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Different sugar residues of the lipopolysaccharide outer core are required for early interactions of *Salmonella enterica* serovars Typhi and Typhimurium with epithelial cells

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ABSTRACT

The role of lipopolysaccharide (LPS) in entry of *Salmonella* Typhimurium into epithelial cells remains unclear. In this study, we tested the ability of a series of mutants with deletions in genes for the synthesis and assembly of the O antigen and the outer core of LPS to adhere to and invade HeLa, BHK, and IB3 epithelial cells lines. Mutants devoid of O antigen, or that synthesized only one O antigen unit, or with altered O antigen chain lengths were as able as the wild type to enter epithelial cells, indicating that this polysaccharide is not required for invasion of epithelial cells *in vitro*. In contrast, the LPS core plays a role in the interaction of *S.* Typhimurium with epithelial cells. The minimal core structure required for adherence and invasion comprised the inner core and residues Glc I–Gal I of the outer core. A mutant of *S.* Typhimurium that produced a truncated LPS core lacking the terminal galactose residue had a significant lower level of adherence to and ingestion by the three epithelial cell lines than did strains with this characteristic. Complementation of the LPS production defect recovered invasion to parental levels. Heat-killed bacteria with a core composed of Glc I–Gal I, but not bacteria with a core composed of Glc I, inhibited uptake of the wild type by HeLa cells. A comparison of the chemical structure of the *S.* Typhi core with the published chemical structure of that of *S.* Typhimurium indicated that the Glc I–Gal I–Glc II backbone is conserved in both serovars. However, *S.* Typhi requires a terminal glucose for maximal invasion. Therefore, our data indicate that critical saccharide residues of the outer core play different roles in the early interactions of serovars Typhi and Typhimurium with epithelial cells.

1. Introduction

Salmonella enterica are enteropathogenic bacteria that cause over one billion infections annually worldwide [1]. Serovars Typhi (herein *S.* Typhi) and Paratyphi are the causative agents of typhoid fever, a systemic disease that affects only humans [2,3]. Non-typhoid serovars, like Typhimurium (herein *S.* Typhimurium) and Enteritidis, have a broad range host specificity and they predominantly cause gastroenteritis in humans [1]. Our current understanding of the molecular pathogenesis of *Salmonella* arises mostly from studies using *S.* Typhimurium [4,5]. However, pairwise comparisons between sequenced *Salmonella* genomes indicate that, despite 97.6%–99.5% identity between housekeeping genes, about 10% of genes are unique to each serovar [6–8]. These genes are likely involved in determining differences in host specificity and pathogenesis among *Salmonella* serovars [9,10].

Whether salmonellosis affects only the intestinal tract or progresses to systemic disease, the ability of bacteria to invade and penetrate the intestinal epithelium is a critical feature of pathogenicity. The type III secretion system encoded within the *Salmonella* pathogenicity island 1 (TTSS-1) mediates interactions with epithelial cells. Type III secreted effector proteins target the actin cytoskeleton and elicit cellular responses that ultimately facilitate entry of *Salmonella* into epithelial cells [11–14]. Full activation of the TTSS-1 requires the contact of the bacterium with the host cell [12]; thus, early bacterial–epithelial cell interactions are necessary before invasion can occur. To date, the nature of these interactions is not fully understood, although it may involve surface structures such as flagella [15–20], fimbriae [21], and the lipopolysaccharide (LPS).

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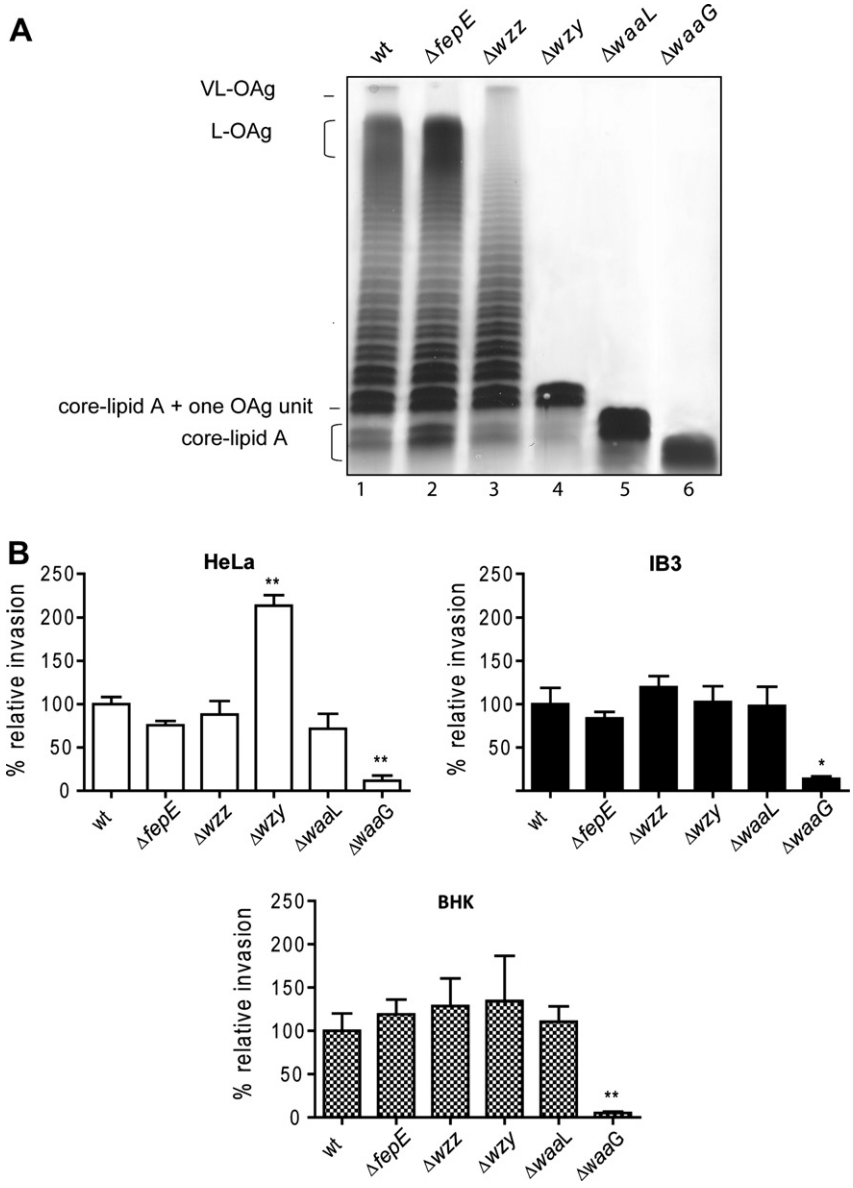

Fig. 1. (A) LPS from *S.* Typhimurium SL1344 wild type (wt) and LPS mutants MTM1138 (Δ*fepE*), MTM5 (Δ*wzz*), MTM1224 (Δ*wzy*), MTM122 (Δ*waaL*) and MTM113 (Δ*waaG*) (lanes 1–6, respectively). LPS samples from equal numbers of bacterial cells (1×10^7 CFU) were loaded in each lane and were analyzed by Tricine-SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining. (B) Invasion of epithelial cells by *S.* Typhimurium SL1344 and LPS mutants. Values represent invasion of each strain relative to invasion of the wild type (wt). Invasion assays were performed in HeLa, BHK and IB3 epithelial cells, in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values that are significantly different from that of the wild-type by the one-way ANOVA test and Dunnett posttest are indicated by asterisks (* = $P < 0.05$; ** = $P < 0.01$).

LPS, the major constituent of the outer leaflet of the bacterial outer membrane, is a complex glycolipid composed of three domains: lipid A, an oligosaccharide core, and O polysaccharide (or O antigen) [22]. Although several studies have established an important role for LPS in *Salmonella* virulence [23], the role of LPS components in the invasion of epithelial cells has not been fully defined. An early report by Tannock et al. [24] indicated that the O antigen was not required for the association with, and invasion of, the ileal mucosa in mice. In contrast, other *in vivo* studies showed that colonization of the mouse intestine was impaired in *S.* Typhimurium LPS-defective mutants [25], and transposon mutants lacking O antigen were attenuated in orally infected mice [26]. More recently, it was reported that a mutant with a relatively short random-length O antigen was attenuated when administered by the oral route of infection [27]. Furthermore, a signature-tagged mutagenesis screening [28] demonstrated that mutants in genes for O antigen biosynthesis were impaired in their ability to colonize chicken and calf intestines.

Not much is known on LPS and invasion of epithelial cells *in vitro*. Studies conducted with spontaneous or transposon-insertion mutants indicated that long-chain O antigen is not required for invasion of cultured HeLa [29,30], Caco-2 [31] or HEp-2 [32] cells by *S.* Typhimurium. Moreover, Holzer et al. [33] showed that two mutants lacking O antigen molecules can adhere and invade HeLa cells more efficiently than the wild type *S.* Typhimurium SL1344.

To date, two reports have addressed the role of the core region of LPS in entry of *S.* Typhimurium into cultured epithelial cells, and their results are contradictory. While Kim et al. [34] showed that a mutant defective in the synthesis of the inner core (deep-rough) was non-invasive, Nagy et al. [35] found that two defined deep-rough mutants could invade intestinal Henle 407 cells in higher amounts than the parental strain.

We previously showed that the O antigen was not required for invasion of HEp-2 cells by *S.* Typhi; instead, an outer core composed of Glc I–Gal I–Glc II was required for efficient bacterial entry. Mutants lacking the exposed Glc II residue were unable to invade the epithelial cells [36]. In this study, we investigated the role of LPS components in the ability of *S.* Typhimurium to invade three tissue culture epithelial cell lines. We show that the presence of O antigen is irrelevant for bacterial invasion of HeLa, BHK or IB3 epithelial cells. In contrast, a core structure consisting of the inner core and the Glc I–Gal I disaccharide of the outer core is necessary for adhesion and subsequent entry of *S.* Typhimurium into epithelial cells. Therefore, the requirement for LPS in the interaction of *Salmonella* with epithelial cells differs between *S.* Typhi and *S.* Typhimurium.

2. Results

2.1. The LPS outer core, but not the O antigen, is required for entry of S. Typhimurium into epithelial cells

To define the LPS components contributing to invasion of epithelial cells by *S.* Typhimurium SL1344, we first constructed precise nonpolar deletions in various genes encoding functions required for the assembly of the O antigen polymer. These included: *fepE* (MTM1138) and *wzzB* (MTM5), encoding the O-antigen chain length regulators; *wzy* (MTM1224) encoding the O-antigen polymerase, and *waaL* (MTM122) encoding the O-antigen ligase. Analyses of the LPS profiles in silver-stained polyacrylamide gels showed that MTM1138 (Δ*fepE*) and MTM5 (Δ*wzzB*) produced LPS lacking very long (VL) and long (L) O antigen, respectively (Fig. 1A, lanes 2 and 3). The mutant in the O-antigen polymerase gene (MTM1224) showed a semi-rough phenotype with a very prominent band of lipid A-core oligosaccharide substituted with one O-antigen unit (Fig. 1A, lane 4), whereas the ligase mutant MTM122 synthesized only the lipid A-core region (Fig. 1A, lane 5).

The ability of each mutant to invade HeLa, BHK and IB3 epithelial cells *in vitro* was compared to that of the parental *S.* Typhimurium SL1344 (Fig. 1B). Strains MTM1138 (Δ*fepE*), and MTM5 (Δ*wzzB*) invaded the three cultured cell lines at similar levels of those of the parental strain (Fig. 1B); indicating that chain-length distribution of the O-antigen molecules is irrelevant to this process. Mutant MTM1224 (Δ*wzy*), which assembles only one O-antigen unit, showed an invasion capacity about 2-fold higher than the wild type in HeLa cells, but in BHK and IB3 cells the invasion levels were similar to the parental strain (Fig. 1B). Finally, strain MTM122 (Δ*waaL*) that lacks O antigen entirely, also invaded the three epithelial cell lines as efficiently as the wild type. These results indicate that the absence of O antigen does not impair entry.

In order to determine whether the outer core region plays a role in the invasion process, as we previously observed in *S.* Typhi, we constructed a deletion mutant in the *waaG* gene, which is responsible for the addition of the first glucose residue (Glc I) to the inner core. This mutant (MTM113) produced a lipid A-core region migrating faster than that of the Δ*waaL* mutant, indicative of a truncated core (Fig. 1A, lane 6). Strain MTM113 was unable to enter to any of the epithelial cells used in this study (Fig. 1B), suggesting that the outer core is necessary for invasion.

2.2. Adhesion and entry of S. Typhimurium to epithelial cells requires an outer core region composed of Glc I–Gal I

To define the sugar residues of the outer core oligosaccharide involved in internalization of *S.* Typhimurium by epithelial cells, we constructed deletions in each of the genes responsible for the assembly of the outer core: *waaK* (MTM115), *waaJ* (MTM102), *waaI* (MTM1015) and *waaB* (MTM109). The published structure of the *S.* Typhimurium outer core oligosaccharide [37] and the LPS profiles of each mutant are shown in Fig. 2A and B, respectively. Analyses of the LPS patterns revealed that the lipid A-core band of strain MTM115 (lane 3) migrated slightly faster than that of the MTM122 (Δ*waaL*) mutant (lane 2), consistent with the absence of the GlcNAc residue in the outer core. Because this residue is essential for the recognition of the core acceptor by the O-antigen ligase [38] this strain also lacked O antigen (lane 3). The absence of Glc II in strain MTM102 produced a more truncated core oligosaccharide (lane 4), and deletion of the *waaI* and *waaG* genes (strains MTM1015 and MTM113, respectively) resulted in further truncations (lanes 5 and 7). Finally, mutant MTM109 (Δ*waaB*) that lacks the Gal II side-chain residue produced a heterogeneous core region. As shown (lane 6) some LPS molecules migrated slightly faster than the core band of mutant MTM1015, whereas other molecules were able to assemble an entire core and attach O-antigen molecules, producing a complete LPS. This result is consistent with previous reports that demonstrated that the requirement of WaaI for the branch galactose is not absolute and thus, the Δ*waaB* mutant has a fraction of complete core molecules that can be substituted with O antigen [39].

The ability of the mutant strains to enter and adhere to epithelial cells (Fig. 3A and B, respectively) was assayed in the three culture cell lines. A mutant in pathogenicity island 1 (ΔSPI-1) was used as negative control of invasion. Levels of association and internalization similar to the parental strain were obtained with strains MTM115 (Δ*waaK*) and MTM102 (Δ*waaJ*) indicating that the branched GlcNAc and the terminal Glc II residues of the outer core are not essential to these processes. Also, MTM109 (Δ*waaB*), which contains some complete LPS molecules, was as adherent and invasive as the wild type. In contrast, deletions in *waaI* and *waaG* (strains MTM1015 and MTM113) reduced significantly the ability of *S.* Typhimurium to associate to and invade epithelial cells. Thus, the Glc I–Gal I backbone

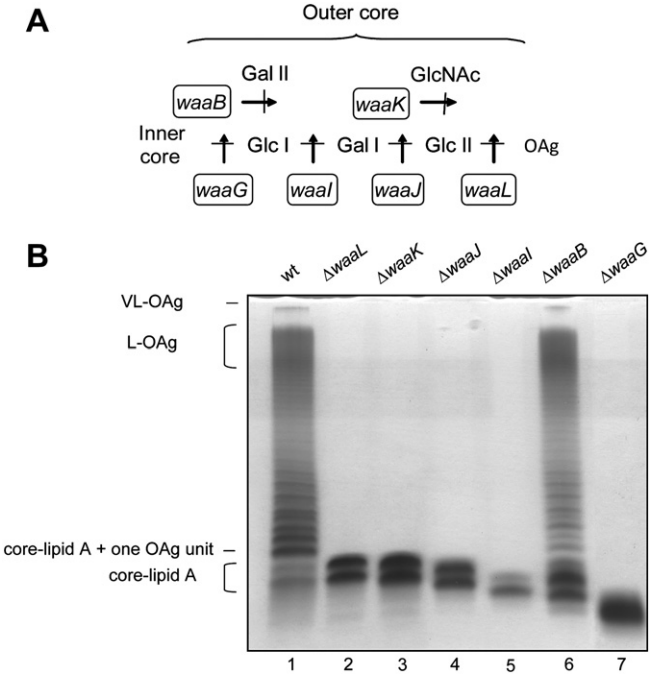

Fig. 2. Published structure of the outer core of serovar Typhimurium (A) and analysis of LPS (B) of *S.* Typhimurium SL1344 wild type (wt) and outer core mutants MTM115 (Δ*waaK*), MTM102 (Δ*waaJ*), MTM1015 (Δ*waaI*), MTM109 (Δ*waaB*) and MTM113 (Δ*waaG*) (lanes 1–6, respectively). LPS samples from equal numbers of bacterial cells (1×10^7 CFU) were loaded in each lane and were analyzed by Tricine/SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining.

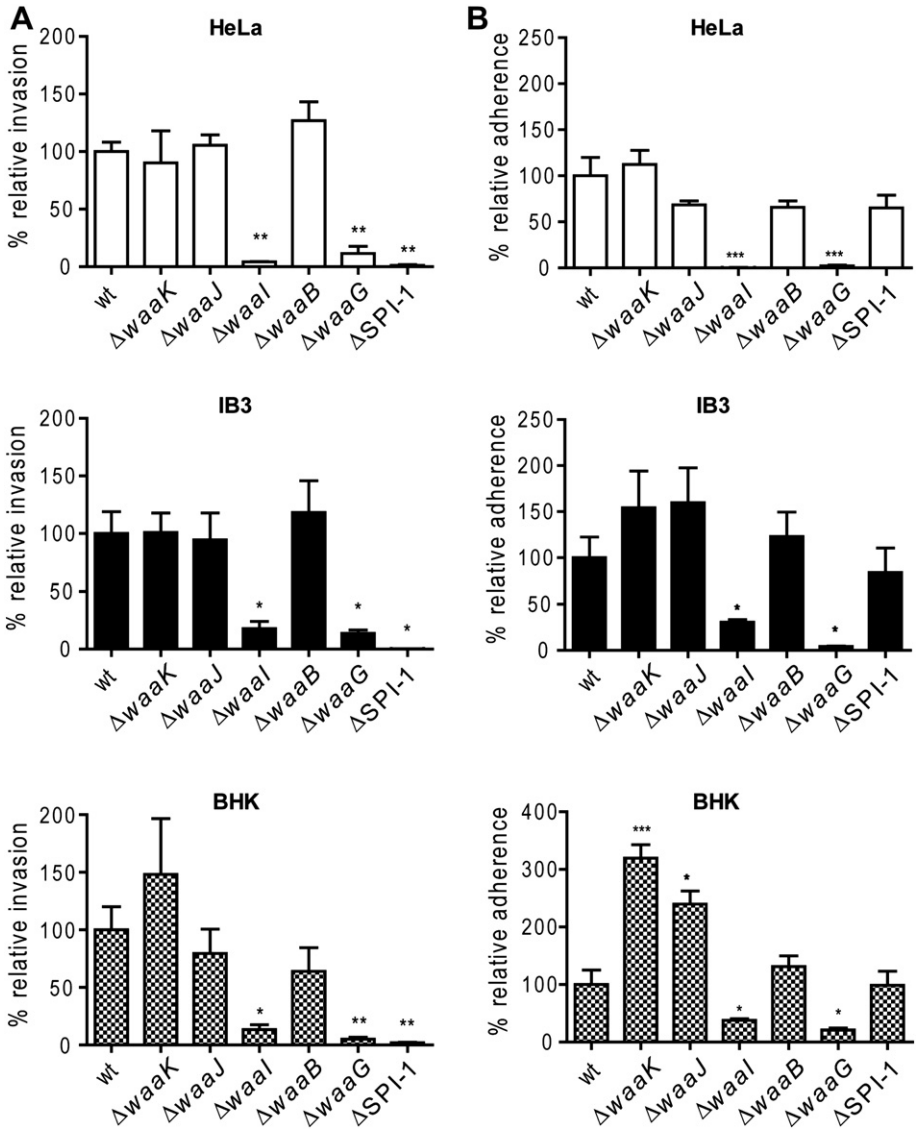

Fig. 3. Invasion (A) and adherence (B) of serovar Typhimurium strain SL1344 wild type (wt) and outer core mutants MTM115 (Δ*waaK*), MTM102 (Δ*waaJ*), MTM1015 (Δ*waaI*), MTM109 (Δ*waaB*) and MTM113 (Δ*waaG*) and a ΔSPI-1 mutant into epithelial cells. Values represent invasion or adherence of each strain relative to the wt. Assays were performed in HeLa, BHK and IB3 cells, in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values that are significantly different from that of the wild-type by the one-way ANOVA test and Dunnett posttest are indicated by asterisks (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

of the outer core is required for adhesion and subsequent entry of *S.* Typhimurium to epithelial cells *in vitro*.

Analyses by light microscopy of monolayers infected with the wild type and the non-invasive mutants were performed. As shown (Fig. 4, Table 2) the cells infected with MTM1015 (Fig. 4B) or MTM113 (Fig. 4C) contained fewer *Salmonella* containing vacuoles (SCV) compared to cells infected with the parental strain (Fig. 4A).

To test whether the LPS mutations affect the function of SPI-1, mutants MTM102 (Δ*waaJ*), MTM1015 (Δ*waaI*) and MTM113 (Δ*waaG*) were transformed with plasmid pSipA/FT carrying the *sipA* gene fused to a FLAG epitope [40]. The wild type strain and the ΔSPI-1 were also transformed as controls. Western blot analyses (Fig. 5) showed that, both the invasive MTM102 and non-invasive MTM1015 secreted the SipA protein when grown under SPI-1-inducing conditions. In contrast, mutant MTM113 was not able to secrete SipA (Fig. 5A) although the protein was expressed intracellularly (Fig. 5B).

To confirm that reduced entry of MTM1015 and MTM113 into epithelial cells was due to the deletion of the corresponding gene, each mutant was transformed with the appropriate intact gene (*waaI* and *waaG*, respectively) cloned into pGEM-T-Easy. LPS analysis revealed that the phenotype of the complemented mutants was identical to the parental profile (Fig. 6A). Also, the complemented strains adhered to and entered epithelial cells as efficiently as the wild-type SL1344 transformed with the control vector (Fig. 6B).

To address whether the Glc I–Gal I backbone of the outer core may be a bacterial ligand used for association with epithelial cells, we tested the ability of heat-killed MTM102 and MTM1015 to inhibit the ingestion of the wild-type strain by HeLa cells. We used a 100-fold excess of heat-killed cells over live wild-type bacteria, because the loss of motility of the killed cells reduces their ability to compete with live bacteria for invasion to the monolayer. As shown (Fig. 7), the addition of bacteria that contain the exposed Gal I residue in the outer core (MTM102) to the bacterial suspension during the invasion assay inhibited uptake of *S.* Typhimurium by HeLa cells. In contrast, addition of MTM1015 had no effect on internalization of the wild-type bacteria.

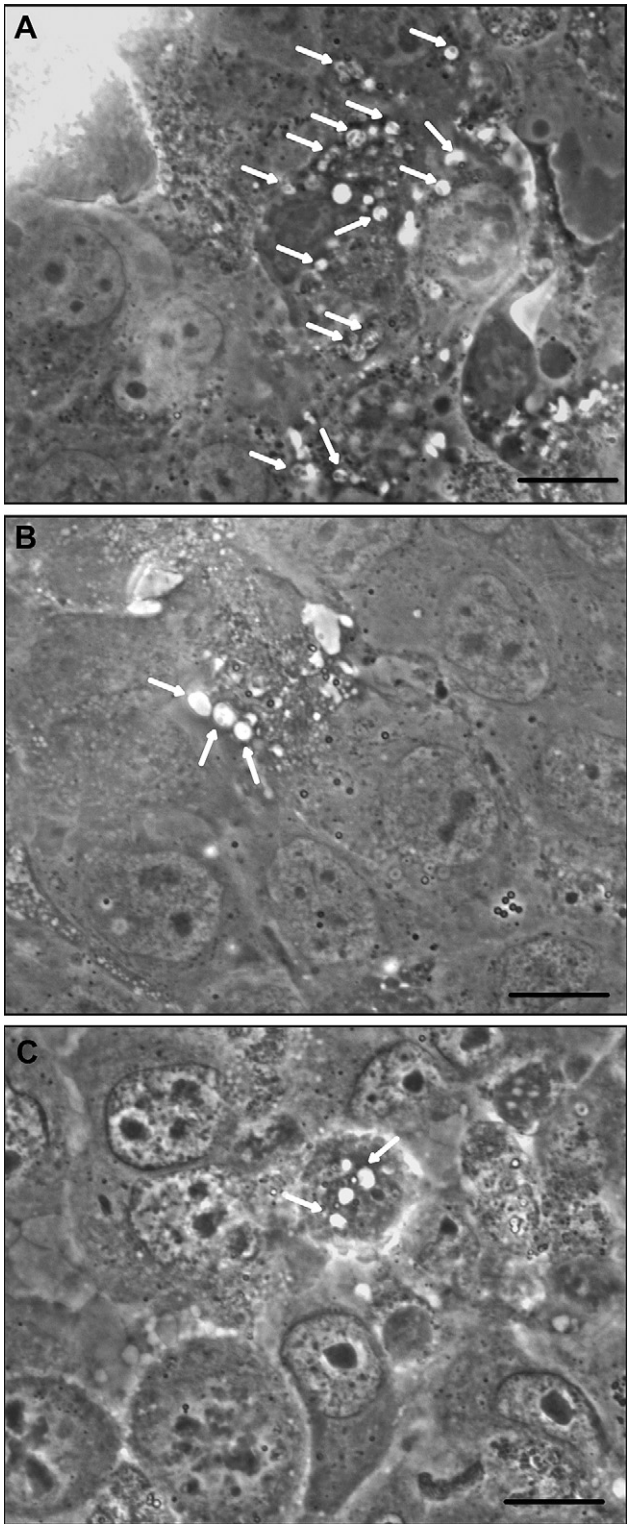

Fig. 4. Representative images of HeLa cells infected with wild-type *S.* Typhimurium SL1344 (A) and truncated core mutants Δ*waaI* (MTM1015) (B) and Δ*waaG* (MTM113) (C). White arrows indicate *Salmonella* containing vacuoles (SCV). Bars = 10 μm

2.3. *S. Typhimurium and S. Typhi require different terminal sugar residues in the outer core for entry into epithelial cells*

Previous results showed that a *S.* Typhi Δ*waaJ* mutant (M102) was unable to invade HEp-2 epithelial cells [36]. In contrast, as described here, a *S.* Typhimurium Δ*waaJ* mutant (MTM102) invades normally (Table 3). To assess whether this difference was due to the cell lines used, we assayed invasion of HeLa, BHK and IB3 cells by *S.* Typhi Ty2 and mutant M102. The results showed that, irrespective of the cell line used, the *S.* Typhi mutant lacking the Glc II residue (M102) did not invade the epithelial cells (Table 3). Thus, although the outer core is necessary for internalization of both serovars, the sugar residues involved in bacteria–cell interaction may be different. Whereas in *S.* Typhi the outer core region

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant properties[b,c,d]	Source or reference
Salmonella Typhimurium strains		
SL1344	*S.* Typhimurium SL1344, wild type	Dr. Guido Mora.
MTM1138	SL1344 Δ*fepE*	This work
MTM5	SL1344 Δ*wzz*	This work
MTM1224	SL1344 Δ*wzy*	This work
MTM122	SL1344 Δ*waaL*	This work
MTM115	SL1344 Δ*waaK*	This work
MTM102	SL1344 Δ*waaJ*	This work
MTM1015	SL1344 Δ*waaI*	This work
MTM109	SL1344 Δ*waaB*	This work
MTM113	SL1344 Δ*waaG*	This work
SL1344/p261	SL1344/pGEM261	This work
MTM102/p102	MTM102/pGEM*waaJ*	This work
MTM1015/p1015	MTM1015/pGEM*waaI*	This work
MTM113/p113	MTM113/pGEM*waaG*	This work
ΔSPI-1	SL1344 ΔSPI-1. Deletion from gene *iagB*(STM2877) to *invH*(STM2900)	Dr. Carlos Santiviago
MTM102/pJC102	SL1344Δ*waaJ*/pGEM*waaJ* from Ty2	This work
MTM1015/pJC1015	SL1344Δ*waaI*/pGEM*waaI* from Ty2	This work
Salmonella Typhi strains		
Ty2	*S.* Typhi Ty2, wild type	I.S.P.[a]
M102	Ty2 Δ*waaJ*	[36]
M102/p102	Ty2 Δ*waaJ*/pGEM*waaJ* from SL1344	This work
M1015/p1015	Ty2 Δ*waaI*/pGEM*waaI* from SL1344	This work
Plamids		
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 $oriT^S$, Amp^R	[46]
pKD4	*bla* FRT *aph* FRT PS1 PS2 ori_{R6K}, Amp^R, Kan^R	[46]
pCP20	*bla cat* cI857 lPR flp pSC101 $oriT^S$, Cam^R, Amp^R	[46]
pGEM-T Easy	TA cloning vector, Amp^R	Promega
p261	261 pb fragment of *rfaH* gene cloned into pGEM-T Easy	C. Blondel
p102	*waaJ* from SL1344 cloned into pGEM-T Easy	This work
p1015	*waaI* from SL1344 cloned into pGEM-T Easy	This work
p113	*waaG* from SL1344 cloned into pGEM-T Easy	This work
pJC102	*waaJ* from Ty2 cloned into pGEM-T Easy	[36]
pJC1015	*waaI* from Ty2 cloned into pGEM-T Easy	[36]
pSipA/FT	*sipA* from *S.* Typhimurium 14028 cloned into pFlagTEM1. Cam^R	[40]

[a] ISP, Institute of Public Health, Santiago, Chile.
[b] Kan^R, kanamycin resistant.
[c] Cam^R, cloramphenicol resistant.
[d] Amp^R, ampicillin resistant.

Table 2
Salmonella containing vacuoles (SCV) in HeLa cells infected with serovar Typhimurium SL1344 (wild type), MTM1015 (SL1344Δ*waaI*) or MTM113 (SL1344Δ*waaG*) strains.

Strains	SCV/field[a]
SL1344	12.9 ± 1.55
S1344LΔ*waaI*	3.05 ± 0.77*
S1344LΔ*waaG*	3.15 ± 0.74*

*Values that are significantly different from the wild type SL1344 ($P < 0.001$) by the one-way ANOVA test and Dunnett posttest.
[a] Values represent the average of SCV counted in five fields from three independent experiments.

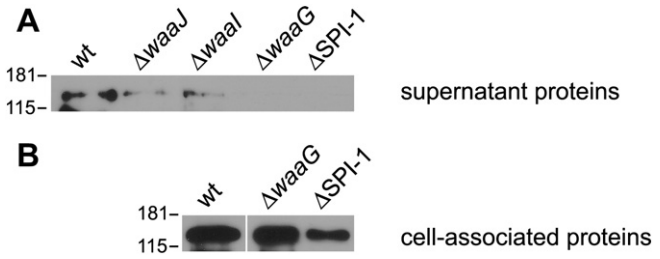

Fig. 5. Analysis of SipA secretion *in vitro* under SPI-1-inducing conditions. Wild type, LPS core mutants and a ΔSPI-1 strain were transformed with pSipA/FT. Bacterial supernatants and bacterial pellets were used to investigate secreted proteins (A) and cell-associated proteins (B). Samples were subjected to SDS-PAGE and the fusion protein was detected by Western blot using anti-FLAG antibodies.

composed of Glc I–Gal I–Glc II is required for invasion, in *S.* Typhimurium an outer core composed of Glc I–Gal I is sufficient for internalization.

These results were unexpected considering the high level of DNA sequence conservation of the *waa* operons. A comparison of the sequences of the *waa* genes in the two serovars revealed 99% identity (www.ncbi.nlm.nih.gov), suggesting that both core structures must be identical. However, to our knowledge, the structure of the LPS core from serovar Typhi has not been elucidated.

To test whether the different invasion abilities of the Δ*waaJ* mutants of Typhi (M102) and *S.* Typhimurium (MTM102) could be attributable to structural differences between the core regions, we performed the structural analysis of the core oligosaccharide in the *S.* Typhi M102 mutant. As shown in Fig. 8A, the experimentally determined structure of *S.* Typhi M102 core is identical to that previously reported for *S.* Typhimurium (Fig. 2) [37]. The only difference found was a partial substitution of the lateral Gal II by Glc (less than 50% of the molecules), which cannot explain the observed

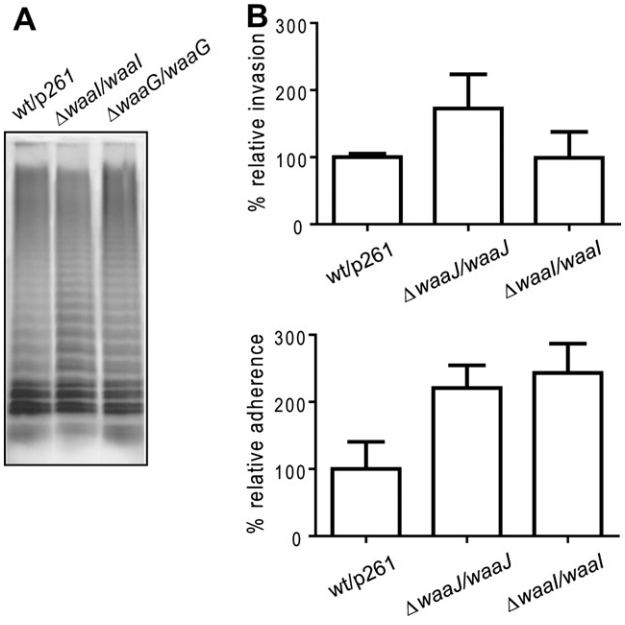

Fig. 6. (A) LPS profiles from wild type *S.* Typhimurium SL1344 transformed with a control plasmid (wt/p261) and complemented core mutants Δ*waaI*/*waaI* (MTM1015/p1015) and Δ*waaG*/*waaG* (MTM113/p113). LPS samples from equal numbers of bacterial cells (1×10^7 CFU) were loaded in each lane and were analyzed by Tricine-SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining. (B) Adherence to and invasion of HeLa cells by wt/p261 and complemented core mutants. Values represent invasion of each strain relative to invasion of wt/p261. Assays were performed in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown.

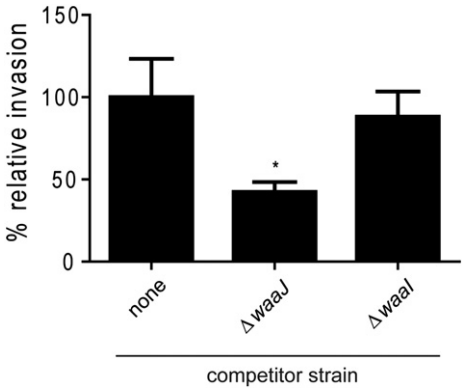

Fig. 7. Inhibition of ingestion of *S.* Typhimurium SL1344 by HeLa cells in the presence of heat-killed MTM1015 (Δ*waaI*) and MTM102 (Δ*waaJ*) strains. The competitor strains were recovered from after a 1 h exposure to epithelial cells, heat killed and incubated to 100 fold excess respect to the wild type. Values represent invasion of the wild type in the presence of the competitor, relative to invasion of the wild type alone. Assays were performed in triplicate on at least three independent occasions. Averages ± standard errors (error bars) are shown. Values that are significantly different from that of the wild type alone ($P < 0.05$) by the one-way ANOVA test and Dunnett posttest are indicated by asterisks.

differences in invasion. The notion that the core regions from both serovars are indeed identical was supported by the results of cross-complementation experiments, in which the Δ*waaI* and Δ*waaJ* mutants from each serovar were transformed with the respective intact gene from *S.* Typhi and *S.* Typhimurium. In all cases, the cross-complemented strains showed a parental LPS phenotype (Fig. 8B). Altogether these results indicate that, although *S.* Typhi and *S.* Typhimurium share the same outer core structure, they require different exposed sugar residues for entry into epithelial cells.

3. Discussion

Despite a number of studies have established an important role for LPS in systemic *S.* Typhimurium virulence [25,41], the requirement for LPS in invasion of epithelial cells remains controversial. In this study, we constructed a series of deletion mutants differing in the production of various parts of the LPS O-antigen and core regions and tested them for their ability to invade three types of cultured epithelial cells. In accordance to previous reports showing that rough mutants (devoid of O antigen) did not have any defect in invasion or adhesion to HeLa [29,30] Caco-2 [31] or HEp-2 [32] cells, we found that *S.* Typhimurium mutants devoid of O antigen, or with altered O antigen chain lengths were as able as the wild type to invade HeLa, BHK and IB3 cell lines. These results are in disagreement with a recent report that showed that mutants lacking O antigen molecules were able to adhere and invade HeLa cells more efficiently than the wild type [33]. We observed a significantly higher level of HeLa cells invasion for the O antigen polymerase mutant, which assembles only one O-antigen unit, suggesting that the absence of polymeric O antigen enhances the ability to enter epithelial cells. However, a similar behavior was not observed either in IB3 or in BHK cells. Thus, in our hands the presence of polymerized O antigen neither impedes nor enhances the ability of *S.* Typhimurium to invade epithelial cells *in vitro*.

In contrast, our results demonstrated that the LPS core plays a role in the interaction of *S.* Typhimurium with epithelial cells. At present, it is not defined whether this region of the LPS molecule is necessary for *S.* Typhimurium internalization, and the available data are conflicting. One report showed that a transposon mutant of *S.* Typhimurium defective in the synthesis of the inner core (deep-rough) was less invasive in HEp-2 and intestinal Henle 407 cells than its isogenic

Table 3
Invasion ability of serovar Typhimurium SL1344 (wild type) and MTM102 (SL1344Δ*waaJ*) strains and serovar Typhi Ty2 (wild type) and M102 (Ty2Δ*waaJ*) strains.

	HeLa	BHK	IB3
SL1344	0.73 ± 0.06	1.57 ± 0.32	4.83 ± 0.91
S1344LΔ*waaJ*	0.77 ± 0.07	1.25 ± 0.33	4.56 ± 1.12
Ty2	0.55 ± 0.05	4.02 ± 0.73	6.87 ± 1.23
Ty2Δ*waaJ*	0.06 ± 0.02*	0.41 ± 0.33*	0.12 ± 0.08*

*Values that are significantly different from that of the wild type Ty2 ($P < 0.001$) by the one-way ANOVA test and Dunnett posttest. Invasion ability was calculated as follows: percent invasion = 100 × (number of bacteria resistant to gentamicin/initial number of bacteria added).

parental strain [34]. In contrast, Nagy et al. [35] reported that a Δ*waaG*::*cat* mutant that produced a complete inner core but lacked the outer core, and a Δ*rfaH*::*cat* mutant that produced a truncated inner core, could invade Henle 407 cells in higher amounts than the parental strain. These differences could be due to the fact that both studies were conducted with mutants that were not rigorously characterized genetically. While in the first report the mutants were obtained by random tranposon insertions, in the second study they were obtained by insertion of an antibiotic-resistant cassette. In both cases, polar effects on genes located downstream the mutation site in the *waa* operon cannot be ruled out.

In this study we have determined that, although an intact LPS core is not required for internalization, a structure consisting of the inner core substituted by the Glc I—Gal I residues of the outer core is essential for adherence and entry of serovar Typhimurium into cultured epithelial cells. The Gal I residue is absolutely required, since the Δ*waaI* mutant showed significantly lower levels of adhesion and entry to the three epithelial cell lines. This defect was not attributable to a defect in the secretion of SPI-1 effectors since this strain was able to secrete the SipA protein under laboratory SPI-1-inducing conditions. On the other hand, an inner core substituted by Glc I is required for proper secretion since the Δ*waaG* strain was unable to secrete the SipA protein (Fig. 5). Also, this mutant showed higher sensitivity to Sodium Dodecyl Sulfate (SDS), indicating that functionality of the outer membrane may be compromised (data not shown).

In a previous study, we demonstrated that the *S.* Typhi outer core terminal glucose residue (Glc II) was necessary for efficient bacterial entry into epithelial cells [36]. In contrast, here we found that the Glc II residue of *S.* Typhimurium's outer core is not required for internalization. These differential requirements for invasion were unexpected, considering that the two serovars supposedly share the same core structures because of the high level of sequence conservation and very similar gene organization of the *waa* operons. The only difference is the presence of ORFs t3798 and t3799 (GenBank accession no. AL513382) inserted between the *waaP* and *waaB* genes in the *S.* Typhi *waa* operon. A deletion mutant that lacked both ORFs showed an identical LPS profile than the wild-type strain, suggesting that these genes are not involved in the biosynthesis of the LPS core (data not shown).

A comparison of the chemical structure of the *S.* Typhi Δ*waaJ* mutant with the published chemical structure of *S.* Typhimurium's outer core [37], indicated that the Glc I—Gal I backbone is conserved in both serovars. In addition, cross-complementation experiments supported the notion that the core regions of *S.* Typhimurium and *S.* Typhi are identical. Thus, the different requirements for LPS of the two serovars to enter epithelial cells cannot be explained by differences in outer core structure or composition. In this context, Pier et al. [42] demonstrated that *S.* Typhi uses the cystic fibrosis transmembrane conductance regulator (CFTR) for entry into epithelial cells. The same group suggested that the bacterial ligand that is recognized by CFTR to mediate invasion of epithelial cells comprise the LPS outer core [43]. On the contrary, invasion of epithelial cells by *S.* Typhimurium was independent of CFTR [42]. These observations are in support of our results, which indicate that critical saccharide residues of the outer core play different roles in the early interactions of serovars Typhi and Typhimurium with epithelial cells. While in *S.* Typhi this structure is required for bacterial entry but not for adhesion [36], the *S.* Typhimurium outer core is needed for adhesion to epithelial cells. To date, the receptor involved in recognition of *S.* Typhimurium LPS outer core to epithelial cells remains unidentified. It has been previously reported that, in phagocytic cells, a GlcNac residue in the outer core of several Gram-negative species, including *S.* Typhimurium, is a ligand recognized by the C-type lectin DC-specific ICAM-grabbing nonintegrin (DC-SIGN) receptor [44]. Therefore, we can speculate that a lectin type receptor could be involved in the interaction of the host cells with the outer core structure composed of (Gal I α1—3 Glu), although to our knowledge, this type of receptor has not been described.

4. Materials and methods

4.1. Bacterial strains, plasmids, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani medium (LB) (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl). Solid medium contained 1.5% (w/v) agar. Ampicillin (Amp, 100 μg/ml), kanamycin (Kan, 50 μg/ml) or Chloramphenicol (Cam, 20 μg/ml) were added when appropriate.

4.2. Cell lines

Baby hamster kidney (BHK) cells expressing the wild-type CFTR with a C-terminal HA epitope (CFTR-HA) [45] and human bronchial epithelial (IB3) cells expressing human wild-type CFTR were provided by Dr. Gergely Lukacs. BHK cells were maintained in 1:1 mixture in Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 Nutrient Mixture medium containing 5% (v/v) fetal bovine serum and 100 μg/ml methotrexate. IB3 cells were maintained in LHC-8 medium containing 5% (v/v) fetal bovine serum and 100 μg/ml Hygromicin. HeLa cells (American Type Culture Collection CCL-2), derived from a cervical carcinoma, were grown in DMEM supplemented with 8% (v/v) fetal bovine serum (FBS).

4.3. Mutagenesis of S. Typhimurium LPS biosynthesis genes

Mutagenesis was performed by the Red/Swap method of Datsenko and Wanner [46] to create chromosomal mutations by homologous recombination using PCR products. Briefly, *S.* Typhimurium SL1344 cells containing plasmid pKD46, which expresses the λ Red recombinase system, were transformed with PCR products that were generated using as template plasmid pKD4, which contains the FRT-flanked kanamycin-resistance gene (*aph*). Each primer pair also carried 25 bases that were homologous to the edges of the gene targeted for disruption. The sequences of the oligonucleotide primers used in this study are available on request. The Kan^r transformants were replica plated in the absence of antibiotic selection at 37 °C and assayed for ampicillin sensitivity to confirm the loss of pKD46. To obtain nonpolar deletion mutants, the antibiotic resistance gene was removed by transforming the gene replacement mutants with pCP20, which encodes the FLP recombinase [47]. Correct insertional gene replacements and the deletion of the antibiotic gene cassettes were confirmed by PCR.

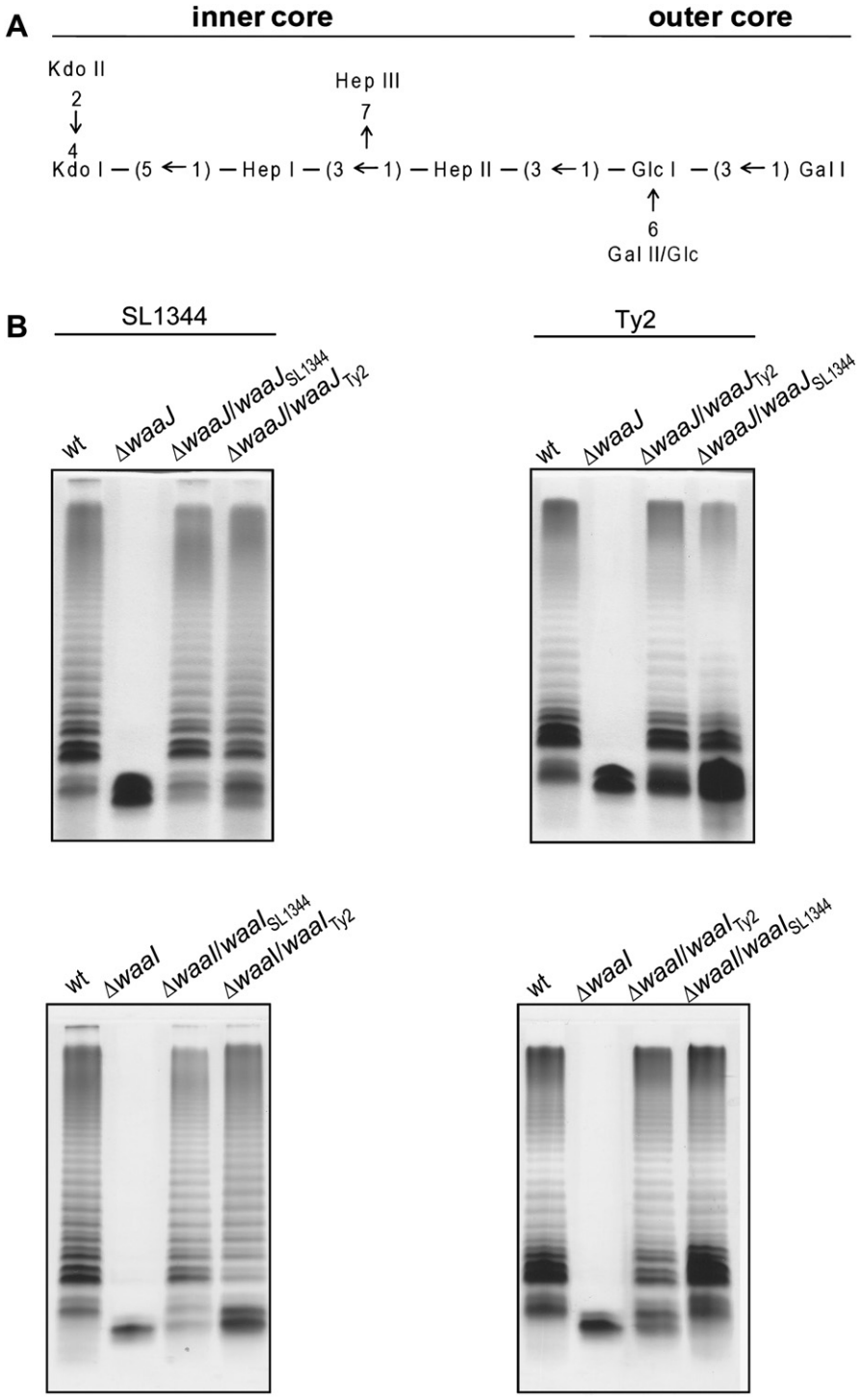

Fig. 8. (A) Structure of the core region of *S.* Typhi mutant strain M102 (Δ*waaJ*) (B) left panel: LPS analysis of *S.* Typhimurium SL1344 wild type and mutants Δ*waaJ* and Δ*waaI* complemented with the corresponding wild type (wt) genes from *S.* Typhimurium and *S.* Typhi. Right panel: LPS analysis of *S.* Typhi Ty2 wild type and Δ*waaJ* and Δ*waaI* mutants complemented with the corresponding wt genes from *S.* Typhi and *S.* Typhimurium. LPS samples from equal numbers of bacterial cells (1×10^7 CFU) were loaded in each lane and were analyzed by Tricine/SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining.

4.4. Complementation experiments

To complement the mutants, the intact gene corresponding to each deletion mutant was PCR amplified from the chromosome of *S.* Typhimurium SL1344, cloned into pGEM-T-Easy and the recombinant plasmid introduced by electroporation into the mutant. For cross-complementation experiments, *S.* Typhi strains M102 and M1015 were complemented with plasmids p102 and p1015, and *S.* Typhimurium strains MTM102 and MTM1015 were complemented with plasmids pJC102 and pJC1015.

4.5. Adhesion and invasion assays

HeLa, IB3 and BHK cells were grown in the corresponding medium and monolayers for infection were prepared by seeding 8×10^4 cells into each well of a 48-multiwell plate and incubating

at 37 °C for 20 h in 5% CO_2 and 95% air. Bacteria were grown aerobically to an optical density at 600 nm of 0.6, washed once with phosphate-buffered saline (PBS), suspended in medium, and added to confluent HeLa, IB3 or BHK cells at a multiplicity of infection of approximately 20. Adhesion assays were performed by allowing bacteria to adhere at 4 °C for 30 min, and then each well was rinsed three times with cold PBS. Then, cells were lysed by incubation with 100 μl of sodium deoxycholate (0.5% w/v in PBS) for 10 min. LB (900 μl) was added, and each sample was vigorously mixed to recover adherent bacteria, which were quantified by plating for colony forming units (CFU) on LB agar plates. Adhesion was calculated as follows: percent adhesion = 100 × (number of cell-associated bacteria/initial number of bacteria added). For invasion assays, HeLa, IB3 or BHK cells were infected as described above, but bacteria were incubated with the monolayers at 37 °C for 30 min after plate centrifugation at 180 × g for 1 min. Then, cells were washed three times with prewarmed PBS and incubated for 1 h in media containing gentamicin (100 μg/ml) to kill extracellular bacteria. Cells were then washed three times with PBS and lysed with sodium deoxycholate (0.5% w/v in PBS). Intracellular bacteria were diluted and plated on LB agar plates. In agreement with a previous report [48], no multiplication of bacteria occurred during the course of the assay. Invasion was calculated as follows: percent invasion = 100 × (number of bacteria resistant to gentamicin/initial number of bacteria added).

For competition assays, HeLa cells were infected with a mixture (1:100) of *S.* Typhimurium SL1344 and heat-killed MTM1015 or MTM102 strains. Heat-killed bacteria were previously exposed to HeLa cells for 1 h at 37 °C; then bacteria were recovered from the culture medium and heated at 65 °C for 2 h.

Data were calculated from at least three independent experiments performed in triplicate and expressed as means ± standard errors. The statistical significance of differences in the data was determined using the one-way analysis of variance test and the Dunnett post test.

4.6. Light microscopy of infected epithelial cells

HeLa cells were grown for 24 h on coverslips and then washed with PBS prior infection with the bacteria for 30 min at 37 °C. Infected cells were washed three times with PBS and coverslips mounted on glass slides for visualization with an Axioscope 2 (Carl Zeiss) microscope with a 100× oil immersion objective. Five fields were observed to quantify *Salmonella* containing vacuoles (SCV), and each experiment was repeated three times. Phase-contrast images were acquired with a Q imaging (Burnaby, British Columbia, Canada) cooled charged-coupled device camera on an Axioscope 2 (Carl Zeiss, Thornwood, NY) microscope with a 100/1.3 numerical aperture Plan-Neofluor objective. Images were digitally processed with the Northern Eclipse version 6.0 imaging analysis software (Empix Imaging, Mississauga, Ontario, Canada).

4.7. Analysis of protein secretion

Bacterial culture supernatants and pellets were obtained to investigate secreted proteins and cell-associated proteins, respectively. Bacteria were transformed with plasmid pSipA/FT and grown in LB broth containing 0.3 M NaCl overnight at 37 °C without aeration (SPI-1-inducing conditions). IPTG (0.5 mM) was added for two additional hours to induce SipA::FLAG-TEM1 expression [40]. The cells were pelleted by centrifugation and the pellets were resuspended in 1 × Laemmli sample buffer and heated at 100 °C for 5 min. The supernatants (2 ml) were collected and filtered (pore size, 0.2 μm). The proteins were precipitated with 10% (w/v) trichloroacetic acid, washed twice in cold acetone and resuspended in 1 × Laemmli sample buffer and heated at 100 °C for 5 min. Proteins were separated by SDS-PAGE (10%) and SipA::FLAG-TEM-1 fusions were detected by Western blot using anti-FLAG monocolonal antibody (1:10000, Stratagene). Primary antibody binding was detected using a goat anti-mouse IgG antibody conjugated to HRP and HRP activity was detected with a chemiluminescent substrate (Pierce).

4.8. LPS analysis

Culture samples were adjusted to an optical density at 600 nm of 2.0 in a final volume of 1.5 ml LB. Cells were centrifuged and the pellets were resuspended in 150 μl of lysis buffer (2% (w/v) SDS and 4% (w/v) β-mercaptoethanol in 0.5 M Tris–HCl buffer pH 6.8) containing proteinase K, followed by hot phenol extraction and a subsequent extraction of the aqueous phase with ether. LPS was separated on 14% (w/v) acrylamide gels using a Tricine-SDS buffer system [49]. Gels were silver stained by a modification of the procedure of Tsai and Frasch [50].

4.9. Structural analysis of LPS

For the structural analysis freeze-dried cells were extracted three times with a mixture of aqueous 90% phenol/chloroform/petroleum ether (2:5:8 v/v/v). After removal of the organic solvents under vacuum, the lipooligosaccharide (LOS) fraction was precipitated from phenol with water, washed first with aqueous 80% phenol, and then three times with cold acetone, and lyophilized with a yield of about 4.3% of the dry mass. For detection of LPS, fractions were analyzed by SDS-polyacrylamide gel electrophoresis stained with silver nitrate.

For isolation of core oligosaccharide, the LPS fraction was completely de-acylated by anhydrous hydrazine and hot KOH. In details, LPS was dissolved in anhydrous hydrazine, stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone, and allowed to precipitate. The precipitate was then centrifuged (6000 × g, 30 min, 4 °C), washed twice with ice-cold acetone, dried, dissolved in water and lyophilized. The product was de-N-acylated with 4 M KOH at 120 °C, overnight. Salts were removed by gel permeation chromatography with Sephadex G-10 (Pharmacia) column (50 × 1.5 cm) to yield the resulting oligosaccharide.

4.10. General analytical methods

Monosaccharides were identified as acetylated O-methyl glycosides derivatives. After methanolysis (2 M HCl/MeOH, 85 °C, 16 h) and acetylation with acetic anhydride in pyridine (85 °C, 30 min) the sample was analyzed by Gas Liquid Chromatography–Mass Spectrometry (GLC–MS). Linkage analysis was carried out by methylation of the complete core region. The sample was hydrolyzed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxyl-reduced, acetylated and analyzed by GLC–MS.

Total fatty acid content was obtained by acid hydrolysis. LOS was first treated with HCl 4 M (4 h, 100 °C) and then neutralized with NaOH 5 M (30 min, 100 °C). Fatty acids were then extracted in $CHCl_3$, methylated with diazomethane and analyzed by GLC–MS.

4.11. NMR analysis

For structural assignments, 1D and 2D ^{1}H-NMR spectra were recorded in D_2O, at 300 K, at pD 7; on Bruker 600 DRX equipped with a cryo probe. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. ROESY and NOESY experiments were recorded using data sets ($t_1 \times t_2$) of 4096 × 256 points with mixing times between 100 ms and 400 ms. Double quantum-filtered phase-

sensitive COSY experiments were performed using data sets of 4096×256 points. TOCSY experiments were performed with spinlock times of 100 ms, using data sets ($t_1 \times t_2$) of 4096×256 points. In all homonuclear experiments the data matrix was zero-filled in both dimensions to give a matrix of $4\,K \times 2\,K$ points and was resolution enhanced in both dimensions by a cosine-bell function before Fourier transformation. Coupling constants were determined by 2D phase-sensitive DQF-COSY. HSQC and HMBC experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048×256 points. Experiments were carried out in the phase-sensitive mode. In all heteronuclear experiments the data matrix was extended to 2048×1024 points using forward linear prediction extrapolation.

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