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IDENTIFICATION AND CHARACTERIZATION OF A CAPSULE IN *SHIGELLA* AND ITS ROLE IN THE PATHOGENESIS

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1. SUMMARY

Background

Gram-negative bacteria of the genus *Shigella* are the etiological agents of human bacterial dysentery, shigellosis. The genus is divided into four serogroups, historically treated as species: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). These species are further divided into serotypes, defined by the structure and the biochemical differences in the oligosaccharide repeating unit (RU) that forms the O antigen (OAg) of the lipopolysaccharide (LPS). At present, *S. flexneri* comprise 14 serotypes and subserotypes, *S. dysenteriae* 15 serotypes, *S. boydii* 20 serotypes and *S. sonnei* a single serotype. *Shigella* spp. are among the most frequent causes of diarrhea. A study by the WHO of 1999 estimated that 164.7 million cases of shigellosis occur per year, of which 1.1 million cases result in death. More recent estimates fix the *Shigella* disease burden at 90 million episodes and 122000 deaths per year. Approximately 99 % of all cases occur in developing countries and, importantly, 69 % of all shigellosis patients are children under the age of five. Among *Shigella* species, *S. flexneri* and *S. sonnei* are endemic and are linked to most of the infections. *Shigella* is transmitted by the fecal-oral route and after passage through the stomach and the small intestine, the bacteria reach the large intestine and invade the colonic and rectal epithelium causing severe tissue damage, abscesses and ulceration and the acute mucosal inflammation characteristic of shigellosis. Clinical symptoms range from mild watery diarrhea to severe inflammatory bacillary dysentery characterized by strong abdominal cramps, fever, nausea, anorexia, dehydration and stools containing blood and mucus. The disease is normally self-limiting but may become life-threatening, especially in immunocompromised patients and malnourished children. Antibiotic resistance and multi-resistant strains are increasing, limiting treatment options. Currently, there is no *Shigella* vaccine available. Natural *Shigella* infection results in protective, serotype-specific immunity. The lipopolysaccharide (LPS) is generally accepted as a protective antigen of *Shigella*. Specifically, the outer domain of the LPS, the O antigen, has been correlated with immunity against *Shigella* infection, as infected patients show protection against reinfection with the homologous strain. Thus, special efforts in *Shigella* vaccine development have focused on developing O antigen containing vaccines, either delivered as live-attenuated vaccine strains or more specifically as O antigen conjugates. In fact, a *S. sonnei* O antigen conjugate showed approximately 70 % efficacy in adults and children 3 years old and older. However in younger children, the conjugate was not effective. Other strategies involve the development of live-attenuated strains. Still, recent candidates are not yet at the stage of efficacy testing. Most of the vaccines currently under development are serotype-specific. There is a widespread agreement that globally useful *Shigella* vaccines must protect against a broad spectrum of *Shigella* serotypes: 16 different serotypes are considered to be globally important for an epidemiologically valid global vaccine, *S. dysenteriae* 1, all 14 types of *S. flexneri*, and the *S. sonnei* single type.

Shigella Project at NVGH

The aim of the *Shigella* project at the Novartis Vaccines Institute for Global Health (NVGH) is to develop a broadly protective, *Shigella* vaccine. Our approach is based on GMMA (Generalized Modules for Membrane Antigens), also known in literature as Outer Membrane Vesicles (OMV). GMMA are naturally secreted proteo-liposomes released by Gram-negative bacteria from the outer envelope. They reflect the outer membrane composition, containing LPS, glycerophospholipids, outer membrane proteins and lipoproteins, and enclose some periplasmic components. Genetic modification of producing bacteria allows the increase of GMMA release, without affecting their resemblance with naturally secreted ones. GMMA are different from detergent-extracted outer membrane vesicles (also called OMV in literature) that are produced by detergent-treatment of homogenized bacteria and show a very different composition and protein profile, enriched in cytoplasmatic and inner membrane proteins. The *Shigella* group has engineered GMMA over-producing *Shigella* strains (*S. sonnei* 53G, Ss) by deleting the *tolR* gene that encodes for a Tol-Pal system protein linking the outer and the inner membranes. This mutation was reported to result in overproduction of GMMA without loss of membrane integrity. With the aim to avoid a serotype-specific immunogenicity of GMMA, the presence of the O side chain has been abolished by deleting genes involved in the synthesis and assembly of LPS. Different approaches to generate O antigen-free GMMA were used. As a first step, *galU* gene was deleted (*Ss ΔgalU*). In this mutant the linkage of the OAg repeating units to the core region of LPS is abolished, leading to a truncated LPS form composed of lipid A and inner core. In addition, mutant strains unable to synthesize the repeating unit of the OAg were generated. In *S. sonnei* the O antigen biosynthetic genes are located on the virulence plasmid (pSS). Therefore we either cured the virulence plasmid from *S. sonnei* 53G (*Ss* phase II, -pSS) or we inactivated the plasmid-encoded OAg gene cluster (*Ss Δwbg*). GMMA were purified from bacterial culture supernatants, characterized in respect to protein content and used as antigen in immunization studies in mice. These studies showed that GMMA are very immunogenic, raising high levels of GMMA-specific IgG.

First results and PhD project hypothesis and aims

The sera raised against GMMA carrying a WT or a mutated LPS were tested for their ability to recognize surface epitopes on live bacteria and to cross react with different *Shigella* serotypes. We observed that the staining of the surface of *Ss* WT *in vitro* was possible only in the presence of OAg-specific antibodies. While other experiments showed that *S. sonnei* GMMA contained conserved proteins that raised an immune response, the respective surface antigens were masked in *in vitro* grown *Shigella* by the presence of exopolysaccharide (EPS) structures and thus were not accessible in the surface staining experiment. This has also been reported for other Gram-negative bacteria. Surprisingly, sera raised against *S. sonnei* *ΔgalU* GMMA, that were thought not to react with the O antigen and so with the WT strain, stained the homologous WT strain in a serotype-specific manner. We hypothesized that the *galU* mutation in *S. sonnei* might not result in complete loss of O antigen with GMMA produced from this mutant still raising an OAg-specific response. Unlike the *S. sonnei* strains lacking the OAg gene cluster, the *galU* mutant is still able to synthesize the OAg repeating units. These units could be assembled on the surface of the bacteria through an alternative pathway that does not involve the linkage of the OAg to the LPS. Recently, several Gram-negative

bacteria, like enteropathogenic *E. coli* (EPEC), *Salmonella enterica* serovar Enteritidis and *Francisella tularensis*, have been shown to express a capsule that based on its mechanisms of synthesis and assembly has been classified as a fourth group of capsule types. A group 4 capsule (G4C) is a high molecular weight (HMW) capsular polysaccharide composed of the same repeating OAg units as the LPS, thus also called OAg capsule. It contains more units than the LPS, which are linked to the cell surface by an unknown mechanism. So far, *Shigella* has been reported to be uncapsulated. However, genes homologous to those identified in EPEC for the group 4 capsule are present in different *Shigella* strains.

Our hypothesis is that *S. sonnei* produces a group 4 capsule and that this capsule is still present in the *galU* mutant. The major aims of the present PhD project were the verification of the presence of a group 4 capsule in *Shigella sonnei* (first year activities were dedicated to this aim), the G4C biochemical characterization and the assessment of its distribution in other *Shigella* serotypes (second year activities), the G4C functional characterization (third year activities).

Verification of the presence of a group 4 capsule in *Shigella sonnei* (First year)

In order to verify that the surface staining of WT *S. sonnei* with the sera raised against Ss $\Delta galU$ GMMA was related to the presence of antibodies to the OAg, we absorbed the sera with a WT LPS extract before the surface staining, to remove OAg-specific antibodies. This pre-absorption abolished the antiserum binding to the surface of WT bacteria, while the binding remained unchanged using a mutated LPS (without OAg). These results indicated that antibodies directed to an OAg-containing material were present in the sera raised against Ss $\Delta galU$ GMMA. With the aim of demonstrating that not only GMMA, but also the parent Ss $\Delta galU$ strain was able to induce an OAg-dependent response, mice were immunized with whole inactivated bacteria of Ss $\Delta galU$. Immune responses equivalent to the responses to the immunization with GMMA were obtained, confirming the expression of an OAg-containing material by the Ss $\Delta galU$ strain. By characterizing the Ss $\Delta galU$ lipopolysaccharide with a monovalent antiserum specific for the Ss OAg, we demonstrated that a low mobility material could be extracted and visualized in western blot analysis. Moreover, the OAg-specific antiserum reacted with the surface of Ss $\Delta galU$ bacteria. Collectively, these results confirmed that, unlike in *S. flexneri*, the *galU* mutation in *S. sonnei* does not result in a complete OAg knock out strain. In fact, Ss $\Delta galU$ possesses immunogenic OAg material, likely not linked to the lipid A-core region of LPS, and this material is incorporated into or attached to GMMA when they are released from the bacterial surface. This is in agreement with our hypothesis that *S. sonnei* possesses an OAg capsule.

Biochemical characterization of the *S. sonnei* group 4 capsule and G4C distribution within the genus (Second year)

The group 4 capsule gene cluster was originally described in pathogenic *E. coli* (EPEC) and was reported to encode for proteins that constitute a polysaccharide secretion and assembly system. *S. sonnei* 53G possesses an intact group 4 capsule gene cluster (*ymcDCBA*, *yccZ*, *etp* (*yccY*), *etk* (*yccC*)) with high homology to the cluster found in EPEC. In order to test if this operon is also involved in capsule formation in *Shigella*, we inactivated the whole operon in our *S. sonnei* WT, producing the Ss Δcps mutant. If the gene cluster is essential for G4C synthesis, we expected that Ss Δcps produces OAg as

LPS side chain but not as capsular polysaccharide. Exopolysaccharides (EPS, containing LPS and potential capsule) were purified from *S. sonnei* WT, *Ss ΔgalU*, *Ss Δcps*, and an OAg-deficient strain, for biochemical analyses. EPS extracts were treated by mild acid hydrolysis to remove the lipid A moiety from the LPS and pure polysaccharide (PS) samples were analyzed by HPLC-SEC in order to evaluate their molecular weight distribution. We observed that *Ss* WT produced an EPS with three populations of higher, medium and lower molecular weight. Interestingly, the HMW polysaccharide was present in the *Ss ΔgalU* extract while it was absent in the polysaccharide sample purified from the *S. sonnei* G4C operon knock out. This suggests that the HMW population is the group 4 capsule polysaccharide. In order to confirm this hypothesis, further sugar composition analyses were performed: WT and *ΔgalU* exopolysaccharide extracts were fractionated by HPLC-SEC and eluted samples were collected to isolate the different populations. The spectra of the HMW fraction analyzed by ¹H-NMR displayed the typical signals belonging to the 2-Acetamido-2-deoxy-L-fucose (FucNAc4N) and the 2-Acetamido-2-deoxy-L-altruronic acid (L-AltNAcA) residues of the *Shigella sonnei* OAg. We then evaluated the presence of LPS core sugars in the PS populations purified from the WT strain, having in mind that the capsule should not be linked to the bacterial surface through the LPS assembly system. 3-deoxy-D-manno-octulosonic acid (Kdo) is one of the sugar residues in the inner core region of the LPS of *Shigella* and represents the reducing end of the LPS polysaccharide side chain upon lipid A removal by acid hydrolysis. We demonstrated that the HMW-PS did not comprise Kdo residues. Subsequently, the surface of the different *S. sonnei* LPS and capsule mutant strains was analyzed by transmission electron microscopy (TEM). A dark and compact layer of electron-dense material was observed at the surface of wild type *S. sonnei* bacteria. This layer likely corresponded to extracellular polysaccharides (LPS O side chain and OAg capsule) as it was completely absent in the OAg-deficient strain *Ss Δwbg*. *S. sonnei Δcps* mutant possessed just a narrow dark layer, probably corresponding to the LPS O side chain. On the contrary, *Ss ΔgalU* had a very thick layer of capsular material. This material protruded from the outer membrane in a loose structure that suggests that the EPS in *Ss ΔgalU* is not tightly attached to the bacterial surface. Together these results showed that the group 4 capsule operon is functional in *Shigella sonnei* and is responsible for the formation of a HMW capsule polysaccharide comprising the same sugars as the LPS O side chain. The G4C is not linked to the bacterial membrane through the lipid A-core region of the LPS since the *Ss ΔgalU* LPS deep rough mutant still possesses it.

With the aim to assess how widespread the group 4 capsule formation might be among different *Shigella* species, we studied the conservation of the group 4 capsule operon in different *Shigella* backgrounds. Genome analysis of several isolates of *S. sonnei*, *S. flexneri*, *S. boydii* and *S. dysenteriae* for the presence of a homologous operon showed that the G4C cluster was highly conserved in *Shigella sonnei*, *S. boydii* and *S. dysenteriae* but not in *S. flexneri*. The functionality of the gene cluster in the other serogroups still needs to be tested. However, we confirmed group 4 capsule production in a different isolate of *S. sonnei*, indicating that it is not a characteristic of a single strain but likely a common feature of most of the *S. sonnei* isolates.

Characterization of the biological function of the capsule in *S. sonnei* (Third year)

Bacterial capsules are well established virulence factors, often acting by protecting the cell from opsonophagocytosis and complement-mediated killing. Moreover they can affect the function of other virulence factors through a shielding effect. We next asked if the G4C in *S. sonnei* had the ability to mask one of the most relevant virulence factors, the type III secretion system (T3SS). We examined the ability of an antibody directed to the tip of the T3SS to react with the surface of the different *S. sonnei* strains. We observed that the accessibility of the T3SS was enhanced in the Ss capsule negative strain, suggesting a possible role for the G4C in modulating the virulence of *S. sonnei*. Therefore, we investigated the impact of the capsule on *S. sonnei* invasive abilities *in vitro*. In an invasion assay using HeLa epithelial cells, the *S. sonnei* G4C mutant was significantly more invasive than the WT bacteria. Complementation of the Ss Δ cps mutant with a plasmid expressing the G4C operon restored the WT-like lower invasiveness or even reduced it. These results suggested that the presence of the capsule polysaccharide accounts for the changes in cell invasion. The increased ability of Ss Δ cps to invade epithelial cells likely results from the fact that in the absence of the capsule, virulence factors, as the T3SS, are rapidly available and exposed, thus triggering higher cell entry proficiency. On the other hand, Ss Δ cps complemented strain was less invasive than the WT strain. This effect is probably caused by 'over-complementation' resulting from the fact that the plasmid used for complementation, and so the G4C gene cluster, is present in multiple copies even though a low copy number vector was used. Thus, the complemented mutant likely expresses a higher amount of capsule polysaccharide showing an attenuated phenotype. Subsequently, the rabbit ligated intestinal loop model of experimental shigellosis was used to investigate whether the G4 capsule contributes to the invasive abilities of *S. sonnei* *in vivo* and to the induction of inflammatory host response during the infection. WT *S. sonnei* infection resulted in histopathological changes of infected loops, including the shortening of villi, influx of inflammatory cells and lesions, similar to the previously reported phenotype of *S. flexneri*. Infection with the Ss Δ cps strain led to much more dramatic alterations of mucosal tissues with rupture and destruction of the intestinal epithelium and several regions of infiltration by inflammatory cells. In contrast, villi of loops infected by the Ss Δ cps complemented strain displayed a decrease in the pattern of pathology. Expression analysis of proinflammatory cytokines relevant in the shigellosis model supported the observation that *S. sonnei* in the absence of the G4 capsule had increased pathogenicity in the gut and triggered higher intestinal inflammation. As discussed above, this likely results from the better exposure and accessibility of virulence factors and of the T3SS. In addition, the recognition of invasive bacteria by the innate immune system of the host could be enhanced in the absence of the capsule. In the same animal model, we studied the influence of the capsule on the ability of *S. sonnei* to translocate and disseminate to systemic sites. Different intestinal loops in the same animal were infected with the same number of bacteria of the different strains and after defined time points, mesenteric lymph nodes (MLN), liver, spleen and blood were collected and the present bacteria were differentiated based on strain-specific characteristics and enumerated. In each of the tested organs we predominantly found *S. sonnei* WT strain (approximately accounting for the 70 % of all recovered bacteria), whereas about 25 % of the bacteria were the Ss Δ cps strain. Interestingly, these data indicated that while in

the intestine the *Ss* Δ *cps* capsule mutant showed the most aggressive/invasive phenotype, it had impaired ability to disseminate compared to *Ss* WT. The reason could be that uncapsulated bacteria are more susceptible to the host immune responses since they lack the protective effect of the capsular polysaccharide. Thus, while the lack of the capsule could be beneficial in the early stages of the infection to very efficiently invade into the intestinal epithelium, in the later stages capsule-deficient bacteria have a disadvantage and succumb to the strong inflammatory response of the host.

Conclusions

OAg capsules are easily overlooked because serological and structural studies are generally interpreted with the expectation that all surface O antigen is LPS-linked. In this study we show that a *S. sonnei* Δ *galU* deep rough LPS mutant still possesses OAg on the surface, indicating a source of OAg linked to the outer membrane by a yet unidentified anchor, not the LPS. We demonstrated that *S. sonnei* forms an O antigen capsule whose assembly is dependent on the G4C operon, previously characterized in pathogenic *E. coli*. G4 capsule expression played a role in pathogenicity of *S. sonnei* both *in vitro* and *in vivo*. The uncapsulated mutant strain was more invasive and triggered stronger inflammation than the WT strain, but possessed impaired abilities to disseminate and establish systemic infection. We propose that the capsule may affect the virulence of *S. sonnei* by shielding other surface virulence factors and, at the same time, by protecting the bacteria from the innate immune response of the host. The G4C gene cluster is highly conserved among different *Shigella* species, suggesting that this could be a common virulence strategy in *Shigella*. This is a new and interesting finding in the *Shigella* field to achieve a better understanding of the pathogenic mechanism of *Shigella* and in addition of the function of extracellular polysaccharides as virulence factors. Finally, the identification of a capsule in *Shigella* could have implications on the design of a vaccine against this enteric disease.

2. RIASSUNTO

Introduzione

I batteri Gram-negativi del genere *Shigella* rappresentano l'agente eziologico della dissenteria bacillare umana, la shigellosi. Il genere è suddiviso in quattro sierogruppi, storicamente trattati come specie: *S. dysenteriae* (sierogruppo A), *S. flexneri* (sierogruppo B), *S. boydii* (sierogruppo C) e *S. sonnei* (sierogruppo D). Tali specie sono ulteriormente suddivise in sierotipi, definiti dalle differenze biochimiche e strutturali nell'unità oligosaccaridica ripetitiva che forma l'antigene O (OAg) del lipopolisaccaride (LPS). Attualmente, *S. flexneri* comprende 14 sierotipi and sottoclassi, *S. dysenteriae* 15 sierotipi, *S. boydii* 20 sierotipi e *S. sonnei* un singolo sierotipo. *Shigella* rappresenta una delle principali cause di diarrea al mondo. Uno studio del WHO del 1999 ha stimato 164.7 milioni di casi di shigellosi all'anno, di cui 1.1 milioni di casi risultano nel decesso degli individui infetti. Stime più recenti hanno riportato un'incidenza di 90 milioni di episodi di shigellosi e 122000 decessi all'anno. Approssimativamente, il 99 % dei casi è rilevato in paesi in via di sviluppo ed il 69 % di individui infetti è costituito da bambini al di sotto dei cinque anni di età. Tra le diverse specie, quelle rilevate più frequentemente sono la *S. flexneri* e la *S. sonnei*. La *Shigella* è trasmessa attraverso la via oro-fecale e, una volta oltrepassato lo stomaco e l'intestino tenue, raggiunge l'intestino crasso invadendo l'epitelio del colon e del retto e causando severe affezioni tissutali, ascessi, ulcerazioni e un'acuta infiammazione mucosale caratteristica della shigellosi. I sintomi clinici variano da una semplice diarrea liquida ad una più severa dissenteria bacillare infiammatoria caratterizzata da forti crampi addominali, febbre, nausea, anoressia, disidratazione e feci contenenti sangue e muco. Normalmente la patologia è auto-limitata ma può diventare mortale, in particolare in soggetti immunocompromessi e bambini malnutriti. Ceppi multi-resistenti agli antibiotici vengono isolati sempre più frequentemente e ciò limita la possibilità di trattamento. Attualmente, non esiste un vaccino efficace contro la *Shigella*. L'infezione da *Shigella* porta naturalmente ad una immunità protettiva e sierotipo-specifica. Il lipopolisaccaride ed, in particolare, il dominio più esterno dell'LPS, l'antigene O, è generalmente riconosciuto come antigene protettivo, dato che pazienti infetti sono protetti da un'ulteriore infezione da parte del ceppo omologo. Pertanto, i tentativi volti allo sviluppo di un vaccino contro la *Shigella* si sono concentrati su prototipi contenenti l'antigene O, sia formulato in vaccini costituiti da ceppi attenuati o come vaccini coniugati. Effettivamente, studi clinici di un vaccino coniugato dell'antigene O di *S. sonnei* hanno dimostrato un'efficacia del 70 %, se somministrato ad adulti e bambini al di sopra dei tre anni di età. Sfortunatamente, il candidato vaccino non è risultato efficace in neonati e bambini più giovani. Strategie alternative prevedono l'utilizzo di ceppi vivi attenuati. In ogni caso, i candidati vaccini più recenti non hanno ancora raggiunto la fase di sviluppo clinico volta a valutarne l'efficacia. La maggior parte dei vaccini in sviluppo è sierotipo-specifica. Globalmente, si crede che un futuro vaccino contro la *Shigella* debba proteggere da un ampio spettro di sierotipi: in particolare, 16 sierotipi sono considerati di importanza globale quali la *S. dysenteriae* tipo 1, tutti i 14 tipi della *S. flexneri*, and l'unico tipo della *S. sonnei*.

Il progetto *Shigella* dell'NVGH

Il principale obiettivo del progetto *Shigella* del Novartis Vaccines Institute for Global Health (NVGH) è quello di sviluppare un vaccino ampiamente protettivo contro la *Shigella*. Il nostro approccio si basa sull'utilizzo di Moduli Generalizzati per Antigeni di Membrana, GMMA (Generalized Modules for Membrane Antigens), anche conosciuti in letteratura come Vescicole di Membrana Esterna, OMV (Outer Membrane Vesicles). Le GMMA sono proteo-liposomi naturalmente rilasciati dalla membrana esterna dei batteri Gram-negativi. Le GMMA riflettono la composizione di membrana esterna e contengono infatti LPS, fosfolipidi, proteine di membrana esterna e lipoproteine e inoltre includono al loro interno alcune componenti periplasmatiche. Attraverso modificazioni genetiche dei batteri produttori è possibile facilitare il rilascio delle GMMA, senza però modificarne la somiglianza con le vescicole naturalmente rilasciate dai batteri. Le GMMA differiscono dalle vescicole estratte tramite trattamento con detergenti. Quest'ultime infatti sono prodotte da preparazioni batteriche omogeneizzate e possiedono una composizione proteica differente, caratterizzata da un arricchimento in proteine citoplasmatiche e di membrana interna. Il gruppo di lavoro sul progetto *Shigella* ha generato ceppi di *Shigella* (in particolare la *S. sonnei* 53G) che producono un'aumentata quantità di GMMA, attraverso la delezione del gene *tolR*. Il gene *tolR* codifica per una proteina del sistema Tol-Pal, un complesso proteico volto al mantenimento del legame tra membrana interna e membrana esterna. Questa mutazione era già stata caratterizzata in precedenza: il risultato è una over-produzione di vescicole che non comporta la perdita dell'integrità di membrana. Inoltre, al fine di evitare una potenziale risposta immunitaria di tipo sierotipo-specifica, i geni responsabili per la sintesi e per la formazione dell'LPS sono stati anch'essi mutati per ottenere GMMA che non possiedono l'antigene O sulla propria superficie. Il primo approccio è stato quello di creare un mutante di delezione nel gene *galU* ottenendo il ceppo *Ss ΔgalU*: tale mutante non possiede la capacità di legare le unità ripetitive dell'OAg alla regione del core dell'LPS, ma possiede quindi un LPS troncato e composto solo dal lipide A e dalla regione del core interno. Il secondo approccio è stato quello di generare ceppi mutanti incapaci di sintetizzare le unità ripetitive dell'OAg. Nella *S. sonnei* il cluster biosintetico dell'OAg risiede sul plasmide di virulenza (pSS). Pertanto abbiamo isolato quelle colonie di *S. sonnei* che avevano perso il pSS (*Ss* -pSS, phase II) o, alternativamente, abbiamo deletato il cluster per la sintesi dell'OAg dal plasmide di virulenza (*Ss Δwbg*). Le GMMA rilasciate da tali mutanti sono state purificate dal supernatante delle colture batteriche, sono state caratterizzate tramite approccio proteomico e sono state utilizzate come antigeni in studi di immunogenicità nel topo. Questi studi hanno dimostrato l'alto potere immunogenico delle GMMA, dato che i sieri immuni contenevano alti livelli di IgG specifici per gli antigeni delle GMMA.

Risultati preliminari, ipotesi del progetto di dottorato e principali obiettivi

I sieri immuni da immunizzazione con GMMA prodotte da ceppi con un LPS wild type (WT) o mutato sono stati testati al fine di stabilire la loro capacità di riconoscere epitopi di superficie di batteri vivi o di cross-reagire con diversi sierotipi di *Shigella*. È stato osservato che la marcatura di superficie di *Ss* WT *in vitro* era possibile solo per mezzo di anticorpi contro l'OAg. Anche se ulteriori esperimenti avevano mostrato che le GMMA di *S. sonnei* contenevano proteine altamente immunogeniche, i rispettivi epitopi di membrana esterna venivano mascherati dalla presenza dei polisaccaridi di superficie e

quindi non risultavano accessibili negli esperimenti di marcatura superficiale di batteri vivi. Simili risultati sono stati riportati in letteratura per altri batteri Gram-negativi. Inaspettatamente, sieri immuni da immunizzazione con GMMA prodotte dal mutante Ss $\Delta galU$, i quali venivano considerati privi di anticorpi diretti contro l'OAg, si sono dimostrati capaci di reagire con la superficie della *Shigella* wild type dello stesso sierotipo. Abbiamo ipotizzato che la delezione del gene *galU* nel sierotipo *sonnei* potesse non risultare nella completa scomparsa dell'OAg dal ceppo produttore e di conseguenza dalle GMMA derivate. Al contrario di quanto accade nei mutanti per i geni coinvolti nella sintesi delle unità ripetitive dell'OAg, il mutante nel gene *galU* può ancora sintetizzare gli zuccheri che compongono l'antigene O. Questi possono essere assemblati sulla superficie dei batteri attraverso un processo che non coinvolge il legame all'LPS. Di recente, in diversi batteri Gram-negativi quali *E. coli* enteropatogeno (EPEC), *Salmonella enterica* sierotipo Enteriditis e *Francisella tularensis*, è stata riscontrata la presenza di una capsula, la quale, secondo il particolare meccanismo di sintesi e formazione, è stata classificata nella quarta classe. Una capsula di gruppo 4 (G4C) è un polisaccaride ad alto peso molecolare composto dalle stesse unità ripetitive dell'OAg dell'LPS, e pertanto viene anche definita 'capsula di OAg'. Una G4C è costituita da una quantità maggiore di unità ripetitive rispetto all'LPS, e queste unità sono legate alla membrana esterna dei batteri attraverso un meccanismo ancora sconosciuto. Al momento, la *Shigella* è descritta in letteratura come batterio non capsulato, d'altronde è stato visto che i geni del cluster responsabile per la formazione della capsula di tipo 4 in EPEC sono presenti anche nel genoma di *Shigella*.

La nostra ipotesi sostiene che la *S. sonnei* produce una capsula di gruppo 4 e che questa capsula è presente anche nel mutante di delezione per il gene *galU*. I principali obiettivi del presente progetto di dottorato sono stati volti innanzitutto alla dimostrazione del fatto che la *S. sonnei* possiede una capsula di gruppo 4 (attività del primo anno di dottorato), successivamente alla caratterizzazione biochimica del polisaccaride capsulare e allo studio di distribuzione di questa caratteristica in altri sierogruppi (attività del secondo anno di dottorato), ed infine alla caratterizzazione funzionale di tale capsula (attività del terzo anno di dottorato).

Verifica della presenza di una capsula di gruppo 4 in *Shigella sonnei*

Abbiamo innanzitutto verificato che la marcatura di superficie dei batteri wild type per mezzo dei sieri da immunizzazione con GMMA prodotte da Ss $\Delta galU$ fosse dovuta alla presenza di anticorpi specifici per l'OAg. Pertanto, abbiamo assorbito tali sieri con un estratto di LPS WT prima dell'esperimento di marcatura, al fine di rimuovere eventuali anticorpi contro l'OAg. In tal modo la capacità dei sieri di legare la superficie dei batteri WT è stata completamente abolita, mentre utilizzando per l'assorbimento un LPS mutato (privo di OAg) la marcatura rimaneva invariata. Da questi risultati abbiamo dedotto la presenza di anticorpi specifici per l'antigene O nei sieri da immunizzazione con GMMA prodotte da Ss $\Delta galU$. Successivamente, per dimostrare che questi particolari risultati immunologici non fossero un artefatto derivante dall'immunizzazione dei topi con le GMMA, abbiamo ripetuto l'esperimento analizzando i sieri derivati dall'immunizzazione con batteri interi inattivati. Anche in questo caso, anticorpi diretti verso l'OAg sono stati rilevati nei sieri derivati da immunizzazione con la *S. sonnei* $\Delta galU$. Il lipopolisaccaride di Ss $\Delta galU$ è stato analizzato in saggi immunologici utilizzando un antisiero monovalente specifico per l'antigene O di *S. sonnei*. Tali saggi

hanno dimostrato la presenza di materiale a ridotta mobilità in corsa elettroforetica monodimensionale che reagiva con l'antisiero monovalente. Non solo l'estratto lipopolisaccharidico, ma anche la superficie dei batteri di *Ss ΔgalU* ha reagito con il siero specifico per l'OAg. In definitiva, questi risultati hanno mostrato che, nonostante il fatto che in letteratura fosse riportato che la *Shigella flexneri* deleta del gene *galU* fosse priva di OAg, un materiale simile all'OAg, ma non legato al core dell'LPS, poteva essere estratto da *Ss ΔgalU*. Inoltre tale materiale era esposto sulla superficie e incorporato nelle GMMA. Queste osservazioni erano in linea con la nostra ipotesi di una capsula di OAg in *S. sonnei*.

Caratterizzazione biochimica della capsula e distribuzione del cluster genico nel genere *Shigella*

Il cluster genico responsabile per la formazione della capsula di tipo 4 era stato precedentemente caratterizzato in un isolato di EPEC ed era stato annotato come codificante per un complesso proteico per la secrezione e la costituzione di polisaccaridi superficiali. Nel genoma del nostro isolato, *S. sonnei* 53G, è presente un cluster genico per la G4C altamente omologo a quello identificato in EPEC. Al fine di valutare se il cluster per la G4C fosse responsabile della formazione della capsula in *S. sonnei*, abbiamo prodotto un mutante di delezione per tale operone, ottenendo il ceppo *Ss Δcps*. Ci aspettavamo che tale mutante possedesse OAg come catena laterale dell'LPS ma non come capsula, nel caso in cui il cluster si fosse dimostrato funzionale nel nostro isolato. I polisaccaridi superficiali (EPS) (contenenti sia l'OAg dell'LPS che l'OAg capsulare) sono stati purificati da *S. sonnei* WT, *Ss ΔgalU*, *Ss Δcps*, e dal mutante nel cluster biosintetico per l'OAg, per poter effettuare saggi biochimici. I vari estratti sono stati trattati con idrolisi acida blanda per rimuovere la porzione lipidica dell'LPS ed i polisaccaridi purificati sono stati analizzati in cromatografia ad esclusione molecolare (HPLC-SEC) per valutare la distribuzione dei pesi molecolari delle specie polisaccaridiche. Abbiamo osservato tre popolazioni ad alto, medio e basso peso molecolare nell'estratto da batteri WT. Una popolazione ad alto peso molecolare, simile a quella identificata nel WT, era presente nel polisaccaride da batteri *ΔgalU* mentre nessuna popolazione a tale peso molecolare era presente nel polisaccaride da batteri *Δcps*. Questo ci suggeriva che la popolazione ad alto peso molecolare fosse il polisaccaride capsulare. Abbiamo quindi effettuato studi di composizione zuccherina e da analisi di NMR monodimensionale abbiamo identificato i picchi caratteristici corrispondenti ai due residui costituenti l'OAg di *S. sonnei* (2-Acetamido-2-deossi-L-fucosio (FucNAc4N), acido 2-Acetamido-2-deossi-L-ialuronico (L-AltNAcA)) nella popolazione ad alto peso molecolare che era stata precedentemente isolata. Inoltre, abbiamo valutato la presenza di residui appartenenti al core dell'LPS, come ad esempio l'acido 2-Cheto-3-deossi-D-manno-ottulosonico (Kdo), nelle varie popolazioni polisaccaridiche. Da questa analisi, il polisaccaride ad alto peso molecolare è risultato privo di Kdo. La superficie dei diversi ceppi mutanti è stata poi caratterizzata tramite analisi di microscopia elettronica a trasmissione (TEM). Uno strato elettronico compatto è stato evidenziato sulla superficie della *Shigella sonnei* WT, corrispondente all'LPS e alla capsula. Questo strato superficiale era assente nel mutante per i geni di sintesi dell'OAg, mentre solo uno strato sottile corrispondente all'LPS è stato evidenziato sulla superficie dei batteri *Δcps*. È stato interessante scoprire che il mutante *galU* possedeva uno strato spesso e non molto compatto, probabilmente legato in

maniera blanda alla membrana esterna. Considerando nel complesso questi risultati, possiamo affermare di aver dimostrato che il cluster codificante per la capsula di gruppo 4 è funzionale in *Shigella sonnei* ed è responsabile della formazione di un polisaccaride ad alto peso molecolare composto dagli stessi residui zuccherini della catena laterale dell'LPS ma non legato alla membrana batterica tramite il core dell'LPS. Si tratta quindi di una capsula di OAg.

Abbiamo poi analizzato i genomi di diversi isolati di *Shigella* alla ricerca di cluster genici omologhi a quello della *S. sonnei* 53G. Abbiamo potuto constatare che il cluster genico per la capsula di gruppo 4 è presente in un alto numero di isolati di *Shigella sonnei*, *S. boydii* e *S. dysenteriae* ma non in *S. flexneri*. In ogni caso, rimane da confermare la funzionalità di tali clusters in isolati di altri sierogruppi.

Caratterizzazione funzionale della capsula in *Shigella sonnei*

Le capsule batteriche sono considerate importanti fattori di virulenza dal momento che proteggono i batteri da meccanismi di opsonofagocitosi e eliminazione mediata dal complemento. Inoltre le capsule possono modulare l'attività di altri fattori di virulenza attraverso meccanismi di mascheramento. Ci siamo chiesti se la capsula di *S. sonnei* avesse l'abilità di mascherare uno dei fattori di virulenza più importanti di *Shigella*, il sistema di secrezione di tipo terzo (T3SS). Abbiamo testato un anticorpo contro la proteina IpaB, all'apice del T3SS, per la sua abilità nel reagire con la superficie dei diversi mutanti di *S. sonnei* e abbiamo notato che l'accessibilità di IpaB era maggiore nel mutante non capsulato. Questo ci ha suggerito un ruolo della G4C nel regolare la virulenza di *Shigella sonnei*. Pertanto abbiamo dapprima saggiato la patogenicità dei diversi mutanti in un esperimento di invasione *in vitro* in cellule epiteliali umane (HeLa). In questo saggio il ceppo non capsulato si è dimostrato altamente invasivo se paragonato al WT. Quando la delezione è stata complementata in trans attraverso il clonaggio del cluster per la G4C in un vettore di espressione, il mutante risultante ha mostrato livelli di invasività pari, se non minori, al ceppo WT. Tali risultati indicano che la presenza della capsula è responsabile della modulazione della virulenza *in vitro*, probabilmente perché nel mutante non capsulato i fattori di virulenza sono più facilmente esposti rendendo il ceppo maggiormente virulento nell'invasione delle cellule ospiti. D'altra parte il fenotipo attenuato del mutante in cui la delezione del cluster per la G4C è complementata in trans può derivare dal fatto che questo ceppo possiede una quantità maggiore di capsula, dal momento che il cluster è presente in più copie rispetto al WT. Successivamente, abbiamo valutato la patogenicità dei diversi mutanti *in vivo* in un modello di infezione di anse intestinali nel coniglio. In questo modello è possibile studiare le abilità invasive dei ceppi testati e la loro capacità di indurre una risposta infiammatoria nell'ospite, tipica dell'infezione da *Shigella*. Mentre le convenzionali lesioni intestinali ed un alto livello di infiammazione intestinale sono stati rilevati dopo infezione con il ceppo WT, una drammatica alterazione della mucosa intestinale e livelli di infiammazione molto elevati sono risultati dall'infezione con il ceppo non capsulato. Al contrario, il mutante in cui la delezione del cluster per la G4C è complementata in trans ha portato ad una patologia più lieve. Di nuovo, questo può essere il risultato del fatto che in assenza della capsula i fattori di virulenza di superficie sono maggiormente esposti tanto da portare ad una maggiore invasività e allo stesso tempo è possibile che il sistema immunitario possa riconoscere più facilmente i ceppi non capsulati così da indurre una maggiore risposta infiammatoria. In fine, abbiamo studiato *in vivo* la

capacità dei diversi mutanti di disseminare a livello sistemico e raggiungere organi periferici. A tal proposito, abbiamo raccolto fegato, milza, linfonodi mesenterici e sangue da animali infettati a livello intestinale contemporaneamente da ceppi capsulati e non capsulati. In ogni organo in esame, abbiamo rilevato prevalentemente la presenza di batteri WT capsulati (circa il 70 % del totale dei batteri trovati). Questi risultati mostrano che i ceppi non capsulati sono più virulenti in una prima fase dell'infezione a livello intestinale, mentre presentano un'incapacità nell'instaurare infezioni a livello sistemico, probabilmente perché in assenza del fattore protettivo conferito dalla presenza della capsula, soccombono alla forte risposta immunitaria innata dell'ospite.

Conclusioni

Le capsule di OAg non sono facilmente identificabili in quanto i dati sierologici e strutturali sono spesso interpretati nell'ottica che l'antigene O di superficie sia completamente legato all'LPS. Nel presente progetto di ricerca abbiamo dimostrato che un mutante incapace di legare l'OAg alla regione del core dell'LPS (Ss $\Delta galU$) possiede ancora OAg sulla sua superficie, non legato all'LPS. Abbiamo dimostrato che *S. sonnei* produce una capsula di OAg la cui formazione è dipendente dalla presenza di un cluster genico, precedentemente caratterizzato per la sua funzione di codificare per un complesso proteico volto alla produzione di una capsula di gruppo 4 in EPEC. La capsula modula la virulenza di *Shigella sonnei* sia *in vitro* che *in vivo*. Ceppi non capsulati sono risultati più virulenti e hanno scatenato una maggiore risposta infiammatoria nell'ospite, ma sono risultati incapaci di instaurare infezioni sistemiche. L'operone codificante per la G4C è altamente conservato in diversi sierogruppi di *Shigella*, pertanto l'espressione di una capsula potrebbe rappresentare una comune strategia di virulenza di tali patogeni umani. In questo lavoro per la prima volta abbiamo identificato e caratterizzato una G4C in *Shigella sonnei*. Questa nuova scoperta ci permette di ottenere una migliore comprensione dei meccanismi di patogenesi della *Shigella* e del ruolo dei polisaccaridi di superficie come fattori di virulenza. Inoltre, l'identificazione di una capsula in *Shigella* potrebbe avere implicazioni significative per lo sviluppo di un vaccino protettivo.

3. INTRODUCTION

3.1 Infectious Diseases and Global Health

Infectious diseases are a major burden of disease and the second most common cause of death worldwide. World Health Organization (WHO) data reported that in 2008 1 billion people worldwide were directly affected by one or more infectious diseases [1]. Moreover, according to the latest published data in 2012, infectious (including parasitic) diseases were responsible for the death of more than 8.7 million people worldwide in 2008 [2]. Infectious diseases do not respect the socioeconomic status as their contribution to the global disease burden has the potential to affect the entire world's population, but the risk is not evenly distributed [3]. People already living in social and economic deprivation have a greater exposure to the risk factors for disease and the impact of diseases is felt not only in the massive loss of life but also in a high-levels of morbidity and the accompanying impact on families, communities and the weak and under-resourced health systems in low and middle-income countries [4]. As a matter of fact, 98.9 % of Disability-Adjusted Life Years (DALYs) and 96.9 % of deaths due to infectious diseases occur in middle and low income countries [5]. Importantly, infectious diseases disproportionately affect the youngest populations. 54 % of DALYs and 43 % of deaths from infectious diseases occur in children less than 5 years of age [5]. According to 2010 estimates by WHO, approximately 7.6 million children die each year before reaching the age of five [6]. As shown in Figure 1, the greatest burden is caused by respiratory diseases, such as pneumonia, diarrheal diseases and malaria [6]. Malnutrition is a contributing factor in more than one third of all child deaths and the links between lack of nutrition and infectious diseases are already well established. Interestingly, this excess of morbidity and mortality in poor and young populations is not seen with non-infectious diseases [5]. Research has played a huge role in efforts to understand, control and prevent the spread of infectious diseases. For some diseases, such as smallpox, vaccination campaigns have led to eradication [7]. However, for many infectious diseases of poverty no effective and affordable treatments exist or existing interventions are not readily available or accessible in communities where the need is greatest [3]. Investment in controlling the spread of infectious and parasitic diseases will have a powerful impact on global human, social and economic development.

Figure 1

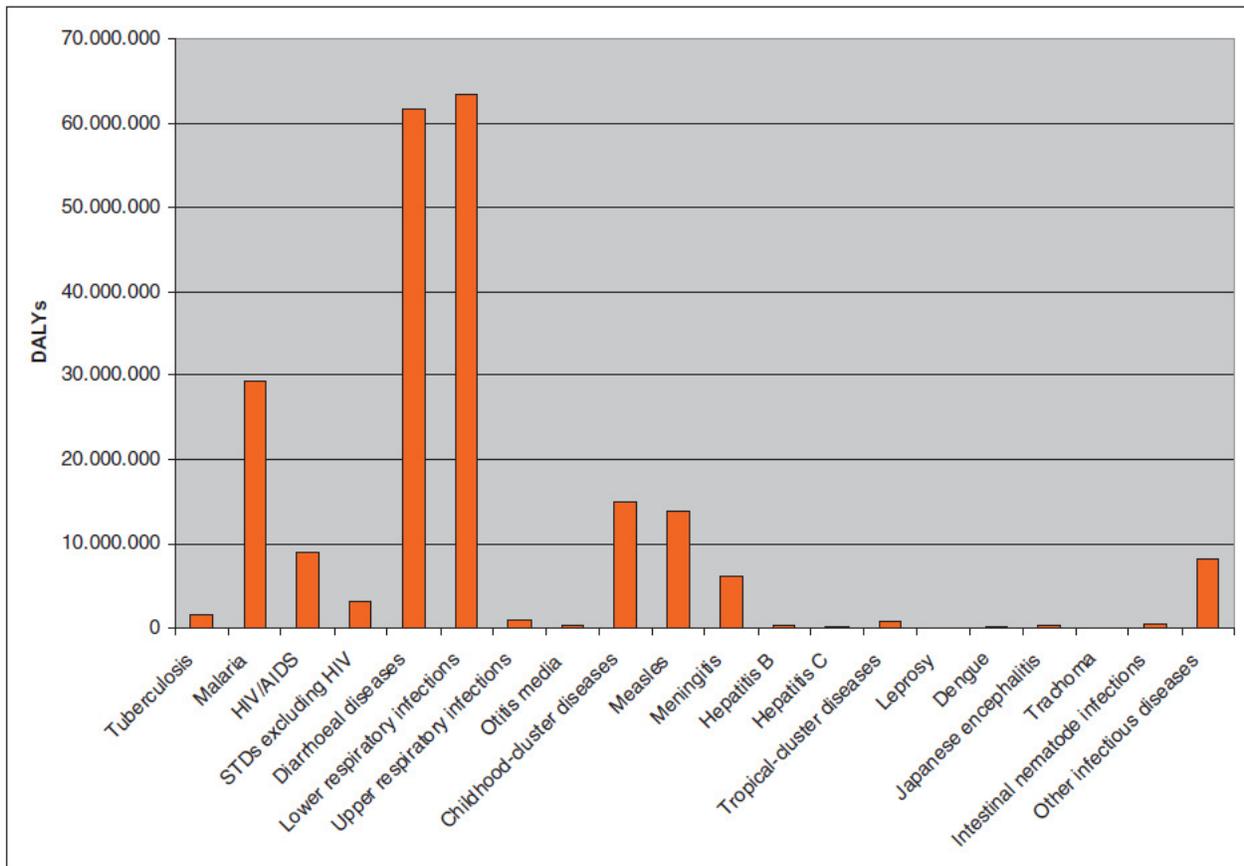


Figure 1: Infectious diseases causing the greatest burden in < 5 year old children. Global DALYs (Reproduced from [8]).

3.1.1 Diarrheal diseases

With the exception of perinatal conditions (e.g. prematurity and low birth weight), diarrhea remains the second leading cause of death among children under 5 years of age [9]. In some settings in developing countries, the average morbidity attack rate can increase from 3.2, estimated globally, to 12 episodes of diarrhea per child per year. Importantly, over the past two decades, treatment and prevention measures including fluid replacement, zinc treatment or promotion of hand washing have been responsible for the decline of mortality from diarrhea from an estimated 5 million deaths among young children to about 1.5 million deaths in 2004 [10]. However, although the number of deaths is decreasing, the high incidence is still a major issue. A contributing factor is that long-term consequences on physical and mental development of children have been reported, as a result of childhood diarrhea [11–13]. Among the principal bacterial agents of diarrheal diseases are *Vibrio cholerae* (cholera), multiple serovars of *Salmonella enterica*, including *S. Typhi* (typhoid fever), and a variety of serotypes of *Shigella* spp., the agents of shigellosis (bacterial dysentery), *Campylobacter* spp. (especially *C. jejuni*) and enteropathogenic *Escherichia coli* strains, including the enterotoxigenic *Escherichia*

coli (EPEC) strains [14]. Among the enteric viruses, rotaviruses remain the leading cause of diarrheal disease in young children in the world [15].

Diarrheal diseases not only represent a major health problem in developing countries but also a high risk to travellers who visit these countries, especially in the tropics [16]. Up to 80 % of diarrheal episodes in travellers are bacterial in nature, caused principally by EPEC strains, followed by *Shigella*, *Campylobacter* and *Salmonella* spp. [14]. The increased frequency of antibacterial drug-resistance among these pathogens is a source of major concern [17,18]. While in the long term access to clean water, better hygiene, adequate nutrition, and improvement of sanitary measures would have the greatest impact on diarrheal diseases, immunization against specific diseases is the best strategy for the short- and mid-term [19]. The development of vaccines against enteric diseases, however, has been a serious challenge because of the large number of pathogens and serotypes, and the requirement to induce mucosal immunity in the gut [20,21].

3.2 *Shigella*

Members of the genus *Shigella* are Gram-negative, non-sporulating, rod-shaped, facultative anaerobe bacteria that belong to the family *Enterobacteriaceae*. The genus is divided into four serogroups, historically treated as species [22]:

- *S. dysenteriae* (serogroup A);
- *S. flexneri* (serogroup B);
- *S. boydii* (serogroup C);
- *S. sonnei* (serogroup D).

These species are further divided into serotypes, on the basis of the antigen specificity, the structure and the biochemical differences of the oligosaccharide repeating unit (RU) that forms the O antigen (OAg), the polysaccharide portion of their lipopolysaccharide (LPS) [23]. At present, the species *S. flexneri* is known to comprise 14 serotypes and subserotypes, *S. dysenteriae* 15 serotypes, *S. boydii* 20 serotypes and *S. sonnei* a single serotype. *Shigella* spp. are facultative intracellular pathogens that show a high specificity for the human host in which they cause bacterial dysentery, shigellosis. The first report on the isolation and characterization of bacteria causing bacillary dysentery, later named *Shigella*, was published by Kiyoshi Shiga at the end of the 19th century [24]. *Shigella* spp. share common characteristics with members of the genus *Escherichia*. In addition, the genetic relatedness suggests that they are a subtype of *E. coli* [25,26]. The whole-genome sequencing of *E. coli* and all four *Shigella* species revealed that they share a common DNA backbone of approximately 3.9 Mb, interrupted by *E. coli*-specific and *Shigella*-specific sequences. The differentiation of *Shigella* and *E. coli* is complicated and the borders appear to be fluid with strains that share characteristics with either species e.g. enteroinvasive *E. coli* (EIEC), sharing biochemical characteristics, essential virulence factors, and clinical symptoms with *Shigella* spp. Comparative genomics indicates that EIEC is genetically more related to *Shigella* than to non-invasive *E. coli* [27] and suggests that they evolved from multiple non-invasive *E. coli* strains by convergent evolution [27,28]. The most striking difference between the genomes of *E. coli* and *Shigella* is the presence of many IS-elements in *Shigella* which contributes to a very dynamic genome [26,29]. The easy acquisition and loss of genes

promote the success of *Shigella* as a pathogen, because fast genetic adaptation is needed to survive in different circumstances in the host [30,31].

3.2.1 Bacteriology, disease burden and global impact

Estimates by the World Health Organization indicate that the world's population suffered from 4.5 billion incidences of diarrhea causing 1.8 million deaths in the year 2002 [32]. Shigellosis is endemic throughout the world and *Shigella* species are among the most frequent causes of diarrhea. A study of the WHO in 1999 estimated that 164.7 million cases of shigellosis occur per year, of which 1.1 million cases result in death [33]. More recent estimates state a global *Shigella* disease burden at 90 million episodes and about 108-122 thousand deaths per year [34,35]. Approximately 99 % of all cases occur in developing countries, where poor hygiene, limited access to clean water and malnutrition promote the spread and the high mortality rate of enteric diseases. Importantly, 69 % of all shigellosis episodes and 61 % of all deaths involve children under the age of five [33]. As mentioned before, *Shigella* spp. are also one of the primary causes of troops and traveler's diarrhea, with about 500 000 cases of shigellosis reported each year among military personnel and travellers from industrialized countries [36]. Three major species are responsible for bacillary dysentery: *S. flexneri* (all 14 serotypes are considered of global importance), *S. sonnei*, and *S. dysenteriae* type I [33]. The fourth species, *S. boydii*, is responsible for scattered disease foci [34]. Distribution of serotype differs between industrialized and developing countries and change over time [37]. *S. flexneri* is endemic in developing countries (60 %) and is the most frequently isolated species worldwide. The predominant serotypes are *S. flexneri* 2a, followed by 1b, 3a, 4a and 6 [34]. Untypable *Shigella* isolates have also recently emerged as a significant cause of diarrhea [38]. *S. sonnei* is the causative agent of most of the shigellosis in industrialized countries where it accounts for 77 % of cases (compared to 15 % in developing countries in 2009) [34]. However, in recent years, it has also become predominant in Thailand and a shift in *Shigella* dominance from *S. flexneri* to *S. sonnei* in developing countries has been documented [34,39,40]. A possible explanation for this shift has been proposed to be related to the decreased prevalence of the environmental bacterial species *Plesiomonas shigelloides*. *P. shigelloides* shares the O antigen composition with *Shigella sonnei*. As natural immunity to *Shigella* is mainly targeted to the O antigen [41] (see following section about Polysaccharide conjugate vaccines), exposure to water contaminated with *Plesiomonas shigelloides* has the potential to cross-protect humans from *S. sonnei* infection [42]. As countries develop economically and water quality improves, humans are less likely to be exposed to *P. shigelloides*. Cross-protection by environmental immunization with *P. shigelloides* therefore declines, which might explain the increase in *S. sonnei* infections [43]. High-case fatality rates of shigellosis have been associated with *S. dysenteriae* type 1 (Sd1) infections that occur in epidemic disease outbreaks. Sd1 is of particular concern since it is the only *Shigella* type carrying a chromosomal gene encoding the 70-KDa heterodimeric protein known as Shiga toxin [44]. Shiga toxin is cytotoxic for a variety of cell types and is responsible for the development of vascular lesions in the colon, the kidney, and the central nervous system [45]. This results in more severe disease manifestation than in shigellosis caused by other serotypes. A major obstacle to the control of Sd1 is its resistance to antimicrobial drugs [46]. However, recent

surveillance data from Bangladesh and India show that Sd1 has not been isolated from these regions in the last years [34].

3.2.2 Shigellosis: definition, transmission and pathogenesis

Shigella spp. are the etiological agents of shigellosis. Shigellosis is an acute intestinal infection that is transmitted by person-to-person contact through the fecal-oral route or indirectly through contaminated food or water. Transmission by house flies has also been documented [47]. *Shigella* is highly infectious, since as few as 10 to 100 microorganisms may be sufficient to cause disease [48]. This low infective dose is in part attributed to bacterial ability to survive the acidity of the host's stomach, through specific acid resistance systems [49]. In addition, *Shigella* can decrease the expression of antibacterial peptides that are constantly released from the mucosal surface of the intestinal tract [50]. After passage through the stomach and the small intestine, the bacteria reach the large intestine and invade the colonic and rectal epithelium causing the acute mucosal inflammation characteristic of shigellosis [51]. The infection is usually confined to the superficial layer of the colonic mucosa where severe tissue damage leads to abscesses and ulceration. Clinical symptoms range from mild watery diarrhea to severe inflammatory bacillary dysentery characterized by strong abdominal cramps, fever, nausea, anorexia, dehydration and stools containing blood and mucus. The disease is normally self-limiting. A combination of oral rehydration and antibiotics administration can reduce the period of bacterial excretion from the patient and leads to the rapid resolution of infection. However, shigellosis may become life-threatening, especially in immunocompromised patients, including neonates and children with underlying immune deficiencies (including HIV infections) or malnutrition, or if there is no adequate medical care. In these cases, patients may develop secondary complications and long term effects can occur, such as peritonitis, septicaemia, the severe Haemolytic Uremic Syndrome (HUS) with kidney failure, reactive arthritis, pneumonia [52].

The current model of *Shigella* pathogenesis is derived from studies of *S. flexneri* [53] (Figure 2). The infection is a multi-step process. When the bacteria reach the mucosa of the terminal ileum and colon, they are taken up by M cells which are specialized epithelial cells that sample the luminal content and deliver it to the underlying mucosal lymphoid tissue in a process called transcytosis. After passing the epithelium, the bacteria are phagocytosed by resident macrophages. However, *Shigella* evades phagosome-mediated killing and induces apoptotic death of the macrophages [54]. Free bacteria, released from dead macrophages, invade the epithelial cells from the basolateral side and spread laterally to adjacent cells. The bacteria multiply in the cytoplasm and eventually kill the host cell while moving towards adjacent epithelial cells by a process of polymerisation/depolymerisation of the actin tubules [55], and through the alteration of the tight-junction proteins composition [56]. Apoptosis of infected macrophages and epithelial cells invasion is accompanied by the release of many pro-inflammatory cytokines, as IL 1 β , IL 18, and IL 8, inducing an acute and massive inflammatory response. How *Shigella* causes epithelial inflammation is still not fully understood. As Gram-negative bacterium, *Shigella* possesses two most studied pathogen-associated molecular patterns (PAMPs): the lipopolysaccharide (LPS) and the peptidoglycan (PGN). Their synthesis is controlled by chromosomal genes in all serogroups but in *S. sonnei*, in which the OAg gene cluster resides on the large virulence plasmid [57,58]. These molecules are located respectively on the bacterial

surface (LPS) and between the inner and outer membrane (PGN). Two major pathways have been shown to be involved in inflammation: sensing of the LPS by the cell-surface expressed receptors TLRs (Toll-like Receptors), and in particular, of the lipid A region of the LPS by TLR4 [59], and sensing of the PGN by the intracellular receptors Nod (Nucleotide oligomerization domain) [60]. IL 1 β and IL 8 induce the recruitment of polymorphonuclear neutrophilic leukocytes (PMNs), that infiltrate the infected site and destabilize the intestinal epithelium [61,62]. The influx of PMN, at a first stage, compromises the integrity of epithelial barrier facilitating the invasion of further organisms from the colonic lumen. Ultimately, however, the PMNs phagocytose and kill *Shigella* contributing to the resolution of infection. This is aided by the generation of an effective antibacterial response which is likely supported by the released IL 18. IL 18 activates natural killer (NK) cells and induces and promotes the production of IFN- γ , responsible for the activation of macrophages, thus amplifying the innate immune response [63,64]. However, the role of IL 18 in shigellosis has not been yet confirmed. The alteration and destruction of tissues in the early stages of the infection causes an impaired adsorption of solutes, nutrients, and water, which is subsequently followed by watery diarrhea and presence of blood and mucus in stools.

Figure 2

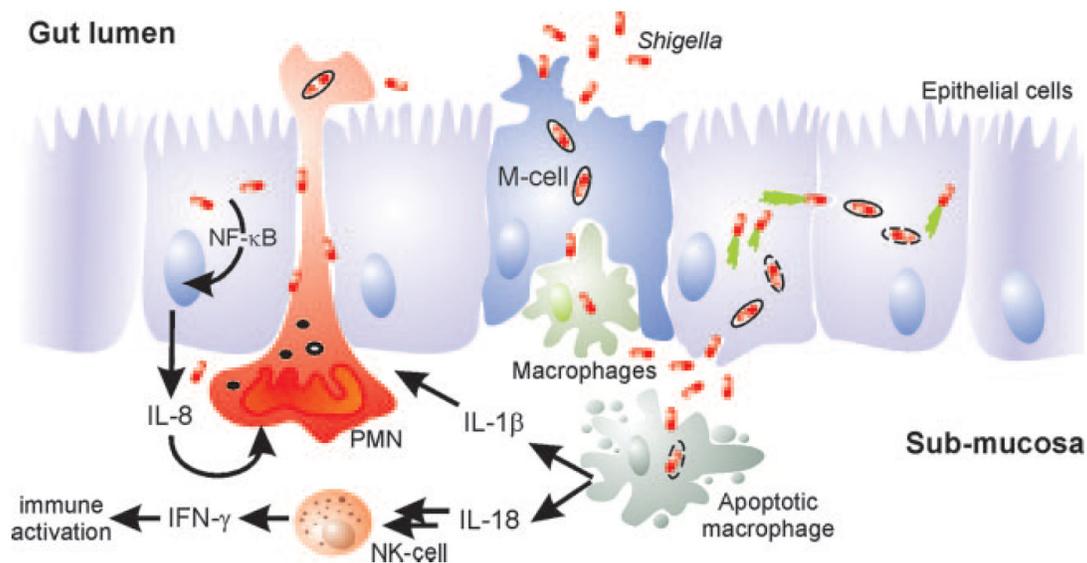


Figure 2: Cellular pathogenesis of *Shigella*. *S. flexneri* passes the epithelial cells (EC) barrier by transcytosis through M cells and encounters resident macrophages. The bacteria evade degradation in macrophages by inducing an apoptosis-like cell death, which is accompanied by proinflammatory signaling. Free bacteria invade the EC from the basolateral side, move into the cytoplasm by vectorial actin polymerization, and spread to adjacent cells. Proinflammatory signaling by macrophages and EC further activates the innate immune response involving NK cells and attracts PMN. The influx of PMN disintegrates the EC lining, which initially exacerbates the infection and tissue destruction by facilitating the invasion of more bacteria. Ultimately, PMN phagocytose and kill *Shigella*, thus contributing to the resolution of the infection. (Figure and figure legend reproduced from [53]).

3.2.3 *Shigella* virulence factors

Shigella are capable of colonizing the intestinal epithelium by exploiting epithelial cell functions and avoiding the host innate immune response. The ability of *Shigella* to invade epithelial cells and cause dysenteric disease is mainly related to the presence of high molecular weight and low copy-numbers virulence plasmids, collectively termed invasion plasmids, pINV, with minor variations between serotypes [65]. These virulence plasmids are approximately 230 Kb large, are non-conjugative [66,67], and encode for the major virulence determinants [68], including invasins (invasive plasmid antigens, Ipa proteins), molecular chaperones, motility factors, regulators, and a specialized type III secretion system (T3SS) [69]. The virulence plasmid also encodes several autotransporters (Type V-secretion-system, T5SS), including SepA and VirG (also referred to with the synonym IcsA) [26]. The T3SS is composed of about 22 proteins (see Figure 3), including a needle-like protein oligomer (composed of MxiH) anchored in the membrane through a complex consisting of a series of protein rings connecting the inner and outer bacterial membranes. The tip of the needle is composed of an IpaB/IpaD complex [70]: in particular, IpaD represents the needle tip forming protein and IpaB together with IpaC, the translocation pore. IpaB and IpaC have been shown to have a strong affinity for the CD44 hyaluronic acid receptor [71], and to form pores in the basolateral membrane of epithelial cells, allowing the bacterium to penetrate into the cytoplasm of the cell [72]. The T3SS also allows for the injection of a set of molecules into the host cells. The functions of those molecules have been characterized to antagonize both innate and adaptive immune responses and of virulence factors, leading to bacterial invasion and its corollary, the inflammatory destruction of the epithelial lining [60,73]. Additional virulence-associated genes are located in pathogenicity islands on the chromosome and contribute directly or indirectly to the pathogenic process [74]. *Shigella*, depending on serotype, also produce one or more toxins that contribute to virulence [75], including *Shigella* enterotoxin 1 (ShET-1), which is encoded by chromosomal gene set present in *S. flexneri* strains 2a [76], enterotoxin 2 (ShET-2), the product of gene *sen*, which is carried by the virulence plasmid [77], and the Shiga toxin, which is encoded by the *stx* gene from a phage of *S. dysenteriae* [78]. The capacity of *Shigella* to carry out virulence functions and manipulate host cells is a counterbalance between the bacteria's attack and the host response. The study of *Shigella* virulence modulation *in vivo* has focused on *Shigella flexneri*. The multi-steps infectious process (See previous paragraph) represents an array of successive environmental conditions to which the bacteria need to successfully adapt. The LPS is a major bacterial surface antigen in face of the adaptive immune response and a main target for the innate immunity. A potential role in pathogenesis, especially for its O side chain, has been proposed [70]. As demonstrated previously in the case of oxygen sensing and T3SS structure and function [79], a crosstalk exists between the activation of various environmental sensors and the T3SS, as well as LPS structural modifications [70,80,81]. The LPS molecules of *S. flexneri* 2a and 5a have O antigen polysaccharides with two modal chain length distributions: short (S) type chains (11–17 OAg repeating units (RUs)), and very long (VL) type chains (>90 OAg RUs) ([80]). It has been shown, for example, that the size distribution pattern of the O antigen changes in favor of longer chains when bacteria are grown at 30 °C, as compared to 37 °C [82]. In addition, OAg chains can mask VirG (IcsA) and interfere with its function in the actin-based motility

(ABM) [83]. These observations suggest that *S. flexneri* has evolved to synthesize LPS with the two OAg modal chain lengths, as S-type OAg chains allow VirG (IcsA) to function in ABM in the presence of VL-type OAg chains that confer resistance to serum [83]. Moreover, *S. flexneri* 5 M90T LPS glucosylation is reported to facilitate invasion of target cells by altering the conformation of LPS to optimize the exposure of TTSS needles while retaining resistance against host defenses [80]. Less is known about *S. sonnei* LPS: the typical O antigen ladder shows a predominant population with 20-25 OAg RUs [84], but studies about the relationship of OAg length distribution and virulence are not documented. With regards of other common surface virulence factors, *Shigella* is characteristically devoid of flagellar expression [85]. Importantly, *Shigella* so far is reported to be uncapsulated.

Figure 3

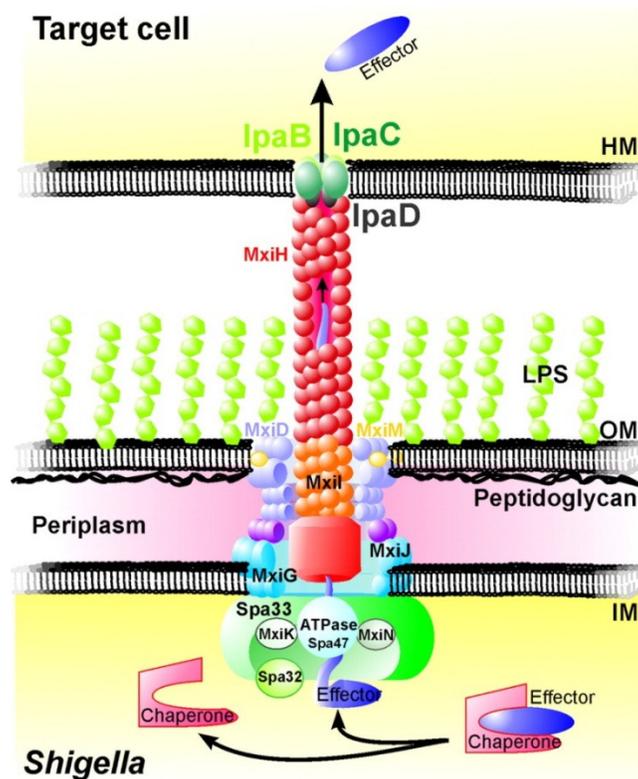


Figure 3: *S. flexneri* PAMPs and T3SS. The T3SS consists of four main parts. The basal body spans the bacterial inner membrane (IM), the periplasm, and the outer membrane (OM). The hollow needle protrudes from the basal body to the bacterial surface. Contact with host cell membranes (HM) triggers the IpaD-guided membrane insertion of the IpaB-IpaC translocon at the needle tip. The T3SS is completed by the cytoplasmic ring, which is comprised of proteins that energize the transport process and mediate the recognition of substrates, chaperone release, and substrate unfolding. The LPS and the peptidoglycan (PGN) molecules are located respectively on the bacterial surface and between the inner and outer membrane with the LPS being a major bacterial surface antigen (Reproduced from [53]).

3.3 Current status of *Shigella* vaccine development

The continuously high incidence rate imply that shigellosis is still an unsolved global health problem [86]. The ever increasing frequency of antimicrobial-resistant *Shigella* strains worldwide has become a major source of concern [46]. Conventionally, the fluoroquinolones, as Ciprofloxacin, have been the mainstay of the treatment for shigellosis and have been recommended as the drug of choice for all the patients, irrespective of their ages [87]. With the emergence of fluoroquinolone resistance among the *Shigellae* [88–93], the choice of the antibiotics had shifted to other alternatives, namely third generation cephalosporins, e.g. ceftriazone. Although not readily available as ceftriaxone has to be administered intravenously, the recent reports of cephalosporin resistance [94–96] have now really limited the options for the therapy. In addition, the extended spectrum beta-lactamases (ESBLs) are on the rise both in terms of frequency of occurrence as well as the type of the class of the enzyme and have been noted in *Shigella* [97,98]. Considering the ability of these organisms to acquire resistance genes on mobile elements, as the ESBL genes are, and the continuous selective pressure which results from the high levels of the antibiotic consumption and the presence of the poor quality unlicensed antibacterial agents in some areas, the likelihood of the development of resistance is enhanced [99]. The emergence of drug resistance strains can become a serious threat to public health, as their spread among a population in which diarrheal diseases are one of the major causes of childhood morbidity and mortality [99]. Vaccination could offer an effective and sustainable preventive measure against this enteric illness. Currently, there is no *Shigella* vaccine available and the large number of relevant serotypes with O antigen diversity makes development of a vaccine challenging [75]. Several strategies have been developed or are under development and are being tested in different clinical phases [100]. Among the vaccine candidates, two different approaches have mainly focused on: attenuated strains of *Shigella* used as live oral vaccines and O polysaccharides of *Shigella* covalently linked to carrier proteins and used as parenteral conjugate vaccines [75]. Other strategies have also been evaluated.

3.3.1 Live attenuated vaccines

Live *Shigella* vaccine candidates are given by needle-free routes and are easier to manufacture than other potential vaccines. However there is only a small margin between excessive reactogenicity, especially in children, caused by insufficient attenuation, and poor immunogenicity, especially in developing countries, by over-attenuation [101]. The *S. flexneri* 2a vaccine strain SC602 developed at the Institut Pasteur, for example, carries deletions in the *virG* (*icsA*) gene involved in cell-to-cell spreading, and in the *iuc* locus, which is involved in iron uptake. SC602 was tested in adult volunteers in the USA and in adults and children in Bangladesh in collaboration with the Walter Reed Army Research Institute (WRAIR) and the International Vaccine Institute (IVI). A remarkable efficacy against challenge was observed in USA volunteers, however, SC602 was safe only at very low doses [102]. In contrast, in adults and children in an endemic population (Bangladesh) even 100-fold higher doses did not induce adverse events but also did not induce any significant immune responses indicating intestinal barriers for live attenuated strains in endemic populations [103]. A live hybrid attenuated *Shigella* strain expressing both *S. flexneri* 2a and *S. sonnei* O

antigens was developed as an oral vaccine using the T32 attenuated *S. flexneri* strain initially produced by Istrati in Romania [104]. Large field studies in China have demonstrated 61-65 % protection against *S. flexneri* 2a and 57-72 % protection against *S. sonnei*. A protective efficacy against heterologous *Shigella* species was also claimed [104]. However, due to the need of three immunizations with high dose of bacteria ($>2 \times 10^{10}$ CFU) the vaccine was never clinically evaluated outside China [101]. Numerous approaches at attenuating *S. flexneri* 2a have been carried out at the Center for Vaccine Development (CVD) in Maryland, USA. The vaccines strain CVD1203 carrying deletions of *aroA* (involved in intracellular proliferation) and *virG* was well tolerated at a dose of 10^6 CFU when tested in a phase I trial in North American adults, but caused unacceptable reactogenicity when administered at doses of 10^8 CFU or 10^9 CFU [105]. CVD1207 vaccine carrying deletions of *virG*, *guaBA* (encoding enzymes involved in guaninenucleotide biosynthesis), *sen* (encoding ShET2) and *set* (encoding ShET1) genes was shown to be over-attenuated and well tolerated in phase I clinical trials with North American volunteers, but was not sufficiently immunogenic [101]. CVD1208 and CVD 1208S vaccine strains, harbouring deletions of *guaBA*, *sen* and *set* genes showed a better balance between clinical acceptability and robust immunogenicity than most attenuated strains tested so far, representing attractive vaccine candidates [75,106]. A bivalent vaccine containing a combination of attenuated *S. flexneri* 2a and 3a was tested in the guinea pig keratoconjunctivitis model [107]. The mixture induced the production of serum and tear antibodies to the included OAg serotypes. Interestingly, the raised sera also cross-reacted with LPS from heterologous *Shigella flexneri* serotypes 1a, 1b, 2b, 4b, 5b, Y but not with LPS from serotype 6. Moreover, the vaccine candidate conferred significant protection against challenge with the homologous serotypes and *S. flexneri* serotypes 1b, 2b, 5b, and Y, but not serotype 6 in accordance with the cross-reactivity data. Thus, despite the fact that natural infection results in predominantly serotype-specific protection, the cross-reactivity could be linked to common antigenic groups in the *S. flexneri* OAg of different serotypes. However, given the antigenic diversity of type and group factors within the *S. flexneri* serotypes, a high variation of cross protection was observed [107]. No protection was observed against serotypes 1a, and 4b despite the confirmation of cross-reactive LPS-antibodies. Thus, the combination of *S. flexneri* 2a and 3a serotypes showed a broad cross protection in the keratoconjunctivitis model, albeit not complete.

3.3.2 Polysaccharide conjugate vaccines

In area where shigellosis is endemic, the children who are less than 5 years of age have the highest rate of incidence. After the age of 5 years, the incidence declines, thus suggesting that a protective immunity develops after the exposure [87,88]. Lipopolysaccharide and specifically the outer domain of the LPS, the O antigen, is generally accepted as a protective antigen of *Shigella* [41]. Epidemiology and volunteer studies have shown that infected patients are protected against reinfection with the homologous *Shigella* strain. This protective immunity is narrowly type-specific. The sero-specificity of natural immunity indicates that immune response to the serotype-specific OAg play a role in protection. Further studies indicated that protection is mediated, at least in part, by serum or mucosal antibodies to the O antigen [41,108,109]. Due to the correlation of antibody responses to the OAg and protection after natural infection, the OAg is an attractive target for subunit vaccine development. However, given its

polysaccharidic nature, O antigen is recognized as a classical T-independent antigen with lower induction of antibody response, immunological memory and affinity of maturation [110,111]. Therefore, in order to overcome these limitations and to induce a T-dependent memory immune response, the O polysaccharide requires conjugation to a protein carrier. O antigen-specific IgG antibodies elicited by conjugate polysaccharide vaccines have been shown to prevent *in vitro* invasion of *Shigella* into Caco-2 cells in culture [112], suggesting that systemic antibodies can interrupt the pathogenic cycle of *Shigella* infection. Based on these observations O antigen-conjugates were developed as vaccine candidates against multiple *Shigella* serotypes. In particular, the O antigen from *S. dysenteriae* type 1 was conjugated to tetanus toxoid, and O antigens from *S. flexneri* and *S. sonnei* were conjugated to the recombinant exotoxin A from *Pseudomonas aeruginosa* (rEPA) or to the CRM9-mutant diphtheria toxin [75]. They were shown to be safe in volunteers in phase I clinical trials. A phase II clinical study in Israeli volunteer soldiers revealed that parenteral vaccines consisting of *S. sonnei* or *S. flexneri* 2a O antigens conjugated to rEPA induced high concentrations of anti-LPS antibodies, similarly to those present after natural infection. The levels of serum IgG and IgA anti-LPS remained elevated 2 years after vaccination [108]. In the following phase III trial, vaccination with *S. sonnei* O antigen-rEPA conjugate protected against homologous infection with 74 % efficacy in adults [113]. In age de-escalation studies both of the *S. sonnei* and *S. flexneri* 2a rEPA-conjugates were shown to be safe and immunogenic in 4-7 years old children, but only *S. flexneri* conjugate elicited a booster effect after the second vaccination [114]. A recent phase III trial in 1-4 years old children revealed that *S. sonnei* and *S. flexneri* O-SP-rEPA conjugates were safe in this age group. However, the immunogenicity and efficacy were age-related, as 71.1 % of efficacy was observed in the 3-4 years old, 35.5 % in the 2-3 years old, while no efficacy was observed in the 1-2 years old children [115]. This data indicate that the effectiveness of *Shigella* O antigen conjugate vaccines has to be improved to be useful in young children in the field. Initial experiments in animals suggests that *Shigella* OAg conjugates with shorter OAg chain length are more immunogenic and thus might provide a strategy for improving the immunogenicity in young children [116]. A similar strategy is followed in the development of polysaccharide conjugates with synthetic oligosaccharides that mimic the O antigen protective epitopes onto appropriate carriers. In a test study, three repeating units of the *S. flexneri* 2a O antigen were shown to induce an efficient anti-LPS antibody response [117]. Another innovative approach to produce a *Shigella* conjugate vaccine is the GlycoVaxyn's technology, that allows the synthesis of bioconjugates via a biological process in *E. Coli*. This *in vivo* glycosylation platform led to the synthesis of GVXN SD133 vaccine, consisting of *Shigella dysenteriae* O1 polysaccharide conjugated to a protein carrier. The GVXN SD133 candidate vaccine underwent through a Phase I clinical study in 2010 to demonstrate its safety and immunogenicity [118].

3.3.3 Other vaccine candidates

Additional strategies to develop a *Shigella* vaccine have been pursued. These include the *Shigella* invasion complex (Invaplex 50), a subcellular macromolecular complex prepared from pathogenic *S. flexneri*, containing LPS and the invasion plasmid antigen B (IpaB), IpaC and IpaD [119]. It showed a robust and protective immune response in a guinea pig model and a clinical Phase I intranasal vaccination study in adult volunteers

revealed that the Invaplex was safe and immunogenic. Studies on dose optimization and improvement of vaccine uptake are currently ongoing [120,121]. Other approaches are based on the association of LPS or O antigen, prepared for example from a *Shigella* strain that has been genetically attenuated by an *msbB* mutation to detoxify the lipid A portion of the *Shigella* endotoxin [122], with proteosomes and ribosomes [101] to amplify the immune response to the carbohydrate. Vaccines containing *S. flexneri* 2a and *S. sonnei* LPS complexed with meningococcal outer membrane protein proteosomes were tested by oral and intranasal vaccination [123]. Immunization studies in mice showed that the proteosomes-LPS complexes induced specific homologous anti-LPS antibodies in serum and mucosal secretions. Further, challenge studies using a keratoconjunctivitis guinea pig model showed that the orally or intranasally administered proteosome-LPS complexes protected against homologous bacteria. An intranasal immunization study conducted in healthy adults showed that *S. flexneri* 2a proteosome-LPS was well tolerated and elicited *S. flexneri* 2a LPS-specific antibodies (IgA, IgG and IgM) [124]. The level of the antibody responses was similar to those observed with live vaccine candidates associated with protective efficacy in human challenge models. As an alternative strategy, several groups have attempted to express *Shigella* O antigens in well-tolerated live vectors such as *E. coli*, generating strain EcSf2a-2, or attenuated *S. Typhi* strain Ty21a, but with only limited efficacy so far [75]. Newer approaches also aim to generate cross-protective responses based on highly conserved virulence factors, such as the T3SS apparatus proteins IpaB and IpaD. Mice immunized in the presence of the double mutant heat-labile toxin (dmLT) from *Escherichia coli*, used as a mucosal adjuvant, with IpaB alone or IpaB combined with IpaD were fully protected against lethal pulmonary infection with *Shigella flexneri* and *Shigella sonnei*, indicating that IpaB and IpaD are promising antigens for the development of a cross-protective *Shigella* vaccine [125].

3.3.4 The lack of animal models

No *Shigella* animal model currently satisfies the ideal criteria of being easy to set up, resembling as closely as possible the conditions met by *Shigella* in the human gut and mimicking the human bacillary dysentery. This lack of a relevant animal model is another obstacle for the development of a successful *Shigella* vaccine. The closest model to human infection is a non-human primate model. In Rhesus macaque monkeys, oral infection with *S. flexneri* without pretreatment with antibiotics or starvation results in the development of an acute colitis similar to the disease observed in humans [101]. However, infection of Rhesus monkeys requires 10^{11} bacteria compared to approximately 100 in humans. In addition, ethical, financial, and logistical considerations limit the testing of vaccine candidates in non-human primates. Therefore, different animals have been considered as possible models for *Shigella* infection: guinea pigs, rabbits, mice [101]. The Sereny test is a keratoconjunctivitis assay conducted on guinea pigs and it was long considered to be the gold standard test for strain virulence and protective immunity upon vaccination. However, this model is irrelevant for the target organ and provides just a quantitative description of the inflammatory response [101]. A currently widely accepted small animal model is the murine model of pulmonary *Shigella* infection. In this model, mice are infected with *Shigella* via the intranasal route resulting in bacterial invasion of bronchial and alveolar epithelia. This leads to the development of acute bronchiolitis and subsequent lethal pneumonia [126]. The characteristics of the

pulmonary lesions observed in infected mice are similar to the colitis developed in humans and non-human primates with shigellosis. In addition, it has been used for the characterization of cellular and humoral immune responses against *Shigella* infection [126], and it has been found to present similar immune responses as those detected in intestinal shigellosis. Thus, even though the lung structure and microenvironment are significantly different compared to the colon, the model has been used to assess protective immune response [101,126] and good protection has been achieved using intranasal immunization with subunit vaccines. More promising models have been developed in small animals to study the pathogenesis of *Shigella* but many of these have limitations to assess active immunization against *Shigella*. The intestine of adult mice is not susceptible to *Shigella* infection (via oral, intragastric and intrarectal inoculation) [60]. However, recently, a model of intragastric infection in newborn mice has been developed. Although mice show inflammatory destruction of the mucosa and infiltration of PMNs into the gut, this model cannot be used to evaluate protective active immunity, as the mice must be infected within 4-5 days after birth [127]. Another model uses the subcutaneous human colon xenograft in severe combined immunodeficient (SCID) mice. Mice express high levels of human IL-1 β and IL-8 and injection of *Shigella* into the lumen of the intestinal xenograft [128] results in a marked infiltration of neutrophils. Thus, this model is a viable option for studying the interactions between *Shigella* and the human intestine. But so far, no protection studies have been reported. The rabbit ligated ileal loop model is permissive to intestinal infection by *Shigella* and results in rupture, invasion and inflammatory destruction of the epithelium. The influx of PMNs observed in this model allows reliable quantification of the invasive phenotype. Therefore, if on one hand it is a good model for the validation of the invasive/inflammatory phenotype of *Shigella* mutants compared to wild type strain [70], on the other hand, the likelihood to use it for protection studies has still to be determined [101]. Recently, a new guinea pig model for bacillary dysentery has been investigated. Guinea pigs were infected by intra-rectal route with *S. flexneri* 2a or 5a strains and developed severe and acute rectocolitis mimicking human shigellosis [129]. In order to test the usefulness of this model for assessing the protective efficacy of *Shigella* vaccines, intra-rectal immunization with the live attenuated SC602 strain was performed and resulted in protection of the guinea pigs against challenge with *S. flexneri* 2a, suggesting that it may be useful for evaluating protective immunity of other *Shigella* vaccine candidates [101]. Finally, the *Shigella* human challenge model has also been used since 1946. The majority of the studies were conducted between 1989 and 1999 and with the aim to evaluate vaccine efficacy [130], although one of the studies was also an infective dose-finding study [131]. An additional study was conducted to assess the cross reactivity of *S. flexneri* 2a antibodies generated subsequent to challenge [132]. However, significant variability in study protocols, including the methods utilized to administer the challenge inoculum, the outcome definitions and variable challenge strains, confound result interpretation. Moreover, experimental challenge studies conducted so far have not been documented in endemic regions where *Shigella* vaccines would be targeted, with the exception of a recent report described in reference [133]. As a matter of fact, as all clinical trials, the human model must weigh the ethical dilemmas involved with placing human subjects at risk against the potential benefit of a future drug, vaccine or other product designed to treat or prevent disease [130].

3.3.5 Need for alternative approaches:

As described in the previous paragraphs, most of the vaccines currently under development are serotype-specific. There is a widespread agreement that future *Shigella* vaccines must protect against a broad spectrum of *Shigella* serotypes: 16 different serotypes are considered to be globally important for an epidemiologically valid global vaccine, *S. dysenteriae* 1, all 14 types of *S. flexneri*, and the *S. sonnei* single type [26,134]. The recently completed GEMS study (Global Enteric Multicenter Study, [135]) will update the knowledge about the current distribution of serotypes and the need of serotypes to be targeted by a *Shigella* vaccine. A combination of different O antigens might possibly be able to cover a large percentage of *Shigella* strains. However, the recent emergence of non typable strains and the possibility of strain replacement will likely require constant updates of a multivalent O antigen vaccine. An alternative strategy to develop a vaccine with broad-coverage will be to focus on bacterial structures that are conserved among different serotypes, such as highly conserved outer membrane proteins. Although attractive vaccines have entered clinical trials, further studies are needed to assess protection in humans and, most importantly, to understand if they can protect pediatric populations in developing countries [75]. As expected, the multifactorial nature of *Shigella* virulence suggests that any vaccine, either alive, inactivated or subunit candidate, must contain a plethora of antigens to lead to the right protective immune response. In addition, considering shigellosis results from an enteric infection, optimal protection is likely to require both mucosal and systemic immune effectors [136].

3.4 Outer membrane particles as Generalized Modules for Membrane Antigens (GMMA)

Pathogenic and non-pathogenic species of Gram-negative bacteria release particles from their surface into the extracellular space. These particles are usually called outer membrane vesicles (OMV) [137]. Naturally secreted OMV are closed spherical blebs of heterogeneous size (10-300 nm diameter) produced by growing cells, not products of cell lysis or death [138] and are characterized by a bilayer membrane containing electron-dense luminal content [139]. They form when a portion of the outer membrane is selectively “blebbed” off into round particles. The particles are proteoliposomes and reflect the composition of the bacterial outer membrane, containing LPS, glycerophospholipids, outer membrane proteins in their natural conformation, and enclosing periplasmic components [140,141]. The release of vesicles has been observed for a wide variety of Gram-negative bacteria including *Escherichia coli*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Shigella*, and *Helicobacter pylori* [142–144] and has been observed in all stages of growth and in a variety of growth environments, including liquid cultures, solid culture, and biofilms [141]. McBroom and Kuehn (in 2007) demonstrated that vesicles production is strongly related to bacterial stress response, and that it increases during periods of bacterial stress, such as the colonization of host tissues [145]. As part of the bacterial envelope, OM blebs are enriched in envelope components and contain also adhesins, toxins, invasins, and immunomodulatory compounds [141]. In many cases, specific proteins have been shown to be enriched or excluded from these blebs, suggesting a specific sorting

mechanism [146,147]. These observations, together with the presence of PAMPs (such as LPS) and virulence factors, suggest different roles for the OMV as secretory pathway for the transport of bacterial lipids, membrane or soluble proteins and virulence factors to host cells and other bacteria and for the modification of extracellular environments [148]. Some bacteria use vesicles to gain a survival advantage over competing bacteria: the presence of periplasmic peptidoglycan hydrolases, for example, can lead to the killing of co-cultured bacteria [149]. Vesicles can fuse to the outer membrane of several Gram-negative bacteria [144] and release their luminal content into the receiving strain, so they can be involved in horizontal transfer of antibiotic resistance proteins [150]. OMV act also as bridging factors in biofilms, mediating the co-aggregation of bacteria and producing a more permissive environment [151]. In addition they are used for communication in environments with mixed pathogen populations, by participating in quorum sensing [152]. The discovery of blebs released from pathogenic bacteria in fluids [153] and tissues [154] of infected hosts, demonstrates that they are able to disseminate away from the direct site of bacterial colonization. Therefore, they may play a role in the pathogenic process, and may be beneficial for the bacteria (e.g. mediating the inflammatory damage, delivering toxins and virulence factors) or for the host (e.g. being an indicator of infection) [140]. Interactions with host cells through a membrane fusion event or via the interaction adhesin/adhesin-receptor molecules present on the surface of the host cells have been reported [140]. Immunomodulatory blebs-associated LPS and outer membrane porins can be recognized by immune cells via Toll-like receptors (TLRs), and other molecules, which are able to activate the host innate and acquired immune response pathways [155,156]. On the other hand, vesicles can allow the escape of the bacteria from the immune detection during colonization. The gingipain protease on *Porphyromonas gingivalis* blebs, for example, degrades the lipopolysaccharide receptor on the surface of human macrophages-like cells deactivating the responsiveness of the immune system in the periodontal disease [157]. The general composition of Gram-negative OM particles seems to modulate the host-pathogen interaction in two synergic but opposite processes: stimulation of pathogen clearance on one side, and enhancing of the virulence of the infection on the other side [140].

Figure 4

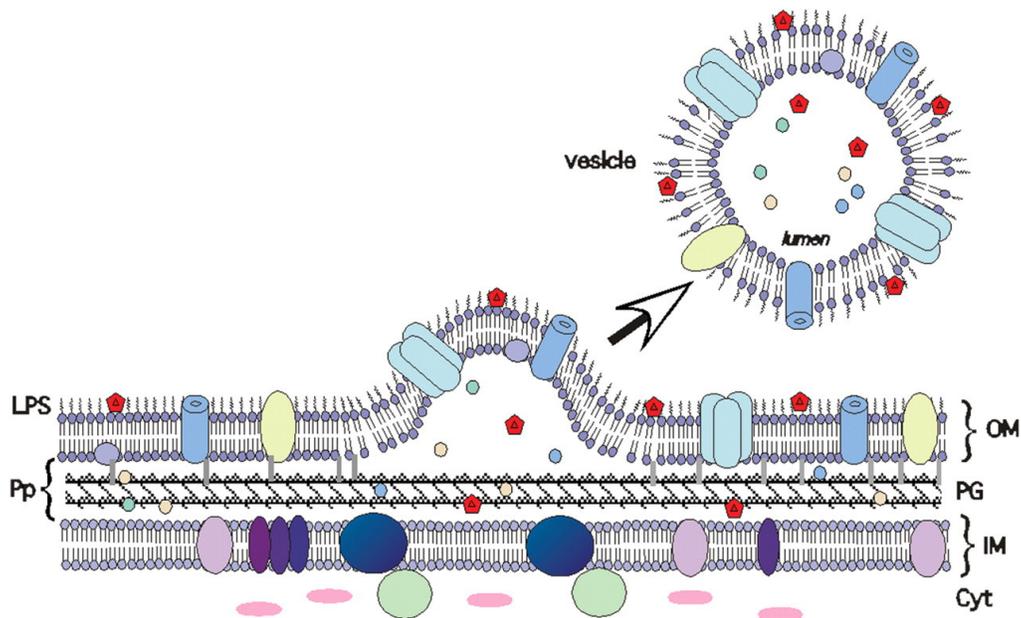


Figure 4: Model of vesicle biogenesis. Naturally secreted outer membrane (OM) vesicles are proteoliposomes consisting of OM phospholipids and LPS, a subset of OM proteins, and periplasmic (Pp) (luminal) proteins. Proteins such as LT (red) [158] that adhere to the external surface of the bacteria are associated with the external surface of vesicles. Proteins and lipids of the inner membrane (IM) and cytosolic content are excluded from OM vesicles. Vesicles are likely to bud at sites where the links between the peptidoglycan (PG) and OM are infrequent, absent, or broken. (Reproduced from [141]).

Proteoliposomes can also be artificially generated by sonication or detergent treatment of bacteria [159]. These detergent-extracted proteoliposomes are usually referred to as bacterial OMV, as the natural secreted ones, or d-OMV (detergent extracted-OMV). While the same term has been used the composition of naturally released blebs and detergent-extracted vesicles are very different. It has been shown that the chemical detergent treatment causes the removal of lipooligosaccharides and lipoproteins and alters the general composition of the vesicles in terms of protein profile, enriching the preparations in cytoplasmic and inner membrane proteins [160]. Interestingly, genetic modification of producing bacteria allows the increase of outer membrane vesicles release, without affecting their resemblance with naturally secreted blebs [160,161]. For example, Extraintestinal pathogenic *E. coli* strains (ExPEC) have been shown to release a high amount of vesicles enriched in outer membrane components when mutated in the Tol-Pal protein complex that cross-links the inner and the outer membranes [162].

Terminology: Generalized modules for Membrane Antigens (GMMA)

As mentioned above, the term OMV has been used for naturally released outer membrane particles, mutant derived-outer membrane vesicles (m-OMV) that are also released from the cell surface and resemble the naturally released blebs, and detergent-extracted outer membrane vesicles with a very different composition. This has resulted in some confusion and often d-OMV are used as example for the development of native

and m-OMV. In our Institute, the Novartis Vaccines Institute for Global Health (NVGH), the use of particles released from the surface of intact *Shigella* bacteria that have been genetically manipulated to increase vesicles release is under investigation as candidate vaccine [163]. In order to differentiate these particles from the substantially different detergent extracted-OMV, we chose the term GMMA, for Generalized Modules for Membrane Antigens [163].

GMMA as vaccine candidates

As GMMA originate from the outer membrane of intact and live bacteria without being modified by chemical treatments, their composition reflects the surface composition of the donor bacteria and this make GMMA good candidates as vaccine agents [160].

Recently, studies focusing on surface-released outer membrane particles and detergent-extracted outer membrane vesicles as vaccination strategies have progressively been receiving more interest [164]. For instance, a d-OMV containing vaccine, the MeNZBTM vaccine, has been successfully used to control *Neisseria meningitidis* type B infections in New Zealand [165]. However, since the dominant antibody response triggered by the MeNZBTM vaccine is directed against the outer membrane protein (OMP) PorA, the coverage is specific to the strains expressing the same PorA variant [166]. On the contrary, *N. meningitidis* GMMA, engineered to over-express protective antigens, have been shown to be more immunogenic and to elicit a broader bactericidal antibody response, than d-OMV [167]. GMMA also represent promising vaccine candidates for several other pathogens such as *Pseudomonas* [168], *Vibrio* [169], *Salmonella* [170]. Several studies revealed that GMMA can additionally be used as delivery system for heterologous antigens and can enhance the protective efficacy of other vaccine candidates through their adjuvant activity [171].

Limitations in the use of GMMA have been related to the endotoxic activity of LPS, the low yield obtained and the determination of defined composition [136]. A detoxified LPS would be needed to administer GMMA especially by parenteral vaccination [137]. A variety of strategies has been examined to attenuate the pyrogenicity of LPS by modifying genes required for the complete acylation of lipid A, e.g. *msbB* and *htrB* in *Shigella* [172] and *E. coli* [173] or *lpxL* in *Neisseria* [174]. By affecting the synthesis of the lipid A portion of the LPS in the GMMA donor bacteria, it is possible to obtain vesicles with reduced reactogenicity that can be safely administered to non-human primates [175]. Regarding the last two aspects, in NVGH, *Shigella* Program, we recently developed an easy and cost-affordable process, based on high-density cultivation of *Shigella* hyper-blebbing strains followed by two-steps tangential flow filtration, providing the basis for a large scale manufacturing process of GMMA that can be applied for production of vaccines from Gram-negative bacteria [163]. Moreover, we performed an extensive qualitative and quantitative proteomic analysis of *Shigella* GMMA, showing that they are highly enriched in OMPs, and we identified immunogenic and conserved surface proteins under native conditions [176]. When tested in a murine model of pulmonary *Shigella* infection, our GMMA produced from *S. sonnei* and *S. flexneri* 2a conferred cross-protection against heterologous serotypes if administered by mucosal route [177]. This work further supports the characterization of GMMA also as a protein-based vaccine against *Shigella*. Other groups are evaluating the employment of GMMA for the production of a *Shigella* vaccine. *S. flexneri* 2a GMMA-candidate containing LPS, lpa proteins, as well as other outer membrane components previously described as immunogens, such as porins and other major OMPs, has been successfully tested in

mice. One single dose of the antigenic complex administered by nasal, ocular or oral route induced very high levels of protection (90–100 %) against the homologous strain [178,179]. In another study, GMMA of *Shigella boydii* type 4 conferred passive protection in neonatal mice against homologous and heterologous strains [180]. In conclusion, the presence of virulence factors and native bacterial surface molecules, together with the adjuvanticity effect make GMMA a good vaccine candidate for broad spectrum protection against Gram-negative bacteria. Importantly, GMMA also represent an ideal tool to study Gram-negative periplasm and outer membrane compartments [162] and to shed light on new mechanisms of bacterial pathogenesis.

4. PhD PROJECT HYPOTHESIS and AIMS

The present work originated from the program at the Novartis Vaccines Institute for Global Health (NVGH) to develop a broadly protective vaccine against *Shigella* using Generalized Modules for Membrane Antigens (GMMA) as candidate antigens. GMMA might induce serotype-specific responses when produced from bacteria expressing a wild type LPS, as the OAg has been reported to be the immunodominant antigen in *Shigella* natural infection. However, the possibility to induce broadly-protective immune responses directed to highly-conserved outer membrane antigens can be investigated by producing GMMA lacking the OAg on their surface. In this context, different *Shigella* LPS mutants were generated and the immunogenic properties of deriving GMMA were evaluated in mice. Surprisingly, an LPS deep rough mutant of *Shigella sonnei* (*S. sonnei galU* null mutant) still elicited a serotype-specific response, indicating the presence of an OAg material, different from the LPS O side chain. A group 4 capsule (OAg capsule, G4C) consisting of the same repeating units as the OAg side chain of the LPS has been recently described in several Gram-negative bacteria. *Shigella* so far is reported to be unencapsulated, but possesses an operon homologous to the one responsible for capsule secretion and assembly in pathogenic *E. coli*.

Our hypothesis is that *S. sonnei* produces a group 4 capsule and that this capsule is still present in the *galU* mutant. The main aims of the present work are:

- the verification of the presence of a group 4 capsule in *Shigella sonnei*;
- the biochemical and structural characterization of the capsule polysaccharide;
- the functional characterization of the capsule, in relation to *S. sonnei* pathogenicity;
- the assessment of the G4 capsule distribution in other *Shigella* species.

The establishment of the expression and the understanding of the role of a capsule polysaccharide is a new and interesting finding in the *Shigella* field. As primary outcome of this research, we can achieve a better comprehension of the pathogenic mechanism of *Shigella* and the function of extracellular polysaccharides as virulence factors. As secondary outcome we can define potential implications of a novel surface polysaccharide on the design of a vaccine against *Shigella* spp.

5. MATERIALS and METHODS

5.1 *Shigella* strains and mutant construction

Shigella sonnei (Ss) 53G [181] wild type (WT) was chosen as parent strain in order to construct the following genetically modified strains (Table 1): Ss $\Delta galU$, Ss -pSS (phasell), Ss -pSS NA^R, Ss Δwbg , Ss Δcps , Ss $\Delta cps(cps)$, Ss $\Delta virG$, Ss $\Delta tolR$, Ss $\Delta tolR/\Delta galU$, Ss $\Delta tolR$ -pSS, Ss $\Delta tolR/\Delta wbg$, Ss $\Delta tolR/\Delta virG$, Ss $\Delta tolR/\Delta virG/\Delta cps$. *Shigella flexneri* 2a (Sf2a) 2457T [25] wild type was chosen as parent strain to produce the following null mutant strains (Table 1): Sf2a $\Delta galU$, Sf2a $\Delta rfbG$.

The deletion of *S. sonnei tolR* [162], *virG* [182,183] and *galU* [184] genes was obtained by substitution of the single gene coding sequence with an antibiotic marker. In particular, kanamycin was used to replace the *tolR* gene giving rise to the hyper-blebbing *tolR::kan* strain [163]; chloramphenicol resistance cassette was used for the *S. sonnei galU::cat* LPS deep rough mutant, and for the *S. sonnei virG (icsA)::cat* mutant in which the presence of the *Shigella sonnei* virulence plasmid (pSS) is selected and the virulence is attenuated by inactivation of the *lcsA* invasion protein [183]. *S. sonnei* Δwbg was produced by substituting genes of the O antigen (OAg) *wbg* gene cluster on the *S. sonnei* virulence plasmid (pSS) [57] with the chloramphenicol resistance gene in the *wbg::cat* LPS rough mutant strain. Erythromycin resistance gene was used to replace the whole group 4 capsule (G4C) gene cluster [185] in the *S. sonnei cps::erm* capsule mutant, and to replace three genes of the OAg *rfb* gene cluster [58] in the *S. flexneri* 2a *rfbG::erm* LPS rough mutant. Antibiotic resistance cassettes used for the generation of gene replacement mutants were obtained by from the following plasmids. The kanamycin cassette was amplified from pUC4K [186], the chloramphenicol gene from pKOBEG [187], and the erythromycin gene from pAT110 [188].

Shigella strains are listed and briefly described in Table 1.

S. sonnei $\Delta tolR$, Ss $\Delta galU$, Ss $\Delta tolR/\Delta galU$, Ss $\Delta tolR$ -pSS were produced in our laboratory (NVGH, Exploratory program, *Shigella* program) as described in reference [163]. *S. flexneri* 2a $\Delta galU$ was obtained by random transposon Tn10 mutagenesis as described in ref. [184] and was kindly provided by the laboratory of Maria Lina Bernardini, Dipartimento di Biologia Cellulare e dello Sviluppo, Università La Sapienza, Rome. The production of the other strains is described in the following paragraphs.

Table 1: List of *Shigella* strains used in this study, their abbreviation and a brief description of their phenotype

STRAIN Abbreviation	DESCRIPTION	SOURCE
Ss WT	<i>S. sonnei</i> 53G, phase I, +pSS	Bibliography [184]
Ss $\Delta galU$	<i>galU</i> deletion, LPS deep rough mutant, +pSS	Bibliography [163]
Ss -pSS	phase II, LPS rough mutant, OAg deficient, -pSS	This study

Ss -pSS NA ^R	phase II, resistant to Nalidixic acid	This study
Ss Δwbg	OAg gene cluster deletion, LPS rough mutant, OAg deficient, + pSS	This study
Ss Δcps	G4C gene cluster null mutant, +pSS	This study
Ss $\Delta cps(cps)$	Ss Δcps mutant complemented with plasmid carrying functional G4C gene cluster, +pSS	This study
Ss $\Delta virG$	<i>virG(icsA)</i> deletion, growth on Cm allows selection for the presence of pSS, attenuated, WT LPS	This study
Ss $\Delta tolR$	<i>tolR</i> deletion, hyperblebbing strain, +pSS	Bibliography [163]
Ss $\Delta tolR/ \Delta galU$	<i>tolR</i> and <i>galU</i> deletion, hyperblebbing strain, LPS deep rough mutant	Bibliography [163]
Ss $\Delta tolR/ -pSS$	<i>tolR</i> deletion, hyperblebbing strain, phase II, LPS rough mutant, OAg deficient, - pSS	Bibliography [163]
Ss $\Delta tolR/ \Delta wbg$	<i>tolR</i> and <i>galU</i> deletion, hyperblebbing strain, LPS rough mutant, OAg deficient, +pSS	This study
Ss $\Delta tolR/ \Delta virG$	<i>tolR</i> and <i>virG(icsA)</i> deletion, hyperblebbing strain, growth on Cm allows selection for the presence of pSS, WT LPS	This study
Ss $\Delta tolR/ \Delta virG/ \Delta cps$	<i>tolR</i> , <i>virG(icsA)</i> and G4C gene cluster deletion, hyperblebbing strain, growth on Cm allows selection for the presence of pSS	This study
Sf2a WT	<i>S. flexneri</i> 2a 2457T	Bibliography [25]
Sf2a $\Delta galU$	Tn10 insertion in the <i>galU</i> gene, LPS deep rough mutant	Bibliography [184]
Sf2a $\Delta rfbG$	OAg gene cluster null mutant, LPS rough mutant, OAg deficient, + pINV	This study

Electrocompetent cells and Recombination-prone *Shigella* cells

Electrocompetent cells were prepared as follows: over night (ON) cultures were diluted 1:50 in 50 mL LB medium and grown until OD₆₀₀= 0.6. Bacteria were collected by centrifugation at 4000 g for 10 min at 4 °C and washed 2 times in cold milliQ water. An additional washing step with cold 10 % glycerol was performed and bacteria were finally resuspended in 500 μ L of 10 % glycerol.

Recombination-prone *Shigella* cells were produced by using the highly proficient homologous λ red recombination system as previously described [163]. λ red recombinase genes under the control of an arabinose inducible promoter (red operon, [189]) are encoded on pAJD434 [190]. pAJD434 was inserted in electrocompetent cells by electroporation. The electrocompetent and recombination-prone cells, carrying the pAJD434, were prepared as follows. Over night cultures were diluted at OD₆₀₀= 0.03 in 150 mL of LB + trimethoprim 100 μ g/mL and grown at 30 °C, until OD₆₀₀= 0.2. 0.2 % arabinose (Sigma-Aldrich) was added to the culture to induce the expression of the recombinases, and cultures were grown until OD₆₀₀= 1.0. Bacteria were then recovered

and washed as described before. After the completion of the genetic mutation requiring homologous recombination, pAJD434 was removed from the mutant strains by ON liquid growths at 37 °C.

***S. sonnei* -pSS (phase II) and -pSS NA^R**

S. sonnei comprises a single serological group expressing the form or phase I O somatic antigen [191]. The O antigen synthetic gene cluster resides on the large virulence plasmid pSS [57]. During cultivation *in vitro* phase I colonies spontaneously lose the pSS and undergo a morphology transition from smooth and even-edged to uneven-edged, rough-appearing phase II colonies. When plated on Congo red TSB agar plates, the color of the *S. sonnei* colonies varies accordingly to the presence of the virulence plasmid from pigmented red (phase I) to white (phase II) [192]. Transition to the form II cell type results in the loss of the form I O antigen and of the virulence [193]. *S. sonnei* -pSS strain used in this study was selected by the white appearance on Congo red plates. The curing of the virulence plasmid was confirmed by the absence of PCR products when probing for the pSS origin of replication and the *wzy* gene [57], using primers pS.so53G.oriF/pS.so53G.oriR and pS.so53G.wzyF/pS.so53G.wzyR, respectively (primer sequences are listed in Table 2).

In order to generate a *S. sonnei* -pSS strain that could be identified using selective medium, experimentally induced Ss -pSS Nalidixic Acid resistant (NA^R) strain [194] was isolated by serial passages of Ss -pSS on LB agar plates supplemented with increasing concentrations of Nalidixic Acid (from 10 to 50 µg/mL). The antibiotic resistant phenotype was confirmed by liquid growth in LB + NA 50 µg/mL.

S. sonnei* Δ*wbg* and Δ*tolR*/ Δ*wbg

A *S. sonnei* O antigen deficient strain still carrying the virulence plasmid was engineered, as alternative to the Ss -pSS phase II strain. This O antigen knock out was obtained by deletion of the plasmid-borne gene cluster encoding the biosynthesis of the O repeating units (RU) [57] from gene *wzz* to *wbgZ* (see Figure 5). The mutant has been abbreviated as Ss Δ*wbg* and was generated as follows. Internal fragments of the genes *wzz* and *wbgZ* were amplified by PCR using primers *wzz*-5_Sac, *wzz*-3_Sam, and *wbgZ*-5_Sma and *wbgZ*-3_Sal, respectively. The *cat* gene was amplified from pKOBEG using primers, EcoRV.Cm.F and EcoRV.Cm.R. PCR products were digested with *Sac*I and *Sma*I (*wzz*), *Sal*I and *Sma*I (*wbgZ*) and EcoRV (*cat*) (Restriction Endonucleases, New England BioLabs®), ligated in a 4-way ligation into pBluscript SK + (Stratagen) vector digested with *Sac*I and *Sal*I, and transformed into *E. coli* DH5αTM-T1R Competent Cells (Invitrogen). The *wzz-cat-wbgZ* fragment of the obtained plasmid pSK-*wbg::cat* was amplified using the primers *wzz*-5_Sac *wbgZ*-3_Sal. The PCR product was transformed into recombination prone *S. sonnei* 53G or *S. sonnei* Δ*tolR* to yield *S. sonnei* Δ*wbg* and *S. sonnei* Δ*tolR*/ Δ*wbg* (primer sequences are listed in Table 2).

Figure 5



Figure 5: Schematic representation of *S. sonnei* plasmid-borne O antigen gene cluster.

***S. sonnei* Δ cps, Δ tolR/ Δ virG/ Δ cps and Δ cps(cps) complemented strain**

Δ cpsKO::*erm* fragment was constructed to delete the whole G4C gene cluster (See Figure 6, [185]) in *S. sonnei* by conventional cloning and homologous recombination. The resulting mutant was abbreviated as Ss Δ cps.

Figure 6

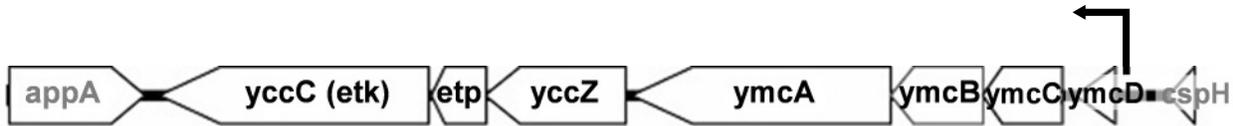


Figure 6: *S. sonnei* G4C operon, schematic representation with flanking genes (*appA* and *cspH*)

Upstream (495 bp) and downstream (526 bp) flanking regions of the G4C gene cluster were amplified from a *S. sonnei* WT colony, by PCR with the EcoRV.cps.3'.F/ XhoI.cps.3'.R and XbaI.cps.5'.F/ EcoRV.cps.5'.R primers, respectively. The two PCR products were digested with EcoRV/ XhoI and XbaI/ EcoRV (Restriction Endonucleases, New England BioLabs®) and cloned into the pBluescript SK + (Stratagen) vector. The ligation was performed in a molar ratio of 1:6 between the plasmid and each fragment using the T4 DNA-Ligase (Roche). 10 μ L of the reaction were transformed in 50 μ L of Max Efficiency® *E. coli* DH5 α TM-T1R Competent Cells (Invitrogen) according to the manufacturer specificities. Transformed colonies were selected on 100 μ g/mL ampicillin and grown ON in LB + amp at 37 °C to do a mini plasmid preparation (QIAprep Spin Miniprep kit, QIAGEN, cat. #27106). The purified plasmid was controlled by XbaI/ XhoI and EcoRV digestions and by PCR with XbaI.cps.5'.F/ XhoI.cps.3'.R primers to contain the G4C flanking regions. The erythromycin resistance gene, *erm* [188] was amplified with primers EcoRV.Ery.F/ EcoRV.Ery.R. After EcoRV digestion, the purified cassette was ligated into the EcoRV site between the G4C operon flanking regions, generating the p Δ cpsKO::*erm* plasmid (see Figure 7). The plasmid was transformed in 50 μ L of DH5 α competent cells and bacteria were selected on LB + 100 μ g/mL erythromycin plates. Minipreps of p Δ cpsKO::*erm* were screened by XbaI/ XhoI and EcoRV digestions and by PCR with XbaI.cps.5'.F/ XhoI.cps.3'.R primers. Finally, KpnI linearized p Δ cpsKO::*erm* was used as template to amplify the linear fragment Δ cpsKO::*erm* (primers XbaI.cps.5'.F/ XhoI.cps.3'.R). 1 μ g of purified Δ cpsKO::*erm* (QIAquick PCR purification kit, QIAGEN, cat. #28106) was used to transform electrocompetent, recombination-prone *Shigella sonnei* WT and Δ tolR/ Δ virG (see following paragraph) cells to generate the G4C knock out strains Ss Δ cps and Ss Δ tolR/ Δ virG/ Δ cps. Mutant colonies were subsequently controlled by PCR with different combinations of primers (cps.ext-5/ cps.ext-3, Ery.ctr.int.F/ cps.ext-3) (primer sequences are listed in Table 2).

Figure 7

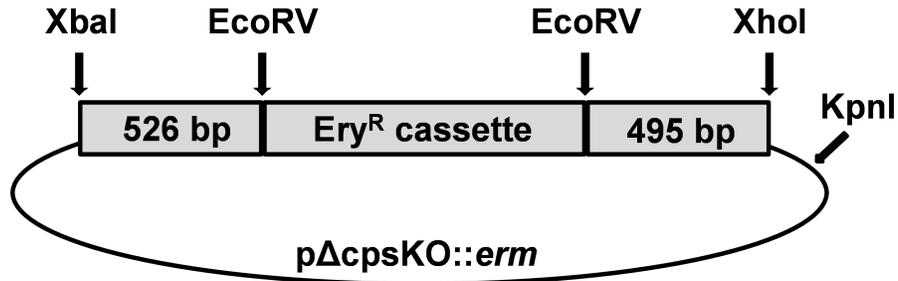


Figure 7: G4C operon knock out. Schema of pBluescript vector carrying the Δ cpsKO::erm construct. Upstream (495 bp) and downstream (526 bp) regions of the group 4 capsule gene cluster are cloned using EcoRV/ XhoI and XbaI/ EcoRV enzymes, erythromycin resistance cassette is subsequently cloned in the plasmid using EcoRV enzyme.

The G4C operon was complemented in the Ss Δ cps KO mutant as follows. The G4C gene cluster with the 280 bp upstream region was amplified using primers XbaI.COMPLcps.5'.F/ Sall.COMPLcps.3'.R (LongRange PCR Kit, QIAGEN, cat. #206401) (primer sequences are listed in Table 2). The XbaI/ Sall digested fragment was cloned into pACYC184 (New England BioLabs®) vector, inactivating the tetracycline resistance cassette while leaving the chloramphenicol cassette intact. Due to the large size of the amplified operon (8032 bp) the ligation was performed in a 1:3 molar ratio of fragment to plasmid, to generate the p Δ cpsCOMPL plasmid (see Figure 8). 10 μ L of the reaction were transformed in DH5 α competent cells, selected on 20 μ g/mL chloramphenicol. Purified plasmid preparations were controlled by double XbaI/ Sall and single Sall digestions. The correct product was used to transform electrocompetent Ss Δ cps cells carrying the G4C cluster deletion to generate the complemented strain called Ss Δ cps(cps). G4C gene cluster complementation was subsequently controlled by different analyses of exopolysaccharide expression.

Figure 8

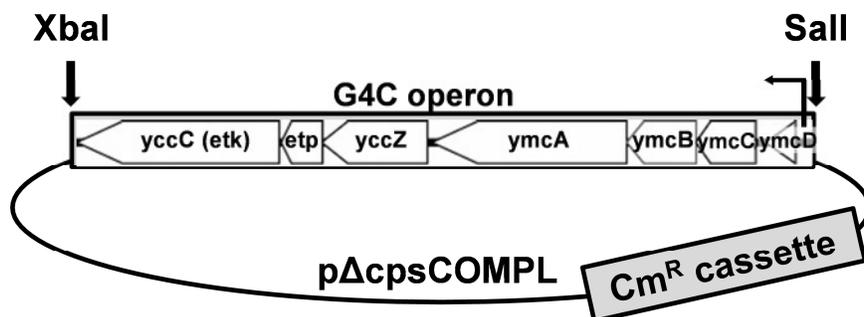


Figure 8: G4C operon complementation. Schema of pACYC184 vector carrying the G4C operon cloned with the 280 bp upstream region using XbaI and Sall enzymes (p Δ cpsCOMPL). Chloramphenicol resistance is used to select positive clones.

***S. sonnei* $\Delta virG$ and $\Delta tolR/ \Delta virG$**

As mentioned before, during cultivation *in vitro*, *S. sonnei* spontaneously loses the virulence plasmid giving rise to phase II OAg deficient colonies [193]. In order to avoid the loss of the pSS and consequently of the ability to synthesize the OAg repeating units, a mutant selectable for the presence of the pSS was produced by inactivating one of the plasmid-encoded virulence factor [183]. Upstream and downstream flanking regions of the *virG* gene were amplified by PCR using primers virGup-5_Sac, virGup-3_Sam and virGdown-5_Sma, virGdown-3_Sal, respectively. The *cat* gene was amplified from pKOBEG using primers, EcoRV.Cm.F and EcoRV.Cm.R. PCR products were digested with SacI and SmaI (upstream flanking region), Sall and SmaI (downstream flanking region) and EcoRV (*cat*), ligated in a 4-way ligation into pBluscript digested with SacI and Sall, and transformed into *E. coli* DH5 α sub-cloning cells, resulting in plasmid pSK-*virG::cat*. The insert containing the *cat* gene and the adjacent flanking regions of *virG* was amplified using primers virGup-5_Sac and virGdown-3_Sal. The PCR product was transformed into recombination prone *S. sonnei* 53G or *S. sonnei* $\Delta tolR$ to yield *S. sonnei* $\Delta virG$ and *S. sonnei* $\Delta tolR/ \Delta virG$ (primer sequences are listed in Table 2).

***S. flexneri* 2a $\Delta rfbG$**

The *S. flexneri* 2a O antigen deficient strain was generated by inactivating three genes in the chromosomal OAg synthetic operon [58], in particular the *rfbG* gene and parts of the flanking genes *rfbF* and *rfc* (see Figure 9). Internal fragments of the genes *rfbF* and *rfc* were amplified by PCR using primers rfbF1-stop_Sma, rfbF-2_Sal, and rfc-1_Sac, rfc-2_XbaSma2, digested with SacI and SmaI (*rfbF*), Sall and SmaI (*rfc*), inserted into pBluscript SK+ digested with SacI and Sall, and transformed into *E. coli* DH5 α , to yield pSK-rfb-flank. The *erm* gene was amplified from pAT110 using primers EcoRV.Ery.F and EcoRV.Ery.R, digested with EcoRV, and inserted into the SmaI site between the *rfbF* and *rfc* fragments in pSK-rfb-flank. The resulting plasmid pSK-rfb::*erm* was used to amplify the rfbF-erm-rfc fragment with primers rfbF-2_Sal and rfc-1_Sac. The PCR product was transformed into recombination prone *S. flexneri* 2a or *S. flexneri* 2a $\Delta tolR$ to yield *S. flexneri* 2a $\Delta rfbG$ and *S. flexneri* 2a $\Delta tolR/ \Delta rfbG$ (abbreviations).

Figure 9



Figure 9: Schematic representation of *S. flexneri* 2a chromosomal O antigen gene cluster.

Primers and plasmids

Shigella mutants were constructed using combinations of primers listed in the following Table 2.

Table 2: List of primers used in this study for generation of *Shigella* mutants

MUTANT/ GENE	PRIMER	SEQUENCE
Ss -pSS	pS.so53G.oriF	CGTAACCGTAATTACAGCCG
	pS.so53G.oriR	GATTTACCTTACCCATCCC
	pS.so53G.wzyF	CGTTGAGTTTTACGTTTTCT
	pS.so53G.wzyR	TTACCAATATAACCCTCCGCA
Ss Δ wbg	wzz-5 Sac	ACTCGAGCTCGGCAGACTCAGCGCAAG
	wzz-3 Sma	CTAACCCGGGCATTGACACAACAATACGTAACCCAG
	wgbZ-5 Sma	CTAACCCGGGTGCGATTTGGTAATGTACTIONCGG
	wgbZ-3 Sall	ACGCGTCGACATTGCTCGCTTGTGATAACAGC
Ss Δ cps	EcoRV.cps.3'.F	AGCTTGATATCCGCATGGACAATACGTACGC
	XhoI.cps.3'.R	CCGCTCGAGACGTGTGGAATTTTGCCGCG
	XbaI.cps.5'.F	CTAGTCTAGAGATCAAGACAGCGTTGACGC
	EcoRV.cps.5'.R	AGCTTGATATCAACTGAAAGTGGCCGGATGC
	cps.ext-5	GCCACCCGTTATTAGATTTG
	cps.ext-3	GCAGAAGTGCTTATACCAGG
Ss Δ cps(cps)	XbaI.COMPLcps.5'.F	CTAGTCTAGACATCCGGCCACTTTCAGTTTTAC
	Sall.COMPLcps.3'.R	ACGCGTCGACCAGCCAGTTATAGTACCACTTG
Ss Δ virG	virGup -5 Sac	ACTCGAGCTCTGTAGTTGATTTGACAGTTGACATCC
	virGup -3 Sma	CTAACCCGGGCACTATATTATCAGTAAGTGGTTGAT AAACC
	virGdown-5 Sma	CTAACCCGGGCGTGTTGATGTCCTGC
	virGdown-3 Sal	ACGCGTCGACAGTTCAGTTCAGGCTGTACGC
Sf2a Δ rfbG	rfbF-1stop Sma	CTAACCCGGGCTAAGCATCTAAGACACCATTCTGTA TC
	rfbF-2 Sall	ACGCGTCGACAATATCCTGGAGCATAACGTGT
	rfc-1 SacI	ACTCGAGCTCACCAATAACGCCTGTTTTCTG CTAGTCTAGAAACCCGGGTTTGATGTGCAACCATAC CTTTT
	rfc-2 XbaSma2	CTTTT
erm	EcoRV.Ery.F	AGCTTGATATCAGAGTGTGTTGATAGTGCAGTATC
	EcoRV.Ery.R	AGCTTGATATCACCTCTTTAGCTTCTTGAAGCT
	Ery.ctr.int.F	CGTACCTTGGATATTCACCGA
cat	EcoRV.Cm.F	AGCTTGATATCTGTGACGGAAGATCACTTCG
	EcoRV.Cm.R	AGCTTGATATCGGGCACCAATAACTGCCTTA
	Cm.verif-5	CATCATGCCGTTTGTGATGG

Plasmids used in this study are listed in the following Table 3.

Table 3: List of plasmids used in this study

PLASMID	Description	SOURCE
pKOBEG	Containing Chloramphenicol resistance cassette	Bibliography [187]
pAT110	Containing Erythromycin resistance cassette	Bibliography [188]
pAJD434	Containing λ red recombinase genes	Bibliography [190]
pBluescript SK +	Cloning vector	Stratagen
pSK- <i>wbg</i> :: <i>cat</i>	pBluescript containing <i>wbg</i> region knock out construct	This study
p Δ cpsKO:: <i>erm</i>	pBluescript containing G4C operon knock out construct	This study
pSK- <i>virG</i> :: <i>cat</i>	pBluescript containing <i>virG</i> knock out construct	This study
pSK- <i>rfbG</i> :: <i>erm</i>	pBluescript containing <i>rfb</i> region knock out construct	This study
pACYC184	Cloning vector	New England BioLabs®
p Δ cpsCOMPL	pACYC184 expressing the G4C operon	This study

5.2 PCR conditions

Standard PCR reactions were performed using the PCR SuperMix (Invitrogen, cat. #10790-020) with 100 pmol of primers. The conditions for the amplification of the products were: initial denaturing step at 94 °C for 30 sec, followed by 30 cycles of denaturing step at 94 °C for 30 sec, annealing step at 52-54 °C for 30 sec, and a variable elongation step at 72 °C (1 minute every 1000 bp), and a final elongation step at 72 °C for 30 sec. For the amplification of the group 4 capsule operon, the LongRange PCR Kit (QIAGEN, cat. #206401) was used accordingly to producer specifications. In particular, the conditions for the amplification were: initial denaturing step at 94 °C for 3 min followed by 35 cycles of denaturing step at 94 °C for 15 sec, annealing step at 62 °C for 30 sec, elongation step at 68 °C for 9 min, and a final elongation step at 72 °C for 30 sec.

5.3 Bacterial growth and fermentation conditions

E. coli and *Shigella* strains were routinely cultured in Luria-Bertani (LB) medium or in 30 g/L tryptic soy broth (TSB, BD BBL™), respectively, at 37 °C, 200 rpm, unless specified in the particular method. Tryptic soy agar (30 g/L TSB, 15 g/L agar) supplemented with 150 mg/L congo red (Sigma-Aldrich) was used to evaluate the presence of the virulence plasmid in *Shigella*. Inactivated bacteria for mice immunization study were produced in low-osmolarity Luria-Bertani medium with NaCl omitted (LBON), containing 10 g/L BactoTryptone (BD BBL™) and 5 g/L yeast extract (BD BBL™), pH adjusted to 7.0 with

NaOH [195]. For the production of GMMA, *Shigella* hyperblebbing strains were grown in 30 g/L TSB, in yeast extract medium (HTMC) or in *S. sonnei* Defined Medium (SSDM) [163]. HTMC was prepared as follows: 30 g/L yeast extract, 5 g/L KH₂PO₄, 20 g/L K₂HPO₄, 1.2 g/L MgSO₄*7H₂O, 15 g/L glycerol, and 0.25 g/L polypropylene glycol (PPG), pH adjusted to 7.2 with 4 M ammonium hydroxide. SSDM was prepared as 30 g/L glycerol, 13.3 g/L KH₂PO₄, 4 g/L (NH₄)₂HPO₄, 1.2 g/L MgSO₄*7H₂O, 1.7 g/L citric acid, 2.5 mg/L CoCl₂*6H₂O, 15 mg/L MnCl₂*4H₂O, 1.5 mg/L CuCl₂*2H₂O, 3 mg/L H₃BO₃, 2.5 mg/L Na₂MoO₄*2H₂O, 13 mg/L Zn(CH₃COO)₂*2H₂O, 2 µM ferric citrate, 50 mg/L thiamine, 10 mg/L nicotinic acid, 2.5 g/L L-aspartic acid, 0.25 g/L PPG, pH maintained at 6.7 with 4 M ammonium hydroxide. For fermentation, starter cultures were grown from glycerol stocks to OD₆₀₀= 0.8 and subsequently transferred to a 5 L fermenter (Applikon) to reach a starting OD₆₀₀ of 0.02. Dissolved oxygen was maintained at 30 % saturation by controlling agitation and setting maximum aeration, pH was maintained at 6.7 by a pH controller and temperature was kept constant at 37 °C. When needed, growth media were supplemented with Kanamycin (Kan, 30 µg/mL), Chloramphenicol (Cm, 20 µg/mL), Erythromycin (Ery, 100 µg/mL), Nalidixic Acid (NA, 50 µg/mL), Trimethoprim (Tmp, 100 µg/mL), Ampicillin (Amp, 100 µg/mL).

5.4 Generalized Modules for Membrane Antigens (GMMA) preparation

GMMA purified from liquid culture supernatant of *Shigella* hyperblebbing strains (*tolR* null mutants) were used as outer membrane preparation to extract the *Shigella* exopolysaccharides (EPS) and as antigens for mice immunization studies. For low density preparations, bacteria were grown in TSB medium in 1 L flasks. Culture supernatants were collected by 10 min centrifugation at 4000 g and sterilized by 0.22 µm filtration. After concentration using a 100 KDa regenerated cellulose membrane (Millipore) in a Stirred Ultrafiltration Cells (Amicon, model 8400), GMMA were separated from soluble proteins by 2 h ultracentrifugation at 186000 g at 4 °C (Optima™ L-series, 45Ti rotor, Beckman Instruments) and resuspended in cold phosphate buffer saline (PBS). Preparations were sterilized by 0.22 µm filtration. For high density productions, GMMA were prepared in flasks or in a 5 L fermenter (Applikon) in HTMC medium or in SSDM and purified from sterile culture supernatants by a two step-Tangential Flow Filtration (TFF) precess [163].

5.5 Preparation of inactivated whole cell *Shigella*

Bacteria inactivated by formalin fixation or by heat inactivation were used as antigens for mouse immunization studies. In particular, formalin fixed (FF) Ss WT, Ss *ΔgalU*, Ss - pSS, Ss *Δwbg*, Sf2a WT, Sf2a *ΔgalU*, Sf2a *ΔrfbG* and heat killed (HK) Ss *ΔgalU* were prepared as follows. ON cultures were grown in LBON supplemented with antibiotics, when needed, starting from colonies selected on congo red plates. The following day, cultures were diluted to OD₆₀₀= 0.07 in 150 mL and grown until OD₆₀₀= 1 (bacterial concentration is 5*10⁸ CFU/mL). For heat inactivation, cells were collected by 10 min centrifugation at 4000 g and concentrated 20 times in PBS (bacterial concentration is 10¹⁰/mL) and the mix was incubated at 100 °C for 2 min. The preparation was stored at

4 °C until use. For formalin fixation, cells were collected by 10 min centrifugation at 4000 g and resuspended in 15 mL Hank's Balanced Salt Solution (HBSS, GIBCO®), supplemented with 0.5 % formalin solution (Sigma-Aldrich). The mixture was incubated for 36 h at room temperature in slow agitation. Subsequently, the inactivated bacteria were collected by centrifugation, resuspended in 7.5 mL PBS (concentration of 10^{10} CFU/mL) and stored at 4 °C until use. 100 µL of each preparation were plated on LB to confirm the absence of bacterial growth.

5.6 Quantification of GMMA-associated proteins

GMMA-associated protein concentration was quantified by Bradford Assay (Bio-Rad Protein Assay, cat. #500-0002). 100 µL of each sample were diluted 1:2 in 6 M guanidine hydrochloride pH 7.8 and boiled at 100 °C for 10 min. Samples were subsequently diluted in the Bradford reagent according to the manufacturer instructions. Bovine Serum Albumin (BSA) was used as standard (Thermo Scientific Pierce, cat. #23209) to build the standard curve (range 1 µg/mL - 9 µg/mL in the assay). Absorbance was measured at wavelength of 595 nm.

5.7 Mouse immunization studies

CD1 female mice of 4 to 6 weeks of age were immunized subcutaneously on days 0, 21, and 35. Blood samples for sera analysis were collected before the first immunization (preimmune sera) and 14 days after the third injection (bleed out, post 3 immune sera). Groups of 8 mice were used for mice immunizations with GMMA. In particular, GMMA obtained from *Ss ΔtolR*, *Ss ΔtolR/ ΔgalU*, and *Ss ΔtolR/ -pSS* hyperblebbing strains were used. Each injection contained 2 µg of GMMA (as quantified by protein amount) formulated in PBS in a final volume of 100 µL. Groups of 5 mice were used when formalin-fixed bacteria were administered. Each injection contained 10^9 CFU formulated in PBS in a final volume of 100 µL. Control mice received PBS alone. The animal experiments complied with the relevant guidelines of Italy and the institutional policies of Novartis. The animal protocol was approved by the Animal Welfare Body of Novartis Vaccines and Diagnostics, Siena, Italy, approval number AEC 2009-05.

5.8 1D SDS-PAGE

GMMA, Formalin Fixed bacterial preparations and hot-phenol LPS extracts (See paragraph) were analyzed by 1D SDS-polyacrylamide gel electrophoresis using an XCell SureLock™ Mini-Cell system (Invitrogen). Samples were boiled for 10 min at 95 °C in NuPAGE® LDS Sample Buffer (Invitrogen) containing 10 mM DL-Dithiothreitol (DTT, Sigma-Aldrich, cat. #D9779) and loaded on NuPAGE® 12 % Bis-Tris gels (10 wells, 1 mm thick, Invitrogen, cat. #NP0341BOX). Precision Plus Protein™ All Blue Standard (Bio-Rad, cat. #161-0373) was used as protein molecular mass marker, and each gel was run in NuPAGE® 3-(N-morpholino)propanesulfonic (MOPS) acid SDS Running Buffer (Invitrogen, cat. #NP0001) at 30 mA per gel. Gels loaded with GMMA or bacterial preparations were stained with Coomassie Brilliant Blue G-250 solution (40 %

ethanol, 10 % acetic acid, 1 g/L G-250, Thermo Scientific Pierce, cat. #20279), and destained with 40 % ethanol, 10 % acetic acid. Gels loaded with LPS extracts were silver stained using the SilverQuest™ Silver Staining Kit (Invitrogen, cat. #LC6070) accordingly to manufacturer instructions.

5.9 WESTERN BLOT

Samples resolved by 1D SDS-PAGE were transferred to nitrocellulose membranes using the iBlot® Gel Transfer Device (Invitrogen, cat. #IB1001). Membranes were washed in PBS, blocked ON with 3 % (w/v) milk in PBS, and after 3 washing steps for 10 min in 3 % milk, 0.05 % (v/v) Tween20 in PBS, they were incubated with the 1:1000 dilution of primary antibody (GMMA polyclonal mice antisera, IpaB H16 mouse monoclonal antibody [196] [kindly provided by the laboratory of Armelle Phalipon, Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, Paris, France] or Ss phase I monovalent rabbit antiserum, Denka Seiken Co., Ltd. cat. #295316) in blocking buffer, for 2h at room temperature. The membranes were then washed as before, and incubated with a 1:5000 dilution in blocking buffer of the appropriate secondary antibodies (Goat anti-Mouse IgG-Alkaline Phosphatase antibody, Sigma-Aldrich, cat. #A3562, or Goat anti-Rabbit IgG-Alkaline Phosphatase conjugate, Invitrogen, cat. #G-21079) for 1 h at room temperature. Immunoblots were washed three times and developed using the SIGMAFAST™ BCIP®/NBT tablet (Sigma-Aldrich, cat. #B5655) solution, as described by the manufacturer.

5.10 Transmission Electron Microscopy (TEM)

GMMA were diluted to a concentration of 100 µg/mL in PBS. 5 µL were placed on copper carbon-coated grids and GMMA were absorbed for 5 min at room temperature. Grids were then washed with 3 consecutive drops of distilled water and blotted with a filter paper. For negative staining the grids were treated with 2 % uranyl acetate for 50 seconds and air-dried. The grids were subsequently analyzed using a TEM (tr) FEI Tecnai G2 Spirit operating at 80 kV equipped with a 2Kx2K CCD camera Olympus SIS Morada.

Bacteria were prepared by processing for TEM and ultrathin resin sectioning. The primary fixation was performed with the addition of alcian blue to highlight polysaccharides. A sample of size=20 was used to measure the thickness of alcian blue stain extending beyond the outer membrane.

5.11 Bacteria surface staining coupled with Flow cytometry (FACS) analysis

Over night *Shigella* cultures were diluted in TSB medium to $OD_{600} = 0.05$ and grown to early exponential phase at $OD_{600} = 0.5$ (bacteria are 2.5×10^8 CFU/mL). Cells were collected and diluted to a concentration of 2×10^7 CFU/mL. For formalin-fixed *Shigella* staining procedure, bacteria were diluted in 0.5 % formalin solution in PBS and fixed in agitation over night at room temperature. Live or fixed bacteria were washed,

resuspended in TSB and aliquoted in 96 well plates (Corning® Costar® cell culture plates, round bottom). The incubation with the desired dilutions of mouse immune sera, IpaB monoclonal antibody (H16, mouse monoclonal Ab, kindly provided by the laboratory of Armelle Phalipon, Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, Paris, France [196]) or the rabbit Ss phase I monovalent antiserum (OAg-specific antiserum) was performed in a final volume of 50 μ L for 1 h, at 4 °C. For competitive staining experiments, prior to bacterial incubation, Ss $\Delta galU$ GMMA immune serum was incubated with 11.25 μ g of LPS (as quantified by phenol-sulfuric assay) extracted from Ss WT or -pSS bacteria, for 1 h at 4 °C, at a 1:1000 dilution in PBS. After washing with 1 % BSA in PBS, bacteria were incubated with Allophycocyanin-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch®) or with Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG (H+L) (Invitrogen) for 1 h, on ice to detect antibody binding. Bacteria were washed, fixed with 4 % formalin in PBS for 20 min, on ice and finally analyzed for cell-bound fluorescence using a FACScanto II flow cytometer (BD Biosciences).

5.12 Hot-phenol extraction of total cellular and outer membrane lipopolysaccharides

Lipopolysaccharides were extracted by the method of Westphal and Jann [197], with some modifications.

For total cellular lipopolysaccharides extraction, ON *Shigella* cultures were diluted in 50 mL LB supplemented with antibiotics, if needed, to OD₆₀₀= 0.1 and grown until OD₆₀₀= 1. Bacteria were collected by centrifugation and the pellet was resuspended in 500 μ L PBS. After 5 min boiling, the mixture was treated with 0.5 μ g/ μ L of Proteinase K (Thermo Scientific Pierce, cat. #17916) at 60 °C ON. An equal volume of saturated phenol solution (pH 8.0) (Sigma-Aldrich, cat. #P4557) was added and incubated for 30 min at 70 °C with occasional mixing. After 1 h centrifugation at 10000 g, the upper aqueous phase was recovered and 2 volumes of absolute ethanol were added. The polysaccharides were allowed to precipitate for 1 h at -70 °C. Samples were centrifuged at 12000 g for 30 min and the pellet containing LPS and capsule polysaccharide was dried in a rotary vacuum drier (SpeedVac) and dissolved in distilled water.

For outer membrane lipopolysaccharides extraction, purified GMMA were used as starting outer membrane preparations. After Proteinase K incubation step, samples were treated as described before.

5.13 Lipid A removal and exopolysaccharides (EPS) isolation

In order to remove the lipid A moiety of LPS, outer membrane lipopolysaccharide samples extracted from GMMA were treated by mild acid hydrolysis with 1 % acetic acid for 1.5 h at 100 °C. The reaction was neutralized by adding few drops of ammonium hydroxide to adjust the pH to 6.5. Samples were centrifuged at 15000 g over night and the supernatant, containing pure polysaccharides, was collected and dialyzed against distilled water.

5.14 Phenol-sulfuric acid assay

Phenol sulfuric acid assay was used for total sugar content quantification, using glucose (Glc) as standard, as previously described in ref. [198].

5.15 High Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

HPLC-SEC analysis was used to analyze the molecular size distribution of EPS populations and to isolate polysaccharide (PS) fractions of different molecular weight. Samples were run on a TSK gel G3000 PWXL column (30 cm x 7.8 mm; particle size 7 μm ; cod. #808021) with a TSK gel PWXL guard column (4.0 cm x 6.0 mm; particle size 12 μm ; cod. #808033) (TosohBioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4 , 5 % CH_3CN , pH 7.2 at the flow rate of 0.5 mL/min (isocratic method for 30 min). Polysaccharides peaks were detected by differential refractive index (dRI).

5.16 ^1H Nuclear Magnetic Resonance (NMR) spectroscopy

NMR analysis was performed to confirm the identity of the PS samples by detecting typical signals of the OAg residues, to confirm the molar ratio of FucNAc4N to L-AltNAcA and to calculate the number of OAg repeating units (RUs) attached to the core [116]. Dried PS samples were solubilized in 650 μL deuterated water (D_2O , Sigma-Aldrich) and transferred to 5 mm NMR tubes. Proton NMR experiments were recorded at 25 $^\circ\text{C}$ on Varian VNMRS-500 spectrometer, equipped with a Pentaprobe. Acquisition time of 5 sec, relaxation delay of 15 sec and number of scans of 64 were set for recording the spectra. For data acquisition and processing VNMRJ ver. 2.2 rev. C and Mestrenova 6.1 (Mestrelab Research) were used respectively. 1D proton NMR spectra were collected using a standard one-pulse experiment. Chemical shifts were referenced to hydrogen deuterium oxide (HDO) at 4.79 ppm.

5.17 KDO quantification by semicarbazide derivatization coupled with HPLC-SEC

Presence of 3-deoxy-D-manno-octulosonic acid (KDO) at the reducing end of isolated EPS population [199] was estimated by HPLC-SEC after derivatization with semicarbazide. Derivatization was performed using the semicarbazide assay for α -ketoacids determination, with minor modifications [200]. EPS samples (100 μL of total volume in water), were added to 100 μL of semicarbazide solution (100 mg semicarbazide hydrochloride + 90.5 mg of sodium acetate anhydrous in 10 mL of water). Samples were heated at 50 $^\circ\text{C}$ for 50 minutes and then analyzed by HPLC-SEC (80 μL injected), on a TSK gel G3000 PWXL column with guard column in 0.1 M NaCl, 0.1 M NaH_2PO_4 , 5% CH_3CN , pH 7.2 mobile phase at the flow rate of 0.5 mL/min (isocratic method for 30 min). Detection was done at 252 nm.

5.18 Gentamicin protection assay (HeLa cells invasion assay)

Invasiveness of the various *S. sonnei* strains was evaluated *in vitro* by using a gentamicin protection assay conducted on HeLa semiconfluent monolayers. Each condition was tested at least in triplicates. Briefly, 5×10^5 HeLa cells/well (HeLa ATCC®, CCL-2™) were seeded ON in 6 well cell culture plates (Corning® Costar®) in Dulbecco's Modified Eagle Medium (D-MEM) high Glucose (Invitrogen, cat. #61965), supplemented with 10 % FBS (New Zealand, Invitrogen). The following day, *Shigella* ON cultures were diluted in TSB medium (supplemented with antibiotics, if needed) and grown to $OD_{600} = 0.5$ (bacteria are 2.5×10^8 /mL). Bacteria were collected, diluted in D-MEM and used to infect HeLa cells with a Multiplicity of Infection of 10 bacteria/cell (MOI 10). After the addition of the bacteria, the infected cells were centrifuged 18 min at 1100 g and 37 °C and incubated for 1 h at 37 °C. Subsequently, the monolayers were washed with PBS and the medium was replaced with D-MEM containing 80 µg/mL gentamicin, to kill extracellular bacteria. After 2 h of incubation in gentamicin-containing medium, cells were washed and lysed by the addition of cold 0.5 % sodium deoxycholate (DOC, Sigma-aldrich) in water. Suitable dilutions were plated in triplicates on congo red agar to determine the number of recovered viable bacteria.

5.19 Rabbit ligated ileal loops model

Virulence of the various *S. sonnei* strains was evaluated *in vivo* by testing their ability to induce a *Shigella*-dependent pathology in the rabbit model of ligated ileal loops [201]. In the single challenge experiment, each loop was infected for 8 h with a single strain dose of 3×10^9 bacteria/loop in 500 µL of physiological saline buffer (0.9 % NaCl). Each condition was tested in 2 rabbits, 3 loops per rabbit. A preliminary experiment was performed in 2 rabbits to establish the lower *S. sonnei* infective dose giving a clear *Shigella*-induced pathology manifestation (doses tested: from 10^9 to 5×10^9 bacteria/loop). Bacteria for the single challenge experiment were prepared as follows: 1.5 mL of $OD_{600} = 0.6$ *Shigella* glycerol stocks were diluted in 50 mL TSB medium (supplemented with antibiotics, if needed), and grown ON at 37 °C, 200 rpm. Bacterial pellets were collected by centrifugation at 4000 g, for 10 min at room temperature, the OD_{600} was adjusted to 12 (bacteria are 6×10^9 /mL) in physiological saline buffer and bacterial suspensions were stored at room temp until use. In the competitive challenge model, each loop was infected for 16 h with two strains resulting in a total dose of 3×10^7 bacteria/loop (1.5×10^7 bacteria of each strain/loop in 250 µL) in a total volume of 500 µL of physiological saline buffer. Each condition was tested in 2 rabbits, in 4 loops per rabbit. Bacteria for the competitive challenge experiments were prepared as follows: 500 µL of $OD = 0.6$ *Shigella* glycerol stocks were diluted in 7 mL TSB medium (supplemented with antibiotics, if needed), and grown ON at 37 °C, 200 rpm. The following day, ON cultures were diluted in 20 mL to start at $OD_{600} = 0.05$ and grown for 5 h. Bacterial pellets were collected, the OD_{600} was adjusted to 0.12 (bacteria are 6×10^7 /mL) in physiological saline buffer and bacterial suspensions were stored at room temp until use. The rabbit ileal loop model protocol was approved by the Comite Regional d'Ethique pour l'Experimentation Animale in Paris 1 (protocol #20070004) and reviewed by the Global Animal Welfare Board of Novartis. Surgery was performed as described previously with

minor modifications [62]. New Zealand white rabbits weighing 2.4–2.6 Kg (Charles River Laboratories, St. Aubin, France) were treated for 7 days with Mucoxid (2.8 g/L sodium sulfadimethoxine salt in the drinking water) (CEVA Sante Animale, France) until the day prior to surgery to minimize infection with *Coccidia* parasites. Rabbits were fasted 24 h before infection, sedated intravenously by ear vein injection with 0.05 mL/Kg Calmivet (Vétoquinol) containing 0.5 % acepromazine and anesthetized by the same route with 0.2 mL/Kg of Imalgene® 1000 (containing 10 % ketamine HCl) (Merial, France). Prior to laparotomy, 2 mL Xylovet (CEVA Sante Animale, France) containing 2.1 % lidocaine was injected intradermally in the abdomen along the site of incision. The small intestine was exteriorized and the cecum was localized. 12 loops of 5 cm segments of ileum starting at the ileum-cecum transition were ligated, avoiding all Peyer's patches, while maintaining the existing vasculature. Each loop was injected with 500 µL of bacterial suspension using a 26-gauge needle. The injection order was randomized for each rabbit. Loops were returned into the abdominal cavity, the abdomen was closed and the animals were returned to their cage for the defined incubation time. Animals were sacrificed by intravenous injection of 120 mg/Kg sodium pentobarbital (Doléthal, Vétoquinol, France). After euthanasia of single challenged rabbits, the exudate of loops was measured and collected to recover bacteria for gene expression analysis. Loops were dissected and processed for RNA extraction, histology and bacterial counting; as control, uninfected tissues were collected as well. Spleen, liver and mesenteric lymph nodes (MLNs) were collected and processed for bacterial counting. After euthanasia of competitive challenged rabbits, loops, spleen, liver and mesenteric lymph nodes were dissected and processed for bacterial counting. Homogenized tissues were serially diluted and appropriate dilutions were plated on selective media to determine the number of recovered viable bacteria.

5.20 Histopathological analyses of tissue samples

For histopathological analysis of *Shigella* infected ileal loops and bacterial immune-localization, intestinal biopsies were fixed at 4 °C in 4 % paraformaldehyde in PBS, embedded in paraffin and sectioned into 7 µm slices using a microtome. Sections were deparaffinated, rehydrated and used for Hematoxylin and Eosin (H&E) staining, GIEMSA staining or for *Shigella* immune staining. Immune staining was performed as follows. Sections were permeabilized for 15 min with the antigen unmasking solution (10 mM Tris, 1 mM EDTA, 0.05 % Tween20, pH 9), treated with 3.3 % H₂O₂ for 10 min and washed. Samples were blocked for 15 min with Ultra V block (Lab Vision Corp; Thermo Scientific, cat. #TA-125UB) and incubated overnight with an in-house mouse polyclonal anti-*S. sonnei* serum (mouse polyclonal antiserum raised against Ss $\Delta toIR$ GMMA). Samples were then incubated with a peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins (DAKO, cat. #K4000) for 1 h, revealed with the 3-amino-9-ethylcarbazole AEC+ Substrate-Chromogen (DAKO, cat. #K3461), counterstained with hematoxylin and mounted with aqueous mounting medium (Merck).

5.21 Real Time-quantitative PCR (RT-qPCR) gene expression analyses of rabbit cytokines and bacterial G4C operon

Real Time-quantitative PCR (RT-qPCR) analyses were performed to measure cytokine genes expression in rabbit infected loops and G4C operon expression in bacteria collected from intestinal fluids.

In order to measure cytokine genes expression, single challenged loop samples were submerged in TRIzol® Reagent (Invitrogen), homogenized with a Precellys tissue homogenizer (Bertin Technologies) and stored at -20 °C until further processed for RNA extraction. A volume of 1 mL of each sample was mixed with 200 µL chloroform, incubated at room temp for 5 min and centrifuged for 2 min at 12000 g at 4 °C. RNA was precipitated by adding 500 µL of the upper aqueous phase to an equal volume of isopropanol. After a centrifugation step, the pellet was washed twice with 800 µL of 70 % ethanol, dried and subsequently dissolved in 100 µL DEPC-treated H₂O (Ambion) by heating the samples at 60 °C for 10 min. Subsequent RNA purification was performed by using the RNeasy Mini Kit (QIAGEN, cat. #74104), including the on-column digestion of DNA. The final RNA concentration was determined using a spectrophotometer (Nanodrop 2000). cDNA synthesis was performed on 10 µg of RNA in a 10 µl sample volume using SuperScript® II Reverse Transcriptase (Invitrogen, cat. #18064-014) as recommended by the manufacturer. The RNA was incubated with 0.5 µg/µL of oligo(dT) 12-18 mer primers (Fermentas, cat. #397Y9) for 10 min at 70 °C and then transferred on ice. 9 mL of a master mix containing 4 µL of SuperScript II buffer, 2 µL of 0.1 M DTT (Invitrogen), 1 µL each of dNTPs stock (10 mM) (Invitrogen), Rnasin (40 UI) (Promega, cat. #N2511) and SuperScript II (Invitrogen) were added to the RNA sample, mixed and incubated at 42 °C for 60 min followed by 5 min at 70 °C to inactivate the enzyme. cDNA was stored at 4 °C until use. Rabbit primer pairs used in this study are described in ref. [202]. RTq-PCR reactions were performed in MicroAmp® Optical 384 Well Reaction Plates with Barcode (Applied Biosystems®) in a total volume of 15 µL containing 12 ng of cDNA, primers (0.2 µM each) and 7.5 µL of FastStart Universal SYBR Green Master (Roche, cat. #4913914001). Reactions were run in duplicate on an ABI PRISM 7900HT (Applied Biosystems) using the universal thermal cycling parameters (2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C; dissociation curve: 15 sec at 95 °C, 15 sec at 60 °C and 15 sec at 95 °C). Results were obtained using the sequence detection software ABI 7900HT SDS2.2 and analyzed using Microsoft Excel. For all samples, dissociation curves were acquired for quality control purposes. The comparative threshold cycle (Ct) method was used for gene expression quantification [203]. First, for each sample, the gene expression levels were normalized to the expression levels of the housekeeping gene encoding Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Δ Ct). Following, the difference between Ct values of samples from loops infected with the different *S. sonnei* mutants and samples from loops infected with *S. sonnei* WT was used to determine the $\Delta\Delta$ Ct. To determine the relative fold increase in gene expression in loops infected with *S. sonnei* mutants versus the condition in WT infected loops, the $\log_2\Delta\Delta$ Ct was calculated.

In order to measure *Shigella* G4C operon expression, exudates from single challenged loops were processed as described before, with minor modifications. For the synthesis of cDNA, purified RNA was incubated with 3 µg/µL random primers

(Invitrogen, cat. #48190-011) instead of oligo(dT) primers. Moreover, for gene expression quantification, the ΔC_t was calculated by normalizing to the expression level of the housekeeping gene encoding the lysine transporter LysP [204]; the $\Delta\Delta C_t$ was the difference between the ΔC_t s of each mutant to the WT. The $\log_2\Delta\Delta C_t$ gave the relative fold increase in gene expression of the mutant strain versus the WT.

Shigella sonnei primer pairs used in this study are listed in Table 4.

Table 4: List of primers used in this study for RTqPCR analysis of *Shigella* G4C operon expression

<i>Shigella</i> TARGET GENE	FORWARD and REVERSE PRIMER SEQUENCES
<i>lysP</i>	lysP.F: CCGAAACTAAAACACTACAGAAGCGC lysP.R: CCTTCTTCAACATAGTTCTGACCG
<i>ymcD</i>	ymcD.F: CTTTCTGCAATCCTTATGGCCTTC ymcD.R: GACGTGGTGGTTGAGGTGTTATG
<i>ymcC</i>	ymcC.F: CACTATTTCTTGCGGGATGTACG ymcC.R: GTCCATTGTGGGTAACCAGCATG
<i>ymcB</i>	ymcB.F: CAGTCGTATTTATTGCGGATGCGC ymcB.R: GACATGTTGGTAGTCTTTAAGCGC
<i>ymcA</i>	ymcA.F: CTTATCTTTTAAGCTGCCTGGCC ymcA.R: CCACTGGCTATATTTGCGTGTGC
<i>yccZ</i>	yccZ.F: GACTGACATTGGCTGTTCTGTC yccZ.R: GATCCCAGACCGTAACCATCAAC
<i>yccY (etp)</i>	yccY.F: CTCAATCCTGGTGGTTTGTACCG yccT.R: GACTCCATTGCCAGAATCAGATC
<i>yccC (etk)</i>	yccC.F: CAGGCAGCACTCAGGAAAATGAG yccC.R: GATTGCAGCAGTTGGATCTCCG

5.22 Statistical analysis

The nonparametric Mann-Whitney test was used for two-way comparison and determination of the statistical significance of the differences in villi atrophy intensity using GraphPad (PRISM) software. Values of $p < 0.001$ were considered statistically significant and are marked on figures as ***.

6. RESULTS

6.1 Generation and immunogenicity of GMMA from *S. sonnei* LPS mutants

The present work originated from the program at the Novartis Vaccines Institute for Global Health (NVGH) to develop a broadly protective vaccine against *Shigella*. Our group has engineered GMMA over-producing *Shigella sonnei* strains by deleting the *tolR* gene that encodes for a Tol-Pal system protein [163] from a wild type *S. sonnei* 53G strain. The Tol-Pal system is a supramolecular complex in Gram-negative bacteria which spans the periplasm and connects the outer and inner membranes, playing a role in maintaining the stability of the outer membrane [205]. Previously, the deletion of *tolR* was reported to result in overproduction of GMMA without loss of membrane integrity in *E. coli* [162]. Studies focused on the characterization of *S. sonnei* GMMA as vaccine candidate against shigellosis are being performed. As already mentioned, GMMA might induce serotype-specific responses when produced from bacteria expressing a wild type LPS, as the OAg has been reported to be the immunodominant antigen in natural infection [41]. However, the possibility to induce broadly-protective immune responses directed to highly-conserved outer membrane antigens can be investigated by producing GMMA lacking the OAg on their surface. Figure 10, shows the schematic structure of *S. sonnei* LPS. The LPS consists of lipid A, core oligosaccharide (OS), and O-specific polysaccharide (O antigen) that is made of repeating saccharide subunits. In particular, the lipid A is embedded in the lipid bilayer of the outer membrane and consists of β -glucosaminyl-1,6-glucosamine units interlinked by pyrophosphate bridges and O-substituted by six fatty acids residues (lauryl-, 3-d-myristoxymyristoyl-, 3-d-hydroxymyristoyl- and palmitoyl- chains) [206]. The core oligosaccharide can be subdivided into inner and outer core domains. The outer core consists of five hexoses, whereas the inner core is composed of three residues of 3-deoxy-D-manno-octulosonic acid (Kdo), three heptoses and a glucosamine (GlcN) residue [207]. Finally, the *S. sonnei* O antigen is composed of a disaccharide repeating unit containing two unusual amino sugars, 2-amino-2-deoxy-L-altruronic acid (LAltNAcA) and 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (2-acetamido-4-amino-2,4-dideoxy-D-fucose, FucNAc4N) [208]. The biosynthesis of LPS is a complex process involving various steps that occur at the plasma membrane, followed by the translocation of LPS molecules to the bacterial cell surface [209]. The core oligosaccharide is assembled on preformed lipid A by sequential glycosyl transfer of monosaccharides, while the O antigen is assembled on undecaprenol-phosphate (Und-P), a polyisoprenoid lipid to which O antigen is linked via a phosphodiester bond. These pathways eventually converge by the ligation of the O antigen onto the outer core domain of the lipid A-core OS acceptor, with the concomitant release of Und-P [210]. Mutations in the core biosynthesis genes [211] lead to deep rough mutants with an incomplete core, which lacks the site for the attachment of O polysaccharides. Mutations in *wb** (formerly *rfb*, [211]) genes, which are involved in the synthesis of the O polysaccharide, give rise to rough mutants lacking the OAg but having a complete core. Typically, *wb** gene clusters encode nucleotide sugar synthases (for biosynthesis of the nucleotide sugar precursors specific to O antigens),

and glycosyltransferases (for the sequential and specific addition of sugars that make the O repeating unit) [210]. Additional genes encoding functions involved in the assembly of the O polysaccharide are also present in the O antigen synthetic clusters, such as *wzy* (O antigen polymerase) and *wzx* (putative O antigen flippase) [210]. In *S. sonnei*, nine genes have been recognized to be essential for the OAg biosynthesis [84]. These genes include two (*wbgW* and *wbgY*) for putative glycosyl transferases and two (*wzx* and *wzy*) for proteins that function in the transport and polymerization of the O repeating units. The remaining five genes (*wbgT*, *wbgU*, *wbgV*, *wbgX*, *wbgZ*) of the cluster function to convert available nucleotide-linked sugars to the FucNAc4N and LAltNAcA-containing precursors of the OAg disaccharide repeating unit [84]. Moreover, an additional gene, *wzz*, participates in the Wzy-dependent biosynthetic pathway, in regulating chain length of LPS-linked glycans.

With the aim to avoid GMMA serotype-specific immunogenicity, different *S. sonnei* LPS mutants were generated by deleting genes involved in LPS core and OAg synthesis and assembly (See Figure 10). Initially, a *galU* deletion mutant ($\Delta galU$) was generated [163]: this mutation blocks the biosynthesis of UDP glucose from glucose-1-P, a precursor needed for the glucosyl residues into the core region [212]. The result is the synthesis of an incomplete LPS core leading to a truncated LPS composed of lipid A and inner core [213]. In literature, *S. flexneri* 2a and 5a $\Delta galU$ have been characterized to be OAg deficient [80,184]. As a second approach, mutant strains unable to synthesize the repetitive unit of the OAg were generated. *S. sonnei* differs from the other *Shigella* strains in having a major deletion in the chromosomal O antigen gene cluster between the genes *galF* and *gnd* (see Figure 9), and a functional gene cluster on the virulence plasmid (pSS) [214]. As mentioned above, *S. sonnei* WT bacteria (expressing the form or phase I O somatic antigen) can spontaneously lose the pSS during cultivation *in vitro*, originating phase II, OAg-negative cells [193]. Therefore, in order to obtain *S. sonnei* LPS rough mutants, we either isolated colonies that had lost the virulence plasmid (*S. sonnei* 53G phase II, abbreviated as Ss -pSS) or we inactivated the OAg gene cluster on the virulence plasmid (Ss Δwbg). These mutations were combined with the deletion of the *tolR* gene with the aim to obtain OAg-negative *S. sonnei* hyper-blebbing strains.

Figure 10

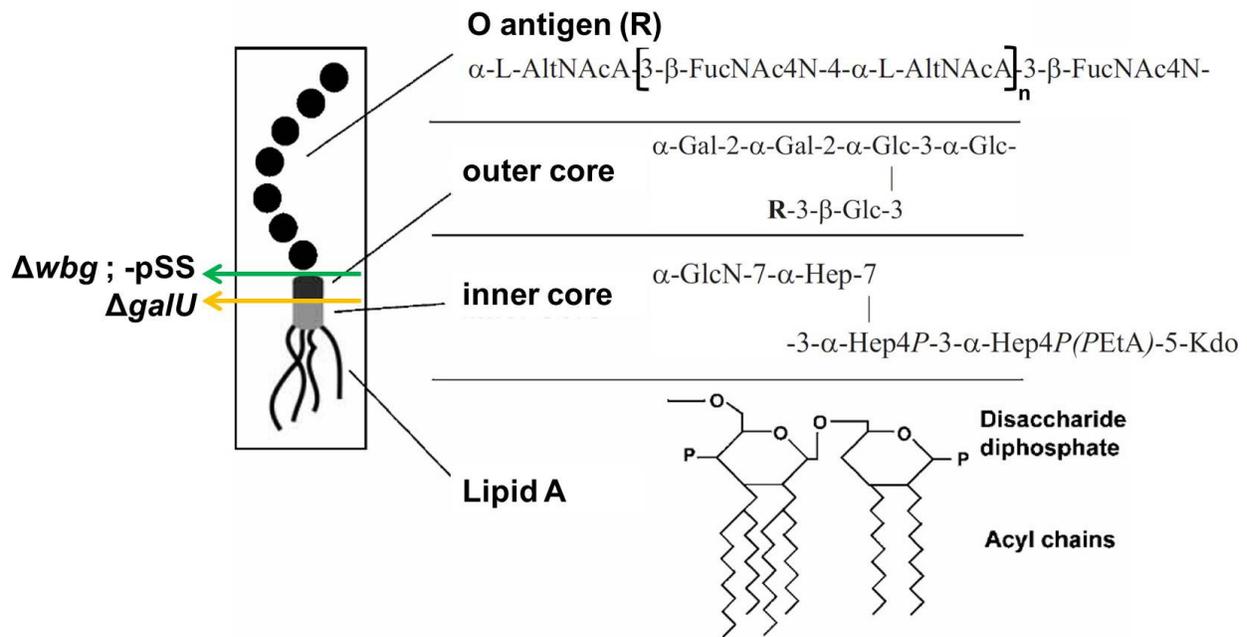


Figure 10: *S. sonnei* LPS structure and sites of genetic modification. Scheme of the structure of the LPS molecule indicating its various regions composition: O antigen, outer core, inner core, and lipid A. LAltNAcA: 2-acetamido-2-deoxy-L-altruronic acid; FucNAc4N: 2-acetamido-4-amino-2,4-dideoxy-D-fucose; Gal: galactose; Glc: glucose; GlcN: glucosamine; Hep: heptose; EtA: ethanolamine; Kdo: 3-deoxy-D-manno-2-octulosonic acid; P: phosphate. *Ss* $\Delta galU$ deep rough mutant has an incomplete core, which lacks the site for the attachment of O polysaccharides; *Ss* Δwbg and -pSS strains do not synthesize the repetitive unit of the OAg (Modified from [210]).

A large amount of GMMA from the different *S. sonnei* mutants was purified from bacterial culture supernatant and characterized in respect to protein content, by proteomic studies. In Figure 11, Panel A, the protein pattern of *S. sonnei* GMMA carrying a WT LPS (*Ss* $\Delta tolR$) or a mutated LPS (*Ss* $\Delta tolR / -pSS$; *Ss* $\Delta tolR / \Delta galU$), is shown in a Coomassie Blue stained-SDS-PAGE. Purified GMMA were also analyzed by transmission electron microscopy, revealing the presence of round particles with a diameter ranging from 50 nm to 80 nm (Figure 11, Panel B).

Figure 11

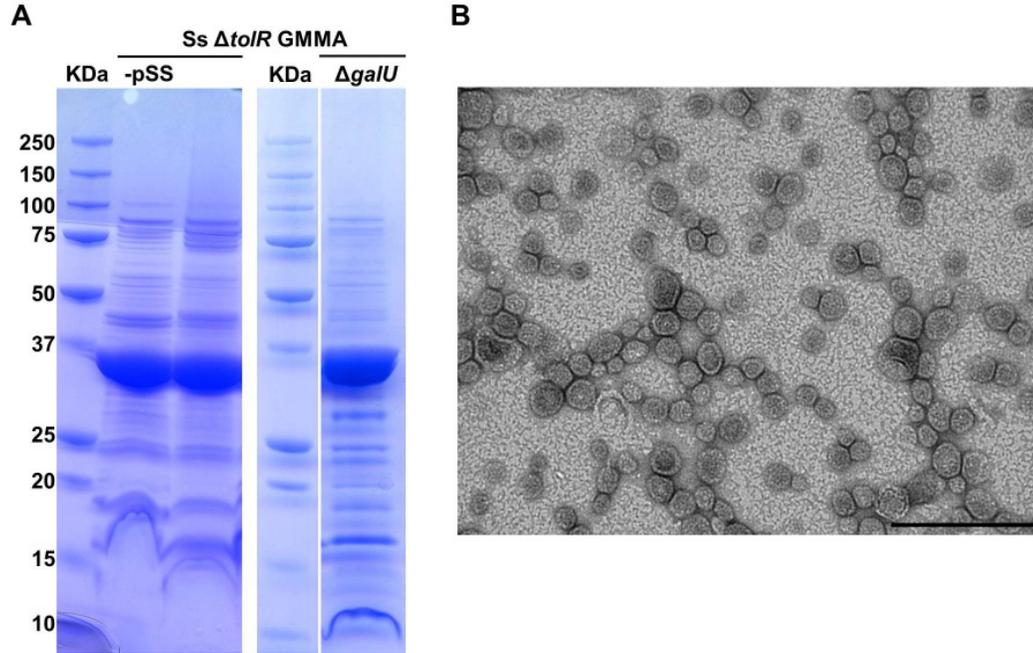


Figure 11: *S. sonnei* GMMA preparations. Panel A) 12 % Bis-Tris SDS-PAGE stained with Coomassie Blue; All Blue molecular weight standard. The gel is loaded with 10 μ g of proteins associated to *S. sonnei* $\Delta tolR$ GMMA with WT LPS, and GMMA from the LPS mutant strains, -pSS and $\Delta galU$. Panel B) Representative electron micrograph of *S. sonnei* $\Delta tolR$ GMMA. Bar length= 200 nm.

S. sonnei GMMA were used as antigens in different immunization studies in mice. Immune sera were firstly analyzed by ELISA to determine antibody titers, showing high levels of GMMA specific IgG [163]. In order to test the ability of the sera raised against GMMA to recognize surface epitopes on live bacteria and to cross react with different *Shigella* serotypes, we performed experiments of surface staining of live homologous (*S. sonnei* 53G) and heterologous (*S. flexneri* 2a) bacteria followed by flow cytometry (FACS) analysis. In particular, immune sera were tested both on *S. sonnei* and *S. flexneri* WT strains and on their OAg-negative isogenic mutants (*Ss* -pSS; *Sf2a* $\Delta rfbG$). In fact, we produced the *S. flexneri* 2a 2457T strain lacking the ability to synthesize the OAg residues, by inactivating three genes (*rfbF*, *rfbG*, *rbc*) (35) of the OAg synthetic gene cluster on the bacterial chromosome (strain abbreviated as *Sf2a* $\Delta rfbG$). The results of the FACS analyses are reported in Fig. 12, Panel A. We observed that sera against *S. sonnei* GMMA with OAg (α -*Ss* $\Delta tolR$ GMMA) stained the surface of *S. sonnei* but not of *S. flexneri* suggesting an O antigen-dependent serotype-specific response. The same sera reacted with O antigen-deficient *S. sonnei* and *S. flexneri* indicating that antibodies against conserved surface structures - likely proteins - were raised but that these proteins were not accessible in the WT strains, in the context of this *in vitro* assay. In accordance with this finding, sera raised against O antigen-deficient GMMA from *S. sonnei* (α -*Ss* $\Delta tolR$ -pSS GMMA) neither stained *S. sonnei* nor *S. flexneri* WT bacteria but only the surface of OAg-deficient strains. Surprisingly, sera against *S. sonnei* $\Delta galU$ GMMA, that were also thought to be O antigen-deficient, stained the homologous WT

strain in a serotype-specific manner. Two key observations and hypotheses result from these experiments.

Firstly, *S. sonnei* GMMA contain conserved and immunogenic proteins but these surface antigens are masked in live *Shigella* grown *in vitro* under the conditions tested so far. Similar results have been obtained with other Gram-negative bacteria in which exopolysaccharide structures (EPS), either capsule or LPS, have been shown to interfere with antibodies binding in *in vitro* experiments, probably by steric hindrance [215]. Therefore, the staining of the surface of Ss WT *in vitro* is likely possible only in the presence of OAg-specific antibodies. Our hypothesis is that the masking effect caused by exopolysaccharides is likely to be temporally and/or spatially regulated *in vivo*. This because surface proteins, e.g. iron uptake systems, must be accessible to allow survival of the bacteria in the host environment [216]. It has also been reported in literature that the accessibility of the T3SS varies on the basis of the oxygen gradient in the host's GI tract [79]. Moreover, in a *Shigella* challenge study, we observed that immune responses raised by GMMA lacking the OAg protected mice from infection with *S. flexneri* 2a, suggesting that surface epitopes other than OAg must be accessible during the infection. Regarding *S. sonnei*, the situation is still unclear. A recent paper showed that mice immunized with IpaB alone or combined with IpaD, two components of the tip of the T3SS, were fully protected against lethal pulmonary infection with *S. flexneri* and *S. sonnei* [125].

The second key observation of the experiment shown in Figure 12 is that GMMA from Ss $\Delta tolR/ \Delta galU$ raised a serotype specific immune response. This response could be a response to proteins of *S. sonnei* that are not present or not conserved in *S. flexneri* and avoid the masking effect described above. Possible candidates for a protein response could be again proteins at the tip of the T3SS that has been reported to extend into the extracellular space beyond the LPS layer, at least in *S. flexneri* [80]. Alternatively, GMMA from Ss $\Delta tolR/ \Delta galU$ raised an OAg response, suggesting that unlike in *S. flexneri*, the *galU* mutation in *S. sonnei* might not result in complete loss of O antigen.

We focused our attention on the unexpected positive signal by FACS analysis obtained when we stained *S. sonnei* WT with sera raised against Ss $\Delta tolR/ \Delta galU$ GMMA. In order to test that this positive signal was due to the presence of antibodies against OAg in the antisera, we performed a competitive FACS experiment. By using the water-phenol extraction method of Westphal and Jann [197], we purified LPS from Ss WT and, as control, from the Ss -pSS strain, lacking the OAg (See Figure 15, Panel A, Lanes b and d, respectively). We used the LPS extracts to pre-absorb Ss $\Delta tolR/ \Delta galU$ GMMA antisera before staining the Ss WT bacteria. As shown in Fig. 12, Panel B, we could observe a shift of the positive signal to the left part of the plot when we tested the serum absorbed with the WT LPS (central histogram, dashed line). This effect was not detected when we used the OAg-lacking LPS extract to absorb the sera (right histogram, dotted line). The pre-absorption with Ss WT LPS extract abolished the antibody binding to the surface of WT bacteria: these results indicate that antibodies against OAg-containing material are present in the sera raised against Ss $\Delta tolR/ \Delta galU$ GMMA. Our hypothesis is that Ss $\Delta galU$ is not completely OAg deficient but possess an immunogenic source of OAg, different from the LPS O side chain.

Figure 12

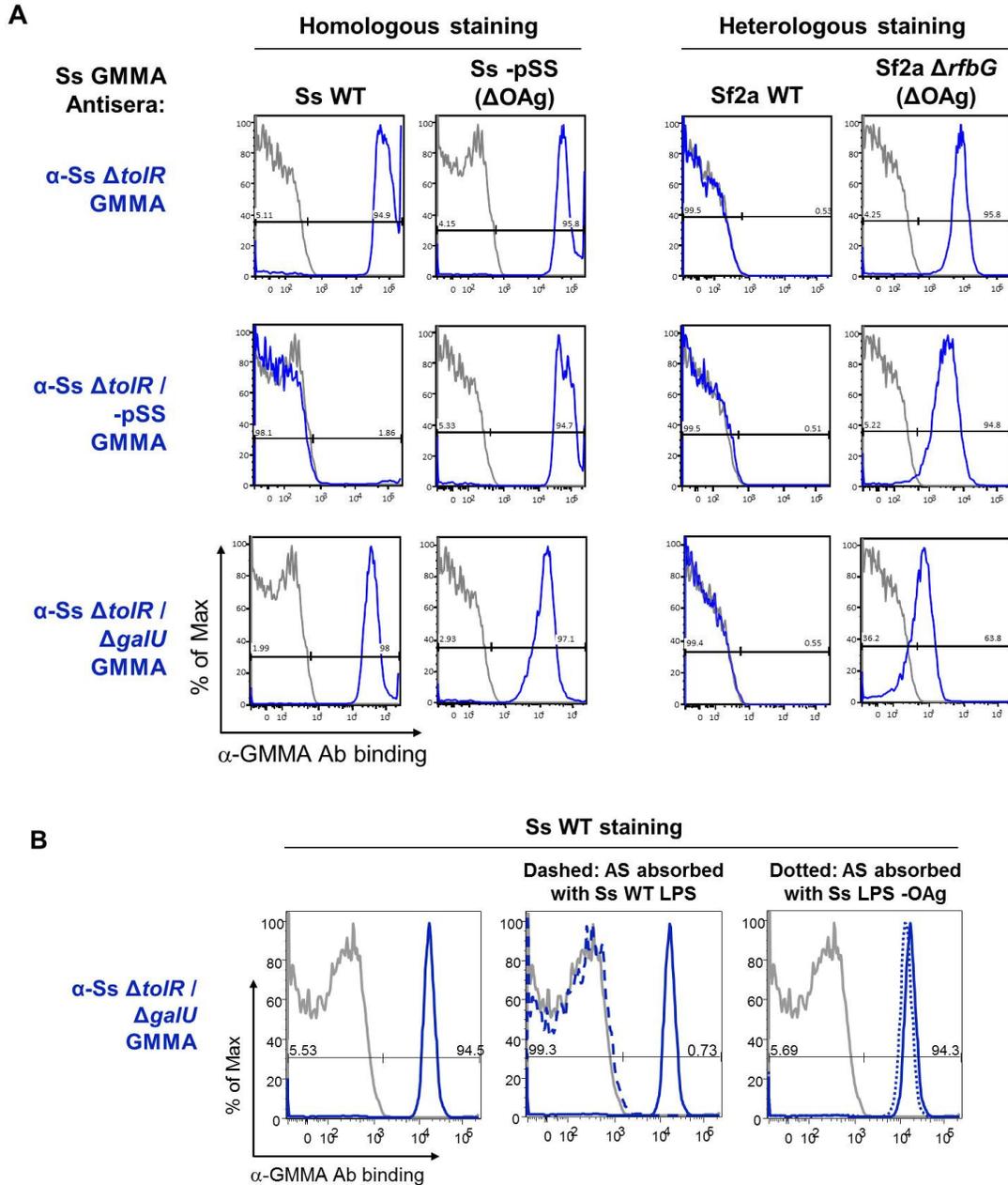


Figure 12: Live *Shigella* surface staining and flow cytometry analysis. Representative results of three experiments are shown. Panel A) Surface staining of Ss WT, Ss -pSS (Δ OAg), Sf2a WT, Sf2a Δ rfbG (Δ OAg) bacteria with immune sera raised against GMMA from Ss Δ tolR (+OAg), Ss Δ tolR/ -pSS (Δ OAg), Ss Δ tolR/ Δ galU strains. Gray profiles: staining with preimmune sera; Blue profiles: staining with GMMA antisera. Sera dilution is 1:1000. Panel B) Surface staining of Ss WT live bacteria with immune sera raised against GMMA from Ss Δ tolR/ Δ galU strain and competitive FACS analysis. Gray profiles: staining with preimmune sera; Blue, solid profiles: staining with Ss Δ galU GMMA antisera; Blue, dashed profile: staining with Ss Δ galU GMMA antisera absorbed with Ss WT LPS extract; Blue, dotted profile: staining with Ss Δ galU GMMA antisera absorbed with Ss -pSS LPS extract; Sera dilution is 1:10000. Bacteria were fixed after staining.

6.2 Immunogenicity of whole cell *S. sonnei* LPS mutants

The previous experiment unexpectedly showed that GMMA derived from the *Ss ΔtolR/ΔgalU* strain are able to raise an immune response to the OAg. We wanted to verify that not only GMMA but also the *S. sonnei ΔgalU* parent strain is able to induce a serotype-specific response by using bacteria instead of GMMA for immunizations. Therefore, we performed an immunogenicity study in which mice received three doses of whole inactivated bacteria (either heat killed, HK, or formalin fixed, FF). In particular, mice were immunized with *S. sonnei* wild type bacteria (positive control for OAg-specific responses), *S. sonnei ΔgalU* and the two *S. sonnei* rough mutants, -pSS and *Δwbg* (negative controls for OAg-specific responses). The rationale for using the two different OAg-negative bacteria was their difference in the presence of the virulence plasmid and thus the control if there is a contribution of virulence plasmid-encoded proteins to the immunogenic properties of the different *S. sonnei* mutants. In addition, one group received *Ss ΔgalU* GMMA from a new lot to confirm our previous results. We were also interested in analyzing if in other *Shigella* serotypes, specifically *S. flexneri 2a*, the LPS mutant strains are able to induce a serotype-specific response as *S. sonnei ΔgalU*. Therefore three groups received inactivated bacteria of *S. flexneri 2a* WT, *ΔgalU* and *ΔrfbG*, respectively. The following Table (Table 5) summarizes the groups of immunization.

Table 5: Immunogenicity of whole *Shigella*, groups of immunization

Mice immunization schema	
Group #	Antigen Name & dose
1	PBS 1X
2	Heat killed (HK) <i>S. sonnei ΔgalU</i> 1 * 10 ⁹
3	Formalin Fixed (FF) <i>S. sonnei ΔgalU</i> 1 * 10 ⁹
4	Formalin Fixed (FF) <i>S. sonnei</i> WT 1 * 10 ⁹
5	Formalin Fixed (FF) <i>S. sonnei Δwbg</i> 1 * 10 ⁹
6	Formalin fixed (FF) <i>S. sonnei</i> -pSS 1 * 10 ⁹
7	GMMA <i>Ss ΔtolR/ΔgalU</i>
8	Formalin fixed (FF) <i>S. flexneri 2a ΔgalU</i> 1 x 10 ⁹
9	Formalin Fixed (FF) <i>S. flexneri 2a</i> WT 1 x 10 ⁹
10	Formalin fixed (FF) <i>S. flexneri 2a ΔrfbG</i> 1 x 10 ⁹

Table 5: Six weeks-old CD1 mice (5 mice/ group) were immunized subcutaneously on days 0, 21 and 35 with three doses of 10⁹ inactivated bacteria or 10 μg of *Ss ΔtolR/ΔgalU* GMMA. Control group received PBS alone. Antisera were collected before the first immunization (preimmune sera) and on day 49 (post 3 sera).

Immune sera obtained from this immunization study were tested for their ability to stain the surface of *Shigella* WT by FACS analysis, as performed in the previous experiments. In Figure 13, Panel A, representative results of live *S. sonnei* WT staining with sera from mice immunized with formalin-fixed homologous WT and mutant bacteria are reported. As expected, sera raised against OAg-carrying bacteria (α -Ss WT) positively reacted with the surface of live *S. sonnei* WT (Panel A, first histogram). Sera raised against OAg-lacking bacteria (α -Ss Δwbg ; α -Ss -pSS) did not stain the surface of *S. sonnei* WT (Panel A, second and fourth histograms), even though they strongly reacted with *S. sonnei* bacteria lacking the OAg (Ss Δwbg , data not shown). Again, in our *in vitro* conditions, the presence of surface polysaccharides on the bacteria used in the FACS experiments appears to interfere with the binding of antibodies recognizing surface proteins. Thus, only OAg-specific antibodies can result in a positive labeling by FACS analysis. This observation is also true for antibodies raised against surface exposed proteins encoded by the *Shigella* virulence plasmid, since both pSS-positive (Δwbg) and pSS-negative (-pSS) OAg-lacking bacteria raised immune sera not able to react with the surface of *S. sonnei* WT. Immune sera raised against the new lot of Ss $\Delta galU$ GMMA confirmed our previous results and strongly reacted with Ss WT (data not shown). Importantly, sera raised against Ss $\Delta galU$ were also able to stain the surface of homologous WT bacteria (Panel A, third histogram), in accordance with the results obtained with Ss $\Delta tolR/ \Delta galU$ GMMA. Therefore, the results described in Figure 12, are not an artifact of GMMA immunization since similar results are obtained when we analyze sera from mice immunized with whole inactivated bacteria. Together these observations support our hypothesis that an OAg-containing material, different from the LPS O side chain, is expressed by *S. sonnei* $\Delta galU$. This material is immunogenic in mice and is incorporated into or attached to the surface during the process of vesicles formation.

In Figure 13, Panel B, representative results of live *S. flexneri* 2a WT staining with sera from mice immunized with formalin-fixed homologous WT and mutant bacteria are reported. As expected, sera raised against OAg-carrying bacteria (α -Sf2a WT) gave positive staining results (Panel B, first histogram), while sera against OAg-lacking bacteria (α -Sf2a $\Delta rfbG$) gave negative staining results (Panel B, second histogram). However, in contrast to the observation that sera raised against Ss $\Delta galU$ bacteria reacted with the homologous WT strain, antisera against Sf2a $\Delta galU$ bacteria were not able to stain the surface of the homologous *S. flexneri* 2a WT bacteria (Panel B, third histogram). This indicates that the *galU* mutation in the *S. flexneri* background results in a complete OAg knock out, as it was previously reported in literature for *S. flexneri* 2a and 5a [80,184,213].

Figure 13

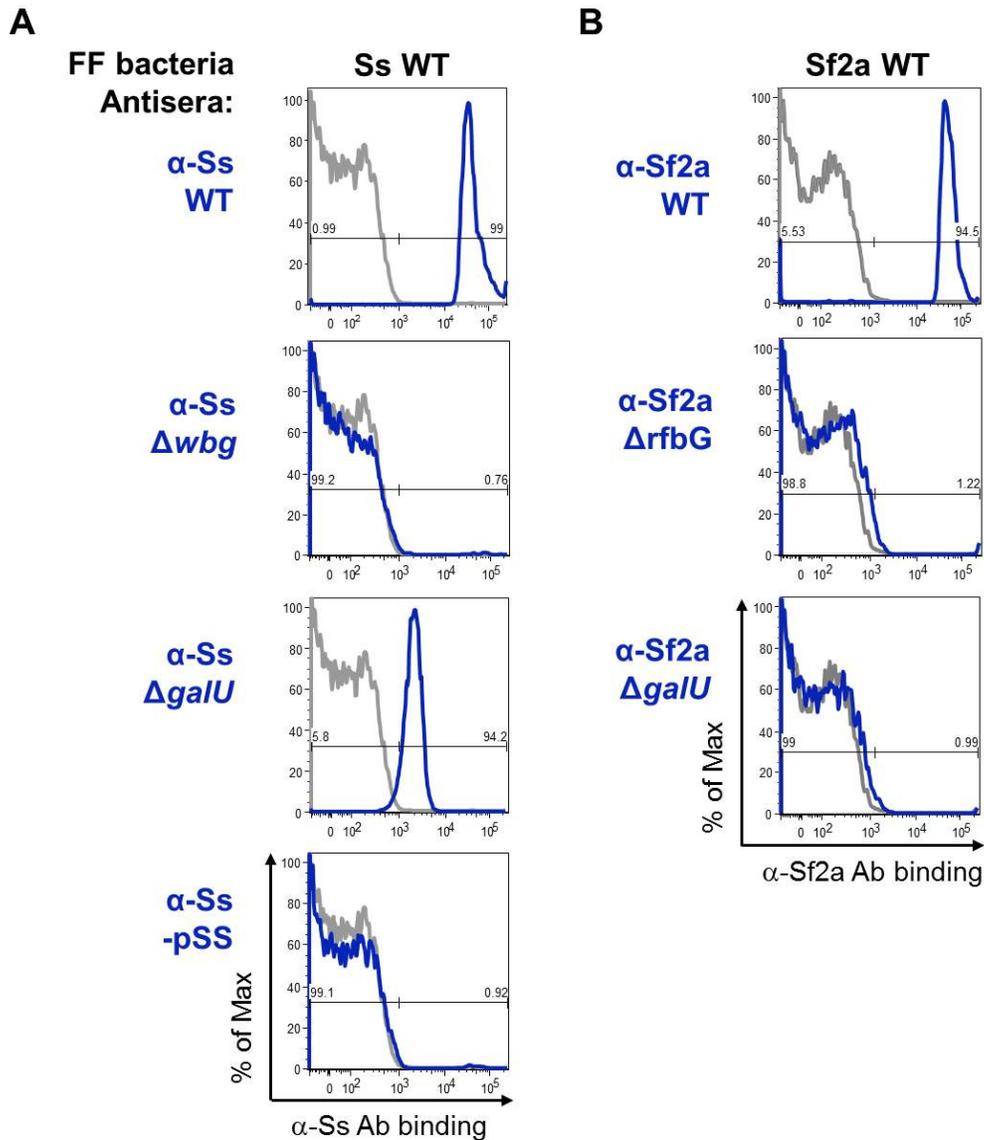


Figure 13: Live *Shigella* surface staining and flow cytometry analysis. Representative results of three experiments are shown. Panel A) Surface staining of Ss WT bacteria with immune sera raised against Ss WT (+OAg), Ss Δwbg (Δ OAg, +pSS), Ss $\Delta galU$, Ss -pSS (Δ OAg) FF-inactivated bacteria. Panel B) Surface staining of Sf2a WT live bacteria with immune sera raised against Sf2a WT (+OAg), Sf2a $\Delta rfbG$ (Δ OAg), Sf2a $\Delta galU$ FF-inactivated bacteria. Gray profiles: staining with preimmune sera; Blue profiles: staining with FF bacteria antisera. Sera dilution is 1:500. Bacteria were fixed after staining.

6.3 *S. sonnei galU* knock out is not a complete OAg-deficient strain

In order to evaluate the presence of OAg in the *S. sonnei galU* null mutant, *S. sonnei* lipopolysaccharides were characterized by different means.

As first step, we tested the reactivity of *S. sonnei* $\Delta galU$ with a commercial antiserum to *S. sonnei* O somatic antigen. The rabbit monovalent antiserum (Denka Seiken) is a typing antiserum for Ss phase I and has been reported to react specifically with the OAg. As the conventional nomenclature, the descriptor 'phase I' indicates a WT strain containing the virulence plasmid and expressing O antigen. As described below, we controlled that the phase I serum is OAg-specific, also in order to ensure that it does not cross-react with *Shigella* proteins. We tested the monovalent antiserum by immunoblots on bacterial lysate (boiled liquid cultures). As shown in Figure 14, it did not react with *S. sonnei* OAg deficient strains, neither the strain carrying the virulence plasmid (Ss Δwbg , Panel B, Lane c) nor the one lacking the plasmid (Ss -pSS, Panel B, Lane e), confirming that the monovalent antiserum does not react with proteins or with the LPS core. As expected, the antiserum strongly reacted with Ss WT lysate (Panel B, Lane b). It also reacted with Ss $\Delta galU$ lysate (Panel B, Lane d), where it recognized a HMW material with low mobility. Giving that the proteins present in Lanes b, c and d are theoretically equivalent and no signal is detected on *S. sonnei* OAg knock out strains lysates, these results show that the commercial antiserum against Ss phase I is OAg-specific. They also indicated that a high molecular weight material is present and is detected in the WT and the $\Delta galU$ mutant. This material is phase I-immune reactive, thus likely contains OAg.

Figure 14

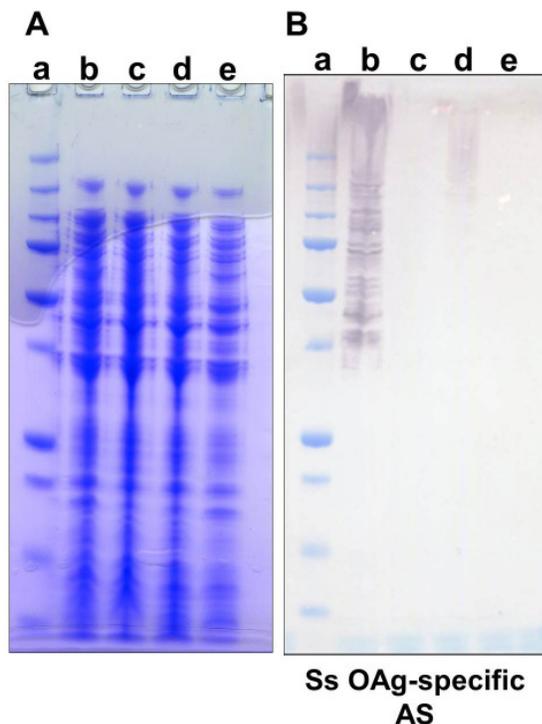


Figure 14: Immunoblot analysis of Ss bacterial lysates with Ss phase I monovalent antiserum (AS) (OAg specific AS). Panel A) 12 % Bis-Tris SDS-PAGE and Coomassie staining of bacterial lysates (5×10^7 bacteria/lane); Panel B) WB with Ss phase I monovalent antiserum (Dilution is 1:1000). Lane a= All Blue molecular weight standard; Lane b= Ss WT; Lane c= Ss Δwbg ; Lane d= Ss $\Delta galU$; Lane e= Ss -pSS.

To characterize the HMW OAg-containing material detected in *S. sonnei* WT and *galU* mutant strains, we then purified the total cellular lipopolysaccharide from *S. sonnei* WT and LPS mutant strains, optimizing a previously described method [197] based on water-phenol extraction followed by ethanol precipitation. In Figure 15, Panel A, the characterization of LPS extracts by silver-stained SDS-PAGE is reported. The LPS extract from Ss WT showed the typical O antigen ladder pattern with the predominant chain length of 20 to 25 repeating units (O units) [84], as detected by silver stain (Fig. 15, Panel A, Lane b). The stain also detected additional material of lower mobility, above the position of 25 O units. As expected, only a band corresponding to the core region was detected in the extract of the *S. sonnei* LPS rough mutant (Ss -pSS, Fig. 15, Panel A, Lane d). Interestingly, the extract from the Ss $\Delta galU$ strain showed slowly migrating material (Fig. 15, Panel A, Lane c). This material is likely not ligated to the core-lipid A portion since this mutant is not able to synthesize the entire core region, but only the inner core region [213]. The LPS extracts were extensively treated with Proteinase K and protein contamination should not be present in a significant concentration. Thus, the low mobility material likely corresponds to a high molecular weight polysaccharide.

LPS extracts were characterized by immunoblot analyses using the Ss phase I monovalent antiserum and the sera raised against *S. sonnei* GMMA that were previously used in FACS experiments (Figure 15, Panel B). As the OAg-specific antiserum detected HMW material in the total lysates of Ss WT and $\Delta galU$ bacteria, our aim was to test if the HMW material detected in the lipopolysaccharide extract from Ss $\Delta galU$ was also recognized by this serum. As expected, the monovalent AS strongly reacted with LPS from phase I Ss WT (Panel B, Lane b) but not with the extract from the phase II rough mutant (Ss -pSS, Panel B, Lane d). Interestingly, the antiserum also recognized the material extracted from Ss $\Delta galU$ (Panel B, Lane c), indicating that the slow migrating material detected by silver stain is phase I immune reactive. This observation is in accordance with our previous results that a HMW OAg-like material is expressed by *S. sonnei* $\Delta galU$ mutant. Subsequently, we performed the immunoblot analysis using the GMMA antisera. The aim was to test if the sera against Ss $\Delta tolR/\Delta galU$ GMMA were able to stain the LPS extract from Ss WT strain. The results are reported in Figure 15, Panel D: Ss $\Delta galU$ GMMA antiserum clearly reacted with the WT extract. This result correlates with the results of the FACS experiments shown above and indicates the presence of antibodies against the OAg in the Ss $\Delta galU$ GMMA immune sera. Interestingly, antisera against Ss $\Delta tolR$ GMMA with a WT LPS (+OAg) reacted with the slowly migrating material of the Ss $\Delta galU$ LPS extract (Fig. 15, Panel C). Considering again that the LPS extracts were extensively treated with Proteinase K, this reaction is an additional indication that OAg-reactive material can be extracted from Ss $\Delta galU$ bacteria.

Figure 15

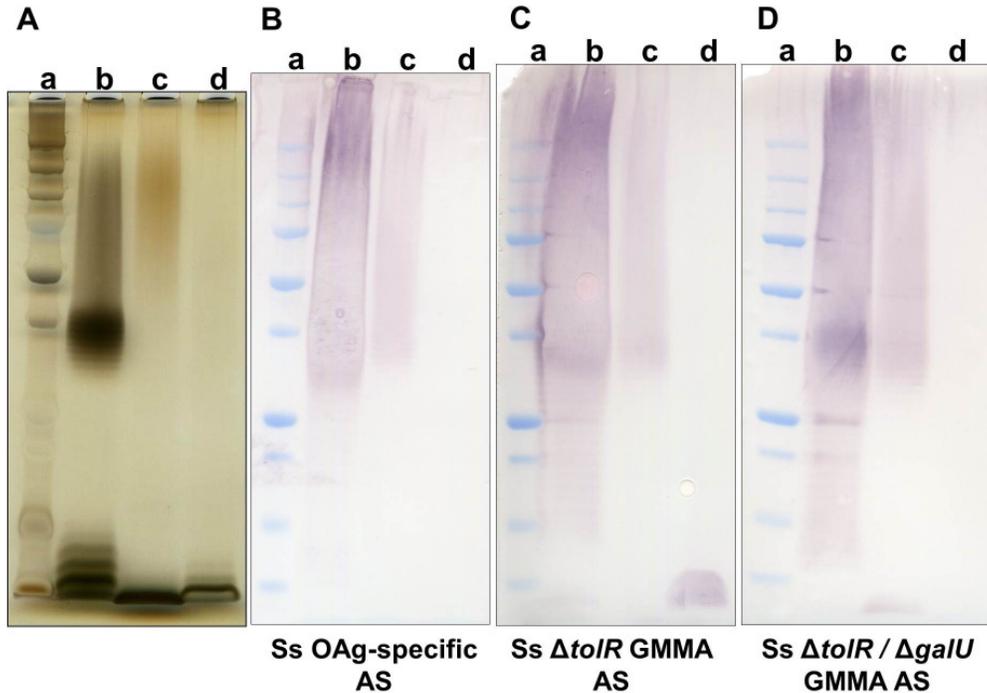


Figure 15: *S. sonnei* LPS extracts and immunoblot analyses. Panel A) 12 % Bis-Tris SDS-PAGE and silver staining; Panel B) WB with *Ss* phase I monovalent antiserum (AS); Panel C) WB with *Ss* $\Delta tolR$ GMMA (+OAg) AS; Panel D) WB with *Ss* $\Delta tolR / \Delta galU$ GMMA AS. Sera dilution is 1:1000. Lane a= All Blue molecular weight standard; Lane b= *Ss* WT LPS; Lane c= *Ss* $\Delta galU$ LPS; Lane d= *Ss* -pSS LPS.

The results described so far indicate that *S. sonnei* $\Delta galU$, although being an LPS deep rough mutant, still possesses OAg. The following experiment further supported this observation. We tested the ability of *Ss* OAg-specific antiserum to stain the surface of *S. sonnei* $\Delta galU$ strain in order to verify that the OAg material previously characterized in SDS-PAGE and Western blot analyses is exposed on the surface of the bacteria. The experiment was performed with formalin fixed bacteria and the results are shown in Fig. 16. As expected, the antiserum strongly reacted with *Ss* WT surface (left panel) while it did not stain the surface of the OAg-deficient strain *Ss* Δwbg (right panel). In the central panel the positive staining of *S. sonnei* $\Delta galU$ by the *Ss* OAg-specific antiserum is shown. This result confirms that *Ss* $\Delta galU$ mutant express an OAg material on the surface, likely not the LPS O side chain. We repeated the surface staining experiment with live bacteria and found that the staining of live *S. sonnei* $\Delta galU$ bacteria with the OAg-specific AS is variable and much less pronounced (data not shown). Not-fixed *S. sonnei* $\Delta galU$ bacteria possibly release the OAg material into the supernatant during the washing steps for FACS analysis, indicating that it may be not tightly attached to the outer membrane.

Figure 16

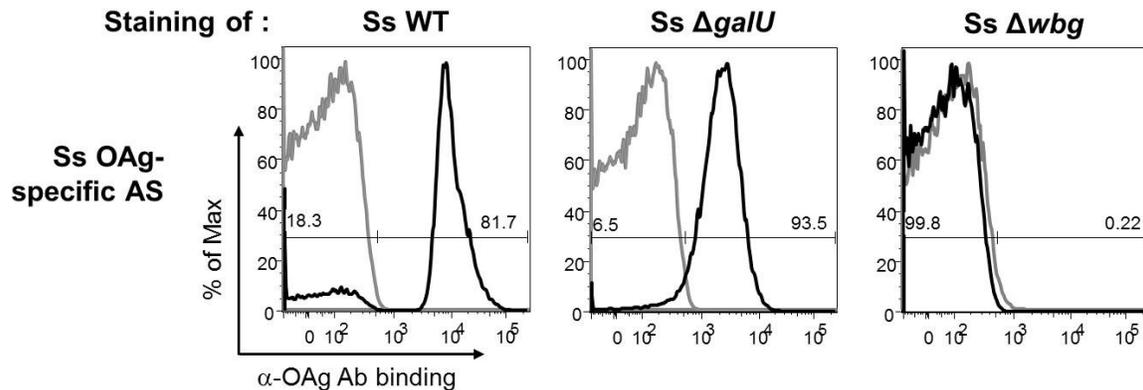


Figure 16: Surface staining of formalin fixed Ss WT, Ss $\Delta galU$ and Ss Δwbg with Ss phase I antiserum (AS) followed by flow cytometry analysis. Gray profiles: staining with uncorrelated antibody (Ab) (*S. flexneri* 2a monovalent AS); Black profiles: staining with Ss phase I monovalent antiserum. Sera dilution is 1:5000.

6.4 Generation of *S. sonnei* 53G group 4 capsule (G4C) mutant

As mentioned in the paragraph 6.1 of the present chapter, unlike the *S. sonnei* LPS rough mutants (Ss -pSS, Ss Δwbg), strains defective in the formation of the core region of LPS, e.g. the *galU* mutant, do not possess acceptor for the O polysaccharide. However, they still have the ability to synthesize the OAg repeating units, since they possess an intact OAg synthetic gene cluster. These O units can be assembled on the surface of the bacteria through an alternative pathway that does not involve the linkage of the OAg to the LPS. Recently, several Gram-negative bacteria, like enteropathogenic *E. coli* (EPEC) [185], *Salmonella enterica* serovar *Enteritidis* [217] and *Francisella tularensis* [218], have been shown to express a capsule that based on its mechanisms of synthesis and assembly has been classified as a fourth group of capsule types (according to the *E. coli* capsules classification) [219]. A group 4 capsule (G4C) is a high molecular weight (HMW) capsular polysaccharide composed of the same repeating units as the LPS O side chain, thus also called 'OAg capsule'. G4 capsules contain more O units than their cognate LPS and are linked to the cell surface by an unknown mechanism [219]. So far, *Shigella* has been reported to be uncapsulated. However genes homologous to those identified in EPEC for the group 4 capsule are present in different *Shigella* strains [185]. The results described above so far indicate that *S. sonnei* $\Delta galU$ possesses a HMW OAg material on the surface, likely not linked to the lipid A-core molecules.

Our hypothesis is that *S. sonnei* produces a group 4 capsule and that this capsule is still present in the *galU* mutant. *S. sonnei* 53G possesses an intact group 4 capsule gene cluster (*ymcDCBA*, *yccZ*, *etp* (*yccY*), *etk* (*yccC*)) [185]. The G4C cluster, also called 22-minute locus [219], is reported to encode for proteins that constitute a polysaccharide secretion and assembly system. Homologs of two proteins of the system, Etk (YccC) and Etp (YccY), are frequently associated with capsule expression [220–222]. In EPEC, all of the seven genes of the G4C operon have been shown to be

required for capsule production [185]. In order to test if the same cluster is also involved in capsule formation in *Shigella*, we inactivated the whole operon in the *S. sonnei* WT, producing the Ss Δ cps mutant. The different steps of mutant generation are summarized in Figure 17. To delete the G4C cluster, upstream and downstream regions of the operon and the erythromycin cassette were amplified by PCR, producing 3 fragments of 495 bp, 526 bp and 1150 bp, respectively, flanked by specific restriction sites (Panel A). The PCR products of the operon flanking regions were inserted into the pBluescript vector and electroporated into *E. coli* sub-cloning cells. The plasmid was purified and the erythromycin cassette was inserted into the EcoRV site between the two flanking regions. The resulting plasmid p Δ cpsKO::*erm* (Panel E) was purified and controlled by enzymatic digestions (Panel C), obtaining fragments of the expected size. The KpnI linearized p Δ cpsKO::*erm* was used as template to amplify the Δ cpsKO::*erm* fragment (2171 bp, Panel B), containing the erythromycin cassette plus the flanking regions. The purified product was electroporated into *S. sonnei* competent cells expressing the lambda red recombination system on pAJD434. A mutant colony, selected on erythromycin, was controlled by PCR using different combinations of primers, according to the scheme described in Panel F. All the combinations produced PCR fragments of the expected size (as negative control we used a colony of Ss WT, Panel D), confirming the integration of the Δ cpsKO::*erm* fragment in the correct locus and the deletion of the G4C cluster in the Ss Δ cps mutant.

Figure 17

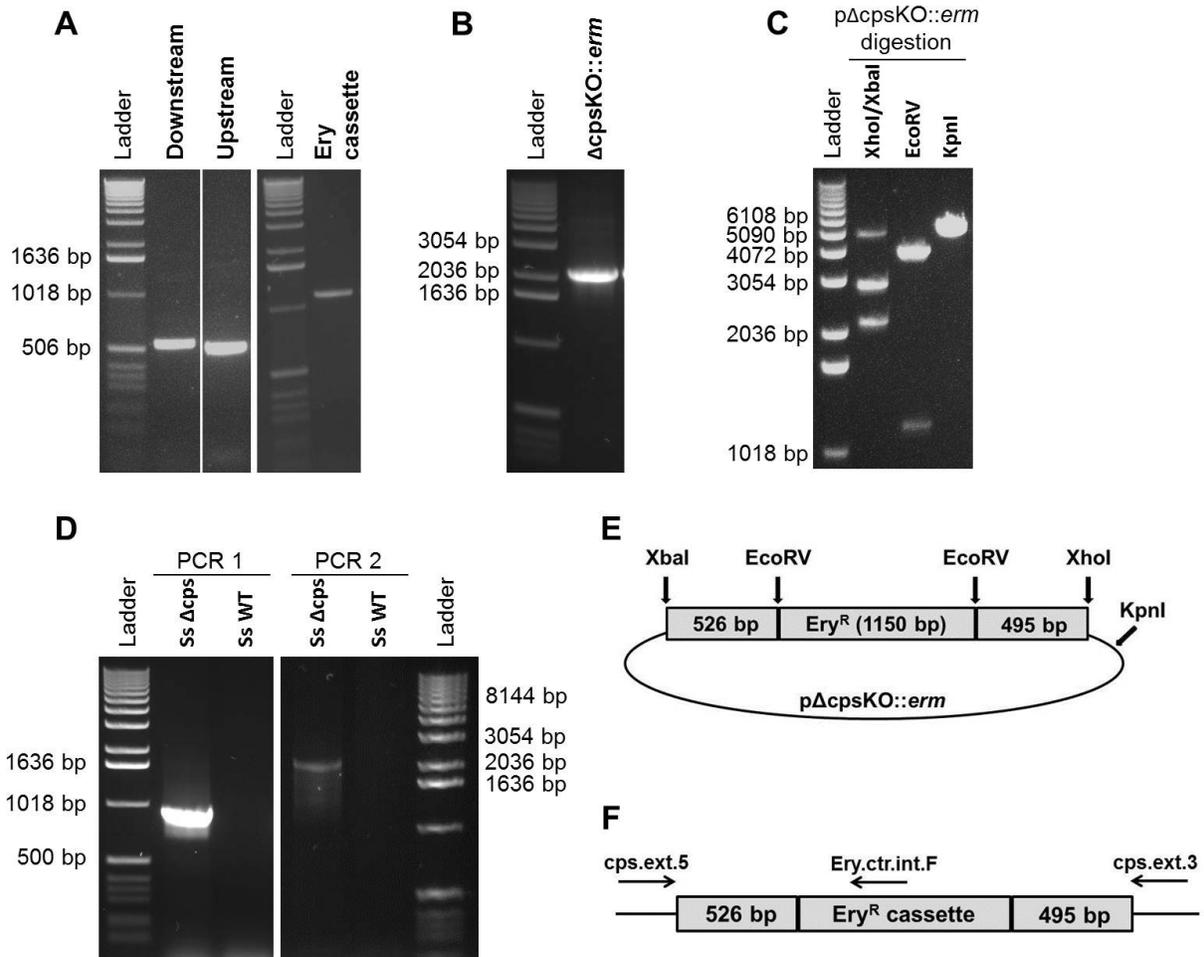


Figure 17: Generation of the *S. sonnei* group 4 capsule gene cluster deletion mutant. A) PCR products of G4C cluster flanking regions (upstream region= 495 bp, downstream region= 526 bp) and erythromycin (ery) cassette (1150 bp). B) PCR amplification of Δ cpsKO::erm construct (F) (expected size ~2171 bp) from p Δ cpsKO::erm (E). C) Enzymatic digestion of p Δ cpsKO::erm. D) Control PCR products of the Ss Δ cps mutant in comparison with a WT colony. PCR1= control primer internal to the erm gene (Ery.ctr.int.F) combined with primer annealing in the external region of the integrated Δ cpsKO::erm construct (cps.ext.5) (expected size for the Ss Δ cps mutant is ~964 bp, for the wild type strain no product is expected); PCR2= primers external to the integrated Δ cpsKO::erm construct (cps.ext.5, cps.ext.3) (expected size for the Ss Δ cps mutant is ~2214 bp, for the wild type strain a fragment of ~8797 bp is expected, however due to the large size, it is unlikely to obtain a product in the conditions used for the PCR reaction). E) Schematic representation of p Δ cpsKO::erm with the restriction sites used for the cloning procedure. F) Schematic representation of Δ cpsKO::erm construct with the primers used to control the *S. sonnei* Δ cps colony.

In addition, we produced a hyper-blebbing *S. sonnei* Δ tolR strain in which the presence of the virulence plasmid, pSS, and therefore the ability to synthesize the OAg units can be selected. We inserted a chloramphenicol resistance cassette in the virulence plasmid locus encoding for the VirG protein [223]. As a result, by growing the

Ss $\Delta tolR/ \Delta virG$ strain on selective medium, we can avoid the spontaneous loss of the pSS that naturally occurs during *S. sonnei* manipulation *in vitro* [193]. In this background we performed the G4C gene cluster knock out, generating the *S. sonnei* $\Delta tolR/ \Delta virG/ \Delta cps$.

If the gene cluster is essential for G4C synthesis, we expect that Ss Δcps still possess OAg as LPS side chain but not as capsular polysaccharide. We previously observed that GMMA from Ss $\Delta tolR/ \Delta galU$ induced an OAg-specific immune response. In the hypothesis that *S. sonnei* possesses a G4C, this would mean that GMMA incorporate the capsular PS expressed by the parent strain during their formation. Again, if the G4C operon is functional in *S. sonnei*, we expect that GMMA produced from the *S. sonnei* $\Delta tolR/ \Delta virG/ \Delta cps$ possess the LPS O side chain but not the OAg capsule. Exopolysaccharides obtained from the different *S. sonnei* mutants were biochemically characterized, and results are reported in the following paragraph.

6.5 Outer membrane polysaccharide analysis: the HMW capsular PS

As mentioned before, GMMA reflect the outer membrane composition of Gram-negative bacteria and are enriched in envelope components as outer membrane proteins and also LPS and exopolysaccharides, while they contain only irrelevant impurities of DNA and cytoplasmic components [140]. Therefore, these outer membrane particles released from intact cells represent an ideal tool to study Gram-negative periplasm and outer membrane compartments [162]. Based on these considerations, we used GMMA as outer membrane enriched-preparations to extract LPS from $\Delta tolR$ mutants of *S. sonnei* with a WT LPS (either the strain with a WT pSS or the $\Delta virG$ strain), from the LPS mutants $\Delta galU$ and Δwbg and from the $\Delta virG/ \Delta cps$ strain. Giving the fact that the mutation in the *virG* gene only has the function to avoid the loss of the pSS in *in vitro* grown *S. sonnei* without affecting the quality of the LPS, the *virG* null mutation is not mentioned in the following paragraphs for simplicity. LPS extracts from the outer membrane particles were then used in further biochemical analyses. In Figure 18, the experimental design is schematically described. Ss WT, ΔOAg (either the Δwbg or the -pSS strain), $\Delta galU$ and Δcps strains are represented on the basis of the expected phenotypes: wild type Ss can form both LPS O side chains (thick black broken line) and O antigen capsule (gray zone around the bacteria). Mutants deficient in repeat unit synthesis or polymerization (*wbg* mutant), are expected to lack both LPS O side chains and O capsule (the thin dotted line represents the smooth outer membrane). G4C gene cluster mutants are expected to be deficient in O-capsule formation but to possess LPS O side chain (represented by the thick black broken line). In the *E. coli* background, it was shown that G4C mutants possess an increased amount of LPS OAg, probably due to the fact that the complex that connects the O side chain to the LPS is no longer competing with the capsule forming system for the same substrates, the O-repetitive units [185,224]. By transferring the LPS/capsule null mutations in the $\Delta tolR$ background, highly concentrated GMMA preparations are obtained from these strains. Lipopolysaccharides are separated in the water phase during the water-phenol extraction and analyzed by SDS-PAGE followed by silver staining and immunoblot, as described before. In order to better characterize the polysaccharides, LPS extracts are then treated by mild acid hydrolysis to remove the lipid A of the LPS [116]. The resulting

samples, containing the exopolysaccharides specifically expressed by each strain, are significantly devoid of impurities such as proteins or nucleic acids, and represent suitable preparations for further biochemical analyses, such as HPLC-Size Exclusion Chromatography (HPLC-SEC) and NMR.

Figure 18

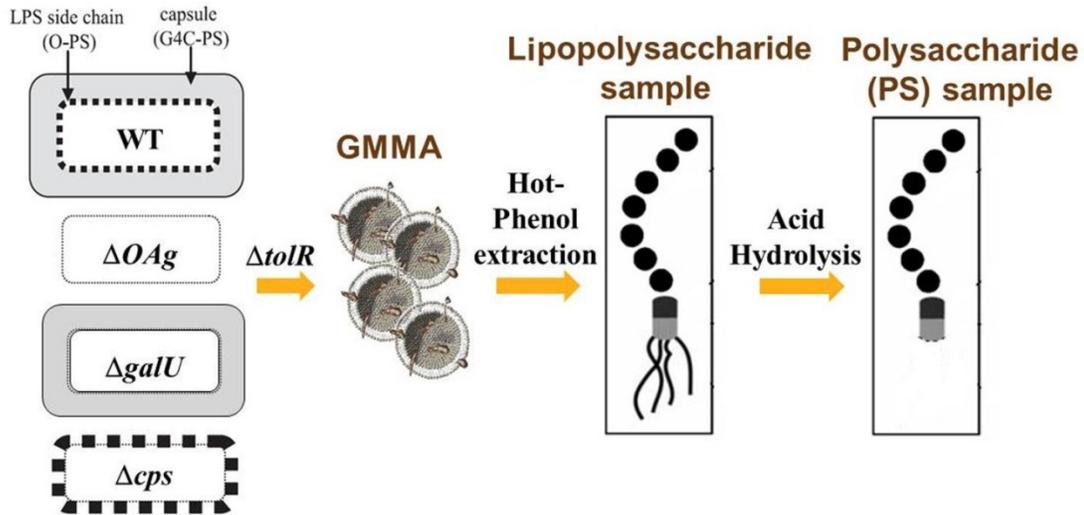


Figure 18: Outer membrane LPS and polysaccharides purification strategy. From the left, schematic representation of the expected phenotype of *Ss* WT and LPS/capsule mutants. LPS/capsule mutations are transferred in the $\Delta toIR$ background to produce highly concentrated GMMA. GMMA are used as outer membrane preparations to extract the lipopolysaccharide samples. Finally, mild acid hydrolysis of LPS leads to pure polysaccharides, characterized by negligible amount of proteins and DNA contamination (Modified from [210,224]).

In Figure 19, the results of the surface polysaccharides (PS) analyses are reported. SDS-PAGE loading material was normalized to GMMA-associated protein concentration of the starting material for the extraction. LPS extracted from *S. sonnei* GMMA showed a similar pattern after silver stain as the LPS from bacterial liquid cultures (Fig. 19, Panel B, compared to Figure 15, Panel A). In particular, the typical LPS ladder is revealed in the *Ss* $\Delta toIR$ GMMA (WT LPS) extract, while only the band corresponding to the entire core or the inner core region is present respectively in the $\Delta toIR/ \Delta wbg$ (LPS rough mutant) and in the $\Delta toIR/ \Delta galU$ (LPS deep rough mutant) GMMA extracts. In this experiment, we did not observe a significant presence of slow migrating material in the $\Delta galU$ extract but we confirmed the presence of HMW phase I immune reactive material by performing immunoblot analyses with the *Ss* OAg-specific monovalent antiserum (data not show). We purified the LPS from *Ss* Δcps bacteria and GMMA and we compared it with the WT LPS pattern. The material extracted from the two different sources looked very similar for the corresponding strains (data not shown). In Figure 19, Panel D, the silver-stained SDS-PAGE analysis of the GMMA-derived LPS is reported. The LPS pattern of *Ss* $\Delta toIR/ \Delta cps$ GMMA showed only slight modifications as compared to the WT pattern, with bands corresponding to low molecular weight (LMW)

LPS species being more intense and with a little shift of the starting site of the smear corresponding to the low mobility material above the 25 O units band. In order to better characterize the polysaccharide distribution in terms of molecular weight, PS extracts were then treated by mild acid hydrolysis in 1 % acetic acid. This reaction was previously characterized in the literature to detach the lipid A from the core-OAg portions of the LPS, without affecting the polysaccharide moiety integrity [116]. After hydrolysis neutralization, we collected the polysaccharide samples, that were analyzed by HPLC-SEC chromatography coupled with a differential refractive index (dRI) detector. Profiles at 214 nm and in the fluorescent emission were also acquired to monitor respectively eventual DNA and protein contaminations, indicating that impurities amount was negligible (data not shown). Results in the dRI are reported in Figure 19, Panels A and C:

- the chromatogram of the *Ss* Δwbg PS (Panel A, dotted line) had a single peak of a low molecular weight population and our hypothesis is that this signal corresponds to the core sugars of the LPS, as this mutant can not synthesize OAg sugars;
- the chromatogram of the *Ss* WT PS (Panel A, solid line) showed the presence of three different populations: the population in the LMW range of the graph (retention time of 17.8 min) partially overlaps with the one present in the *Ss* Δwbg PS and could correspond to the core sugars linked to few repeating units of the OAg. The major peak in the medium molecular weight (MMW) range (retention time of 15.1 min) could correspond to the predominant OAg population of 20-25 repeating units, as reported in literature [84]. The peak with the lowest elution time of 12.3 min corresponds to a HMW-PS population, likely composed of OAg sugars as well, since both the MMW and the HMW populations are absent in the PS from the OAg-deficient strain;
- two peaks were revealed in the chromatogram of the *Ss* $\Delta galU$ PS (Panel A, dashed line): one had a higher retention time than the signal obtained from the *Ss* Δwbg PS and we hypothesize that it could match with the shorter core region of the LPS (inner core) that the *Ss* $\Delta galU$ possesses. Interestingly, the other peak had a similar retention time as the HMW population of the *Ss* WT PS. Therefore this peak could correspond to the HMW capsule polysaccharide.

However, one of the most decisive proofs of the presence of a G4C in *Shigella sonnei* was obtained from HPLC-SEC analysis of the hydrolyzed EPS from *Ss* Δcps GMMA (Panel C, dashed-dotted line).

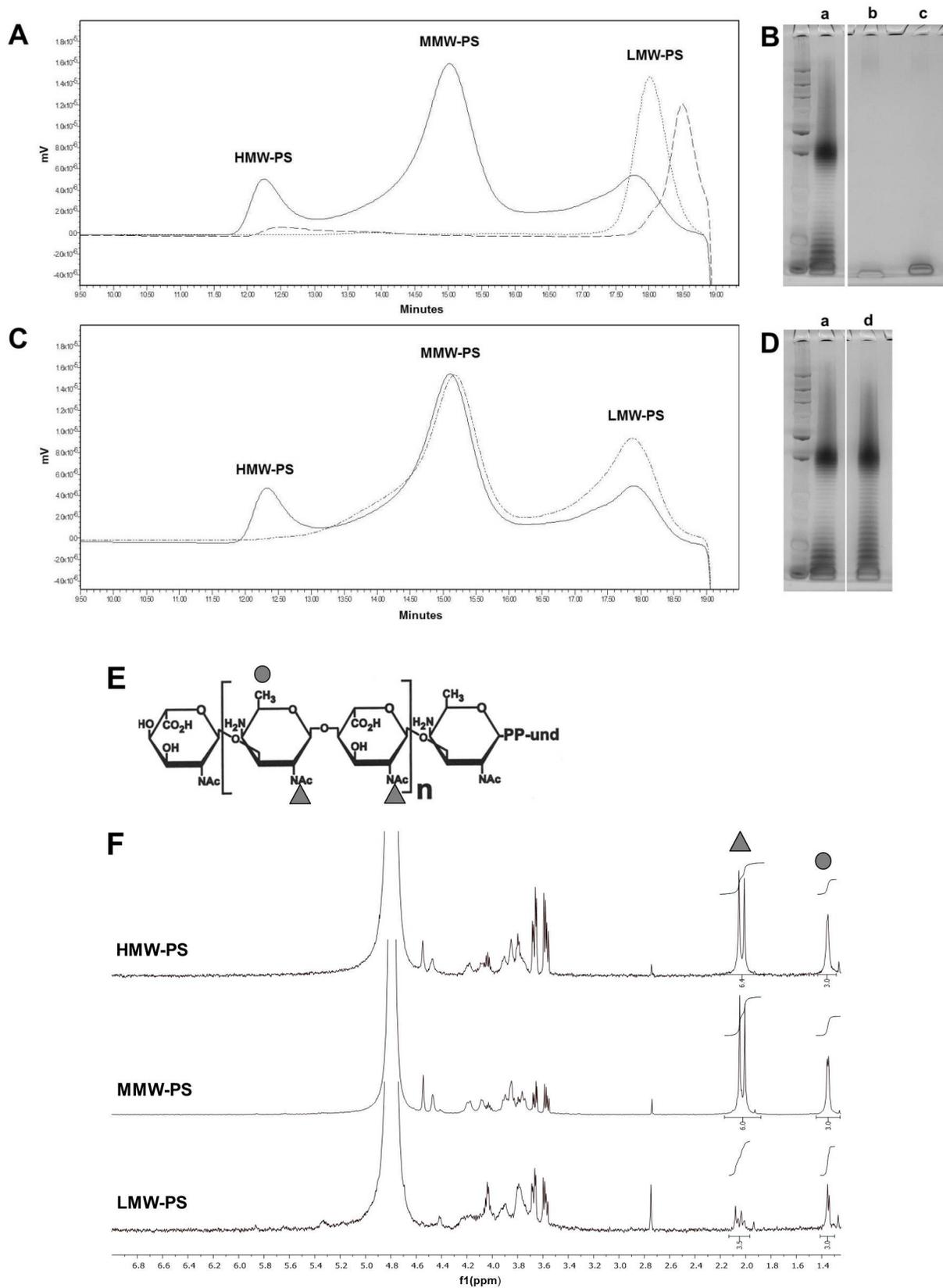
- While the chromatogram of the WT PS (solid line) revealed the presence of three major populations, the chromatogram of the Δcps PS (dashed-dotted line) revealed only the MMW and LMW populations, eluting at the same time as the corresponding populations of the WT sample. More importantly, the peak corresponding to the HMW-PS population was completely absent in the chromatogram of the *Ss* Δcps PS. These results clearly support the hypothesis that *S. sonnei* express a capsule, a HMW polysaccharide that is no more present in the null mutant for the G4C secretion and assembly system. As the *Ss* Δcps mutant can still synthesize the OAg side chain of the LPS, the major peak in the MMW range is likely raised by the most representative population of the OAg of the LPS linked to the core, while the LMW population represents a shorter polysaccharide composed of few OAg repeating units and core sugars. The LMW-PS is more pronounced in the Δcps PS in terms of

relative amount: as it was shown in literature for EPEC G4C mutants, in the absence of the capsular PS the amount of OAg on the LPS seems to increase [185].

If the HMW-PS population is the G4 capsule, it should comprise OAg residues. Following sugar composition analyses further confirmed our hypothesis. WT and $\Delta galU$ exopolysaccharide extracts were fractionated by HPLC-SEC and eluted samples were collected to isolate the different PS populations (HMW-PS= from 11.5 min to 13 min; MMW-PS= from 14 min to 16 min; LMW-PS= from 17 min to 18 min). 1H -NMR analysis was performed on isolated fractions in order to understand their composition in terms of OAg residues. The structure of the *S. sonnei* OAg repeating units is reported in Figure 19, Panel E, and the composition of the Ss LPS core is reported in Figure 10 [84,116]. The spectra of the high, medium and low molecular weight fractions composing the WT PS contain the typical signals belonging to protons of the 2-Acetamido-2-deoxy-L-fucose (FucNAc4N) and the 2-Acetamido-2-deoxy-L-altruronic acid (L-AltNAcA) residues of the *Shigella sonnei* OAg (Figure 19, Panel F) [116,208]. In particular, signals at 1.34-1.36 ppm belong to protons of the methyl group of the FucNAc4N (3 protons – round symbol), while signals at 2.00-2.04 ppm belong to protons of the N-acetyl groups (2 groups with 3 protons each – triangular symbol) of the FucNAc4N and the L-AltNAcA. Integration of these signals in the spectra of the HMW-PS and the MMW-PS fractions confirmed the expected ratio of 6:3 in terms of number of protons per signal. The spectrum of the LMW-PS was not optimally resolved, however it likely comprises few OAg units and LMW degradation products. By NMR we were able to demonstrate that the three PS populations of the Ss WT extract are made up of OAg residues. Similar results were obtained when we analyzed the HMW-PS of the $\Delta galU$ extract (data not shown). Importantly the HMW-PS that we think represents the group 4 capsule polysaccharide, comprises OAg residues.

For the most concentrated fraction corresponding to the MMW-PS we could quantify the number of OAg repeating units per core unit in an analysis of 1H -NMR with an increased acquisition time (Figure 19, Panel G). Anomeric signals of outer core α -Gal in the terminal position (5.82 ppm) and α -Gal in the internal position (5.62 ppm) were detected. The ratio between the signals of the FucNAc4N and the L-AltNAcA N-acetyl groups (2.00-2.04 ppm) on the signals of the outer core α -Gal residues (5.82-5.62 ppm) gives an estimation of the number of the OAg repeating units attached to the core. We determined that the MMW-PS fraction possesses about 23 OAg RUs. This result is in line with what reported in literature for the predominant OAg species of the *S. sonnei* LPS side chain [84].

Figure 19



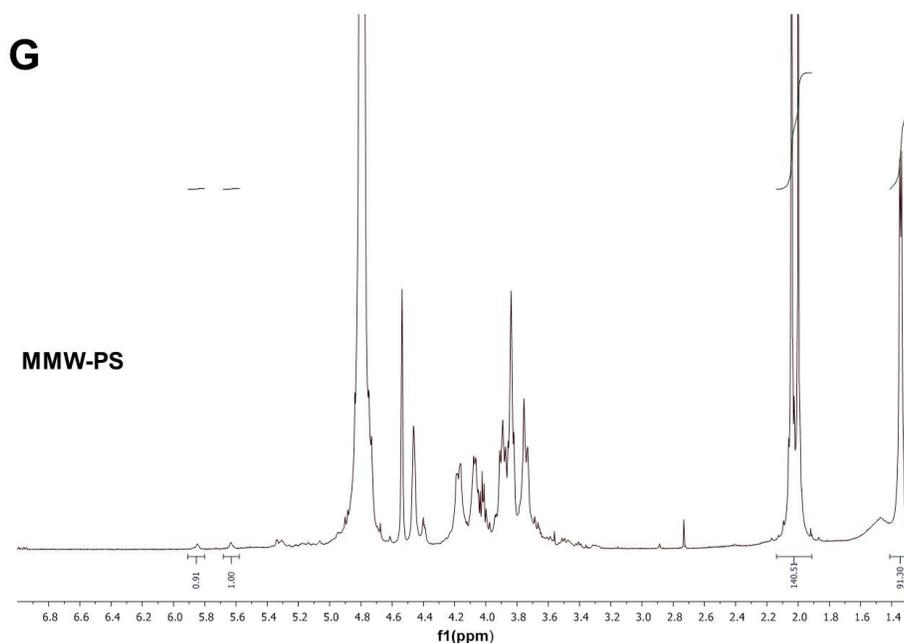


Figure 19: *S. sonnei* surface lipopolysaccharide and exopolysaccharide (EPS) analyses. Panels A) and C) HPLC-SEC analysis of outer membrane preparations of *S. sonnei* EPS after mild acid hydrolysis; EPS from GMMA of *Ss ΔtolR/ ΔvirG* (WT EPS) (solid line), *Ss ΔtolR/ ΔgalU* (dashed line), *Ss ΔtolR/ Δwbg* (dotted line), *Ss ΔtolR/ ΔvirG/ Δcps* (dashed-dotted line). Polysaccharide samples run on TSK gel G3000 PWXL-CP column and peaks are detected by differential refractive index (dRI). Panels B) and D) 12 % Bis-Tris SDS-PAGE and silver staining of LPS extracts from GMMA of *Ss ΔtolR/ ΔvirG* (WT LPS, lane a), *Ss ΔtolR/ ΔgalU* (Lane b), *Ss ΔtolR/ Δwbg* (Lane c), *Ss ΔtolR/ ΔvirG/ Δcps* (Lane d). Panel E) *S. sonnei* OAg repeating unit; round and triangular symbols highlight the groups detected by NMR analyses. Panel F) ¹H-NMR spectra of *S. sonnei* WT exopolysaccharide fractions; integrals of signals of the N-acetyl groups of the FucNAc4N and the L-AltNAcA OAg residues at 2.00 and 2.04 ppm (triangular symbol) and of the FucNAc4N methyl group at 1.34–1.36 ppm (circular symbol) are reported (symbols refer to residues in Panel E); Panel G) Determination of the number of OAg RUs attached to the core: ¹H-NMR spectrum of *S. sonnei* WT MMW fraction; integrals of the signals of the N-acetyl groups of the FucNAc4N and the L-AltNAcA OAg residues at 2.00 and 2.04 ppm, of the FucNAc4N methyl group at 1.34–1.36 ppm and of the core α-Gal residues at 5.82 and 5.62 ppm are reported.

It has been mentioned before that OAg repeating units that are part of the capsule are assembled and linked to the surface of bacteria independently of the LPS [219]. Therefore a pure group 4 capsule polysaccharide should not comprise sugars of the core region of the LPS. 3-deoxy-D-manno-octulosonic acid (Kdo) is one of the sugar residues in the inner core region of the LPS of *Shigella* and represents the reducing end of the LPS polysaccharide side chain upon lipid A removal by acid hydrolysis [207,225]. We optimized a Kdo quantification method [199] to be suitable for HPLC-SEC analysis and we estimated the presence of Kdo moiety in the three PS fractions isolated from the *S. sonnei* WT EPS. After the derivatization with a semicarbazide reagent, polysaccharides containing Kdo absorb at 252 nm (Figure 20, square box). We demonstrated that the MMW-PS (Fig. 20, dashed line) and the LMW-PS (Fig. 20, dotted line) gave a signal at 252 nm, indicating that they comprise Kdo residues, hence they likely derive from the LPS side chain. Conversely, the HMW-PS (Fig. 20, solid line) did not absorb at 252 nm, so it doesn't seem to contain Kdo moieties. It is important to

notice that the results of this assay are concentration-dependent and further analyses should be performed in order to confirm this observation and to exclude an underestimation of the Kdo content in the HMW-PS. However, the current results from the semicarbazide assay, together with the NMR results are in accordance with our hypothesis that the HMW population of the Ss WT polysaccharide is the G4C polysaccharide.

Figure 20

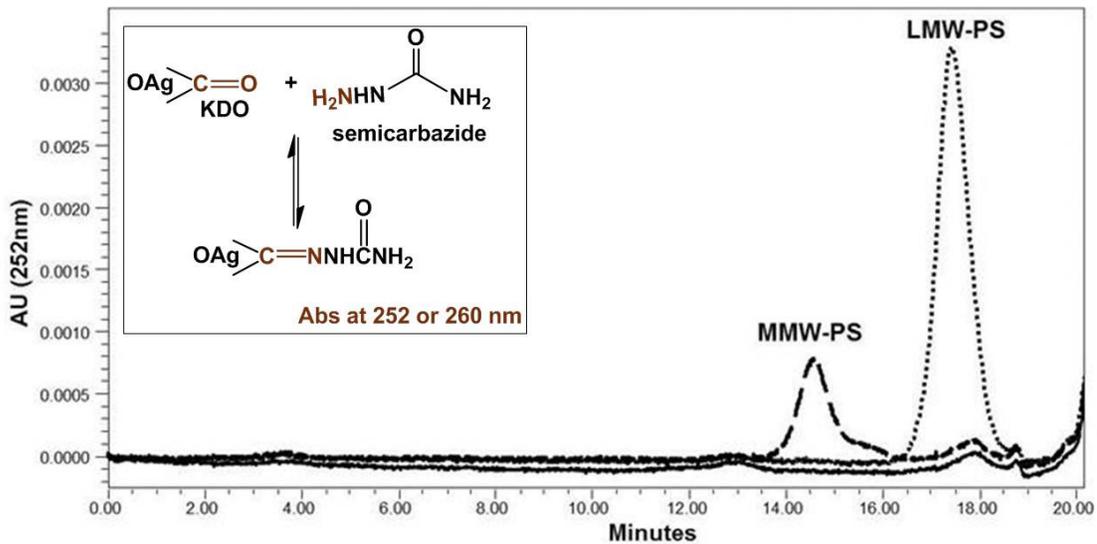


Figure 20: Estimation of Kdo moiety in the *S. sonnei* WT polysaccharide populations. Chromatograms of PS fractions derivatized with semicarbazide (reaction is described in the square box) and analyzed by HPLC-SEC on TSK gel G3000 PWXL-CP column (peaks are detected at 252 nm); solid line: HMW-PS; dashed line: MMW-PS; dotted line: LMW-PS.

6.6 Electron Microscopy analysis of *S. sonnei* LPS and capsule mutants

All together results described so far indicate that *S. sonnei* possess a HMW-capsule polysaccharide, comprising the same residues as the LPS O side chain and therefore classified in the fourth group of the OAg capsules. The formation of the G4C in *S. sonnei* does not depend on the LPS assembly, since the $\Delta galU$ LPS deep rough mutant still possess the capsule polysaccharide. The protein complex encoded by the 22-minute locus [219] is responsible for the assembly of the G4C in *S. sonnei*, as it was reported for pathogenic *E. coli* [185]. To further support these notions, we examined the LPS and capsule mutants by transmission electron microscopy (TEM) and compared them with the WT strain. Representative electron micrographs are reported in Figure 21. A thick dark layer of electron-dense material was seen at the surface of wild type *S. sonnei* bacteria (left, upper panel). This layer likely corresponds to extracellular polysaccharides

(LPS O side chain and OAg capsule) as it was completely absent in the OAg-deficient strain *Ss* Δwbg (left, lower panel). *S. sonnei* Δcps mutant possessed just a narrow dark layer (right, lower panel), likely corresponding to the LPS O side chain. On the contrary, *Ss* $\Delta galU$ had a very thick layer (right, upper panel), likely corresponding to the capsular polysaccharide. This material protruded from the outer membrane in a way that suggests that the EPS in *Ss* $\Delta galU$ is not tightly attached to the bacterial surface. This would be in agreement with our previous observation according which the surface staining of live *S. sonnei* $\Delta galU$ bacteria with the OAg-specific antiserum is variable and much less pronounced of the staining of formalin-fixed bacteria, as *S. sonnei* $\Delta galU$ tends to easily release the capsular PS in the environment.

Figure 21

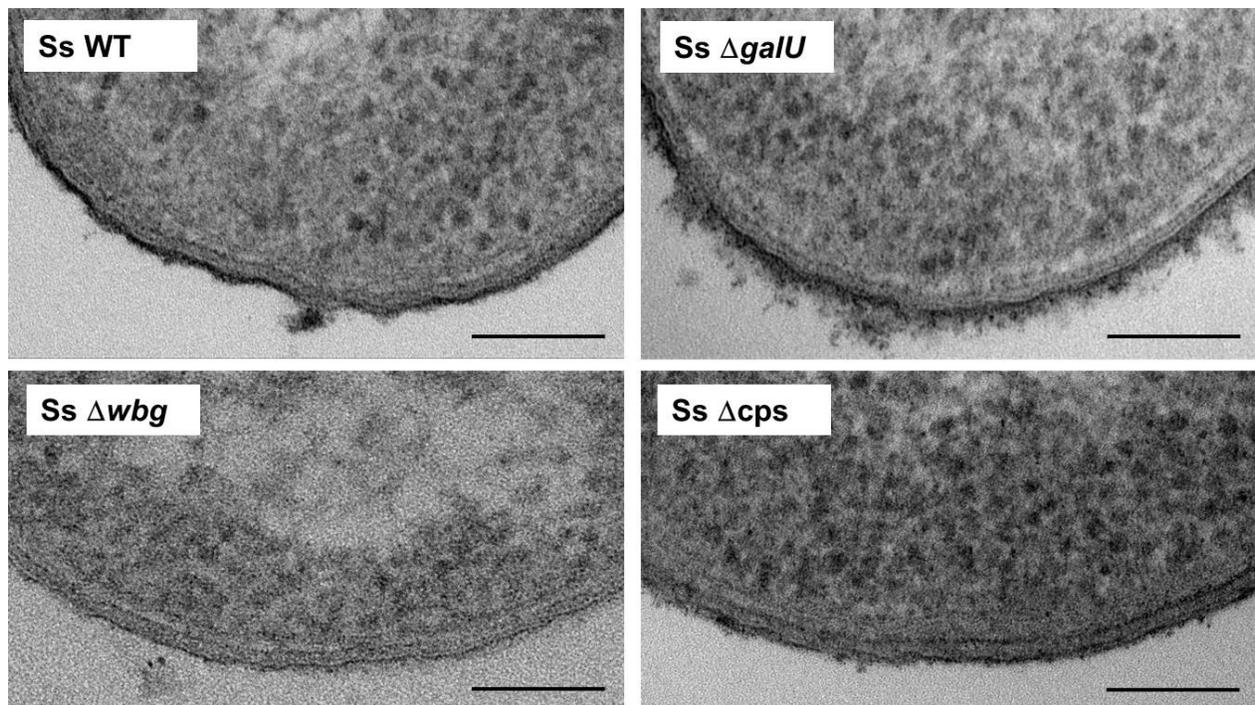


Figure 21: Surface analysis of *S. sonnei* WT and LPS/capsule mutants. Representative micrographs of negative staining-Transmission Electron Microscopy (TEM) and ultrathin resin sectioning analysis after samples fixation and treatment with Alcian blue. The electron-dense material at the bacterial surface corresponds to extracellular polysaccharides. Scale bars: 100 nm.

The extension of the dark layer from the surface of the different strains has been estimated as the average thickness measured in different positions along the bacterial membrane. As it is reported in Table 6, the absolute values were not so impressive, probably due to an artifact of the EM technique used in this study, that doesn't maintain the EPS structure in its native form. However the relative differences between the strains support the characteristics of the expected phenotypes. The WT polysaccharide layer resulted about two times thicker than the Δcps one, while, as expected, the layer was not detected in the Δwbg OAg-negative strain. *Ss* $\Delta galU$ had a very remarkable

surface phenotype with an average layer thickness of about 25 nm. Two hypotheses could explain why we observed this big difference with the WT strain (7 nm layer thickness): it could be that, with the impossibility of a linkage with the core-lipid A molecules, all the O units are assembled to form a higher amount of the capsule polysaccharide in *Ss ΔgalU*. Alternatively, it could be that in the presence of the LPS side chain, the OAg forms a highly ordered structure with the capsule giving rise to a more condensed EPS (as observed in the WT strain), while in the absence of the LPS side chain, the capsule protrudes chaotically and with a variable extent from the outer membrane (as observed in the *ΔgalU* strain).

Table 6: Thickness of the outer layer

<i>S. sonnei</i> strain	Outer layer average thickness (nm)
WT	7.0
<i>Δcps</i>	3.8
<i>ΔgalU</i>	25.6
<i>Δwbg</i>	0.0

Table 6: Estimated thickness of the EPS outer layer measured on electron micrographs of *S. sonnei* WT and LPS and capsule mutant strains (nm, average of 20 different measurements).

6.7 *S. sonnei* capsule and type III secretion system (T3SS) exposure

The present study is the first report of a capsule in *Shigella*. Bacterial capsules are well established virulence factors, often acting by protecting the cell from opsonophagocytosis and complement-mediated killing [219]. They have also been recognized to play an important role in affecting the functionality of other virulence factors. For example, capsule can mask the function of *E. coli* short adhesins [226] and this phenomenon is not restricted to adhesins but it was also reported for other factors in different bacteria, including *Klebsiella pneumoniae* fimbriae [227] and *Neisseria pili* [228]. The virulence of *Shigella* is mainly mediated by the activity of an array of plasmid-encoded virulence factors among which the type III protein secretion system (T3SS) is one of the most important [69]. To establish whether the presence of the capsule affects the exposure of T3SS molecules on the surface of *S. sonnei*, we examined bacteria by FACS analysis after immunolabeling with a monoclonal antibody against IpaB, which is both secreted and exposed at the T3SS tip [70]. Results are reported in Figure 22, Panel A: IpaB monoclonal antibody did not react with the surface of *S. sonnei* WT bacteria, while it reacted with *S. flexneri*. It is important to notice that the IpaB antibody used in this assay was raised against *S. flexneri* 5a M90T protein (H16, mouse monoclonal Ab, [196]). Even if the epitope used to generate the antibody differs only of one amino acid if compared to the corresponding epitope in *S. sonnei*, it is possible that the affinity of the antibody for *S. sonnei* IpaB is too low and the surface-bound

fluorescence was under the detection limit. This could also explain the weak reaction of the Ab with the *S. sonnei* lysate observed in the western blot analysis, as compared to the stronger reaction with the *S. flexneri* lysate (Panel B, 62 KDa band). Another possibility is that *S. sonnei* possesses less T3SS molecules on the surface per cell, as compared to *S. flexneri*. In any case, when we tested the IpaB Ab on the *S. sonnei* capsule negative strain (Δ cps), a small shift of the fluorescent signal towards the positive gate of the plot was observed, indicating that, regardless the level of affinity of the antibody for the *S. sonnei* IpaB protein, the T3SS tip was more accessible in the absence of the capsule polysaccharide. The accessibility of the IpaB antigen was even increased in the *S. sonnei* and *flexneri* strains lacking both the LPS O side chain and the OAg capsule (*Ss* Δ wbg, *Sf2a* Δ rfg). As expected, *S. sonnei* -pSS was not recognized by the IpaB antibody. Studies on *S. flexneri* showed that glucosylation of the LPS O antigen, via a bacteriophage-encoded enzyme, shortens the OAg and thus enhances the T3SS function, indicating that the WT strain has evolved a phenotype in which the structure of the surface polysaccharides is optimized for virulence [80]. For *S. sonnei* there is no characterization of the role of exopolysaccharides in relationship to other surface virulence factors. The flow cytometry analysis of *in vitro* grown *S. sonnei* suggests that the presence of a capsule, specifically in *S. sonnei*, can affect the exposure of the T3SS and thus the virulence abilities of the pathogen. Further studies are being performed to study more in deep the T3SS accessibility in the different mutants by immunogold labeling and TEM analysis. However results reported in this paragraph are in agreement with what reported for Enterohemorrhagic and Enteropathogenic *E. coli* (EHEC and EPEC): it has been shown that the group 4 capsule can transiently shield the type three secretion systems having an impact on the host-pathogen interaction [224].

Figure 22

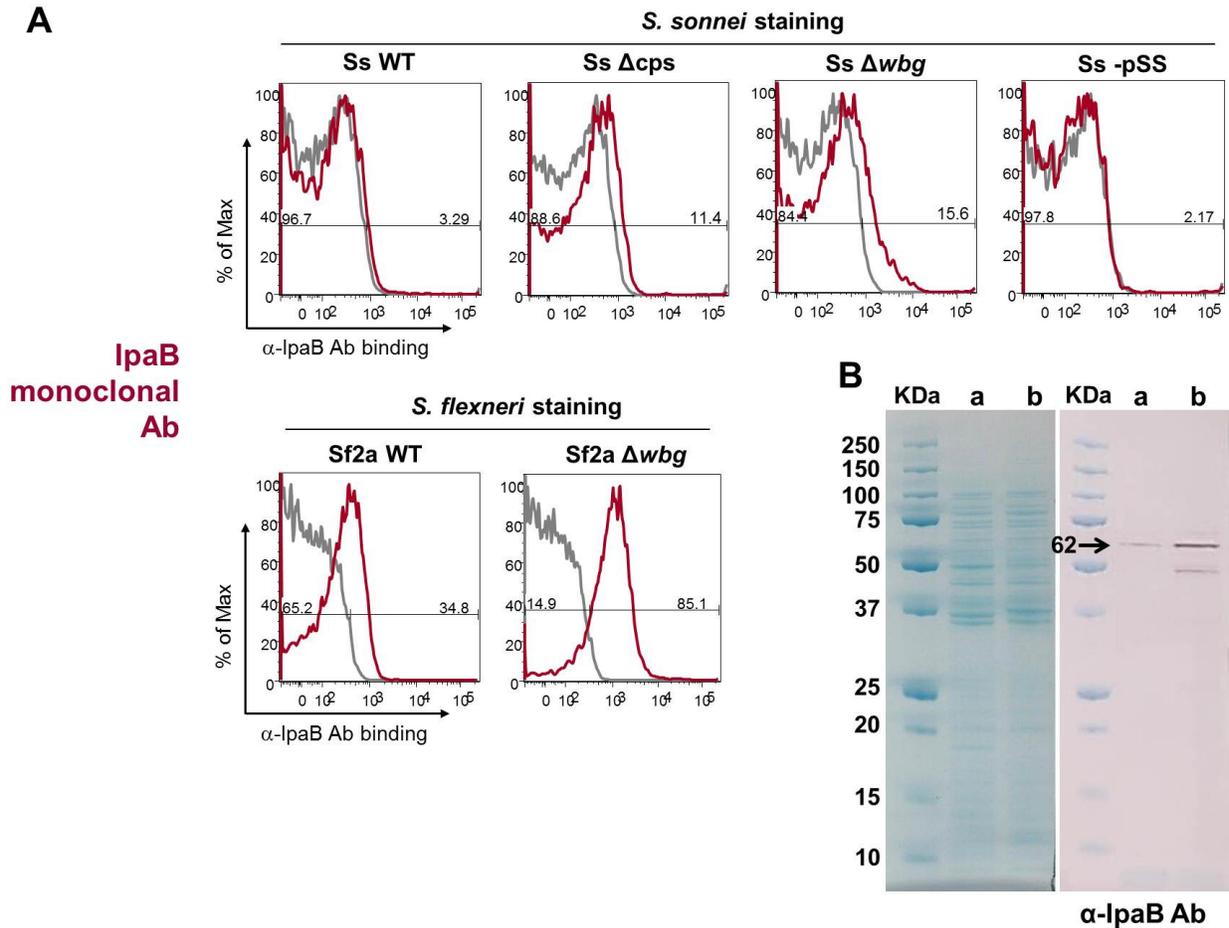


Figure 22: IpaB exposure on the surface of *S. sonnei* and *S. flexneri*. Panel A) Surface staining of *S. sonnei* and *S. flexneri* WT and mutant strains with a monoclonal antibody (Ab) against IpaB and FACS analysis (red profiles). Ab dilution is 1:50. Gray profiles: staining with the secondary Ab. Panel B) 12 % Bis-Tris SDS-PAGE and Coomassie staining of bacterial lysates (5×10^7 bacteria/lane) and WB analysis with the monoclonal Ab against IpaB (Dilution is 1:1000). All Blue molecular weight standard; Lane a= *Ss* WT; Lane b= *Sf2a* WT.

6.8 Complementation of the G4C operon in *S. sonnei* Δ cps strain

The *S. sonnei* capsule mutant complemented with a plasmid expressing the group 4 capsule gene cluster was produced. In particular, the complementing plasmid p Δ cpsCOMPL (Figure 23, Panel A) was generated by cloning the whole G4C cluster [185] with its promoter region in the low copy number vector pACYC184. The G4C operon was amplified with the 280 bp upstream region by using the LongRange PCR Kit (PCR product of 8032 bp, Fig. 23, Panel B) and it was cloned in the XbaI and Sall restriction sites of the cloning vector (Panel C). After a passage into *E. coli* sub-cloning cells, the plasmid was purified by miniprep and controlled by enzymatic digestions (Panel D), obtaining fragments of the expected size. The purified p Δ cpsCOMPL was

electroporated into *S. sonnei* Δ cps competent cells. The complemented colony, named Ss Δ cps(cps), was selected on chloramphenicol and on Congo-red supplemented agar, in order to verify the compatibility of the pSS virulence plasmid with the complementing plasmid. The LPS of the Ss Δ cps(cps) strain was purified, as described before, and compared with the WT LPS: the typical LPS pattern was revealed by silver staining in the complemented strain extract, with the smear corresponding to the low mobility material starting at the very high molecular weight region of the SDS-PAGE, as for the WT sample (data not shown). Moreover, a preliminary HPLC-SEC analysis on the total cellular polysaccharide confirmed the restoration of the expression of the HMW-PS population (data not shown). The Ss Δ cps(cps) possesses a higher copy number of the G4C cluster with respect to the WT strain. Electron microscopy analyses are being performed in order to verify that it express a WT level, if not a higher amount, of capsule polysaccharide.

Figure 23

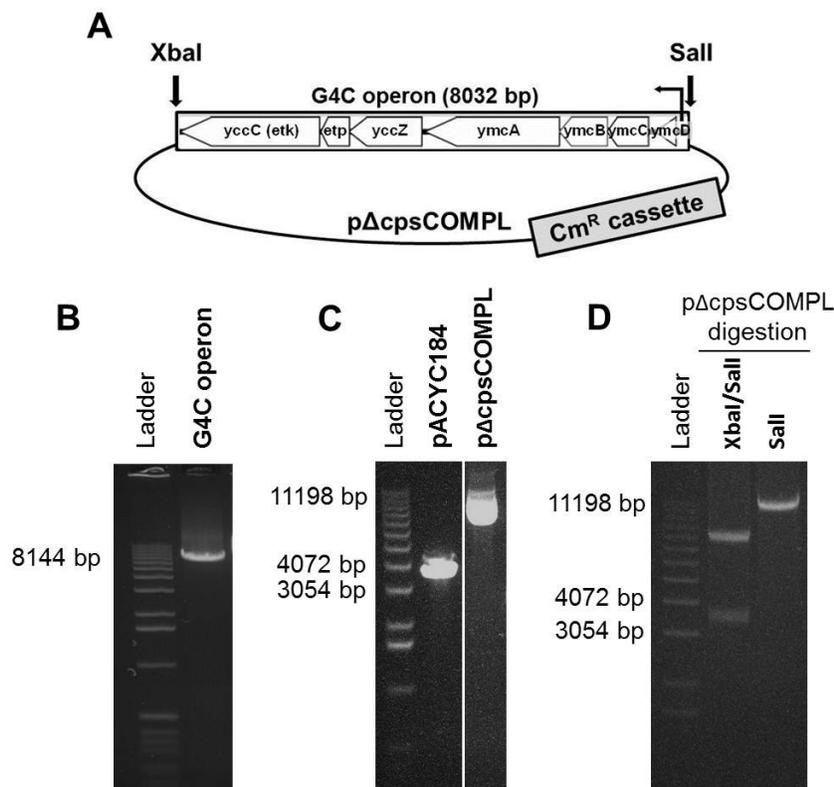


Figure 23: Generation of *S. sonnei* Δ cps strain complemented with a plasmid expressing the G4C gene cluster with its promoter region. A) Schematic representation of p Δ cpsCOMPL complementing plasmid, with the restriction sites (XbaI, SmaI) used for the cloning procedure and carrying the G4C operon (8032). The tetracycline cassette of the cloning vector is inactivated by the insertion of the G4C operon, while the chloramphenicol cassette is used for colony screening. B) PCR amplification of the G4C operon by LongRange PCR kit from a WT colony of *S. sonnei*. C) p Δ cpsCOMPL miniprep product, compared to parent pACYC184 cloning vector. D) Control enzymatic digestion of p Δ cpsCOMPL and deriving products of the expected size.

6.9 G4C functional characterization: *in vitro* HeLa cells invasion assay

We hypothesized that the expression of the group 4 capsule contributes to the virulence of *S. sonnei* through an effect on its invasive potential. Therefore, to investigate whether the capsule plays a role in *S. sonnei*-host cell interaction, we infected *in vitro* human epithelial cells and we compared the invasiveness of Ss Δ cps and Ss Δ cps(cps) with the WT strain. Semi-confluent HeLa cell layers were incubated with a multiplicity of infection (MOI) of 10 bacteria/cell for 1 h. After killing extracellular bacteria by adding gentamicin, intracellular bacteria were collected by lysing the infected cells. CFU were counted and results are reported in Fig. 24. The *S. sonnei* G4C mutant exhibited significant increased invasiveness, being about 100 times more invasive than the WT strain. Complementing the Δ cps mutant with a plasmid expressing the G4C operon restored the WT-like lower invasiveness and even reduced it up to 3 folds. As expected, the Ss phase II (-pSS) strain was completely avirulent and did not invade HeLa cells [193]. These interesting results suggest that the presence of the capsule polysaccharide accounts for the changes in cell invasion: Ss Δ cps increased invasive ability could derive from the fact that in the absence of the capsule, virulence factors, as the T3SS, are rapidly available and exposed and the strain might result in higher cell entry proficiency. On the other hand, Ss Δ cps(cps) complemented strain is less invasive than the WT strain and we hypothesize that it could be so because this strain is “over-complemented”, expressing a higher amount of capsule polysaccharide and resulting in an attenuated phenotype. These speculations correlate with several other findings. First of all, if we compare *S. sonnei* and *S. flexneri* for their invasive potential *in vitro*, we always observe that *S. sonnei* is much less invasive than *flexneri*, with at least 10 folds of difference (data not shown). The attenuated phenotype of Ss WT could derive not only from its natural tendency to lose the virulence plasmid, but also to its peculiar ability to express a capsular polysaccharide. It has been reported in literature, for example, that the expression of Vi capsule PS is implicated in reducing the invasiveness of *S. enterica* serotype Typhi [229]. Moreover, a recent paper published by Shifrin, Y. et al. reported that EPEC mutants in the G4C operon similarly exhibited increased invasiveness at early time points postinfection [224]. In the same paper, it has been shown that the capsule can transiently shield the T3SS apparatus and other virulence factors. This masking inhibits the attachment of EPEC to tissue-cultured epithelial cells and attenuates TTSS-mediated protein translocation into host cells. The authors demonstrated that reciprocal regulation of capsule and surface virulence factors resulted in a more efficient pathogenic phenotype not only *in vitro* but more importantly also *in vivo* [224].

Figure 24

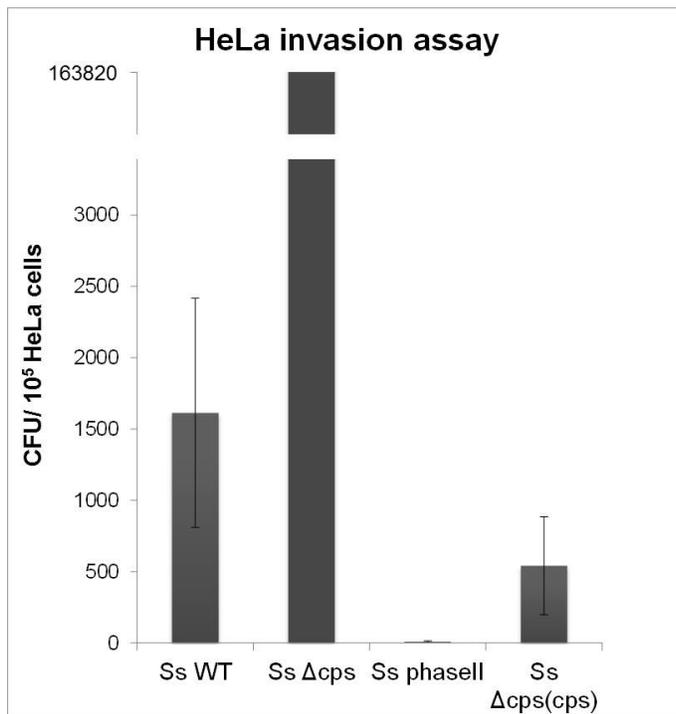


Figure 24: HeLa cells invasion assay. Y axis: *Shigella* CFU per 10⁵ cells. HeLa cells were infected with *S. sonnei* WT, Δcps, phase II (-pSS), Δcps(cps).

6.10 G4C functional characterization: *in vivo* rabbit model of ligated ileal loops

Shigellosis is an acute infection of the large intestine of the human being, characterized by severe mucosal inflammation, bacterial invasion in the epithelial layer, abscesses formation and tissues disruption [53]. The rabbit ligated ileal loop model is permissive to intestinal infection by *Shigella* and results in rupture, invasion and inflammatory destruction of the epithelium typical of shigellosis. The influx of PMNs observed in this model allows reliable quantification of the invasive phenotype, therefore it represents a good model for the validation of the invasive/inflammatory phenotype of *Shigella* mutants compared to wild type strain [70]. In this study, the rabbit ligated intestinal loop model of experimental shigellosis was used to investigate whether the G4 capsule contributes to the invasive abilities of *S. sonnei in vivo* and to the induction of inflammatory host response during the infection. In this model, severity of tissue inflammation can be evaluated by recording qualitative and quantitative alterations of the intestinal mucosa and by measuring pro-inflammatory host gene expression. Moreover, local and peripheral invasiveness can be estimated by bacterial counts in infected tissues. First of all, we determined the lower infectious dose giving rise to the typical *Shigella*-induced pathology by testing increasing doses (from 10⁹ to 5*10⁹ bacteria/loop) of different *S. sonnei* strains. We ended up with an optimal dose of 3*10⁹ bacteria/loop,

an amount very similar, even if a little bit higher, to what is commonly used in the *S. flexneri* model [202]. Then, the animal study was designed to perform both a single challenge experiment and a competitive challenge experiment. In the single challenge experiment, ligated loops were infected with 3×10^9 bacteria/loop of *S. sonnei* WT, or Ss Δ cps mutant, or Ss Δ cps(cps) strains. As negative control, we also tested the Ss phase II (-pSS) strain. Each condition was repeated in at least 6 loops, divided in two different animals. The infection was conducted for 8 h, after that infected tissues were collected for bacterial count, histopathology analysis and gene expression analysis. In the competitive challenge experiment, each loop was inoculated with a total dose of 3×10^7 bacteria of a 1:1 combination of two *S. sonnei* strains (Ss WT + Ss Δ cps, Ss WT + Ss Δ cps(cps), Ss WT + Ss -pSS). Each condition was tested in at least 8 loops, divided in two different animals. The infection was conducted for 16 h, after that infected tissues were collected for bacterial count on selective media.

Data obtained from the single challenge experiment describe the specific behavior of each strain in the host environment. The analysis starts with loops observation at the moment of biopsies collection: the invasive phenotype of wild type *S. sonnei* correlated with the presence of fluids and blood in the infected loops whereas the non-invasive strain (Ss -pSS) barely caused fluid production and loops looked flat as the uninfected tissue. Interestingly, Ss Δ cps strain caused maximum fluid and blood accumulation, with loops having pale outer surface and showing a very intense inflammatory pathology. On the contrary, the Ss Δ cps(cps) complemented strain elicited an intermediate phenotype, very often less severe than the WT.

Histopathological analysis supported the qualitative data provided above. Representative pictures of Hematoxylin and Eosin (H&E) stained slices showed alterations of villi (Figure 25, Panel A). In the characteristic lesions observed after infection by the wild type strain (Panel A, first picture), the villi were shortened and enlarged, with several indentations forming the characteristic Christmas tree-like shape, typical of intestinal inflammation. Several regions of tissue disruption were observed and infiltration of inflammatory cells was also detected within the lamina propria and in the edematous submucosal tissues. Notably, infection of ileal loops by the Ss Δ cps strain led to dramatic alterations of mucosal tissues with rupture and destruction of the intestinal epithelium (Panel A, second picture). The lesions included extensive zones of epithelial detachment and loss of villi with tissue necrosis, leaving crypts as the only identifiable epithelial structure. Often, submucosal tissues were strongly edematous, with a large area infiltrated by inflammatory cells between the residual mucosae and the muscular layer. In contrast, villi of loops infected by the Ss Δ cps(cps) complemented mutant showed a decrease in the pattern of pathology (Panel A, third picture). Villi remained in general of a length and a width very similar to those observed following infection with the avirulent strain (Ss phase II, fourth picture). A limited edema was observed with few cells infiltrating the lamina propria. Regions of tissue lesions were rather scarce. Finally, villi of loops infected with the avirulent strain Ss phase II (-pSS) appeared long and narrow, with no evidence of rupture of the epithelial lining, no significant infiltrate of the lamina propria, and no submucosal edema (Panel A, fourth picture). As control, the section of uninfected tissue is reported (Panel A, fifth picture) to show the appearance of a normal rabbit epithelial architecture.

Bacteria were detected by immunostaining with an in-house polyclonal anti-*Shigella sonnei* serum. As shown in Fig. 25, Panel B, a massive amount of bacteria was

associated with the lamina propria and the epithelium of the villi, particularly in areas of abscesses, rupture/destruction of the epithelial lining and of villi indentation, following infection with both Ss WT and Ss Δ cps (Panel B, first and second pictures). This indicates that the invasive abilities of *S. sonnei* in the intestinal epithelium were not impaired by the lack of the capsule but rather enhanced, as expected from the *in vitro* data presented in the previous paragraph. Under similar conditions, Ss phase II showed no invasion at all, as no bacteria were detected within the villi epithelium (Panel B, fourth picture). In intestinal loops infected by Ss Δ cps(cps) (Panel B, third picture), an interesting pattern of staining was shown that matched the previous observations: the reduced severity of tissue damage observed in H&E-stained slices correlated with a reduced amount of bacteria invading the intestinal epithelium as the majority of Ss Δ cps(cps) bacteria were seen in the lumen. Moreover, no significant lesion was observed in regions in which bacteria were found both in the lamina propria and in epithelial cells. This might indicate that infected tissues are more tolerant to the presence of invasive *S. sonnei* that express a high amount of capsule polysaccharide, as it was shown for *Salmonella* Typhi expressing Vi capsule [230].

Histopathological alterations were quantified and recorded as the average ratio between the length and the width (L/W) of the villi that measures villi atrophy on H&E-stained and GIEMSA-stained (data not shown) tissue sections (Figure 25, Panel C). This quantitative estimation is inversely proportional to the inflammatory status of the epithelium and reflects the qualitative analysis described above. Again, Ss Δ cps strain had the more aggressive phenotype and induced villi atrophy with a significantly higher degree than what was measured in Ss WT-infected samples (average L/W ratio of 3.6 and 5.3, respectively). On the other hand, the entity of Ss Δ cps(cps)-induced atrophy was reduced if compared to both the WT-induced and Δ cps-induced atrophy (average L/W ratio of 7.0). Ss phase II did not induce significant villi alteration as the estimated atrophy value was very similar to the L/W ratio of uninfected tissues (average L/W ratio of 7.7 and 8.4, respectively).

Summarizing the results reported so far, qualitative biopsies observation and histopathological analysis of infected rabbit intestinal tissues indicate that the lack of the G4 capsule polysaccharide in the Ss Δ cps strain caused a dramatic increase of pathogenicity, that is particularly characterized by the augmentation of mucus and blood production in the infected loop, of rupture and destruction of the epithelial lining and of tissue inflammatory manifestations. On the contrary, trans-complemented Ss Δ cps(cps) strain, showed an attenuated phenotype *in vivo*, with less bacteria invading the epithelium and less tissue inflammation, likely also due to a higher host tolerance to encapsulated bacteria.

Figure 25

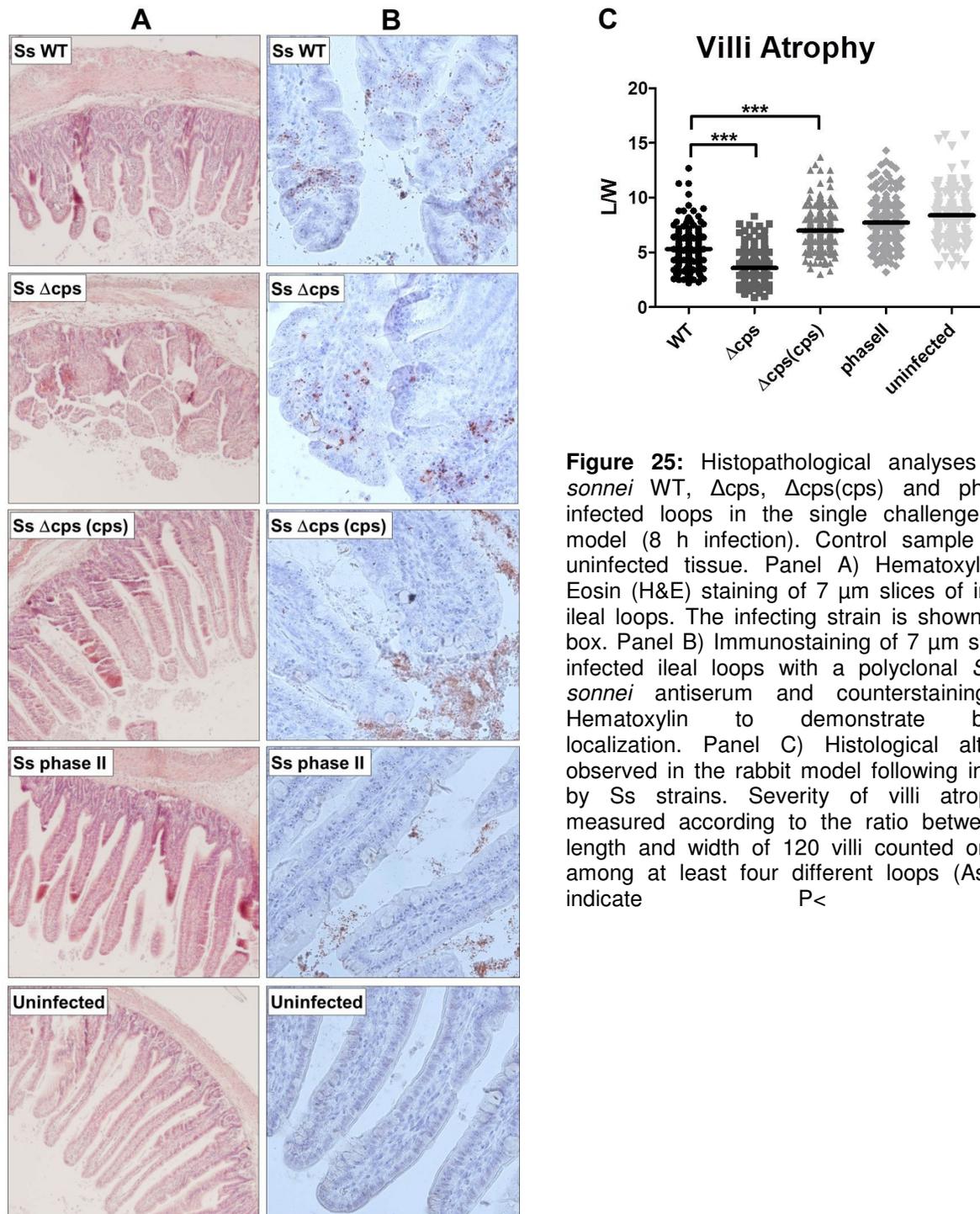


Figure 25: Histopathological analyses of *S. sonnei* WT, Δ cps, Δ cps(cps) and phase II infected loops in the single challenge rabbit model (8 h infection). Control sample is the uninfected tissue. Panel A) Hematoxylin and Eosin (H&E) staining of 7 μ m slices of infected ileal loops. The infecting strain is shown in the box. Panel B) Immunostaining of 7 μ m slices of infected ileal loops with a polyclonal *Shigella sonnei* antiserum and counterstaining with Hematoxylin to demonstrate bacteria localization. Panel C) Histological alteration observed in the rabbit model following infection by *Ss* strains. Severity of villi atrophy is measured according to the ratio between the length and width of 120 villi counted on each among at least four different loops (Asterisks indicate $P < 0.001$).

Further analyses were performed to evaluate the host inflammatory changes to infection by the different *S. sonnei* strains. Portions of the infected ligated loops were processed in order to measure mRNA levels of several genes encoding proinflammatory cytokines and chemokines [202]. In particular, we focused our attention on those interleukins whose mRNA levels are most prominently induced or are most relevant in response to *Shigella* infection [53]: IL 6, IL 8, IL 1 β . Real Time-quantitative PCR (RT-qPCR) analyses were performed to calculate the fold induction of these genes in the *S. sonnei* mutants-infected loops over the WT-infected loops. Results are reported in Figure 26, first, second and third plots. All the three interleukins studied, showed a similar trend, consistent with what observed in histopathological analyses. Compared to the wild type Ss strain, the Ss capsule negative strain elicited increased levels of IL 6, 8 and 1 β , while the Ss Δ cps(cps) complemented strain triggered lower expression levels of these proinflammatory genes. As expected, Ss phase II avirulent strain did not relevantly induce inflammatory factors, with measured levels similar to those of the uninfected tissue. However, these differences were not statistically significant, also due to the high variability among the different replicates of the same infective condition and between the different animals. It is interesting to notice that differences in the expression of proinflammatory cytokines between the Ss WT-infected samples and the Ss Δ cps-infected samples became more relevant with the increase of the infective dose (Results for IL 8 are reported as example in Figure 26, fourth plot): in the samples infected with a Ss Δ cps dose of $5 \cdot 10^9$ bacteria/loop the highest expression levels of IL 8 were measured, as compared to the samples infected with lower doses and to the samples infected with the same higher dose of Ss WT. Even if not statistically significant, collectively these results, together with the previous results obtained from the *in vivo* model, indicate that *S. sonnei* in the absence of the G4 capsule shows increased pathogenicity in the gut and triggers higher intestinal inflammation. This could be the result of the fact that virulence factors are no more shielded by the capsule polysaccharide and this might facilitate on one hand the bacterial adhesion and entry in the epithelial cells, and on the other hand the recognition of invasive bacteria by the innate immune system of the host.

Figure 26

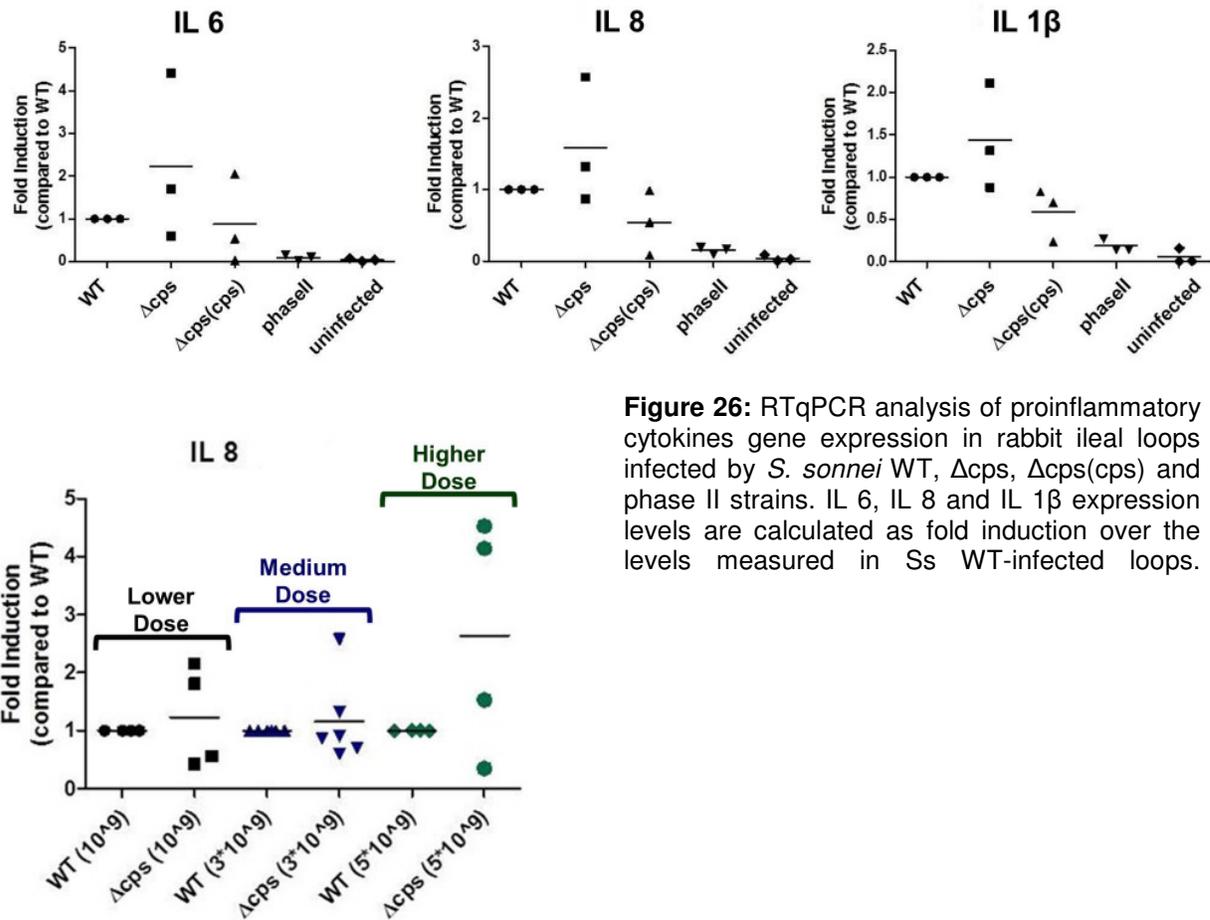


Figure 26: RTqPCR analysis of proinflammatory cytokines gene expression in rabbit ileal loops infected by *S. sonnei* WT, Δ cps, Δ cps(cps) and phase II strains. IL 6, IL 8 and IL 1 β expression levels are calculated as fold induction over the levels measured in *Ss* WT-infected loops.

We next investigated how the G4C might contribute to bacterial fitness and host colonization. Bacterial translocation is a phenomenon in which enteric bacteria cross the intestinal barrier and reach the mesenteric lymph nodes. From there, dissemination throughout the body, to liver, spleen and general circulation may occur and cause severe consequences as sepsis and multisystem organ dysfunction [231]. We studied the influence of the capsule on the ability of *S. sonnei* to translocate and disseminate to establish systemic infection by collecting mesenteric lymph nodes (MLN), liver, spleen and blood from animals infected concomitantly with the different *S. sonnei* strains. Infected organs were processed in order to count viable bacteria on selective media. The relative percentage of the different *S. sonnei* strains recovered from MLN, liver, spleen and blood is reported in Figure 27: as it is shown in the graph, in each peripheral organ we predominantly found *S. sonnei* WT strain (as about 70 % of all the bacteria recovered). *S. sonnei* capsule mutant strain accounted for about the 26 % of the total, while a negligible amount of the other strains (*Ss* Δ cps(cps), *Ss* phase II) was measured. Similar results were obtained by analyzing tissues from rabbits infected for 16 h during the competitive challenge experiment (data not shown). Interestingly, these data indicate that while in the intestine the *Ss* Δ cps capsule mutant showed the most aggressive/invasive phenotype, it had impaired ability to translocate and disseminate to

establish peripheral infection, as compared to Ss WT. A possible reason could be that acapsulated bacteria are more susceptible to the host immune responses since they lack the protective effect of the capsular polysaccharide. So if in a first moment they invade very efficiently the intestinal epithelium, then they might succumb to the strong inflammatory response of the host. This aspect has been demonstrated for other pathogens [232]. On the other hand, probably due to the fact that the Ss Δ cps(cps) complemented strain is less invasive in the gut, we do not find it in peripheral organs.

Figure 27

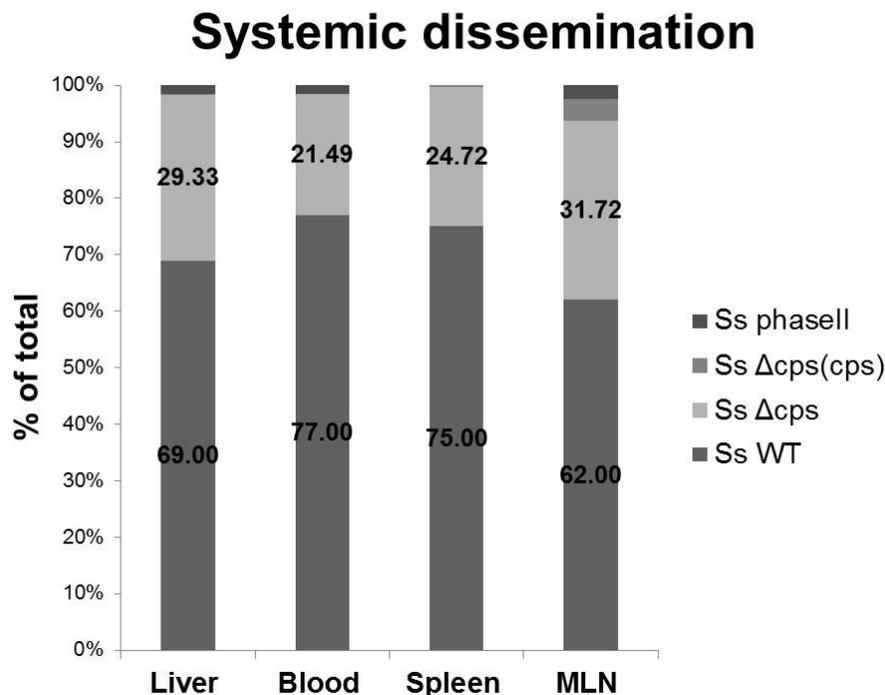


Figure 27: Systemic spread of *S. sonnei* WT, Δ cps, Δ cps(cps), phase II strains in liver, blood, spleen and mesenteric lymph nodes (MLN). Data are reported as relative percentage of the total bacteria collected in each organ in the rabbit model following 8 h of infection.

Finally, to support our hypothesis that the group 4 capsule has an important role in affecting the invasive abilities of *S. sonnei* and the host response during infection, we needed to prove that the G4C operon was indeed expressed by bacteria *in vivo*. Therefore, we measured G4C gene cluster expression in bacteria isolated from infected loops. Real Time-quantitative PCR (RT-qPCR) analyses were performed to calculate the fold induction of each gene of the cluster in *S. sonnei* mutants over the levels of the WT strain. In Figure 28, the results of *etp* and *etk* genes expression analysis are reported (similar results were obtained for the other genes of the cluster, data not shown). Ss WT bacteria expressed the G4C operon at a similar level as the housekeeping gene encoding for the lysine transporter LysP [26]. Moreover, we saw that in the complemented strain, the operon was 5 to 6 times more expressed, than in the WT

strain. The data are not normalized to gene copy number, however this result supports the hypothesis of the ‘over-complementation’ of the *Ss* Δ cps(cps). *S. sonnei* phase II had lower expression levels, suggesting a mechanisms of gene repression in the absence of the OAg units substrates. *Ss* Δ cps do not possesses the cluster, so no expression was detected.

Figure 28

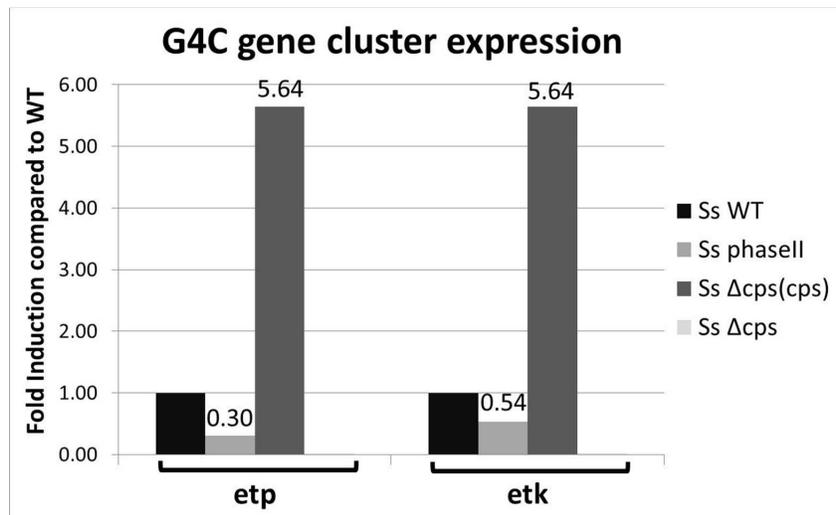


Figure 28: RT-qPCR expression analysis of *etp* and *etk* genes of the G4C operon in *S. sonnei* WT, Δ cps, Δ cps(cps) and phase II bacteria from infected loops. Expression levels are calculated as fold induction over the levels measured in *Ss* WT.

6.11 G4C operon conservation in other *Shigella* species

Finally, we studied the conservation of the group 4 capsule operon in different *Shigella* species. Genomes of several isolates of *S. boydii*, *flexneri*, *sonnei* and *dysenteriae* were analyzed for the presence of a G4C cluster homologous to the *S. sonnei* 53G one. In general, we found that the G4C cluster was highly conserved in all the *Shigella* background, but in *S. flexneri* (except for the *S. flexneri* 6 serotype) (See Table 7). About the 77 % of the genomes of *S. boydii*, *sonnei* and *dysenteriae* analyzed possessed a cluster with a 99 % score of homology. On the contrary, *S. flexneri* very often possessed the single genes of the operon, but these genes were not clustered together in the same position of the genome. This observation is in agreement with our results showing that *S. flexneri* 2a does not express an OAg capsule, as its Δ galU mutant is completely OAg-deficient.

Table 7: *Shigella* genome comparison

Genomes	Operon hits > 99 % (% of total)
<i>S. boydii</i>	74.7
<i>S. flexneri</i> (other than 6)	7.8
<i>S. flexneri</i> 6	100
<i>S. sonnei</i>	73.3
<i>S. dysenteriae</i>	82.1

Table 7: *Shigella* genome comparison to evaluate group 4 capsule gene cluster distribution within the genus.

7. DISCUSSION

In this thesis we describe the identification and characterization of a group 4 capsule in *Shigella sonnei* important for bacterial pathogenesis *in vitro* and *in vivo*.

Exopolysaccharides, comprising LPS O side chain and all other surface exposed polysaccharides [219], play critical roles in interactions between bacteria and their immediate environments. In particular, capsules are high molecular weight surface-enveloping polysaccharides structure attached to the surface of the cells through an uncharacterized mechanism. They are important protecting factors that help bacteria surviving to desiccation [217] and are well established virulence factors, often acting by protecting the cell from opsonophagocytosis and complement-mediated killing [219]. Gram-negative bacterial capsules, in particular the exemplifying *E. coli* capsules, have been classified into four groups, based on genetic and biochemical criteria [222]. The group 4 capsules (G4C) subclass comprises a subset of former group I K antigens and surface polysaccharides known as 'O antigen capsules'. G4C were first identified in *E. coli* serotype O111 [233], where 50 % of the O111 antigen is in the smooth LPS fraction and the remainder is in a LPS-unlinked capsular form. From there, G4 capsules have been found in *E. coli* isolates that cause intestinal infections, including representatives of enteropathogenic (EPEC), enterotoxigenic (ETEC) [185] and enterohemorrhagic (EHEC) [224] *E. coli*, as well as in *Salmonella enterica* serovar Enteritidis [217] and *Francisella tularensis* [218]. The full distribution of G4 capsules is unknown and many may have been overlooked because early structural and serological analyses were interpreted with the expectations that surface O antigen is only found as LPS side chain, in a lipid A-core-linked form [219]. Although *E. coli* OAg capsules have been characterized from long time [234], this is the first report about a capsule in *Shigella*. Its existence has been overlooked, again probably because of its similarity to the cognate LPS O side chain and because it can not be identified by standard serological tests. The presence of the G4C in *S. sonnei* has been uncovered by the immunological characterization of the LPS deep rough mutant, *S. sonnei galU* knock out (*Ss ΔgalU*). In this mutant, the linkage of the OAg repeating units to the lipid A-core region of the LPS is abolished [213]. In *S. flexneri* 2a and 5a backgrounds, deletion of the *galU* gene has been reported to result in OAg deficiency [80,184]. However, we demonstrated that *Ss ΔgalU* and its deriving outer membrane particles (GMMA) were still able to induce an OAg-specific response in mice. A low mobility material, not linked to the LPS, could be extracted from *Ss ΔgalU* and could be visualized in immunoblot analyses with an OAg-specific antiserum. In 2002, De-Qi, Xu and colleagues [84] firstly observed the presence of a phase I-reactive material in immunoblot analyses, above the position 25 OAg repeating units, in the context of the characterization of polysaccharides expressed by a *Salmonella enterica* serovar Typhi live vaccine vector candidate. By cloning the *S. sonnei* OAg synthetic operon in the vaccine strain, they found most of the OAg material in a LPS-unlinked form. The authors speculated about the possibility of the presence of a capsule-like material in *S. sonnei*. However no further studies followed to clarify this hypothesis. In the present study, the surface localization of the OAg-containing material in *Ss ΔgalU* was demonstrated by an experiment of OAg surface staining. Moreover, we

showed that *S. sonnei* WT and $\Delta galU$ possess a high molecular weight polysaccharide comprising the same residues of the LPS O side chain (FucNAc4N, L-AltNAcA).

Generally, capsule synthesis requires nucleotide diphospho-sugar precursors available in the cytoplasm and concludes with an assembled polymer at the periplasmic face of the plasma membrane. Capsule formation is completed by surface translocation and export, two steps that follows reactions which are unique to capsular polysaccharides [219] (see Figure 29). The genes required for G4C formation are organized into two loci (for details about proteins involved in G4C biosynthesis, see Table 8). The first is responsible for the synthesis of Wzy-dependent O units (see paragraph 6.1) [235,236] and determines both the LPS and the capsular O antigen portions [237,238]. In this case, the lipopolysaccharide O antigen and the capsule antigen are composed of the same repeating units, this is the reason why G4C have also been termed 'O-antigen capsules' [211]. The genes in the Wzy-dependent synthetic locus are required for the synthesis of the repeating unit, its delivery to the periplasm, and its polymerization by Wzy into both O antigen and G4C polysaccharide (Fig. 29). Accordingly, mutants deficient in this locus are expected to be both rough and uncapsulated. *S. sonnei* OAg synthetic cluster is composed of nine genes, including the one encoding for the Wzy polymerase (*wbgT*, *wbgU*, *wzx*, *wzy*, *wbgV*, *wbgW*, *wbgX*, *wbgY* and *wbgZ*) [84]. Mutants in the OAg biosynthetic operon (*Ss* -pSS or *Ss* Δwbg) resulted to lack both the LPS side chain and the capsule, as expected in case of a Wzy-dependent synthetic pathway typical of group 4 capsules [219]. The second locus that is involved in G4C formation is the G4C operon, also termed the '22-minute locus' because it is mapped to min 22 in the *E. coli* K-12 genetic map [239]. This operon encodes for seven proteins (YmcDCBA, YccZ, Etp (YccY), Etk (YccC) [219]. The functionality of the G4C operon has been first demonstrated in EPEC serotype O127, in which all seven genes in this transcriptional unit are required for group 4 capsule assembly [185]. The presence of G4C analogue clusters is also evident in other Gram-negative pathogens such as *K. pneumoniae* [240] and *Salmonella* [217]. Regulation of capsular biosynthesis in a number of both Gram-positive and Gram-negative bacteria is mediated via a phosphoregulatory system, consisting of bacterial-tyrosine kinases (BY-kinases), and their cognate phosphatases [219]. In Gram-negative bacteria, the BY-kinase is expressed as a single membrane-spanning polypeptide, also known as a PCP 2a protein [236], with the C-terminal kinase domain on the cytoplasmic side. The kinase and phosphatase activities of Etk (homologous to Wzc) and Etp (homologous to Wzb), two proteins of the G4C operon, have been confirmed [219] and their role in repeating unit polymerization into G4C-PS and in capsule assembly has been shown [237,241,242]. The G4C locus contains the four additional genes *ymcABCD*, also required for capsule expression. All four are predicted to encode for exported proteins, with YmcA and YmcC may being lipoproteins [243]. Their function has not been elucidated yet, although databases searches identify hypothetical proteins from polysaccharide systems in other bacteria. We demonstrated that the formation of the HMW-PS founded in *S. sonnei* was dependent on the presence of an operon, highly homologous (98 % homology) to the G4C cluster identified in EPEC [185]. Peleg, A. et al. already suggested in 2005 the existence of an intact G4C gene cluster in some genomes of *Shigella*. Here, we showed that the *S. sonnei* 53G G4C operon is functional and encodes for the formation of an LPS-independent HMW OAg polysaccharide, thus a group 4 capsule.

Table 8: Proteins involved in group 4 capsule formation, their predicted localization and function

PROTEIN	LOCATION	FUNCTION
Wzy	Integral inner membrane	Und-PP-linked oligosaccharide repeating unit (RU) polymerase
Wzx	Integral inner membrane	O-unit flippase, required for export of und-PP-linked RUs in Wzy-dependent synthesis
YmcD	Inner membrane	Putative transposase
YmcC	Outer membrane (OM) lipoprotein	Putative regulator
YmcB	Unknown	Putative capsule polysaccharide protein
YmcA	OM lipoprotein	Putative capsule polysaccharide protein
YccZ	Outer membrane	Forms a multimeric putative translocation channel and interacts with the periplasmic domain of Etk (homologue of Wza)
YccY (Etp)	Cytoplasm	Protein tyrosine phosphatase; dephosphorylates Etk (homologue of Wzb)
YccC (Etk)	Inner membrane with a large periplasmic domain and cytosolic N and C termini	Cryptic autophosphorylating protein tyrosine kinase, participates in high-level polymerization of capsular polysaccharide and forms part of a trans-envelope capsule translocation complex (homologue of Wzc)

Figure 29

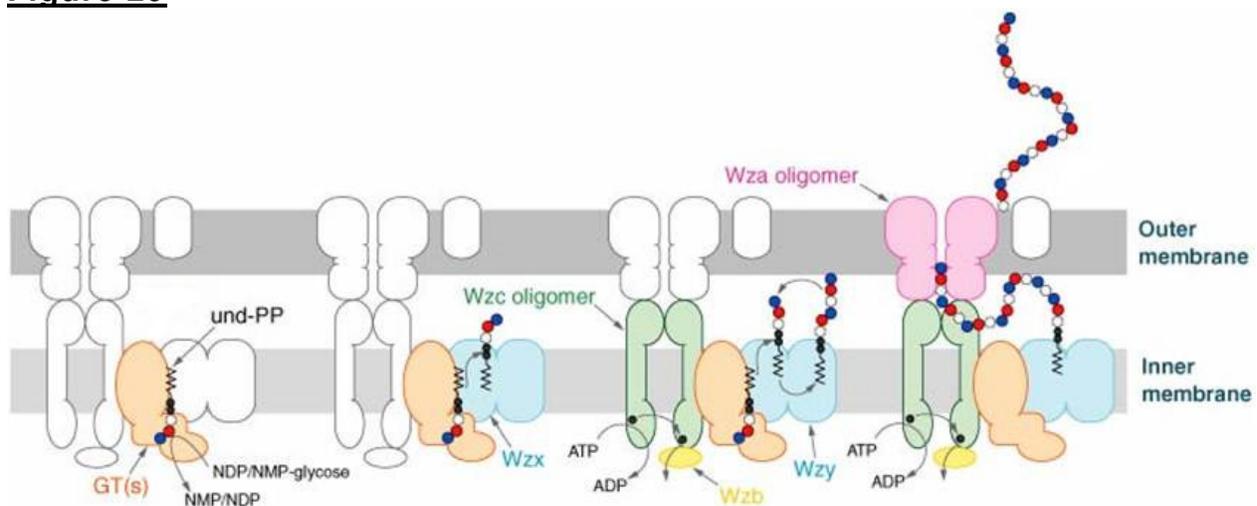


Figure 29: A model for biosynthesis and assembly of group 4 capsules. Beginning at the left, und-PP-linked repeat units are assembled at the interface between the cytoplasm and the inner membrane. Newly synthesized und-PP-linked repeats are then flipped across the membrane in a process requiring Wzx. This provides the substrates for Wzy-dependent polymerization wherein the polymer grows by transfer of the growing chain to the incoming und-PP-linked repeat unit. Continued polymerization requires transphosphorylation of C-terminal tyrosine residues in the Wzc oligomer and dephosphorylation by the Wzb phosphatase. Polymer is translocated by Wza, which likely acts as a channel (Figure and Legend reproduced from [219]).

As mentioned before, the linkage of the G4 capsules to the bacterial membrane is still not well characterized [219]. We performed compositional analyses and we did not identify Kdo, a residue of the LPS core region, as a component of the capsular antigen. This again suggests that the G4C is not linked to the bacterial membrane through the LPS. The G4 capsule of *Salmonella* has been shown to possess a lipid anchor [244]. A lipidic nature of the carrier of the G4C in *S. sonnei* could explain the mobility of the hot-phenol surface polysaccharide extracts that we observed in SDS-PAGE. However, studies to clarify this hypothesis have not been performed yet.

The function of the G4C as potential virulence factor of *S. sonnei* was investigated both *in vitro* and *in vivo*. Our results showed that the presence of the capsule polysaccharide accounted for changes in cell invasion ability *in vitro* since the G4C mutant (Ss Δ cps) resulted significantly more invasive than the WT on HeLa cells. Complementing the Δ cps mutant with a plasmid expressing the G4C operon restored the WT-like lower invasiveness or even reduced it. Ss Δ cps increased invasiveness could derive from the fact that in the absence of the capsule, virulence factors, as the T3SS, are rapidly available and exposed and the strain might result in higher cell entry proficiency. On the other hand, Ss Δ cps complemented strain was less invasive than the WT strain. We hypothesized that it could be so because this strain is 'over-complemented', as a result of the fact that the plasmid used for complementation, and so the G4C gene cluster, is present in multiple copies even though a low copy number vector was used. Thus, the complemented mutant likely expressed a higher amount of capsule polysaccharide and consequently showed an attenuated phenotype. These results are in accordance with the findings reported for EPEC and EHEC, when the invasiveness of encapsulated and unencapsulated strains was compared in a similar HeLa cells invasion assay [224]. It has been shown that at early time points postinfection, the G4C appeared to mask surface structures including intimin and the T3SS and this masking inhibited the attachment of EPEC and EHEC to tissue-cultured epithelial cells, diminished their capacity to induce the formation of actin pedestals, and attenuated T3SS-mediated protein translocation into host cells [224]. Masking by capsule of *E. coli* virulence factors has been frequently reported [224,226]. We also observed that the accessibility of the IpaB protein at the tip the type III secretion system was reduced *in vitro* in the Ss WT bacteria, as compared to the unencapsulated strain. Together, *in vitro* studies indicated a role of the capsule in affecting the pathogenic abilities of *S. sonnei* with the WT strain having a disadvantage in invasiveness associated with capsule formation. However, the fact that the bacteria maintain the capsule indicates that this surface structure contributes to bacterial fitness under some conditions. In support of this notion, the rabbit model of intestinal ileal ligated loops was used to evaluate *in vivo* the G4 capsule contribution to the invasive abilities of *S. sonnei* and to the induction of inflammatory host responses during the infection. If compared to the characteristic intestinal pathology induced by *S. sonnei* WT, Ss capsule mutant infection led to a more severe intestinal disease and a stronger inflammatory response. In contrast, infection by the Ss Δ cps complemented strain displayed a decrease in the pattern of pathology. We hypothesized that in the unencapsulated bacteria, virulence factors are no more shielded by the capsule polysaccharide and this might facilitate bacterial adhesion and entry in the host cells, as it was observed in the *in vitro* model of infection. Concomitantly, the recognition of invasive bacteria by the innate immune system of the host could be enhanced in the absence of the capsule. We then investigated the influence of the

capsule on the ability of *S. sonnei* to translocate and disseminate to establish systemic infection, in the same rabbit animal model. Interestingly, our results indicated that the presence of the capsule is needed for *S. sonnei* capabilities to disseminate. While in the intestine the Ss Δ cps capsule mutant showed the most aggressive/invasive phenotype, it had impaired ability to disseminate compared to Ss WT. The reason could be that uncapsulated bacteria are more susceptible to the host immune responses since they lack the protective effect of the capsular polysaccharide. Thus, while the lack of the capsule could be beneficial in the early stages of the infection to very efficiently invade into the intestinal epithelium, in the later stages capsule-deficient bacteria have a disadvantage in translocation and dissemination and succumb to the strong inflammatory response of the host (see Figure 30). This hypothesis is supported by literature reports that demonstrated that the expression of the T3SS and the G4C is conversely and temporally regulated to be optimized for efficient host colonization by EHEC [224]. As demonstrated for EPEC and EHEC [224], the shielding concept leaves the bacteria with an obvious dilemma. They cannot translocate proteins or invade host cells without the assistance of virulence factors, such as adhesin proteins and the T3SS, but at the same time, the polysaccharide shields contribute to bacterial fitness by providing protection against countermeasures at the disposal of a mammalian host. As an example, *Yersinia* solved the shielding dilemma by careful adjustment of the respective lengths of the T3SS needle and the YadA adhesin, such that the length of the T3SS needle is the minimum that still allows efficient protein translocation through the YadA shield [245]. We reasonably think that *Shigella sonnei* has evolved fine regulation mechanisms to optimize its virulence during host infection. In our study, we showed that the WT, encapsulated bacteria possessed the optimized phenotype which is balanced both for the invasive abilities and for the persistence and spread in the host. Studies on the regulation of the *S. sonnei* capsule expression in different stages of the infection would shed light on this important aspect. Genome analysis of several isolates of *S. sonnei*, *S. flexneri*, *S. boydii* and *S. dysenteriae* for the presence of a homologous operon showed that the G4C cluster is highly conserved in *Shigella sonnei*, *S. boydii* and *S. dysenteriae*, suggesting that this could be a common virulence strategy in *Shigella*. An exception is *S. flexneri* where the LPS O side chain has been characterized to be the shielding effector. *S. flexneri* has optimized its virulence phenotype through OAg glucosylation, via a bacteriophage-encoded enzyme, that shortens the O side chain 3D structure and thus enhances the T3SS function without compromising the protective properties of the LPS [80]. Regarding the other serogroups, the functionality of the G4C gene cluster still needs to be tested. However, we confirmed group 4 capsule production in a different isolate of *S. sonnei* (*S. sonnei* 25931, data not shown), indicating that the *S. sonnei* 53G capsule is not a characteristic of a single strain, but likely a common feature of most of the *S. sonnei* isolates.

Figure 30

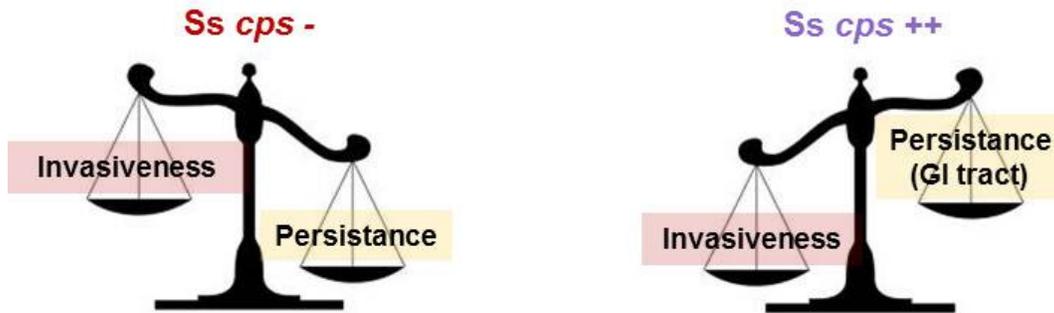


Figure 30: The expression of a G4 capsule (cps) affects the invasive abilities of *S. sonnei* both *in vitro* and *in vivo*. The *Ss* WT encapsulated strain seems to possess the optimized phenotype to efficiently invade the epithelial cells but also to persist in the host and establish systemic infection. Uncapsulated (*Ss* Δ cps, cps-) or hyper-capsulated (*Ss* Δ cps(cps), cps++) strains are both at a competitive disadvantage through a tradeoff between their invasive capacity and their ability to survive innate immune responses.

In addition to their capacity to mask surface virulence factors, it has been shown that capsules possess anti-inflammatory properties. One example is the anti-inflammatory effect of Vi capsule polysaccharide of *Salmonella enterica* serotype Typhi [246]. Recently, animals infected with a Vi-positive *S. Typhimurium* chimera, harbouring the *Salmonella* locus that encodes the Vi polysaccharide, showed a significant reduction in cellular trafficking of innate immune cells, including PMN and NK cells, as compared to animals infected with a Vi-negative strain [247]. The presence of the Vi capsule also correlated with induction of the anti-inflammatory cytokine IL 10 *in vivo*, a factor that impacted on chemotaxis and the activation of immune cells *in vitro* [247]. What makes *S. sonnei* polysaccharide even more interesting in its ability to regulate the host immune response to bacterial infection is its potential to act as a zwitterionic immunomodulating polysaccharide. Zwitterionic polysaccharides (ZPS) are in fact a novel class of major histocompatibility complex class II (MHC II)- dependent antigens characterized by a high molecular weight [248] and by the presence of positive and negative charges within a repeating unit, leading to a helical 3D structure of the polysaccharide [249]. The HMW *S. sonnei* G4C (and less likely, the MMW LPS O side chain) possesses this requisite since an amino group and a carboxylic group are present on the C-4 of the FucNAc4N and on the C-6 of the L-AltNAcA, respectively. These groups are likely to be in the protonated form in physiological conditions. ZPS have been already characterized in other bacteria, e.g. *B. fragilis* (capsular PSA) [250] and *S. pneumoniae* (capsular SP1) [251], and they have shown immunomodulatory properties such as the abilities to induce immunological tolerance through the activation of IL 10 producing- T_{reg} cells, to modulate the balance between Th_1 and Th_2 host responses and to affect the Th_{17} response and proinflammatory signaling, e.g. during *B. fragilis*-induced abscess formation [249]. *In vitro* and *in vivo* studies to evaluate the host adaptive immune response would be needed to understand how the *S. sonnei* capsule polysaccharide modulates the host response in relation to its zwitterionic nature. These studies could give an important contribution in the understanding of *S. sonnei*-mediated tuning of the host immunity during infection. Based on the OAg sugar composition reported for all the other *Shigella*

strains [208], it seems that the zwitterionic nature is a peculiar feature of the *S. sonnei* polysaccharide. As mentioned in paragraph 3.3.2, the main response in natural immunity to *Shigella* is driven by the OAg, thus it represents a valid antigen for the development of a vaccine against *Shigella*. A study to evaluate the IgG subclass response to *Shigella sonnei* and *Shigella flexneri* 2a was performed in subjects naturally exposed to these organisms. It was reported that IgG2 was the major component in the anti-*S. flexneri* subclass response, as observed also in studies performed on *S. dysenteriae* infected patients, while the anti-*S. sonnei* pattern of IgG subclass response was unique among the genus *Shigella*, being predominantly composed of the IgG1 subclass [252]. The relationship between this distinctive immune response and the zwitterionic nature of the *S. sonnei* polysaccharide has not been investigated yet, but could be a reasonable explanation. Conventionally, the immune response to bacterial carbohydrate antigens is considered to be T cell-independent, and the IgG antibodies elicited are primarily restricted to the IgG2 subclass [110]. Therefore, for the development of a saccharide-based vaccine, conjugation of the antigen to a carrier protein is needed to induce a T-cell dependent response. The zwitterionic properties of the *S. sonnei* capsule in the context of a conjugated molecule could magnify the immune response to the potential candidate vaccine. As a support to this proposition, in a recent paper by Gallorini, S. et al., the authors showed that ZPS engineered-glycoconjugates induce higher T-cell and Ab responses to carrier and PS, respectively, compared to control PS-glycoconjugates made with the native polysaccharide form [253].

In conclusion, this study describes a new and interesting finding in the *Shigella* field to achieve a better understanding of the pathogenic mechanism of *Shigella* and in addition of the function of extracellular polysaccharides as virulence factors. Results reported in this thesis will compose a manuscript about the first isolation, and the biochemical and functional characterization of a group 4 capsule in *Shigella sonnei* 53G. Wzy-dependent capsule polysaccharides have been proposed as target for the development of antimicrobial compounds against different pathogens [254]. The discovery of new targets for virulence seems even more important in Gram-negative pathogens, which are traditionally more difficult to treat due to the presence of the outer membrane. Moreover, capsular polysaccharides are important factors in bacterial pathogenesis and have been the target of a number of successful vaccines [255,256]. Therefore, the identification of a capsule in *Shigella* could have implications on the design of new-generation anti-*Shigella* drugs and also on the development of preventive measures, as a vaccine against this enteric disease.

8. ANNEX

Communications

Poster Presentations:

- O antigen of *Shigella* masks surface proteins *in vitro*. Mariaelena Caboni, Pesce, I., Maggiore, L., Berlanda Scorza, F., Colucci, A. M., Saul, A., Gerke, C. (Novartis Research Days 2010, NV&D, Novartis campus, Siena, Italy).
- Polysaccharides on the surface – a new coat for *Shigella*? Mariaelena Caboni, Pesce, I., Berlanda Scorza, F., Maggiore, L., Saul, A., Gerke, C. (EBI–Wellcome Trust Summer School in Bioinformatics 2011, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK).
- Polysaccharides on the surface – a capsule for *Shigella*? Mariaelena Caboni, Pesce, I., Berlanda Scorza, F., Maggiore, L., Saul, A., Gerke, C. (Novartis Research Days 2011, NV&D, Novartis campus, Siena, Italy).
- A deep rough mutant of *Shigella sonnei* reveals the expression of an O antigen capsule. Mariaelena Caboni, Giannelli, C., Maggiore L., Berlanda Scorza F., Saul A., Gerke C. (Life Science Symposium LSS 2012 – Global Health meets Infection Biology, Swiss Federal Institute of Technology EPFL, Lausanne, Switzerland).

Oral Presentations:

- Polysaccharides on the surface – a new coat for *Shigella*? Mariaelena Caboni (Novartis PhD students Workshop 2010, NV&D, Novartis campus, Siena, Italy).
- Protection against *Shigella* in mice by a novel approach using outer membrane particles. Christiane Gerke, Berlanda Scorza, F., Colucci, A. M., Maggiore, L., Pesce, I., Caboni, M., Di Cioccio, V., Bloink, K., Saul, A. J. (Vaccines For Enteric Diseases (VED) 2011, Cannes, France).
- Polysaccharides on the surface – a capsule for *Shigella*? Mariaelena Caboni (Novartis PhD students Workshop 2011, NV&D, Novartis campus, Siena, Italy).
- Developing a *Shigella* vaccine based on generalized modules for membrane antigens. Christiane Gerke, Berlanda Scorza, F., Colucci, A. M., Cole, R., Schrandt, T. A., Maggiore, L., Rossi, O., Pesce, I., Caboni, M., Vandenhoeck, J., Casanova, H., Norais, N., Di Cioccio, V., Bloink, K., Saul, A.J. (Phacilitate Vaccine Forum Washington 2012, Washington D.C., USA).
- A deep rough mutant of *Shigella sonnei* uncovers the expression of an O antigen capsule. Mariaelena Caboni (Novartis PhD students Workshop 2012, NV&D, Novartis campus, Siena, Italy).

Publication:

High Yield Production Process for Shigella Outer Membrane Particles. Francesco Berlanda Scorza, Colucci, A. M., Maggiore, L., Sanzone, S., Rossi, O., Ferlenghi, I., Pesce, I., Caboni, M., Norais, N., Di Cioccio, V., Saul, A., Gerke, C. PLoS ONE (2012), 7(6):e35616.

Courses, Conferences and Scientific Visits

Courses:

- Enhancing your presentation skills (NIBR training course, 2010, Novartis campus, Siena).
- EBI–Wellcome Trust Summer School in Bioinformatics (Wellcome Trust Genome Campus, 2011, Hinxton, Cambridge, UK).
- 1st Scientific Training Course: Vaccinology (ALTA - EIMID^{ITN}, 2011, Novartis campus, Siena).
- How to write a paper (Novartis Academy course, 2012, Keith Veitch, Head of Publications, Novartis Pharma, Novartis campus, Siena).

Conferences:

- Ageing and immunity: Addressing immune senescence to ensure healthy ageing (Novartis conference, 2010, Novartis campus, Siena).
- The importance of population biology in developing bacterial vaccines (Novartis Academy workshop, 2010, Prof. Richard Moxon, Novartis campus, Siena).
- A chemist's overview of traditional and modern vaccines (Novartis Academy workshop, 2010, Prof. Neil Ravenscroft, Novartis campus, Siena).
- Moving Conjugate Vaccines into the future (Novartis workshop, 2010, Novartis campus, Siena).
- Novartis-Harvard meeting: Minisymposium on Vaccines and Host-Pathogen Interactions (Novartis Academy-Harvard medical school symposium, 2012, Novartis campus, Siena).
- Life Science Symposium LSS 2012: Global Health meets Infection Biology (Swiss Federal Institute of Technology EPFL, 2012, Lausanne, Switzerland).

Scientific Visits:

- July, 23rd – August, 5th, 2012: Visiting PhD student, Unité de Pathogénie Microbienne Moléculaire, Institute Pasteur, Paris. Supervisor: Prof. Philippe J. Sansonetti.
- September, 26th – October, 26th, 2012 : Visiting PhD student, Unité de Pathogénie Microbienne Moléculaire, Institute Pasteur, Paris. Supervisor: Prof. Philippe J. Sansonetti.

High Yield Production Process for *Shigella* Outer Membrane Particles

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Abstract

Gram-negative bacteria naturally shed particles that consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. These particles have been proposed for use as vaccines but the yield has been problematic. We developed a high yielding production process of genetically derived outer membrane particles from the human pathogen *Shigella sonnei*. Yields of approximately 100 milligrams of membrane-associated proteins per liter of fermentation were obtained from cultures of *S. sonnei* Δ*tatR* Δ*galU* at optical densities of 30–45 in a 5 L fermenter. Proteomic analysis of the purified particles showed the preparation to primarily contain predicted outer membrane and periplasmic proteins. These were highly immunogenic in mice. The production of these outer membrane particles from high density cultivation of bacteria supports the feasibility of scaling up this approach as an affordable manufacturing process. Furthermore, we demonstrate the feasibility of using this process with other genetic manipulations e.g. abolition of O antigen synthesis and modification of the lipopolysaccharide structure in order to modify the immunogenicity or reactivity of the particles. This work provides the basis for a large scale manufacturing process of Generalized Modules of Membrane Antigens (GMMAs) for production of vaccines from Gram-negative bacteria.

Citation: Berlanda Scorza F, Colucci AM, Maggiore L, Sanzone S, Rossi O, et al. (2012) High Yield Production Process for *Shigella* Outer Membrane Particles. PLOS ONE 7(6): e35616. doi:10.1371/journal.pone.0035616

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Introduction

Shigella spp. are Gram-negative bacteria that infect the intestinal epithelium and cause dysentery. In 1999 the World Health Organization estimated an annual burden of 164.7 million shigellosis cases throughout the year of which 163.2 occur in developing countries, including 1.1 million deaths, mostly in children younger than 5 years of age [1]. Four serogroups have been identified: *S. dysenteriae* (15 serotypes), *S. boydii* (20 serotypes), *S. flexneri* (14 serotypes) and *S. sonnei* (1 serotype) [2]. No vaccine is currently available. So far, vaccine candidates based on O antigen conjugates and live attenuated strains have been shown in clinical trials to protect against homologous strains [3–6]. Vaccines using inactivated bacteria or subcellular components are at various stages of development [3,6].

Gram-negative bacteria naturally shed outer membrane particles consisting of outer membrane lipids, outer membrane proteins, and enclosed periplasmic proteins [7–9]. Unlike most unilamellar biological vesicles, outer membrane particles are formed by blebbing and not by invagination of the membrane.

Thus, the orientation of components in the membrane of the outer membrane particles is the same as in the bacterial outer membrane and the components in the outer face of the bacterial outer membrane are also in the outer face of the outer membrane particles [7]. Outer membrane particles are naturally shed at low concentration. Mutations such as the deletion of gene *gua33* in *Neisseria meningitidis* [10] or modifications of the *tat-pal* pathway of *Escherichia coli*, *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium [11,12] can increase the level of shedding. Especially, deletion of the *tatR* gene in *E. coli* has been shown to result in substantial overproduction of outer membrane particles without loss of membrane integrity [11,13]. Studies have characterized the protein content of these outer membrane particles [10,13], and unlike conventional detergent-extracted outer membrane vesicles derived from homogenized bacteria they are almost free of cytoplasmic and inner membrane components and maintain lipoproteins. The outer membrane particles used for those proteomic studies have been derived in small quantities from cells grown to low cell density.

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