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A UDP-HexNAc:Polyprenol-P GalNAc-1-P Transferase (WecP) Representing a New Subgroup of the Enzyme Family▽†

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The *Aeromonas hydrophila* AH-3 WecP represents a new class of UDP-HexNAc:polyprenol-P HexNAc-1-P transferases. These enzymes use a membrane-associated polyprenol phosphate acceptor (undecaprenyl phosphate [Und-P]) and a cytoplasmic UDP-D-*N*-acetylhexosamine sugar nucleotide as the donor substrate. Until now, all the WecA enzymes tested were able to transfer UDP-GlcNAc to the Und-P. In this study, we present *in vitro* and *in vivo* proofs that *A. hydrophila* AH-3 WecP transfers GalNAc to Und-P and is unable to transfer GlcNAc to the same enzyme substrate. The molecular topology of WecP is more similar to that of WbaP (UDP-Gal polyprenol-P transferase) than to that of WecA (UDP-GlcNAc polyprenol-P transferase). WecP is the first UDP-HexNAc:polyprenol-P GalNAc-1-P transferase described.

The lipopolysaccharide (LPS) is one of the major structural and immunodominant molecules of the outer membrane in Gram-negative bacteria. It consists of three moieties: lipid A, core oligosaccharide, and O-specific antigen or O side chain. The O antigen is the external component of LPS, and its structure consists of a polymer of oligosaccharide repeating units. The genetics of O-antigen biosynthesis in the *Enterobacteriaceae* have been intensively studied, and it has been shown that the *wb** clusters usually contain genes involved in biosynthesis of activated sugars, glycosyl transferases, O-antigen polymerases, and O-antigen export (for a review, see reference 32). Despite the heterogeneity in the structures of the O antigens, only three pathways for the assembly of O antigens have been recognized: these are the Wzy-dependent pathway, the ABC transporter-dependent pathway, and the synthase-dependent pathway (14, 32). All of them initiate O-antigen LPS biosynthesis with an integral membrane protein that catalyzes the transfer of glucose (Glc)/galactose (Gal)-1-phosphate (WbaP) or *N*-acetylhexosamine (HexNAc)-1-phosphate onto undecaprenyl phosphate (Und-P) (38).

The UDP-HexNAc:polyprenol-P HexNAc-1-P transferase enzyme family, which includes both eukaryotic and prokaryotic transmembrane proteins, catalyzes the biosynthesis of polyprenol-linked oligosaccharides (26). These transferase-catalyzed reactions involve a membrane-associated polyprenol phosphate acceptor (Und-P) and a cytoplasmic UDP-D-*N*-acetylhexosamine sugar nucleotide as the donor substrate. Four subgroups of bacterial enzymes have been identified within the family, based on their specific substrate preference: these are MraY, WecA/TagO, WbcO/WbpL, and RgpG (5). MraY-type transferases are highly specific for UDP-*N*-acetylmuramate-pentapeptide (UDP-MurNAc-pp), whereas WecA proteins are selective for UDP-*N*-acetylglucosamine (UDP-GlcNAc) (31). The WbcO/WbpL substrate specificity has not yet been determined, but the structures of their biosynthetic end products imply that UDP-*N*-acetyl-D-fucosamine (UDP-FucNAc) and/or UDP-*N*-acetyl-D-quinovosamine (UDP-QuiNAc) is used (10, 41). Similar reasoning suggests that the RgpG subgroup is composed of relatively nonspecific transferases that can use either UDP-FucNAc or UDP-GlcNAc (40).

Mesophilic *Aeromonas* sp. serotype O34 strains have been recovered from moribund fish and from clinical specimens (17); O34 is the single most common *Aeromonas* serotype, accounting for 26.4% of all *Aeromonas* infections (18). Previous investigations have documented O34 strains as important causes of infections in humans. The *Aeromonas hydrophila* wb_{O34} gene cluster contains genes necessary for the production of O34-antigen LPS, and a role for the majority of these genes was previously attributed according to the chemical O34-antigen LPS structure obtained from strain AH-3 (Fig. 1) (20). By mutation and sequence homology, ORF15 from the *A. hydrophila* wb_{O34} gene cluster was putatively annotated as an undecaprenol-sugar-P-transferase (WecP, formerly WecA) able to initiate the assembly of the O34-antigen units, and the assembly of the O34 antigen was characterized as a Wzy-dependent pathway (20). In this study, we report the characterization of *Aeromonas* WecP, which represents a new class of UDP-HexNAc:polyprenol-P HexNAc-1-P transferases, according to its specific substrate preference.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *Aeromonas* was grown in either tryptic soy broth (TSB) or tryptic soy agar (TSA), and *Escherichia coli* or *Klebsiella pneumoniae* was grown in Luria-Bertani (LB) Miller broth and

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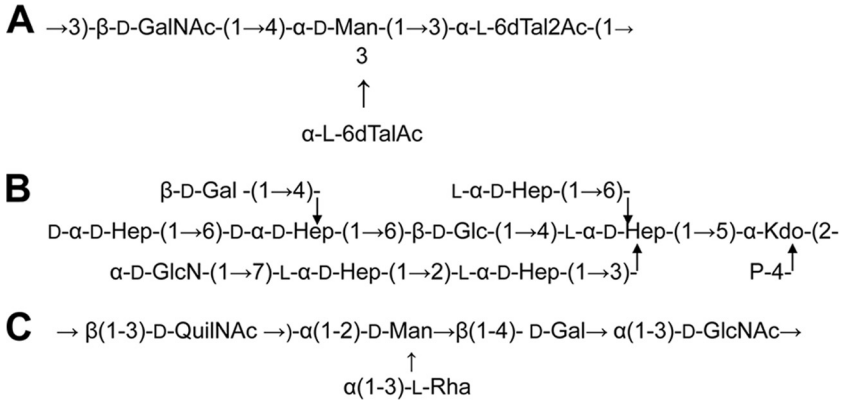

FIG. 1. Chemical structures of *Aeromonas* O34-antigen LPS (A) and LPS core (B) from *A. hydrophila* AH-3 and the O7-antigen LPS from *E. coli* strain VW187 (C).

LB Miller agar. Spectinomycin (50 μg/ml), tetracycline (20 μg/ml), chloramphenicol (25 μg/ml), gentamicin (20 μg/ml), kanamycin (50 μg/ml), or ampicillin (100 μg/ml) was added to the different media when required.

General DNA methods. Standard DNA manipulations were done essentially as described previously (37). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

DNA sequencing and computer analysis of sequence data. Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method (39) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Oligonucleotides used for genomic DNA amplifications and DNA sequencing were purchased from Sigma-Aldrich. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) were inspected. Deduced amino acid sequences were compared with those from DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST (2) network service at the National Center for Biotechnology Information and the European Biotechnology Information. ClustalW was used for multiple-sequence alignments (6).

Mutant construction. The *K. pneumoniae* 52145ΔwecA mutant was constructed by performing an *in vitro* in-frame deletion of the gene (27) as previously

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)[b]	Source or reference
E. coli strains		
DH5α	F⁻ *endA hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* ϕ80d*lacZ*ΔM15	13
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F⁻ *proAB lacI*^{q}ZDM15 Tn*10*)	Stratagene
S17-1	*hsdR pro recA*, RP4-2 in chromosome; Km::Tn*7* (Tc::Mu)	20
BL21(λD3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*(λD3)	Novagen
VW187	O7:K1; clinical isolate	M.A. Valvano
MV501	VW187; *wecA*::Tn*10* Tc^r	M.A. Valvano
A. hydrophila strains		
AH-3	O34, wild type	20
AH-405	AH-3, spontaneously Rif^r	20
AH-3ΔwecP[a]	AH-3 *wecP* mutant in frame with pDM4	20
S. enterica strains		
LT2	Wild type	M.A.Valvano
MSS2	LT2, Δ*wbaP*::Cat, Cm^r	M.A. Valvano
K. pneumoniae strains		
52145	Wild-type O1:K2	30
52145ΔwecA	51245 *wecA* mutant in frame with pKO3	This study
Plasmids		
pRK2073	Helper plasmid, Spc^r	20
pKO3	Cm^r *sacB* temp-sensitive suicide vector	27
pGEMT-Gne	pGEM-T vector with complete *gne* of AH-3	8
pBAD33	Arabinose-inducible expression vector, Cm^r	ATCC
pBAD33-Gm	pBAD33 vector with Gm^r	20
pBAD33-WecP$_{Ah}$	pBAD33-Gm with *A. hydrophila* AH-3 *wecA*	This study
pBAD33-WecA$_{Ec}$	pBAD33-Gm with *E. coli* VW187 *wecA*	This study
pBAD33-WecA$_{Kp}$	pBAD33-Gm with *K. pneumoniae* 52145 *wecA*	This study
pBAD33-WbaP$_{Se}$	pBAD33-Gm with *S. enterica* LT2 *wbaP*	This study
pET-30 Xa/LIC	IPTG-inducible expression vector, Km^r	Novagen
pET-30-WecP$_{Ah}$	pET-30 Xa/LIC with *A. hydrophila* AH-3 *wecP*	This study

[a] Formerly named AH-3ΔwecA.
[b] IPTG, isopropyl-β-D-thiogalactopyranoside.

described (16). The mutant was constructed using 52145 chromosomal DNA and primers 52145wecA-A (5′-GA**AGATCT**GTATAATGGCGCCGGATAG-3′), 52145wecA-B (5′-CCCTACCACTAAACTTAAACACGAGGCGTGAAGGAATATAA-3′; bold type indicates restriction sites), 52145wecA-C (5′-TGTTTAAGTTTAGTGGATGGGGCGTTTGTCCTGATTACCC-3′), and 52145wecA-D (5′-GA**AGATCT**CGACTATCCAGCCTTTTCC-3′) in two sets of asymmetric PCRs to amplify DNA fragments AB and CD), respectively. DNA fragments AB and CD were annealed at their overlapping region (underlined letters in the 52145wecA-B and -C primer sequences) and amplified by PCR as a single fragment, using primers 52145wecA-A and -D. The fusion product was purified, BamHI digested, ligated into BamHI-digested and phosphatase-treated pKO3 vector, electroporated into *E. coli* DH5α, and plated on chloramphenicol LB agar plates at 30°C to obtain plasmid pKO3Δ*wecA*$_{Kp}$. The mutated gene was transferred to the chromosome by homologous recombination using the temperature-sensitive suicide plasmid pKO3, containing the counterselectable marker *sacB*. The plasmid with the engineered in-frame deletion (pKO3Δ*wecA*$_{Kp}$) was transferred into *K. pneumoniae* 52145 by transformation. Mutants were selected based on growth in LB agar containing 10 to 15% sucrose and loss of the chloramphenicol resistance marker of vector pKO3 (25). Mutation was confirmed by sequencing of the whole constructs in amplified PCR products.

Plasmid constructions and mutant complementation studies. For complementation studies, the *A. hydrophila* AH-3 *wecP*, *E. coli* VW187 *wecA*, *K. pneumoniae* 52145 *wecA*, and *Salmonella enterica* LT2 *wbaP* genes were PCR amplified by using specific primer pairs and ligated to the plasmid pBAD33-Gm (22) (see the list of primers in Table SP1 in the supplemental material). The plasmid constructions were transformed into *E. coli* DH5α by electroporation, plated on gentamicin LB agar plates and incubated at 30°C. Plasmids with the amplified genes were independently transferred into the corresponding mutants by triparental mating using the mobilizing strain HB101/pRK2073 in *Aeromonas* or transformation by electroporation into *E. coli*, *S. enterica*, or *K. pneumoniae*. Transconjugants were selected on plates containing gentamicin (and rifampin for *Aeromonas*) and confirmed by PCR. Each gene was expressed from the arabinose-inducible and glucose-repressible pBAD33 promoter (P_{BAD}). Repression from the *araC* promoter was achieved by growth in medium containing 0.2% (wt/vol) D-glucose, and induction was obtained by adding L-arabinose to a final concentration of 0.2% (wt/vol) (21).

Purification of *A. hydrophila* AH-3 His$_6$-WecP. For *wecP* overexpression, the pET-30 Xa/LIC vector (Novagen) and AccuPrime (Invitrogen) high-fidelity polymerase were used. The *A. hydrophila* AH-3 *wecP* was amplified from genomic DNA by using primers PET-A3wec-for (5′-GGTATTGAGGGTCGCATGCTTGCTGTGTGTTTACC-3′) and PET-A3wec-rev (5′-AGAGGAGAGTTAGAGCCATTCCTTTCTTCCCAAAGC-3′), and the PCR product was ligated into pET-30 Xa/LIC (Novagen) and electroporated into *E. coli* BL21(λDE3). The His$_6$-WecP protein was overexpressed and cell lysates were obtained as previously reported for other proteins (8, 22). The total membrane fraction was obtained by ultracentrifugation (200,000 × *g* for 30 min at 10°C), the His$_6$-WecP protein was solubilized and purified with a Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) as previously reported (1). When needed, the His$_6$-WecP protein was concentrated using a Centriplus 10-ml YM-30 centrifugal filter device (Amicon Bioseparations), and typical protein preparations contained yields of 0.07 to 0.08 g/ml as determined by the Bio-Rad Bradford assay.

LPS isolation and electrophoresis. Cells were grown in LB, washed with water, and dehydrated by sequential washing with methanol-chloroform (1:1) (3 volumes), ethanol, acetone (2 volumes), and diethyl ether. The LPS was extracted from dehydrated cells after evaporation of the last dissolvent at room temperature. The phenol-chloroform/light petroleum ether method (11) was used for strains producing rough LPS (without O antigen), while the phenol-water procedure (48) was used for the strains producing the O-antigen domain (smooth LPS). For screening purposes, LPS was obtained after proteinase K digestion of whole cells (15). LPS samples were separated by SDS-PAGE or *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)-SDS-PAGE and visualized by silver staining as previously described (42).

Preparation of oligosaccharides. The LPS preparations (20 mg) were hydrolyzed in 1% acetic acid (100°C for 120 min), and the precipitate was removed by centrifugation (8,000 × *g* for 30 min) and lyophilized to give lipid A. The supernatants were fractionated on a column (56 by 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer, pH 4.5, with monitoring using a differential refractometer to obtain the oligosaccharide fractions.

GC-MS analysis. Partially methylated alditol acetates and methyl glycoside acetates were analyzed on an Agilent Technologies 5973N mass spectrometry (MS) instrument equipped with a 6850A gas chromatograph (GC) and an RTX-5 capillary column (30-m by 0.25-mm inner diameter [i.d.]; flow rate, 1 ml/min; helium as the carrier gas; Restek). Acetylated methyl glycoside analysis was performed with the following temperature program: 150°C for 5 min, increase from 150°C to 250°C at 3°C/min, and 250°C for 10 min. For partially methylated alditol acetates, the temperature program was 90°C for 1 min, increase from 90°C to 140°C at 25°C/min, increase from 140°C to 200°C at 5°C/min, increase from 200°C to 280°C at 10°C/min, and 280°C for 10 min.

Isolation of cell envelope fractions. The cultures were grown on TSB at 30°C. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction (membrane fraction) was collected by centrifugation at 100,000 × *g* for 2 h.

WecP transferase assay. The reaction mixture used for the *in vitro* transferase assay contained approximately 50 μg of the total protein membrane fraction from *A. hydrophila* AH-3 or *A. hydrophila* AH-3ΔwecP(pBAD33-WecP$_{Ah}$) (providing the enzyme and the endogenous Und-P acceptor), 10 mM $MgCl_2$, and 131.4 pmol of UDP-[^{14}C]GalNAc (237 mCi/mmol) or UDP-[^{14}C]GlcNAc (230 mCi/mmol) (NEN Life Science), in 250 μl of 10 mM Tris-HCl (pH 8.5). The mixture was incubated for 30 min at 37°C, and the lipid fraction was extracted twice with the same volume of 1-butanol. The extracts were washed at least three times with the same volume of distilled water, and the amount of radioactivity incorporated into the lipid fraction was measured in a scintillation counter. Radioactive counts were normalized to the background value by using 50 μg of the total protein membrane fraction from the AH-3ΔwecP mutant (lacking the enzyme) instead of the wild-type membrane fraction. The assay was also performed using purified His$_6$-WecP (0 to 10 ng) and 50 μg of the total protein membrane fraction from *A. hydrophila* AH-3ΔwecP (providing the endogenous Und-P acceptor). One unit of enzyme activity was defined as 10^{-3} pmol of GalNAc/GlcNAc incorporated into the lipid fraction per min per mg of protein in the reaction.

RESULTS

***A. hydrophila* WecP characterization.** A BLAST-X analysis of *A. hydrophila* AH-3 WecP revealed similar proteins (identities around 45 to 50% and similarities around 60 to 70%) in a number of bacteria like *Leptospira* or *Oceanobacter* spp. besides the genus *Aeromonas* (>90% similarity). However, the well-characterized *Enterobacteriaceae* WecA protein showed low-level similarities with *A. hydrophila* WecP (identities below 30% and similarities below 50%) (data not shown).

Computer programs such as TMHHM 2.0 (prediction of transmembrane helices in proteins, from the Center for Biological Sequence Analysis, Denmark [http://www.cbs.dtu.dk/services/TMHMM/]) have been used to predict transmembrane helices and membrane topology models for proteins involved in the transfer of sugar-phosphate residues to undecaprenol phosphate (Und-P), such as WecA (25) and WbaP (36). The same computer program was used to model the *A. hydrophila* AH-3 WecP molecular topology, revealing three predicted domains that could be clearly delineated: these were an N-terminal region containing a cluster of four transmembrane helices, a large central periplasmic loop, and a C-terminal cytosolic tail (Fig. 2). The *A. hydrophila* AH-3 WecP molecular topology model resembles that of *Salmonella enterica* WbaP, which catalyzes the transfer of Gal-1-P from UDP-Gal onto Und-P (34). The WbaP membrane topology model (Fig. 2) also consists of an N-terminal region containing a cluster of four transmembrane helices, a large central periplasmic loop, and a C-terminal cytosolic tail (36). Previous work (45) has shown that the C-terminal half of WbaP carries the Gal-1-P transferase activity. In contrast, the topological model for *E. coli* WecA (Fig. 2), which is responsible for the transfer of GlcNAc-1-P from UDP-GlcNAc to polyprenol-P, consists of 11 transmembrane helices, five cytosolic loops, and five periplasmic loops (25).

Because of the differences between *Aeromonas* WecP and

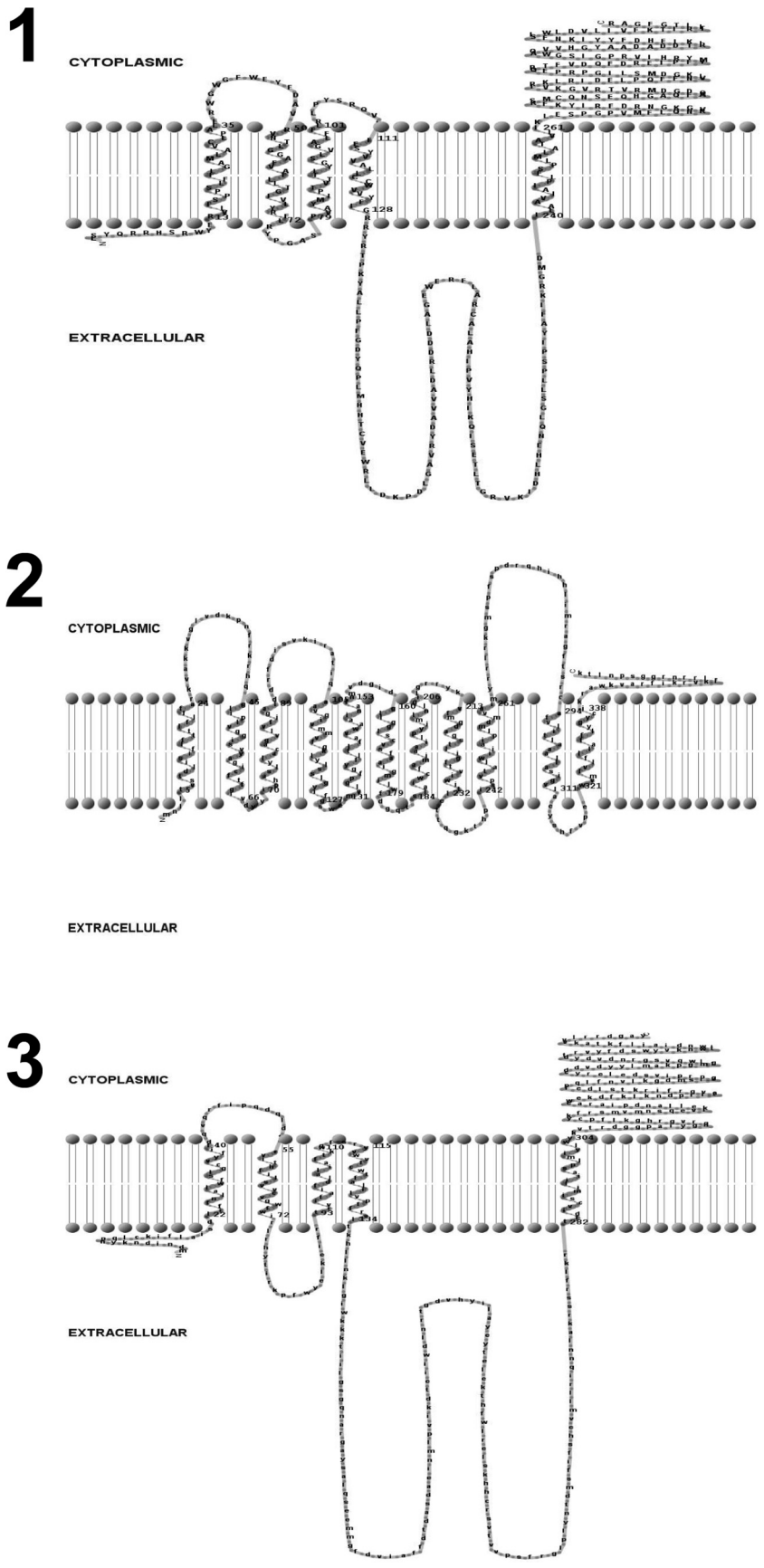

FIG. 2. Membrane topological models for *S. enterica* LT2 WbaP (panel 1), *E. coli* VW187 WecA (panel 2), and *A. hydrophila* AH-3 WecP (panel 3).

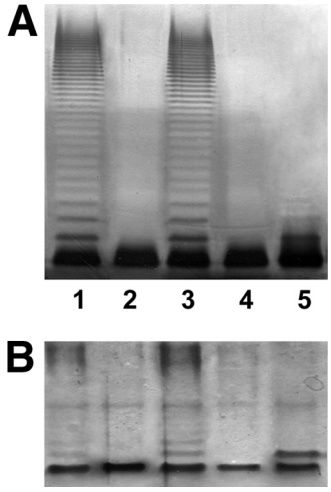

FIG. 3. Polyacrylamide gels showing the migration of LPS from AH-3ΔwecA and its complementation. The LPS samples were separated on SDS-PAGE (A) and SDS-Tricine-PAGE (B) and visualized by silver staining. Shown are LPS samples from *A. hydrophila* AH-3 (wild type [WT]) (lanes 1), the AH-3ΔwecP mutant (lanes 2), AH-3ΔwecP(pBAD33-$WecP_{Ah}$) (lanes 3), AH-3ΔwecP(pBAD33-$WbaP_{Se}$) (lanes 4), and AH-3ΔwecP(pBAD33-$WecA_{Ec}$) (lanes 5).

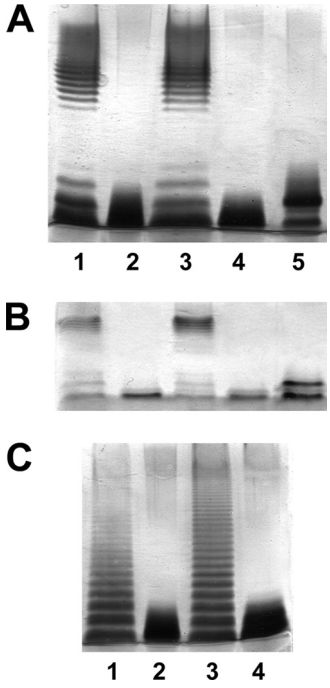

FIG. 4. Polyacrylamide gels showing the migration of LPS from *E. coli* WecA (MV501) and *S. enterica* WbaP (MSS2) mutants and its complementation. The LPS samples were separated on SDS-PAGE (A and C) and SDS-Tricine-PAGE (B) and visualized by silver staining. (A and B) Shown are LPS samples from *E. coli* VW187 (WT) (lanes 1), MV501 (lanes 2), MV501(pBAD33-$WecA_{Ec}$) (lanes 3), MV501(pBAD33-$WecP_{Ah}$) (lanes 4), and MV501(pBAD33-$WecP_{Ah}$)(pGEMT-Gne) (lanes 5). (C) Shown are LPS samples from *S. enterica* LT2 (WT) (lane 1), MSS2 (lane 2), MSS2(pBAD33-$WbaP_{Se}$) (lane 3), and MSS2(pBAD33-$WecP_{Ah}$)(pGEMT-Gne) (lane 4).

Enterobacteriaceae WecA, we suggest the name of WecP for this protein in *Aeromonas* (Wec for being a putative UDP-HexNAc:polyprenol-P HexNAc-1-P transferase and P for the similarity in the molecular topology to WbaP). Note that the names WecB, -C, and -D are already used (29). In order to identify the sugar donor for WecP, we decided to introduce well-known sugar–Und-P transferases into an *A. hydrophila wecP* mutant as well as this WecP protein into well-characterized *wecA* and *wbaP* mutants.

Complementation studies of the AH-3ΔwecP mutant. As we previously published, the AH-3ΔwecP mutant was completely unable to biosynthesize the O34-antigen LPS (20). The mutant harboring plasmid pBAD33-$WecP_{Ah}$ (carrying the gene from strain AH-3) showed an LPS profile identical to that of the wild-type strain, while no changes could be observed when the mutant carried the plasmid vector alone or plasmid pBAD33-$WbaP_{Se}$ carrying the *Salmonella enterica* LT2 *wbaP* (Fig. 3). When plasmid pBAD33-$WecA_{Ec}$ (carrying the *E. coli* VW187 [O7] *wecA*) was introduced in the mutant and expressed with arabinose, we could see two bands on LPS gels (the band corresponding to the mutant plus an additional band with reduced motility) (Fig. 3). Isolated LPS of the AH-3ΔwecP mutant with plasmid pBAD33-$WecA_{Ec}$ grown under expressing conditions (with arabinose) was devoid of high-molecular-mass O-antigen polysaccharide (O-antigen PS). Sugar analysis of LPS (see Materials and Methods) from the AH-3ΔwecP mutant renders the following monosaccharides: glucose (Glc), Gal, glucosamine (GlcN), L-*glycero*-D-*manno*-heptopyranose and D-*glycero*-D-*manno*-heptopyranose (L,D-Hep and D,D-Hep, respectively), and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), corresponding to the LPS core (Fig. 1). However, the sugar analysis of LPS from AH-3ΔwecP mutant with plasmid pBAD33-$WecA_{Ec}$ grown under expressing conditions (with arabinose) showed the same monosaccharides as described above plus 6-deoxy-talose (L-6dTal) and mannose (D-Man), in agreement with the reported O34-antigen LPS (Fig. 1) (20, 23), but with GlcNAc instead of *N*-acetyl-galactosamine (GalNAc), which is the other sugar from the O34-antigen LPS in the wild-type strain (Fig. 1). The amount of GlcNAc is similar to the amounts of D-Man and D-Glc and about half of the amount of L-6dTal. From these results, it seems that the additional band with reduced motility observed in the LPS gels for the AH-3ΔwecP mutant with plasmid pBAD33-$WecA_{Ec}$ grown under expressing conditions (with arabinose) corresponds to an LPS molecule with a single O-antigen repeating unit. No GlcNAc was detected in the LPS of AH-3ΔwecP mutant alone or with plasmid vector pBAD33.

The AH-3ΔwecP mutant was complemented by different pBAD33 plasmids with several *Aeromonas wecP* homologues from strains like *A. hydrophila* PPD134/91 (20), *A. caviae* Sch3N (12), *A. veronii* biovar Sobria AH-41 (34), and *A. salmonicida* subsp. *salmonicida* A449 (21), according to their LPS profiles (data not shown).

Complementation studies of *E. coli* MV501 (WecA) and *S. enterica* MSS2 (WbaP) mutants. Plasmid pBAD33-$WecP_{Ah}$ (carrying the gene from strain AH-3) was unable to change the *S. enterica* MSS2 or *E. coli* MV501 LPS profile (Fig. 4). One possible reason the LPS profile not to change could be the absence of UDP-GalNAc in *S. enterica* strain MSS2 or *E. coli* strain MV501. Generation of UDP-GalNAc from UDP-GlcNAc requires the presence of a Gne (UDP-GalNAc 4-epi-

merase) homologue. Thus, we introduced the *A. hydrophila gne*-containing plasmid pGEMT-Gne (7) into *S. enterica* strain MSS2 and *E. coli* strain MV501. No changes in LPS profile were observed in *S. enterica* strain MSS2 carrying *A. hydrophila* AH-3 *wecP* and *gne*. However, when both genes were introduced into the *E. coli* MV501 mutant, we could see an LPS profile showing two bands (one with the same motility as the mutant and the other one with a reduced motility) (Fig. 4A and B, lanes 5). LPS isolated from the *E. coli* MV501 mutant with plasmids pBAD33-$WecP_{Ah}$ and pGEMT-Gne together grown under expressing conditions (with arabinose) was sugar analyzed. Among the different sugars (Kdo, L,D-Hep, Glc, Gal, GlcNAc, Man, rhamnose [Rha], and QuiNAc), GalNAc was detected in ratios similar to Man (a unique sugar in the reported *E. coli* O7-antigen LPS structure [Fig. 1] [28, 47]). The presence of Man could not be detected in isolated LPS of *E. coli* MV501, LPS of *E. coli* MV501 carrying pGEMT-Gne alone, or LPS of *E. coli* MV501 carrying pBAD33-$WecP_{Ah}$ alone. GalNAc has not been described as being present in LPS of *E. coli* strains 32 with O7-antigen LPS (Fig. 1) (25, 47) or LPS core (4) and was not detected in the LPS of *E. coli* MV501 with or without plasmid vector pBAD33, pBAD33-$WecP_{Ah}$, or pGEMT-Gne. Because it is only a single additional band in the LPS profile of the *E. coli* MV501 mutant with plasmids pBAD33-$WecP_{Ah}$ and pGEMT-Gne together grown under expressing conditions (with arabinose) and the Man or GalNAc values for this LPS are always about three times less than that of Glc (a monosaccharide from the LPS core but not from the O7-antigen LPS), this additional band could account for LPS molecules with a single O-antigen repeating unit.

All these results suggest that *A. hydrophila* WecP is a UDP-HexNAc:polyprenol-P GalNAc-1-P transferase in a Wzy-dependent pathway of O-antigen assembly.

***K. pneumoniae* 52145 (O1:K2) WecA mutant and complementation.** WecA initiates O-unit synthesis in many *Enterobacteriaceae*, *Klebsiella pneumoniae* O1 being one of them (9). Also, this O1 antigen is an example of those using an ABC transporter-dependent pathway for assembly (38). By using the methodology described in Materials and Methods, we constructed an in-frame *wecA* mutant of strain 52145 (O1:K2) (30), which was devoid of the O1-antigen LPS banding pattern in gels (Fig. 5). The LPS of the 52145ΔwecA mutant harboring plasmid pBAD33-$WecA_{Kp}$ or pBAD33-$WecA_{Ec}$ showed in gels the same banding pattern exhibited by the wild-type LPS. In contrast, the LPS of the 52145ΔwecA mutant harboring the plasmid vector alone (data not shown), with plasmid pBAD33-$WecP_{Ah}$ from *A. hydrophila* AH-3, or with pBAD33-$WbaP_{Se}$ from *S. enterica* LT2 did not show changes in its LPS profile (Fig. 5). In *K. pneumoniae* O1:K2, no generation of UDP-GalNAc from UDP-GlcNAc is possible, due to the lack of the epimerase enzyme, like in *E. coli* VW187 (O7). Therefore, when we included plasmid pGEMT-Gne together with plasmid pBAD33-$WecP_{Ah}$ from *A. hydrophila* AH-3 in the 52145ΔwecA mutant grown under inducing conditions, the O1-antigen banding pattern was rescued, according to SDS-PAGE (Fig. 5). As has been previously described (34) for the LPSs of the strains using the ABC-transporter dependent pathway, like in *K. pneumoniae* O1, the initial sugar for the O-antigen LPS is retained in their structure. The initial sugar for the wild-type strain is GlcNAc, as reported for *K. pneumoniae* (43, 44). When we examined the sugar composition of the LPS from 52145ΔwecA mutant with plasmids pBAD33-$WecP_{Ah}$ and pGEMT-Gne together grown under inducing conditions, we observed a large amount of Gal (in agreement with the reported O1-antigen LPS structure [9]) and minor GalNAc content. No GlcNAc could be detected in this LPS sample. As previously reported for strain 52145, no GalNAc was present either in the LPS core or in the *K. pneumoniae* O1 antigen (9, 33, 43, 44). All these results prompted us to study the *in vitro* enzymatic activity of WecP.

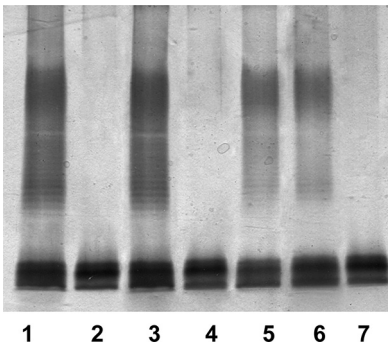

FIG. 5. Polyacrylamide gels showing the migration of LPS from the 52145ΔwecA mutant and its complementation. The LPS samples were separated on SDS-PAGE and visualized by silver staining. Shown are LPS samples from *K. pneumoniae* 52145 (lane 1), 52145ΔwecA (lane 2), 52145ΔwecA(pBAD33-$WecA_{Kp}$) (lane 3), 52145ΔwecA(pBAD33-$WecP_{Ah}$) (lane 4), 52145ΔwecA(pBAD33-$WecP_{Ah}$)(pGEMT-Gne) (lane 5), 52145ΔwecA(pBAD33-$WecA_{Ec}$) (lane 6), and 52145ΔwecA(pBAD33-$WbaP_{Se}$) (lane 7).

UDP-HexNAc:polyprenol-P HexNAc-1-P transferase assay. The assay was performed as described in Materials and Methods by using the total membrane fractions of *A. hydrophila* AH-3 and *A. hydrophila* AH-3ΔwecP(pBAD33-$WecP_{Ah}$) as the source of both enzyme and the endogenous Und-P acceptor. As controls, the membrane fractions of *A. hydrophila* AH-3ΔwecP and *A. hydrophila* AH-3ΔwecP (pBAD33) were used. The membrane fraction from *A. hydrophila* AH-3 catalyzed the incorporation of GalNAc-1-P from UDP-GalNAc with an specific activity similar to that obtained when using the membrane fraction of *A. hydrophila* AH-3ΔwecP(pBAD33-$WecP_{Ah}$) grown under noninducing conditions (Table 2). Membrane fractions obtained from AH-3ΔwecP(pBAD33-$WecP_{Ah}$) grown in the presence of L-arabinose (inducing conditions for P_{BAD}) resulted in about a 10-fold increase in specific activity (Table 2). None of the membrane preparations described above was able to catalyze the transfer of GlcNAc-1-P from UDP-GlcNAc at significant levels. As expected, experiments performed using a membrane preparation from *A. hydrophila* AH-3ΔwecP or from AH-3ΔwecA(pBAD33), both under inducing and noninducing conditions, did not catalyze the incorporation of GalNAc or GlcNAc.

To prove the role of WecP, we amplified the *wecP* gene from *A. hydrophila* AH-3 and cloned it into pET-30 Xa/LIC (see Materials and Methods). The construction was made to obtain a WecP protein with an extra amino-terminal sequence of 47 amino acid residues, with 6 histidine residues in positions 2 to 7. This His_6-WecP was overproduced in *E. coli* BL21(λD3) harboring pET-30-$WecP_{Ah}$ and purified from the membrane fraction as previously reported for WecA (1). The transferase

TABLE 2. *In vitro* WecP transferase assay using UDP-[^{14}C]GlcNAc or UDP-[^{14}C]GalNAc[d]

Membrane fraction or protein	Incorporation (U/mg/min) of[a]:	
	GlcNAc	GalNAc
A. hydrophila AH-3 (wild type)	<1	8 ± 1
AH-3ΔwecP mutant	ND	ND
AH-3ΔwecP mutant + pBAD33-WecP$_{Ah}$		
Noninduced[b]	<1	5 ± 2
Induced[c]	<1	76 ± 6
AH-3ΔwecP mutant + pBAD33		
Noninduced[b]	<1	<1
Induced[c]	<1	<1
His$_6$-WecP (ng)		
0	<1	<1
5	<1	150 ± 12
10	<1	224 ± 17

[a] Standard deviations shown were obtained from three independent experiments. ND, not detected.

[b] Cultures were grown in the absence of L-arabinose, not inducing the P_{BAD} promoter.

[c] Cultures were grown in the presence of L-arabinose, inducing the P_{BAD} promoter.

[d] Reaction mixtures used for the *in vitro* transferase assay contained approximately 50 μg of the total protein membrane fraction (providing the enzyme and the endogenous Und-P acceptor), 10 mM $MgCl_2$, and the corresponding radiolabeled UDP-HexNAc in 10 mM Tris-HCl (pH 8.5). In assays using purified His$_6$-WecP, 50 μg of the total protein membrane fraction from *A. hydrophila* AH-3ΔwecP was used as the source of Und-P. The mixture was incubated for 30 min at 37°C, and the lipid fraction was extracted twice with the same volume of 1-butanol. The extracts were washed at least three times with the same volume of distilled water, and the amount of radioactivity incorporated into the lipid fraction was measured in a scintillation counter. All the values are normalized to the background obtained using the lipid extract from the membrane fraction of AH-3ΔwecP mutant.

activity was determined as described above by using the total membrane fraction of *A. hydrophila* AH-3ΔwecP as the source of the endogenous Und-P acceptor and by using 1 to 10 ng of purified His$_6$-WecP. In control experiments without added His$_6$-WecP, neither GlcNAc-1-P nor GalNAc-1-P was incorporated from UDP-GlcNAc or UDP-GalNAc. In contrast, in the presence of His$_6$-WecP, GalNAc-1-P was incorporated from UDP-GalNAc but no GlcNAc-1-P was incorporated when using UDP-GlcNAc. The specific activity of GalNAc-1-P incorporation was dependent on the amount of His$_6$-WecP added to the reaction (Table 2). These results strongly suggest that the WecP protein is indeed a UDP-GalNAc:polyprenol-P GalNAc-1-P transferase.

DISCUSSION

The *A. hydrophila* AH-3 WecP represents a new class of UDP-HexNAc:polyprenol-P HexNAc-1-P transferases. These transferase-catalyzed reactions involve a membrane-associated polyprenol phosphate acceptor and a cytoplasmic UDP-D-*N*-acetylhexosamine sugar nucleotide as the donor substrate. Four subgroups of bacterial enzymes have been identified based on their specific substrate preference (31). However, until now, all the WecA enzymes tested were able to transfer UDP-GlcNAc to the Und-P. It has been indicated that WecA can also transfer *N*-acetylgalactosamine-1-phosphate (25), as it is also essential for the synthesis of O antigens containing *N*-acetylgalactosamine (46). However, reference 46 only suggested that in a study of the *E. coli* O157 *wb** cluster, WecA transfers GalNAc-phosphate to Und-P to initiate O-unit synthesis as well as initiating enterobacterial common-antigen synthesis by transfer of GlcNAc-phosphate. Assuming this suggestion, they subsequently identified the genes for all the required steps for synthesis of the O157 O-antigen (46). In no case is there any biochemical proof for the transfer of GalNAc. Furthermore, recently, a novel biosynthetic pathway for the production of GalNAc-P-P-Und by epimerization after the formation of GlcNAc-P-P-Und by WecA in *E. coli* O157 has been described (35).

In this study, we present *in vitro* and *in vivo* proofs that *A. hydrophila* AH-3 WecP transfers GalNAc to Und-P and is unable to transfer GlcNAc to the same enzyme substrate. The *in vitro* assays clearly indicated that [^{14}C]GalNAc but not [^{14}C]GlcNAc is incorporated in the lipid fraction of the protein membrane (endogenous Und-P acceptor) by *A. hydrophila* AH-3 WecP.

The LPS profiles in gels together with the sugar analysis of the mutants used in this study carrying well-defined sugar-Und-P transferases clearly indicate the *in vivo* transfer of GalNAc to the Und-P by WecP. In these studies, it is important to point out that in *A. hydrophila* AH-3 or *E. coli* O7, assembly of O antigen is through the Wzy-dependent pathway, while in the case of *K. pneumoniae* O1, it is through the ABC transporter-dependent pathway (32). In the first case for synthesis of O antigens, monomers are assembled on a lipid carrier (Und-P) by enzymes encoded in the *wb* gene cluster before their incorporation into the LPS molecule through the O-antigen polymerase and the flippase activity (Wzy and Wzx). In the second case, the complete O-antigen LPS is assembled on a lipid carrier (Und-P) before its incorporation into the LPS molecule through an ABC-2-type pathway (Wzm-Wzt). These differences could explain why we found a partial complementation in LPS gels in *A. hydrophila* AH-3ΔwecP carrying *E. coli* WecA or in *E. coli* MV501 (WecA mutant) carrying *A. hydrophila* WecP plus Gne, while the complementation was fully achieved, based on LPS gels, in *K. pneumoniae* 52145ΔwecA carrying *A. hydrophila* WecP plus Gne. The presence of GalNAc in the sugar analysis of LPS from *E. coli* or *K. pneumoniae* complemented strains, and the presence of GlcNAc in LPS from AH-3ΔwecP carrying *E. coli* O7 WecA instead of GalNAc, fully supported *in vivo* the transfer of GalNAc to the Und-P by WecP.

The WecP enzyme (UDP-GalNAc:polyprenol-P GalNAc-1-P transferase) differs from WecA (UDP-GlcNAc:polyprenol-P GlcNAc-1-P transferase) in membrane topology. The WecP protein membrane topology model resembles that of the WbaP enzyme (UDP-Gal:polyprenol-P Gal-1-P transferase), with the two enzymes sharing three predicted domains: an N-terminal region containing four transmembrane helices, a central periplasmic loop, and a C-terminal domain containing the last transmembrane helix and a large cytoplasmic tail which carries the sugar-phosphate transferase activity. In addition, the C-terminal putative catalytic domain of WecP showed a considerable degree of conservation with that of WbaP (Fig. 6A), in agreement with the fact that WbaP showed this conservation with a broad spectrum of bacterial species (36). The other two WbaP domains showed a low degree of conservation

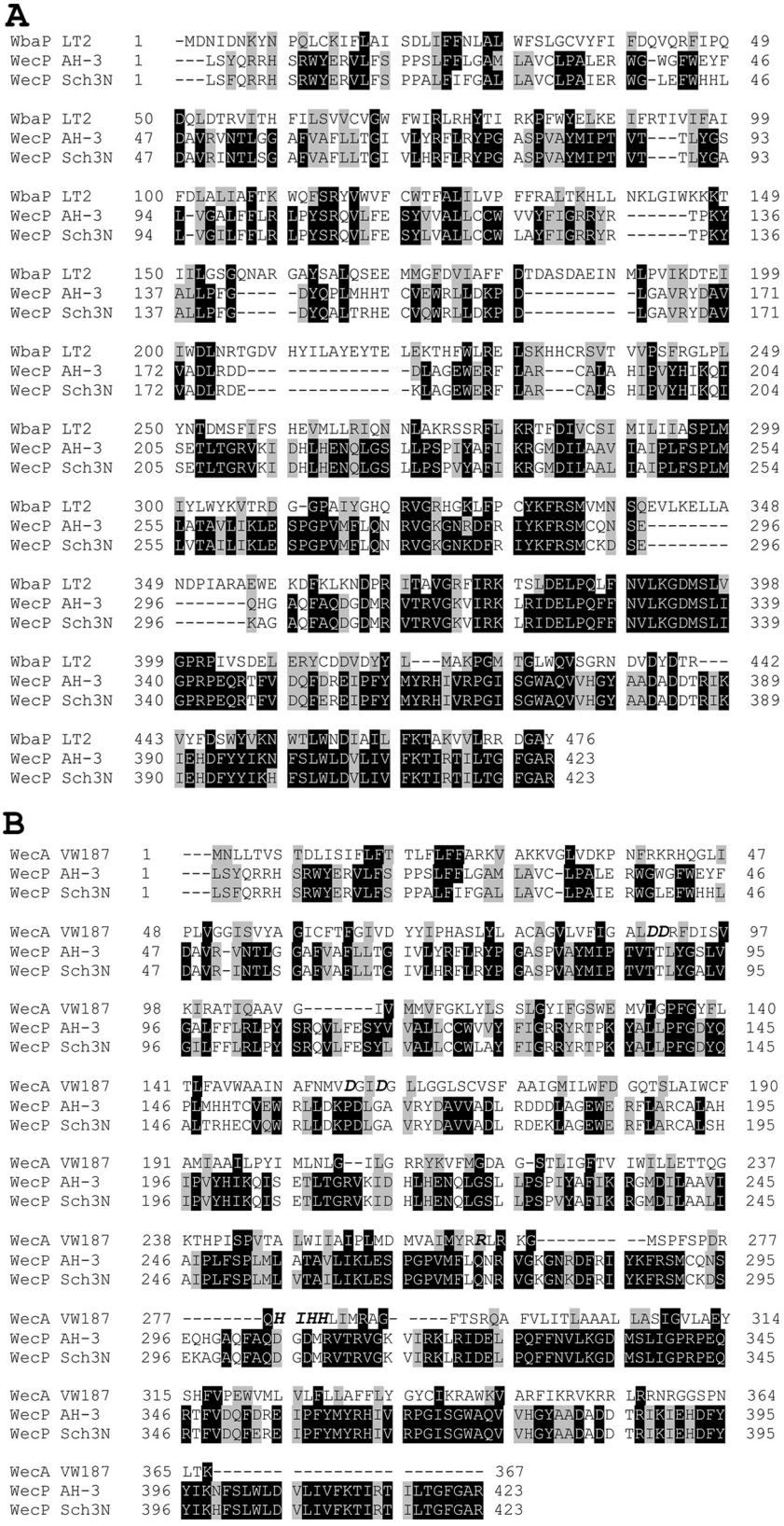

FIG. 6. Multiple-sequence alignments of *S. enterica* strain LT2 WbaP with *A. hydrophila* AH-3 WecP and *A. caviae* Sch3N (A) and *E. coli* strain VW187 WecA with the same *Aeromonas* WecP proteins mentioned above (B). Identical (black) and similar (gray) residue conservations are shown. Amino acids of functional importance in the transfer of GlcNAc-1-P to Und-P (26) are italic and labeled in boldface for *E. coli* strain VW187 WecA (B).

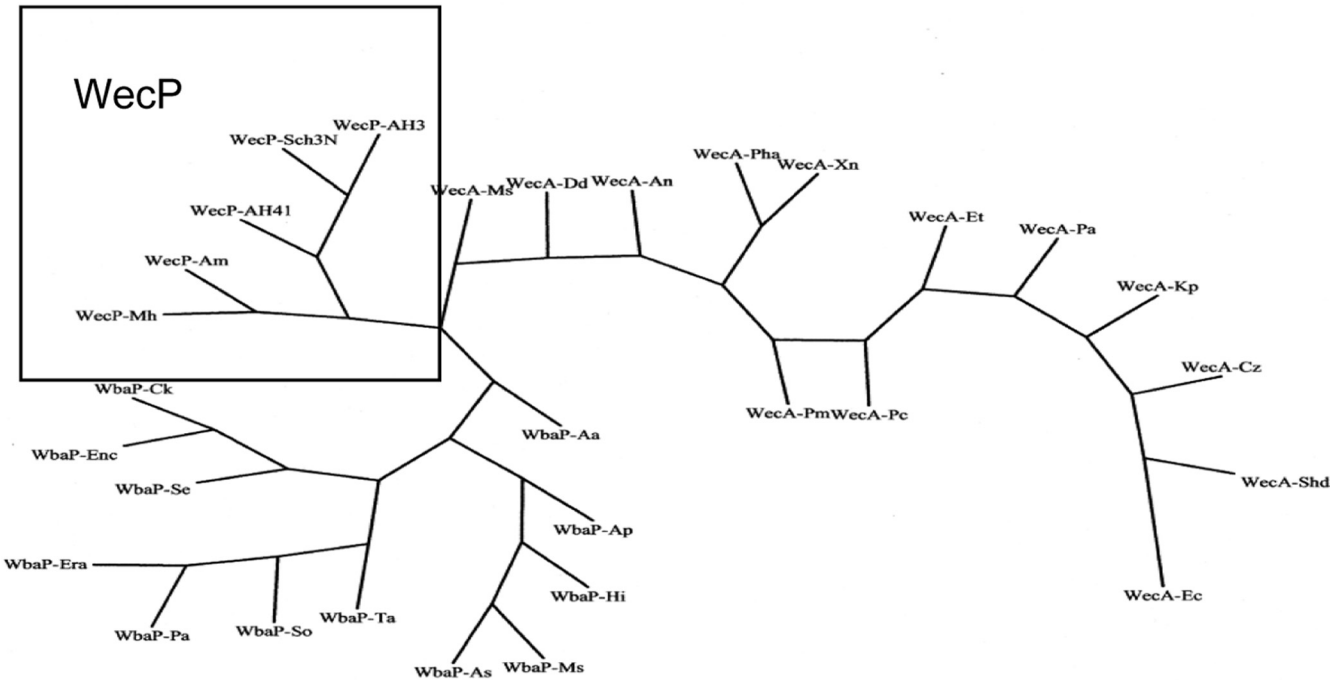

FIG. 7. Phylogenetic tree of selected WecP, WecA, and WbaP homologues. The tree was generated by using the Protpars program (parsimony method) in the PHYLIP package, using default parameters and bootstrap values. WecP proteins shown are from *A. hydrophila* strains AH-3 (WecP-AH3) (EU274663) and AH-41 (WecP-AH41), *A. caviae* Sch3N (WecP-Sch3N), *Actinobacillus minor* 202 (WecP-Am) (ZP_03611743), and *Mannheimia haemolytica* PHL213 (WecP-Mh) (ZP_04978354). WecA proteins shown are from *E. coli* VW187 (WecA-Ec), *K. pneumoniae* 52145 (WecA-Kp), *Shigella dysenteriae* Sd 197 (WecA-Shd) (YP_405383), *Cronobacter sakazakii* ATCC BAA-894 (WecA-Cz) (YP_001439802), *Pantoea annanatis* LMG 620103 (WecA-Pa) (YP_003518442), *Xenorhabdus nematophila* ATCC 19061 (WecA-Xn) (YP_003710698), *Photorhabdus asymbiotica* (WecA-Pha) (YP_003042971), *Arsenophonus nasoniae* (WecA-An) (CBA71837), *Proteus mirabilis* HI4320 (WecA-Pm) (YP_002153001), *Pectobacterium carotovorum* WP114 (WecA-Pc) (ZP_03829856), *Edwardsiella tarda* ATCC 23685 (WecA-Et) (ZP_06716201), *Dickeya dadantii* 3937 (WecA-Dd) (YP_003885016), and *Mannheimia succiniciproducens* MBEL55E (WecA-Ms) (YP_088103). WbaP proteins shown are from *S. enterica* serovar Typhimurium LT2 (WbaP-Se) (NP_461027), *Enterobacter cloacae* ATCC 13047 (WbaP-Enc) (YP_003613836), *Citrobacter koseri* ATCC BAA-895 (WbaP-Ck), *Erwinia amylovora* ATCC BAA-2158 (WbaP-Era) (CBX81132), *Pantoea annanatis* LMG 620103 (WbaP-Pa) (YP_003520801), *Serratia odorifera* 4Rx13 (WbaP-So) (ZP_06189355), *Tolumonas auensis* DSM 9187 (WbaP-Ta) (YP_002893229), *Actinobacillus succinogenes* 130Z (WbaP-As) (YP_001344127), *Mannheimia succiniciproducens* MBEL55E (WbaP-Ms) (YP_087854), *Haemophilus influenzae* RdKW20 (WbaP-Hi) (NP_439033), *Actinobacillus pleuropneumoniae* L20 (WbaP-Ap) (YP_0010541601), and *Aggregatibacter aphrophilus* NJ8700 (WbaP-Aa) (YP_002893229) (designations following the protein names represent GenBank accession numbers).

compared to WecP (Fig. 6B), in agreement with the reduced homology to a number of proteins previously described (36).

WecA is localized in discrete regions within the membrane, and five cytosolic loops have been determined using computer programs and some experimental data (25). Specific amino acid residues of functional importance in the transfer of GlcNAc-1-P to Und-P have been identified in cytosolic loops 2, 3, and 5 of WecA (25). Replacement of Asp90 and Asp91 in cytosolic loop 2 and Asp156 and Asp159 in cytosolic loop 3 with other amino acids affected the function of WecA, as demonstrated by the lack of *in vitro* enzymatic activity or reduced *in vitro* enzymatic activity of the mutated proteins, which could not mediate O-antigen LPS production *in vivo* (3). Alignment between WecP and WecA did not show Asp residues equivalent to Asp90, Asp91, Asp156, and Asp159 (Fig. 6B). The differences in substrate specificity and membrane topology between WecP and WecA suggest that their phylogenetic relationship is not very close. This was checked by running the phylogenetic inference software Protpars from the PHYLIP package (J. Felsenstein, PHYLIP [Phylogeny Inference Package] version 3.5c, Department of Genetics, University of Washington, Seattle, WA, 1993), using WecP, WecA, and WbaP homologues (Fig. 7). This approach suggests that each of these enzyme classes constitutes a different phylogenetic branch.

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