserum that had been adsorbed with bacterial cells of O5 and O18 to make it specific for O-acetylated LPS, indicating that like serotype O20, the O antigen of the transformant contained O-acetylated sugars. These observations were substantiated by high-performance liquid chromatography analysis, which showed that LPS isolated from the transformant contained 45-fold more mild base-labile acetate than the wild-type control, PAO1 LPS (39). This increase corresponds to levels found in LPS from the D3 lysogen, *P. aeruginosa* strain 1380. When the *oac* gene was disrupted, LPS of the transformants did not show any detectable levels of O acetylation. The *oac* gene could not be used individually in the complementation tests with the PAO1 background strain, since it was discovered that Oac can acetylate only β-linked O-antigen repeat units (39).

In light of these findings, Newton et al. (39) proposed that *P. aeruginosa* serotypes O2 and O16 may have acquired a β-polymerase gene from bacteriophage D3 or another closely related phage and that this gene lies outside of the *wbp* cluster. This *P. aeruginosa* Wzy_{β} protein would then be responsible for the β-linkages observed in these serotypes. Southern hybridization experiments showed that a probe generated from D3 wzy_{β} bound to *P. aeruginosa* genomic DNA from serotypes O2 and O16, but not O5, O18, or O20 (39). Interestingly, an *iap* probe did not hybridize with any of the wild-type *P. aeruginosa* O2 serogroup serotypes. The only strain that bound the *iap* probe was the transformant containing the entire seroconverting cassette. Therefore, it was postulated that the native α-polymerase (Wzy_{α}) in the *wbp* cluster of serotypes O2 and O16 is either not functional or not being expressed (39).

Until this study, there was no direct experimental evidence that the O2 and O16 serotypes possess a functional β-polymerase in their chromosomes. We were able to identify, clone, and characterize wzy_{β} from the chromosomal DNA of O2 and O16. We also obtained evidence that wzy_{α} is transcribed in both serotypes O2 and O16 and that this gene is functional, i.e., capable of complementing a wzy_{α} mutant derived from serotype O5 (strain PAO1). Although previous studies suggested that *iap* was absent from the *P. aeruginosa* genome, we were successful in amplifying it by PCR using O2 and O16 chromosomal DNAs as templates. These observations further the understanding of the mechanisms responsible for the diversity in *P. aeruginosa* serotypes, in particular, the cause of α- versus β-glycosidic linkages in the O antigen.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype, phenotype or properties	Reference or source
E. coli		
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	51
ONE Shot Mach 1 T1	F$^-$ ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(r_K^- m_K^-) Δ*recA1398 endA1 tonA* (confers resistance to phage T1); chemically competent	Invitrogen
SubCloning efficiency DH5α competent cells	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
SM10	*thi-1 thr leu tonA lacy supE recA* RP4-2-Tc::Mu Km^r	44
P. aeruginosa		
O5	Wild-type strain PAO1; IATS serotype O5; A$^+$ B$^+$	17
O2	Wild-type strain 5934; IATS serotype O2 (ATCC 33349); A$^+$ B$^+$	33
O16	Wild-type strain 170003; IATS serotype O16 (ATCC 33363); A$^-$ B$^+$	33
O5-wzy$_\alpha$-Gm^R	O5 *wzy*$_\alpha$::Gm^r; O5 mutant with a gentamicin cassette inserted into the *wzy*$_\alpha$ gene	14
O16-wzy$_\beta$-Gm^R	O16 *wzy*$_\beta$::Gm^r; O16 mutant with a gentamicin cassette inserted into the *wzy*$_\beta$ gene	This study
O5-wzy$_\alpha$-Gm^R-27	O5-wzy$_\alpha$ mutant transformed with the pUCP27 plasmid	This study
O5-wzy$_\alpha$-Gm^R-comp-O5wzy$_\alpha$	O5-wzy$_\alpha$ mutant complemented with pUCP27 containing *wzy*$_\alpha$ from O5	This study
O5-wzy$_\alpha$-Gm^R-comp-O2wzy$_\alpha$	O5-wzy$_\alpha$ mutant complemented with pUCP27 containing *wzy*$_\alpha$ from O2	This study
O5-wzy$_\alpha$-Gm^R-comp-O16wzy$_\alpha$	O5-wzy$_\alpha$ mutant complemented with pUCP27 containing *wzy*$_\alpha$ from O16	This study
O5-wzy$_\alpha$-Gm^R-comp-O2wzy$_\beta$	O5-wzy$_\alpha$ mutant complemented with pUCP27 containing *wzy*$_\beta$ from O2	This study
O16-wzy$_\beta$-Gm^R-27	O16 *wzy*$_\beta$::Gm^r mutant transformed with the pUCP27 plasmid	This study
O16-wzy$_\beta$-Gm^R-comp-O2wzy$_\alpha$	O16 *wzy*$_\beta$::Gm^r mutant complemented with pUCP27 containing *wzy*$_\alpha$ from O2	This study
O16-wzy$_\beta$-Gm^R-comp-O2wzy$_\beta$	O16 *wzy*$_\beta$::Gm^r mutant complemented with pUCP27 containing *wzy*$_\beta$ from O2	This study
Plasmid		
pEX18Ap	Ap^r/Cb^r *oriT*$^+$ *sacB*; gene replacement vector with multiple cloning site from pUC18	20
pEX18Ap-O2wzy$_\beta$	pEX18Ap with *wzy*$_\beta$ inserted into SacI and PstI sites	This study
pEX18Ap-O16 wzy$_\beta$-Gm^R	pEX18Ap with *wzy*$_\beta$ with gentamicin cassette inserted in the EcoRI site of *wzy*$_\beta$	This study
pPS856	Ap^r Gm^r; 0.83-kb blunt-ended SacI fragment from pUCGM ligated into the EcoRV site of pPS854	20
pQE80	4.8-kb histidine-tagged expression vector with RBS; Ap^r	QIAGEN
pUCP27	4.9-kb pUC18-based broad-host-range vectors; Tc^r	50
pUCP27-RBS-O5wzy$_\alpha$	*wzy*$_\alpha$ from O5 with RBS from pQE80 cloned into EcoRI and PstI sites of pUCP27	This study
pUCP27-RBS-O2wzy$_\alpha$	*wzy*$_\alpha$ from O2 with RBS from pQE80 cloned into EcoRI and PstI sites of pUCP27	This study
pUCP27-RBS-O16wzy$_\alpha$	*wzy*$_\alpha$ from O16 with RBS from pQE80 cloned into EcoRI and PstI sites of pUCP27	This study
pUCP27-O2wzy$_\beta$	*wzy*$_\beta$ from O2 with RBS from pQE80 cloned into EcoRI and PstI sites of pUCP27	This study
TOPO-O2wzy$_\beta$-topfrag	5′ PCR fragment of O2 *wzy*$_\beta$ cloned into pCR-Blunt II-TOPO vector	This study
TOPO-O16wzy$_\beta$-topfrag	5′ PCR fragment of O16 *wzy*$_\beta$ cloned into pCR-Blunt II-TOPO vector	This study
TOPO-O2wzy$_\beta$-bottomfrag	3′ PCR fragment of O2 *wzy*$_\beta$ cloned into pCR-Blunt II-TOPO vector	This study
TOPO-O16wzy$_\beta$-bottomfrag	3′ PCR fragment of O16 *wzy*$_\beta$ cloned into pCR-Blunt II-TOPO vector	This study
TOPO-O2iap	*iap* from *P. aeruginosa* O2 cloned into pCR-Blunt II-TOPO vector	This study
TOPO-O16iap	*iap* from *P. aeruginosa* O16 cloned into pCR-Blunt II-TOPO vector	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Serotypes were confirmed by slide agglutination tests as described previously (29), using polyclonal antisera from the serotyping kit for *P. aeruginosa* (manufactured by Chengdu Institute of Biological Products, Ministry of Public Health, Chengdu, Sichuan, People's Republic of China) (34). Bacterial strains were cultured in Luria-Bertani broth (Invitrogen Canada Inc., Burlington, Ontario, Canada) at 37°C. *Pseudomonas* isolation agar (Difco) and resolving media were used for mating experiments. The resolving media consisted of 1.0% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 1.5% Bacto agar (Difco), 5% sucrose, and gentamicin. The following antibiotics were used: kanamycin, 50 μg/ml for *Escherichia coli*; tetracycline, 10 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; gentamicin, 150 and 300 μg/ml for *P. aeruginosa*; and carbenicillin, 600 μg/ml for *P. aeruginosa* (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada).

DNA procedures. Plasmid DNA was isolated using the GenElute plasmid miniprep kit (Sigma-Aldrich) following procedures recommended by the manufacturer. Calf intestinal alkaline phosphatase was purchased from New England Biolabs Ltd. (Mississauga, Ontario, Canada). Ligation of digested DNA was performed using T4 DNA ligase (New England Biolabs) at 16°C overnight (16 h). Alternatively, ligations were performed using the Rapid DNA ligation kit (Roche Applied Science, Laval, Quebec, Canada). The Zero-Blunt TOPO PCR cloning

kit (Invitrogen) was used for cloning blunt-ended PCR-amplified DNA into the pCR-Blunt II-TOPO vector. PCRs were performed in GeneAmpPCR System thermocyclers (models 2400 and 9700; Applied Biosystems) using *Pwo*I polymerase (Roche Applied Science, Quebec, Canada). PCR amplification of the *iap* (for inhibitor of α-polymerase) genes from *P. aeruginosa* serotypes O2 and O16 was performed using primers specific for the D3 *iap* 5′ and 3′ sequences: iapFwd (5′-GTGCATTTAAAATTTTCAATCATAG-3′) and iapRev (5′-CATATGTC TTGGTAGTAAGTTGC-3′), respectively. Oligonucleotide primers were purchased from the University of Guelph Molecular Supercenter. DNA extraction from agarose gels was performed using the UltraClean 15 DNA purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA). DNA in solution was purified using the High Pure PCR product purification kit (Roche) and eluted in 50 to 100 μl H_2O. Plasmid DNA was introduced into *P. aeruginosa* serotype O5 by electroporation using a Gene Pulser instrument (Bio-Rad).

Sequencing and in silico analysis. Sequencing of plasmid constructs, PCR products, and genomic DNA was performed at the Guelph Molecular Supercenter (University of Guelph, Guelph, Ontario, Canada). Sequences were analyzed using GENERUNNER for Windows (Hastings Software, NY), as well as the online servers Basic Local Alignment Search Tool (3, 4), CLUSTALW (48), EMBL-EBI (European Bioinformatics Institute), TMHMM Server v. 2.0 (45), SOSUI (18), and the MEMSAT3 prediction method of the PSIPRED Protein Structure Prediction Server (7, 21, 37).

Construction of plasmids for complementation. Complementation vectors were made by PCR amplifying the *P. aeruginosa* wzy_{α} gene using wzyfwd (5′-CGTTGA CGAAT**TCTAGA**ATGTATATAC-3′) and Rev/wzy/stopO5,O2,O16 (5′-GGTTGA TAAAAG**CTGCAG**TCATAG-3′) (restriction sites are shown in boldface). The fragment was then cloned into the pUCP27 vector (50). Once cloning was verified by sequencing, the fragment was excised with SstI and PstI and cloned into pQE80. To obtain the ribosome binding site (RBS) from pQE80, the fragment was excised with EcoRI and PstI and cloned into pUCP27. The same method was used for wzy_{α} from O5, O2, and O16. Since wzy_{β} contains an internal EcoRI site, this strategy could not be used for that gene. Instead, plasmid pUCP27-RBS-O5wzy_{α} was digested with XbaI and PstI to remove the O5 wzy_{α} gene, and a wzy_{β} gene amplified with flanking XbaI and PstI sites was cloned in.

Generation of a wzy_{β} chromosomal knockout mutant. The *P. aeruginosa* chromosomal mutant was generated using the method of Hoang et al. (20). The *P. aeruginosa* O2 wzy_{β} gene was amplified with flanking SacI and PstI restriction sites using wzybetafwdSacI (5′-AATA**GAGCTC**ATGAATAGGACCAAGCTT CCG-3′) and wzybetarevPstI (5′-GA**CTGCAG**TTAATTATCCTCGATTTTAA GATTGG-3′). The gene was then cloned into the suicide vector pEX18Ap, which contains the *sacB* gene of *Bacillus subtilis*. A gentamicin resistance (Gm^r) cassette from pPS856 was excised by SmaI digestion and then subcloned into the EcoRV site of the wzy_{β} insert DNA. The orientation of the Gm^r cassette was confirmed by restriction digests and sequencing. The resulting plasmid was transformed into the mobilizer strain *E. coli* SM10 and then conjugally transferred into *P. aeruginosa* serotype O16 according to the method of Simon et al. (44). After they had mated, the cells were spread plated on Pseudomonas Isolation Agar containing 150 μg/ml gentamicin. Colonies that grew on the gentamicin-containing medium were picked and streaked on resolving media.

LPS preparation for SDS-PAGE, silver staining, and Western blotting. LPS was prepared using the method of Hitchcock and Brown (19) and analyzed by separation on standard discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% running gels (27). LPS was visualized by silver staining using the method of Fomsgaard et al. (15) and by Western blotting using the standard procedure described by Towbin et al. (49) and Burnette (8), with minor modifications. The primary antibodies used in immunoblotting were MAb MF15-4 (specific for O5), MAb MF71-2 (specific for O2), and MAb MF47-4 (specific for O16). All the MAbs used have been described previously (30, 31). The secondary antibody was a goat anti-mouse $F(ab')_2$-alkaline phosphatase conjugate (Jackson ImmunoResearch). The blots were developed using a substrate containing 0.033% nitroblue tetrazolium and 0.016% BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 0.1 M sodium bicarbonate buffer, pH 9.8.

Immunofluorescence microscopy. Immunofluorescence microscopy was performed using the method described by Clarke et al. (12). Bacteria were viewed on a Zeiss Axiovert 200 microscope using a 100× objective lens and oil immersion, and the images were processed using Openlab software (Improvision). MAb MF15-4 was used for visualization of LPS antigen of O5, MAb MF71-2 for O2, and MAb MF47-4 for O16. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated AffiniPure $F(ab')_2$, at a 1:50 dilution in 0.85% NaCl containing 1% bovine serum albumin (Bio/Can Scientific).

Reverse transcription (RT) PCR. RNA was harvested from *P. aeruginosa* serotypes O5, O2, and O16 using the PURESCRIPT RNA isolation kit (Gentra Systems, Minneapolis, MN) as recommended by the manufacturer. To eliminate contaminating genomic DNA, the RNA solution was incubated at 37°C overnight with RQ1 RNase-Free DNase (Promega). RNA was converted to cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) as recommended by the manufacturer. PCR analysis was performed using primers specific for a 264-bp internal fragment of wzy_{α}. The primers used were alphaRTPCRfwd (5′-GAGGTGTTTGGCTATTCATTCTTG-3′) and alphaRTPCRrev (5′-CCCCCACATAACCCAACTGC-3′). A BLAST search was performed to confirm that the primers would bind specifically to wzy_{α}. The PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide for visualization under UV light. One microliter of extracted RNA was used as a negative control, while positive controls for the PCR consisted of *P. aeruginosa* genomic DNA as the template. Further, positive controls used primers specific for a 177-bp fragment of the *P. aeruginosa* housekeeping gene, *rpoD*. The primers used were rpoDf (5′-GGGCGAAGAAGGAAATGGTC-3′) and rpoDr (5′-CAGGTGGCGTAGGTGGAGAA-3′) (42). It has been shown that the *rpoD* gene is constitutively transcribed in all *P. aeruginosa* serotypes (42).

Nucleotide sequence accession numbers. The nucleotide sequences of wzy_{β} from *P. aeruginosa* O2 and O16 strains have been submitted to the NCBI-GenBank databases under the accession numbers EF153452 and EF153453, respectively.

RESULTS

***wzy_{α}* genes from *P. aeruginosa* serotypes O2 and O16 are functional in vivo and encode O-antigen polymerases.** The wzy_{α} gene was amplified from serotypes O2, O5, and O16 and sequenced. The nucleotide sequence of wzy_{α} from O16 had a different sequence than that reported in the NCBI databases. The sequence was identical to that of the O2 wzy_{α} (accession number AF498412). Results from antibody agglutination tests, as well as analysis of LPS by SDS-PAGE and Western blotting, confirmed that our strain was indeed serotype O16. Genomic DNA was reisolated from this strain, and wzy_{α} was amplified, cloned, and resequenced. Once again, the sequence corresponded to the O2 wzy_{α} sequence, indicating that our strain (ATCC 33363) was different than the O16 strain sequenced by Raymond et al. (40), accession number AF498408.

The O5 wzy_{α} mutant was then complemented using plasmids containing wzy_{α} genes from *P. aeruginosa* serotypes O2 and O16, as well as from serotype O5 as a positive control. wzy_{α} genes from all three strains were able to restore the smooth (S) LPS phenotype, as shown by the banding patterns of LPS of the complemented strains, which resembled that of the wild-type O5 strain (Fig. 3A). Results from Western immunoblotting revealed that LPS from wild-type serotype O5 and the complemented strain showed reactivity only with an O5-specific MAb (MF15-4). An O2-specific MAb (MF71-2) reacted strongly with LPS from wild-type serotype O2 but no other LPS. Similarly, an O16-specific MAb (MF47-4) reacted strongly with LPS from wild-type serotype O16 and weakly with serotype O2 LPS and showed no reactivity with the complemented strains. These results showed that regardless of the origin of the wzy_{α} gene from any of the three serotypes used in the complementation experiments, α-linked O antigen was restored in the O5 wzy_{α} mutant (Fig. 3B to D).

Immunofluorescence microscopy was used to further substantiate the results obtained by Western blotting. The complemented strains showed fluorescence when reacted with O5-specific MAb (MF15-4), followed by incubation with the FITC-labeled goat-anti-mouse Fab_2 secondary antibody. Immunofluorescence was observed on the outer surfaces of cells, which is the location of the receptor for the serotype-specific antibodies. Cells of the knock-

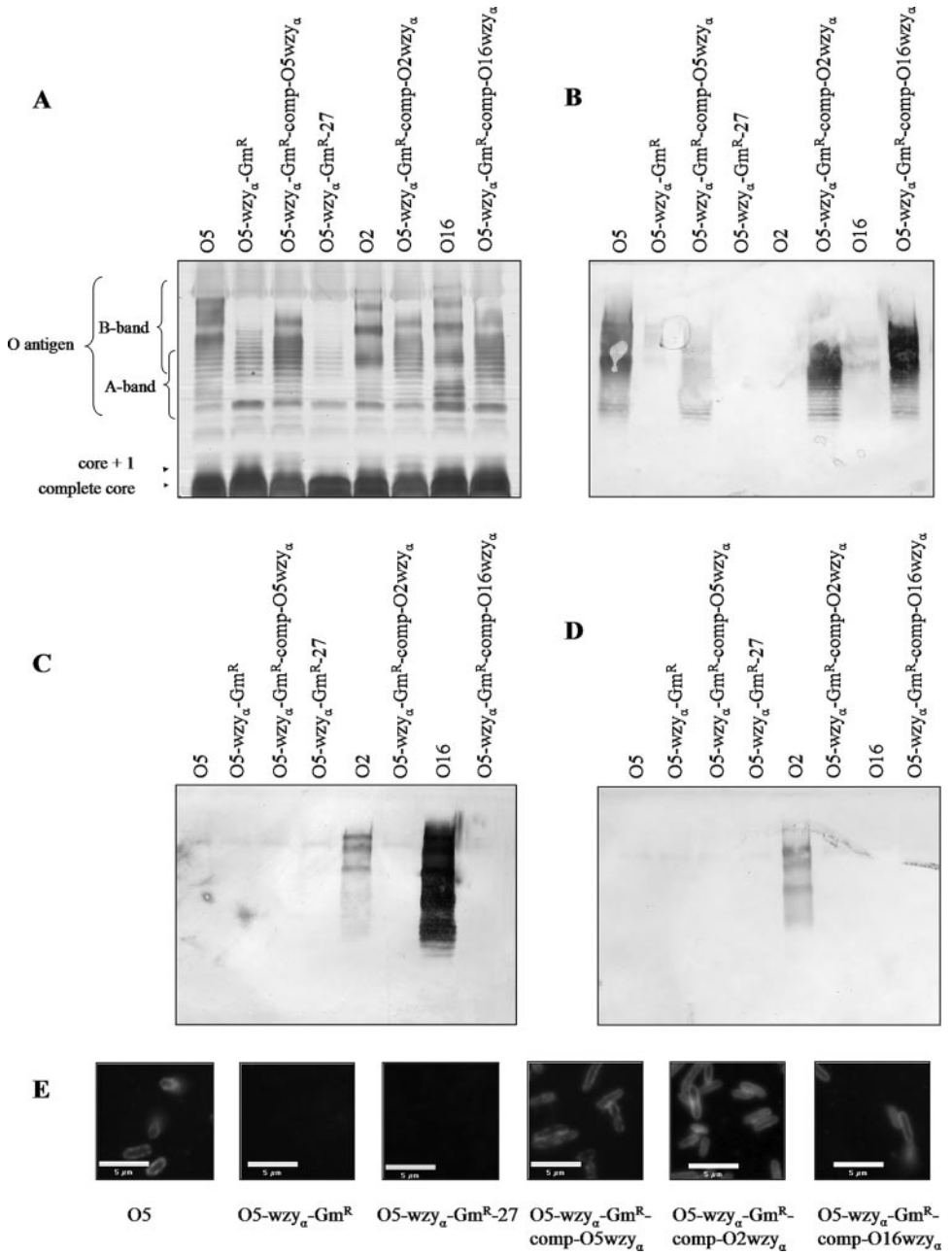

FIG. 3. *wzy*$_\alpha$ genes from *P. aeruginosa* O5, O2, and O16 restored alpha-linked O antigen in a *wzy*$_\alpha$ knockout mutant. (A) Silver-stained SDS-PAGE gel of LPS from wild-type serotype O5 (strain PAO1), an O5 *wzy*$_\alpha$ knockout mutant, a *wzy*$_\alpha$ mutant complemented with O5 *wzy*$_\alpha$, a *wzy*$_\alpha$ mutant transformed with pUCP27 (control), serotype O2 (wild type), a *wzy*$_\alpha$ mutant complemented with O2 *wzy*$_\alpha$, serotype O16 (wild type), and a *wzy*$_\alpha$ mutant complemented with O16 *wzy*$_\alpha$. Note that regardless of the origin of the *wzy*$_\alpha$ gene used in the complementation experiment, an O5 LPS banding modality was restored. (B) Western blots of the genes from panel A reacted with O5-specific MAb. (C) Western blots of the genes from panel A reacted with O16-specific MAb. (D) Western blots of the genes from panel A reacted with O2-specific MAb. Only O5-specific MAbs reacted with LPS from the complemented strains, indicating that they have α-linked O antigen. (E) Immunofluorescence micrographs showing that *wzy*$_\alpha$ genes from *P. aeruginosa* O5, O2, and O16 could restore α-linked O antigen in a *wzy*$_\alpha$ knockout mutant. All cells were reacted with O5-specific primary MAb (MF15-4) and FITC-linked secondary antibody. Only those cells with a functional *wzy*$_\alpha$ gene fluoresced. Each of the scale bars represents 5 μm.

out mutant and the negative control did not react with any antibodies and consequently did not show any fluorescence (Fig. 3E).

Analysis of the transcription of *wzy*$_\alpha$ of serotypes O2 and O16. To determine whether *wzy*$_\alpha$ was being transcribed in *P. aeruginosa* serotypes O2 and O16, RT-PCR was performed. RNA prepared from serotype O5 was used as a positive control. A band at approximately 264 bp, which is the predicted size of the *wzy*$_\alpha$ fragment, was amplified from all three serotypes. This indicated that *wzy*$_\alpha$ was being transcribed in all three serotypes (Fig. 4A).

Amplification and cloning of *P. aeruginosa* chromosomal *wzy*$_\beta$. Using primers specific for the D3 *wzy*$_\beta$ gene, amplifica-

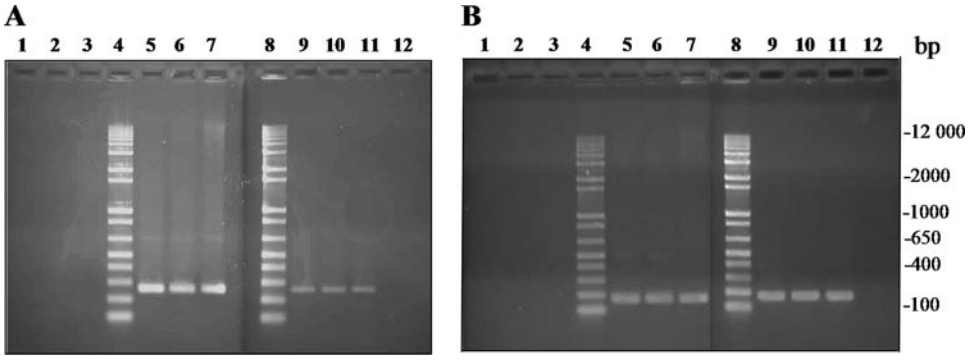

FIG. 4. RT-PCR detection of wzy_{α} and *rpoD* mRNA transcripts. (A) RT-PCR mRNA detection of wzy_{α} (264-bp) transcripts. (B) RT-PCR mRNA detection of *rpoD* (177-bp) transcripts. Lanes 1, PCR of O5 RNA (negative control); lanes 2, PCR of O2 RNA (negative control); lanes 3, PCR of O16 RNA (negative control); lanes 4, 1-kb Plus DNA ladder; lanes 5, PCR of O5 genomic DNA (positive control); lanes 6, PCR of O2 genomic DNA (positive control); lanes 7, PCR of O16 genomic DNA (positive control); lanes 8, 1-kb Plus DNA ladder; lanes 9, PCR of O5 cDNA; lanes 10, PCR of O2 cDNA; lanes 11, PCR of O16 cDNA; lanes 12, PCR without template (negative control).

tion of the 5′ end of the gene from serotypes O2 and O16 was easily achieved. The 3′ end, however, could not be amplified, likely due to the fact that D3-specific primers did not bind in that region, suggesting that the 3′ O2/O16 wzy_{β} sequence was significantly different than the 3′ D3 wzy_{β} gene sequence. A number of strategies were attempted to determine the sequence of the 3′ region of wzy_{β}, including designing new primers specific for various regions of the 3′ end, as well as downstream regions (primer sequences available upon request). Ultimately, data obtained from sequencing analysis performed by the Guelph Molecular Supercenter (University of Guelph, Guelph, Ontario, Canada) generated a partial sequence of the 3′ end of the *P. aeruginosa* wzy_{β} gene, as well as a short sequence downstream. Despite gaps in the sequencing results, sufficient information was obtained to allow the design of a downstream primer, wzybetaRev8 (5′-GCCCACCAAGAGC GGAATC-3′), that was used along with wzy-betaFwd3 (5′-C GCCCTCTGTTACTTGC-3′) to amplify the remainder of wzy_{β} from both O2 and O16 *P. aeruginosa*. PCR products from these reactions were obtained and cloned into the pCR-Blunt II-TOPO vector. Sequencing of both the O2 and O16 wzy_{β} genes revealed that they share 100% sequence identity. As expected, attempts to amplify wzy_{β} from *P. aeruginosa* serotype O5 did not yield a product DNA band. These results substantiate the absence of a wzy_{β} gene within the whole genome sequence database of PAO1 (http://v2.pseudomonas.com; 47).

A region of 60 bp upstream of wzy_{β} in O2 and O16 was sequenced and found to have high similarity to the same locus in the D3 genome. This region is not part of the seroconverting operon but appears to have been horizontally transferred along with wzy_{β}. The sequence of 170 bp downstream of wzy_{β} in O2 and O16 was also determined but did not show similarity to D3 or any other known sequence. No gene homologous to D3 *oac* could be identified in this region, even though in the D3 seroconverting operon, *oac* is located 75 bp downstream of wzy_{β}. This indicated that modifications had occurred after the genes involved in seroconversion were integrated into the chromosomes of O2 and O16 strains.

In silico analysis of *P. aeruginosa* wzy_{β}. The *P. aeruginosa* O2/O16 wzy_{β} gene is predicted to encode a protein of 41.8 kDa. When subjected to a BLAST database search, the only DNA sequence that showed similarity was the D3 phage wzy_{β}. The *P. aeruginosa* wzy_{β} nucleotide sequence was 87.42% identical to that of the D3 wzy_{β}. The primary amino acid sequence of *P. aeruginosa* Wzy_{β} was 90.41% identical and 97.15% similar to that of Wzy_{β} from the D3 phage. The mol% G+C for wzy_{β} from *P. aeruginosa* is 43.99%, which is strikingly similar to the 43.32% G+C from D3 wzy_{β}. A PSI-BLAST search was performed with the O2/O16 Wzy_{β} sequence, and iteration 1 showed similarities to the hypothetical protein H16 B0028 of *Ralstonia eutropha* H16 (accession number YP_728199), with an E value of 1e−15, as well as similarity to the hypothetical protein ELI 13300 from *Erythrobacter litoralis* HTCC2592 (accession number YP_459549), with an E value of 2e−13. Further iterations using the default parameters did not produce any new hits.

Analyses of the protein topography of Wzy_{β} were performed by the online programs TMHMM, SOSUI, and MEMSAT3. All three programs predicted high degrees of similarity between the *P. aeruginosa* and D3 Wzy_{β} topographies. Comparisons of the *P. aeruginosa* Wzy_{α} protein to the *P. aeruginosa* and D3 Wzy_{β} proteins are summarized in Table 2.

A wzy_{β} knockout mutant does not produce O antigen. A chromosomal mutant of wzy_{β} in serotype O16 was produced.

TABLE 2. Comparison of the *P. aeruginosa* Wzy_{α}, *P. aeruginosa* Wzy_{β}, and D3 bacteriophage Wzy_{β} O-antigen polymerases

Property	Value		
	P. aeruginosa Wzy_{α}	*P. aeruginosa* Wzy_{β}	D3 phage Wzy_{β}
Total no. of bp	1,317	1,155	1,161
Total no. of amino acids	438	384	386
Molecular mass (kDa)[a]	48.9	41.8	42.6
% G+C[a]	44.8	44.0	43.3
% Leucine, isoleucine, and phenylalanine[a]	30.82	31.00	29.28
Predicted no. of transmembrane segments			
TMHMM	12	9	9
SOSUI	10	10	9
MEMSAT3	10	11	11

[a] Based on nucleotide sequence.

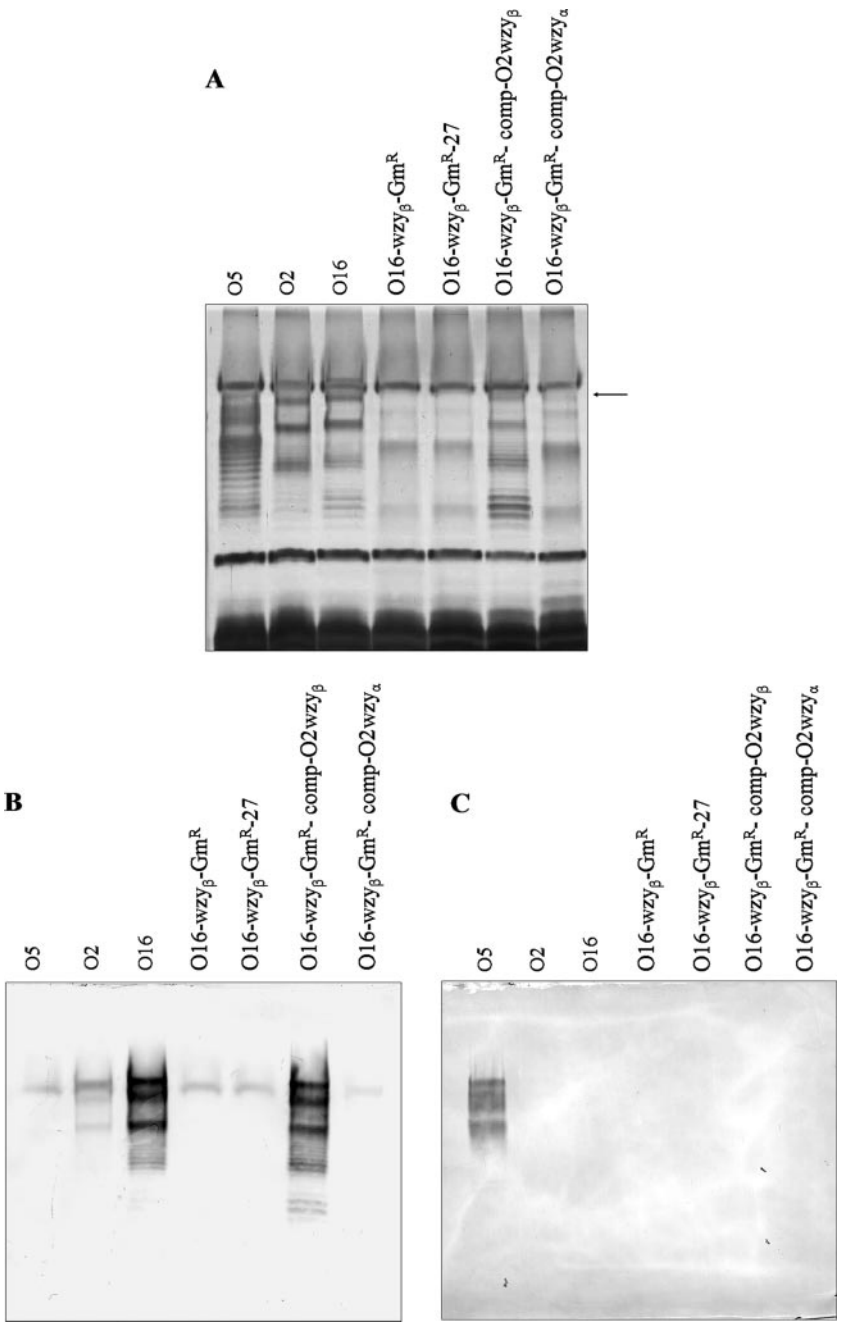

FIG. 5. B-band LPS production can be restored by a wzy_β gene, but not by a wzy_α gene, in a wzy_β knockout mutant. (A) Silver-stained SDS-PAGE gel of LPS from serotype O5 (wild type), serotype O2 (wild type), serotype O16 (wild type), an O16 wzy_β knockout mutant, an O16 wzy_β mutant transformed with pUCP27 (control), an O16 wzy_β mutant complemented with O2 wzy_β, and an O16 wzy_β mutant complemented with O2 wzy_α. Note that the banding modality of the O16 wzy_β mutant complemented with O2 wzy_β is very similar to that of wild-type O16, indicating that S-LPS was being restored. O2 wzy_α could not restore S-LPS in the O16 wzy_β mutant strain, except for one HMW band, which is indicated by an arrow. (B) Western blots of the samples in panel A reacted with O16-specific MAb. O16-specific MAb reacts with LPS from the O16 wzy_β mutant complemented with O2 wzy_β, but not with LPS from the O16 wzy_β mutant complemented with O2 wzy_α, indicating that wzy_α cannot restore B-band production in the O16 wzy_β knockout mutant. (C) Western blots of the samples in panel A reacted with O5-specific MAb. O5-specific MAb can react only with α-linked O antigen, which in this blot was found exclusively in the LPS from O5.

The insertion of the gentamicin cassette into the O16 wzy_β gene was verified by PCR-based screening (data not shown). LPS was isolated from this mutant strain (O16-wzy$_\beta$-Gm^r) and analyzed using SDS-PAGE and silver staining (Fig. 5A) and Western blotting (Fig. 5B and C). The mutant was found to lack high-molecular-weight (HMW) B-band LPS, which is consistent with the semirough phenotype. The LPS from wild-type O16 showed an LPS banding pattern with molecules of heterogeneous sizes. This is consistent with the S-LPS phenotype. When the wzy_β mutant was complemented in *trans* with a wzy_β

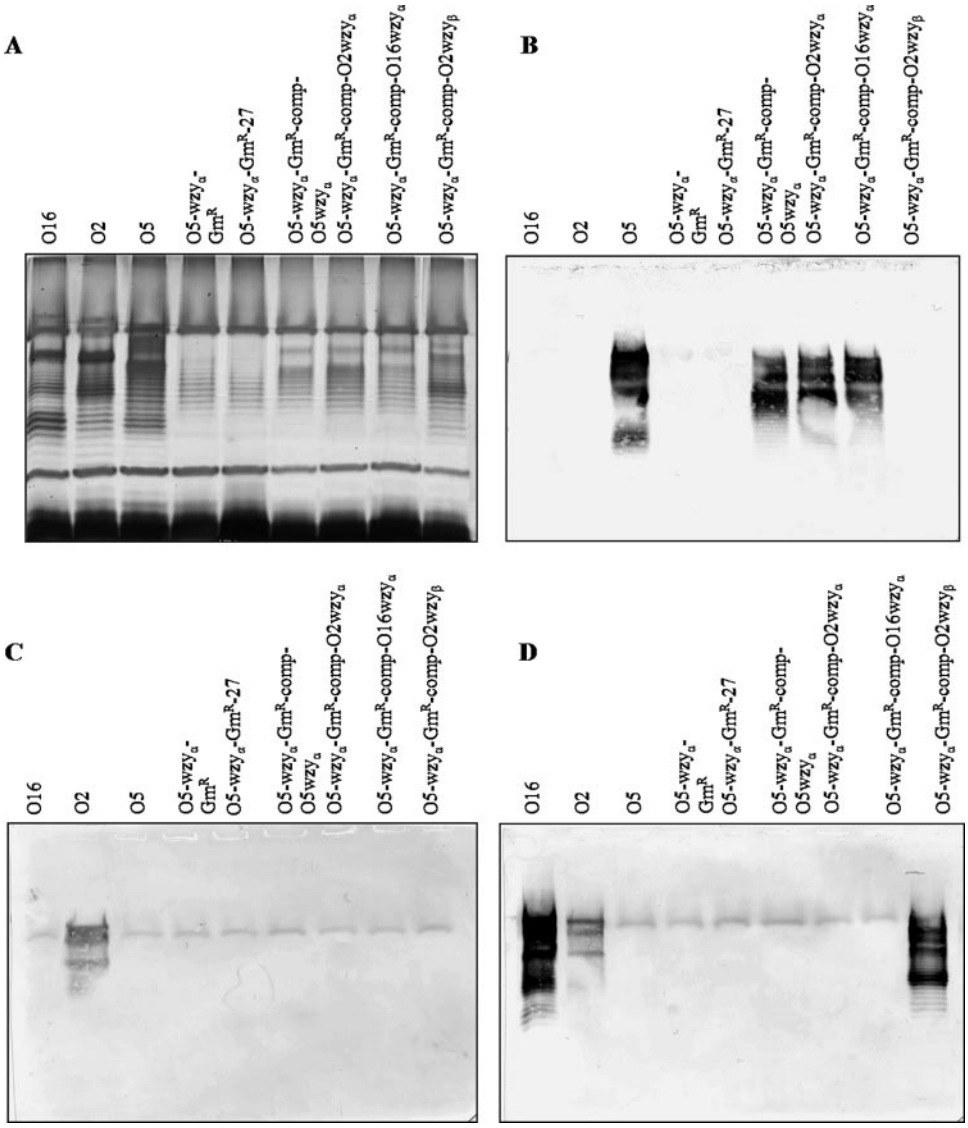

FIG. 6. Complementation of a serotype O5-derived wzy_α knockout mutant by wzy_β restored a LPS banding modality similar to that of wild-type serotype O16. (A) Silver-stained SDS-PAGE gel of LPS from serotype O16 (wild type), serotype O2 (wild type), serotype O5 (wild type), a wzy_α knockout mutant, a wzy_α mutant transformed with pUCP27 (control), a wzy_α mutant complemented with O5 wzy_α, a wzy_α mutant complemented with O2 wzy_α, a wzy_α mutant complemented with O16 wzy_α, and a wzy_α mutant complemented with O2 wzy_β. (B) Western blots of LPS reacted with O5-specific MAb. (C) Western blots of LPS reacted with O2-specific MAb. (D) Western blots of LPS reacted with O16-specific MAb.

gene, S-LPS was restored. When anti-O16 MAb (MF47-4) was used in Western blotting, there was a strong reaction with both the wild-type O16 LPS and the LPS from the O16 wzy_β mutant complemented with wzy_β (O16-wzy$_\beta$-Gm^R-comp-O2wzy$_\beta$). The anti-O16 MAb (MF47-4) showed weak cross-reactivity with wild-type serotype O2 LPS and no reactivity with the O16 wzy_β mutant LPS (see Fig. 5B). Anti-O5 MAb (MF15-4) reacted only with wild-type O5 LPS bands (Fig. 5C). Likewise, anti-O2 MAb (MF71-2) reacted only with wild-type serotype O2 LPS bands (data not shown).

Transformation of the O16 wzy_β mutant with a plasmid containing wzy_α did not restore S-LPS. Attempts to complement the O16 wzy_β knockout mutant with wzy_α from *P. aeruginosa* serotype O2 did not restore B-band O-antigen production. Upon careful examination of the LPS bands by SDS-PAGE and silver staining, one HMW LPS band was observed, but the overall LPS banding modality did not resemble that of wild-type O16. Instead, the LPS banding pattern was most similar to those of the O16-wzy_β mutant and the negative control (O16-wzy$_\beta$-Gm^R-27) (Fig. 5A). Results from Western blotting showed that none of the LPS bands observed in the SDS-PAGE gels were B-band LPS, as they were not reactive with either the O5-specific MAb (MF15-4) or the O16-specific MAb (MF47-4) (Fig. 5B and C).

Transformation of the O5 wzy_α mutant with wzy_β produced β-linked O antigen. The O5-wzy$_\alpha$-Gm^R strain was transformed with a pUCP27-wzy_β gene, and LPS from the cross-complemented strain was analyzed by silver staining (Fig. 6A) and Western blotting (Fig. 6B to D). LPS from the complemented strain exhibited a banding modality more similar to that of wild-type serotype O16 than to wild-type O5, and the LPS

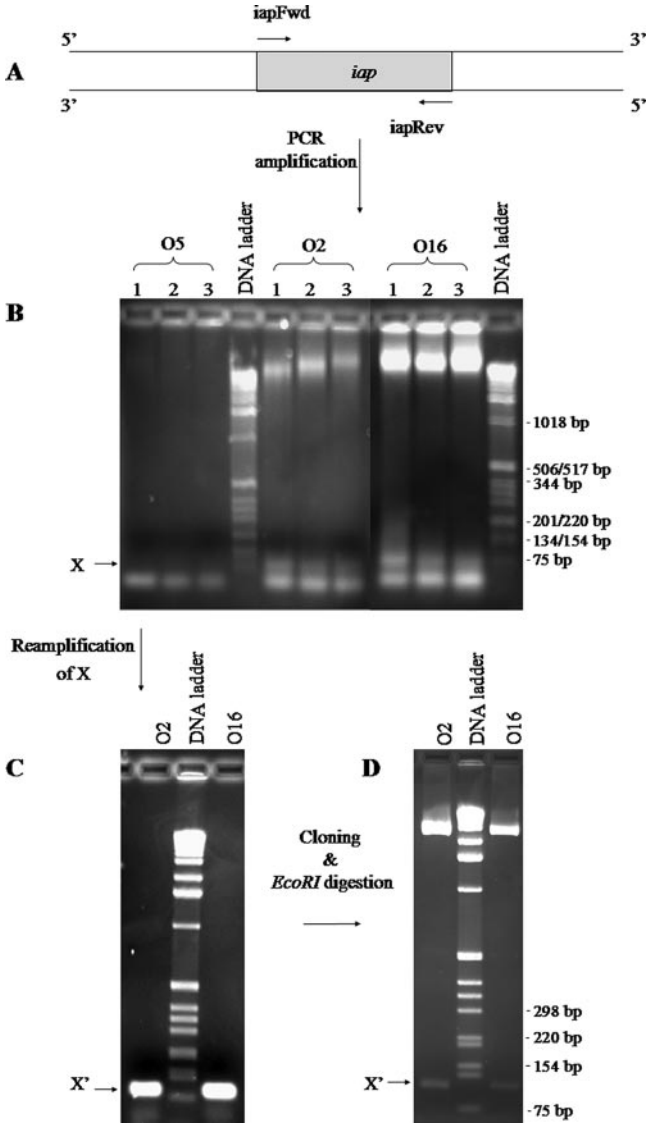

FIG. 7. PCR amplification of *iap*. (A) Primers iapFwd and iapRev were designed to bind to the 5′ and 3′ ends of D3 *iap*, respectively. (B) Genomic DNAs from serotypes O5, O2, and O16 were used as templates for PCRs. Three different $MgSO_4$ concentrations (1 mM, 1.5 mM, and 2 mM) were used and are indicated by the numbers 1, 2, and 3, respectively. The arrow points to bands corresponding to sizes of ~95 bp that were present in the O2 and O16 samples but missing in O5. (C) These bands from the O2 and O16 lanes were gel excised and reamplified using primers iapFwd and iapRev. (D) The product, labeled X′, was cloned into a pCR-Blunt II-TOPO vector. Plasmids were extracted from two clones, purified, and digested with EcoRI.

bands reacted with an O16-specific MAb (MF47-4). This indicates that a β-linked O antigen was restored. Furthermore, an O5-specific MAb (MF15-4) reacted only with wild-type O5 and the wzy_α mutant strains that had been complemented with wzy_α.

Amplification and cloning of *P. aeruginosa* chromosomal *iap*. Using primers designed based on the D3 bacteriophage *iap* sequence (Fig. 7A), a faint band at approximately 95 bp was amplified from chromosomal DNA of O2 and O16, but not from O5 (Fig. 7B). When the faint band was excised and the DNA was extracted and reamplified using the same primers, a bright band at approximately 95 bp, consistent with the predicted size of *iap*, was clearly visible (Fig. 7C). After gel purification, TOPO cloning (Fig. 7D), and sequencing, our results showed that the entire region between primer-binding locations (47 bp) was identical in O2 and O16 and that it was also identical to that of the D3 *iap* gene. In addition, the D3-specific iapFwd and iapRev primers were able to anneal to O2 and O16 chromosomal DNA in the PCR; it is likely that the 5′ and 3′ ends of the O2 and O16 *iap* genes are similar, if not identical, to those of the D3 *iap*.

DISCUSSION

The results of the complementation studies with wzy_α from serotypes O2 and O16 supported the hypothesis that another gene or process must be involved in the formation of β-linked O antigen in the O2 and O16 serotypes, since the wzy_α genes in both serotypes are clearly responsible for the α-linkages in O polysaccharides in *P. aeruginosa*. The wzy_α genes from O2 and O16 are identical, while the O5 wzy_α differs from these two by only 1 nucleotide, with the amino acid encoded by that codon being conserved. Thus, they are the same protein with the same substrate specificity.

Results from RT-PCR experiments of wzy_α from *P. aeruginosa* serotypes O5, O2, and O16 clearly showed that wzy_α is transcribed in all three serotypes. Transcription of wzy_α in O5 was expected, since the O antigen of this serotype contains α-linkages, but it was surprising that transcription was also occurring in serotypes O2 and O16, since these serotypes produce LPS with β-linked O antigens. One possible explanation is that the O-antigen polymerase activity of Wzy_α is inhibited in serotypes O2 and O16. We hypothesized that the D3 bacteriophage *iap* gene, which has been shown to inhibit Wzy_α in *P. aeruginosa* (39), was also horizontally transferred into these two serotypes. Although the presence of *iap* could not be detected in *P. aeruginosa* by Southern blotting (39), it is important to consider that *iap* is only 96 bp in length, which may have been too short for adequate probe binding. Indeed, by means of PCR, we were able to successfully amplify *iap* from *P. aeruginosa* serotype O2 and O16 chromosomal DNAs. The presence of *iap* in these two serotypes supported our hypothesis that the product Iap is responsible for the inhibition of Wzy_α. The inability to amplify *iap* from serotype O5 DNA was expected, since serotype O5 produces α-linked O antigen. It is predicted that inhibition occurs at the translation or protein function level, since the wzy_α genes continue to be successfully transcribed in all serotypes tested. The *rfb* locus of *Salmonella enterica* serovar Anatum group E1 has been shown to contain two putative *wzy* genes, namely, *orf9.6* and *orf17.4*, by McConnell et al. (36). *orf9.6* apparently encodes Wzy_α, responsible for the α1-6 glycosidic linkage in the O10 O antigen of group E1 bacteria. The latter gene, *orf17.4*, when transformed into a spontaneous *Salmonella* mutant defective in O polymerase, caused the transformant to produce O15 LPS with β-linked O-antigen units. The authors suggested that *orf17.4* might be derived from a phage origin, but the mechanism for only *orf9.6* to be functional in vivo was not investigated. In contrast, wzy_β in serotypes O2 and O16 is clearly localized elsewhere in the *P. aeruginosa* chromosome and not within the

wbp-rfb locus. Evidence that wzy_β might have a D3 bacteriophage origin has been presented.

A chromosomal knockout mutant of wzy_β in *P. aeruginosa* O16 was produced in this study, and this mutant is important for characterizing the function of the gene. Despite the experience of our group in generating knockout mutants of various genes in this species, making the O16 wzy_β mutant was not straightforward. It appeared that the *P. aeruginosa* O16 strain was spontaneously developing resistance to gentamicin, compromising the screening technique of the protocol. This spontaneous resistance was also accompanied by a loss of fluorescence of the bacterial colonies. It has since been discovered that lower concentrations of gentamicin, such as 30 μg/ml and 40 μg/ml, are more effective at selecting true mutants because they discourage the occurrence of spontaneous gentamicin-resistant mutations in *P. aeruginosa* (Craig Daniels, Toronto Sick Children Hospital, personal communication). LPS prepared from the O16 wzy_β mutant complemented with wzy_β and reacted exclusively with O16-specific MAb (MF47-4) in Western immunoblotting. These results showed that Wzy_β is responsible for the formation of β-linked O antigen. In addition, an O2 wzy_β gene could be used for the complementation, since it is 100% identical to the O16 wzy_β gene. The LPS banding modality of the O5 wzy_α knockout mutant cross-complemented with O2 wzy_β was similar to that of wild-type O16. The minor differences can be explained by the fact that wzy_β was introduced in a high-copy number plasmid, which could affect the stoichiometry of the ratio between Wzy_β and Wzz in the membrane complex for LPS assembly. An altered ratio of the two proteins is expected to affect the length of the O antigen, thereby causing a change in the modality of the LPS bands visualized in the SDS-PAGE gels (13). If Wzy_β was restoring β-linked O antigen in the mutant, it is not surprising that the LPS banding modality of the complemented strain resembled that of wild-type O16, since the serotype O5 and O16 O antigens have the same sugar composition and differ only by linkage type. Essentially, a switch to β-linkage caused the conversion of serotype O5 to O16. This was substantiated by results from Western immunoblotting experiments, since the O5 wzy_α mutant cross-complemented with wzy_β was reactive with the anti-O16 MAb (MF47-4).

Transformation of the O16 wzy_β mutant with wzy_α did not restore the production of HMW B-band LPS. Only one of the HMW bands appeared to have been restored upon analysis by SDS-PAGE and silver staining, but the overall modality did not resemble that of wild-type O16 (Fig. 5A). The partial restoration of O antigen is consistent with the theory that the *iap* gene we were able to amplify by PCR encodes a functional O antigen inhibitor in this serotype. The O16 wzy_β knockout mutant already has a functional wzy_α gene that is being inhibited. Since the exogenous wzy_α gene was being introduced in a high-copy-number plasmid (pUCP27), the amount of Wzy_α being expressed in the cell would have been much higher than normal. This supersaturation of Wzy_α likely exhausted all Wzy_α inhibitor (Iap) binding sites, allowing a small degree of α-linked O antigen to be produced. Analysis of the Western blots, however, revealed no reaction between this complemented strain and the O5-specific MAb (MF15-4) (Fig. 5C). The reason for this is not known.

The *P. aeruginosa* wzy_β gene is most likely a xenolog of the D3 phage wzy_β gene, since a BLAST search revealed no homology to other known genes or proteins. Likewise, the *P. aeruginosa iap* gene is also predicted to be a xenolog of the D3 *iap* gene, since our sequencing results show that they are identical. It is important to consider that the G+C content of the D3 genome is 59%, which is considerably higher than that of the D3 3.6-kb serotype-converting cassette (43%) containing wzy_β and *iap* (39). The possibility that D3 acquired wzy_β and *iap* from *P. aeruginosa* is unlikely, since the G+C content of the *P. aeruginosa* genome is 67% (39). Newton et al. (39) have also speculated that the D3 serotype conversion genes may be "morons," a term used to describe portable expression units in the genomes of lambda-like phages with reduced G+C percentages (22). These genes, therefore, may have been acquired from yet another source at a relatively recent point in evolution, with D3 acting as a shuttle vector between them (39). Based on PSI-BLAST results, it is plausible that Wzy_β shares an evolutionary relationship with the hypothetical proteins of *R. eutropha* H16, *E. litoralis* HTCC2594, or the phage that infect these gram-negative bacterial species.

The differences in sequence between *P. aeruginosa* wzy_β and D3 wzy_β indicate that evolutionary modification occurred after wzy_β was integrated into the *P. aeruginosa* chromosome. Based on the sequences of the O2 and O16 *wbp* gene clusters reported by Raymond et al. (40), we are certain that wzy_β and *iap* are elsewhere. Until the whole genome sequences of these strains are available, their exact locus in the chromosome is presently unknown. At this time, it is not clear whether the wzy_β and *iap* genes integrated on their own or as part of the 3.6-kb seroconverting operon. Although the upstream sequence of wzy_β in O2 and O16 is similar to that of the wzy_β upstream sequence in D3, the downstream sequence showed no similarity. If the entire seroconverting operon had been integrated into O2 and O16, the *oac* gene would have been detectable in this downstream sequence. However, it is also plausible that the *oac* gene has undergone drastic changes in sequence and may no longer be easily identifiable by PCR or Southern hybridization. Further sequencing of the regions flanking wzy_β in *P. aeruginosa*, as well as sequencing of the *P. aeruginosa iap* upstream and downstream regions, would be of great interest, as it may uncover *attP* sites, *int* genes, or insertion sequences. These would suggest that a select few genes from D3 have been permanently acquired, or "fixed," by *P. aeruginosa*. Alternatively, it is possible that the entire D3 genome is present in O2 and O16 as a viable prophage. This could be investigated by induction and characterization of phage from serotypes O2 and O16. Our initial attempts to induce D3 from these serotypes with mitomycin C were unsuccessful.

The fixation of the seroconverting genes into a bacterial host chromosome has been observed before. *Shigella flexneri* serotype 1a strain Y53 has been found to contain a fixed chromosomal version of an SfV phage-derived serotype conversion cassette. It was found that this cassette encoded factors that mediate the conversion of a serotype Y strain to serotype 1a (2, 5). The organization of the 5.8-kb fragment, as well as its upstream and downstream regions (10.6 kb in total), suggested that this operon was originally an Sf1 lysogen, although it appears that a large portion of the phage genome was deleted (2). Southern hybridization analyses revealed that this frag-

ment was also present in three other natural isolates of *S. flexneri* (2). Another study by Adams et al. (1) revealed that a 3.8-kb fragment from *S. flexneri* serotype 4a strain NCTC 8296 contains three putative O-antigen modification genes. Phage-derived seroconversion genes have also been found in the chromosomes of *E. coli* strains (6, 16, 35).

It has become increasingly apparent that phage and their bacterial hosts influence each others' evolution (10, 22, 38, 43). Many important bacterial virulence factors are encoded by prophage or have a phage origin. This makes *P. aeruginosa* Wzy_{β} particularly interesting to study, as it likely contributes greatly to *P. aeruginosa* virulence. The question of whether Wzy_{β} plays a role in virulence or other physiological functions provides an important avenue for future research. It is imperative to locate the *oac* gene in the *P. aeruginosa* chromosome and to characterize its function. To provide further clues to Iap's function, wild-type O5 could be transformed with the wzy_{β} gene to see if both α- and β-linked O antigens are concomitantly produced. In the long run, these studies can be expanded to include serotypes outside of the O2 serogroup. Ultimately, the serotype-converting genes from the D3 phage can be used together with other genes to create a strain expressing multiple (possibly all 20) types of O antigen. This strain could potentially be useful for the development of a multivalent vaccine.

ACKNOWLEDGMENTS

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REFERENCES

1. **Adams, M. M., G. E. Allison, and N. K. Verma.** 2001. Type IV O antigen modification genes in the genome of *Shigella flexneri* NCTC 8296. Microbiology **147:**851–860.
2. **Adhikari, P., G. Allison, B. Whittle, and N. K. Verma.** 1999. Serotype 1a O-antigen modification: molecular characterization of the genes involved and their novel organization in the *Shigella flexneri* chromosome. J. Bacteriol. **181:**4711–4718.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
5. **Bastin, D. A., A. Lord, and N. K. Verma.** 1997. Cloning and analysis of the glucosyl transferase gene encoding type I antigen in *Shigella flexneri*. FEMS Microbiol. Lett. **156:**133–139.
6. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
7. **Bryson, K., L. J. McGuffin, R. L. Marsden, J. J. Ward, J. S. Sodhi, and D. T. Jones.** 2005. Protein structure prediction servers at University College London. Nucleic Acids Res. **33:**W36–W38.
8. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
9. **Burrows, L. L., D. F. Charter, and J. S. Lam.** 1996. Molecular characterization of the *Pseudomonas aeruginosa* serotype O5 (PAO1) B-band lipopolysaccharide gene cluster. Mol. Microbiol. **22:**481–495.
10. **Canchaya, C., G. Fournous, and H. Brussow.** 2004. The impact of prophages on bacterial chromosomes. Mol. Microbiol. **53:**9–18.
11. **Clarke, A. J., N. T. Blackburn, and H. Strating.** 2000. Pathways for the *O*-acetylation of bacterial cell wall polysaccharides. Kluwer Academic Publishers, New York, NY.
12. **Clarke, B. R., L. Cuthbertson, and C. Whitfield.** 2004. Nonreducing terminal modifications determine the chain length of polymannose O antigens of *Escherichia coli* and couple chain termination to polymer export via an ATP-binding cassette transporter. J. Biol. Chem. **279:**35709–35718.
13. **Daniels, C., C. Griffiths, B. Cowles, and J. S. Lam.** 2002. *Pseudomonas aeruginosa* O-antigen chain length is determined before ligation to lipid A core. Environ. Microbiol. **4:**883–897.
14. **de Kievit, T. R., T. Dasgupta, H. Schweizer, and J. S. Lam.** 1995. Molecular cloning and characterization of the *rfc* gene of *Pseudomonas aeruginosa* (serotype O5). Mol. Microbiol. **16:**565–574.
15. **Fomsgaard, A., M. A. Freudenberg, and C. Galanos.** 1990. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. J. Clin. Microbiol. **28:**2627–2631.
16. **Guan, S., and N. K. Verma.** 1998. Serotype conversion of a *Shigella flexneri* candidate vaccine strain via a novel site-specific chromosome-integration system. FEMS Microbiol. Lett. **166:**79–87.
17. **Hancock, R. E., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat-2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140:**902–910.
18. **Hirokawa, T., S. Boon-Chieng, and S. Mitaku.** 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14:**378–379.
19. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
20. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
21. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1994. A model recognition approach to the prediction of all-helical membrane protein structure and topology. Biochemistry **33:**3038–3049.
22. **Juhala, R. J., M. E. Ford, R. L. Duda, A. Youlton, G. F. Hatfull, and R. W. Hendrix.** 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. J. Mol. Biol. **299:**27–51.
23. **Knirel Yu, A., and N. K. Kochetkov.** 1994. Structure of lipopolysaccharides from gram-negative bacteria. III. Structure of O-specific polysaccharides. Biokhimiia **59:**1784–1851.
24. **Knirel Yu, A., E. V. Vinogradov, N. A. Kocharova, N. A. Paramonov, N. K. Kochetkov, B. A. Dmitriev, E. S. Stanislavsky, and B. Lanyi.** 1988. The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa*. Acta Microbiol. Hung. **35:**3–24.
25. **Kropinski, A. M.** 2000. Sequence of the genome of the temperate, serotype-converting, *Pseudomonas aeruginosa* bacteriophage D3. J. Bacteriol. **182:**6066–6074.
26. **Kuzio, J., and A. M. Kropinski.** 1983. O-antigen conversion in *Pseudomonas aeruginosa* PAO1 by bacteriophage D3. J. Bacteriol. **155:**203–212.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
28. **Lam, J. S., L. L. Graham, J. Lightfoot, T. Dasgupta, and T. J. Beveridge.** 1992. Ultrastructural examination of the lipopolysaccharides of *Pseudomonas aeruginosa* strains and their isogenic rough mutants by freeze-substitution. J. Bacteriol. **174:**7159–7167.
29. **Lam, J. S., M. Y. Handelsman, T. R. Chivers, and L. A. MacDonald.** 1992. Monoclonal antibodies as probes to examine serotype-specific and cross-reactive epitopes of lipopolysaccharides from serotypes O2, O5, and O16 of *Pseudomonas aeruginosa*. J. Bacteriol. **174:**2178–2184.
30. **Lam, J. S., L. A. MacDonald, and M. Y. Lam.** 1987. Production of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. Infect. Immun. **55:**2854–2856.
31. **Lam, J. S., L. A. MacDonald, M. Y. Lam, L. G. Duchesne, and G. G. Southam.** 1987. Production and characterization of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. Infect. Immun. **55:**1051–1057.
32. **Lightfoot, J., and J. S. Lam.** 1993. Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. Mol. Microbiol. **8:**771–782.
33. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33:**256–264.
34. **Liu, P. V., and S. Wang.** 1990. Three new major somatic antigens of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **28:**922–925.
35. **Mavris, M., P. A. Manning, and R. Morona.** 1997. Mechanism of bacteriophage SfII-mediated serotype conversion in *Shigella flexneri*. Mol. Microbiol. **26:**939–950.
36. **McConnell, M. R., K. R. Oakes, A. N. Patrick, and D. M. Mills.** 2001. Two functional O-polysaccharide polymerase *wzy* (*rfc*) genes are present in the *rfb* gene cluster of Group E1 *Salmonella enterica* serovar Anatum. FEMS Microbiol. Lett. **199:**235–240.
37. **McGuffin, L. J., K. Bryson, and D. T. Jones.** 2000. The PSIPRED protein structure prediction server. Bioinformatics **16:**404–405.
38. **Miao, E. A., and S. I. Miller.** 1999. Bacteriophages in the evolution of pathogen-host interactions. Proc. Natl. Acad. Sci. USA **96:**9452–9454.

39. **Newton, G. J., C. Daniels, L. L. Burrows, A. M. Kropinski, A. J. Clarke, and J. S. Lam.** 2001. Three-component-mediated serotype conversion in *Pseudomonas aeruginosa* by bacteriophage D3. Mol. Microbiol. **39:**1237–1247.
40. **Raymond, C. K., E. H. Sims, A. Kas, D. H. Spencer, T. V. Kutyavin, R. G. Ivey, Y. Zhou, R. Kaul, J. B. Clendenning, and M. V. Olson.** 2002. Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. J. Bacteriol. **184:**3614–3622.
41. **Rocchetta, H. L., L. L. Burrows, and J. S. Lam.** 1999. Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. Microbiol. Mol. Biol. Rev. **63:**523–553.
42. **Savli, H., A. Karadenizli, F. Kolayli, S. Gundes, U. Ozbek, and H. Vahaboglu.** 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. J. Med. Microbiol. **52:**403–408.
43. **Shi, S. Y., X. H. Cai, and D. F. Ding.** 2005. Identification and categorization of horizontally transferred genes in prokaryotic genomes. Acta Biochim. Biophys. Sin. (Shanghai) **37:**561–566.
44. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad-host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotecchnology **1:**784–791.
45. **Sonnhammer, E. L., G. von Heijne, and A. Krogh.** 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc. Int. Conf. Intell. Syst. Mol. Biol. **6:**175–182.
46. **Stanislavsky, E. S., and J. S. Lam.** 1997. *Pseudomonas aeruginosa* antigens as potential vaccines. FEMS Microbiol. Rev. **21:**243–277.
47. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
48. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
49. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
50. **West, S. E., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81–86.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.