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lfnA from *Pseudomonas aeruginosa* O12 and *wbuX* from *Escherichia coli* O145 Encode Membrane-Associated Proteins and Are Required for Expression of 2,6-Dideoxy-2-Acetamidino-L-Galactose in Lipopolysaccharide O Antigen▽†

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The rare sugar 2,6-dideoxy-2-acetamidino-L-galactose (L-FucNAm) is found only in bacteria and is a component of cell surface glycans in a number of pathogenic species, including the O antigens of *Pseudomonas aeruginosa* serotype O12 and *Escherichia coli* O145. *P. aeruginosa* is an important opportunistic pathogen, and the O12 serotype is associated with multidrug-resistant epidemic outbreaks. O145 is one of the classic non-O157 serotypes associated with Shiga toxin-producing, enterohemorrhagic *E. coli*. The acetamidino (NAm) moiety of L-FucNAm is of interest, because at neutral pH it contributes a positive charge to the cell surface, and we aimed to characterize the biosynthesis of this functional group. The pathway is not known, but expression of NAm-modified sugars coincides with the presence of a *pseA* homologue in the relevant biosynthetic locus. PseA is a putative amidotransferase required for synthesis of a NAm-modified sugar in *Campylobacter jejuni*. In *P. aeruginosa* O12 and *E. coli* O145, the *pseA* homologues are *lfnA* and *wbuX*, respectively, and we hypothesized that these genes function in L-FucNAm biosynthesis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and nuclear magnetic resonance analysis of the *lfnA* mutant O-antigen structure indicated that the mutant expresses 2,6-dideoxy-2-acetamido-L-galactose (L-FucNAc) in place of L-FucNAm. The mutation could be complemented by expression of either His_6-tagged *lfnA* or *wbuX* in *trans*, confirming that these genes are functional homologues and that they are required for NAm moiety synthesis. Both proteins retained their activity when fused to a His_6 tag and localized to the membrane fraction. These data will assist future biochemical investigation of this pathway.

Pseudomonas aeruginosa is a ubiquitous gram-negative microorganism and an important opportunistic pathogen which causes life-threatening infections in compromised animals and plants. It accounts for approximately 1 in 10 reported cases of common hospital-acquired infections (22) and is a principal cause of morbidity in cystic fibrosis patients (10).

One of the major virulence determinants for *P. aeruginosa* is a cell surface carbohydrate polymer known as B-band O antigen, which is a component of lipopolysaccharide (LPS). There are 20 major serotypes of *P. aeruginosa* which differ in the structure of their B-band O antigen. The chemical structure of O antigen in each of these serotypes has been elucidated by Knirel and his colleagues (36), and the gene clusters encoding B-band biosynthesis in all 20 serotypes have been cloned and sequenced (49). O antigen confers resistance to complement-mediated killing (16), and mutants deficient in O antigen are avirulent in a burnt mouse model of infection (14).

The predominant *P. aeruginosa* serotypes in clinical specimens, regardless of geographic location, are O1, O6, and O11 (3, 46). In contrast, the prevalence of O12 among clinical isolates varies depending upon geographical location (58) and can change over time (1); however, this serotype has made up 20% of all *P. aeruginosa* isolates in some clinical studies (1, 39). O12 is associated with multidrug-resistant epidemic outbreaks in European hospitals (3, 21, 30), including infection of cystic fibrosis patients (43) and burn wounds (46), and the prevalence of O12 among clinical isolates in Europe may be due to expansion of a few highly successful clones (47, 48).

Enterohemorrhagic *Escherichia coli* (EHEC) is a causative agent of human gastrointestinal disease with life-threatening complications in the very young or elderly. The principal characteristic feature of EHEC is expression of Shiga toxin (reviewed in reference 61). The most common *E. coli* O-antigen serotype associated with these pathogenic *E. coli* strains is O157, but one of the classic non-O157 serotypes commonly associated with Shiga toxin production is O145 (4, 5, 51). O145 strains have been identified as the etiologic agent in cases of bloody diarrhea (19), hemorrhagic colitis (8), and hemolytic-uremic syndrome (20, 28, 57).

Both the *P. aeruginosa* O12 and the *E. coli* O145 O-antigen repeats contain a 2,6-dideoxy-2-acetamidino-L-galactose (L-FucNAm) residue (12, 24, 37). This sugar is rare in nature and is found only in bacteria. Besides these etiological agents of human disease, L-FucNAm is also expressed by pathogens of

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animals (*Salmonella enterica* subsp. *arizonae* serotypes O21 [59] and O61 [60]) and fish (*Yersinia ruckerii* O1 [6] and *Flavobacterium columnare* ATCC 43622 [40]).

The biosynthetic pathway for expression of L-FucNAm is unknown, but synthesis of the acetamidino group on the 5-acetamido-7-acetamidino-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-nonulosonic acid (Pse5NAc7Am) residue of *Campylobacter jejuni* flagellin glycan requires the *pseA* gene, and homologues of this gene exist in the *P. aeruginosa* O12 and the *E. coli* O-antigen biosynthesis loci (*lfnA* and *wbuX*, respectively). A *pseA* mutant glycosylates flagellin with 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-nonulosonic acid (Pse5NAc7NAc) where Pse5NAc7Am usually is, and analysis of the sugar-nucleotide pool in this mutant indicates that PseA probably operates at a late stage in Pse5NAc7Am biosynthesis, possibly catalyzing amidotransfer to CMP-Pse5NAc7NAc (42). A *pseA* homologue (*wbpG*) is also present in the O-antigen locus of *P. aeruginosa* O5 which possesses the acetamidino-modified sugar 2,3-dideoxy-2-acetamido-3-acetamidino-D-mannuronic acid (D-ManNAc3NAmA) in its O antigen. The coincidence of acetamidino sugars and genes encoding PseA homologues suggests that these may be the enzymes responsible for acetamidino group generation. In each case, the sugars containing the acetamidino group are different, implying that despite a common catalytic chemistry, the enzymes may not necessarily share the same substrate specificity. These genes all encode proteins in the so-called PP-loop superfamily and are therefore predicted to bind ATP (9). A conserved domain search (41) indicates that 52% of the Pfam00733 motif (C-terminal domain of asparagine synthase) is present in the LfnA sequence (see Fig. S1 in the supplemental material). Information from the Pfam database (25) suggests that proteins with this domain use ATP to activate their substrates toward amidotransfer. Such a function would be consistent with the involvement of this protein in biosynthesis of L-FucNAm.

Here we report the genetic investigation of *lfnA* from *P. aeruginosa* O12, which was previously known as *orf17* in the O-antigen biosynthesis locus. A *lfnA* mutant continues to produce O antigen, but with an altered structure. Results from nuclear magnetic resonance (NMR) analysis of purified polysaccharides from this mutant indicate that the O antigen lacks the acetamidino group usually present on the L-FucNAm residue, thus expressing L-FucNAc in its place. These findings indicate a role for *lfnA* in the formation of the acetamidino group in synthesis of L-FucNAm in *P. aeruginosa* O12. We also demonstrate that *wbuX* from *E. coli* O145 can cross-complement an *lfnA* mutation implicating *wbuX* in L-FucNAm biosynthesis in *E. coli* O145.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely propagated in Luria-Bertani (LB) broth or on LB agar containing 1.5% Bacto Agar (Difco). All strains were grown at 37°C with the following antibiotics added as appropriate: ampicillin (100 μg ml^{-1}), carbenicillin (300 μg ml^{-1}), kanamycin (50 μg ml^{-1}), gentamicin (15 μg ml^{-1} for *E. coli*; 150 μg ml^{-1} for *P. aeruginosa*), and tetracycline (10 μg ml^{-1} for *E. coli*; 100 μg ml^{-1} for *P. aeruginosa*).

DNA methods. Standard methods were used for DNA manipulations. Unless otherwise stated, enzymes and reagents were purchased from New England Biolabs or Invitrogen and used according to the manufacturer's instructions. Oligonucleotides were supplied by the University of Guelph Molecular Supercenter. PCR was performed using KOD Hot Start (Novagen) or *Pwo* (Roche) DNA polymerases.

Mutagenesis of *lfnA* in the O-antigen locus of *P. aeruginosa* serotype O12. *lfnA* (*orf17*) from the O-antigen biosynthesis locus of *P. aeruginosa* O12 was PCR amplified using the primers 5′-AGGAAAAACATATGAGCAACCACAGCTACAAG-3′ (NdeI site underlined) and 5′-TAAGGATCCTCATCTGAATAACCTACGTTCC-3′ (BamHI site underlined) and ligated without prior restriction digestion into SmaI-cut pEX100T. The resulting plasmid was cut within the *lfnA* coding sequence by digestion with PpuMI, then blunted by filling in the ends with the Klenow fragment, and ligated with the SmaI-digested *aacC1* (gentamicin resistance [Gm^r]) cassette from pUCGm. This generated the gene knockout vector pKOLD2. This vector was transformed into *P. aeruginosa* O12 by conjugation between log-phase cultures of the *P. aeruginosa* acceptor strain and *E. coli* SM10 harboring pKOLD2 as the donor (54). Transconjugants were initially selected on *Pseudomonas* isolation agar (Difco) containing gentamicin to isolate clones in which plasmid DNA was incorporated into the chromosome. Colonies from these plates were then streaked onto LB agar plates (but without NaCl), supplemented with 10% sucrose to select for double recombinants that contained a chromosomal copy of the mutant *lfnA*::*aacC1* allele but lacked the counterselection marker *sacB*, which is lethal to cells grown on sucrose (29). Allelic exchange in putative *lfnA* knockout strains was confirmed by PCR.

Construction of the complementation plasmid pUCP20-rbs-*his*$_6$-*lfnA*. The *lfnA* PCR product described above was digested with NdeI and BamHI and ligated into pET28a to generate plasmid pFuc41 in which *lfnA* is fused to the plasmid-encoded N-terminal His_6 tag (His_6-*lfnA*). This gene fusion, together with the upstream ribosome-binding site (rbs), was excised with XbaI and HindIII and ligated into the shuttle vector pUCP20 to generate the complementing vector, pUCP20-rbs-*his*$_6$-*lfnA*.

Construction of the His_6-*lfnA* protein expression plasmid pVLT31-rbs-*his*$_6$-*lfnA*. The His_6-*lfnA* fusion, together with the upstream rbs, was excised from pFuc41 using XbaI and HindIII and cloned into these restrictions sites in the broad-host-range vector pVLT31. The resulting plasmid, pVLT31-rbs-*his*$_6$-*lfnA*, places the His_6-*lfnA* fusion under the control of the *Ptac* promoter in a broad-host-range vector.

Construction of the *wbuX* protein expression plasmids pBa1ET28a-*wbuX* and pBf3ET28a-*his*$_6$-*wbuX*. *wbuX* was PCR amplified using the primers 5′-TTTTTTTTGCTAGCTCCATGGAAAATAAAAATTATCAAATT-3′ (NheI and NcoI sites underlined) and 5′-TTTTTTTTGGATCCTCATCTGAAATACCTTTTTTC-3′ (BamHI site underlined). Cloning this fragment into pET28a using the NcoI and BamHI consensus sites generated a vector (pBa1ET28a-*wbuX*) for expression of wild-type, untagged WbuX in *E. coli*. Cloning into the NheI and BamHI sites of pET28a generated the vector pBf3ET28a-*his*$_6$-*wbuX* for expression of His_6-tagged WbuX (His_6-WbuX) in *E. coli*.

Construction of the *wbuX*-based cross-complementation plasmids pCr1UCP20-rbs-*wbuX* and pCs2UCP20-rbs-*his*$_6$-*wbuX*. *wbuX*-containing XbaI-HindIII fragments from pBa1ET28a-*wbuX* and pBf3ET28a-*his*$_6$-*wbuX* were ligated into XbaI-and-HindIII-digested pUCP20 to generate the cross-complementation vectors pCr1UCP20-rbs-*wbuX* and pCs2UCP20-rbs-*his*$_6$-*wbuX*, respectively.

Electroporation protocol for *P. aeruginosa*. DNA was introduced into *P. aeruginosa* using a novel protocol, based on the methods of Helmark and coworkers (32) and Choi and coworkers (13). An overnight culture of *P. aeruginosa* was used to inoculate 50 ml of growth medium (LB supplemented with 0.25 M sucrose, 27.8 mM glucose, and 0.75% [wt/vol] glycine) to give a starting optical density at 600 nm (OD_{600}) of 0.03. The culture was shaken at 37°C until the OD_{600} reached approximately 0.1 when cells were harvested by centrifugation at 10,000 × *g* for 10 min at room temperature. The pellet was washed once in 5 ml of sterile wash solution (0.25 M sucrose, 10% [vol/vol] glycerol) at room temperature and then suspended in wash solution to give an OD_{600} of approximately 30. A 50-μl aliquot of this suspension was combined with 5 μg of plasmid DNA and pulsed in a 2-mm-gap electroporation cuvette (2.5 kV, 25 μF, 400 Ω) using a Gene Pulser apparatus (Bio-Rad). Immediately, 950 μl of LB was added, and the cells were recovered with shaking at 37°C for 3 h before plating on selective medium.

LPS preparation. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, LPS was prepared using the proteinase K digestion method of Hitchcock and Brown (34). For structural analysis, LPS was prepared using the hot aqueous phenol extraction method of Westphal and Jann (64).

SDS-PAGE. SDS-PAGE was performed with a discontinuous gel system and 12.5% resolving gels (38). Proteins were visualized using SimplyBlue SafeStain (Invitrogen). LPS was visualized using the rapid silver staining method of Fomsgaard and coworkers (26) or by Western blotting.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
E. coli		
JM109	e14$^-$ (McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36*$^+$ *proAB*$^+$ *lacI*^{q}*ZΔM15*]	Stratagene
DH5α	F$^-$ ϕ80d*lac*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA*	Invitrogen
SM10	*thi-1 thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r	54
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λDE3(*lacI lacUV5*-T7 gene 1 *ind-1 sam-7 nin-5*)	Novagen
N 00 6496	Wild type; O145:NM	Mohamed Karmali
N 01 2051	Wild type; O145:NM	Mohamed Karmali
N 02 5149	Wild type; O145:NM	Mohamed Karmali
P. aeruginosa		
PAO1	Wild-type strain, IATS[a] serotype O5 (ATCC 33351)	31
O4	Wild-type strain, IATS serotype O4 (ATCC 33351)	ATCC
O11	Wild-type strain, IATS serotype O11 (ATCC 33358)	ATCC
O12	Wild-type strain, IATS serotype O12 (ATCC 33359)	ATCC
O12 *lfnA*	O12 *lfnA*::*aacC1*; Gm^r	This work
Plasmids		
pEX100T	Counterselectable, mobilizable suicide vector backbone; contains *oriT* and *sacB*; Ap^r	53
pUCGm	Source of small, broad-host-range gentamicin cassette *aacC1*; Ap^r Gm^r	52
pET28a	Protein expression vector for creating thrombin-cleavable N-terminal His_6 tag gene fusions; Km^r	Novagen
pUCP20	*Escherichia-Pseudomonas* shuttle vector derived from pUC18; Ap^r Cb^r	63
pVLT31	Broad-host-range vector containing the *Ptac* promoter for induction of downstream gene expression; Tc^r	17
pKOLD2	Suicide vector pEX100T containing the mutant *lfnA* allele *lfnA*::*aacC1*; Ap^r Gm^r	This work
pFuc41	pET28a-derived expression vector encoding expression of His_6-Lfn; Km^r	This work
pUCP20-rbs-*his*$_6$-lfnA	XbaI-HindIII fragment from pFuc41, containing the ribosome binding site, N-terminal His_6 tag, and *lfnA* coding sequence cloned into pUCP20; Ap^r Cb^r	This work
pVLT31-rbs-*his*$_6$-lfnA	XbaI-HindIII fragment from pFuc41, containing the ribosome binding site, N-terminal His_6 tag, and *lfnA* coding sequence cloned into pVLT31; Tc^r	This work
pBa1ET28a-*wbuX*	Untagged *wbuX* cloned into the NcoI and BamHI sites of pET28a; Km^r	
pBf3ET28a-*his*$_6$-wbuX	*wbuX* cloned into the NheI and BamHI sites of pET28a, generating an N-terminal His_6 tag fusion; Km^r	This work
pCr1UCP20-rbs-*wbuX*	XbaI-HindIII fragment from pBa1ET28a-*wbuX*, which contains the ribosome binding site and wild-type *wbuX* cloned into pUCP20; Ap^r Cb^r	This work
pCs2UCP20-rbs-*his*$_6$-wbuX	XbaI-HindIII fragment from pBf3ET28a-*his*$_6$-*wbuX*, which contains the ribosome binding site and His_6-*wbuX* fusion cloned into pUCP20; Ap^r Cb^r	This work

[a] IATS, International Antigenic Typing Scheme.

Western blotting. Western transfer of proteins and LPS was performed to BioTraceNT nitrocellulose membranes (Pall) according to standard protocols with minor modifications (11, 56). His-tagged proteins were detected using His-Probe H-3 primary antibodies (Santa Cruz Biotechnology). Rabbit polyclonal antibodies specific for *P. aeruginosa* O4, O11, and O12 serotypes (from a *Pseudomonas* typing kit prepared by Chengdu Institute of Biological Products, Ministry of Public Health, Chengdu, Sichuan, China) were used to detect the presence of O-antigen-containing LPS in these bacteria. Blots were incubated with alkaline phosphatase-conjugated secondary antibodies and then visualized by incubation with nitroblue tetrazolium (7) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to standard protocols.

NMR spectroscopy. ^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500-MHz spectrometer for samples in D_2O solutions at 25°C for the oligosaccharides with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences. Experiments included correlation spectroscopy (COSY), total correlation spectroscopy (mixing time 120 ms), nuclear Overhauser effect spectroscopy (mixing time 200 ms), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) (optimized for an 8-Hz coupling constant).

Mild acid hydrolysis of LPS. LPS (25 mg) was hydrolyzed with 2% acetic acid (AcOH) (2 ml, 3 h, 100°C), and the precipitates of the lipid components were removed by centrifugation at top speed in a microcentrifuge (12,000 rpm). Soluble products were separated by gel chromatography on a Sephadex G50 column. Polymeric and oligosaccharide fractions were obtained. Oligosaccharide (core) fraction was further separated by anion-exchange chromatography.

Anion-exchange chromatography. Anion-exchange chromatography was performed on a Hitrap Q column (Pharmacia). The column was washed with water for 10 min and then eluted with a linear gradient of 0 to 1 M NaCl over 60 min, with a flow rate of 3 ml min^{-1} and UV detection at 220 nm. Fractions were desalted by gel chromatography on a Sephadex G-15 column.

Monosaccharide analysis. The polysaccharide, core, or LPS (0.5 mg) was hydrolyzed (0.2 ml of 3 M trifluoroacetic acid, 120°C, 2 h) and evaporated to dryness under a stream of air. The residue was dissolved in water (0.5 ml), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with AcOH (0.3 ml), and dried, and methanol (1 ml) was added. The mixture was dried twice by the addition of methanol, and the residue was acetylated with acetic anhydride (0.5 ml, 100°C, 30 min), dried, and analyzed by gas chromatography on a HP1 capillary column (30 m by 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) and a temperature gradient of 170 (4 min) to 260°C at 4°C min^{-1}.

Gel filtration chromatography. Prior to NMR analysis, carbohydrates derived from LPS were purified on Sephadex G-50 (2.5- by 80-cm) or Sephadex G-15 (1.6- by 80-cm) columns using pyridinium acetate buffer (pH 4.5) (4 ml pyridine and 10 ml AcOH in 1 liter water) as the eluent, with monitoring of the eluate by a refractive index detector.

Protein expression and subcellular localization by differential centrifugation. His_6-LfnA and His_6-WbuX expression in the *P. aeruginosa lfnA* mutant was done at basal expression levels from the plasmids pUCP20-rbs-*his*$_6$-*lfnA* and pCs2UCP20-rbs-*his*$_6$-*wbuX*, respectively. Cultures were grown overnight in LB, and isopropyl-β-D-thiogalactopyranoside (IPTG) was not added to the growth medium.

His_6-Lfn expression in *P. aeruginosa* from the pVLT31-based expression plasmid was induced once the cultures attained an OD_{600} of approximately 0.3 by adding IPTG to a final concentration of 0.4 mM. The cultures were then incubated for 3 hours at 37°C.

His_6-WbuX was expressed in *E. coli* BL21(DE3) according to protocols in the pET system manual (Novagen). Expression of His_6-WbuX from the pET vector was induced, once the OD_{600} reached 0.4, by adding IPTG to 0.4 mM. The induced culture was then incubated overnight at 15°C, before the cells were harvested.

The cells were harvested by centrifugation (10,000 × *g*, 10 min) and suspended in 50 mM sodium phosphate, pH 8.0, and 300 mM NaCl. Bacterial cells were lysed on ice with 5-s ultrasound bursts generated by a model 500 sonic dismembrator (Fisher Scientific) fitted with a macrotip at 35% power for a total of 2 minutes. Cell debris was removed by centrifugation at 30,000 × *g* for 20 min, and membranes were isolated by ultracentrifugation at 100,000 × *g* for 1 to 2 h. The membrane pellets were gently washed twice with water and then suspended in SDS loading buffer (62.5 mM Bis-Tris [pH 6.8], 0.5% [wt/vol] SDS, 10% [wt/vol] glycerol, 0.005% bromophenol blue, 250 mM $NaHSO_3$). Proteins were considered to be soluble if they remained in solution after the ultracentrifugation step.

Bioinformatics. Protein sequences were analyzed, and sequence identities were calculated based upon alignments obtained using the position-specific iterative basic local alignment search tool (PSI-BLAST) (2).

RESULTS

Mutation of *lfnA* increases the relative mobility of LPS bands on SDS-PAGs. To characterize the function of *lfnA* in O-antigen biosynthesis, the chromosomal copy of the gene was replaced with a mutant allele in which the coding sequence is disrupted by a gentamicin resistance cassette. LPS prepared from this *lfnA* knockout mutant was compared to that of wild-type bacteria using SDS-PAGE and silver staining. B-band LPS in several *P. aeruginosa* strains exhibits a bimodal or multimodal chain length distribution. In the *P. aeruginosa* serotype O5 strain, PAO1, this pattern is determined by the operation of two chain-length-regulating proteins Wzz_1 and Wzz_2 (15). In SDS-PAGs, this modal O-antigen length distribution is visible as clusters of intense bands in the top portion of the gel (Fig. 1). When the *lfnA* mutant LPS is examined using SDS-PAGE, these clusters of bands migrate faster through the gel than those of the wild-type LPS (Fig. 1A). This mutation does not interfere, however, with recognition of the O antigen by serotype O12-specific polyclonal antibodies (Fig. 1B). This suggests that the mutation has caused either a shortening of the modal O-antigen chain lengths or a change in O-antigen repeat unit structure or both. The spacing between individual bands in the SDS-PAGE profile indicates the change in electrophoretic mobility due to each additional O unit. Compared to LPS from the wild-type O12 strain, LPS from the *lfnA* mutant exhibited reduced spacing between bands, suggesting that this mutation caused a change in the structure of each O-antigen repeat.

A novel electroporation protocol enabled complementation of the *lfnA* mutation. We wished to complement the *lfnA* mutation by expressing the gene from plasmid pUCP20. This vector does not have an *oriT* and cannot therefore be transferred into *P. aeruginosa* by conjugation. Transformation of plasmid DNA into *P. aeruginosa* O12 strain ATCC 33359 using standard electroporation protocols usually employed for *P. aeruginosa* (18, 23, 55) has proven to be difficult. To alleviate this problem, we developed a novel method for preparation of electrocompetent *P. aeruginosa* by combining steps from two methods in the literature (13, 32). Cells were grown according to the method of Helmark and coworkers (32) but harvested at room temperature according to the protocol of Choi and coworkers (13).

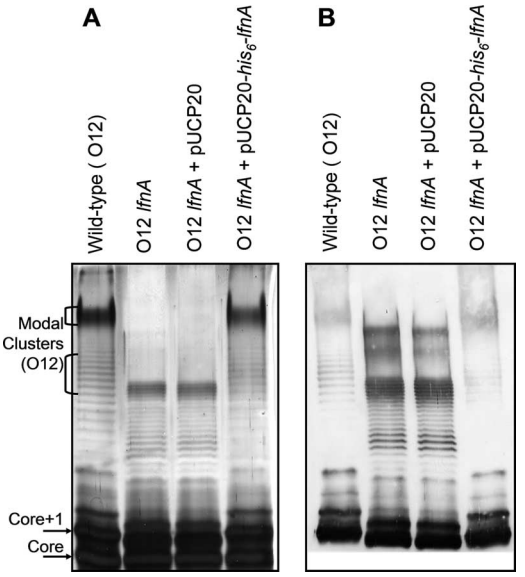

FIG. 1. SDS-PAGE analysis of LPS from wild-type *P. aeruginosa* serotype O12, the *lfnA* mutant, and the *lfnA* mutant containing either the complementing plasmid pUCP20-*his*$_6$-*lfnA*, or the empty vector pUCP20. LPS was visualized by silver staining (A) and by Western blotting with polyclonal, anti-O antigen primary antibodies (B). The empty vector has no effect on LPS phenotype, whereas the complementing plasmid restores the wild-type LPS banding pattern. The positions of modal clusters of O-antigen-containing LPS in the wild-type lanes are indicated. The bands representing unsubstituted LPS core and core plus one O-antigen repeat (Core+1) are also indicated. The Core+1 is the smallest molecule to be recognized by the O-antigen-specific antibodies.

Using this method, we introduced the complementing plasmid into the *lfnA* mutant strain, and LPS in the complemented strain showed a phenotype similar to that observed in the wild-type strain (Fig. 1).

The *lfnA* mutant produces LPS which contains L-FucNAc in the O-antigen repeat unit, instead of L-FucNAm. To establish the structural basis for the closer spacing between O-antigen bands in SDS-PAGE analysis of the *lfnA* mutant LPS, we purified LPS from the mutant and from the complemented strain and analyzed the O-antigen repeat structure by NMR.

Oligosaccharides from the mutant and complemented strains were isolated from LPS by mild acid hydrolysis. Monosaccharide analysis confirmed the presence of fucosamine and quinovosamine in both products. In the case of the wild-type strain, mild acid hydrolysis gave a polysaccharide, although to some extent the O chain was depolymerized. The mutant LPS produced a trisaccharide and no polymer.

The polysaccharide of the wild-type strain was studied using two-dimensional NMR (COSY, total correlation spectroscopy, nuclear Overhauser effect spectroscopy, HSQC, and HMBC) (Table 2) and was confirmed to have the previously described structure of serotype O12 O antigen (12) (Fig. 2D). In particular, the amidine group was identified by its characteristic signals at 20.4 (methyl group carbon) and 169 ppm (carbonyl carbon). The amidine group was connected to N-2 of fucosamine, which was determined from HMBC correlation be-

TABLE 2. NMR data for the *Pseudomonas aeruginosa* serotype O12 polysaccharide (40°C) and the *lfnA* mutant repeating unit trisaccharide (25°C)[a]

Unit[b]	NMR spectrum	Signal (ppm) at the following position[c]:								
		1	2/3ax	3(3eq)	4	5	6	7	8	9
PS 8eLeg5Ac7Ac (A)	^{1}H		1.68	2.64	3.54	3.71	4.10	3.95	3.72	1.33
	^{13}C	173.7	105.0	42.4	69.2	53.8	73.6	54.7	73.2	14.9
PS 8eLeg5Ac7Ac, Ac (2x)	^{1}H		1.98							
	^{13}C	175.3	23.1							
TS 8eLeg5Ac7Ac (A)	^{1}H		1.87	2.30	3.94	3.75	4.12	3.98	3.75	1.11
	^{13}C	174.5	97.1	40.6	68.4	54.0	71.7	54.0	73.7	15.7
PS FucNAm (B)	^{1}H	5.13	3.98	4.07	4.02	4.43	1.22			
	^{13}C	96.7	52.3	75.6	72.4	67.3	17.1			
PS FucNAm, Am	^{1}H		2.29							
	^{13}C	167.5	20.4							
TS FucNAc (B)	^{1}H	4.95	4.08	3.87	3.79	4.41	1.18			
	^{13}C	98.6	50.8	69.1	72.4	67.9	16.7			
TS FucNAc, Ac	^{1}H		2.01							
	^{13}C	175.7	23.5							
PS QuiNAc (C)	^{1}H	4.84	4.11	3.63	3.32	3.65	1.32			
	^{13}C	94.1	54.6	76.7	74.6	69.6	18.0			
PS QuiNAc, Ac	^{1}H		2.08							
	^{13}C	175.0	23.3							
TS QuiNAc (C)	^{1}H	4.79	4.03	3.57	3.24	3.63	1.27			
	^{13}C	94.6	54.8	76.8	74.9	69.7	18.0			

[a] Acetyl signals of the residues A and C in the trisaccharide (^{1}H/^{13}C): 1.95/23.0; 1.98/23.4; 2.03/23.4; all C-1 at 175.4 ppm.
[b] Abbreviations: PS, wild-type polysaccharide; Ac (2x), two acetyl groups; TS, the *lfnA* mutant repeating unit trisaccharide; Am, acetimldoyl.
[c] 3ax, axial proton in 8eLeg5Ac7Ac residue; 3eq, equatorial proton in 8eLeg5Ac7Ac.

tween the amidine CO and H-2 of the fucosamine residue from HMBC correlation. The monosaccharide sequence was determined on the basis of nuclear Overhauser effect and HMBC correlations. Thus, the polysaccharide has the following structure:

→8)-α-8eLeg5Ac7Ac-(2→3)-α-L-FucNAc-(1→3)-α-D-QuiNAc-(1→

A B C

where 8eLeg5Ac7Ac is 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid and QuiNAc is 2-acetamido-2,6-dideoxyglucose.

The trisaccharide obtained by mild acid hydrolysis of the *lfnA* mutant LPS had the nonulosonic acid at the reducing end in the anomeric configuration opposite the configuration observed in the wild-type polysaccharide, as could be concluded from the difference in position of ^{1}H signals of its methylene group (Table 2). This trisaccharide contained four N-acetyl groups and no amidine (Table 2). The difference in the stability of the polymers with and without the amidine group, with respect to depolymerization in the conditions of mild hydrolysis, may be due to a stabilizing influence of the positively charged amidine group on the nearby anomeric center of the nonulosonic acid residue. The structure of the mild acid-released trisaccharide is therefore:

α-L-FucNAc-(1→3)-α-D-QuiNAc-(1→8)-β-8eLeg5Ac7Ac

B C A

The trisaccharide structure represents the chemical repeating unit of the polysaccharide which is different from the biological repeating unit (12). Using these data, the structure of the *lfnA* mutant O polysaccharide was deduced (Fig. 2C).

The amidine signal which is absent in NMR analysis of the *lfnA* mutant O polysaccharide was restored in the NMR spectrum of the O polysaccharide from the complemented mutant (Fig. 2B), verifying that the O-antigen structure was restored to the wild-type polysaccharide by supplying *lfnA* in *trans*.

Expression of His$_6$-*lfnA* in other *P. aeruginosa* serotypes has no apparent effect on the L-FucNAc sugars in their respective O-antigen repeats. The B-band O-antigen repeats of *P. aeruginosa* serotypes O4 and O11 contain L-FucNAc residues (as well as other sugars). Since mutation of *lfnA* in *P. aeruginosa* O12 resulted in expression of L-FucNAc instead of L-FucNAm within the O repeat, we were interested to see whether introduction of *lfnA* into serotypes O4 and O11 would cause a change from L-FucNAc to L-FucNAm in the O-antigen repeats in these strains. The *lfnA* complementation plasmid pUCP20-*his$_6$-lfnA* enabled us to answer this question: after electroporation of this plasmid into these strains, no discernible changes in the LPS were observed based upon silver-stained SDS-PAGE and Western blot analyses (data not shown).

Cross-complementation of the *lfnA* mutation with *wbuX* from enterohemorrhagic *E. coli* O145. One of the closest homologues of *lfnA* in the DNA databases is *wbuX* from *E. coli* O145. The predicted proteins encoded by these genes share 71% sequence identity. Since *E. coli* O145, like *P. aeruginosa* O12, also expresses L-FucNAm, we hypothesized that the two genes are functionally interchangeable. *wbuX* was PCR amplified from three *E. coli* O145 strains, N 00 6496, N 01 2051, and N 02 5149. The *wbuX* sequence in all three strains was identical

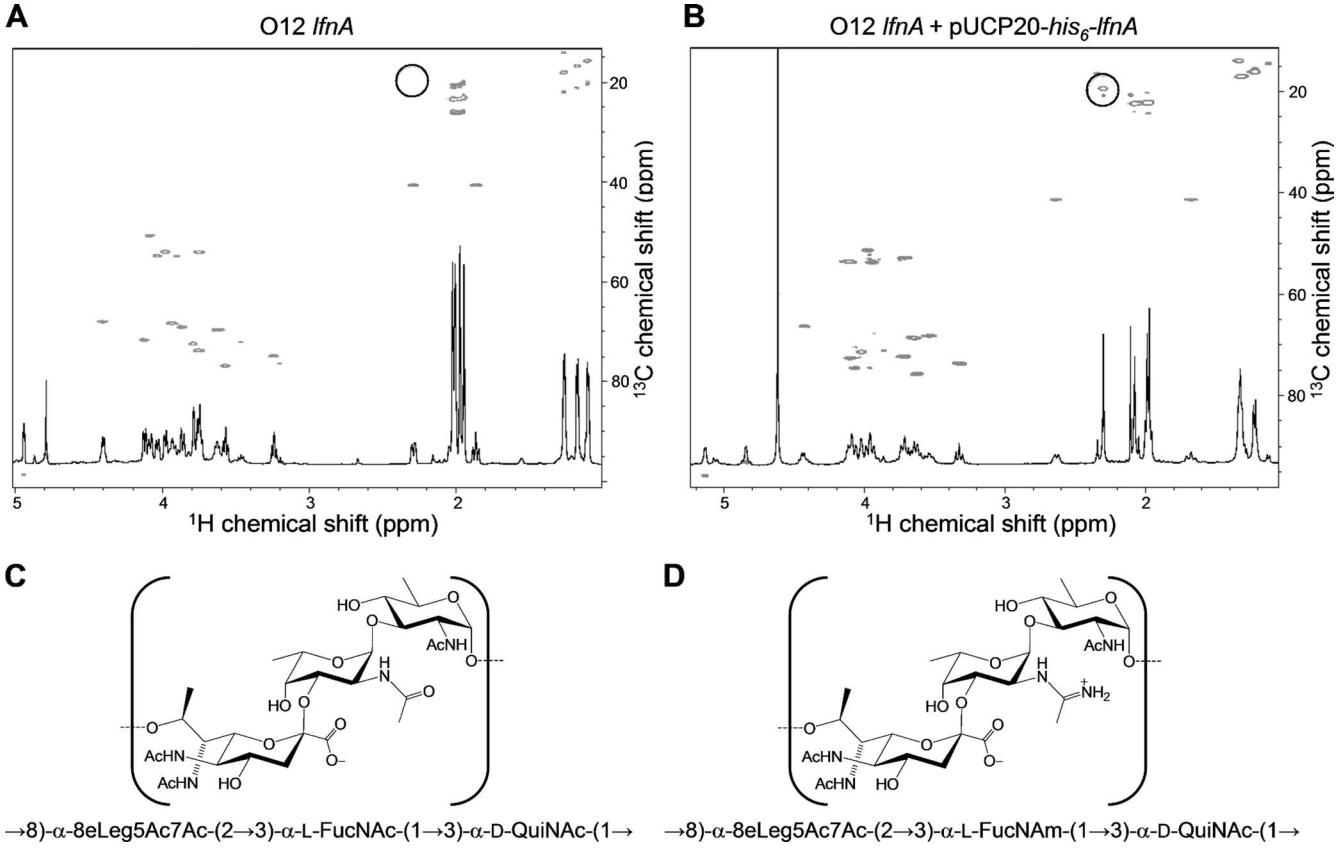

FIG. 2. ^{13}C-^{1}H correlated HSQC NMR spectra of the repeating unit trisaccharide of the *lfnA* mutant (A) and of the polysaccharide of the complemented *lfnA* mutant (B). Circles highlight the position in panel B of the characteristic methyl signal of the acetamidino group, which is not observed in the *lfnA* mutant. (C) Deduced structure of the *lfnA* mutant O polysaccharide. (D) Structure of the wild-type (or complemented *lfnA* mutant) O polysaccharide.

to that previously reported (NCBI protein ID AAV74532.1) (24). When *wbuX* was supplied on the shuttle vector pUCP20 in the *lfnA* mutant, it restored the wild-type LPS profile in SDS-PAGE analyses (Fig. 3). The gene was also able to complement the mutation with codons for an N-terminal His_6 tag at its 5′ end (Fig. 3).

His_6-LfnA and His_6-WbuX localize to the membrane fraction. During the process of expression and purification of His_6-LfnA in *E. coli*, we observed that the protein localized predominantly to the membrane fraction (data not shown). Therefore, in order to establish whether a membrane location was due to intrinsic properties of the protein or just an artifact of overexpression in *E. coli*, we wanted to investigate the cellular localization of His_6-LfnA in *P. aeruginosa*. We examined the subcellular localization of His_6-LfnA when it was expressed from the complementing vector pUCP20-rbs-*his$_6$-lfnA* in the *lfnA* mutant strain. The complementation experiments indicated that in these conditions, the His_6-*lfnA* gene is able to express active protein. Cellular fractions were isolated by differential centrifugation, and His_6-tagged proteins were detected by Western blotting (Fig. 4). A band of the correct apparent molecular weight and which reacted with the anti-His_6 tag antibody was observed in the membrane fraction, but no His_6-LfnA was detected in the soluble fraction. To examine the localization of this protein when overexpressed, His_6-*lfnA*

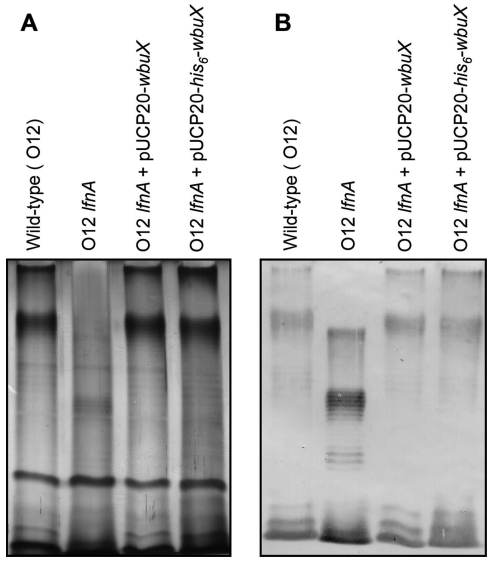

FIG. 3. Cross-complementation of *lfnA* mutation by expression of *wbuX* in *trans*. LPS was analyzed by SDS-PAGE and silver staining (A) or by Western blotting using serotype O12-specific, polyclonal, primary antibodies (B). Maintenance of either one of the cross-complementation plasmids, pUCP20-*wbuX* or pUCP20-*his$_6$-wbuX*, within the *lfnA* mutant restores the wild-type O-antigen banding pattern.

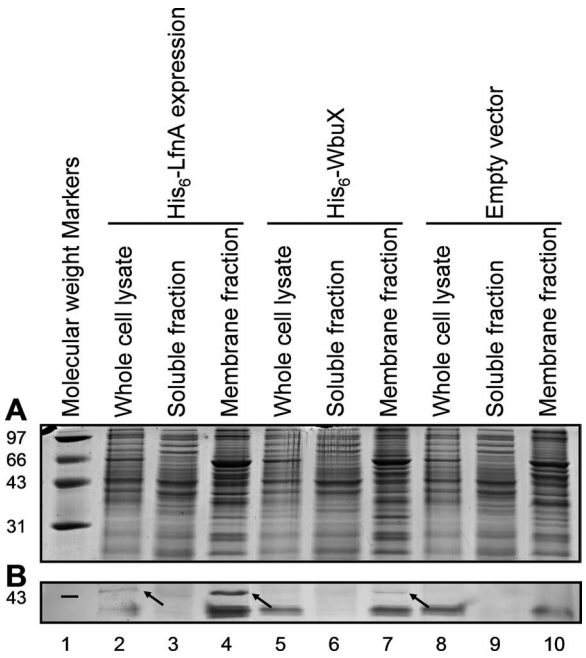

FIG. 4. Localization of His_6-LfnA and His_6-WbuX expressed in the *P. aeruginosa* serotype O12 *lfnA* mutant. Cells were grown harboring the complementation vector pUCP20-rbs-*his*$_6$-*lfnA* (lanes 2 to 4), the cross-complementation vector pCs2UCP20-rbs-*his*$_6$-*wbuX* (lanes 5 to 7), or the empty vector, pUCP20 (lanes 8 to 10). Cellular fractions were isolated by differential centrifugation and then analyzed by SDS-PAGE. Proteins were visualized by SimplyBlue SafeStain staining of the gel (A) or by Western blotting using anti-His_6-tag primary antibodies (B). Arrows indicate the positions of His_6-LfnA and His_6-WbuX in the blot. Exposure of the blot to the developing solution for a longer time did not reveal any His_6-tagged proteins in the soluble fraction lanes (data not shown). The positions of molecular weight markers (in thousands) are shown to the left of the gels.

was subcloned into the *Pseudomonas* protein expression vector pVLT31, and expression of His_6-LfnA was induced in *P. aeruginosa* strain PAO1. In these conditions also, His_6-LfnA localized to the membrane fraction (data not shown).

With a view to future in vitro studies on these proteins, we also investigated the localization of His_6-WbuX. Had His_6-WbuX proven to be a soluble protein, this would have facilitated purification of this LfnA functional homologue for biochemical studies. His_6-WbuX expressed from the cross-complementing plasmid pCs2UCP20-rbs-*his*$_6$-*wbuX* in the *P. aeruginosa lfnA* mutant also localized to the membranes (Fig. 4), as did His_6-WbuX overexpressed from the pBf3ET28a-*his*$_6$-*wbuX* vector in *E. coli* BL21(DE3) (data not shown).

DISCUSSION

We have characterized the phenotype of a *P. aeruginosa* serotype O12 *lfnA* mutant by SDS-PAGE and Western blot analysis of LPS and at the molecular level by elucidating the chemical structure of the O antigen using NMR techniques. The phenotype of this mutant, which produces L-FucNAc instead of L-FucNAm in its O antigen, is similar to that of the *pseA* mutant in *C. jejuni* (42), which lacked the acetamidino group normally present on the flagellin glycan. While the *pseA* mutant was not complemented and therefore possible polar effects of that mutation cannot be ruled out, we have complemented the *lfnA* mutation by expression of His_6-*lfnA* from a plasmid and therefore present the first unequivocal evidence that genes in this family are required for the expression of the acetamidino moieties found in bacterial glycans.

Mutation of another close homologue of *lfnA*, *wbpG* from *P. aeruginosa* serotype O5, results in expression of semirough LPS, i.e., the lipid A-core is substituted with only a single O-antigen unit (50). This phenotype resembles that of an O-antigen polymerase mutant, *wzy*, and the implication has been drawn that the acetamidino modification of the nonreducing terminal sugar in the O-antigen repeat in this serotype (2,3-dideoxy-2-acetamido-3-acetamidino-D-mannuronic acid) is required for recognition by Wzy (50). In *P. aeruginosa* serotype O12, the acetamidino modification is located on the middle sugar of the O repeat, and it may be that the polymerase exhibits more-relaxed substrate specificity with respect to residues which are more distant from the sites of Wzy-catalyzed glycosidic bond formation.

Successful complementation of the *lfnA* mutation with *wbuX* verifies the hypothesis that *lfnA* and *wbuX* are functional homologues in *P. aeruginosa*. The successful complementation with His_6-tagged versions of both genes demonstrates that these fusions are functional in vivo and that these constructs will prove useful for future purification and in vitro characterization of these two proteins.

Neither LfnA nor WbuX has a predicted transmembrane helix, nor any other motifs which would indicate membrane association; therefore, neither was anticipated to localize to the membrane fraction. This subcellular localization may occur as a result of protein-protein interactions with an integral membrane protein. His_6-WbuX remains entirely membrane associated, however, even when overexpressed from the pET28a vector and therefore likely to vastly outnumber potential interaction partners. This suggests that the membrane association is more likely due to intrinsic properties of the protein itself. Monotopic membrane proteins, such as prostaglandin H_2 synthase, can associate with lipid bilayers through membrane binding domains which do not span the membrane but have high local concentrations of positively charged and aromatic residues (27, 45, 62). An alignment of LfnA and WbuX sequences shows several conserved stretches of sequence which are particularly enriched for these amino acid side chains. These patches are distributed throughout the sequence, and therefore, it would probably be far from trivial to engineer these proteins to make a soluble, yet still functional truncated enzyme. As a consequence, in vitro studies will require detergent solubilization of the protein to facilitate purification. While this is beyond the scope of the present work, knowledge of the LfnA and WbuX membrane localization will be useful for future studies of these proteins.

Expression of His_6-*lfnA* in other *P. aeruginosa* serotypes, which produce O-antigen repeats containing L-FucNAc residues, did not affect the LPS-banding profile in SDS-PAGE analysis in these strains. Mutation of the O-antigen repeat by knocking out *lfnA* in *P. aeruginosa* O12 dramatically increased the electrophoretic mobility of O-antigen-containing LPS molecules. The most important factor causing this band shift is probably a change in the charge of the LPS molecule. The pK_a of the amidine group is expected to be approximately 11 (33)

so that at neutral pH, and in the SDS-PAGE running conditions, the FucNAm sugars will be positively charged, therefore retarding migration toward the anode. In this context, it is very likely that incorporation of L-FucNAm into serotype O4 and O11 LPS would result in a band shift in SDS-PAGE. Therefore, we conclude that L-FucNAm is not incorporated as a result of His_6-*lfnA* expression in these strains. This may indicate that the *lfnA* substrate is not present in these serotypes. The substrate for *lfnA* may be UDP-L-FucNAc. This is the O-antigen precursor synthesized from UDP-D-GlcNAc by WbjB, WbjC, and WbjD in *P. aeruginosa* O11 (35, 44) These three genes are present in the O12 B-band locus (49), and the acetamidino group could conceivably be synthesized by direct amidotransfer to the UDP-L-FucNAc acetamido group. At present, however, there is no experimental evidence that UDP-L-FucNAc is the LfnA substrate, and amidotransfer may occur at a later stage in O-antigen biosynthesis. Alternatively, a proportion of the UDP-L-FucNAc in the sugar nucleotide pool may be converted to UDP-L-FucNAm in these experiments, but the L-FucNAm is not incorporated into LPS by downstream enzymes due to substrate specificities of the glycosyl transferase, O-antigen flippase, or O-antigen polymerase or all three. Certainly, the sugar-nucleotide pool must retain sufficient quantities of UDP-L-FucNAc in these experiments to enable expression of wild-type LPS. Another possibility is that expression of His_6-*lfnA* alone in the other *P. aeruginosa* serotypes is not sufficient for amidotransfer to occur; other factors may be required.

In conclusion, we have presented genetic data to indicate the roles of *lfnA* and *wbuX* in L-FucNAm biosynthesis and substantiated these with NMR analysis of LPS prepared from the *lfnA* mutant. We have demonstrated that His_6-tagged fusions of LfnA and WbuX retain their function and shown that these proteins localize to the membranes. This will facilitate biochemical studies of this pathway. These genes may be particularly amenable to in vitro studies, since their putative substrate UDP-L-FucNAc can by synthesized in vitro from UDP-D-GlcNAc using WbjB, WbjC, and WbjD in a well-characterized pathway (35, 44), and therefore, enzyme-substrate assays should be possible in the future.

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