Role of Gne and GalE in the Virulence of *Aeromonas hydrophila* Serotype O34[∇]

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The mesophilic *Aeromonas hydrophila* AH-3 (serotype O34) strain shows two different UDP-hexose epimerases in its genome: GalE (EC 3.1.5.2) and Gne (EC 3.1.5.7). Similar homologues were detected in the different mesophilic *Aeromonas* strains tested. GalE shows only UDP-galactose 4-epimerase activity, while Gne is able to perform a dual activity (mainly UDP-*N*-acetyl galactosamine 4-epimerase and also UDP-galactose 4-epimerase). We studied the activities in vitro of both epimerases and also in vivo through the lipopolysaccharide (LPS) structure of *A. hydrophila gne* mutants, *A. hydrophila galE* mutants, *A. hydrophila galE-gne* double mutants, and independently complemented mutants with both genes. Furthermore, the enzymatic activity in vivo, which renders different LPS structures on the mentioned *A. hydrophila* mutant strains or the complemented mutants, allowed us to confirm a clear relationship between the virulence of these strains and the presence/absence of the O34 antigen LPS.

Mesophilic *Aeromonas* spp. are ubiquitous waterborne bacteria and pathogens of reptiles, amphibians, and fish (3). They can be isolated as a part of the fecal flora of a wide variety of other animals, including some used for human consumption, such as pigs, cows, sheep, and poultry. In humans, *Aeromonas hydrophila* isolates belonging to hybridization groups 1 and 3 (HG1 and HG3), *Aeromonas veronii* biovar *sobria* (HG8/HG10), and *Aeromonas caviae* (HG4) have been associated with gastrointestinal and extraintestinal diseases, such as wound infections of healthy humans, and less commonly with septicemias of immunocompromised patients (23). The pathogenicity of mesophilic aeromonads has been linked to a number of different determinants, such as toxins, proteases, outer membrane proteins (35), lipopolysaccharide (LPS) (29), flagella (30, 37), and the type III secretion system (43).

Mesophilic *Aeromonas* sp. strains of serotype O34 typically express smooth LPS on the surface. We fully characterized chemically the O-antigen and the core LPS of *A. hydrophila* strain AH-3 (serotype O34) (Fig. 1) (24, 25). Mesophilic *Aeromonas* strains from this serotype are the most frequently isolated in clinical sources (28). A single mutation in a gene that codes for UDP *N*-acetylgalactosamine 4-epimerase (*gne*) renders a strain with the O[−] phenotype (LPS without O-antigen molecules) in serotype O34 (9). No changes were observed for the LPS core in a *gne* mutant from strain *A. hydrophila* AH-3 (serotype O34). O34 antigen LPS contains *N*-acetyl galactosamine (GalNAc), while no such sugar residue forms part of the LPS core from *A. hydrophila* AH-3. The *A. hydrophila* AH-3 *gne* mutants are drastically reduced in some pathogenic features (serum resistance or adhesion to Hep-2 cells) and less virulent for fish and mice than the wild-type strain. Strain AH-3, like other mesophilic *Aeromonas* strains, possesses two

kinds of flagella (18), and the lack of the O34-antigen molecules by the *gne* mutation in this strain reduced the motility without any effect on the biogenesis of both polar and lateral flagella. *gne* is present in all the mesophilic *Aeromonas* strains tested and is able to fully complement all the phenotypes mentioned above (9).

GalE (EC 3.1.5.2) is the enzyme able to epimerize UDP galactose, always present in all the bacteria studied, while Gne (EC 3.1.5.7.) is a UDP *N*-acetylgalactosamine 4-epimerase (9). However, in some cases a single protein could perform GalE and Gne activities (6), while two different proteins have been reported in other cases (5). We decided to investigate the presence of a *galE* gene in *Aeromonas* strains of serotype O34 and to fully characterize the role in vitro and in vivo of both enzymatic activities linked to bacterial virulence, especially through the role of these epimerases in LPS biogenesis.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and growth conditions. Bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. *Aeromonas* sp. strains were routinely grown on tryptic soy broth or tryptic soy agar at 30°C, while *Escherichia coli* and *Salmonella enterica* serotype Typhimurium strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C. When required, the strains were cultured in Davis minimal medium with glucose or galactose as the unique carbon source. Bacteriophages Br2, Ffm, and P22 were grown and tested on *S. enterica* serotype Typhimurium strains as previously described (45). Kanamycin (50 µg ml^{−1}), ampicillin (100 µg ml^{−1}), tetracycline (20 µg ml^{−1}), rifampin (100 µg ml^{−1}), chloramphenicol (20 µg ml^{−1}), or gentamicin (20 µg ml^{−1}) was added to the different media.

General DNA methods. General DNA manipulations were done essentially as described elsewhere (40). 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 (41) with the ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). Oligonucleotides used for genomic DNA amplifications experiments and for 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 (2, 4) network service at the National Center for Biotechnology Information and the

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FIG. 1. Chemical structures of the O34 antigen LPS (A) and the LPS core (B) from *A. hydrophila* strain AH-3 (24, 25). The O34 antigen LPS is linked to the Gal residue (in italic letters) of the LPS core (25).

European Biotechnology Information websites, respectively. Clustal W was used for multiple sequence alignments.

Dot blot hybridizations. 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 using the enhanced chemiluminescence labeling and detection system (Amersham) according to the manufacturer's instructions.

Isolation of *Aeromonas galE* and double *galE* gene mutants. To obtain nonpolar *galE* mutants, the gene was amplified by PCR, ligated into the vector pGEMTeasy (Promega), and transformed into *E. coli* XL1-Blue. The Tn5-derived kanamycin resistance cartridge (*npIII*) from pUC4-KIXX was inserted into the gene. The cartridge contains an outward-reading promoter that ensures the expression of downstream genes when inserted in the correct orientation (8); however, such insertion will alter the regulation of such genes. The SmaI-digested cassette was inserted into a restriction site internal to each gene, and the presence of a single HindIII site in the SmaI-digested cassette allowed its orientation to be determined. The construct containing the mutated gene was ligated to suicide vector pDM4 (31), electroporated into *E. coli* MC1061 (λ *pir*), and plated on chloramphenicol plates at 30°C. Plasmid with the mutated gene (pDM4-GALE) was transferred into rifampin-resistant (Rif^r) *A. hydrophila* AH-405 by triparental mating using *E. coli* MC1061 (λ *pir*) containing the insertion construct and the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing chloramphenicol, kanamycin, and rifampin. PCR and Southern blot 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 adding 5% sucrose to the agar plates. The pDM4 vector contains *sacB*, which produces an enzyme that converts sucrose into a product that is toxic to gram-negative bacteria. One of the rifampin-resistant kanamycin-resistant and chloramphenicol-sensitive (Cm^r) transconjugants surviving on plates with 5% sucrose was chosen and confirmed by PCR as a *galE* mutant (AH-2804).

To construct a *galE* gene double mutant, we used the suicide plasmid pGM-GNE (Table 1) to inactivate *galE* of AH-2804 (*galE*). To construct pGM-GNE, first the kanamycin resistance cassette in pFS100 was replaced with the gentamicin resistance cassette from pUCGm/ox (36) to get plasmid pGM100 (Table 1). Then, an internal fragment of *gne* was amplified by PCR, ligated into pGEM-Teasy (Promega), and transformed into *E. coli* XL1-Blue. The DNA insert was recovered by EcoRI restriction digestion and was ligated into EcoRI-digested and phosphatase-treated pGM100. The ligation was transformed into *E. coli* MC1061 (λ *pir*) and selected for gentamicin resistance, and one of the resulting recombinant plasmids was named pGM-GNE. Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer pGM-GNE into Rif^r Km^r *A. hydrophila* AH-2804 to obtain a defined *gne* insertion mutant (AH-2806; *galE gne* double mutant), selecting for Rif^r, Km^r, and Gm^r.

Complementation studies. Complementation analysis of the different *Aeromonas* mutants was performed by conjugal transfer of wild-type *galE* or *gne* cloned in pACYC-GALE or pACYC-GNE. Recombinants were selected on LB agar containing tetracycline, and LPS was isolated and analyzed in gels.

Plasmid constructions for gene overexpression. For *galE* and *gne* overexpression, the pET-30 Xa/LIC vector (Novagen) and AccuPrime polymerase (high fidelity; Invitrogen) were used. The *galE* gene was amplified from plasmid DNA COS-GALE using primers AFw (5'-GGTATTGAGGGTCGCATGAAAGTAC TGGTACC-3') and ARv (5'-AGAGGAGAGTTAGAGCCTGTTACGCCT

CGTACCC-3'), while the *gne* gene was amplified with primers Bfw (5'-GGTA TTGAGGGTCGCATGACAATATTGGTCACG GG-3') and BRv (5'-AGAG GAGAGTTAGAGCCATGAATAAATGAGGCTCCC-3') from plasmid COS-GNE. The 1,017- and 1,065-nucleotide PCR products, respectively, were electrophoresed in agarose, and the DNA bands were recovered and purified. Purified amplicons were treated with T4 DNA polymerase in the presence of 2.5 mM dGTP for 30 min at 22°C to generate single-stranded ends (underlined letters above) complementary to Xa/LIC single-stranded ends in pET-30 Xa/LIC. After inactivation of the T4 DNA polymerase, each amplicon was mixed with pET-30 Xa/LIC vector and electroporated into NovaBlue GigaSingles competent cells and plated on LB supplemented with kanamycin (30 µg/ml). Trans-formants were analyzed for proper construction of pET30-GalE and pET30-Gne by PCR amplification and sequencing. These plasmids express GalE and Gne with an in-frame His₆ tag fused to their N terminus.

Preparation of cell-free extracts containing GalE and Gne. The His₆-GalE and His₆-Gne proteins were overexpressed from *E. coli* BL21(ΔDE3) containing pET30-GalE and pET30-Gne, respectively. Both strains were grown for 18 h at 37°C in LB medium supplemented with kanamycin. The culture was then diluted 1:100 in fresh medium, and incubation was continued until the culture reached an *A*₆₀₀ of 0.6. Expression of the fusion proteins was induced by adding isopropyl-1-thio-β-D-galactopyranoside to the culture (1 mM final concentration) and an additional 3-h incubation. The cells were harvested, washed once with 50 mM HEPES (pH 7.5), and then frozen until needed. The cell pellet was resuspended in 50 mM HEPES (pH 7.5) and sonicated on ice (for a total of 2 min using 10-s bursts followed by 10-s cooling periods). Unbroken cells, cell debris, and the membrane fraction were removed by ultracentrifugation at 100,000 × *g* for 60 min. For comparison, a soluble extract was prepared using the same protocol from *E. coli* BL21(ΔDE3) cells containing the pET30 vector. Protein expression was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein contents of the pET30 Xa/LIC, pET30-GalE, and pET30-Gne lysates were determined using the Bio-Rad Bradford assay as directed by the manufacturer.

Purification of His₆-GalE and His₆-Gne. Lysates containing the His₆-GalE or His₆-Gne proteins were prepared as described above, except that the pellet was washed and resuspended in 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl (buffer A). His₆-GalE and His₆-Gne were purified on an ÄKTA Explorer 100 system (Amersham Biosciences) using a 1-ml HiTrap chelating HP column (Amersham Biosciences) previously loaded with nickel sulfate and equilibrated with buffer A as recommended by the manufacturer. The column was washed with 5 mM imidazole in buffer A for 10 column volumes, and a step gradient of 50 to 500 mM imidazole in buffer A was then applied. His₆-GalE and His₆-Gne were eluted from the column at an imidazole concentration of 300 mM. The buffer was exchanged into 50 mM HEPES (pH 7.5) with 50 mM NaCl using a HiPrep 26/10 desalting column (Amersham Biosciences) according to the manufacturer's instructions. The protein was concentrated using a Centrifuplus 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.

Cell surface isolation and analyses. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in². Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected by centrifugation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium *N*-laurylsarcosinate (14), and the outer membrane (OM)

TABLE 1. Bacterial strains, bacteriophages, and plasmids used

Strain, plasmid, or bacteriophage	Genotype or phenotype ^a	Source or reference
<i>A. hydrophila</i> strains		
AH-3	O34, wild type	35
AH-405	AH-3, spontaneous Rif ^r	35
AH-2767	AH-3 <i>gnc</i> :mini-Tn5Km1 Rif ^r Km ^r	9
AH-2804	AH-3 <i>galE</i> mutant with pDM4, Km ^r	This study
AH-2806	AH-3 <i>galE-gnc</i> double mutant, Km ^r Gm ^r	This study
<i>E. coli</i> strains		
S17-1	<i>hsdR pro recA</i> , RP4-2 in chromosome, Km::Tn7 (Tc::Mu)	13
XL1-Blue	<i>endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^sΔM15 Tn10</i> (Tet ^r)]	Stratagene
DH5α	F ⁻ <i>endA hsdR17 (r_K⁻ m_K⁺) supE44 thi-1 recA1 gyrA96 φ80lacZ</i>	19
MC1061	<i>thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara-14 xy-15 supE44, λ pir</i>	39
SM10	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^r λpir</i>	39
<i>S. enterica</i> serotype Typhimurium strains		
SL3770	Expresses smooth LPS	27
SL1306	<i>galE503</i> , produces rough LPS of Rc chemotype	27
Plasmids		
pRK2073	Helper plasmid, Spc ^r	39
pLA2917	Cosmid vector, Km ^r Tc ^r	1
COS-GALE	pLA2917 cosmid with the complete <i>gal</i> region, Tc ^r	This study
pGEM-T	PCR cloning vector, Amp ^r	Promega
pGEMT-GNE	pGEMT with complete <i>gnc</i> from AH-3, Amp ^r	9
pBluescriptSK	Ap ^r ori of ColE1	Stratagene
pFS100	pGP704 suicide plasmid, <i>pir</i> dependent, Km ^r	39
pGM100	pFS100 suicide plasmid, <i>pir</i> dependent, Gm ^r	This study
pGM-GNE	pGM100 with an internal fragment of <i>gnc</i> , Gm ^r	This study
pACYC184	Plasmid vector, Cm ^r Tc ^r	20
pACYC-GALE	pACYC184 with complete <i>galE</i> of AH-3, Tc ^r	This study
pACYC-GNE	pACYC184 with complete <i>gnc</i> of AH-3, Tc ^r	9
pDM4	Suicide plasmid, <i>pir</i> dependent with <i>sacAB</i> genes	
	oriR6K, Cm ^r	31
pDM4-GALE	pDM4 with AH-3 <i>galE::Km</i> , Cm ^r Km ^r	This study
pET30Xa/LIC	Vector for overexpressing His-tagged proteins, Km ^r	Novagen
pET30-GalE	pET30 derivative overexpressing His ₆ -GalE, Km ^r	This study
pET30-Gne	pET30 derivative overexpressing His ₆ -Gne, Km ^r	This study
Bacteriophages		
Br2	Specific for the Rc chemotype	45
Ffm	Lyses only cells expressing rough LPS	45
P22	Lyses cells with smooth LPS	45

^a Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; Tc, tetracycline; Spc, spectinomycin; Gm, gentamicin.

fraction was collected as describe above. OM proteins were analyzed by SDS-PAGE by the Laemmli procedure (26). Protein gels were fixed and stained with Coomassie blue. Cultures for analysis of LPS were grown in TSB at 37°C. LPS was purified by the method of Galanos et al. (17), resulting in a 2.3% yield. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (12). LPS samples were separated by SDS-PAGE or SDS-Tricine-PAGE and visualized by silver staining as previously described (12, 20).

Isolation of oligosaccharides. LPS (20 mg) was hydrolyzed with 1% AcOH (100°C for 1 h). The resulting precipitate was removed by centrifugation, and the supernatant was analyzed by mass spectrometry. Another sample of LPS was deacylated and purified as already described (22), obtaining 6 mg of alditol oligosaccharides mixture.

LPS chemical analysis. For chemical analysis, either purified LPS or core LPS oligosaccharides samples were hydrolyzed with 1 N trifluoroacetic acid for 4 h at 100°C. Alditol acetates and methyl glycoside acetates were analyzed on an Agilent Technologies 5973N mass spectrometry instrument equipped with a 6850A gas chromatograph and an RTX-5 capillary column (Restek; 30 m by 0.25 inner diameter; flow rate, 1 ml min⁻¹; He as carrier gas). Acetylated methyl glycoside analysis was performed with the following temperature program: 150°C for 5 min, 150° to 250°C at 3° min⁻¹, and 250°C for 10 min. Acetylated methyl ester lipid analysis was performed as follows: 150° for 3 min, 150° to 280°C at 10°C min⁻¹, and 280°C for 15 min. The alditol acetates mixture was analyzed

with the following temperature program: 150°C for 5 min and 150°C to 300°C at 3°C min⁻¹. For partially methylated alditol acetates, the temperature program was 90°C for 1 min, 90°C to 140°C at 25°C min⁻¹, 140°C to 200°C at 5°C min⁻¹, 200°C to 280°C at 10°C min⁻¹, and 280°C for 10 min.

Mass spectrometry studies. Positive- and negative-ion reflectron, matrix-assisted time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source. Ion acceleration voltage was 25 kV, grid voltage was 17 kV, the mirror voltage ratio was 1.12, and delay time was 150 ns. Samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed nitrogen laser. Post-source decay was performed using an acceleration voltage of 20 kV. The reflectron voltage was decreased in 10 successive 25% steps. Mass calibration was obtained with a maltooligosaccharide mixture from corn syrup (Sigma). A solution of 2,5-dihydroxybenzoic acid in 20% CH₃CN in water at a concentration of 25 mg/ml was used as the MALDI matrix. One μl of matrix solution was deposited on the target, followed by loading of 1 μl of the sample. The droplets were allowed to dry at room temperature. Spectra were calibrated and processed under computer control using the Applied Biosystems Data Explorer software.

Methylation analysis. A core oligosaccharide sample (1 mg) obtained from 1% AcOH hydrolysis was first reduced with NaBH₄ and then methylated using the Ciucanu-Kerek procedure (10). Linkage analysis was performed as follows: the methylated sample was carboxymethyl reduced with lithium triethylborohydride

(Super-Hydride; Aldrich), mildly hydrolyzed to cleave ketosidic linkage, reduced by means of NaBD₄, and then totally hydrolyzed, reduced with NaBD₄, and finally acetylated as described previously (16).

Cell extract production and enzymatic activity measurements in cell extracts or purified recombinant proteins (Gal and GalNAc 4-epimerase assays). Suspensions of bacteria (25%, wt/vol), washed in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM MgCl₂, were disrupted in a Branson model 350 sonifier at 0°C. Disrupted bacteria were subjected to high-speed centrifugation (180,000 × *g* for 2 h) at 5°C to obtain cell extracts. Protein concentrations of extracts were determined by using the Bio-Rad Bradford assay as directed by the manufacturer with bovine serum albumin as the standard.

The assay for UDP-galactose 4-epimerase was performed as previously described (7) by adding 20 to 50 µl of cell extract (approximately 100 to 250 µg of protein) or 1 µl of purified recombinant proteins (approximately 7 to 8 µg of protein) to 1 ml of a solution containing 50 mM Tris-HCl buffer (pH 8.0), 5 mM MgCl₂, 1 mM NAD⁺, and 0.03 U of NAD⁺-dependent uridine 5'-diphosphoglucose dehydrogenase (Sigma). The assay was begun by addition of 0.5 mM UDP-Gal, and the increase in A₃₄₀ was followed in a Beckman DU 640 spectrophotometer. Initial rates of NADH formation were determined by using the kinetics program installed in the instrument. A molar extinction (E₃₄₀) of 6,220 M⁻¹ cm⁻¹ was assumed in all calculations.

The assay for UDP-*N*-acetylglucosamine 4-epimerase was performed as previously described (11, 44). In this procedure, the conversion of UDP-GalNAc to UDP-GlcNAc is measured after acid hydrolysis by the 3.6-fold increase in the color (A₅₈₅) of free GlcNAc over GalNAc in the Morgan-Elson reaction. The reactions were carried out by adding 20 µl of cell extract or 1 µl of purified recombinant protein to a volume containing 0.5 ml of 10 mM glycine, 1 mM MgCl₂, 0.1 mM EDTA, and 0.1 mM UDP-GalNAc. Enzyme activity was halted after 5 and 10 min of incubation at 37°C by the addition of 0.8 µl of concentrated HCl. Following hydrolysis and completion of the Morgan-Elson reaction, color development was measured at 585 nm. Control assays with extract or purified recombinant protein alone and with substrate only were run simultaneously. All assays were performed in triplicate. Product formation (i.e., GlcNAc formed by hydrolysis of UDP-GlcNAc) was measured from standard plots prepared by subjecting UDPGlcNAc, UDP-GalNAc, GlcNAc, and GalNAc (Sigma-Aldrich, St. Louis, Mo.) to the same procedures.

Virulence for fish and mice. The virulence of the strains grown at 20°C was measured by monitoring their 50% lethal dose (LD₅₀) by the method of Reed and Muench, as previously described (38).

(i) **Fish.** Rainbow trout (12 to 20 g) were maintained in 20-liter static tanks at 17 to 18°C. The fish were injected intraperitoneally with 0.05 ml of the test samples (approximately 10⁹ viable cells). Mortality was recorded up to 2 weeks; all the deaths occurred within 2 to 8 days.

(ii) **Mice.** Albino Swiss female mice (5 to 7 weeks old) were injected intraperitoneally with 0.25 ml of the test sample (approximately 5 × 10⁹ viable cells). Mortality was recorded for up to 1 week; all the deaths occurred within 2 to 5 days.

Nucleotide sequence accession number. The nucleotide sequence of the *A. hydrophila* AH-3 *galE* region described here has been assigned the following GenBank accession number: DQ860510.

RESULTS

Identification and cloning of the *A. hydrophila* AH-3 *galE* gene. Grown in the presence of galactose, *Salmonella enterica* serotype Typhimurium *galE* mutants accumulate intracellular Gal-1-P and UDP-Gal, leading to bacteriostasis and bacteriolysis (33, 34). On the basis of the sensitivity of *galE* mutants to galactose, an *A. hydrophila* AH-3 gene bank in pLA2917 previously obtained (35) was electroporated into *S. enterica* serotype Typhimurium SL1306. This strain is a *galE* mutant, and so growth on minimal medium containing galactose as the sole carbon source occurs only if a functional *galE* gene is present. Approximately 30 galactose-resistant colonies were obtained, and a representative recombinant plasmid was chosen for further study (COS-GALE). Rough LPS characteristic of the Rc chemotype is observed for *S. enterica* serotype Typhimurium SL1306 alone or carrying the plasmid vector. A ladder banding pattern consistent with smooth LPS is visualized when COS-

FIG. 2. A. LPS from *S. enterica* serotype Typhimurium SL3770 (wild type), mutant SL1306 (*galE*), and mutant SL1306 complemented with plasmids COS-GALE and pACYC-GALE, respectively (lanes 1, 2, 3, and 4). B. LPS from *S. enterica* serotype Typhimurium SL3770 (wild type), mutant SL1306 (*galE*), and mutant SL1306 complemented with plasmids pACYC-GNE and pGEMT-GNE, respectively (lanes 1, 2, 3, and 4). LPS was extracted and analyzed by SDS-PAGE (12%) according to the methods of Darveau and Hancock (12) and silver stained (12, 20).

GALE is present in strain SL1306 (Fig. 2A). Phage sensitivity assays with phages Br2, Ffm, and P22 were used to characterize LPSs on the outer membranes of *S. enterica* serotype Typhimurium L1306 and SL3770 with and without various plasmids. Phage Br2 is specific for the Rc chemotype, phage Ffm lyses only cells expressing rough LPS, and phage P22 specifically lyses cells with smooth LPS. The phage sensitivity pattern of *S. enterica* serovar Typhimurium SL1306 carrying COS-GALE is identical to that of *S. enterica* serovar Typhimurium SL3770 wild type (resistant to Br2 and Ffm and sensitive to P22), while the mutant, with or without plasmid vector, was sensitive to Br2 and Ffm and resistant to P22. Slide agglutination tests with *Salmonella* antiserum specific for group B factors showed that agglutination was observed only with *S. enterica* serovar Typhimurium SL3770 or *S. enterica* serovar Typhimurium SL1306 carrying COS-GALE. Gas chromatography-mass spectrometry analysis of LPSs from *S. enterica* serovar Typhimurium SL1306, *S. enterica* serovar Typhimurium SL3770, and *S. enterica* serovar Typhimurium SL1306 carrying COS-GALE showed that the relative sugar ratios for the latter two samples were essentially identical (data not shown). These data demonstrate that the *galE* mutation in *S. enterica* serovar Typhimurium SL1306 was complemented by a functional *galE* gene from *A. hydrophila* AH-3.

DNA sequence of an *A. hydrophila* AH-3 genomic region coding for GALE (COS-GALE). The DNA sequence of COS-GALE showed the presence of five relevant ORFs (Fig. 3A). *galE* was followed downstream by three genes transcribed in the same direction with high homology with *galT*, *-K*, and *-M*; upstream, a gene with high homology with *galR* transcribed in the opposite direction to *galE* was found (Fig. 3B). A gene with high homology with several oxidoreductase genes from *Yersinia*

FIG. 3. A. Genetic organization of the *A. hydrophila* AH-3 *galE* region from the COS-GALE plasmid. Transcription direction and stops (lollipop) are indicated. B. Main homologies of the ORFs as well as their sizes in nucleotides and amino acid (aa) residues are shown.

sp. was found and named *orf1*. This genetic organization and the transcriptional direction for these genes are typical for the *gal* operon. Complementation studies of *S. enterica* serotype Typhimurium SL1306 by COS-GALE were confirmed, since reintroduction of the single *galE* gene (plasmid pACYC-GALE) rescues the complete wild-type phenotype in the mutant as the one obtained by COS-GALE (Fig. 2A, LPS) and by the phage sensitivity pattern and slide agglutination test.

***galE* and *gne* are different in *A. hydrophila* AH-3.** Comparison of nucleotide and deduced amino acid sequences indicated that *gne* and *galE* are different genes in *A. hydrophila* AH-3. Using the plasmid pACYC-GNE, we tried to complement the *S. enterica* serotype Typhimurium SL1306 *galE* mutant. Figure 2B shows that the LPS from this strain was only partially complemented according to the O-antigen LPS expression by this plasmid, neither having a high copy number (pGEMT-GNE). However, strain SL1306 harboring plasmid pACYC-GNE or pGEMT-GNE was able to grow on minimal medium containing galactose as the single carbon source; besides that,

an increase in the replication time could be observed with respect to the wild-type strain (SL3770). Furthermore, some changes in their sensitivity to bacteriophages Br2, Ffm, and P22 could be observed when *A. hydrophila gne* was expressed in this strain (some sensitivity to phages Br2 and Ffm and resistance to P22). Also, weak positive slide agglutination with antiserum specific for group B factors could be observed when *A. hydrophila gne* was expressed in SL1306. All the results obtained are in agreement with the presence of some O-antigen LPS molecules (not being the same number as for the wild-type strain SL3770) when SL1306 contains *A. hydrophila gne*. None of these results are obtained when the plasmid vectors alone are introduced in strain SL1306.

Distribution of *galE* and *gne* among mesophilic *Aeromonas* sp. We studied the presence of *galE* in different mesophilic *Aeromonas* strains ($n = 50$) where previously *gne* was identified (9). PCR fragment DNA amplification used genomic DNAs from these strains and oligonucleotides (AFw and ARv; see Materials and Methods) binding to regions flanking *galE*. In all

FIG. 4. A. LPS in SDS-PAGE gels from mesophilic *Aeromonas* strains. Lane 1, *A. hydrophila* AH-3 (wild type); lane 2, *A. hydrophila* AH-3 *galE* mutant (AH-2804); lane 3, *A. hydrophila* AH-3 *gne* mutant (AH-2767); lane 4, *A. hydrophila* AH-3 *galE gne* double mutant (AH-2806); lane 5, AH-2806 mutant complemented with pACYC-GNE (*A. hydrophila* AH-3 *gne*); lane 6, AH-2806 mutant complemented with pACYC-GALE (*A. hydrophila* AH-3 *galE*). B. SDS-Tricine-PAGE gels from LPS cores of mesophilic *Aeromonas* strains. Lane 1, *A. hydrophila* AH-3 (wild type); lane 2, *A. hydrophila* AH-3 *galE* mutant (AH-2804); lane 3, *A. hydrophila* AH-3 *galE gne* double mutant (AH-2806); lane 4, AH-2806 mutant complemented with pACYC-GALE (*A. hydrophila* AH-3 *galE*).

the strains tested, except one, a single 1,017-bp band was amplified. To confirm that *galE* was indeed present, the nucleotide sequence of the DNA-amplified fragments from several *Aeromonas* strains was determined. The negative strain by PCR was positive in a dot blot using the 1,017-bp band as a DNA probe. Thus, either *galE* or *gne* was found in all the mesophilic *Aeromonas* strains tested.

***Aeromonas galE* mutants.** The previously obtained results prompted us to generate a *galE* nonpolar mutant in *A. hydrophila* AH-3 (AH-2804) with plasmid pDM4-GALE. This mutant showed an identical LPS profile to the wild-type strain (Fig. 4A); it was able to cross-react with anti-O34 serum and also was able to grow in minimal medium with galactose as a single carbon source. However, this growth was delayed in comparison to wild-type strain AH-3 growth or that of the rifampin-resistant mutant (AH-405) under the same conditions. Similar results were obtained when *galE* mutants were isolated in several ($n = 5$) mesophilic *Aeromonas* strains with *galE* and *gne* (data not shown). The introduction of the *A. hydrophila* AH-3 *galE* gene (plasmid pACYC-GALE) in the *A. hydrophila* AH-3 *gne* mutant (AH-2767) did not rescue the presence of O34-antigen LPS, as when plasmid pACYC-GNE was introduced (9).

***Aeromonas galE gne* double mutant and complementation studies.** The fact that no changes in the *A. hydrophila* AH-3 *galE* mutant LPS could be observed and also that the LPS core from the *A. hydrophila* AH-3 *gne* mutant (AH-2767) does not lack galactose (9) prompted us to obtain a double *A. hydrophila* AH-3 *galE gne* mutant. From the *galE* mutant (AH-2804; Km^r) we derived a *gne* mutant using the same technique previously described (9) but with plasmid pGM100 in order to use gen-

tamicin resistance for the selection. The *galE gne* double mutant (AH-2806) shows an LPS profile devoid of the O34 antigen LPS and also a slightly faster migration of the LPS core in gels (Fig. 4). Mutant AH-2806 was completely unable to grow in minimal medium with galactose as the single carbon source.

LPS obtained from mutant AH-2806 (*galE gne*) was subjected to mild acid hydrolysis (1% acetic acid) to cleave the acid-labile ketosidic linkage between Kdo (3-deoxy-D-manno-octulosonic acid) and lipid A. The lipid A fraction was removed by high-speed centrifugation, and the core oligosaccharides were recovered by Sephadex G-50 chromatography. Chemical composition analysis of the core oligosaccharide fraction by gas chromatography-mass spectrometry of acetylated methyl glycosides revealed the absence of D-galactose and the presence of the remaining residues found in the wild-type core LPS (L-glycero-D-manno-heptose, D-glycero-D-manno-heptose, D-glucose, D-glucosamine, and Kdo), suggesting that in this mutant the core LPS is truncated at the D-galactose level. To further prove this point, the oligosaccharide fractions from AH-2806 were analyzed by negative-ion reflectron MALDI-TOF. This experiment revealed the presence of a major signal at 1,712.7 m/z (Fig. 5). This signal is in agreement with a structure similar to the wild-type strain (Kdo-Hep6-GlcN-Glc-Gal) but deficient in D-galactose (Kdo-Hep6-GlcN-Glc; 1,714.5 Da) (25). The signal at 1,695.0 m/z (Fig. 5) could correspond to the oligosaccharide anhydro form, as previously described for LPS samples hydrolyzed with acetic acid (25). In addition, the signal at 1,735.55 m/z probably corresponds to a sodium adduct. Furthermore, the oligosaccharide sequence was determined using the MALDI-postsource decay technique (unpublished data) and confirmed that the *galE gne* double mutation results in a D-galactose-deficient core oligosaccharide. This result was also confirmed by a methylation analysis experiment of the N-acetylated oligosaccharide alditols mixture obtained from the AH-2806 mutant. This experiment showed the presence of 3,4,6-linked Hep, terminal Hep, and GlcN but did not detect a terminal Gal residue.

Introduction of the *galE* gene from the wild-type strain (plasmid pACYC-GALE) into double mutant AH-2806 resulted in a core LPS essentially identical to that of the wild-type strain (Fig. 6; major signal at m/z 1,874.41 corresponding to Kdo-Hep6-GlcN-Glc-Gal) but lacking the O34 antigen (Fig. 4A), with the same electrophoretic moiety (Fig. 4B) and able to grow in minimal medium with galactose as a single carbon source. The result about the LPS core structure was also confirmed by determining the core oligosaccharide sequence from the *galE*-complemented AH-2806 mutant (unpublished data). A methylation analysis experiment with the N-acetylated oligosaccharide alditols mixture confirmed the presence of a terminal Gal residue as the complete LPS core from the wild-type strain.

Introduction of the *gne* gene from the wild-type strain (plasmid pACYC-GNE) into double mutant AH-2806 resulted in a complete LPS with O34 antigen (Fig. 4A) and the ability to grow with some delay in minimal medium with galactose as a unique carbon source.

Gal and GalNAc 4-epimerase enzymatic activities in cell extracts and purified recombinant proteins (GalE and Gne). The genetic analysis as well as the LPS chemical structure of the mutants prompted us to study the enzymatic activities that allow the production of UDPGal from UDPGlc (Gal 4-epim-

FIG. 5. Negative-ion MALDI-TOF spectrum of acid-released core LPS oligosaccharide from *A. hydrophila* AH-2806 (*galE gne* double mutant).

erase) and the production of UDPGalNAc from UDPGlcNAc (GalNAc 4-epimerase). *A. hydrophila* AH-3 (wild-type strain) as previously indicated (9) showed high Gal and GalNAc 4-epimerase activities, grown in glucose or galactose, when measured as described in Materials and Methods (Table 2). However, mutant strain AH-2804 (*galE*) with or without the plasmid vector (pACYC184) showed a four- to fivefold decrease in its Gal 4-epimerase activity but not a complete lack, while no differences could be observed in the GalNAc 4-epimerase activity with respect to the wild type. This decrease in Gal 4-epimerase activity in strain AH-2804 can be rescued by the reintroduction of the single gene

using plasmid pACYC-GALE. Furthermore, strain AH-2806 (*galE gne* double mutant) with or without plasmid vector showed a complete lack of both enzymatic activities (Gal 4- and GalNAc 4-epimerases) when grown in glucose and was unable to grow in galactose as a single carbon source (Table 2). The introduction of pACYC-GALE (*galE*) in strain AH-2806 restored the Gal 4-epimerase activity but not the GalNAc 4-epimerase activity, while the introduction of plasmid pACYC-GNE (*gne*) in strain AH-2806 fully restored the GalNAc 4-epimerase activity and partially the Gal 4-epimerase activity (similar levels obtained for strain AH-2804 *galE* mutant) (Table 2).

FIG. 6. Negative-ion MALDI-TOF spectrum of acid-released core LPS oligosaccharide from *A. hydrophila* AH-2806 (*galE gne* double mutant) complemented with *A. hydrophila galE* (pACYC-GALE).

TABLE 2. Gal and GalNAc 4-epimerase activities in cell extracts of wild-type *A. hydrophila* AH-3 and mutant strains

Strain	Carbon source ^a	4-Epimerase activity	
		Gal ^b	GalNAc ^c
AH-3, wild type	Glucose	91.2 ± 1.7	24.6 ± 2.2
	Galactose	34.5 ± 1.5	12.3 ± 1.6
AH-405, AH-3 rifampin resistant	Glucose	90.7 ± 1.9	22.4 ± 2.5
	Galactose	33.6 ± 1.4	12.0 ± 1.1
AH-2804, <i>galE</i> mutant	Glucose	22.6 ± 1.7	23.7 ± 2.0
	Galactose	12.8 ± 1.3	13.4 ± 0.9
AH-2804 + plasmid pACYC184	Glucose	21.8 ± 2.0	23.9 ± 1.1
	Galactose	13.1 ± 1.4	12.6 ± 1.2
AH-2804 + plasmid pACYC-GALE	Glucose	90.7 ± 1.1	23.0 ± 1.8
	Galactose	33.3 ± 1.7	11.8 ± 1.9
AH-2806; <i>galE gne</i> double mutant	Glucose	<0.12 (ND) ^d	<0.03 (ND)
	Galactose	No growth	No growth
AH-2806 + plasmid pACYC184	Glucose	<0.09 (ND)	<0.03 (ND)
	Galactose	No growth	No growth
AH-2806 + plasmid pACYC-GALE	Glucose	78.9 ± 2.7	<0.03 (ND)
	Galactose	29.1 ± 1.8	<0.03 (ND)
AH-2806 + plasmid pACYC-GNE	Glucose	24.3 ± 2.1	22.7 ± 2.0
	Galactose	15.6 ± 1.3	13.1 ± 1.5

^a Each strain was grown in Davis minimal medium containing glucose or galactose as a unique carbon source.

^b Nanomoles of UDP-Gal converted to UDP-Glc min⁻¹ mg protein⁻¹.

^c Nanomoles of UDP-GalNAc converted to UDP-GlcNAc min⁻¹ mg protein⁻¹.

^d ND, no detectable activity.

The recombinant Gne and GalE proteins obtained as described in Materials and Methods, instead of cell extracts, were assayed for Gal and GalNAc 4-epimerase enzymatic activities. The recombinant *Aeromonas* GalE shows a clear UDPGal 4-epimerase activity, while no UDPGalNAc 4-epimerase activity was observed. The recombinant *Aeromonas* Gne shows a clear UDPGalNAc 4-epimerase activity and also a UDPGal 4-epimerase activity in these assays. The recombinant *Aeromonas* GalE UDPGal 4-epimerase activity was at least five times higher than the same enzymatic activity to the same protein concentration produced by the recombinant *Aeromonas* Gne.

Virulence studies. We tested the virulence of the wild-type strain and mutants (LD₅₀), as described in Materials and Methods. No differences were found in mortality between the wild-type strain and the *A. hydrophila* AH-3 *galE* mutant (AH-2804) (10^{5.3} and 10^{7.4} LD₅₀ in fish and mice for the wild-type strain, respectively). However, the *galE gne* double mutant AH-2806 showed a higher LD₅₀ (an increase of 1 to 2 log units) in both fish and mice than the wild-type strain did. This result is similar to the virulence previously observed for an *A. hydrophila* AH-3 *gne* mutant (9). Complementation of this double mutant with pACYC-GNE (carrying the single *gne*) completely restored the virulence for fish or mice (LD₅₀ similar to the wild-type strain), while no changes were observed with the plasmid vector alone or with the plasmid carrying the *A. hydrophila galE*. These results suggested that O34 antigen LPS is an important virulence factor for mesophilic *Aeromonas* serogroup O34 pathogenesis.

DISCUSSION

Mesophilic *Aeromonas* sp. strains like strain AH-3 (serotype O34) showed at least two different UDP-sugar epimerases: GalE, with the unique enzymatic activity of UDP-Gal epimerase responsible for the conversion of UDPGlc to UDPGal,

and Gne, with two enzymatic activities, UDP-Gal and UDPGalNAc 4-epimerase, responsible for the conversion of UDPGlcNAc to UDPGalNAc. It is clear that these epimerases are critical for the biosynthesis of the different surface carbohydrates, like capsular polysaccharide, O-antigen, and core LPS. While in several enterobacteria like *E. coli* or *S. enterica* serovar Typhimurium GalE is the unique UDP-Gal epimerase, and their mutations correlate with changes in the LPS chemical structure (in many cases with the lack of the O-antigen LPS), no changes were observed in *A. hydrophila* AH-3 when this mutation occurred. The reason is that Gne (an enzyme not found in many enteric bacteria) is able to perform UDP-Gal epimerization as well as UDPGalNAc 4-epimerization. As we demonstrated, *A. hydrophila* Gne is able to show both enzymatic activities either in vitro or in vivo, but with a UDP-Gal epimerization in vitro it is at least five times less effective than *A. hydrophila* GalE. The reasons that allow us to conclude that *A. hydrophila* Gne shows UDP-Gal epimerization activity in vivo are the following.

(i) *A. hydrophila galE* mutants showed no changes in LPS and also are able to grow in minimal medium with galactose as a single energy and carbon source.

(ii) The *A. hydrophila galE gne* double mutant shows an LPS devoid of the O34 antigen LPS and also lacks the Gal residue of the LPS core (Fig. 1). This Gal residue is the one where O34-antigen LPS is linked to the LPS core and is present in the LPS core of the *A. hydrophila gne* single mutant (9).

(iii) When the *A. hydrophila galE gne* double mutant is complemented by *A. hydrophila galE* alone, the Gal residue of the LPS core is rescued; in addition, no O34 antigen is found (as we previously demonstrated, O34 antigen requires the UDPGalNAc 4-epimerization to form UDP-GalNAc [9]). When the *A. hydrophila galE gne* double mutant is complemented by *A. hydrophila gne* alone, the complete LPS with O34

FIG. 7. Multiple sequence alignments of UDP-hexose 4-epimerases. GalE proteins are from *Trypanosoma brucei* (Tb-CAD23117-GalE), *T. cruzi* (Tc-CAE17296-GalE), *Escherichia coli* (Ec-AAC73846-GalE), *Salmonella enterica* serovar Typhimurium (Se-Typhi-GalE), *A. hydrophila* (Ah-GalE), *Campylobacter jejuni* (Cj-GalE-Bif); *Homo sapiens* (Hs-Q14376-GalE), and *Bacillus subtilis* (Bs-NP_391765-GalE); Gne proteins are from *A. hydrophila* (Ah-Gne) and *Yersinia enterocolitica* (Ye-AAC60777-Gne); WbpP is from *Pseudomonas aeruginosa* (AAM27817WbpP-Pa); WbgU is from *Plesiomonas shigelloides* (AAG17409WbgU-Psh). Biochemically confirmed UDPGal 4-epimerase activity enzymes (bold letters), UDPGalNac 4-epimerase enzymes (italic letters), and bifunctional enzymes (underlined) are shown. Residue conservation shading in more than 50% of the sequence: black, identical; dark gray, similar; light gray, conserved. Box 1, GXXGXXG motif; boxes 2 to 7, predicted substrate binding pocket residues.

antigen is rescued. This is because both enzymatic activities are performed by *A. hydrophila* Gne in vivo.

(iv) *A. hydrophila* Gne is able to partially complement (not fully) the *S. enterica* serotype Typhimurium *galE* mutant, as can be judged by LPS gels, phage sensitivity, and growth in minimal medium with galactose.

In recent reports, a single and unique protein was able to perform both enzymatic activities (UDP-Gal and UDPGalNAc 4-epimerase) in vitro and in vivo, as in *Bacillus subtilis* (42) and *Campylobacter jejuni* (6). In other bacteria, such as many enteric bacteria, including *S. enterica* serovar Typhimurium, only GalE exists with a unique UDP-Gal epimerase activity and no *gne* could be detected or identified in sequenced genomes. However, other UDP-hexose epimerases, like *Yersinia enterocolitica* Gne (5) or *Pseudomonas aeruginosa* WbpP (15), a protein with mainly UDPGalNAc 4-epimerase activity and some UDP-Gal epimerase activity in vitro, could be detected as described. In both cases, because their genomes or similar have been sequenced, *galE* seems to be present. It is tempting to speculate, according to the results obtained in *A. hydrophila*, that bacteria with two proteins like GalE and Gne have two possibilities for UDP-Gal epimerization in vivo: a major one based on GalE and a second one depending on the dual performance of Gne. This suggestion is in agreement that in some bacteria *galE* mutants showed, at least for the phenotypes studied, identical characteristics as the wild-type strain, like in *Vibrio cholerae* (32). The presence of GalE and Gne homologues in the same genome can be deduced from the *Vibrio* genomes already completely sequenced.

Both *A. hydrophila* GalE and Gne are members of the UDP-hexose 4-epimerase family of enzymes (Fig. 7). It has been recently proposed that the substrate specificity of these enzymes for UDP-Glc/UDP-Gal or UDP-GlcNAc/UDPGalNAc lies in six key amino acid residues in the saccharide binding pocket (21). Amino acid residues L/K-S-Y-N-H/N-L/Y were proposed to be involved in recognizing UDP-Glc/UDP-Gal. Amino acid residues G-S-Y-N-a-s are present in WbpP from *P. aeruginosa* and would be involved in recognizing UDP-GlcNAc/UDPGalNAc. Residues K-S-Y-N-N-c are present in the bifunctional human GalE using as substrate both UDP-Glc/UDP-Gal and UDP-GlcNAc/UDPGalNAc (lowercase letters indicate similar amino acid residues). Nevertheless, it should be noted that the bifunctional enzyme from *C. jejuni* key residues (I-T-Y-N-L-L) (6) (Fig. 7) differs from those of other bifunctional 4-epimerases. By contrast, *A. hydrophila* GalE and Gne contain the same six key amino acid residues in their putative saccharide binding pocket, K-S-Y-N-N-C (Fig. 7), and according to mentioned work (21) they should be bifunctional enzymes recognizing both UDP-Glc/UDP-Gal and UDP-GlcNAc/UDPGalNAc. The bifunctionality of the *A. hydrophila* Gne enzyme was demonstrated by us, but this is not the case for the *A. hydrophila* GalE enzyme, which seems to be able to use only UDP-Glc/UDP-Gal as substrates. Our results indicate that the saccharide binding pocket is not the only determinant allowing the recognition of UDP-Glc/UDP-Gal, UDP-GlcNAc/UDPGalNAc, or both.

The results obtained in the performed virulence studies are in agreement with the presence or absence of O34 antigen LPS. As previously indicated (9), the O34 antigen LPS is essential in *Aeromonas* serotype O34 pathogenesis. Then, an *A.*

hydrophila galE mutant was as virulent as the wild-type strain (presence of O34 antigen LPS), and furthermore, the same result was obtained when the *A. hydrophila galE gne* double mutant was complemented with *Aeromonas gne* alone (presence of O34 antigen LPS). All of the other *A. hydrophila* mutants (*gne* or *galE gne*) and the complemented double mutant with *Aeromonas galE* alone showed a reduction in virulence (absence of O34 antigen LPS).

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REFERENCES

- Allen, L. N., and R. S. Hanson. 1985. Construction of broad-host-range cosmid cloning vector: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *J. Bacteriol.* **161**:955–962.
- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Austin, B., and C. Adams. 1996. Fish pathogens, p. 197–243. In B. Austin, M. Altwegg, P. J. Gosling, and S. W. Joseph (ed.), *The genus Aeromonas*. John Wiley and Sons, New York, NY.
- Bateman, A., E. Birney, L. Cerruti, R. Durbin, L. Etwiler, S. R. Eddy, S. Griffiths-Jones, K. L. Howe, M. Marshall, and E. L. Sonnhammer. 2002. The Pfam protein families database. *Nucleic Acids Res.* **30**:276–280.
- Bengoechea, J. A., E. Pinta, T. Salminen, C. Oertelt, O. Holst, J. Radziejewska-Lebrecht, Z. Piotrowska-Seget, R. Venho, and M. Skurnik. 2002. Functional characterization of Gne (UDP-N-acetylglucosamine-4-epimerase), Wzz (chain length determinant), and Wzy (O-antigen polymerase) of *Yersinia enterocolitica* serotype O:8. *J. Bacteriol.* **184**:4277–4287.
- Bernatchez, S., C. M. Szymanski, N. Ishiyama, J. Li, H. C. Jarrell, P. C. Lau, A. M. Berghuis, N. M. Young, and W. W. Wakarchuk. 2005. A single bifunctional UDP-GlcNAc/Glc-4 epimerase supports the synthesis of three cell surface glycoconjugates in *Campylobacter jejuni*. *J. Biol. Chem.* **280**:4792–4802.
- Boels, I. C., A. Ramos, M. Kleerebezem, and W. M. de Vos. 2001. Functional analysis of the *Lactococcus lactis galU* and *galE* genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis. *Appl. Environ. Microbiol.* **67**:3033–3040.
- Bott, M., M. Meyer, and P. Dimroth. 1995. Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. *Mol. Microbiol.* **18**:533–546.
- Canals, R., N. Jiménez, S. Vilches, M. Regué, S. Merino, and J. M. Tomás. 2006. A gene (uridine diphosphate N-acetylglucosamine 4-epimerase) is essential for mesophilic *Aeromonas* serotype O34 virulence. *Infect. Immun.* **74**:537–548.
- Ciucanu, I., and F. Kerek. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131**:209–217.
- Creuzenet, C., M. Belanger, W. W. Wakarchuk, and J. S. Lam. 2000. Expression, purification, and biochemical characterization of WbpP, a new UDP-GlcNAc C4 epimerase from *Pseudomonas aeruginosa* serotype O6. *J. Biol. Chem.* **275**:19060–19067.
- Darveau, R. P., and R. E. Hancock. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* **155**:831–838.
- De Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
- Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *J. Bacteriol.* **115**:717–722.
- Filling, C., K. D. Berndt, J. Benach, S. Knapp, T. Prozorovski, E. Nordling, R. Ladenstein, H. Jornvall, and U. Oppermann. 2002. Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J. Biol. Chem.* **277**:25677–25684.
- Forsberg, L. S., U. Ramadas Bhat, and R. W. Carlson. 2000. Structural characterization of the O-antigenic polysaccharide of the lipopolysaccharide from *Rhizobium etli* strain CE3. *J. Biol. Chem.* **275**:18551–18563.
- Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245–249.
- Gavin, R., A. A. Rabaan, S. Merino, J. M. Tomás, I. Gryllos, and J. G. Shaw.

2002. Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. *Mol. Microbiol.* **42**:383–397.
19. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
20. Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269–277.
21. Ishiyama, N., C. Creuzenet, J. S. Lam, and A. M. Berghuis. 2004. Crystal structure of WbpP, a genuine UDP-N-acetylglucosamine 4-epimerase from *Pseudomonas aeruginosa*. *J. Biol. Chem.* **279**:22635–22642.
22. Izquierdo, L., N. Coderch, N. Piqué, E. Bedini, M. Corsaro, S. Merino, S. Fresno, J. M. Tomás, and M. Regué. 2003. The *Klebsiella pneumoniae* wabG gene: its role in the biosynthesis of the core lipopolysaccharide and virulence. *J. Bacteriol.* **185**:7213–7221.
23. Janda, J. M., and S. L. Abbott. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. *Clin. Infect. Dis.* **27**:332–344.
24. Knirel, Y. A., A. S. Shaskov, S. N. Senchenkova, S. Merino, and J. M. Tomás. 2002. Structure of the O-polysaccharide of *Aeromonas hydrophila* O34: a case of random O-acetylation of 6-deoxy-L-talose. *Carbohydr. Res.* **337**:1381–1386.
25. Knirel, Y. A., E. Vinogradov, N. Jimenez, S. Merino, and J. M. Tomás. 2004. Structural studies on the R-type lipopolysaccharide of *Aeromonas hydrophila*. *Carbohydr. Res.* **339**:787–793.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
27. MacLachlan, P. R., and K. E. Sanderson. 1985. Transformation of *Salmonella typhimurium* with plasmid DNA: differences between rough and smooth strains. *J. Bacteriol.* **161**:442–445.
28. Merino, S., S. Camprubí, and J. M. Tomás. 1993. Incidence of *Aeromonas* spp. serotypes O34 and O:11 among clinical isolates. *Med. Microbiol. Lett.* **2**:48–55.
29. Merino, S., X. Rubires, A. Aguilar, J. F. Guillot, and J. M. Tomás. 1996. The role of the O-antigen lipopolysaccharide on the colonization *in vivo* of the germfree chicken gut by *Aeromonas hydrophila* serogroup O34. *Microb. Pathog.* **20**:325–333.
30. Merino, S., X. Rubirés, A. Aguilar, and J. M. Tomás. 1997. The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O34 strains. *FEMS Microbiol. Lett.* **151**:213–217.
31. Milton, D. L., R. O'Toole, P. Horstedt, and H. Wolf-Watz. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.* **178**:1310–1319.
32. Nesper, J., M. C. Lauriano, K. E. Klose, D. Kapfhammer, A. Kraiß, and J. Reidl. 2001. Characterization of *Vibrio cholerae* O1 El Tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization and biofilm formation. *Infect. Immun.* **69**:435–445.
33. Nikaído, H. 1961. Galactose sensitive mutants of *Salmonella*. I. Metabolism of galactose. *Biochim. Biophys. Acta* **48**:460–469.
34. Nikaído, H. 1961. Galactose sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. *Biochim. Biophys. Acta* **48**:470–479.
35. Noguerras, M. M., S. Merino, A. Aguilar, V. J. Benedí, and J. M. Tomás. 2000. Cloning, sequencing and role in serum susceptibility of porin II from mesophilic *Aeromonas* sp. *Infect. Immun.* **68**:1849–1854.
36. Quénée, L., D. Lamotte, and B. Polack. 2005. Combined *sacB*-based negative selection and *cre-lox* antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. *BioTechniques* **38**:63–67.
37. Rabaan, A. A., I. Gryllos, J. M. Tomás, and J. G. Shaw. 2001. Motility and the polar flagellum are required for *Aeromonas caviae* adherence to Hep-2 cells. *Infect. Immun.* **69**:4257–4267.
38. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent end points. *Am. J. Hyg.* **27**:493–497.
39. Rubirés, X., F. Saigí, N. Piqué, N. Climent, S. Merino, S. Albertí, J. M. Tomás, and M. Regué. 1997. A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivatives. *J. Bacteriol.* **179**:7581–7586.
40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
41. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
42. Soldo, B., C. Scotti, D. Karamata, and V. Lazarevic. 2003. The *Bacillus subtilis* Gne (GneA, GalE) protein can catalyse UDP-glucose as well as UDP-N-acetylglucosamine 4-epimerisation. *Gene* **319**:65–69.
43. Vilches, S., C. Urgell, S. Merino, M. R. Chacón, L. Soler, G. Castro-Escarpulli, M. J. Figueras, and J. M. Tomás. 2004. Complete type III secretion system of a mesophilic *Aeromonas hydrophila* strain. *Appl. Environ. Microbiol.* **70**:6914–6919.
44. Wang, L., S. Huskic, A. Cisterne, D. Rothmund, and P. R. Reeves. 2002. The O-antigen gene cluster of *Escherichia coli* O55:H7 and identification of a new UDP-GlcNAc C4 epimerase gene. *J. Bacteriol.* **184**:2620–2625.
45. Wilkinson, R. G., P. Gemski, and B. A. D. Stocker. 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. *J. Gen. Microbiol.* **70**:527–554.