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Highlights

- The *Plesiomonas shigelloides* 302-73 strain (serotype O1) *wb* gene cluster.
- The cluster encodes 15 proteins in agreement with chemical structure.
- The mutants lacking this O1-antigen LPS unable to survive in serum or adhere.
- First report on genetics from a *P. shigelloides* O-antigen LPS not shared by *Shigella*.

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The *Plesiomonas shigelloides* wb_{O1} gene cluster and the role of O1-antigen LPS in pathogenicity

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ABSTRACT

The *Plesiomonas shigelloides* 302-73 strain (serotype O1) *wb* gene cluster encodes 15 proteins which are consistent with the chemical structure of the O1-antigen lypopolysaccharide (LPS) previously described for this strain. The *P. shigelloides* O1-antigen LPS export uses the Wzy-dependent pathway as correspond to heteropolysaccharides structures. By the isolation of two mutants lacking this O1-antigen LPS, we could establish that the presence of the O1-antigen LPS is crucial for to survive in serum mainly to become resistant to complement. Also, it is an important factor in the bacterial adhesion and invasion to some eukaryotic cells, and in the ability to form biofilms. This is the first report on the genetics from a *P. shigelloides* O-antigen LPS cluster (*wb*) not shared by *Shigella* like *P. shigelloides* O17, the only one reported until now.

1. Introduction

Plesiomonas shigelloides is a Gram-negative, flagellated, rod-shaped bacterium. This ubiquitous and facultative anaerobic organism has been isolated from such sources as freshwater, surface water, and many wild and domestic animals [1]. *P. shigelloides* is a bacterium associated with diarrheal disease in humans [2]. The organism has been reported to cause several types of gastroenteritis, including acute secretory gastroenteritis [3], an invasive shigellosis-like disease [4], and a cholera-like illness [5]. Extraintestinal infections, such as meningitis, bacteremia [6], and pseudoappendicitis [7], are also associated with *P. shigelloides* infection. The bacterium has remained within the family *Vibrionaceae* until molecular studies carried out by Martinez-Murcia et al. [8] indicated that *P. shigelloides* is phylogenetically related to the genus *Proteus*. Furthermore, Huys and Sings [9] in an evaluation of the amplified fragment length polymorphism technique for genotyping *Aeromonas* spp. found that *P. shigelloides* clearly falls out of the major *Aeromonas* cluster. In the light of these recent findings the genus *Plesiomonas* has been moved to the family *Enterobacteriaceae*, and is the only oxidase-positive member of this family [10]. Unlike other phenotypic methods, serology has more successfully been used for distinguishing different strains of *P. shigelloides*. There are mainly two major serotyping schemes, which are based on somatic (O) and flagellar (H) antigens. At the present moment, 102 somatic antigens and 51 flagellar antigens have been recognized [11].

In Gram negative bacteria the lipopolysaccharide (LPS) is one of the major structural and immunodominant molecules of the outer membrane. It consists of three moieties: lipid A, core oligosaccharide, and O-specific antigen or O side chain. The O-antigen is the external component of LPS and, its structure consists on a polymer of oligosaccharide repeating units. Another interesting feature is the high chemical variability shown by the O-antigen LPS leading to a similar genetic variation in the genes involved in their biosynthesis, the so called *wb* cluster [for a review see Ref. [12]]. The genetics of the O-antigen biosynthesis has been intensively studied in the *Enterobacteriaceae*, and it has been shown that the *wb* clusters usually contain genes involved in biosynthesis of activated sugars, glycosyl transferases, O-antigen polymerases, and O- antigen export. Q1

Despite the emerging importance of this pathogenic microorganism, to date only seven LPS structures out of 102 O-serotypes of *P. shigelloides* were investigated. As first structures, the O-specific polysaccharides of *P. shigelloides* strains 22074 and 12254 were determined in 1995 by Linnerborg et al. [13]. So far, only two complete LPS molecules isolated from *P. shigelloides* CNCTC 113/92 (serotype O54) and CNCTC 144/92 (serotype O74) [14,15] were elucidated. Additionally the structures of the core oligosaccharide substituted with the O-specific chains from strain 302-73 (serotype O1) [16,17] and the O-specific polysaccharide from strain CNCTC 110/92 (serotype O51) [18] and AM36565 [19] were identified.

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Recently, Kubler-Kielb et al. reported for the first time on the structure of the core oligosaccharide substituted with one repeating unit (RU) of the O-specific PS isolated from LPS of *P. shigelloides* strain 7-63 (serotype O17) [20]. It was known that its O-antigen structure is identical to that of *Shigella sonnei* phase I [21], a causative agent of dysentery. Both species acquired virulence plasmid with gene cluster coding O17-antigen [22].

In this work we study the genetics of *P. shigelloides* O1-antigen LPS, being the first *Plesiomonas* O-antigen not shared with *Shigella* described. We also study their biological role in some pathogenic features.

2. Results

2.1. Mutant isolation and analyses

P. shigelloides wild type strain 302-73 (serogroup O1 [16],) rifampicin-resistant spontaneous mutant was isolate (302-73R). After mutagenesis of 302-73R as described in Materials and Methods, we selected mini:Tn5 insertion mutants (Km^r) unable to cross react by colony blot with specific antiserum against *Plesiomonas* O1-antigen LPS.

Mutants 302-A and 302-B were selected among 1200 mutants initially screened for it's completely inability to cross react with the *Plesiomonas* O1-antigen LPS antiserum. SDS-PAGE showed that both mutants lack the O1-antigen LPS by their gel profile (Fig. 1). Purified LPS from both mutants also showed a completely absence of deoxy sugars characteristics of O1-antigen LPS and the presence of all the monosaccharides from the LPS-core (Kdo, Hep, Gal, GalA, GlcN, [17]) (Fig. 2).

Southern blot analysis using a specific probe for the transposon demonstrated that mutants 302-A and 302-B had a single copy of the minitransposon in its genome. Cloning of the minitransposon-containing fragment from the genomic DNA of the mutants using different approaches previously used in other bacteria was unsuccessful.

A cosmid-based genomic library of *P. shigelloides* 302-73 was constructed and introduced into *Escherichia. coli* DH5α as indicated in Material and Methods, and tetracycline-resistant (20 μg/ml) clones were obtained. We found two recombinant positive clones

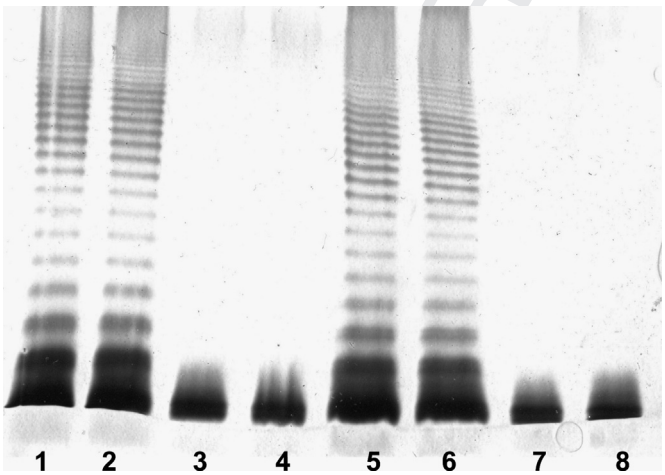

Fig. 1. Polyacrylamide gels showing the migration of LPS from several *P. shigelloides* strains, mutants and its complementation. The LPS samples were separated on SDS-PAGE and visualized by silver staining [36,37]. Shown are LPS samples from 302 to -73 wild type (lane 1), 302-73R wild type rifampicin resistant (lane 2), 302-A mutant strain (lane 3), 302-B mutant strain (Lane 4), 302-A mutant strain + plasmid COS-PO1 (lane 5), 302-B mutant strain + plasmid COS-PO2 (lane 6), 302-A mutant strain + plasmid pLA2917 (lane 7), and 302-B mutant strain + plasmid pLA2917 (lane 8).

→3)-α-L-PneNAc4*O*Ac(1→4)-α-L-FucNAc(1→4)-α-L-Fuc-NAc(1→4)-α-L-FucNAc(1→3)-β-D-QuiNAc4NHb(1→

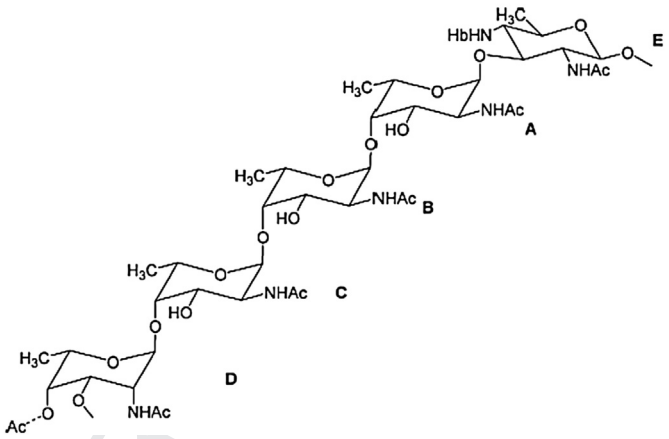

Fig. 2. *P. shigelloides* O1-antigen LPS chemical structure [16]. Residues A, B, and C are FucNAc = *N*-acetylfucosamine; residue D is PneNAc = 2-acetamido-2,6-dideoxy-talose or *N*-acetylpneumosamine; and residue E is QuiNAcHb = *N*-acetylquinovosamine plus (*S*)-3-hydroxybutanoyl.

(COS-PO1 and COS-PO2) that were able to complement *P. shigelloides* 302-A and 302-B mutants, respectively, by positive cross reactivity with specific antiserum. Furthermore, SDS-PAGE showed that both mutants complemented with COS-PO1 and COS-PO2, respectively, recover the O1-antigen LPS by their gel profile (Fig. 1). Also, purified LPS from both mutants 302-A and 302-B complemented with COS-PO1 and COS-PO2, respectively, showed the presence of deoxy sugars characteristics of O1-antigen LPS and all the monosaccharides from the LPS-core [17] (Fig. 2). No such complementation was achieved when the mutants harbor the plasmid vector alone (see Fig. 1).

2.2. *P. shigelloides 302-73 wb gene cluster and analysis of the ORF's deduced amino acid sequence*

The nucleotide sequence of the plasmids COS-PO1 and COS-PO2 DNA inserts were determined in order to identify the *P. shigelloides* 302-73 genes able to complement both mutants. A nucleotide sequence of 19057 bp was determined in both directions by using oligonucleotides complementary to pLA2917 sequences' flanking the DNA inserts. Other sequence-derived oligonucleotides were purchased (Sigma–Aldrich) and used to complete the nucleotide sequence (GenBank accession number: KC702805). Analysis of the sequenced regions altogether showed 15 ORFs (Fig. 3A) transcribed in the same direction. Upstream or downstream of the last ORF no other ORFs involved in LPS biosynthesis were identified. These results suggest that this sequence corresponds to the O1-antigen *wb* gene cluster. In all cases, putative Shine Dalgarno sequences were found upstream of all ORFs start codons. Computer analysis of the wb_{O1} gene cluster sequence revealed a conserved JUMPstart sequence with the 8 bp *ops* (*o*peron *p*olarity *s*upressor) sequence (GGCGGTAG) 67 bp upstream from ORF1 required. The *ops* sequence is recognized by the bacterial antiterminator RfaH, which can be recruited by the transcription elongation complex to reduce pausing and termination at intergenic sites of polycistronic operons, allowing RNA polymerase to conclude transcription of the distal genes in large operons. A putative promoter-like sequence upstream of ORF1 could be observed, and a rho-independent possible transcriptional terminator sequence downstream of ORF15 was detected (Fig. 3A).

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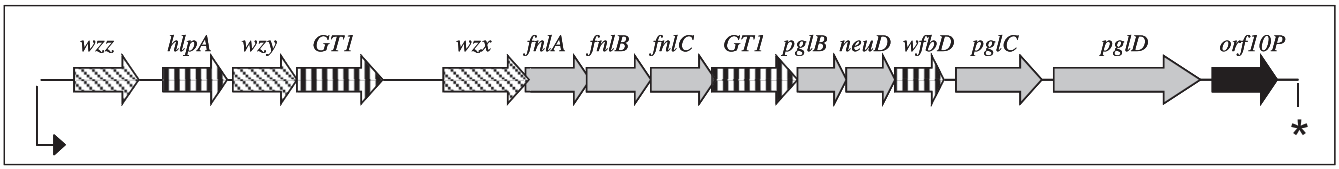
A



B

ORF	Protein size	Predicted function	Homologous gene	Percentage Identity/Similarity	Start/Stop
1	367	O antigen chain length determinant protein	*wzz Plesiomonas shigelloides*	89/93	106/1209
2	356	protein containing nucleotide-diphospho-sugar transferase domain	*hlpA Pseudomonas putida*	55/75	2220/3290
3	336	O-antigen polymerase	*wzy E. coli*	25/47	3295/4305
4	405	glycosyl transferase group 1	Uncharacterized *Pseudomonas, Clostridium*	41/62	4302/5519
5	414	Flippase	*wzx Providencia alcalifacien*	44/63	7392/8636
6	349	UDP-N-acetyl-D-glucosamine 4,6-dehydratase, 5-epimerase, and 3-epimerase	*fnlA Vibrio*	90/95	8717/9754
7	368	UDP-*N-acetyl*-L-pneumosamine 4 reductase	*fnlB Vibrio*	86/93	9755/10861
8	376	UDP-N-acetyl-L-(2)-fucosamine epimerase	*fnlC Vibrio*	92/97	10878/12008
9	420	glycosyl transferase group 1	Uncharacterized *Vibrio, Pseudomonas.*	67/82	12075/13283
10	198	UDP-N-acetylgalactosaminyltransferase	*pglB Neisseria*	79/88	13280/13876
11	228	acetyltransferase	*neuD Acinetobacter*	57/76	13869/14510
12	140	MaoC-like dehydratase	*wfbD E. coli*	68/83	14518/14940
13	391	pilin glycosylation protein	*pglC Neisseria*	85/91	14953/16128
14	639	UDP-D-quinovosamine 4-dehydrogenase	*pglD Neisseria*	61/78	16217/18136
15	233	Aquaporin Z	*orf10P Plesiomonas shigelloides*	100/100	18255/18956

Fig. 3. A. Schematic diagram of the *P. shigelloides* wb_{O1}. The arrow indicates the putative promoter-like sequence upstream of ORF1 and * the rho-independent transcriptional terminator sequence downstream of ORF15. B. The table shows the percentage of identity/similarity of the *P. shigelloides* 302-73 ORFs with homologous genes with predicted protein functions.

The DNA sequence was translated in all six frames, and all ORFs were inspected. Computer database searching was carried out to tentatively identify the sequenced genes. Proteins similar to each ORF gene product were analyzed to determine the levels of similarity and identity (Table on Fig. 3B). Analysis of the sequenced region showed 15 complete ORFs apparently transcribed in the same direction.

Once the DNA fragment was completely sequenced, we used several primers derived from the DNA sequence to establish that the miniTn5 of 302-A mutant was located in a gene with great similarity to *pglD* from different bacteria (ORF14), and of 302-B mutant in a gene with great similarity to *fnlA* from different bacteria (ORF6). In order to prove that mutations in these genes were responsible for the phenotype observed in both mutants, we decided to isolate independently defined insertional mutants using the replication λ*pir*-dependent plasmid pSF100 [23], as previously described.

Plasmids pFS-ORF14 and pSF-ORF6 were constructed carrying internal fragments of *pglD* and *fnlA*, respectively. Plasmids were transferred by mating independently to *P. shigelloides* 302-73R, and Rif^r and Km^r colonies were selected. We obtained mutants 302 *pglD* and 302 *fnlA*, were the insertions were confirmed by Southern blotting using appropriate DNA probes. Both independent mutants showed the same phenotype: no cross reactivity with specific O1-antigen LPS antiserum and lack of O-antigen in SDS-PAGE gel, as it happens with 302-A and 302-B mutants. Complementation of these mutants (302 *pglD* and 302 *fnlA*) with COS-PO1 and COS-PO2 carrying genes from the *P. shigelloides wb* gene cluster, respectively, was fully achieved (ability to produce O1-antigen LPS), but not when the plasmid vector alone was introduced. Furthermore, COS-PO2 DNA was obtained and digested with *Sph*I and *Pst*I restriction enzymes, and the 2.659 bp from the four different generate fragments containing only the whole *pglD* gene was ligated to a pLA2917 vector, previously digested with *Bgl*II and dephosphorylated, to generate pLA-pglD plasmid. Amplification by PCR using CSpLA (gactgggcggttttatgg) and MpLA (aatagcagccagtcccttc) primers allowed confirming the presence of *pglD* and verifying its orientation. pLA-pglD plasmid was able to fully complemented the 302 *pglD* mutant as judged by their ability to produce O1-antigen LPS.

2.3. Serum killing

The serum survival of the *P. shigelloides* strains is shown in Fig. 4. *P. shigelloides* wild type strain 302-73 was resistant to the bactericidal activity of non immune human serum (NHS) (approximately

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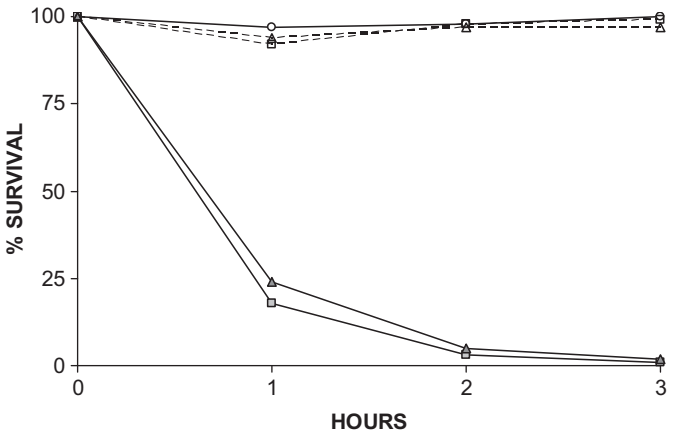

Fig. 4. *P. shigelloides* strains survival in NHS [40]. (○) 302-73 wild type, (▲) 302-A *pglD* mutant, (■) 302-B *fnlA* mutant, (Δ) 302-A *pglD* mutant + COS-PO1, and (□) 302-B *fnlA* mutant + COS-PO2.

100% survival after 3 h of incubation with NHS), while the mutants (302-A and B) lacking the O1-antigen LPS showed sensitivity (less than 3% survival after 3 h of incubation with NHS). The complementation of the mutants with COS-PO1 and COS-PO2, respectively, renders them resistant to the bactericidal activity of NHS (>96% survival after 3 h of incubation with NHS). Values found are similar to the wild type strain. No changes were observed in the mutants by the introduction of the plasmid vector alone. Control experiments using heat-decomplemented NHS render more than 95% survival for all the mutant strains tested.

2.4. Adherence and invasion to INT 407 and Caco-2 cells

P. shigelloides wild type strain 302-73 is able to adhere and invade INT 407 and Caco-2 cells (Tables 2 and 3). However, mutants lacking the O1-antigen LPS derived from this strain showed a drastic reduction (>77%) in their capacity to adhere these eukaryotic cells versus the wild type strain. In all the cases it seems to be a better adhesion to INT 407 cells than Caco-2 cells (Table 2). Table 3 shows that the degree of invasion to INT 407 and Caco-2 cells by *P. shigelloides* mutants lacking the O1-antigen LPS was drastically reduced (>85%) in comparison with the wild type strain. Again, in all the cases it seems to be a slight better invasion to INT 407 cells than Caco-2 cells (Table 3). As can be observed in Tables 2 and 3 a full recovery of the ability to adhere and invade INT 407 and Caco-2 cells was achieved when the mutants (302-A and B) were complemented with COS-PO1 and COS-PO2, respectively, when they recover the O1-antigen LPS. No restoration of adhesion or invasion was found when mutants harbor the plasmid vector alone.

2.5. Biofilm formation

P. shigelloides 302-73 is able to form biofilms in an *in vitro* system [24]. Table 4 shows that mutants lacking the O1-antigen LPS are quite unable to form biofilms while the wild type strain is able to do it. However, when the mutants were complemented (production of O1-antigen LPS) they are able to again form biofilms in this system as the wild type strain. When the mutants carried the plasmid vector alone no changes could be observed in the ability of the mutants to form biofilms.

3. Discussion

The *P. shigelloides* O1-antigen *wb* gene cluster showed a % G + C of 40.0 lower than the 51% reported for the *P. shigelloides* strains, characteristic of some *wb* clusters that usually possess a G + C content at least 10% below the species average. The encoded proteins are consistent with the chemical structure of the O1-antigen LPS (Fig. 2), like biosynthetic genes for the production of monosaccharide residues corresponding to the UDP-deoxyamino sugars (L-FucNAc, L-PneNAc, and D-QuiNAc). ORFs 6-7-8 encoding for FlnA-B-C, respectively, which from UDP-GlcNAc led to the formation of UDP-L-FucNAc through also the formation of UDP-L-PneNAc by the following enzymatic reactions: UDP-GlcNAc→UDP-2-acetoamido-2,6-dideoxy-β-L-*lyxo*4-hexulose→UDP-L-PneNAc→UDP-L-FucNAc. The ORFs 10-13-14 encoding for PglB-C-D, respectively, which are basic for the biosynthesis of the UDP-2,4-diacetoamido-2,4,6-trideoxyhexoses being D-QuiNAc (bacillosamine) the most characteristic of them.

Table 1
Bacterial strains, cosmids and plasmids used.

Strain, cosmid, or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F− *endA hsdR17* (rk− mk+) *supE44 thi-1 recA1 gyr-A96 80lacZ*	[43]
S17-1λpirKm1	*thi thr1 leu tonA lacY supE recA*::*RP4-2* (*Tc*::*Mu*) *Km*^{r} *λpir* with miniTn5Km-1	[29]
MC1061λpir	*thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ *pir*	[23]
P. shigelloides		
302-73	Wild type, serotype O12:K80	(our lab, [16])
302-73R	302-73, spontaneous Rif^r	This study
302-A	302-73*pglD*:mini-Tn5Km-1 Rif^r Km^r	This study
302-B	302-73*fnlA*:mini-Tn5Km-1 Rif^r Km^r	This study
Plasmids		
pLA2917	Tc^r, Km^r	[31]
COS-PO1	pLA2917 with 20-kb chromosomal 302-73 *Sau*3A insert carrying part of the *wb* cluster, Tc^r	This study
COS-PO2	pLA2917 with 20-kb chromosomal 302-73 *Sau*3A insert carrying part of the *wb* cluster, Tc^r	This study
pRK2073	Helper plasmid, Sp^r	[23]
pGEM-T	PCR cloning vector, Amp^r	Promega
pFS100	pGP704 suicide plasmid, *λpir*-dependent, Km^r	[23]
pFS-ORF14	pFS100 with an internal fragment of *pglD*, Km^r	This study
pFS-ORF6	pFS100 with an internal fragment of *fnlA*, Km^r	This study

[a] r = resistant.

Table 2
The adhesion of different *P. shigelloides* strains to INT 407 and Caco-2 eukaryotic cells.

Strain	Mean number of bacteria % reduction per INT 407 cell		Mean number of bacteria % reduction per Caco-2 cell	
302-73 (wild type)	17 ± 2.3	[a]	15 ± 2.0	[a]
302-73R (rifampicin^r)	17 ± 2.1		15 ± 1.7	
302-A O1-antigen LPS$^-$	4 ± 1.6	77	3 ± 1.2	80
302-B O1-antigen LPS$^-$	4 ± 1.8	77	3 ± 1.5	80
302-A + COS-PO1 (O1-antigen LPS$^+$)	16 ± 1.9	6	13 ± 2.5	14
302-B + COS-PO2 (O1-antigen LPS$^+$)	16 ± 1.7	6	13 ± 1.8	14
302-A + pLA2917	4 ± 1.2	77	3 ± 1.3	80
302-B + pLA2917	4 ± 1.6	77	3 ± 1.1	80

[a] Student's *t*-test, $P < 0.001$.

Table 3
The *P. shigelloides* strains invasion to INT 407 and Caco-2 cells.

Strain	Invasion to[a]			
	INT 407 cells	(% Reduction)	Caco-2 cells	(% Reduction)
302-73 wild type	0.97 ± 0.012 [b]		0.86 ± 0.010 [b]	
302-73R (rifampicin^r)	0.96 ± 0.009		0.85 ± 0.008	
302-A O1-antigen LPS$^-$	0.13 ± 0.006	87	0.10 ± 0.005	89
302-B O1-antigen LPS$^-$	0.14 ± 0.005	86	0.11 ± 0.007	88
302-A + COS-PO1 (O1-antigen LPS$^+$)	0.92 ± 0.009	6	0.82 ± 0.010	5
302-B + COS-PO2 (O1-antigen LPS$^+$)	0.95 ± 0.011	3	0.84 ± 0.006	3
302-A + pLA2917	0.14 ± 0.007	86	0.10 ± 0.007	89
302-B + pLA2917	0.14 ± 0.005	86	0.09 ± 0.004	90

[a] Percentage invasion is the percentage input bacteria surviving, after extensive washing, gentamycin treatment. Numbers represent the mean ± standard deviation. The negative control for invasion was *E. coli* DH5α which showed 0.04 ± 0.004% invasion.
[b] Student's *t*-test, $P < 0.001$.

The putative acetyltransferase (ORF11) could be the one that acetylates the L-PneNAc. It is tempting to speculate the need of at least four glycosyltransferases to form the O1-antigen LPS according to their chemical structure (Fig. 2), being the clear putative candidates ORFs 4 and 9, and more speculative ORFs 2 and 12. Two main known pathways for O-antigen export, besides that there are four reported, have been established [25]: the Wzy-dependent pathway for heteropolysaccharides structures and the ABC 2 transporter-dependent pathway mainly for homopolysaccharides. The presence of ORF5 (Wzx) and ORF3 (Wzy) showed that *P. shigelloides* O1-antigen LPS belongs to the first pathway. Furthermore, ORF1 is a Wzz protein able to regulate the O-antigen chain length, characteristic of the Wzy-dependent pathway, as it has been described in other bacteria [25].

To synthesize O-antigens, monomers are assembled on a lipid carrier (undecaprenol phosphate) by enzymes encoded or not in the *wb* gene cluster before their incorporation into the LPS molecule. No *P. shigelloides* wb_{O1} ORF seems to render homology with undecaprenol-sugar-P-transferase [25], then the gene codifying this enzyme seems to be outside the cluster. We could not at that time localize this gene, as far as today no complete *P. shigelloides* genome is available.

The presence of aquaporins in the previously gene cluster reported coding O17-antigen LPS [22] and this one (ORF15) could be an important characteristic for these high hydrophobic LPSs. Aquaporins are a large family of transmembrane channel proteins that are present throughout all domains of life and their malfunction has been implicated in several human disorders [26]. Different permeability to water and other solutes and variations in the amino acid composition of the ar/R region has led to the classification of aquaporins into two main functional sub-families [27,28]. The first are strict aquaporins, which only allow the passage of water molecules like *E. coli* AqpZ. The second are aquaglyceroporins, less selective aquaporins that can transport water and other solutes, such as glycerol, urea and other uncharged small molecules like *E. coli* GlpF. The occurrence of deoxy sugars as hydrophobic substituent's in all of the *P. shigelloides* O-chain structures so far characterized could explain the need for aquaporins in these strains with highly hydrophobic LPS.

Table 4
Biofilm values of several *P. shigelloides* strains using the method of Pratt and Kolter [24].

Strain	Value[a]
302-73	1.2 ± 0.09
302-73R (rifampicin^r)	1.2 ± 0.07
302-A O1-antigen LPS$^-$	0.3 ± 0.03
302-B O1-antigen LPS$^-$	0.3 ± 0.05
302-A + COS-PO1 (O1-antigen LPS$^+$)	1.1 ± 0.11
302-B + COS-PO2 (O1-antigen LPS$^+$)	1.0 ± 0.08
302-A + pLA2917	0.3 ± 0.04
302-B + pLA2917	0.3 ± 0.06

[a] Student's *t*-test, $P < 0.001$.

The presence of the O1-antigen LPS for *P. shigelloides* is crucial for survive in serum, mainly to become resistant to complement. Also, the *P. shigelloides* O1-antigen LPS is important either for the adhesion and invasion to different eukaryotic cells. It is possible that the O1-antigen LPS is not the only factor promoting adhesion and invasion of *P. shigelloides*, but is a relevant factor for these pathogenic characteristics. The presence of the *P. shigelloides* O1-antigen LPS is also important in the ability to form biofilms tested according to Pratt and Kolter [24]. As it happens in other *Enterobacteriaceae* the O-antigen LPS is relevant for the pathogenicity of this bacterium. Nevertheless, there is a special characteristic for *P. shigelloides* LPS: their hydrophobicity. It is tempting to speculate that the occurrence of hydrophobic substituents in all of the O-chain structures so far characterized rendering a high hydrophobic LPS (found in the phenol phase in their isolation, [16]), altogether with the presence of aquaporins, could suggest a method adopted by this bacterium to adhere to host cells in aqueous environment.

Finally, this is the first report on the genetics from a *P. shigelloides* O-antigen LPS cluster (*wb*) not shared by *Shigella* like *P. shigelloides* O17, the only reported until now [22]. Furthermore, this study outlined the importance in *P. shigelloides* of this polysaccharide structure in several pathogenic features for the first time.

4. Materials and methods

4.1. Bacterial strains, plasmids and growth conditions

The bacterial strains, cosmids and plasmids used are listed on Table 1. Bacteria were grown in TSB broth and TSA medium supplemented with kanamycin (25 μg/ml), tetracycline (20 μg/ml), ampicillin (100 μg/ml) and rifampicin (100 μg/ml) when needed.

4.2. MiniTn5Km-1 mutagenesis

Conjugal transfer of transposition element miniTn5Km-1 from *E. coli* S17-1λ*pir*Km-1 [29] to *P. shigelloides* 302-73R (wild type strain rifampicin-resistant) was carried out in a conjugal drop incubated for 6 h at 30 °C with the ratio 1:5:1 corresponding to S17-1λ*pir*Km-1, 302-73R and HB101 with pRK2073 (helper plasmid), respectively. Serial dilutions of the mating mix were plated on TSA supplemented with rifampicin and kanamycin, in order to select mutants.

4.3. Construction of a P. shigelloides genomic library

P. shigelloides strain 302−73 (serotype O1) genomic DNA was isolated and partially digested with *Sau3A* as described by Sambrook et al. [30]. Cosmid pLA2917 [31] was digested with *Bgl*II, dephosphorylated and ligated to *Sau*3A genomic DNA fragments. DNA packaging by using Gigapack Gold III (Stratagene) and infection of *E. coli* DH5α were carried out as previously described [32]. Recombinant clones were selected on TSA plates supplemented with tetracycline.

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4.4. General DNA methods

General DNA manipulations were done essentially as described [30]. DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

4.5. Southern blot hybridizations

Southern blotting was performed by capillary transfer [30] onto Hybond N1 (Amersham) nylon membrane. Probe labeling, hybridization, and detection were carried out using the enhanced chemiluminescence labeling and detection system (Amersham) according to the manufacturer's instructions.

4.6. DNA sequencing and computer analysis of sequence data

Double-stranded DNA sequencing was performed by using the dideoxy-chain termination method [33] with the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystem). Oligonucleotides used for genomic DNA amplifications experiments and for DNA sequencing were purchased from Sigma–Aldrich. 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 [34,35] network service at the National Center for Biotechnology information and the European Biotechnology Information, respectively. Clustal W was used for multiple sequence alignments.

4.7. Complementation studies

Complementation analysis of the different mutants was performed by conjugal transfer of recombinant clones from the genomic library. Recombinants were selected on TSA containing tetracycline and kanamycin.

4.8. LPS isolation and SDS-PAGE

For screening purposes LPS was obtained after proteinase K digestion of whole cells [36]. LPS samples were separated by SDS-PAGE and visualized by silver staining as previously described [36,37]. For large-scale isolation LPS was extracted from bacterial dried cells using phenol/chloroform/light petroleum (2:5:8 by vol.) [38],. The residual material was then extracted with a 1:1 mixture of phenol and water at 68 °C [39]. Both the water and the phenol phases were dialyzed (cut-off 3500) and lyophilized. The LPS was recovered from the phenol phase. Monosaccharides were analyzed as alditol acetates as well as acetylated methyl glycosides. Alditol acetates were obtained from the LPS (1 mg). The LPS was hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2 h), reduced with $NaBD_4$ and acetylated with Ac2O and pyridine. Acetylated methyl glycosides were obtained from the crude LPS. Methanolysis was performed in 1 M MeOH/HCl (0.5 ml, 80 °C, 20 h) and the sample was extracted twice with hexane. The methanol layer was concentrated and the residue was dried and acetylated. In order to obtain the O-chain polysaccharide, the LPS was mildly hydrolyzed with 1% aqueous AcOH. The lipidic portion of the LPS was removed by centrifugation and the supernatant was fractionated with a Sephadex G-50 column (Pharmacia), eluting with pyridine/acetate buffer. The fractions containing the O-chain were collected, dialyzed in water, and freeze dried.

4.9. Antiserum

Anti-*P. shigelloides* O1-antigen LPS serum was obtained and assayed as previously described for other LPSs, but in this case using the fractions containing the O1-chain as mentioned in the previous section [40,41].

4.10. Colony immunoblotting

For colony blot, the colonies were transferred to a filter membrane by capillarity and air dried. The membranes were then blocked and incubated sequentially with 1% bovine serum albumin, specific anti-O serum (1:500), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, and 5-bromo-4-chloro-indolyl-phosphate disodium-nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline were included after each incubation step. Colony blotting was performed using *P. shigelloides* O1-antigen LPS serum.

4.11. Serum killing

The survival of exponential-phase bacteria in non immune human serum was measured as previously described [40].

4.12. Adhesion and invasion assays

A stock culture of INT 407 cells was obtained from the American Type Culture Collection. This cell line was cultured in MEM with 10% FBS (fetal bovine serum) at 37 °C in a humidified, 5% CO_2 incubator. Caco-2 cells from our lab stock were grown to confluence with MEM supplemented with 20% FBS at 37 °C in a humidified, 5% CO_2 incubator. For experimental assays, each well of a 24-well tissue culture tray was seeded with 1×10^5 cells per well and incubated for 18 h at 37 °C in a humidified, 5% CO_2 incubator.

The adherence an invasion assays assay methods were adapted from Oelschaleger et al. [42]. Briefly, approximately 5×10^7 bacterial cells were layered onto confluent monolayers of approximately 1×10^5 INT 407 or Caco cells suspended in Hank's balanced salt solution (HBSS) per well in 24-well plates, and incubated at 37 °C in 5% CO_2 for different times. For determination of adherence, the cells were washed extensively with HBSS with strong agitation for 2 min, prior to lysis of the monolayer with 0.01% Triton X-100 and enumeration of total bacteria by plate count in TSA. For determination of invasion, the monolayer was washed extensively with HBSS, and fresh, prewarmed medium containing gentamycin (100 μg/ml) was added to kill extracellular bacteria. After 1 h incubation, the monolayer was washed twice with HBSS, and cells lysed with 0.01% Triton X-100 for 30 min; the released intracellular bacteria were enumerated by plate counting. The invasive ability was expressed as the percentage of the inoculum surviving the gentamycin treatment; adherent bacteria were expressed as the total number of bacteria enumerate without antibiotic treatment. In some experiments coverslips were used in the 24-h well trays, and after incubation the coverslips were washed, fixed in 70% methanol and stained with Giemsa. Coverslips were then mounted on glass microscope slides and the adherence was assessed by bright-field microscopy. For each assay, bacteria adhering to 30 randomly selected cells from each of two monolayers were counted. The assays were performed at least in triplicate.

4.13. Biofilm formation

Quantitative biofilm formation was performed in a microtiter plate as described previously [24], with minor modifications.

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Briefly, bacteria were grown on TSA and several colonies were gently resuspended in TSB (with or without the appropriated antibiotic); 100 µl aliquots were place in a microtiter plate (polystyrene) and incubated 48 h at 37 °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.

4.14. Statistical analysis

The data obtained in several assays were analyzed by the *t*-test using Microsoft Excel software.

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