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Molecular Analysis of Three *Aeromonas hydrophila* AH-3 (Serotype O34) Lipopolysaccharide Core Biosynthesis Gene Clusters▽†

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By the isolation of three different *Aeromonas hydrophila* strain AH-3 (serotype O34) mutants with an altered lipopolysaccharide (LPS) migration in gels, three genomic regions encompassing LPS core biosynthesis genes were identified and characterized. When possible, mutants were constructed using each gene from the three regions, containing seven, four, and two genes (regions 1 to 3, respectively). The mutant LPS core structures were elucidated by using mass spectrometry, methylation analysis, and comparison with the full core structure of an O-antigen-lacking AH-3 mutant previously established by us. Combining the gene sequence and complementation test data with the structural data and phenotypic characterization of the mutant LPSs enabled a presumptive assignment of all LPS core biosynthesis gene functions in *A. hydrophila* AH-3. The three regions and the genes contained are in complete agreement with the recently sequenced genome of *A. hydrophila* ATCC 7966. The functions of the *A. hydrophila* genes *waaC* in region 3 and *waaF* in region 2 were completely established, allowing the genome annotations of the two heptosyl transferase products not previously assigned. Having the functions of all genes involved with the LPS core biosynthesis and most corresponding single-gene mutants now allows experimental work on the role of the LPS core in the virulence of *A. hydrophila*.

In gram-negative bacteria, the lipopolysaccharide (LPS) is one of the major structural and immunodominant molecules of the outer membrane. It consists of three domains: lipid A, core oligosaccharide, and O-specific polysaccharide or O antigen. The genetics of O-antigen biosynthesis has been intensively studied in the *Enterobacteriaceae* and some other gram-negative bacteria. In studies of several *Enterobacteriaceae*, like *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*, genes involved in LPS core biosynthesis are usually found clustered in a region of the chromosome, the *waa* gene cluster (13, 29). This gene arrangement is not always present in other gram-negative bacteria (28); e.g., in *Bordetella* species, the genes involved in the biosynthesis of the O antigen and core are present in the same gene cluster (2). On the other hand, a careful analysis of several fully sequenced genomes suggested that the genes for the LPS core biosynthesis may not be clustered and may be distributed between several regions, e.g., as in *Yersinia pestis* (24).

The overwhelming majority of the LPSs studied (15) contain at least one residue of 3-deoxy-D-*manno*-oct-2-ulosonic (ketodeoxyoctonic) acid (Kdo), which links the core to the lipid A moiety (KdoI). The second characteristic sugar of the core is L-*glycero*-D-*manno*-heptose (LD-Hep), although there are a few LPSs that contain D-*glycero*-D-*manno*-heptose (DD-Hep) or lack any heptose (8). In those containing LD-Hep, the presence of a Hep-α-(1→5)-Kdo disaccharide is a characteristic feature. In several gram-negative bacteria, like *Haemophilus* or *Vibrio* species, KdoI is phosphorylated at position 4 (11, 35), whereas in *S. enterica*, *E. coli*, and some others, KdoI is glycosylated at O-4 with a second Kdo residue (KdoII). Either KdoI (in *Acinetobacter* species) or KdoII (e.g., in *Burkholderia cepacia* and *Yersinia pestis* [15]) can be replaced with D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko). The biosynthesis pathway of the latter and regulation of its expression in the LPS remain unknown.

Mesophilic, motile *Aeromonas* species are opportunistic and primary pathogens of a variety of aquatic and terrestrial animals, including humans; the clinical manifestations range from gastroenteritis to soft-tissue infections, including septicemia and meningitis (4, 20). The varied clinical picture of *Aeromonas* infections and gastroenteric illness in particular suggests that complex pathogenic mechanisms are involved in this bacterium. Serotype O34 is most common among mesophilic *Aeromonas* spp. (19), accounting for 26.4% of all infections. Previous investigations have documented O34 strains as important causes of infections in humans (18, 19). Recently, we have established the structures of the O34 antigen (22) and the LPS core of *Aeromonas hydrophila* AH-901 (23), an O-antigen-lacking mutant derived from a typical wild-type strain, AH-3, belonging to serotype O34 (Fig. 1).

Now we report the identification and characterization of three genomic regions in *A. hydrophila* AH-3 (serotype O34) involved in the LPS core biosynthesis. Combining these data together with the structure elucidation of the LPS core in mutants in each gene from the three gene clusters enabled a

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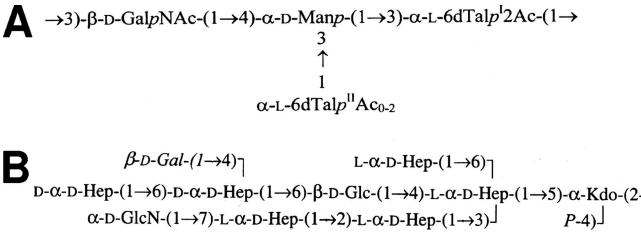

FIG. 1. Chemical structures of O34-antigen LPS (A) and the LPS core (B) of *A. hydrophila* strain AH-3 (22, 23). O34-antigen LPS is linked to the Gal residue (shown in italics) of the LPS core (23).

presumptive assignment of all LPS core biosynthesis gene functions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Aeromonas* strains were routinely grown in tryptic soy broth (TSB) or on tryptic soy agar at 30°C unless stated otherwise. *E. coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C. Kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), tetracycline (20 μg ml^{-1}), rifampin (100 μg ml^{-1}), or chloramphenicol (25 μg ml^{-1}) was added to the different media.

Mini-Tn*5*Km-1 mutagenesis. The conjugal transfer of transposition element mini-Tn*5*Km-1 from *E. coli* S17-1 λ*pir*Km-1 (7) to *A. hydrophila* AH-405 (AH-3 rifampin resistant) was carried out in a conjugal drop incubated for 6 h at 30°C in 1:5:1 ratios of S17-1 λ*pir*Km-1 to AH-405 to HB101/pRK2073 (helper plasmid), respectively. The serial dilutions of the mating mix were plated on tryptic soy agar supplemented with rifampin and kanamycin in order to select mutants.

General DNA methods. General DNA manipulations were done essentially as previously described (32). 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 (33) with the ABI Prism dye terminator cycle sequencing kit (Perkin Elmer). Oligonucleotides used for genomic DNA amplifications and DNA sequencing were purchased from Pharmacia LKB Biotechnology. 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 of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST (3, 5) network service at the National Center for Biotechnology Information and the European Biotechnology Information, respectively. ClustalW was used for multiple-sequence alignments.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
DH5α	F$^-$ *endA hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* φ80*lacZ*M15	12
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F$^-$ *proAB lacI*^{q}*Z*ΔM15 Tn*10*)	Stratagene
S17-1	*hsdR pro recA*, RP4-2 in chromosome Km::Tn*7* (Tc::Mu)	7
MC1061	*thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara-14 xyl-5 supE44* λ*pir*	31
A. hydrophila		
AH-3	O34; wild type	25
AH-405	AH-3; spontaneous Rif^r	25
AH-3005	AH-3 ORF6.1:mini-Tn*5*Km-1; Rif^r Km^r	This study
AH-3006	AH-3 ORF3.2:mini-Tn*5*Km-1; Rif^r Km^r	This study
AH-3007	AH-3 ORF1.3:mini-Tn*5*Km-1; Rif^r Km^r	This study
AH-3Δ2.1	AH-3 ORF2.1 mutant in frame with pDM4	This study
AH-3Δ3.1	AH-3 ORF3.1 mutant in frame with pDM4	This study
AH-3Δ4.1	AH-3 ORF4.1 mutant in frame with pDM4	This study
AH-3Δ5.1	AH-3 ORF5.1 mutant in frame with pDM4	This study
AH-3Δ2.2	AH-3 ORF2.2 mutant in frame with pDM4	This study
AH-3Δ7.1	AH-3 ORF7.1 insertion mutant with pFS100; Km^r	This study
AH-3Δ4.2	AH-3 ORF4.2 insertion mutant with pFS100; Km^r	This study
Plasmids		
pRK2073	Helper plasmid; Spc^r	7
pLA2917	Cosmid vector; Km^r Tc^r	27
COS-CORE2	pLA2917 cosmid with AH-3 core region 2; Tc^r	This study
COS-CORE3	pLA2917 cosmid with AH-3 core region 3; Tc^r	This study
pGEM-T	PCR cloning vector; Ap^r	Promega
pGEMT-ORF2.3	pGEM-T with AH-3 ORF2.3; Ap^r	This study
pGEMT-ORF2.3-1.2	pGEM-T with AH-3 ORF2.3 and ORF1.2; Ap^r	This study
pDM4	*pir* dependent with *sacAB* genes, oriR6K; Cm^r	26
pDM4Δ2.1	pDM4 with AH-3ΔORF2.1; Cm^r	This study
pDM4Δ3.1	pDM4 with AH-3ΔORF3.1; Cm^r	This study
pDM4Δ4.1	pDM4 with AH-3ΔORF4.1; Cm^r	This study
pDM4Δ5.1	pDM4 with AH-3ΔORF5.1; Cm^r	This study
pDM4Δ2.2	pDM4 with AH-3ΔORF2.2; Cm^r	This study
pFS100	pGP704 suicide plasmid, *pir* dependent; Km^r	31
pFS-7.1	pFS100 with an internal fragment of ORF7.1; Km^r	This study
pFS-4.2	pFS100 with an internal fragment of ORF4.2; Km^r	This study
pBAD33	Arabinose-inducible expression vector	ATCC
pABD-ORF	pBAD33 with the corresponding ORF; Cm^r	This study

[a] Rif, rifampin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Ap, ampicillin; Spc, spectinomycin.

Southern and dot blot hybridizations. Southern blotting was performed by capillary transfer (32). For dot blot hybridizations, the DNA was denatured by boiling for 5 min, chilled on ice for another 5 min, and spotted onto Hybond-N1 (Amersham) nylon membrane. Probe labeling, hybridization, and detection were carried out by using the enhanced chemiluminescence labeling and detection system (Amersham) according to the manufacturer's instructions.

Mutant construction. The chromosomal in-frame AH-3Δ2.1, AH-3Δ3.1, AH-3Δ4.1, AH-3Δ5.1, and AH-3Δ2.2 deletion mutants were constructed by allelic exchange as described by Milton et al. (26). The primers used to obtain the mutants are listed in Table SP1 in the supplemental material. Two asymmetric PCRs were carried out to obtain two DNA fragments (A-B and C-D) that were annealed at their overlapping regions and PCR amplified as a single DNA fragment using primers A and D. The amplified in-frame deletion gene products were purified, SalI or BglII digested, ligated into SalI- or BglII-digested and phosphatase-treated pDM4 vector, electroporated into *E. coli* MC1061 (λ*pir*), and plated on chloramphenicol LB agar plates at 30°C to obtain the pDM4 plasmids with the corresponding deleted genes. Each mutated gene was transferred to the chromosome by homologous recombination using the λ*pir*-dependent suicide plasmid pDM4, which contained the counterselectable marker *sacB*. Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the plasmids containing the engineered in-frame deletions into the *A. hydrophila* AH-405 rifampin-resistant strain. Transconjugants were selected on plates containing chloramphenicol and rifampin at 30°C. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. To complete the allelic exchange, the integrated suicide plasmid was forced to recombine out of the chromosome by growth on agar plates containing 15% sucrose. Mutants were selected based on their survival on plates containing 15% sucrose and the loss of the chloramphenicol-resistant marker of vector pDM4. The mutations were confirmed by sequencing the whole constructs in amplified PCR products.

Mutants AH-3Δ7.1 and AH-3Δ4.2 were obtained as defined insertion mutants by the integration of suicide plasmid pFS100 (31), carrying an internal fragment from the gene. Primers and restriction sites used are indicated in Table SP1 in the supplemental material. Mutants AH-3Δ6.1 (AH-3005), AH-3Δ3.2 (AH-3006), and AH-3Δ1.3 (AH-3007) were obtained by mini-Tn*5* insertion.

Plasmid constructions and mutant complementation studies. For complementation studies, the *A. hydrophila* AH-3 genes (ORF2.1, ORF3.1, ORF4.1, ORF5.1, ORF6.1, ORF7.1, ORF2.2, ORF3.2, ORF4.2, and ORF1.3) were PCR amplified by using specific primer pairs and ligated to plasmid pBAD33 (see the list of primers in Table SP2 in the supplemental material). The plasmid constructs were transformed into *E. coli* LMG194 by electroporation, plated on chloramphenicol LB agar plates, and incubated at 30°C. Plasmids with the amplified genes were independently transferred into the corresponding mutants by triparental mating by using the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing chloramphenicol and rifampin and confirmed by PCR. Each gene was expressed from the arabinose-inducible and glucose-repressible pBAD33 promoter. 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). The cultures were grown for 18 h at 30°C in TSB medium supplemented with chloramphenicol and 0.2% glucose, diluted 1:100 in fresh medium (without glucose), and grown until they reached an A_{600} of about 0.2. Then, L-arabinose was added, and the cultures were grown for another 2 h. Repressed controls were maintained in glucose-containing medium.

To obtain the pGEMT-ORF2.3-1.2 plasmid, the *A. hydrophila* AH-3 *kdkA* (ORF2.3) gene and its putative promoter site were amplified by PCR using chromosomal DNA with primers 2.3-F (5′-GTGACAACAATCCCCGATG-3′) and 2.3-R (5′-ATCAGCGCCAGATCAAACT-3′). The amplified DNA fragment (1,099 bp) was ligated into the vector pGEM-T (Promega) to obtain the pGEMT-ORF2.3 plasmid and transformed into *E. coli* XL1-Blue. Transformants were selected on LB plates containing ampicillin. The *A. hydrophila* AH-3 *waaA* (ORF1.2) gene was PCR amplified from chromosomal DNA with primers 1.2-F (5′-ACGCGTCGACCCGATCGTGCTGCAAGTG-3′) and 1.2-R (5′-ACGCGTCGACCACGACCTTCAGCGACTC-3′). The PCR product (1,519 bp) was digested with SalI (sites underlined above) and ligated to the SalI-digested and phosphatase-treated pGEMT-ORF2.3 to generate the pGEMT-ORF2.3-1.2 plasmid, which was transformed into *E. coli* XL1-Blue.

LPS isolation and SDS-PAGE. For LPS analysis, cultures were grown in TSB at 20 or 37°C. LPS was isolated by the method of Galanos et al. (10), resulting in a 2.3% yield. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (9). LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or SDS-Tricine-PAGE and visualized by silver staining as previously described (9, 14).

Large-scale isolation and mild-acid degradation of LPS. Dry bacterial cells of each mutant in 25 mM Tris-HCl buffer containing 2 mM $CaCl_2$, pH 7.63 (10 ml g^{-1}), were treated at 37°C with RNase, with DNase (24 h, 1 mg g^{-1} each), and then with proteinase K (36 h, 1 mg g^{-1}). The suspension was dialyzed and lyophilized, and the LPS was extracted by the phenol-water procedure (36).

A portion of the LPS (~50 mg) from each strain was heated with aqueous 2% acetic acid (6 ml) at 100°C for 45 min. The precipitate was removed by centrifugation (13,000 × *g*, 20 min) and the supernatant was 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 by using a differential refractometer (Knauer, Germany). An oligosaccharide fraction was obtained in a yield of 9 to 20%, depending on the strain.

Methylation analysis. A sample of each oligosaccharide (0.5 mg) was dissolved in 1 ml dimethyl sulfoxide. An excess of powdered NaOH was added, and the reaction glass was flushed with dry N_2 and sealed. After the mixture was stirred at 20°C for 1 h, 0.5 ml of cold CH_3I was added and the mixture was stirred at 20°C for 1 h. Then, water was added, the methylated product was extracted with $CHCl_3$, and the extract was washed with water and evaporated with a stream of dry nitrogen. The methylated oligosaccharide was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h), and acid was removed with a stream of nitrogen. The methylated monosaccharides were conventionally reduced with $NaBH_4$, acetylated with acetic oxide in pyridine, and analyzed by gas-liquid chromatography (GLC) on a Hewlett-Packard 5880 chromatograph and by GLC-mass spectrometry (MS) on a Hewlett-Packard HP 5989A instrument using an HP-5ms capillary column and a temperature gradient of 150°C (3 min) to 320°C with an increase of 5°C min^{-1}.

MS. Negative-ion electrospray ionization with Fourier transform ion cyclotron resonance MS was performed on an APEX II instrument (Bruker Daltonics) equipped with an actively shielded 7-T magnet and an Apollo ion source. Mass spectra were acquired by using standard experimental sequences as provided by the manufacturer. The mass scale was calibrated externally with rough LPS of known structure. Samples (~10 ng μl^{-1}) were dissolved in 50:50:0.001 (vol/vol/vol) mixtures of 2-propanol, water, and triethylamine with a pH of ~8.5 and sprayed at a flow rate of 2 μl min^{-1}. The capillary entrance voltage was set to 3.8 kV, and the drying gas temperature was set to 150°C. The capillary exit voltage was set to −100 V and in some cases was increased to −200 V to get a better signal intensity. The spectra showing several charge states for each component were charge deconvoluted using Bruker XMASS 6.0.0 software, and the mass numbers given refer to monoisotopic molecular masses.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the three *A. hydrophila* AH-3 chromosomal regions containing LPS core biosynthesis genes described here have been assigned the following GenBank accession numbers: EU296246, EU296247, and EU296248.

RESULTS

After the mutagenesis of a rifampin-resistant isolate (AH-405) of the *A. hydrophila* wild-type strain AH-3 (serotype O34) (25), mutants (Km^r) were screened for their inability to react with specific serum against O34-antigen LPS (7). Mutants AH-3005, AH-3006, and AH-3007 were among 3,000 mutants that were initially screened (1.7% serum-resistant mutants) and showed a lack of O34 antigen and a faster mobility of R-type LPS (lipid A and core) in gel (Fig. 2). The AH-3007 LPS moved faster than that of AH-3006, which moved faster than the AH-3005 LPS, thus indicating that genes implicated in the biosynthesis of the AH-3 LPS core could be affected by the transposon. Southern blot analysis using a specific probe for the transposon demonstrated that each mutant had a single copy of a minitransposon in the genome (data not shown). After the cloning of the minitransposon containing a fragment from flanking DNA from the genome of each mutant (EcoRV digestion) in pBCSK (Stratagene), sequence analysis was performed using the oligonucleotides from the mini-Tn*5*Km-1 flanking regions, 5′-AGATCTGATCAAGAGACAG-3′ and 5′-ACTTGTGTATAAGAGTCAG-3′, and the universal oligonucleotides from the plasmid vector, T3 and T7. Three different ORFs (one for each mutant) encoding proteins, all

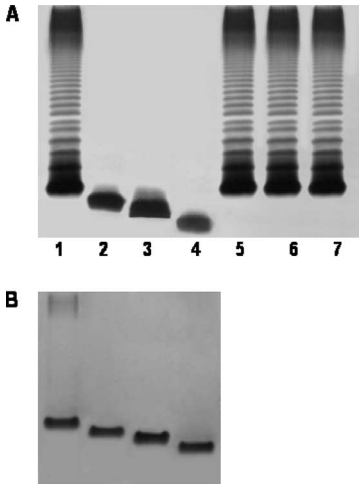

FIG. 2. (A) LPS samples were extracted and analyzed by SDS-PAGE (12%) as described by Darveau and Hancock (9). Results for SDS-PAGE-extracted LPSs from *A. hydrophila* AH-3 (wild type; lane 1), mutants AH-3005, AH-3006, and AH-3007 (lanes 2, 3, and 4, respectively), and mutants complemented with plasmids pBAD-ORF6.1, pBAD-ORF3.2, and pBAD-ORF1.3 (lanes 5, 6, and 7, respectively) are shown. (B) SDS-Tricine-PAGE gels from analysis of the LPS cores of mesophilic *Aeromonas* strains (lanes 1 to 4, same as for panel A). The strains with pBAD33 plasmids were grown under induced conditions.

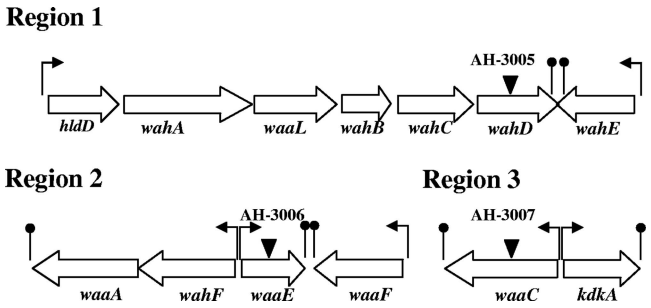

FIG. 3. Genetic organization of three *A. hydrophila* AH-3 chromosomal regions containing genes for LPS core biosynthesis. Regions 2 and 3 are from COS-CORE2 and COS-CORE3 plasmids, respectively. Transcription direction and stops (lollipops) are indicated. The locations of mini-Tn*5* insertions (for AH-3005, AH-3006, and AH-3007) are shown.

similar to heptosyl transferases from different bacteria, were identified. These sequence data were used to synthesize three internal DNA probes from the *A. hydrophila* AH-3 genome (named 1, 2, and 3 for the heptosyl transferases of AH-3005, AH-3006, and AH-3007, respectively).

Cloning and sequencing of the three *A. hydrophila* AH-3 genomic regions comprising the *waa* genes in *E. coli* K-12 strains. *A. hydrophila* AH-3 is a typical strain of serotype O34 (25). A cosmid-based genomic library of *A. hydrophila* AH-3 was constructed and introduced into *E. coli* DH5α (27). Tetracycline-resistant clones were screened by colony blotting using the DNA probes previously obtained from the regions close to the mini-Tn*5* location in the different mutant strains. An independent recombinant positive clone was found for probes 2 and 3, named COS-CORE2 and COS-CORE3, respectively. No recombinant positive clone was obtained for probe 1 after extensive analysis of either more than 5,000 clones or a clone able to hybridize with more than one probe. With the genome DNA region of probe 1, the nucleotide sequence was spanned, either downstream or upstream of the region, by genome-walking PCR (37). The analysis of sequenced region 1 showed seven genes involved in the LPS core biosynthesis (Fig. 3), and the corresponding analysis of the proteins encoded by these ORFs with their homologies is presented in Table 2.

The nucleotide sequences of the DNA inserts of plasmids COS-CORE2 and COS-CORE3 were determined in order to identify the *A. hydrophila* AH-3 genes conferring the LPS core production. The complete nucleotide sequence was determined in both directions by using oligonucleotides complementary to cosmid pLA2917 sequences flanking the COS-CORE2 and COS-CORE3 DNA inserts. Other sequence-derived oligonucleotides were purchased (Amersham-Pharmacia Biotech) and used to complete the nucleotide sequence. The analysis of the sequenced regions showed genes involved in LPS core biosynthesis in both regions (named 2 and 3 for COS-CORE2 and COS-CORE3, respectively), as indicated in Fig. 3. The corresponding analysis of the proteins encoded by these ORFs in both regions with their homologies is presented in Table 2. All the homologies in Table 2 included only proteins with confirmed functions.

Mutant isolation, phenotypic characterization, and complementation. Mutants were named according to the ORF number and the region number; for instance, AH-3Δ2.1 indicates a mutant with a mutation in ORF2 of region 1. As judged by using SDS-PAGE, mutants AH-3Δ3.1, AH-3Δ4.1, AH-3Δ6.1, AH-3Δ2.2, AH-3Δ3.2, AH-3Δ4.2, and AH-3Δ1.3 completely lacked O34-antigen LPS, and R-type LPS from all mutants but AH-3Δ3.1 (a putative *waaL* mutant) migrated faster to different degrees than the R-type LPS of the wild-type strain (Fig. 4A). Mutants AH-3Δ2.1, AH-3Δ5.1, and AH-3Δ7.1 showed different amounts of O34-antigen LPS in gels, but some changes in the migration of the R-type LPS compared to that of the wild-type LPS were observed (Fig. 4B and C). No AH-3Δ1.2 or AH-3Δ2.3 mutants were obtained for two genes similar to those obtained for Kdo transferase (*waaA*) and Kdo kinase (*kdkA*).

Complementation with the corresponding wild-type gene expressed in the vector plasmid pBAD33 restored the complete LPS in all mutants, as revealed by SDS-PAGE. No such complementation was achieved with the plasmid vector alone. As an example, Fig. 5A shows the complementation of AH-3Δ3.1 and AH-3Δ2.1 mutants with plasmids pBAD-ORF3.1 and pBAD-ORF2.1, respectively. In addition, plasmid pBAD-ORF3.2 was fully able to complement the *Klebsiella pneumoniae* 52145Δ*waaE* mutant (16), and plasmid pBAD-ORF4.2 was the only one carrying different putative heptosyltransferase genes able to complement the *K. pneumoniae* 52145Δ*waaF* mutant (17) (Fig. 5B). None of the pBAD plasmid vectors carrying different putative heptosyltransferases was able to complement *K. pneumoniae* 52145Δ*waaC* or *E. coli waaC* mutants (17). A similar result was obtained for the *K. pneumoniae* mutant 52145Δ*waaQ*.

E. coli strain CJB26 harbors a kanamycin determinant inserted in the *waaA* gene and its wild-type *waaA* gene in a temperature-sensitive plasmid (pJSC2), leading to a growth-temperature-sensitive phenotype (6). Plasmid pGEMT-1.2

TABLE 2. Characteristics of the three *A. hydrophila* AH-3 regions containing genes for LPS core biosynthesis

Region	ORF		Nucleotide position	Protein size (no. of amino acids)	Homologous protein (species)	Accession no.	% Identity/ % similarity
	No.	Protein encoded					
1	1	HldD	591–1550	319	ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (*Klebsiella pneumoniae*)	Q9XCA1	71/83
	2	WahA	1601–3358	586	Glycosyltransferase (WavL) (*Vibrio cholerae*)	AAL77362	64/77
	3	WaaL	3368–4492	374	Lipid-A-core, O-antigen ligase from different bacteria	ZP_00202015	23/45
	4	WahB	4577–5242	221	Glycosyltransferase (*Actinobacillus pleuropneumoniae*)	AAD30156	50/68
	5	WahC	5328–6356	342	ADP-heptose-LPS heptosyltransferase, either I, II, or III from different bacteria	YP_109261	28/42
	6	WahD	6411–7520	370	ADP-heptose-LPS heptosyltransferase (*Actinobacillus pleuropneumoniae*)	AAD30157	26/48
	7	WahE	8526–7462	355	ADP-heptose-LPS heptosyltransferase (*Fusobacterium nucleatum*)	ZP_00145125	27/54
2	1	WaaA	1585–320	421	3-deoxy-D-*manno*-octulosonic acid transferase (*Vibrio cholerae* and other species)	AAF93409	53/71
	2	WahF	2741–1587	384	ADP-heptose-LPS heptosyltransferase II (*Vibrio parahaemolyticus*)	NP_796591	57/73
	3	WaaE	2944–3732	262	α-L-*glycero*-D-*manno*-heptose β-1,4-glucosyltransferase (*Vibrio fischeri*)	YP_203517	56/70
	4	WaaF	4837–3794	347	Probable ADP-heptose-LPS heptosyltransferase II (*Vibrio cholerae* and other species)	AAF93399	59/73
3	1	WaaC	1476–421	351	ADP-heptose-LPS heptosyltransferase (*Vibrio cholerae* and other species)	ZP_00757651	58/73
	2	KdkA	1661–2374	273	3-deoxy-D-*manno*-octulosonic acid kinase (*Vibrio alginolyticus* and other species)	ZP_01261295	44/65

(carrying ORF1.2 alone) was unable to complement *E. coli* CJB26 (it did not change the growth-temperature-sensitive phenotype), whereas plasmid pGEMT-ORF2.3-1.2 (carrying ORF1.2 and ORF2.3 together) was fully able to abolish the growth-temperature-sensitive phenotype. This experiment strongly suggests that ORF1.2 and ORF2.3 encode the Kdo transferase WaaA and the Kdo kinase KdkA of the *A. hydrophila* LPS inner-core biosynthesis pathway.

Structure elucidation of the mutant LPSs. The LPSs were isolated by the phenol-water extraction of the enzymatically digested cells of each mutant. The LPS samples were degraded with a mild acid and the released core oligosaccharides were isolated by using gel permeation chromatography on Sephadex G-50. They were studied by high-resolution electrospray ionization MS and, when necessary, methylation analysis. The latter method enabled not only the determination of the linkage positions in most sugar residues but also differentiation between DD-Hep and LD-Hep residues, whose derivatives have different retention times in GLC.

AH-3Δ3.1 and AH-3Δ4.1. The oligosaccharide sample from the first mutant was found to be essentially identical to that from the R-type LPS of *A. hydrophila* AH-901 (20). The major molecular ion peak at *m*/*z* 1,857.63 in its mass spectrum (see Figure SA in the supplemental material) corresponded to the full core (calculated molecular mass, 1,857.61 atomic mass units), whose structure is shown in Fig. 6. The mutant AH-3Δ4.1 showed a structure identical (Fig. 6) to that of the AH-3Δ3.1 mutant but lacked the Gal residue (major molecular ion peak at *m*/*z* 1,695.57).

AH-3Δ7.1 and AH-3Δ5.1. The mass spectrum of the core from AH-3Δ7.1 (see Figure SA in the supplemental material) was similar to that of AH-3Δ3.1, but the masses were lower by 192 atomic mass units, which corresponded to the lack of a heptose residue. Methylation analysis showed that the lost residue was the terminal LD-HepIV, whereas the terminal DD-HepII was retained. In contrast, in AH-3Δ5.1, the terminal LD-HepIV was conserved and the terminal DD-HepII was lost. Based on the mass spectrum (see Figure SA in the supplemen-

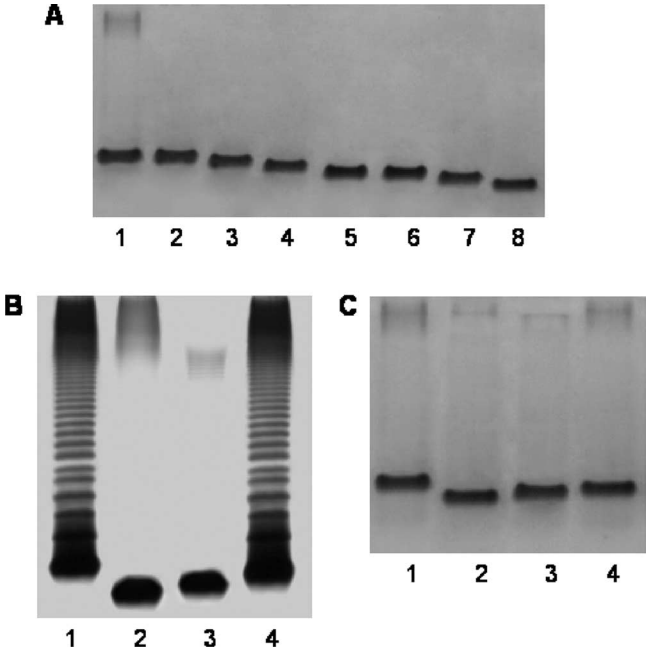

FIG. 4. (A) SDS-Tricine-PAGE gels for the wild type, rifampin-resistant strain AH-405 (lane 1), and the LPS core mutants lacking O34-antigen LPS, namely, AH-3Δ3.1, AH-3Δ4.1, AH-3Δ6.1, AH-3Δ2.2, AH-3Δ3.2, AH-3Δ4.2, and AH-3Δ1.3 (lanes 2 to 8, respectively). SDS-PAGE (B) and SDS-Tricine-PAGE (C) gels of the wild type, rifampin-resistant strain AH-405 (lane 1), and the LPS core mutants showing different amounts of O34-antigen LPS, namely, AH-3Δ2.1, AH-3Δ5.1, and AH-3Δ7.1 (lanes 2 to 4, respectively).

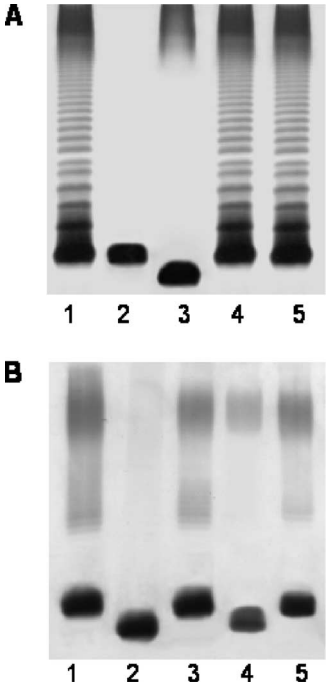

FIG. 5. SDS-PAGE gels of LPSs from strains of *A. hydrophila* (A) and *Klebsiella pneumoniae* (B). (A) Lanes: 1, AH-405 (wild type); 2, AH-3Δ3.1 mutant; 3, AH-3Δ2.1 mutant; 4, AH-3Δ3.1 complemented with the pBAD-ORF3.1 plasmid; and 5, AH-3Δ2.1 complemented with the pBAD-ORF2.1 plasmid. (B) Lanes: 1, 52145 (O1:K2, wild type); 2, 52145Δ*waaF* mutant (13); 3, 52145Δ*waaF* complemented with the pBAD-ORF4.2 plasmid; 4, 52145Δ*waaE* mutant (12); and 5, 52145Δ*waaE* complemented with the pBAD-ORF3.2 plasmid. The strains with pBAD33 plasmids were grown under induced conditions.

tal material), the major core glycoform in AH-3Δ5.1 was also devoid of galactose (molecular ion peak at *m*/*z* 1,503.53). Therefore, the core oligosaccharides from these two mutants have the structures shown in Fig. 6.

AH-3Δ2.1. The core from AH-3Δ2.1 was found to lack GlcN. The major glycoforms were also devoid of Gal and either one or three Hep residues (the latter is shown as a major glycoform in Fig. 6), as determined from the appearance in the mass spectrum of intense molecular ion peaks at *m*/*z* 1,342.43 (see Fig. SA in the supplemental material) and 958.30 (not shown), respectively.

AH-3Δ6.1. For the AH-3Δ6.1 mutant, the mass spectrum showed the lack of the outer-core trisaccharide fragment consisting of Gal, DD-HepII, and DD-HepI (see Fig. SA in the supplemental material). Methylation analysis confirmed the conservation of the terminal LD-HepIV, the absence of Gal and any DD-Hep, and the appearance of the terminal Glc.

AH-3Δ2.2 and AH-3Δ3.2. The cores from the AH-3Δ2.2 and AH-3Δ3.2 mutants showed similar mass spectra (see Fig. SA in the supplemental material). Both contained only one molecular ion peak at *m*/*z* 796.24 or 796.25 for an oligosaccharide consisting of one Kdo and three heptose residues. Methylation analysis of the former showed the presence of only terminal LD-Hep, whereas both terminal and 2-substituted LD-Hep residues were revealed in the latter. Based on these data, the branched and linear structures were assigned to the oligosaccharides from AH-3Δ2.2 and AH-3Δ3.2, respectively (Fig. 6).

AH-3Δ4.2. For AH-3Δ4.2, a further-truncated core containing one Kdo and two heptose residues was identified by the presence in the mass spectrum of an intense molecular ion peak at *m*/*z* 604.19 (see Fig. SA in the supplemental material).

AH-3Δ1.3. As the LPS from the AH-3Δ1.3 mutant gave no oligosaccharide upon mild-acid degradation, it was studied as a whole. The mass spectrum (see Fig. SB in the supplemental material) showed that the core region is restricted to a Kdo phosphate.

DISCUSSION

The isolation by transposon mutagenesis of three different mutants from *A. hydrophila* AH-3 devoid of O34-antigen LPS and altered R-type LPS migration in gels allowed the characterization of three different genomic regions with LPS core biosynthesis genes. Region 1 contained seven genes flanked by genes encoding a putative regulator, TetR, and a hypothetical protein, region 2 contained four genes flanked by genes encoding an integral membrane protein of unknown function and CoaD, and region 3 contained two genes flanked by genes encoding a guanylate kinase and a protein with a GGDEF domain. The three regions and the genes contained are in complete agreement with those recently sequenced for *A. hydrophila* ATCC 7966 (34), in which region 1 (AHA4232 to AHA4226), region 2 (AHA0170 to AHA0167), and region 3 (AHA0042 to AHA0043) are flanked by the same putative

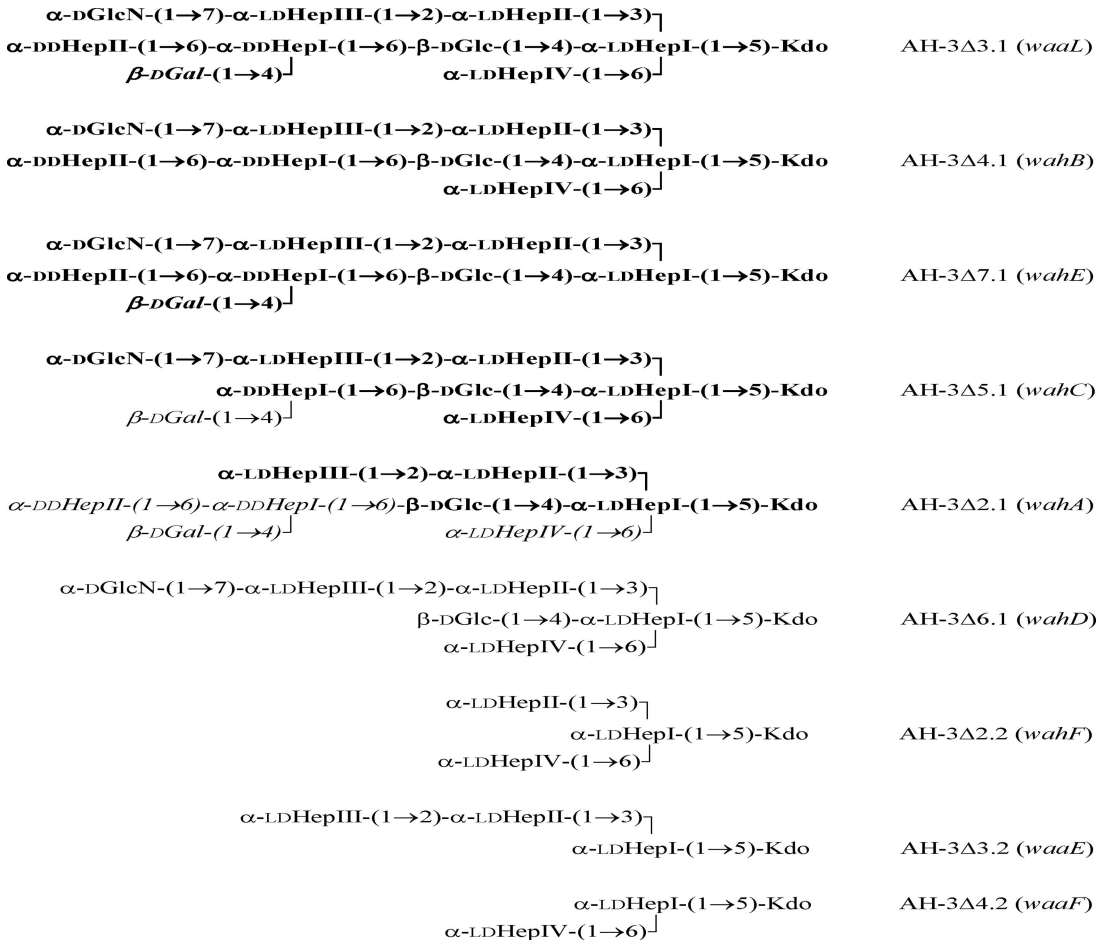

FIG. 6. Proposed structures of the core oligosaccharides released by mild-acid hydrolysis from the LPSs of the *A. hydrophila* mutants. When several oligosaccharides were obtained, components present in a nonstoichiometric quantity are shown in italics and the major glycoform is shown in bold. The assigned names of the mutated genes in this work are shown in parentheses.

genes. While the chemical structure of the *A. hydrophila* AH-3 LPS is already known (22, 23), this is not the case for *A. hydrophila* ATCC 7966. The presumptive assignment of all genes involved in the LPS core biosynthesis of *A. hydrophila* AH-3, as well as the distribution of inner- and outer-core biosynthesis genes between regions 1, 2, and 3, is shown in Fig. 7.

Region 3. Two genes of region 3, ORF1 and ORF2, encode L-D-HepI transferase WaaC and Kdo kinase KdkA, respectively. The function of the *waaC* gene was confirmed by the mass spectrum of the whole LPS of the mutant AH-3Δ1.3. The *A. hydrophila* AH-3 LPS consists of a phosphorylated Kdo residue (most likely, Kdo 4-phosphate) as in *Vibrio* spp. (23, 35), which is in agreement with the similarity between *A. hydrophila* WaaC and a heptosyl transferase of *Vibrio* (not determined to be WaaC). This also accounts for the failure of *A. hydrophila* WaaC complementation in *Enterobacteriaceae*, which possess a nonphosphorylated Kdo in the core. Furthermore, the failure of isolation of a *kdkA* mutant could be due to the lethality of a mutation in this gene encoding Kdo kinase.

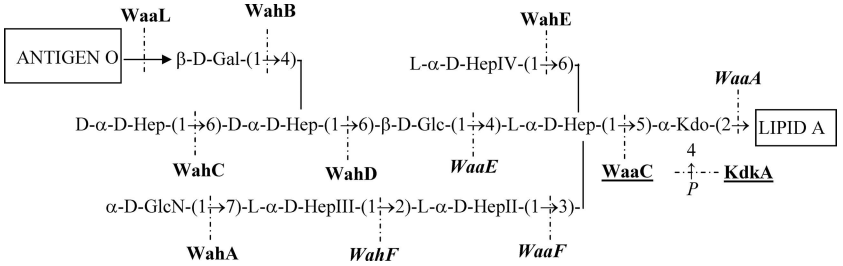

FIG. 7. Complete presumable assignment of the genes involved in the LPS core biosynthesis of *A. hydrophila* AH-3. Proteins encoded by genes from different regions are shown in roman (region 1), italic (region 2), or underlined (region 3) type.

Region 2. Region 2 includes four genes, ORF1 to ORF4, which encode Kdo transferase WaaA, a new heptosyl transferase that we named WahF, glucosyl transferase WaaE, and LD-HepII transferase WaaF, respectively. Mutation in the *A. hydrophila* gene *waaA* seems to be lethal, as it has been reported for the majority of gram-negative bacteria (28). In *E. coli*, Kdo is not phosphorylated. *A. hydrophila* WaaA is able to complement *E. coli* strain CJB26 (a temperature-sensitive *waaA* mutant) only when coexpressed with the *A. hydrophila* Kdo kinase KdkA, thus confirming the functions of both proteins. WaaE is L-*glycero*-D-*manno*-heptose β-1,4-D-glucosyltransferase not only by homology but also by its ability to complement the *K. pneumoniae* 52145Δ*waaE* mutant. The core oligosaccharide in the *waaE* mutant AH-3Δ3.2 represents a remaining linear fragment of the core consisting of Kdo and three LD-Hep residues (Fig. 6). This result suggests that the transfer of the Glc residue precedes the incorporations of the outer core, GlcN, and LD-HepIV residues. ORF4 encodes LD-HepII transferase WaaF, as this is the only heptosyltransferase able to fully complement the *K. pneumoniae* 52145Δ*waaF* mutant. Its function was confirmed by the mass spectrum of the core oligosaccharide from the *waaF* mutant AH-3Δ4.2, which showed a further-truncated core containing only Kdo and two LD-Hep residues (Fig. 6). Hence, the incorporation of LD-HepII precedes that of Glc. WahF was assigned as L-*glycero*-D-*manno*-heptose α-1,2-L-*glycero*-D-*manno*-heptosyltransferase (LD-HepIII transferase) since the corresponding mutant, AH-3Δ2.2, has a truncated core lacking LD-HepIII but containing the three other LD-Hep residues, as shown in Fig. 6. Accordingly, WahF was unable to complement the *K. pneumoniae* 52145ΔwaaF mutant, and an *A. hydrophila* AH-3 *waaE wahF* double mutant has a trisaccharide LPS core consisting of Kdo, LD-HepI, and LD-HepII (data not shown).

Region 1. From seven genes present in region 1, ORF1 was identified as ADP-D-*glycero*-β-D-*manno*-heptose-6-epimerase due to the fact that an extremely high homology was observed. This enzyme converts the DD-Hep derivative into the corresponding LD-Hep derivative (21). ORF2 was named WahA and assigned as bifunctional L-*glycero*-D-*manno*-heptose α-1,7-*N*-acetylglucosaminyltransferase/*N*-deacetylase. The WahA protein showed two domains: a glycosyltransferase group 1 domain (amino acid residues 156 to 303) and a polysaccharide deacetylase domain (amino acid residues 404 to 543). The core from the *wahA* mutant AH-3Δ2.1 lacked GlcN but an oligosaccharide with all other core components present was minor, whereas the major oligosaccharides were also devoid of a part of the outer core or the whole outer core and one of the LD-Hep residues. The AH-3Δ2.1 mutant LPS still showed some O34 antigen in gel, but it was a smaller amount than the O34 antigen of the wild-type strain.

ORF3 was assigned as WaaL since the *waaL* mutant AH-3Δ3.1 produced an LPS with the full core but was completely devoid of the O34 antigen. As in the wild-type strain, the terminal Gal was present in a nonstoichiometric amount. WaaL showed 11 transmembrane helices between amino acid residues 15 and 31, 40 and 56, 69 and 85, 90 and 106, 113 and 132, 149 and 165, 178 and 194, 199 and 215, 222 and 238, 331 and 347, and 354 and 370, as is characteristic for lipid-A-core, O-antigen ligases (1). The ORF4 product was named WahB and designated D-*glycero*-D-*manno*-heptose β-1,4-galactosyltransferase based on the fact that the LPS of the *wahB* mutant AH-3Δ4.1 lacks only galactose from the core and the O34 antigen. The ORF5 product, named WahC, was designated D-*glycero*-D-*manno*-heptose α-1,6-D-*glycero*-D-*manno*-heptosyltransferase, as the core from the *wahC* mutant AH-3Δ5.1 was devoid of DD-HepII. The presence of DD-HepII seems to be important for the incorporation of Gal into the core since only a minor amount of Gal and, consequently, a reduced amount of the O34 antigen were found in the mutant LPS.

The ORF6 product, named WahD, was assigned as D-glucose α-1,6-D-*glycero*-D-*manno*-heptosyltransferase (DD-HepI transferase) based on the lack of O34 antigen and the outer core from the LPS of the corresponding mutant AH-3Δ6.1. The ORF7 product was named WahE and assigned as L-*glycero*-D-*manno*-heptose α-1,6-L-*glycero*-D-*manno*-heptosyltransferase (LD-HepIV transferase) since the core from the *wahE* mutant differed from the full core only in its lack of LD-HepIV. This assignment is consistent with the presence of the O34 antigen in the mutant AH-3Δ7.1.

The data obtained show that the full inner core consisting of Kdo, three LD-Heps (I, II, and III), and Glc, i.e., the LPS domain synthesized by inner-core gene regions 2 and 3, is absolutely necessary for the addition of the outer-core monosaccharides. The absence of GlcN significantly affected the incorporation of the outer-core sugars, too. While the contributions of lipid A and O antigen to pathogenesis have been well studied, so far, little is known about the role of the LPS core domain. The LPS core domain seems to contribute to pathogenesis or to adaptation to several body sites, as suggested by the prevalence of some core types within clinical isolates. The interchanging of two core types of *K. pneumoniae* has a measurable effect on its virulence in an animal model of experimental infection (30). Mutation in either of the genes for biosynthesis of the inner-core heptose region induced the susceptibility of *Yersinia pestis* to the bactericidal action of normal human serum (24). Having determined the functions of all genes involved with the *A. hydrophila* LPS core biosynthesis pathways and most corresponding single-gene mutants now allows experimental work on the role of the LPS core in the virulence of this bacterium.

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