The inner-core lipopolysaccharide biosynthetic *waaE* gene: function and genetic distribution among some *Enterobacteriaceae*

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To determine the function of the *waaE* gene in the biosynthesis of the inner-core LPS of *Klebsiella pneumoniae*, a *waaE* non-polar mutant has been constructed. Data obtained from the comparative chemical analysis of LPS samples obtained from the wild-type, the mutant strain and the complemented mutant demonstrated that the *waaE* gene is involved in substitution of α -L-glycero-D-manno-heptopyranose I (L,D-Heppl) at the O-4 position by a β -D-glucopyranose (β -D-Glcp) residue. In addition, DNA amplification and nucleotide sequence determination studies revealed that *waaE* homologues located between the *waaA* and *coaD* genes are present in clinical isolates of *Enterobacteriaceae* containing the structure β -D-Glcp-(1→4)- α -L,D-Heppl (*K. pneumoniae*, *Proteus mirabilis* and *Yersinia enterocolitica*), as well as in strains of *Serratia marcescens* and *Enterobacter aerogenes* of unknown LPS-core structures. Complementation studies using non-polar *waaE* mutants prove that all the *waaE* homologues perform the same function. Furthermore, *K. pneumoniae*, *Ser. marcescens* and *P. mirabilis* non-polar *waaE* mutants showed reduced adhesion and pathogenicity. In addition, the *Ser. marcescens* and *P. mirabilis* *waaE* mutants showed reduced swarming motility and ability to form biofilms *in vitro*. All these characteristics were rescued by reintroduction of the *waaE* gene independently of its origin. An easy DNA amplification method to detect this gene was established, which also helps in finding the potential presence of this structural feature [β -D-Glcp-(1→4)- α -L,D-Heppl] in the inner-core LPS of *Enterobacteriaceae* members with unknown LPS-core structures.

Keywords: LPS, *waaE* homologues, non-polar mutant characterization, cross-complementation

INTRODUCTION

In Gram-negative bacteria LPS is one of the major structural and immunodominant molecules of the outer membrane. It consists of three domains: lipid A, core

Abbreviations: α -D-Gal(p)A, α -D-galacturonic acid; GlcN(p), glucosamine; β -D-Glc(p), β -D-glucopyranose; L,D-Hep(p), α -L-glycero-D-manno-heptopyranose; Kdo(p), 3-deoxy-D-manno-octulopyranosonic acid; BATH, bacterial adherence to hydrocarbons; HIC, hydrophobic interaction chromatography; NHS, non-immune human serum.

The GenBank accession number for the *waaE* gene sequences of *P. mirabilis* CECT170, *Y. enterocolitica* R102 and *Ent. aerogenes* CECT684 reported in this paper are AY075039, AY075041 and AY075040, respectively.

oligosaccharide and O-specific antigen or O side chain. The genetics of LPS core biosynthesis have been studied in some genera of the *Enterobacteriaceae* and in other Gram-negative bacteria (Allen & Maskell, 1996; Kalher *et al.*, 1996; Filiatrault *et al.*, 2000; Regué *et al.*, 2001). The genes involved in LPS core biosynthesis in *Escherichia coli* and *Salmonella enterica* are usually found in the *waa* (*rfa*) gene cluster (Schnaitman & Klena, 1993; Heinrichs *et al.*, 1998). [The nomenclature proposed by Reeves *et al.* (1996) for proteins and genes involved in core LPS biosynthesis is used in this work.]

Comparison of the known structures of core LPS from different *Enterobacteriaceae* reveals that the inner core

structure contains two residues of 3-deoxy-D-manno-octulopyranosonic acid (Kdop) and three residues of L-glycero-D-manno-heptopyranose (L,D-HeppI, L,D-HeppII and L,D-HeppIII) (Radziejewska-Lebrecht *et al.*, 1994; Heinrichs *et al.*, 1998; Vinogradov *et al.*, 2000; Vinogradov & Perry, 2001). In addition, some *Enterobacteriaceae*, like *E. coli*, contain a phosphoryl group modification at the L,D-HeppI residue (Heinrichs *et al.*, 1998). In other species, like *Klebsiella pneumoniae*, a substitution of L,D-HeppI at the O-4 position by a β -D-glucopyranose residue (β -D-Glcp) is found (Vinogradov & Perry, 2001). Furthermore, in *K. pneumoniae*, *Proteus mirabilis* and *Yersinia enterocolitica* a substitution of L,D-HeppII at the O-3 position by an α -D-galacturonic acid residue (α -D-GalpA) residue is found (Radziejewska-Lebrecht *et al.*, 1994; Vinogradov *et al.*, 2000; Vinogradov & Perry, 2001), while a substitution of L,D-HeppII at the O-3 position by a Glcp residue is found in *E. coli* (Heinrichs *et al.*, 1998).

The *waaE* gene is present in *K. pneumoniae* and *Serratia marcescens waa* gene clusters, but not in *waa* gene clusters from *E. coli* and *Sal. enterica* (Guasch *et al.*, 1996; Regué *et al.*, 2001). In this study we present results showing that the *K. pneumoniae waaE* gene is responsible for the attachment of the branched Glcp residue to L,D-HeppI. In addition, the presence of *waaE* homologues is determined for representative species of *Enterobacteriaceae*, and we show by complementation studies that all the *waaE* homologues perform the same function in inner core biosynthesis. We also investigated *waaE* gene function in some pathogenic features, as well as its role in motility and biofilm formation.

METHODS

Bacterial strains, plasmids and growth conditions. *Serratia marcescens* N28b (Guasch *et al.*, 1996) and *Klebsiella pneumoniae* 889, (serovar O:8) (Hansen *et al.*, 1999), as well as *Escherichia coli*, *Salmonella enterica*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Proteus mirabilis* and *Yersinia enterocolitica* strains isolated from clinical samples were used in this study. As controls the following strains from the Spanish Culture Collection were used: *Y. enterocolitica* R102 (O:8), *P. mirabilis* CECT170 (O:3) and *Enterobacter aero-*

genes CECT684. Bacterial strains were grown in LB Miller broth and LB Miller agar (Miller, 1972). Plasmids used in this study and their characteristics are shown in Table 1. LB media was supplemented with ampicillin (100 μ g ml⁻¹ or 3 mg ml⁻¹) and chloramphenicol (25 μ g ml⁻¹) when required.

General DNA methods. General DNA manipulations were done as described by Sambrook *et al.* (1989). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment) and alkaline phosphatase were used as recommended by the suppliers.

LPS isolation and analysis. Cultures for analysis of LPS were grown in TSB at 37 °C. LPS was purified by the method of Westphal & Jann (1965). For screening purposes LPS was obtained after proteinase K digestion of whole cells (Hitchcock & Brown, 1983). LPS samples were separated by SDS-PAGE or SDS-Tricine-PAGE and visualized by silver staining as described previously (Tsai & Frasch, 1982; Pradel & Schnaitman, 1991). For chemical analyses, purified LPS was deacylated by treatment with acetic acid (Süsskind *et al.*, 1998) and the core region was purified by column chromatography on Sephadex G50. The core fractions were hydrolysed with 1 M trifluoroacetic acid for 4 h at 100 °C. Monosaccharides were analysed as their alditol acetate derivatives by GLC on a Rtx-2330 column (Restek). Alditol acetate monosaccharides were obtained by a procedure described by Vinogradov *et al.* (1992). Colorimetric analysis of Kdo was performed by the method of Karkhanis *et al.* (1978). Methylation of carboxyl-reduced core oligosaccharides was performed according to the method of Ciucanu & Kerek (1984). Briefly, the methylated samples were hydrolysed in 2 M trifluoroacetic acid at 100 °C for 2 h, reduced, acetylated and analysed using GLC-MS.

DNA sequencing and computer analysis of sequence data. Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method (Sanger *et al.*, 1977) with the ABI Prism dye terminator cycle sequencing kit (Perkin Elmer). The relevant PCR-amplified genomic DNAs were sequenced by using oligonucleotides used in the amplification reactions. Other sequence-derived oligonucleotides were used to extend the nucleotide sequence. Primers used for genomic DNA amplification experiments and for DNA sequencing were purchased from Pharmacia LKB Biotechnology. The DNA sequence was translated in all six frames and all ORFs were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from non-redundant GenBank and EMBL databases by using the BLAST (Altschul *et al.*, 1990, 1997) and FASTA (Pearson &

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics*	Source or reference
pKO3	Cm ^r <i>sacB</i> temperature-sensitive replication	Link <i>et al.</i> (1997)
pKO3 Δ waaE	pKO3 containing the engineered <i>waaE</i> deletion	Regué <i>et al.</i> (2001)
pGEMT	Ap ^r plasmid vector	Promega
pGEMT-WaaE _{sm}	<i>waaE</i> gene from <i>Ser. marcescens</i> in pGEMT	This work
pGEMT-WaaE _{Kp}	<i>waaE</i> gene from <i>K. pneumoniae</i> in pGEMT	Regué <i>et al.</i> (2001)
pGEMT-WaaE _{pm}	<i>waaE</i> gene from <i>P. mirabilis</i> in pGEMT	This work
pGEMT-WaaE _{ye}	<i>waaE</i> gene from <i>Y. enterocolitica</i> in pGEMT	This work
pGEMT-WaaE _{ea}	<i>waaE</i> gene from <i>Ent. aerogenes</i> in pGEMT	This work

* Ap^r, Ampicillin resistance; Cm^r, chloramphenicol resistance.

Lipman, 1988) network service at the National Center for Biotechnology Information and the European Biotechnology Information, respectively. CLUSTAL W (Thompson *et al.*, 1994) was used for multiple sequence alignments.

DNA amplification. Genomic DNA from different strains of representative *Enterobacteriaceae* was isolated and used as template in PCR experiments using primers designed to amplify DNA fragments containing the 3' end of the *waaA* gene and the 3' end of the *fpg* gene, respectively, at their ends. Oligonucleotide pairs Pr2 (5'-GATGGCGGGGAAC-3') and Fpg2 (5'-CATTGCCAACATGGTGAA-3'), kdtb1 (5'-GTC-ATGGGATCGAACGTC-3') and Yer3 (5'-CTGTTGACCG-ACGAAGACTA-3'), and Nuk9 (5'-TGCTTTATACCACT-CTACT-3') and Nuk10 (5'-GATAAACGACCACTCTTTG-3') were used to amplify and to subclone the *waaE* homologues from *P. mirabilis*, *Y. enterocolitica* and *Ent. aerogenes*, respectively, in vector pGEMT.

Complementation studies. Complementation analysis of relevant *waaE* mutants was performed by transformation of *waaE* genes from different *Enterobacteriaceae* cloned in pGEMT. Transformants were selected on LB agar containing ampicillin (100 µg ml⁻¹) and LPS was isolated and analysed by Tricine-SDS-PAGE.

Serum killing. The survival of exponential-phase bacteria in non-immune human serum was measured as described previously (Merino *et al.*, 1992).

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by two different methods. The first method used was hydrophobic interaction chromatography (HIC) on phenylsepharose as described by Källénus *et al.* (1985). Briefly, bacteria were suspended in 10 mM PBS (pH 7.4) to an OD₄₇₀ of 1.0, applied to a phenylsepharose column and eluted with 4 M NaCl. The eluate was collected and its OD₄₇₀ determined. The second method used was bacterial adherence to hydrocarbons (BATH) as described previously by Rosenberg *et al.* (1980). Briefly, cells were washed twice in phosphate/urea/magnesium buffer (pH 7.1), suspended in the same buffer at an OD₄₀₀ of 1.0 and vortexed with various volumes of hydrocarbon. The OD₄₀₀ of the aqueous phase was expressed as a percentage of the OD₄₀₀ of a standard volume of untreated cells.

Bacterial adherence assay. The adherence assay using *K. pneumoniae* strains to uroepithelial cells (UEC) was done as described previously (Falkowski *et al.*, 1986; Merino *et al.*, 1997).

Urinary tract infections in rats. The bacterial strains used to establish infection were grown overnight in LB agar supplemented with antibiotics when needed and gently resuspended in PBS to the appropriate concentration. In each experiment, 12 female Wistar rats (200–250 g; strain CFHB; Interfauna UK) were used. Ten animals were infected and two were used as controls. The infections were established and quantified as described previously (Camprubí *et al.*, 1993).

Swimming and swarming motility assay. Freshly grown bacterial colonies were transferred with a sterile toothpick into the centre of motility agar (1% tryptone, 0.5% NaCl, 0.25% agar) or swarm agar (LB base, 0.6% agar). The plates were incubated at 37 °C for 16–24 h and motility was assessed by examining the migration of bacteria through the agar from the centre towards the periphery of the plate.

Transmission electron microscopy. Bacterial suspensions were placed on Formvar-coated copper grids and negatively stained with a 2% solution of potassium phosphotungstate. Bacteria were then viewed on a Phillips EM 400 transmission electron microscope.

Biofilm formation. Quantitative biofilm formation was performed in a microtitre assay as described by Pratt & Kolter (1998) with minor modifications. Briefly, bacteria were grown on trypticase soy agar (TSA) and several colonies were gently resuspended in trypticase soy broth (TSB) with or without the appropriate antibiotics; 100 µl aliquots were placed in a microtitre polystyrene plate and incubated for 48 h at 30 °C without shaking. After the bacterial cultures were poured out, the plate was washed extensively with water, fixed with 2.5% glutaraldehyde, washed once with water and stained with 0.4% crystal violet solution. After solubilization of the crystal violet with ethanol/acetone (80/20, v/v) the absorbance was determined at 570 nm.

RESULTS

Construction and analysis of a *K. pneumoniae* 889 *waaE* mutant

We have reported previously the presence of the *waaE* gene in *K. pneumoniae* and *Ser. marcescens* N28b (Guasch *et al.*, 1996; Regué *et al.*, 2001). Monosaccharide composition analysis and complementation experiments with the *Haemophilus ducreyi* *lgtF* gene suggested that the *waaE* gene encodes the glucosyltransferase involved in the attachment of a Glc residue by a β1,4 linkage to L,D-HeppI in the LPS inner core. To confirm this proposed function *K. pneumoniae* 889 (O8:K69) (Hansen *et al.*, 1999) was used because its chemical core LPS structure has been recently updated

Fig. 1. Conserved region in the core LPS structure of *K. pneumoniae* (Vinogradov & Perry, 2001).

Fig. 2. SDS-Tricine PAGE analysis of LPS samples from *K. pneumoniae* 889 (lane 1), *K. pneumoniae waaE* derivative (889-1; 2), *K. pneumoniae* 889-1(pGEMT-WaaE_{Kp}) (3), *K. pneumoniae* 889-1(pGEMT-WaaE_{sm}) (4), *K. pneumoniae* 889-1(pGEMT-WaaE_{pm}) (5), *K. pneumoniae* 889-1(pGEMT-WaaE_{ve}) (6), *K. pneumoniae* 889-1(pGEMT-WaaE_{ea}) (7), *Ser. marcescens* wild-type N28b (8), *Ser. marcescens waaE* derivative (N28b-16; 9), *Ser. marcescens* N28b-16(pGEMT-WaaE_{sm}) (10), *Ser. marcescens* N28b-16(pGEMT-WaaE_{Kp}) (11), *Ser. marcescens* N28b-16(pGEMT-WaaE_{pm}) (12), *Ser. marcescens* N28b-16(pGEMT-WaaE_{ve}) (13) and *Ser. marcescens* N28b-16(pGEMT-WaaE_{ea}) (14).

using the non-encapsulated mutant NRC6121 (Fig. 1) (Vinogradov & Perry, 2001). To construct a *K. pneumoniae* 889 *waaE* non-polar mutant an in-frame tagged deletion was used. Plasmid pKO3ΔwaaE, containing the engineered deletion, was used to introduce the *waaE* deletion into *K. pneumoniae* 889 by double recombination as described previously (Link *et al.*, 1997; Regué *et al.*, 2001). Candidate mutants were screened by PCR and one of them, strain 889-1, was proved to contain the

desired mutation by DNA nucleotide sequence determination of the *waaA-coaD* region. LPS from strains 889 (wild-type) and 889-1 (*waaE*) were extracted and analysed by SDS-Tricine-PAGE. The core LPS from strain 889-1 migrates faster than that of the wild-type strain and it appears that the mutant LPS still contains O antigen, although in smaller amounts than wild-type LPS (Fig. 2, lanes 1 and 2). This behaviour is identical to that found for other *K. pneumoniae waaE* mutants (Regué *et al.*, 2001). LPS samples from the wild-type strain and the *waaE* mutant were deacylated by mild acid hydrolysis and the core regions were recovered on Sephadex G50 chromatography. Monosaccharide analysis of the core oligosaccharides from the LPS of *K. pneumoniae* 889 revealed the presence of Glc, GalA, glucosamine (GlcN), L,D-Hep and Kdo. The absence of Glc was the most relevant change found in the core oligosaccharides from the LPS of *K. pneumoniae* 889-1.

The core oligosaccharides were methylated as described by Ciucanu & Kerek (1984) and subjected to GLC-MS analysis. As expected from the known *K. pneumoniae* core structure (Vinogradov & Perry, 2001) 2,3,4-tri-O-methylglucitol, 2,3,4,6-tetra-O-methylgalactitol, 2,3,6-tri-O-methylgalactitol, 2,4,6-tri-O-methyl-L-glycero-D-manno-heptitol and 2,6,7-tri-O-methyl-L-glycero-D-manno-heptitol were identified among the methylation products obtained from the wild-type core LPS (Table 2). The *waaE* mutant core LPS fraction was characterized by the absence of 2,3,4-tri-O-methylglucitol and 2,3,4,6-tetra-O-methylgalactitol, and the presence of 2,4,6,7-tetra-O-methyl-L-glycero-D-manno-heptitol instead of 2,6,7-tri-O-methyl-L-glycero-D-manno-heptitol (Table 2). No differences in core LPS composition and methylated products were found between the wild-type strain and the *waaE* mutant complemented with the wild-type *waaE* gene (Table 2). These results show that

Table 2. Methylation analysis of core oligosaccharides released by mild acid hydrolysis from wild-type *K. pneumoniae* 889, 889-1 (*waaE* mutant) and 889-1 harbouring plasmid pGEMT-WaaE_{Kp}

Data are reported as relative amounts of major methylated products. ND, Not detected.

Major methylated products	Derived from:	Strain		
		889	889-1	889-1(pGEMT-WaaE _{Kp})
2,3,6-Me ₃ -Gal	D-GalApII	0.8	0.2	0.8
2,4,6-Me ₃ -Hep	L,D-HepII	1.0	1.0	1.0
2,3,4,6,7-Me ₅ -Hep	L,D-HepIII	0.75	0.7	0.8
2,6,7-Me ₃ -Hep	L,D-HepI*	0.7	ND	0.8
2,3,4-Me ₃ -Glc	D-Glcp	1.1	ND	1.0
2,3,4,6-Me ₄ -Gal	D-GalApI	0.7	ND	0.8
2,4,6,7-Me ₄ -Hep	L,D-HepI†	ND	0.8	ND

* Derived from L,D-HepI substituted at the O-4 position by D-Glcp.

† Derived from L,D-HepI non-substituted at the O-4 position.

Fig. 3. Organization of the distal end of the *waa* gene cluster in members of the *Enterobacteriaceae* deduced from PCR genomic DNA amplification and determination of the nucleotide sequence of at least one strain from each species.

the major effect of the *waaE* mutation is to preclude the attachment of a Glcp residue by a β 1,4 linkage to L,D-HeppI in the LPS inner core (Fig. 1). The *waaE* mutant LPS is devoid of Glcp and GalA1p residues, explaining its faster mobility in SDS-Tricine-PAGE. These results are in agreement with the initially proposed glucosyl-transferase function for the *K. pneumoniae* WaaE protein (Regué *et al.*, 2001).

Distribution of the *waaE* gene in members of the *Enterobacteriaceae*

In both *K. pneumoniae* and *Ser. marcescens* the *waaE* gene was found located between the *waaA* and *coaD* genes (Guasch *et al.*, 1996; Regué *et al.*, 2001). In the *Enterobacteriaceae* studied the *coaD* and *fpg* genes have been found just downstream from the *waa* gene cluster (Sofia *et al.*, 1994; Heinrichs *et al.*, 1998; Regué *et al.*, 2001). To determine the extent of the presence of the *waaE* gene in clinical isolates of different *Enterobacteriaceae*, PCR DNA amplification experiments using genomic DNA and primers annealing to the *waaA* and *fpg* genes were used. Oligonucleotides NUE1 (5'-TACCGCCATCCGCTAAAC-3') and NUE2 (5'-CGCTGGCAGAGTGTGAATT-3') were designed from the *E. coli* genomic sequence. Using genomic DNA from several *E. coli* strains, as well as several *Sal. enterica* serovars and *Shi. dysenteriae*, *Shi. flexneri* and *Shi. sonnei* strains as template, oligonucleotide pair NUE1/NUE2 generated PCR amplification DNA fragments of about 2.0 kb. The DNA nucleotide sequence of the amplified products was determined for at least one strain of each genus confirming the gene order *waaA-coaD-fpg* and the absence of the *waaE* gene in the tested strains of these species (Fig. 3).

In addition, we designed oligonucleotides CNN5 (5'-AGGCGTGGTGGCAAACAAGAT-3') and FpgI (5'-AACCGTGGCTGATGGATAA-3') from the nucleotide sequence of the *waa* gene clusters from *K. pneumoniae* and *Ser. marcescens*. Using genomic DNA from *Y. enterocolitica* strains as template, primer pair CNN5-

FpgI generated PCR amplification DNA fragments of about 3.0 kb. Oligonucleotide pairs KdtAF (5'-GGAAACAGAACTGTGGC-3') and FpgI, and Nuk9 (5'-TGCTTTATACCACCCTACT-3') and Nuk10 (5'-GATAAACGACCACTCTTTG-3'), generated PCR amplification DNA fragments of about 3.0 kb when using *P. mirabilis* and *Ent. aerogenes* DNA as template, respectively. The DNA nucleotide sequence of the amplified products was determined for *P. mirabilis* CECT170, *Y. enterocolitica* R102 and *Ent. aerogenes* CECT684. In these three strains *waaE* homologues were found between the *waaA* and *coaD* genes (Fig. 3).

An alignment of the known *Enterobacteriaceae* WaaA proteins was used to design degenerate oligonucleotide MRI (5'-ATGGRACYGARCTITGGCC-3'). The oligonucleotide pair MRI/Fpg1 allowed the amplification of the *waaA-fpg* region in all the strains of *Enterobacteriaceae* tested in this study, using genomic DNA as template. Amplified fragments of about 1.7 kb were obtained when using genomic DNA from strains of different serovars of *Sal. enterica*, *E. coli*, *Shi. dysenteriae*, *Shi. flexneri* and *Shi. sonnei* as template. In addition, amplified fragments of about 2.7 kb were obtained when using genomic DNA from different strains of *K. pneumoniae*, *Ser. marcescens*, *P. mirabilis*, *Y. enterocolitica* and *Ent. aerogenes* as template. Representative results of such amplification experiments are shown in Fig. 4. Thus, this oligonucleotide pair can be used to detect the presence of the *waaE* gene in members of the *Enterobacteriaceae* and to predict the presence of a Glcp residue attached via a β 1,4 linkage to the L,D-HeppI residue in the inner-core LPS (Fig. 1).

Construction and preliminary analysis of *Ser. marcescens* N28b and *P. mirabilis* CECT170 *waaE* mutants

To construct *waaE* non-polar mutants in *Ser. marcescens* N28b and *P. mirabilis* CECT170, an in-frame tagged deletion was constructed using the previously mentioned plasmid pKO3. Plasmid pKO3 Δ waaE was

Fig. 4. PCR-amplified DNA products obtained using oligonucleotides MRI and FPG1 and genomic DNA from *E. coli* (lane 1), *Sal. enterica* serovar Enteritidis (2), *Shi. sonnei* (3), *K. pneumoniae* (4), *Ser. marcescens* (5), *P. mirabilis* (6), *Y. enterocolitica* (7) and *Ent. aerogenes* (8). Lane 0, molecular mass standard (1 kb plus DNA ladder; Gibco-BRL).

used to introduce the mutation into *Ser. marcescens* N28b and *P. mirabilis* CECT170 by double recombination (Link *et al.*, 1997; Regué *et al.*, 2001). Candidate mutants were screened by PCR and *Ser. marce-*

scens N28b-16 and *P. mirabilis* CECT170-8 were shown to contain the desired mutation by DNA nucleotide sequence determination of the *waaA-coaD* region. LPS from strains N28b (wild-type) and N28b-16 (*waaE*) were extracted and analysed by SDS-Tricine-PAGE. The core LPS from strain N28b-16 migrates faster than that of the wild-type strain and it appears that the mutant LPS still contains O antigen, although in smaller amounts than wild-type LPS (Fig. 2, lanes 8 and 9). This behaviour is identical to that found for *K. pneumoniae waaE* mutants (Regué *et al.*, 2001). Similar results were obtained for *P. mirabilis* strains CECT170 (wild-type) and CECT170-8 (*waaE*).

Cross complementation among *waaE* homologues

Alignment of the known *Enterobacteriaceae* WaaE proteins revealed that all these proteins share a significant level of similarity (76%) and identity (53%). This similarity strongly suggests that these proteins perform the same function in the biosynthesis of the inner-core LPS. To confirm this functional relationship, the *waaE* gene homologues from *Ent. aerogenes*, *Y. enterocolitica* and *P. mirabilis* were cloned in vector pGEM-T. These recombinant plasmids were introduced into the *waaE* mutant strains *K. pneumoniae* 889-1 and NC16 (Regué *et al.*, 2001), *Ser. marcescens* N28b-16 and *P. mirabilis* CECT170-8. The LPS from mutant strains 889-1 and N28b-16 transformed with pGEMT-WaaE_{Kp}, pGEMT-WaaE_{Sm}, pGEMT-WaaE_{Pm}, pGEMT-WaaE_{Ye} and pGEMT-WaaE_{Ea} showed a wild-type migration pattern (Fig. 2). Similar results were obtained for *K. pneumoniae* NC16 and *P. mirabilis* CECT170-8. These results

Table 3. Survival of exponential-phase *K. pneumoniae* strains in NHS

All the assays were done at least in triplicate.

Strain	Percentage inoculum surviving at:		
	1 h	2 h	3 h
889 (O8:K69, wild-type)	98 ± 3	103 ± 6	115 ± 7
889-1 (<i>waaE</i>)	18 ± 2	5 ± 0.8	0.8 ± 0.4
889-1 (<i>waaE</i>)(pGEMT)	17 ± 1	3 ± 1.2	0.4 ± 0.2
889-1(pGEMT-WaaE _{Kp})	95 ± 2	98 ± 4	103 ± 8
889-1(pGEMT-WaaE _{Sm})	92 ± 4	99 ± 5	100 ± 7
889-1(pGEMT-WaaE _{Pm})	89 ± 8	94 ± 3	99 ± 12
889-1(pGEMT-WaaE _{Ye})	99 ± 5	104 ± 4	110 ± 10
889-1(pGEMT-WaaE _{Ea})	94 ± 2	97 ± 5	105 ± 4
52145 (O1:K2, wild-type)	106 ± 11	117 ± 17	140 ± 22
NC16 (<i>waaE</i> from 52145)	15 ± 4	7 ± 2	0.6 ± 0.3
NC16 (pGEMT)	14 ± 8	5 ± 3	0.4 ± 0.2
NC16(pGEMT _{Kp})	98 ± 9	107 ± 12	127 ± 15
NC16(pGEMT _{Sm})	100 ± 5	109 ± 14	121 ± 21
NC16(pGEMT _{Pm})	96 ± 7	104 ± 9	113 ± 16
NC16(pGEMT _{Ye})	99 ± 4	111 ± 6	122 ± 17
NC16(pGEMT _{Ea})	93 ± 11	102 ± 11	120 ± 13

confirm that the *waaE* gene homologues perform the same role in inner-core LPS biosynthesis.

Contribution of the *waaE* gene to some pathogenic features

Since all the *waaE* homologues studied appear to have the same function, we decided to investigate the contribution of *waaE* genes to some pathogenic features involving surface molecules by using the set of isogenic mutants obtained. The *waaE* mutant strains *K. pneumoniae* 889-1 and NC16, *Ser. marcescens* N28b-16 and *P. mirabilis* CECT170-8 were sensitive to the bactericidal activity of NHS (non-immune human serum). The respective wild-type strains, as well as the mutants complemented with the *waaE* gene homologues, were resistant to this activity. As an example, Table 3 shows the results obtained for *K. pneumoniae* strains. Surface hydrophobicity of *K. pneumoniae waaE* mutant strain 889-1, measured by both the HIC and the BATH methods, was higher than the wild-type strain or the *waaE* mutant complemented with the *waaE* gene homologues. The *Ser. marcescens* and *P. mirabilis waaE* mutants were also more hydrophobic than their respective wild-type strains and the complemented *waaE* mutants.

The physico-chemical properties of the bacterial surface are basic in the interactions (association or adhesion) between bacteria and the eukaryotic cells of the host tissues. Since *K. pneumoniae*, *P. mirabilis* and *Ser. marcescens* are important causes of nosocomial urinary tract infections, the adhesion of these strains to uroepithelial (UEC) cells was measured (Table 4). We found that *waaE* mutants showed an approximately two-fold reduction in their ability to adhere to UEC cells in comparison to their respective wild-type strains or the complemented mutants, again independent of *waaE* origin.

To test if the changes in adhesion resulted in a lesser ability to infect and colonize, a urinary tract rat experimental infection model was used because it has been shown previously to be a good model to assay the *in vivo* colonization of some *K. pneumoniae* strains (Merino *et al.*, 2000). In this assay the total number of infected animals and the viable bacteria found in kidney, bladder or urine were measured. The *waaE* mutants showed a drastic reduction in their ability to infect or colonize the urinary tract compared to the wild-type strains or the mutants complemented with the different *waaE* gene homologues (Table 4).

The role of the *waaE* gene in swarming motility and formation of biofilms

The results obtained in colonization of the rat urinary tract prompted us to examine if the lack of *waaE* was able to alter other surface molecules involved in bacterial colonization besides the LPS, for instance flagella. Using only the *waaE* mutants from *Ser. marcescens* and *P. mirabilis*, we studied their motility either on swimming

Table 4. The adhesion of different *K. pneumoniae*, *Ser. marcescens* and *P. mirabilis* strains to UEC cells

Strain	Percentage bacteria adhered*
<i>K. pneumoniae</i> 889 (O8:K69, wild-type)	41.3 ± 3.7
889-1 (<i>waaE</i> from 889)	19.8 ± 3.2
889-1 (<i>waaE</i>)(pGEMT)	18.7 ± 3.6
889-1(pGEMT-WaaE _{Kp})	39.6 ± 3.9
889-1(pGEMT-WaaE _{Sm})	38.8 ± 3.7
889-1(pGEMT-WaaE _{Pm})	40.3 ± 3.2
889-1(pGEMT-WaaE _{Ve})	39.0 ± 3.6
889-1(pGEMT-WaaE _{Ea})	37.9 ± 4.3
<i>K. pneumoniae</i> 52145 (O1:K2, wild-type)	52.3 ± 6.2
NC16 (<i>waaE</i> from 52145)	23.8 ± 3.9
NC16 (<i>waaE</i>)(pGEMT)	22.6 ± 4.1
NC16(pGEMT-WaaE _{Kp})	50.1 ± 5.5
NC16(pGEMT-WaaE _{Sm})	50.6 ± 5.3
NC16(pGEMT-WaaE _{Pm})	48.9 ± 6.3
NC16(pGEMT-WaaE _{Ve})	49.7 ± 4.8
NC16(pGEMT-WaaE _{Ea})	50.8 ± 5.0
<i>Ser. marcescens</i> N28b (wild-type)	33.7 ± 2.6
N28b-16 (<i>waaE</i>)	12.8 ± 3.2
N28b-16(pGEMT)	11.7 ± 2.2
N28b-16(pGEMT-WaaE _{Sm})	30.9 ± 4.5
N28b-16(pGEMT-WaaE _{Kp})	32.1 ± 2.8
N28b-16(pGEMT-WaaE _{Pm})	31.3 ± 3.0
<i>P. mirabilis</i> CECT170 (wild-type)	42.7 ± 3.8
CECT170-8 (<i>waaE</i>)	18.0 ± 4.3
CECT170-8(pGEMT)	19.2 ± 3.6
CECT170-8(pGEMT-WaaE _{Pm})	42.0 ± 5.1
CECT170-8(pGEMT-WaaE _{Sm})	39.7 ± 4.3
CECT170-8(pGEMT-WaaE _{Kp})	40.6 ± 3.7

* The percentage of bacteria adhered to UEC cells ± SD was calculated as described by Merino *et al.* (1997). All the assays were done at least in triplicate (Student's *t*-test, *P* < 0.01).

or swarming agar plates. No differences could be observed in swimming plates, but a large swarming reduction of the *waaE* mutants in comparison to their wild-type strains or the mutants complemented with the different *waaE* gene homologues was found on the appropriate plates (see Fig. 5 for *P. mirabilis* results). However, when we studied the swarming cells using light or electron microscopy no differences were found between the *waaE* mutants and the wild-types or the *waaE*-complemented mutants. All the strains showed characteristic elongated and hyperflagellated cells.

In addition, the *Ser. marcescens* and *P. mirabilis waaE* mutants were also impaired in their ability to form biofilms when assayed by the method of Pratt & Kolter (1998), with decreases of approximately 40 % compared to their wild-types (Table 6). The introduction of the different *waaE* gene homologues into the mutants led to a full recovery of biofilm formation. In all these assays the same results were obtained for the *waaE* mutants

Table 5. Experimental urinary tract infection in rats by different *K. pneumoniae*, *Ser. marcescens* and *P. mirabilis* strains

Strain (infection dose, c.f.u. per rat)	Sample	Percentage infected rats and viable counts*	
		Percentage infected†	Mean $\pm$ SD‡
<i>K. pneumoniae</i> 51245 (O1:K2, wild-type) (0.8×10^9)	Kidney	100	6.9 ± 0.6
	Bladder	100	6.7 ± 0.5
	Urine	100	8.4 ± 0.4 (6)
NC16 (<i>waaE</i> from 51245) (1.0×10^9)	Kidney	40	4.1 ± 0.5
	Bladder	30	3.2 ± 0.6
	Urine	30	5.9 ± 0.4 (3)
NC16(pGEMT-WaaE _{Kp}) (1.1×10^9)	Kidney	90	6.7 ± 0.5
	Bladder	90	6.1 ± 0.6
	Urine	100	8.0 ± 0.5 (6)
NC16(pGEMT-WaaE _{Sm}) (0.8×10^9)	Kidney	100	6.8 ± 0.7
	Bladder	90	6.3 ± 0.4
	Urine	100	8.5 ± 0.2 (6)
NC16(pGEMT-WaaE _{Pm}) (0.8×10^9)	Kidney	90	6.4 ± 0.7
	Bladder	100	6.5 ± 0.3
	Urine	100	8.2 ± 0.6 (6)
<i>Ser. marcescens</i> N28b (wild-type) (1.2×10^9)	Kidney	90	6.3 ± 0.7
	Bladder	90	6.0 ± 0.8
	Urine	100	7.9 ± 0.8 (4)
N28b-16 (<i>waaE</i>) (1.1×10^9)	Kidney	20	3.9 ± 0.4
	Bladder	10	3.2
	Urine	20	6.6 ± 0.3 (2)
N28b-16 (<i>waaE</i>)(pGEMT _{Sm}) (0.8×10^9)	Kidney	80	5.7 ± 0.8
	Bladder	80	5.4 ± 1.2
	Urine	100	6.9 ± 0.6 (6)
<i>P. mirabilis</i> CECT170 (wild-type) (1.0×10^9)	Kidney	100	7.0 ± 0.8
	Bladder	90	6.5 ± 0.4
	Urine	100	8.3 ± 0.2 (6)
CECT170-8 (<i>waaE</i>) (1.2×10^9)	Kidney	40	4.0 ± 0.3
	Bladder	50	3.3 ± 0.7
	Urine	40	6.2 ± 0.5 (4)
CECT170-8(pGEMT-WaaE _{Pm}) (1.4×10^9)	Kidney	100	7.4 ± 0.6
	Bladder	100	7.5 ± 0.4
	Urine	100	8.0 ± 0.2 (6)

* A total of 20 kidneys and 10 bladders were studied in each group. Numbers in parentheses represent urine samples studied.

† Percentage of positive cultures. The lowest number of organisms detectable by the method was 50 c.f.u. g^{-1} (kidney or bladder) or ml^{-1} (urine).

‡ Values represent the mean $\log(\text{c.f.u. g}^{-1}$ or $\text{ml}^{-1} \pm \text{SD})$ of positive cultures. All the assays were done at least in triplicate.

and the corresponding mutants transformed with pGEMT.

DISCUSSION

Structural studies of the core region of LPS from *K. pneumoniae* have revealed that all of them have very similar core structures with minor differences among different serovars (Vinogradov & Perry, 2001). In all the *K. pneumoniae* studied the core structure is charac-

terized by the substitution of L,D-HeppI at the O-4 position by a Glcp [$\beta\text{-D-Glcp-(1}\rightarrow\text{4)-}\alpha\text{-L,D-HeppI}$] and by the substitution of the L,D-HeppII at the O-3 position by an $\alpha\text{-Kdo-(2}\rightarrow\text{6)-}\alpha\text{-D-GlcN-(1}\rightarrow\text{4)-}\alpha\text{-D-GalA}$ trisaccharide (Vinogradov & Perry, 2001) (Fig. 1). Previously, we have suggested that the *K. pneumoniae waaE* gene encodes a glucosyltransferase involved in the transfer of a Glc residue by a $\beta\text{1,4}$ linkage to L,D-HeppI in its inner-core LPS. This suggestion was based on the compositional analysis of a *waaE* mutant and comple-

Fig. 5. The swarming motility phenotype of *P. mirabilis* strains CECT170 (wild-type, a) and CECT170-8 (*waaE* mutant, b) assayed as described in Methods.

Table 6. Biofilm formation of *Ser. marcescens* and *P. mirabilis* strains quantified according to Pratt & Kolter (1998)

Strain	Biofilm quantification (OD ₅₇₀) [*]
<i>Ser. marcescens</i> N28b (wild-type)	1.12 ± 0.07 (100 %)
N28b-16 (<i>waaE</i>)	0.67 ± 0.08 (40 %)
N28b-16(pGEMT)	0.71 ± 0.05 (37 %)
N28b-16(pGEMT-WaaE _{sm})	1.08 ± 0.08 (< 5 %)
N28b-16(pGEMT-WaaE _{Kp})	1.10 ± 0.06 (< 5 %)
N28b-16(pGEMT-WaaE _{Pm})	1.06 ± 0.09 (< 5 %)
<i>P. mirabilis</i> CECT170 (wild-type)	1.56 ± 0.12 (100)
CECT170-8 (<i>waaE</i>)	0.92 ± 0.08 (41 %)
CECT170-8(pGEMT)	0.90 ± 0.11 (40 %)
CECT170-8(pGEMT-WaaE _{Pm})	1.50 ± 0.06 (< 5 %)
CECT170-8(pGEMT-WaaE _{sm})	1.47 ± 0.15 (< 5 %)
CECT170-8(pGEMT-WaaE _{Kp})	1.53 ± 0.07 (< 5 %)

^{*} Percentage of OD₅₇₀ reduction taking the OD₅₇₀ of the wild-type strain as 100 %. All the assays were done at least in triplicate (Student's *t*-test, *P* < 0.01).

mentation analysis of the *waaE* mutant with the *Haemophilus ducreyi* *lgtF* gene (Regué *et al.*, 2001). In this work we confirmed *waaE* function by additional experimental evidence based on comparative composition and methylation analysis of the core region between wild-type *K. pneumoniae* 889 (O:8) and a non-polar *waaE* mutant (889-1). As expected from the *K. pneumoniae* core LPS structure, the major methylated products obtained from the core LPS of *K. pneumoniae* 889 corresponded to methylation products derived from residues L,D-HeppI (2,6,7-tri-O-methyl-D-glycero-L-manno-heptitol), L,D-HeppII (2,4,6-tri-O-methyl-D-glycero-L-manno-heptitol), L,D-HeppIII (2,3,4,6,7-penta-O-methyl-D-glycero-L-manno-heptitol), Glcp (2,3,4-tri-O-methylglucitol), GalpAI (2,3,4,6-tetra-O-methylgalactitol) and GalApII (2,3,6-tri-O-methylgalactitol) (Table 2). The presence of 2,3,4,6-tetra-O-methylgalac-

titol showed that strain 889 contains a substitution of Glcp at the O-6 position by a GalAp residue, a feature described for some strains of *K. pneumoniae* (Vinogradov & Perry, 2001). The absence of 2,3,4-tri-O-methylglucitol (Glcp) and 2,3,4,6-tetra-O-methylgalactitol (GalpAI) in core LPS obtained from strain 889-1 (*waaE* non-polar mutant) indicates that the major effect of the *waaE* mutation is to preclude the attachment of the Glcp residue to L,D-HeppI. Furthermore, 2,4,6,7-tetra-O-methyl-L-glycero-D-manno-heptitol, derived from O-4 non-substituted L,D-HeppI, instead of 2,6,7-tri-O-methyl-L-glycero-D-manno-heptitol, derived from O-4 substituted L,D-HeppI, was found in the core LPS of the *waaE* mutant. In addition, strain 889-1 transformed with pGEMT-WaaE_{Kp} generated core LPS methylated products identical to those obtained with core LPS isolated from the wild-type strain, as well as the presence

of Glc. These results agree with a glucosyltransferase function for the WaaE protein.

Studies on the genetic organization of the *Enterobacteriaceae* genes involved in core LPS biosynthesis have showed that the *waa* gene cluster contains two conserved regions. One is in the proximal end of the cluster with three conserved genes, *gmhD*, *waaF* and *waaC*, encoding the last enzyme in the biosynthesis of L,D-HepP, L-glycero-D-manno-heptosyltransferase II and L-glycero-D-manno-heptosyltransferase I, respectively (Heinrichs *et al.*, 1998; Regué *et al.*, 2001). There are two different models for the distal end of the *waa* gene cluster. In the five known *E. coli* core LPS types and in *Sal. enterica* serovar Typhimurium the *waa* clusters end with the *waaA* gene encoding the bifunctional Kdo transferase (Sofia *et al.*, 1994; Heinrichs *et al.*, 1998). In *K. pneumoniae* and *Ser. marcescens* *waa* clusters the *waaE* gene is found just downstream from the *waaA* gene (Guasch *et al.*, 1996; Regué *et al.*, 2001). In both models the *coaD* and *fpg* genes are found just downstream from the last *waa* gene, either *waaA* or *waaE*. A PCR assay for *waaE* homologues among clinical isolates of several species of *Enterobacteriaceae* suggested the presence of an extra gene in strains of *P. mirabilis*, *Y. enterocolitica* and *Ent. aerogenes*. No such *waaE* extra gene was detected among clinical isolates of *E. coli*, *Sal. enterica* serovars Typhimurium, Enteritidis and Infantis, and *Shi. dysenteriae*, *Shi. flexneri* and *Shi. sonnei* (Figs 3, 4). Furthermore, nucleotide sequence determination for a representative strain for each species confirmed the presence of *waaE* homologues flanked by the *waaA* gene and the *coaD-fpg* genes in *P. mirabilis*, *Y. enterocolitica* and *Ent. aerogenes* (Fig. 3). Analysis of the corresponding sequence strongly suggests that the *waaA*, *waaE* and *coaD* genes are cotranscribed and that *fpg* is transcribed in the opposite direction; these two features have also been found in *K. pneumoniae* (Regué *et al.*, 2001). Genetic complementation studies showed that the identified *waaE* homologues are able to complement non-polar *K. pneumoniae* and *Ser. marcescens* *waaE* mutant strains, thus proving that all the *waaE* homologues perform the same function in inner-core LPS biosynthesis. These results agree with core LPS structural studies in *P. mirabilis* and *Y. enterocolitica* where a substitution of L,D-HepPI at the O-4 position by a Glcp [β -D-Glcp-(1 $\rightarrow$ 4)- α -L,D-HepPI] has been found (Radziejewska-Lebrecht *et al.*, 1994; Vinogradov *et al.*, 2000). From the *Enterobacteriaceae* *waa* gene cluster distal end sequence data a set of primers was designed to amplify genomic DNA containing the 3' end of the *waaA* gene up to the 3' end of the *fpg* gene. These primers allow rapid screening for the presence of *waaE* homologues and can be used to predict the presence of a Glcp residue attached by a β 1,4 linkage to L,D-HepPI in the inner-core LPS of members of the *Enterobacteriaceae*, such as *Ser. marcescens* and *Ent. aerogenes*.

The contribution of the *waaE* gene to the different pathogenic features studied could be explained by the fact that these mutants showed a clear reduction in O-antigen LPS molecules (Fig. 2). It seems clear, at least in

K. pneumoniae, that the O-antigen LPS is critical for serum resistance (Reid & Sobel, 1984; Figueroa & Densen, 1991; Merino *et al.*, 2000), changes in surface hydrophobicity (Marshall, 1984; Merino *et al.*, 2000), adhesion to UEC cells (Merino *et al.*, 1997, 2000), and urinary tract infection and colonization in rats (Nevola *et al.*, 1987; Finlay & Falkow, 1989; Licht *et al.*, 1996; Russo *et al.*, 1996; Merino *et al.*, 2000). We may conclude also that these facts could be extended to other enterobacteria, such as *Ser. marcescens* and *P. mirabilis*.

It has been reported that some *Sal. enterica* serovar Typhimurium mutants unable to show swarming motility (efficient colonization of the growth surface) with functional flagella (essential for this form of motility), showed defects in LPS biosynthesis (Toguchi *et al.*, 2000). These authors suggested that the O-antigen LPS improves surface wettability required for swarm colony expansion. The role of the *waaE* gene in *P. mirabilis* and *Ser. marcescens* swarming phenotype could be attributed to O-antigen LPS reduction (surface wettability reduction) observed in the *waaE* mutants, while the flagella in both bacteria remain functional. Furthermore, we suggest that this altered swarming phenotype with a reduction of surface wettability could explain the reduced biofilm formation ability of the *waaE* mutants. Recently, Watnick *et al.* (2001) demonstrated that at least exopolysaccharide synthesis and flagella are needed for the efficient formation of a three-dimensional biofilm in *Vibrio cholerae* O:139.

All these roles that we attribute to the *waaE* gene are supported by the fact that well genetically characterized isogenic mutants were used, because all the *waaE* gene homologues were able to complement the *waaE* mutants, independently of their origin, and because the complemented mutants recovered the wild-type strain characteristics.

We cannot exclude that the inner-core change observed in the *waaE* mutants, the lack of substitution of L,D-HepPI at the O-4 position by β -D-Glcp, could also play a role in swarming motility, biofilm formation and deficient pathogenic phenotypes showed by these mutants. Interestingly, both the WaaP and WaaE proteins are involved in substitution of L,D-HepPI at the O-4 position by a phosphate or pyrophosphoethanolamine (Yethon *et al.*, 1998, 2000) and by a β -D-Glcp residue, respectively. The phenotype of the *waaE* mutants shares some similarities to *E. coli* F470 and *Sal. enterica* serovar Typhimurium *waaP* mutants. In all these cases a faster migrating LPS band is seen in Tricine-SDS-PAGE, the core LPS appears to be capped with O-antigen and the amount of O antigen is reduced in the mutants. In addition, in *Sal. enterica* serovar Typhimurium a *waaP* mutant showed reduced virulence *in vivo* (Yethon *et al.*, 2000).

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REFERENCES

- Allen, A. G. & Maskell, D. J. (1996). The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. *Mol Microbiol* **19**, 37–52.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- 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.
- Camprubí, S., Merino, S. & Tomás, J. M. (1993). The role of the O-antigen lipopolysaccharide and capsule on an experimental *Klebsiella pneumoniae* infection of the rat urinary tract. *FEMS Microbiol Lett* **111**, 9–14.
- Ciucanu, I. & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* **131**, 209–217.
- Falkowski, W., Edwards, M. & Schaeffer, A. J. (1986). Inhibitory effect of substituted aromatic hydrocarbons on adherence of *E. coli* to human epithelial cells. *Infect Immun* **52**, 863–866.
- Figuerola, J. E. & Densen, P. (1991). Infectious diseases associated with complement deficiencies. *Clin Microbiol Rev* **4**, 359–395.
- Filiatrault, M. J., Gibson, B. G., Schilling, B., Sun, S., Munson, R. S. & Campagnari, A. A. (2000). Construction and characterization of *Haemophilus ducreyi* lipooligosaccharide (LOS) mutants defective in expression of heptosyltransferase III and beta 1,4-glucosyltransferase: identification of LOS glycoforms containing lactosamine repeats. *Infect Immun* **68**, 3352–3361.
- Finlay, B. & Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiol Rev* **53**, 210–230.
- Guasch, J. F., Piqué, N., Climent, N., Ferrer, S., Merino, S., Rubirés, X., Aguilar, A., Tomás, J. M. & Regué, M. (1996). Cloning and characterization of two *Serratia marcescens* genes involved in core lipopolysaccharide biosynthesis. *J Bacteriol* **178**, 5741–5747.
- Hansen, D. S., Mestre, F., Albertí, S., Hernández-Allés, S., Alvarez, D., Domenech Sanchez, A., Gil, J., Merino, S., Tomás, J. M. & Benedí, V. J. (1999). *Klebsiella pneumoniae* lipopolysaccharide O-typing: revision of prototype strains and O-group distribution among clinical isolates of different sources and countries. *J Clin Microbiol* **37**, 56–62.
- Heinrichs, D. E., Yethon, J. A. & Whitfield, C. (1998). Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* **30**, 221–232.
- Hitchcock, P. J. & Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **154**, 269–277.
- Kahler, C. M., Carlson, R. W., Rahman, M. M., Martin, L. E. & Stephens, D. S. (1996). Two glycosyltransferase genes, *lgtF* and *rfaK*, constitute the lipooligosaccharide (ice inner core extension) biosynthesis operon of *Neisseria meningitidis*. *J Bacteriol* **178**, 6677–6684.
- Källénus, G., Korkhonen, T. K., Väisänen-Rhen, V. & Svenson, S. B. (1985). Adherence assays. In *Enterobacterial Surface Antigens*, pp. 321–331. Edited by T. K. Korkhonen, E. A. Dawes & P. H. Mäkelä. Amsterdam: Elsevier.
- Karkhanis, Y. D., Zeltner, J. Y., Jackson, J. J. & Carlo, D. J. (1978). A new improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. *Anal Biochem* **85**, 595–601.
- Licht, T. R., Krogfelt, K. A., Cohen, P. S., Poulsen, L. K., Urbance, J. & Molin, S. (1996). Role of lipopolysaccharide in colonization of the mouse intestine by *Salmonella typhimurium* studied by *in situ* hybridization. *Infect Immun* **64**, 3811–3817.
- Link, A. J., Phillips, D. & Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179**, 6228–6237.
- Marshall, K. C. (1984). Glossary. In *Microbial Adhesion and Aggregation*, pp. 397–399. Edited by K. C. Marshall. New York: Springer.
- Merino, S., Camprubí, S., Albertí, S., Benedí, V. J. & Tomás, J. M. (1992). *Klebsiella pneumoniae* resistance mechanisms to complement mediated killing. *Infect Immun* **60**, 2529–2535.
- Merino, S., Rubires, X., Aguilar, A. & Tomás, J. M. (1997). The role of O1-antigen in the adhesion to uroepithelial cells of *Klebsiella pneumoniae* grown in urine. *Microb Pathog* **23**, 49–53.
- Merino, S., Altarriba, M., Izquierdo, L., Nogueras, M. M., Regué, M. & Tomás, J. M. (2000). Cloning and sequencing of the *Klebsiella pneumoniae* O5 *wb* gene cluster and its role in pathogenesis. *Infect Immun* **68**, 2435–2440.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.
- Nevola, J. J., Laux, D. C. & Cohen, P. S. (1987). *In vivo* colonization of the mouse large intestine and *in vitro* penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimurium* and its lipopolysaccharide-deficient mutant. *Infect Immun* **55**, 2884–2990.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**, 2444–2448.
- Pradel, E. & Schnaitman, C. A. (1991). Effect of the *rfaH* (*sfrB*) and temperature on the expression of the *rfa* genes of *Escherichia coli* K-12. *J Bacteriol* **173**, 6428–6431.
- Pratt, L. A. & Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* **30**, 285–293.
- Radziejewska-Lebrecht, J., Shashkov, A. S., Stroobant, V., Wartenberg, K., Wart, C. & Mayer, H. (1994). The inner core region of *Yersinia enterocolitica*. *Eur J Biochem* **221**, 343–351.
- Reeves, P. R., Hobbs, M., Valvano, M. A. & 8 other authors (1996). Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **4**, 495–503.
- Regué, M., Climent, N., Abitau, N., Coderch, N., Merino, S., Izquierdo, L., Altarriba, M. & Tomás, J. M. (2001). Genetic characterization of the *Klebsiella pneumoniae waa* gene cluster, involved in core lipopolysaccharide biosynthesis. *J Bacteriol* **183**, 3564–3573.
- Reid, G. & Sobel, J. D. (1984). Bacterial adherence in the pathogenesis of urinary tract infections: a review. *Rev Infect Dis* **9**, 470–487.
- Rosenberg, M., Gutnick, D. & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9**, 29–33.
- Russo, T. A., Brown, J. J., Jodush, S. T. & Johnson, J. R. (1996). The O4 specific antigen moiety of lipopolysaccharide but not the K54 group 2 capsule is important for urovirulence of an extraintestinal isolate of *Escherichia coli*. *Infect Immun* **64**, 2343–2348.

- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schnaitman, C. L. & Klena, J. D. (1993).** Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* **57**, 655–682.
- Sofia, H. J., Burland, V., Daniels, D. L., Plunkert, G., III & Blattner, F. R. (1994).** Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. *Nucleic Acids Res* **22**, 2576–2586.
- Süsskind, M., Brade, L., Brade, H. & Holst, O. (1998).** Identification of a novel heptoglycan of β 1 α 2-linked D-glycero-D-manno-heptopyranose: chemical and antigenic structure of lipopolysaccharides from *Klebsiella pneumoniae* ssp. *pneumoniae* rough strain R20 (O1⁻:K20⁻). *J Biol Chem* **273**, 7006–7017.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Toguchi, A., Siano, M., Burkhart, M. & Harshey, R. M. (2000).** Genetics of swarming motility in *Salmonella enterica* serovar Typhimurium: critical role of lipopolysaccharide. *J Bacteriol* **182**, 6308–6321.
- Tsai, C. M. & Frasch, C. E. (1982).** A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. *Anal Biochem* **119**, 115–119.
- Vinogradov, E. & Perry, M. B. (2001).** Structural analysis of the core region of the lipopolysaccharides from eight serotypes of *Klebsiella pneumoniae*. *Carbohydr Res* **335**, 291–296.
- Vinogradov, E. V., Holst, O., Thomas-Oates, J. E., Broady, K. W. & Brade, H. (1992).** The structure of the O-antigenic polysaccharide from lipopolysaccharide of *Vibrio cholerae* strain H11 (non-O1). *Eur J Biochem* **210**, 491–498.
- Vinogradov, E., Radziejewska-Lebrecht, J. & Kaca, W. (2000).** The structure of the carbohydrate backbone of core-lipid A region of the lipopolysaccharides from *Proteus mirabilis* wild-type strain S1959 (serotype O3) and its Ra mutant R110/1959. *Eur J Biochem* **267**, 262–268.
- Watnick, P. I., Laurino, C. M., Klose, K. E., Croal, L. & Kolter, R. (2001).** The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Mol Microbiol* **39**, 223–235.
- Westphal, O. & Jann, K. (1965).** Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr Chem* **5**, 83–91.
- Yethon, J. A., Heinrichs, D. E., Monteiro, M. A., Perry, M. B. & Whitfield, C. (1998).** Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *J Biol Chem* **273**, 26310–26316.
- Yethon, J. A., Gunn, J. S., Ernst, R. K., Miller, S. I., Laroche, L., Malo, D. & Whitfield, C. (2000).** *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence *in vivo*. *Infect Immun* **68**, 4485–4491.

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