Mesophilic *Aeromonas* UDP-glucose pyrophosphorylase (GalU) mutants show two types of lipopolysaccharide structures and reduced virulence

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A mutation in *galU* that causes the lack of O34-antigen lipopolysaccharide (LPS) in *Aeromonas hydrophila* strain AH-3 was identified. It was proved that *A. hydrophila* GalU is a UDP-glucose pyrophosphorylase responsible for synthesis of UDP-glucose from glucose 1-phosphate and UTP. The *galU* mutant from this strain showed two types of LPS structures, represented by two bands on LPS gels. The first one (slow-migrating band in gels) corresponds to a rough strain having the complete core, with two significant differences: it lacks the terminal galactose residue from the LPS-core and 4-amino-4-deoxyarabinose residues from phosphate groups in lipid A. The second one (fast-migrating band in gels) corresponds to a deeply truncated structure with the LPS-core restricted to one 3-deoxy-D-manno-oct-2-ulonic acid (Kdo) and three L-glycero-D-manno-heptose residues. *galU* mutants in several motile mesophilic *Aeromonas* strains from serotypes O1, O2, O11, O18, O21 and O44 were also devoid of the O-antigen LPS. The *galU* mutation reduced to less than 1 % the survival of these *Aeromonas* strains in serum, decreased the ability of these strains to adhere and reduced by 1.5 or 2 log units the virulence of *Aeromonas* serotype O34 strains in a septicemia model in either fish or mice. All the changes observed in the *galU* mutants were rescued by the introduction of the corresponding single wild-type gene.

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INTRODUCTION

Mesophilic *Aeromonas* are ubiquitous water-borne bacteria and pathogens of reptiles, amphibians, fish and humans

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Abbreviations: Ara4N, 4-amino-4-deoxyarabinose; COSY, correlation spectroscopy; ESIMS, electrospray ionization mass spectrometry; gHMBC, gradient-enhanced heteronuclear multiple band correlation; HMBC, heteronuclear multiple-band correlation; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; Kdo, 3-deoxy-D-manno-oct-2-ulonic acid; LD-Hep, L-glycero-D-manno-heptose; NOESY, nuclear Overhauser effect spectroscopy; OESY, Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

The GenBank/EMBL/DDBJ accession no. for the nucleotide sequence of the *A. hydrophila* AH-3 *galU* region described in this paper is EF123040.

A table of ¹H and ¹³C NMR data for oligosaccharide fractions 1 and 2 is available with the online version of this paper.

(Austin & Adams, 1996; Janda & Abbott, 1998). The pathogenicity of mesophilic aeromonads has been linked to a number of different determinants, such as toxins, proteases, outer-membrane proteins (Nogueras *et al.*, 2000), lipopolysaccharide (LPS) (Merino *et al.*, 1996), flagella (Merino *et al.*, 1997; Rabaan *et al.*, 2001) and type III secretion system (Vilches *et al.*, 2004). The O-antigen LPS plays an important role as a virulence factor in many Gram-negative bacteria (see Finlay & McFadden, 2006 for a review). We have fully characterized chemically the O-antigen and the core LPS of *Aeromonas hydrophila* strain AH-3 (serotype O34) (Fig. 1; Knirel *et al.*, 2002, 2004), this serotype being frequently found among mesophilic *Aeromonas* strains from clinical sources (Merino *et al.*, 1993). Mutants devoid of the O34-antigen LPS are drastically reduced in some pathogenic features (serum resistance or adhesion to HEp-2 cells) and less virulent for fish and mice in comparison to the wild-type strain (Canals *et al.*, 2006a), thus the O34-antigen LPS is essential for the virulence of mesophilic *Aeromonas* O34 strains. Strain

Table 1. Bacterial strains and plasmids used

Strain/plasmid	Genotype/phenotype*	Source/reference
Strains		
<i>Aeromonas hydrophila</i>		
AH-3	O34, wild-type	Nogueras <i>et al.</i> (2000)
AH-405	AH-3, spontaneous Rif ^R	Nogueras <i>et al.</i> (2000)
AH-2886	AH-3galU: miniTn5Km1 Rif ^R Km ^R	This study
<i>Escherichia coli</i>		
S17-1	<i>hsdR pro recA, λpir</i> , RP4-2 in chromosome Km::Tn7 (Tc::Mu)	De Lorenzo <i>et al.</i> (1990)
XL1-Blue	<i>endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac</i> [F' <i>proAB lacI</i> ^q ΔM15 Tn10 (<i>tet</i> ^R)]	Stratagene
DH5α	F [−] <i>endA hsdR17</i> (r _K [−] m _K ⁺) <i>supE44 thi-1 recA1 gyr-A96 φ80lacZ</i>	Hanahan (1983)
MC1061	<i>thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44, λpir</i>	Rubirés <i>et al.</i> (1997)
Plasmids		
pRK2073	Helper plasmid, Spc ^R	Rubirés <i>et al.</i> (1997)
pLA2917	Cosmid vector, Km ^R Tc ^R	Allen & Hanson (1985)
COS-GALU	pLA2917 cosmid with the complete region, Tc ^R	This study
pGEM-T	PCR cloning vector, Amp ^R	Promega
pBluescriptSK Ap ^r ori of ColE1	Stratagene	
pFS100	pGP704 suicide plasmid, <i>λpir</i> -dependent, Km ^R	Rubirés <i>et al.</i> (1997)
pFS-GALU	pFS100 with an internal fragment of <i>galU</i> , Km ^R	This study
pET30 Xa/LIC	Vector for overexpressing His-tagged proteins, Km ^R	Novagen
pET30-GalU	pET30 derivative overexpressing His ₆ -GalU, Km ^R	This study
pACYC184	Plasmid vector, Cm ^R Tc ^R	Nogueras <i>et al.</i> (2000)
pACYC-GALU	pACYC184 with complete <i>galU</i> of AH-3, Cm ^R	This study

*R, resistant; Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; Tc, tetracycline; Spc, spectinomycin.

DNA by PCR using primers galU-A and galU-B (5'-ACG-GCATATTCCACGTGG-3' and 5'-CTGGAAAAACGGGTCAAGC-3') and subcloned in vector pGEMT, digested with *Eco*RI and subcloned in the *pir* replication-dependent plasmid pFS100 (Rubirés *et al.*, 1997). This plasmid construction (pFS-GALU) was used to obtain *galU*-deficient mutants from several *Aeromonas* strains by a single recombination event as previously described (Rubirés *et al.*, 1997). Plasmid pFS-GALU was isolated, transformed into *E. coli* MC1061 (*λpir*) (Rubirés *et al.*, 1997), and transferred by conjugation from *E. coli* MC1061 to the different rifampicin-resistant (Rif^R) *Aeromonas* strains (from our laboratory collection) as previously described (Rubirés *et al.*, 1997). Chromosomal DNA from 10 transconjugants obtained from each of the different mesophilic *Aeromonas* strains was analysed by Southern blot hybridization with appropriate *galU* DNA probe to obtain defined insertion *galU* mutants as previously described (Nogueras *et al.*, 2000).

Complementation studies. Complementation analysis of the different *galU* mutants was performed by conjugal transfer of wild-type *galU* (pACYC-GALU) cloned in pACYC184 vector. Recombinants were selected on LB agar containing chloramphenicol and rifampicin, and their LPS was isolated and analysed in gels (Darveau & Hancock, 1983).

Plasmid constructions for gene overexpression. For *galU* overexpression, the pET-30 Xa/LIC vector (Novagen) and AccuPrime (Invitrogen, High-fidelity Taq) polymerase were used. The *galU* gene was amplified from plasmid DNA pACYC-GALU using primers AFw (5'-GGTATTGAGGGTCGCATGAAAAAGACACCGCTCGT-3') and ARv (5'-AGAGGAGAGTTAGAGCCCAGCTATTACAACGCTTGACG-3'). The 918 nt PCR product was

electrophoresed in agarose and the DNA band 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, the 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^{−1}). Transformants were analysed for proper construction of pET30-GalU by PCR amplification and sequencing. The plasmid expresses GalU with an in-frame His₆ tag fused to the N terminus.

Preparation of cell-free extracts containing GalU. The His₆-GalU protein was overexpressed from *E. coli* BL21(ΔDE3) (Novagen) containing pET30-GalU by growing the strain 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 OD₆₀₀ 0.6. Expression of the fusion protein 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 at 0.9 W). 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) containing the pET30 vector. Protein expression was monitored by SDS-PAGE, and protein contents of the pET30 and pET30-GalU lysates were determined using the Bio-Rad Bradford assay as directed by the manufacturer.

Purification of His₆-GalU. Lysate containing the His₆-GalU protein was 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). The His₆-GalU was 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 50–500 mM imidazole in buffer A was then applied. His₆-GalU was 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 Centriplus 10 ml YM-30 centrifugal filter device (Amicon Bioseparations), and typical protein preparations contained yields of 0.07–0.08 g ml⁻¹ 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 p.s.i (110.4 MPa). 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 (Filip *et al.*, 1973), and the outer-membrane fraction was collected as described above. Outer-membrane proteins were analysed by SDS-PAGE by the Laemmli procedure (Nogueras *et al.*, 2000). Protein gels were fixed and stained with Coomassie blue. Cultures for chemical analysis of LPS were grown in TSB at 37 °C. LPS was purified by the method of Westphal & Jann (1965), resulting in a 2.3 % yield. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (Darveau & Hancock, 1983). LPS samples were separated by SDS-PAGE or SDS-Tricine-PAGE and visualized by silver staining as previously described (Darveau & Hancock, 1983).

Chemicals. All chemicals used in the LPS isolation and analyses were purchased from Sigma-Aldrich. Sephadex G-50 (S) was from Amersham Biosciences; phosphoglucomutase and glucose-6-phosphate dehydrogenase were from Sigma.

Isolation and mild acid degradation of the LPS. Bacterial cells grown in 14 l TSB for 18 h at 30 °C (5.57 g dry weight) were digested with DNase, RNase (24 h, 1 mg g⁻¹ each) and Proteinase K (36 h, 1 mg g⁻¹) in water (60 ml) at 37 °C with stirring; the suspension was dialysed against distilled water and freeze-dried. Digested cells were extracted with aqueous 45 % phenol at 68 °C (Westphal & Jann, 1965); the extract was dialysed against tap water without separation of the layers and, after removal of residual cells by centrifugation, freeze-dried to give the LPS (300 mg).

Degradation of the LPS

***N,O*-Deacylation.** A LPS sample (100 mg) was deacylated with 4 M potassium hydroxide (4 ml) in the presence of sodium borohydride (20 mg) by heating at 100 °C for 16 h using a block heater. The product was fractionated by high-performance anion-exchange chromatography on a CarboPac PA1 column (Dionex; 250 × 9 mm) using a linear gradient of 0.1→0.8 M sodium acetate in 0.1 M sodium hydroxide at a flow rate of 3 ml min⁻¹ for 1 h. Oligosaccharide fractions 1 and 2 were eluted at a concentration of sodium acetate ~0.5 M and desalted on a column (1.6 × 80 cm) of Sephadex G-15.

O-Deacylation. A LPS sample (20 mg) was treated with anhydrous hydrazine (1 ml) for 1 h at 50 °C. The mixture was cooled down to 0 °C and poured into cooled acetone (200 ml) with stirring. The

precipitated material was collected by centrifugation, dissolved in water and lyophilized to yield O-deacylated LPS (15 mg).

Mild acid degradation. A LPS sample (40 mg) was treated with aqueous 2 % acetic acid (100 °C, 2 h), the precipitate was removed by centrifugation, and the supernatant was fractionated by gel-permeation chromatography on a column (80 × 2.5 cm) of Sephadex G-50 (S) using pyridinium acetate buffer (4 ml pyridine and 10 ml acetic acid in 1 l water) as the eluant and a Waters differential refractometer (USA) for monitoring. Two core oligosaccharide fractions, 3 and 4 (12 and 3 mg, elution volumes 2.84 and 2.98 related to the column void volume, respectively) were obtained as well as a monosaccharide fraction, which was not studied further.

Monosaccharide analysis. A sample of the O-deacylated LPS (2 mg) was *N*-acetylated with acetic anhydride (0.1 ml) in aqueous saturated sodium hydrogen carbonate (1 ml) in the presence of an excess of solid sodium hydrogen carbonate with stirring for 30 min, desalted on Sephadex G-15, hydrolysed with 4 M trifluoroacetic acid (100 °C, 2 h), dried under a stream of nitrogen and conventionally reduced with sodium borohydride. After adding acetic acid and methanol (2 × 1 ml), the sample was dried, acetylated with acetic anhydride (0.5 ml, 100 °C, 20 min), dried and analysed by GLC.

NMR spectroscopy and mass spectrometry. ¹H and ¹³C NMR spectra were recorded using a Varian UNITY/Inova 500 spectrometer in deuterium oxide solutions at 25 °C with acetone standard (δ 2.225 for ¹H and 31.5 for ¹³C) using software from the manufacturer and standard pulse sequences COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy; mixing time 120 ms), NOESY (nuclear Overhauser effect spectroscopy; mixing time 300 ms), ¹H, ³¹P HMQC (heteronuclear multiple-quantum coherence), ¹H, ¹³C HSQC (heteronuclear single-quantum coherence), gHMBC (gradient-enhanced heteronuclear multiple-band correlation; optimized for 5 Hz coupling constant) and HSQC-TOCSY (mixing time 80 ms).

Electrospray ionization mass spectrometry (ESIMS) was performed in the negative ion mode using a Micromass Quattro spectrometer with direct injection in aqueous 50 % acetonitrile with 0.2 % formic acid at a flow rate of 15 µl min⁻¹.

Cell-free extract production and enzymic activity measurements in cell-free extracts or purified recombinant protein (UDP-glucose pyrophosphorylase). Suspensions of bacteria (25 %, w/v), 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-free extracts. Protein concentrations of extracts were determined by using the Bio-Rad Bradford assay as directed by the manufacturer with BSA as the standard.

The activity of UDP-glucose pyrophosphorylase was determined by the procedure of Albrecht *et al.* (1966) with some modification. The reaction mixture contained 100 mM Tris/HCl, pH 8.0, 2.0 mM MgCl₂, 1.0 mM 2-mercaptoethanol, 1.0 mM UDP-glucose, 1.0 mM NADP, 1.0 mM sodium pyrophosphate, 8.0 µM glucose 1,6-bisphosphate, 5 units each of phosphoglucomutase and glucose-6-phosphate dehydrogenase, and the protein sample (either cell-free extract or purified recombinant protein). In a final volume of 1 ml, the reaction was started by the addition of the protein sample, and the change of corresponding absorbance at 340 nm was determined for 1 h. Specific activity was expressed as the amount of NADPH synthesized per min per mg protein at 25 °C.

Measurements of UDP-glucose in dried cells or cell-free extracts. Dried bacterial cells were weighed and then suspended

[50 mg dried cells (ml acid)⁻¹] at 0 °C in 0.02 M HCl. The suspensions were centrifuged at 8900 g for 10 min at 4 °C, and supernatants collected for analyses. UDP-glucose was measured by a modification of the fluorometric assay described by Passonneau & Lowry (1992). Briefly, the HCl extracts were neutralized with NaOH, then after adding 0.1 mM EDTA [100 µl (ml extract)⁻¹], the extracts were boiled for 5 min and centrifuged at 8900 g for 20 min at 4 °C. Aliquots of these supernatants (500 µl) or cell-free extracts (500 µl) were added to 500 µl solutions containing 2 mM MgCl₂, 2 mM NAD⁺, 0.016 unit (ml UDP-glucose dehydrogenase)⁻¹, and 50 mM Tris/HCl, pH 8.1. The samples were incubated for 2 h at 24 °C, then the NADH generated was measured fluorometrically. After subtracting blank values from incubations conducted without UDP-glucose dehydrogenase, the UDP-glucose content of the samples was determined from a standard curve generated with known concentrations of UDP-glucose.

Study of some pathogenic features. Bacterial survival in human serum and adherence assay to HEp-2 cells were performed as previously described (Canals *et al.*, 2006a).

Virulence for fish and mice. The virulence of the strains grown in TSB for 24 h at 20 °C was measured by monitoring their LD₅₀ by the method of Reed and Muench, as previously described (Canals *et al.*, 2006a).

RESULTS

After mini-Tn5 mutagenesis of a rifampicin-resistant isolate of the *A. hydrophila* wild-type strain AH-3 (serotype O34), mutants (Km^r) were screened for resistance to bacteriophage PM1 (a specific bacteriophage for the O34-antigen LPS; Merino *et al.*, 1992) in order to obtain mutants devoid of the O34-antigen LPS. Mutant AH-2886 was one of the 36 amongst 1500 colonies that were initially screened and showed a complete resistance to bacteriophage PM1 when compared to the wild-type (sensitive to this bacteriophage). Whole cells of mutant AH-2886 were unable to react in an enzyme immunoassay with specific antiserum against O34-antigen LPS and, as can be observed in Fig. 2 (lane 2), the LPS of strain AH-2886 was devoid of O34-antigen. The LPS-core showed two bands with a faster mobility in comparison with the wild-type strain. Neither whole AH-2886 cells nor its purified LPS were able to react with specific antiserum against O34-antigen LPS in enzyme immunoassays (Benedí *et al.*, 1989). Strain AH-2886 was unable to grow in minimal medium with galactose as sole carbon source while the wild-type strain was fully able to do so. No differences between the wild-type and the mutant were observed using glucose as sole carbon source. No major differences were found in the growth rate (in Davis minimal medium) between mutant strain AH-2886 (generation time 45 min) and the wild-type strain AH-3 (generation time 40 min). Comparative SDS-PAGE analysis of the outer-membrane protein profile indicated that the mutant and the wild-type strain showed essentially the same protein bands (data not shown). Southern blot analysis using a specific probe for the transposon demonstrated that mutant AH-2886 had a single copy of the mini-transposon in its genome (data not shown). In order to identify the gene(s) responsible for the observed phenotype,

Fig. 2. LPS in SDS-PAGE gels from mesophilic *Aeromonas* strains: lane 1, *A. hydrophila* AH-3 (wild-type); lane 2, *A. hydrophila* AH-3 *galU* mutant (AH-2886); lane 3, AH-2886 mutant complemented with COS-GALU; lane 4, AH-2886 mutant complemented with pACYC-GALU; lane 5, AH-2886 mutant complemented with pLA2917; and lane 6, AH-2806 mutant complemented with pACYC184. O, O-antigen LPS; C, LPS-core.

genomic DNA was isolated from mutant AH-2886, partially digested with *EcoRV*, and DNA fragments of about 5.0 kb were ligated to vector pBluescript SK. The DNA ligation mixture was transformed into *E. coli* DH5 α ; colonies growing on kanamycin and ampicillin plates were recovered, and analysed again by Southern blotting with the mini-Tn5-specific probe to identify clones containing the transposon and surrounding chromosomal DNA from mutant AH-2886. The nucleotide sequence of the insert of one such recombinant plasmid was determined by using oligonucleotides 5'-AGATCTGATC-AAGAGACAG-3' and 5'-ACTTGTGTATAAGAGTCAG-3' (from the mini-Tn5Km-1 flanking regions), and the oligonucleotides T3 and T7 from the plasmid vector. Analysis of the sequence allowed the identification of an ORF, interrupted by the mini-Tn5, which encoded a protein with identity/similarity (73/85 %, respectively) to several GalU proteins from different Gram-negative bacteria. For this reason we named this ORF *galU*.

Cloning and sequencing of an *A. hydrophila* AH-3 genomic region encoding *galU* in *E. coli* K12 strains

A cosmid-based genomic library of *A. hydrophila* AH-3 in *E. coli* DH5 α was constructed as previously described (Nogueras *et al.*, 2000). This library was screened by colony blotting using a DNA probe to the *galU* ORF of *A. hydrophila* AH-3. Several positive recombinant clones were identified, of which clone COS-GALU was chosen for further analysis because it was able to completely complement the AH-2886 mutation by rescuing the wild-type strain pattern of bacteriophage sensitivity, growth in minimal medium with galactose as sole carbon source, and complete LPS as shown by SDS-PAGE (Fig. 2). The DNA sequence of *galU*, where the mini-Tn5 was inserted,

as well as the surrounding region in cosmid COS-GALU, indicates the presence of two flanking ORFs (ORF1 and ORF2) to *galU* (Fig. 3). The upstream incomplete ORF1 transcribed in the same direction as *galU* after a stop codon was found to be similar/identical (71/75 %) in the BLASTX to several dipeptide/tripeptide permeases from *Photobacterium* and *Vibrio* species. The downstream complete ORF2 transcribed in the same direction showed identity/similarity (50/67 %) (in the helix–hairpin–helix region) to competence proteins ComEA of several Gram-negative bacteria), such as *Actinobacillus pleuropneumoniae* and *Shewanella* sp. This genetic organization and the transcriptional direction for these genes (Fig. 3) strongly suggest that the mutant AH-2886 phenotype is attributable only to *galU* insertion mutation, since no polar effects on downstream genes should be expected. This was confirmed since reintroduction of the single *galU* (plasmid pACYC-GALU) rescues the complete wild-type phenotype in mutant AH-2886, i.e. phage sensitivity, growth in minimal medium with galactose as sole carbon source, and presence of O34-antigen LPS (Fig. 2).

Distribution of *galU* among mesophilic *Aeromonas*

We studied the presence of *galU* in 50 different mesophilic *Aeromonas* strains by PCR fragment DNA amplification using genomic DNAs from these strains and oligonucleotides (*galU*for and *galU*rev, see Methods) binding to regions flanking *galU* (Fig. 3). The mesophilic *Aeromonas*

Fig. 3. (a) Genetic organization of the *A. hydrophila* AH-3 *galU* region from plasmid COS-GALU. Transcription direction, stops (lollipops) and primers are indicated. (b) Main homologies of the ORFs, and their size in nucleotides and amino acids.

strains used were environmental strains ($n=15$) isolated mainly from water samples ($n=10$) and shellfish ($n=5$) by using ampicillin dextrin agar or Tergitol agar, and clinical strains ($n=35$) isolated from blood agar supplemented or not with ampicillin depending upon their intestinal ($n=25$) or extraintestinal ($n=10$) origin. All the strains (not belonging to serotype O34) were identified to the species level by 16S rDNA restriction fragment length polymorphism analysis (Figueroa *et al.*, 2000). In all the strains tested a single 1274 bp band was amplified. To confirm that *galU* was indeed present, the nucleotide sequence of the DNA-amplified fragments from several of the *Aeromonas* strains was determined. Thus, *galU* was found in all the mesophilic *Aeromonas* strains tested.

To determine if *galU* is also involved in the production of non-O34-antigen LPSs we constructed *galU*-defined insertion mutants in several mesophilic *Aeromonas* reference strains belonging to different O serotypes (O1, O2, O11, O18, O21 and O44). LPS from the reference rifampicin-resistant strains O1, O2, O11, O18, O21 and O44 showed O-antigen LPS on SDS-PAGE (Fig. 4). All the *galU* mutants obtained with plasmid pFS-GALU from these strains are in agreement with the AH-2886 mutant phenotype: they lack the O-antigen LPS as shown by SDS-PAGE, have a LPS core with two bands with higher migration than the unique wild-type LPS-core band, and are unable to grow in minimal medium with galactose as sole carbon source. All the *galU* mutants can be complemented by the reintroduction of *galU* in plasmid pACYC-GALU as judged by recovery of the O-antigen LPS in gels (Fig. 4) or the ability to grow in minimal medium with galactose as sole carbon source.

Characterization of the mutant AH-2886 LPS

SDS-PAGE analysis of the LPS from mutant AH-2886 strain, growing in either TSB or minimal medium with glucose as sole carbon source, showed the absence of the O34 antigen LPS. Furthermore, a higher mobility of the two LPS-core bands could be observed when compared with the corresponding unique band of the wild-type LPS (Fig. 2, lanes 1 and 2). This suggested that the mutant AH-2886 LPS has a truncated core moiety.

The LPS from mutant AH-2886 was fully deacylated by strong alkaline hydrolysis, and the product was fractionated by high-performance anion-exchange chromatography on CarboPac PA1 to give two oligosaccharides 1 and 2. They were studied by ^1H , ^{13}C and ^{31}P NMR spectroscopy, including full assignment of the signals and fixation of chemical shifts correlations by two-dimensional NMR techniques, including ^1H , ^1H COSY, TOCSY, NOESY, ^1H , ^{31}P HMQC, ^1H , ^{13}C HSQC, HMBC and HSQC-TOCSY experiments according to the published methodology (Duus *et al.*, 2000) (for chemical shift assignments see supplementary Table S1, available with the online version of this paper). The results showed that the core region in the major compound 1 has almost the same structure as the

Fig. 4. LPS in SDS-PAGE gels from mesophilic *Aeromonas* wild-type rifampicin-resistant strains O1, O2, O11, O18, O21 and O44 (lanes 1, 4, 7, 10, 13, 16, respectively); *galU* insertional mutants constructed with pFS-GALU plasmid from strains O1, O2, O11, O18, O21 and O44 (lanes 2, 5, 8, 11, 14, 17, respectively); and *galU* mutants from strains O1, O2, O11, O18, O21 and O44 with pACYC-GALU (lanes 3, 6, 9, 12, 15, 18, respectively). O and C as in Fig. 2.

corresponding oligosaccharide obtained in a similar manner from the R-type LPS of mutant AH-901 strain studied by us earlier (Knirel *et al.*, 2004). The only difference between the two was the lack of the terminal galactose residue from 1, whereas both Gal-containing and Gal-lacking compounds were isolated from the LPS of mutant AH-901 (Knirel *et al.*, 2004). The minor oligosaccharide 2 had a deeply truncated structure with the core restricted to one 3-deoxy-D-manno-oct-2-ulonic acid (Kdo) and three L-glycero-D-manno-heptose (LD-Hep) residues. The structure of 2 was determined by NMR spectroscopy, and it was confirmed to be a part of the major oligosaccharide 1. The structures of oligosaccharides 1 and 2 are shown in Fig. 5.

The mutant AH-2886 LPS was O-deacylated with anhydrous hydrazine and the product analysed by ESIMS. The mass spectrum showed the major peak for a compound with the molecular mass 2729.2 Da, which corresponded to compound 1 with two additional N-linked 3-hydroxy-myristic groups in the lipid A moiety. The oligosaccharide from the mutant AH-2886 LPS differs from the

corresponding product from the AH-901 LPS in the lack not only of Gal from the core but also of 4-amino-4-deoxyarabinose (Ara4N) residues from the lipid A backbone [both phosphate groups in the AH-901 LPS were glycosylated with Ara4N to a significant extent (Knirel *et al.*, 2004)]. The absence of Gal from this compound was finally confirmed by monosaccharide analysis, which revealed Glc, GlcN, DD-Hep and LD-Hep in approximate ratios 1:1:2:4.

The mass spectrum of the O-deacylated LPS contained no peak corresponding to the minor compound 2, probably because of its low content. However, ESIMS analysis of the products obtained by mild acid degradation of the mutant AH-2886 LPS showed the presence of both expected oligosaccharides 3 and 4 (molecular masses 1696.6 and 797.3 Da, respectively, Fig. 5) thus showing that 2 was no artefact induced by alkaline degradation of the LPS. The two types of the LPS core structures are evidently represented by two bands shown by the mutant AH-2886 LPS in SDS-PAGE (Fig. 2, lane 2).

Fig. 5. Structures of the oligosaccharides obtained by strong alkaline (1 and 2) and mild acid (3 and 4) degradations of the LPS from mutant AH-2886. For sugar abbreviations see legend to Fig. 1.

UDP-glucose pyrophosphorylase activity

The genetic analysis as well as the LPS chemical structure of the mutants prompted us to study the enzymic activity that allows the production of UDP-glucose from glucose 1-phosphate (UDP-glucose pyrophosphorylase). *A. hydrophila* AH-3 (wild-type strain) grown on glucose or galactose showed a high enzymic activity (Table 2), when measured as described in Methods. However, mutant strain AH-2886 with or without the plasmid vector (pACYC184) showed a marked reduction in enzymic activity (Table 2), which could be rescued by the reintroduction of the single gene using plasmid pACYC-GALU. As described for other *galU* mutants (Nesper *et al.*, 2001), AH-2886 (or with plasmid vector) was unable to grow on minimal medium with galactose, while the same strain plus plasmid pACYC-GALU was able to do so.

The purified recombinant GalU protein obtained and assayed as described in Methods showed clear UDP-glucose pyrophosphorylase activity, confirming that *galU* does indeed encode this enzyme.

Measurements of UDP-glucose in dried cells and cell-free extracts

The neutralized HCl extracts from dried cells obtained according to Methods from the wild-type strain AH-3 had UDP-glucose contents of 25 ± 3.0 nmol (mg dry weight)⁻¹. However, the values obtained for the mutant strain AH-2886 (either with or without the plasmid vector) were always about 10 ± 0.4 nmol UDP-glucose (mg dry weight)⁻¹. When strain AH-2886 was complemented with plasmid pACYC-GALU, it had a similar UDP-glucose content [24 ± 2.0 nmol (mg dry weight)⁻¹] to that of the wild-type strain. Also, the cell-free extracts from strain AH-3 (wild-type) or the mutant complemented with plasmid pACYC-GALU had UDP-glucose contents at least three times higher than those obtained with mutant strain AH-2886 with or without the plasmid vector.

Table 2. UDP glucose pyrophosphorylase activity in cell extracts of wild-type *A. hydrophila* AH-3 and mutant strains

Strain*	UDP-glucose pyrophosphorylase activity†
AH-3; wild-type	167 ± 12
AH-405; AH-3 rifampicin-resistant	170 ± 15
AH-2886; <i>galU</i> mutant	<0.1 (ND)
AH-2886/pACYC184	<0.1 (ND)
AH-2886/pACYC-GALU	185 ± 20

*Each strain was grown in minimal medium containing glucose as sole carbon source.

†Nanomoles of NADPH synthesized per min per mg protein (means ± SD of three replicates). ND, No detectable activity.

Serum killing

Wild-type mesophilic *Aeromonas* strains belonging to serotypes O1, O2, O11, O18, O21, O34 and O44 were resistant to the bactericidal activity of non-immune human serum (NHS) (98 % survival after 3 h incubation with NHS), while their respective *galU* insertion mutants or the same mutants with plasmid vector pACYC184 were sensitive (less than 1 % survival after 3 h incubation with NHS). The introduction of *galU* (pACYC-GALU) rendered *galU* insertion mutants resistant to the bactericidal activity of NHS (>90 % survival after 3 h incubation with NHS), as found for their respective wild-type strains.

Adherence to HEp-2 cells

Table 3 shows the adhesion of O34 wild-type strain, *galU* mutant and complemented mutant to HEp-2 cells. The wild-type strains showed a high percentage of adherence to HEp-2 cells, while the *galU* mutant (either with the plasmid vector alone) showed a large decrease (60 % reduction) in the percentage adhesion to HEp-2 cells. The introduction of *galU* (pACYC-GALU) rescued completely the adherence to HEp-2 cells in the mutant strain. As can be observed in Table 3, all the *galU* mutants obtained from several mesophilic *Aeromonas* strains (serotypes O1, O2, O11, O18, O21 and O44) showed a large reduction in the adhesion to HEp-2 cells.

Virulence studies

We tested the virulence of strain AH-3 (wild-type) and the corresponding *galU* insertion mutant by LD₅₀, as shown in Table 4. The *galU* mutant AH-2886 showed a higher LD₅₀ (an increase of 1.5–2 log units) in both fish and mice in comparison with the wild-type LD₅₀. No differences were found in mortality between the wild-type strain and its rifampicin-resistant mutant. Complementation of the defined insertion mutant (AH-2886) with pACYC-GALU (carrying the single *galU*) completely restored its virulence for fish and mice (similar LD₅₀ to the wild-type strain; Table 4), while no changes were observed with the plasmid vector alone.

DISCUSSION

In this work, we identified a mutation in *galU* that caused the spontaneous bacteriophage PM1-resistant phenotype of *A. hydrophila* strain AH-3. The *galU* gene product is responsible for synthesis of UDP-glucose from glucose 1-phosphate and UTP, and *galU* mutants are unable to synthesize UDP-glucose by this reaction (Priebe *et al.*, 2004). We proved that *A. hydrophila* GalU is a UDP-glucose pyrophosphorylase responsible for the chemical reaction mentioned above. Besides its function as a substrate for glucosyltransferases resulting in glucosylated surface structures, UDP-glucose plays a well-established biochemical role as a glycosyl donor in the enzymic

Table 3. Adhesion of different *A. hydrophila* strains to HEp-2 cells

Strain and main characteristics	Mean no. of bacteria per HEp-2 cell ($\pm$ SD, $n=3$)	Reduction in adhesion (%)*
AH-3; wild-type	18.3 $\pm$ 2.2	
AH-405; AH-3 rifampicin-resistant mutant	17.9 $\pm$ 2.0	2†
AH-2886; <i>galU</i> insertion mutant from AH-405	5.9 $\pm$ 0.6	68
AH-2886/pACYC-GALU; <i>galU</i> mutant complemented with the single <i>galU</i>	18.1 $\pm$ 2.0	1
AH-2886/pACYC184; <i>galU</i> mutant with plasmid vector alone	6.2 $\pm$ 0.9	66
O1 wild-type	15.7 $\pm$ 1.6	
<i>galU</i> insertion mutant from O1	4.4 $\pm$ 0.5	72
O2 wild-type	16.8 $\pm$ 2.0	
<i>galU</i> insertion mutant from O2	5.3 $\pm$ 0.7	68
O11 wild-type	25.6 $\pm$ 0.1	
<i>galU</i> insertion mutant from O11	7.0 $\pm$ 1.2	73
O18 wild-type	19.3 $\pm$ 2.2	
<i>galU</i> insertion mutant from O18	6.3 $\pm$ 0.8	67
O21 wild-type	15.5 $\pm$ 0.7	
<i>galU</i> insertion mutant from O21	5.1 $\pm$ 0.6	67
O44 wild-type	18.7 $\pm$ 1.4	
<i>galU</i> insertion mutant from O44	5.8 $\pm$ 0.9	69

*The level of adhesion of strain AH-3 was taken as 100 %.

†Student's *t* test; *P*, 0.001.

biosynthesis of carbohydrates. Some examples in Gram-negative bacteria are synthesis of the osmoprotectants trehalose (under conditions of high osmolarity) and membrane-derived oligosaccharide (under conditions of low osmolarity) in *E. coli* (Csonka & Epstein, 1996); synthesis of UDP-4-amino-4-deoxy-L-arabinose, which is incorporated into lipid A of *Salmonella enterica* serovar Typhimurium to protect against antimicrobial cationic peptides, especially polymyxin B (Ernst *et al.*, 1999); and synthesis of UDP-galactose, which serves as a donor for several surface structures, including glycosylated pili in *Neisseria meningitidis* (Stimson *et al.*, 1995). It is less clear whether UDP-glucose additionally acts as an intracellular signal molecule. For one study, it was reported that UDP-glucose controls the expression of σ^S in *E. coli* (Böhlinger *et al.*, 1995).

The LPS from several *galU* mutants in SDS-PAGE, growing in either TSB or minimal medium with glucose as sole carbon source, showed the absence of the O-antigen and a higher mobility of the LPS-core two bands as compared with the corresponding unique band of their respective wild-type strain's LPS-core. Taking advantage of the complete determined LPS structure of strain AH-3 (serotype O34), we could establish that the *galU* mutant from this strain showed two types (represented by two bands on LPS gels) of LPS structures by several chemical analyses: one (the major product representing the LPS band on the gels with slow migration), which is the complete LPS-core but devoid of galactose residue in the outer core where the O34-antigen LPS is linked; and a

second one (the minor product representing the LPS band on the gels with fast migration), which is a deeply truncated structure with the core restricted to one Kdo and three LD-Hep residues (see Figs 1 and 5). The low amount of UDP-glucose in the mutant compared to the wild-type could explain its inability to complete the LPS-core structure. In approximately 10 % of the LPS molecules the mutant is unable to transfer the glucose residue to the inner core, and the LPS-core is not further extended (minor LPS product, oligosaccharide 2 in Fig. 5). In the rest of the LPS molecules (90 %), the mutant is able to incorporate the glucose residue to the inner core and the outer core is added (major LPS product, oligosaccharide 1 in Fig. 5). However, in this case also the mutant cannot complete the LPS-core because it is always unable to incorporate the final Gal residue. Of course the lack of galactose residue explains why the mutant is unable to express the O34-antigen LPS. Furthermore, not only the lack of the galactose residue from the outer core but also the lack of Ara4N residues from the lipid A backbone is due to the low level of UDP-glucose. In view of the results obtained in other *galU* mutants from different *Aeromonas* strains, these facts about LPS seem to be general, at least from what we observed in LPS gels (two bands in the core LPS region lacking the O-antigen LPS). Complementation with the single *A. hydrophila galU* gene completely restored all the LPS defects. To our knowledge this is the first time that well-characterized LPS structural defects have been correlated with a reduced level of a nucleotide diphosphate sugar (UDP-glucose) in the intracellular pool.

Table 4. Virulence for rainbow trout and mice of several *A. hydrophila* AH-3 (serogroup O34)

Strain and main characteristics	LD ₅₀ * for:	
	Rainbow trout	Mice
AH-3; wild-type	10 ^{5.3}	10 ^{7.4}
AH-405; AH-3 rifampicin-resistant mutant	10 ^{5.4}	10 ^{7.6}
AH-2886; <i>galU</i> insertion mutant from AH-405	10 ^{7.5}	10 ^{8.7}
AH-2886/pACYC-GALU; <i>galU</i> mutant complemented with the single <i>galU</i>	10 ^{5.5}	10 ^{7.4}
AH-2886/pACYC184; <i>galU</i> mutant with plasmid vector alone	10 ^{7.7}	10 ^{8.8}

*The values are means of three independent experiments, and the maximum deviation was always <10^{0.4}.

The *Aeromonas galU* mutation would prevent the growth on galactose as sole carbon source for similar reasons to those established in other bacteria. In *E. coli*, galactose is converted to galactose 1-phosphate, which in turn leads to the formation of glucose 6-phosphate, which enters the glycolytic pathway. To convert galactose 1-phosphate to glucose 1-phosphate, the GalT enzyme needs UDP-glucose. In the absence of GalU, not enough UDP-glucose would be present to form glucose 1-phosphate. In addition, the accumulation of galactose 1-phosphate could have a toxic effect and explain the inability to grow in minimal medium with galactose as sole carbon source (Priebe *et al.*, 2004). Furthermore, the low amount of UDP-glucose found in *Aeromonas galU* mutants could not be explained by GalE (UDP-glucose epimerase) or by GalT, due to the inability of these mutants to grow in minimal medium with galactose as sole carbon source. Since there are several reactions where UDP-glucose is used in biosynthesis [e.g. trehalose production in *E. coli* (Purvis *et al.*, 2005)], perhaps some of these reactions are reversible enough in *Aeromonas galU* mutants to generate the low amount of UDP-glucose needed to introduce a single glucose residue into the LPS-core.

The *galU* mutation also drastically affected the serum susceptibility of several *Aeromonas* strains from different serotypes, reduced the ability of these strains to adhere, and decreased the virulence in a septicemic model either in fish or mice. The O-antigen LPS from several Gram-negative bacteria (smooth strains) impedes the attachment of C3b (complement opsonin) to the membrane, and thus the strain becomes resistant to the bactericidal activity of serum (Taylor, 1988). Furthermore, the *Aeromonas* O-antigen LPS has been described as an adhesin which facilitates the attachment to eukaryotic cells (Francki & Chang, 1994).

Results obtained with *waaL* mutants of *P. aeruginosa* (lacking the O-antigen LPS) indicated that LPS of *P.*

aeruginosa plays a role in flagellar biogenesis in this species (Abeyrathne *et al.*, 2005). However, this is not the case in *A. hydrophila galU* mutants because the flagellar biogenesis is unaffected. The alteration in motility observed for these mutants could be attributed to the lack of a hydrophilic surface component such as O-antigen LPS. It is well known that the O-antigen LPS contributes to the wettability of the bacterial surfaces (Toguchi *et al.*, 2000).

All the changes observed in the *galU* mutants in these virulence experiments are rescued by the introduction of the corresponding single wild-type gene, but not by the introduction of the plasmid vector alone, i.e. by the recovery of O-antigen LPS. Thus, O-antigen LPS is essential for mesophilic *Aeromonas* virulence. To our knowledge this is the first report relating the intracellular level of UDP-glucose to virulence through the LPS structure.

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REFERENCES

- Abeyrathne, P. D., Daniels, C., Poon, K. K. H., Matewish, M. J. & Lam, J. S. (2005). Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J Bacteriol* **187**, 3002–3012.
- Albrecht, G. J., Bass, S. T., Seifert, L. L. & Hansen, R. G. (1966). Crystallization and properties of uridine diphosphate glucose pyrophosphorylase from liver. *J Biol Chem* **241**, 2968–2975.
- Allen, L. N. & Hanson, R. S. (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., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Boca Raton, FL: CRC Press.
- Austin, B. & Adams, C. (1996). Fish pathogens. In *The Genus Aeromonas*, pp. 197–243. Edited by B. Austin, M. Altwegg, P. J. Gosling & S. W. Joseph. New York: Wiley.
- Bateman, A., Birney, E., Cerruti, L., Durbin, R., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M. & Sonnhammer, E. L. (2002). The Pfam protein families database. *Nucleic Acids Res* **30**, 276–280.
- Benedí, V. J., Ciurana, B. & Tomás, J. M. (1989). Isolation and characterization of *Klebsiella pneumoniae* unencapsulated mutants. *J Clin Microbiol* **27**, 82–87.
- Böhringer, J., Fischer, D., Mosler, G. & Hengge-Aronis, R. (1995). UDPglucose is a potential intracellular signal molecule in the control

- of expression of σ^S -dependent genes in *Escherichia coli*. *J Bacteriol* **177**, 413–422.
- Canals, R., Jiménez, N., Vilches, S., Regué, M., Merino, S. & Tomás, J. M. (2006a). A gene (uridine diphosphate N-acetylgalactosamine 4-epimerase) is essential for mesophilic *Aeromonas* serotype O34 virulence. *Infect Immun* **74**, 537–548.
- Canals, R., Ramirez, S., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M. & Merino, S. (2006b). Polar flagella biogenesis in *Aeromonas hydrophila*. *J Bacteriol* **188**, 542–555.
- Canals, R., Altarriba, M., Vilches, S., Horsburgh, G., Shaw, J. G., Tomás, J. M. & Merino, S. (2006c). Analysis of the lateral flagella gene system of *Aeromonas hydrophila* AH-3. *J Bacteriol* **188**, 852–862.
- Chang, H. Y., Lee, J. H., Deng, W. L., Fu, T. F. & Peng, H. L. (1996). Virulence and outer membrane properties of a *galU* mutant of *Klebsiella pneumoniae* CG43. *Microb Pathog* **20**, 255–261.
- Csonka, L. N. & Epstein, W. (1996). Osmoregulation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1210–1223. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Darveau, R. P. & Hancock, R. E. (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., Herrero, M., Jakubzik, U. & Timmis, K. N. (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.
- Duus, J. Ø., Gotfredsen, C. H. & Bock, K. (2000). Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. *Chem Rev* **100**, 4589–4614.
- Ernst, R. K., Guina, T. & Miller, S. I. (1999). How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *J Infect Dis* **179** (Suppl. 2), S326–S330.
- Evans, D., Kuo, T., Kwong, M., Van, R. & Fleiszig, S. (2002). *Pseudomonas aeruginosa* strains with lipopolysaccharide defects exhibit reduced intracellular viability after invasion of corneal epithelial cells. *Exp Eye Res* **75**, 635–643.
- Figueras, M. J., Soler, L., Chacón, M. R., Guarro, J. & Martínez-Murcia, A. J. (2000). Extended method for discrimination of *Aeromonas* spp. by 16S rDNA-RFLP. *Int J Syst Evol Microbiol* **50**, 2069–2073.
- Filip, C., Fletcher, G., Wulff, J. L. & Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *J Bacteriol* **115**, 717–722.
- Finlay, B. B. & McFadden, M. (2006). Anti-immunology: evasion of host immune system by bacterial and viral pathogens. *Cell* **124**, 767–782.
- Francki, K. T. & Chang, B. J. (1994). Variable expression of O-antigen and the role of lipopolysaccharide as an adhesin of *Aeromonas sobria*. *FEMS Microbiol Lett* **122**, 97–102.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Janda, J. M. & Abbott, S. L. (1998). Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. *Clin Infect Dis* **27**, 332–344.
- Knirel, Y. A., Shaskov, A. S., Senchenkova, S. N., Merino, S. & Tomás, J. M. (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.
- Knirel, Y. A., Vinogradov, E., Jimenez, N., Merino, S. & Tomás, J. M. (2004). Structural studies on the R-type lipopolysaccharide of *Aeromonas hydrophila*. *Carbohydr Res* **339**, 787–793.
- Komeda, Y., Icho, T. & Iino, T. (1977). Effects of *galU* mutation on flagellar formation in *Escherichia coli*. *J Bacteriol* **129**, 908–915.
- Merino, S., Camprubí, S. & Tomás, J. M. (1992). Characterization of an O-antigen bacteriophage from *Aeromonas hydrophila*. *Can J Microbiol* **38**, 235–240.
- Merino, S., Camprubí, S. & Tomás, J. M. (1993). Incidence of *Aeromonas* spp serotypes O34 and O:11 among clinical isolates. *Med Microbiol Lett* **2**, 48–55.
- Merino, S., Rubires, X., Aguilar, A., Guillot, J. F. & Tomás, J. M. (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.
- Merino, S., Rubires, X., Aguilar, A. & Tomás, J. M. (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.
- Nesper, J., Lauriano, C. M., Klose, K. E., Kapfhammer, D., Kraiss, A. & Reidl, J. (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.
- Nogueras, M. M., Merino, S., Aguilar, A., Benedí, V. J. & Tomás, J. M. (2000). Cloning, sequencing and role in serum susceptibility of porin II from mesophilic *Aeromonas* sp. *Infect Immun* **68**, 1849–1854.
- Passonneau, J. V. & Lowry, O. H. (1992). *Enzymatic Analysis: a Practical Guide*. Clifton, NJ: Humana Press.
- Priebe, G. P., Dean, C. R., Zaidi, T., Meluleni, G. J., Coleman, F. T., Coutinho, Y. S., Noto, M. J., Urban, T. A., Pier, G. B. & Goldberg, J. B. (2004). The *galU* gene of *Pseudomonas aeruginosa* is required for corneal infection and efficient systemic spread following pneumonia but not for infection confined to the lung. *Infect Immun* **72**, 4224–4232.
- Purvis, J. E., Yomano, L. P. & Ingram, L. O. (2005). Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress. *Appl Environ Microbiol* **71**, 3761–3769.
- Rabaan, A. A., Gryllos, I., Tomás, J. M. & Shaw, J. G. (2001). Motility and the polar flagellum are required for *Aeromonas caviae* adherence to Hep-2 cells. *Infect Immun* **69**, 4257–4267.
- Rioux, S., Galarneau, C., Harel, J., Frey, J., Nicolet, J., Kobisch, M., Dubreuil, J. D. & Jacques, M. (1999). Isolation and characterization of mini-Tn10 lipopolysaccharide mutants of *Actinobacillus pleuropneumoniae* serotype 1. *Can J Microbiol* **45**, 1017–1026.
- Rubirés, X., Saigí, F., Piqué, N., Climent, N., Merino, S., Albertí, S., Tomás, J. M. & Regué, M. (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.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sandlin, R. C., Lampel, K. A., Keasler, S. P., Goldberg, M. B., Stolzer, A. L. & Maurelli, A. T. (1995). Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. *Infect Immun* **63**, 229–237.
- Sanger, F., Nicklen, S. A. & Coulson, R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.
- Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H. R., Payne, G., Saunders, J. R., Jennings, M. P., Barker, S. & other authors (1995). Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamid-2,4,6-trideoxyhexose. *Mol Microbiol* **17**, 1201–1214.
- Taylor, P. W. (1988). Bacterial resistance to complement. In *Virulence Mechanisms of Bacterial Pathogens*, pp. 107–120. Edited by J. A. Roth. Washington, DC: American Society for Microbiology.

Toguchi, A., Siano, M., Burkart, M. & Harshey, R. M. (2000). Genetics of swarming motility in *Salmonella enterica* serovar typhimurium: critical role for lipopolysaccharide. *J Bacteriol* **182**, 6308–6321.

Vilches, S., Urgell, C., Merino, S., Chacón, M. R., Soler, L., Castro-Escarpulli, G., Figueras, M. J. & Tomás, J. M. (2004). Complete type III secretion system of a mesophilic *Aeromonas hydrophila* strain. *Appl Environ Microbiol* **70**, 6914–6919.

Westphal, O. & Jann, K. (1965). Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. In *Methods in Carbohydrate Chemistry*, vol. 5, *General Polysaccharides*, pp. 83–91. Edited by R. L. Whistler. New York: Academic Press.

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