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Shigella
A model of virulence regulation in vivo

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Much is known about the molecular effectors of pathogenicity of gram-negative enteric pathogens, among which Shigella can be considered a model. This is due to its capacity to recapitulate the multiple steps required for a pathogenic microbe to survive close to its mucosal target, colonize and then invade its epithelial surface, cause its inflammatory destruction and simultaneously regulate the extent of the elicited innate response to likely survive the encounter and achieve successful subsequent transmission. These various steps of the infectious process represent an array of successive environmental conditions to which the bacteria need to successfully adapt. These conditions represent the selective pressure that triggered the "arms race" in which Shigella acquired the genetic and molecular effectors of its pathogenic armory, including the regulatory hierarchies that regulate the expression and function of these effectors. They also represent cues through which Shigella achieves the temporo-spatial expression and regulation of its virulence effectors. The role of such environmental cues has recently become obvious in the case of the major virulence effector of Shigella, the type three secretion system (T3SS) and its dedicated secreted virulence effectors. It needs to be better defined for other major virulence components such as the LPS and peptidoglycan which are used as examples here, in addition to the T3SS as models of regulation as it relates to the assembly and functional regulation of complex macromolecular systems of the bacterial surface. This review also stresses the need to better define what the true and relevant environmental conditions can be at the various steps of the progression of infection. The "identity" of the pathogen differs depending whether it is cultivated under in vitro or in vivo conditions. Moreover, this "identity" may quickly change during its progression into the infected tissue. Novel concepts and relevant tools are needed to address this challenge in microbial pathogenesis.

Introduction

Shigella is a Gram-negative bacterium of the enterobacteriaceae family and the etiological agent of bacillary dysentery or shigellosis. Shigella encompasses four subgroups (*S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*). Within each subgroup, different serotypes are identified based on the structure of the O-antigen repeats that comprise the polysaccharide moiety of the lipopolysaccharide (described below).[1] There is a large variety of serotypes among Shigella isolates; *S. dysenteriae* encompasses 15 serotypes, *S. flexneri*, 14 serotypes, *S. boydii*, 20 serotypes and *S. sonnei* a single serotype. Brutal epidemics of bacillary dysentery can be caused by *S. dysenteriae 1* which produces the Shiga toxins, whereas the endemic form of the disease is caused essentially by *S. flexneri* and *S. sonnei*.[2]

Bacillary dysentery is marked by fever, violent abdominal cramps and rectal urgencies. Dysenteric stools characteristically contain erythrocytes, polymorphonuclear neutrophils (PMNs) and mucus, reflecting the invasive character of Shigella. Severe complications may occur such as intestinal perforations, septicemia and toxic megacolon. Resistance to multiple antibiotics has recently been observed, including fluoroquinolones,[3] which increases the threat of the occurrence of severe disease forms due to the lack of efficient treatments. Unfortunately, no vaccine against shigellosis is available despite multiple and diverse vaccine design strategies (live-attenuated strains,[4] glycoconjugate molecules[5]).

Shigella chromosomal genes control the two most studied pathogen-associated molecular patterns (PAMPs): the lipopolysaccharide (LPS) and the peptidoglycan (PGN). These molecules are located respectively on the bacterial surface and between the inner and outer membrane. Shigella is characteristically devoid of flagellar expression and expresses a LDC (lysine decarboxylase) negative phenotype.[6] Shigella virulence is based on the presence of a large virulence plasmid,[7,8] encoding a Type III-secretion-system (T3SS), and a set of secreted proteins that are thevirulence effectors associated in some cases with their dedicated chaperones.[9] The virulence plasmid also encodes several autotransporters (Type V-secretion-system, T5SS), including SepA and IcsA. The T3SS is composed of about 22 proteins, including a needle-like protein oligomer (MxiH) anchored in the membrane through a needle 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. At the base of the inner membrane, a basal body and a soluble complex are sequentially assembled.

T3SS are responsible for the injection of virulence factors into host colonic epithelial cells, leading to bacterial invasion and its corollary, the inflammatory destruction of the epithelial lining.

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They also allow for the injection of a set of molecules whose function is clearly to antagonize both innate and adaptative immune responses (as reviewed in refs.10 and 11). How Shigella causes epithelial inflammation is still not fully understood. Two major pathways have been shown to be involved: sensing of the PAMPs by the cell-surface expressed receptors TLRs (Toll-like Receptors) and the intracellular receptors Nod (Nucleotide oligomerization domain). In fact, Shigella has been instrumental in the discovery of the molecules involved in the intracellular sensing of PAMPs[12] and pyroptosis.[13]

A large number of studies have led to the description of the composition and structure of these components (T3SS, LPS and PGN). However, most of these studies were performed in vitro using purified molecules. These approaches do not take into account the putative fine molecular tuning which likely occurs during the infectious process in vivo, especially at the intestinal surface which is still largely terra incognita. Indeed, Shigella has acquired, through co-evolution, the ability to adapt to different environmental conditions (i.e., pH, temperature, osmolarity or oxygen) due to the expression of a set of transcriptional regulators. As demonstrated previously in the case of oxygen sensing and T3SS structure and function,[14] it is anticipated that crosstalk exists between various environmental sensors activation and the T3SS, but also LPS or PGN structural modifications. The physiological analysis of the modification of the structure and the function in vivo will provide essential information to further characterize these "missing links" and improve our understanding of Shigella's "identity" during the infectious process.

In this review we present recent discoveries in the field of Shigella virulence modulation in vivo, with special emphasis on *Shigella flexneri*. We specifically focus on structure-function modifications of secretion systems and PAMPs on the gut epithelium surface in response to environmental cues. This emerging field of research is an ambitious challenge aimed at characterizing Shigella as it is encountered by the human host during infection.

Investigating Shigella Physiopahtology In Vivo

Physio-pathology of shigellosis. *Activation of the host inflammatory response.* The main steps of colonic infection by Shigella are adhesion, invasion, intracellular replication and cell-to-cell spread. Each of these steps is likely to be regulated by environmental cues. Shigella invasion of the host colonic epithelium is T3SS-dependent. It is associated with a massive recruitment of PMN, which is in part elicited by IL-8 release by infected epithelial cells and leads to tissue destruction. Shigella induces a controlled inflammatory response which includes release of both inflammatory (IL6, IL-8, IL1β, TNFα and β) and anti-inflammatory cytokines (IL-10 and TGFβ).[15]

The modulation of the adaptive immune response by Shigella is currently under investigation, and is focused on the sensitivity of B cells and T cells to the microorganism. It has recently been shown that T-cell priming and motility is modulated by *S. flexneri*.[16,17] Further investigations are required to understand the weak immune response elicited by natural shigellosis as observed in the infected human colon, which is the natural site of Shigella invasion (reviewed in ref. 18). The effect of environmental cues on the control of the inflammatory response by Shigella is largely unexplored, although it has recently been demonstrated that the hypoxia-inducing-factor (HIF-1α) controls the balance between regulatory T cells (Treg/TH17) in response to oxygen tension modulation.[19]

The modulation of the innate and adaptive immune responses is not described in this review, but is an appealing field of research which remains relatively unexplored. In this context, it is crucial to study this pathogen in conditions which replicate as closely as possible the human colon for in vivo analysis and the choice of an appropriate animal model is a prerequisite..

Animal models of Shigellosis. What is the optimal animal model in which to explore virulence regulation? Ideally, this model should be easy to set up and should restitute as closely as possible the conditions met by Shigella in the human gut. No model currently satisfies these criteria.

The macaque monkey model is the most appropriate as it allows intragastric inoculation,[20] resulting in the development of an acute colitis similar to the disease observed in humans. This model is not widely used, however, on account of its cost and surrounding ethical issues. On the other hand, the rabbit ileal loop model is permissive to intestinal infection by Shigella and results in rupture, invasion and inflammatory destruction of the epithelial lining. The influx of PMNs observed in this model in zones of bacterial invasion allows reliable quantification of the invasive phenotype. This model was key to the identification of the anti-inflammatory function of some Shigella effectors. Additionally, this model has been used as a standard assay to validate the invasive/inflammatory phenotype of Shigella mutants, and to monitor their adaptation and ability to colonize the intestinal lumen compared with the wild-type strain through Competitive Index calculation (i.e., Signature Tagged Mutagenesis screen; see refs. 21 and 14). However, the microenvironments of the ileum and of the colon differ particularly with respect to oxygen tension, pH, nature of the nutriments and of the local microbiota. Furthermore, while the ileal mucus layer is thin (< 300 μm), the thickness of the colon mucus layer can reach 1 mm. In addition, in both cases, the backbone of the mucus layer is mainly composed of Muc2, but it shows differential glycosylation profiles in each environment being enriched in either sialylated or sulfated oligosaccharide species respectively in the ileum and the colon.[22,23] These specificities might play a crucial role in mucus colonization by the microbiota or Shigella and adaptation of this model for studies in the colon is warranted.

More recently a Guinea pig model of colonic infection was proposed[24] and confirmed by different teams, including ours (Nigro G et al., unpublished data). Young animals (weight < 250 g) develop shigellosis following intracolic inoculation by wild-type Shigella (10^{11} CFU (Colony-forming Unit)/animal). Epithelial destruction associated with neutrophil recruitment and acute inflammation is observed 7 h after inoculation. Weight loss may be assessed after 24 h of infection.[24] This model, despite the current shortage of genetic and immunological tools, represents

an appealing perspective because it is simple and relevant to the site of development of shigellosis. It allows for better characterization of Shigella infection, especially regarding environmental parameters (such as pH, oxygen, osmolarity), although development of tools to measure these parameters in vivo is still required. While this animal model of shigellosis does not allow for oral infection, it iscurrently the only model that allows environmental adaptation in the colon.

A large amount of work has been invested in the development of a mouse model of infection. For reasons which are not yet clear (as discussed in ref. 11) the intestine of adult mice is not susceptible to Shigella infection (via oral, intragastric and intrarectal inoculation). It is not impossible, however, that resistance is due in part to the extreme susceptibility of Shigella to murine epithelial defense molecules. Newborn mice are susceptible to Shigella and develop lethal enteritis until 5 d. At this stage, acquisition of the resistance status is correlated with the appearance of Paneth cells and the concurrent expression of anti-microbial peptides, particularly α-defensins, lectins and enzymes such as lysozyme, phospholipases and proteases.[25] This model may help address a limited set of issues, but does not address all of the characteristics of shigellosis, while the age restricted susceptibility indicates that the bacterium is not exposed to the full spectrum of host innate defenses.

The subcutaneous human colon xenograft has been used as a model of infection in a limited number of studies.[26] Although complex and logistically demanding, this model allows for the study of infection in a species-specific context, at least for the epithelial lining. It permits studies of the early phases of epithelial invasion, but does not allow for immunological studies, due to the immuno-deficient background of the transplanted mouse. Its complexity and limited capacity indicate that it is not entirely suitable for regulatory studies that require a fair amount of screening capacity.

In contrast, the murine pulmonary model of infection has been used in a large number of studies following intranasal inoculation. The advantage of this model resides essentially in its accessibility and in the availability of a large array of immunological probes and of numerous lines of KO and transgenic mice, allowing for the analysis of innate and adaptive immune responses to Shigella wild type and various mutants. However, the lung structure and microenvironment (including mucus composition, epithelium surface oxygen tension), the triggering and development of inflammation and the nature of the epithelial barrier are significantly different compared with the colon. Consequently, this diminishes its degree of relevance for the study of regulatory processes because the cues that are imposed are largely irrelevant.

In summary, the rabbit ligated ileal loop model, even if not ideal, has been extremely useful for pathogenesis studies and enabled the identification and characterization of several virulence phenotypes. The guinea pig model now represents a more appealing model to study the environmental modulation of Shigella virulence because it reflects the more natural human situation. It remains to be proven whether it possesses sufficient sensitivity to detect the effect of key regulatory cues. More to colonizing the colonic mucosa, Shigella faces and adapts to various local environments which are described below.

Environmental changes during the colonization process by Shigella. *Crossing the GI tract.* After oral infection, Shigella encounters gastric acidic pH (pH 1–2) before reaching the duodenum, the jejunum, the ileum and finally the colon, which is the site of colonization. The duodenal pH is 6–6.5, the jejunal and ileal pH are 7–8 and the colonic pH is rather alkaline (pH 8). Shigella propagates in the GI tract at 37°C until reaching the colonic epithelial surface. Intestinal invasion leads to an inflammatory response which causes an acute colitis and a fever that can peak at 40°C.[33] Bile acids released upon shigellosis seem to increase the levels of fecal steroids in infected patients, including cholic acid and chenodeoxycholic acid, but also 7-α-dehydroxylated derivatives (deoxycholic and lithocholic acid). Simultaneously, cholesterol derivatives level decrease.[27]

Interface with the colonic epithelium. Upon reaching the colon, Shigella colonizes a thick mucus layer which covers and protects the epithelial surface. It is relatively unexplored becuase of its fragility upon extraction and further in vitro investigation is warranted. Better characterization requires development of novel techniques for direct in vivo observation (i.e., 2-photon microscopy) or optimized histological techniques that preserve its organization and composition.[28] For example, the thickness of the human colonic mucus layer in vivo is estimated around 1 mm, with the factors involved in thickness control largely unknown.[29] This layer is composed of 10% glycoproteins, with a majority of mucins (Muc2, Muc5ac, Muc5b and Muc6) secreted by goblet cells. Since mucus detection in patient stools has a diagnostic element, it is proposed that Shigella has the ability to disorganize and disrupt this cell-surface gel-forming layer. It has been demonstrated that cell-surface expressed serine proteases, such as Pic, contribute to this property (see below). Shigella reaches and invades epithelial cells in a T3SS-dependent manner (for T3SS structure description, see below). In vitro, Shigella invades epithelial cells, lyses the membrane-bound phagocytic vacuole,[30] escapes autophagy,[31] grows within the cytoplasmic compartment, and spreads from cell-to-cell by exploiting the host cell cytoskeleton as a motor.[32]

Reactive oxygen and nitrogen species (ROS and NO) are produced during the infection process, through increased expression of the epithelial inducible nitric oxide synthase (iNOS) and in association with the specific PMN myeloperoxidase (MPO) activity.[34–37] Intracellular Shigella also triggers the mitochondrial Nod-like receptor NLRX1, which is indirectly involved in ROS production through the amplification of NFκB activation.[38]

Additionally, Shigella infection tends to be confined to the epithelial layer of the colonic mucosa, where it may be associated with mucosal abscesses and ulcerations and leads to severe tissue damage that accounts for the clinical manifestations of dysentery.[39,40] However, the physiology of Shigella within abscesses has not been studied, especially as relates to the innate response efficiency in this microenvironment.

Altogether these observations point to the critical question of the physiological adaptation of Shigella in the infected colon. This aspect is largely unexplored as changes of cues such as the

pH or the oxygen availability have not so far been characterized upon infection, even if they are likely to occur and to play a critical role in Shigella virulence.

Sensing of the environment by Shigella. Sensing environmental changes is mostly mediated by transcriptional regulators. Inactivation of a single master bacterial sensor leads to the attenuation of the corresponding mutants in vivo (e.g., the oxygen sensor FNR (fumarate-nitrate reductase regulator)[14] and virF[41]). The diversity of transcriptional regulators activated throughout the infectious process reflects the variety of environmental conditions Shigella faces during infection of the colon. The most studied cues to which Shigella is exposed are listed below and include pH, osmolarity, temperature, oxygen tension, magnesium, ROS, NO and intracellular conditions.

pH. The resistance of Shigella to the gastric acidic pH is based on RpoS.[42] It is a RNA polymerase sigma factor controlling the expression of *hdeAB*, which is a cistron encoding the periplasmic charperone-like proteins HDEA and HDEB which mediate the resistance of Shigella to low pH.[43,44] CpxA/cpxR is a stress response-activated two-component system that is also involved in pH sensing. It modulates the expression of *virF* upon sensing the appropriate environmental pH (activated at pH 7.4 and repressed at acidic pH)[45,46] (**Fig. 1**).

Osmolarity. Low osmolarity and low temperature are sensed as inhibitory conditions for the expression of a set of virulence genes (e.g., Ipas, icsA, mxiC), through the activation of the H-NS repressor (nucleoid-structuring protein).[47,48] In turn, H-NS is responsible for the temperature-dependent repression of *virF* expression at low temperatures.[49] This result was confirmed-by the discovery of novel H-NS like proteins (Sfh, StpA) involved in the repression of *virF*.[50,51]

Temperature. VirF is a global transcriptional regulator activating *virB* expression which in turn controls the expression of several operons encoding the invasion genes (reviewed in ref. 52). Shigella shows optimal activation of the expression of a set of virulence factors at body temperature (37°C), while nothing is

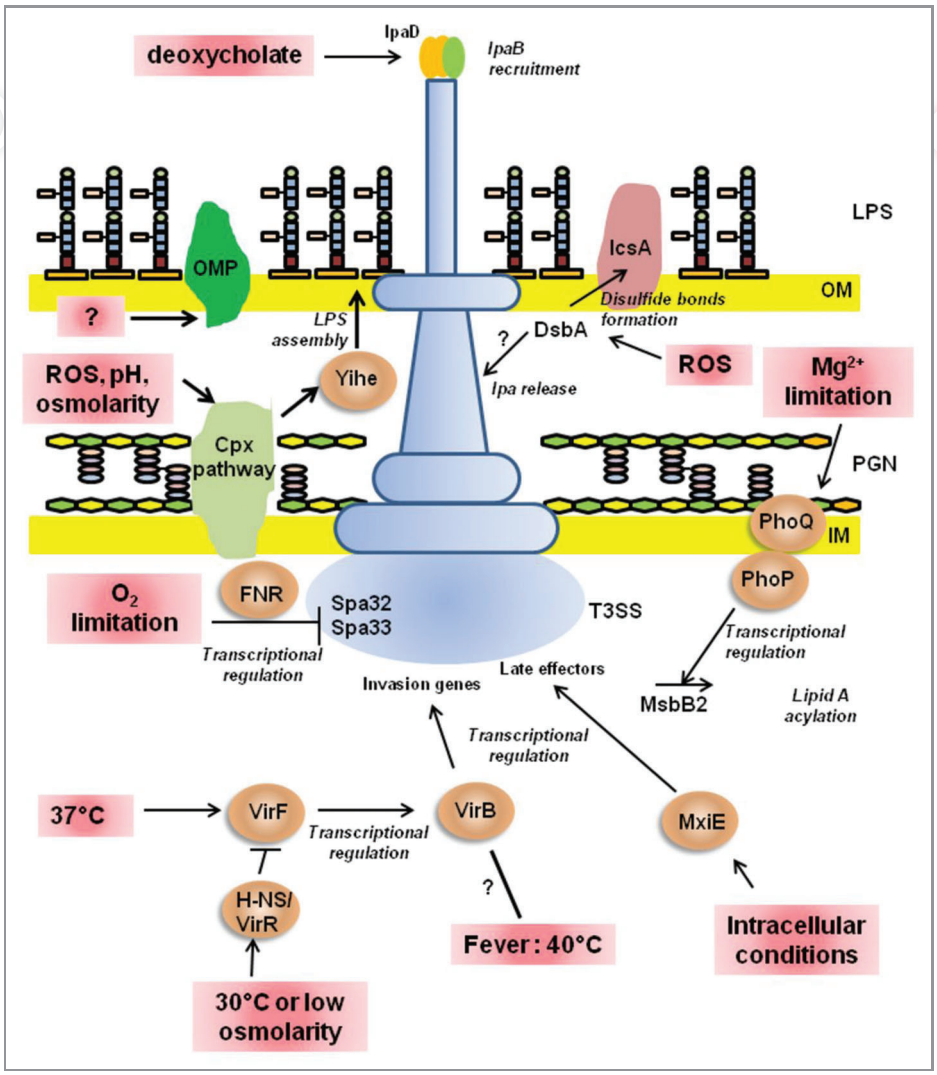

Figure 1. In vivo modulation of Shigella T3SS and PAMPS structure and function. Schematic representation of environmental cues action on T3SS and PAMPS structure and function modulation.

known concerning virulence genes expression at the higher temperatures of 40°C, which may occur during acute shigellosis (**Fig. 1**).

Oxygen. Oxygen availability in the colonic lumen should be low; however, oxygen has been observed diffusing from the epithelial surface through the thick mucus layer.[14] The transcriptional regulator FNR has been shown to mediate the adaptation of Shigella to low-oxygen environments, controlling the metabolic shift necessary for bacterial survival in anaerobic conditions but also modulating the structure and the function of the T3SS (**Fig. 1**).[14] Nevertheless, no quantitative experiment of the oxygen tensions has yet been performed in vivo.

Magnesium. Magnesium sensing is mediated by the PhoP/PhoQ two-component global regulator system[53] which has been shown to be essential for Shigella survival in the host cell vacuole in phagocytes. PhoP/PhoQ-mediated regulation of virulence in the context of the colonic surface remains to be characterized (**Fig. 1**).

ROS and NO. ROS and NO production have been reported during shigellosis on the infected epithelial surface (see above). Shigella resistance to ROS appears to be mediated by OxyR and SoxRS,[54] even though the relevance of these mechanisms remains to be demonstrated in vivo.

Intracellular conditions. After epithelial cell invasion, Shigella encounters intracytoplasmic conditions when the intracellular vacuole is ruptured. It has been demonstrated that MxiE, a transcriptional regulator belonging to the AraC/Xyl regulator family, specifically activates the expression of a set of effector genes—including *ospB*, *ospC1*, *ospE2*, *ospF*, *virA* and *ipaH (9.8)*[55,56]—upon perception of the T3SS activity, a situation that reflects engagement of a target cell.[57] This likely occurs during the entry process and in the intracellular compartment, an environment in which the cues that may affect *mxiE* expression and the activity of its product are largely unknown.Although the identification of specific transcriptional regulators involved in the sensing of environmental parameters is a direct approach, it has also been reported that the structural properties of several cellular components are sensitive to environmental cues. For example, DsbA, the periplasmic oxidoreductase, is involved in ROS sensing.[58,59] The T3SS needle component MxiH is sensitive to temperature and pH,[60] which might have consequences in vivo on Shigella virulence at different sites of the gastro-intestinal tract.

This list will probably expand over the next years as studies on in vivo adaptation are actively in progress (e.g., Nitrosylation, etc.).

The Virulence Determinants of Shigella and Their Modulation in Response to Environmental Changes In Vivo

In the second part of this review, we present most well-characterized bacterial surface components involved in the interaction with host cells, focusing on the Type III and V secretion systems (T3SS, T5SS) and on the two main PAMPS, the LPS and the PGN. The synthesis and assembly of these components are well-studied, multi-step pathways, which are likely to be simultaneously regulated by several transcriptional factors, sensing and responding to environmental cues. However, most of these putative modulations are probably unknown. For this reason, we begin by summarizing and updating recent discoveries on the assembly of these components before describing their role in bacterial-host interaction.

The Type III secretion system (T3SS). *Secretion across three membranes.* The delivery of virulence factors into host cells by Gram-negative bacteria are required for the evolution and selection of complicated pathways necessary to bypass physical barriers consisting of three membranes: the inner- and outer-membrane of the bacterium and the cell cytoplasmic membrane. Some of these mechanisms likely evolved from bacterial surface organelles like flagellum or pili. The secretion systems form a continuous secretion mechanism that takes place without a periplasmic intermediate.[61] Among them the T3SS and the Type IV Secretion system (T4SS) are well-established pathways dedicated to crosstalk by delivery of bacterial factors (proteins or DNA) to the cytosol of eukaryotic cells.[62-64]

T3SS architecture and conserved features. T3SS are encoded by large operons, usually 20–40 kb long, that are found on pathogenicity islands.[63] The virulence of Shigella is defined by a virulence plasmid allowing the expression of the T3SS components.[8] T3SS is a multicomponent apparatus spanning both bacterial membranes with a needle-like extracellular appendage that delivers effectors. This extracellular appendage is built by a unique protein subunit, $MxiH^{SctF}$ (in this review, species-specific names are used, followed by standard T3SS nomenclature proposed by Hueck[66] in superscript), that is itself a T3SS secreted substrate. The integral bacterial membrane part of the T3SS apparatus consists of a series of rings that strengthen the attachment of this appendage to the bacterium and are together termed the T3SS needle complex. The protein that forms the outer membrane ring belongs to the secretin protein family, which are common to the Type II Secretion System (T2SS), the main terminal branch of the general secretion pathway (GSP), as well as to the Type IV pili system (T4PS).[65] Outer membrane integrated secretins consist of various domains with the C-terminal being the most conserved. The N-terminal domains of secretins are less conserved, due to different contact with various inner membrane proteins of each system as they form periplasmic rings. The $MxiD^{SctC}$ secretin of the T3SS of *S. flexneri* is shown in red in **Figure 2**. In most systems, secretins have specific roles in the system biogenesis.[67] Secretins possess an N-terminal signal that mediates their secretion into the periplasmic space through GSP. They are thought to be delivered to the outer membrane through a specific small lipidated protein termed pilotin via the Lol (lipoprotein outer membrane localization) pathway.[68] Pilotin proteins from various systems possess no detectable sequence similarity despite their common function. The MxiM pilotin of *S. flexneri* possesses a different fold from the T3SS pilotin ExsB from *Pseudomonas aeruginosa* or the T2SS pilotins from *Neisseria meningitis* or *P. aeruginosa.*[69] This trait is probably an outcome of the interaction of pilotins with the non-conserved C-terminal tail of various secretins. In *S. flexneri*, the last 46 residues of the $MxiD^{SctC}$ secretin are

structured upon binding to MxiM.[68] The inner membrane rings in Shigella are formed by MxiG^{SctD} and MxiJ^{SctJ}, the latter being homologs of the FliF protein of the bacterial flagellum (external blue rings in **Fig. 2A**). MxiG^{SctD} is a single-pass inner membrane protein that oligomerizes to form the most external inner membrane ring of T3SS. The central density observed in the inner membrane rings of a T3SS needle complex reconstruction map from *Salmonella enterica* sv Typhimurium is attributed to the SpaP^{SctR}, SpaQ^{SctT}, SpaR^{SctS}, SpaS^{SctU} and InvA^{SctV} proteins.[70] Corresponding homologs of Shigella are shown in **Figure 2** in blue (Spa24^{SctR}, Spa29^{SctT}, Spa9^{SctS}) or pink (Spa40^{SctU}, MxiA^{SctV}).

In this T3SS core structure, numerous cytosolic components which are recruited to orchestrate the secretion of various T3SS substrates based on priorities imposed by T3SS biogenesis and scope of action. Almost all the cytosolic components have their homologs in the bacterial flagellum. Indeed the FliM/FliN pair is responsible for the formation of a large cytoplasmic ring (C-ring) in the flagellum.[71] These cytosolic components are mainly represented by a unique polypeptide in most T3SS (in **Fig. 2** the cytoplasmic ring is represented diagrammatically in green). Electron microscopy analysis in Shigella revealed that the Spa33^{SctQ} protein is localized beneath the T3SS needle complex via interactions with MxiG^{SctD} and MxiJ^{SctJ}.[72] However, the structure of the C-ring could not be characterized. Recently, Lara-Tejero and colleagues[73] reported the presence of a large platform in *S. enterica* sv Typhimurium T3SS sorting substrates prior to secretion. This cytoplasmic platform consists of SpaO^{SctQ}, OrgA^{SctK} and OrgB^{SctL}. InvC^{SctN} possesses the α/β-like subunit-like fold of the F_0-F_1 ATPase allowing anchoring, possibly, through a γ-like F_0-F_1 ATPase subunit (SctO protein family[74,75]).

T3SS substrates and switches. Three groups of T3SS substrates can be defined with respect to their differential secretion timing, leading to the T3SS injectisome gradual assembly prior the effectors secretion. After the localization of various T3SS rings in bacterial membranes and subsequent integration of the T3SS core proteins (SctR,S,T) in the inner membrane, the MxiH^{SctF} protein in Shigella builds the needle (shown in yellow in **Fig. 2**) and MxiI^{SctI} builds the inner rod (shown in orange in **Fig. 2**). They are the first secreted substrates. Both of these proteins are small enough to be exported without the involvement of the cytoplasmic export gate, as opposed to larger following substrates after its activation by an external signal.[76] This substrate specificity switching involves Spa40^{SctU} and Spa32^{SctP} in Shigella. Spa40^{SctU} was found to interact with several cytoplasmic proteins including Spa32^{SctP}, Spa33^{SctQ}, Spa47^{SctN}, MxiK^{SctK} and MxiN^{SctL} but also with the inner-membrane associated protein MxiA^{SctV}. These interactions depend on Spa40^{SctU} autoprocessing in its NPTH conserved motif. The inactivation of *spa40^{sctU}* abolishes protein secretion and leads to needle negative T3SS[77] while the

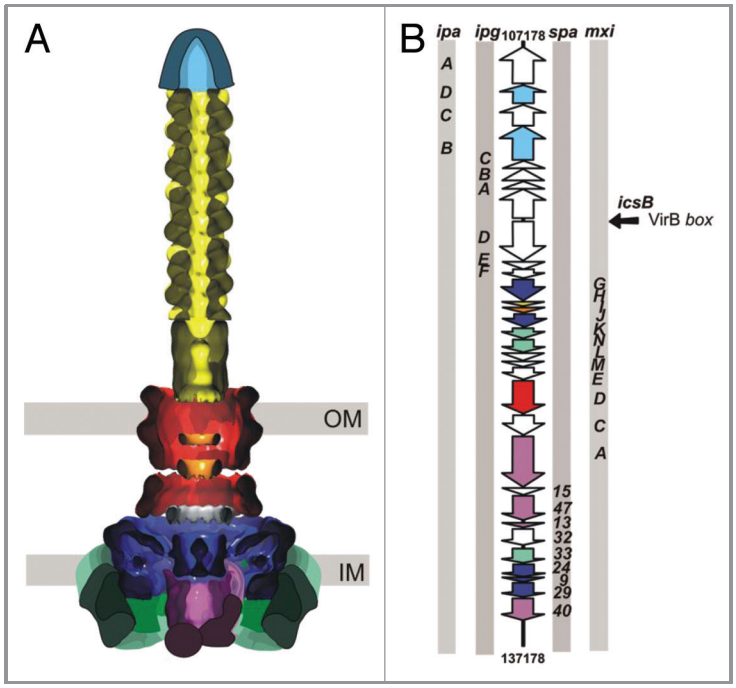

Figure 2. Overall architecture of the *S. flexneri* T3SS injectisome and genetic organization of the gene cluster coding for the various subunits. (A) Overall architecture of the *S. flexneri* T3SS injectisome. The needle complex is represented as a cut through view of the 3D-reconstraction map (EMDB_1617) based on negative staining electron microscopy data and presented here in a contour level of 1.58. Various parts of the needle complex are depicted in different colors using the Color Blob tool of Chimera (see ref. 149). The inner membrane rings, consisting of the MxiG and MxiJ proteins, as well as the central part (probably consisting of the core T3SS proteins Spa24, Spa9 and Spa29) are depicted in blue. Genes responsible for the production of these proteins are also depicted in blue in B. Red blobs represent the density attributed to the T3SS secretin rings formed by the MxiD protein. Small rings central to the secretin rings depicted in orange are reminiscent of the inner rod structure that forms a continuous conduit to deliver proteins from the T3SS base to the needle. The needle is depicted in yellow. The lower featureless part of the needle is portion of the EMDB_1617 map, while the upper helical part is an autonomous needle helical reconstruction map (EMDB_1416) based on negative staining electron microscopy data and verified by X-ray fiber diffraction methods (see ref. 150). On top of the needle the needle-tip is usually found, which consists mainly of the IpaD protein and possibly IpaB, here depicted diagrammatically in light blue. The hypothetical T3SS cytoplasmic sorting platform (see ref. 73) of the *S. enterica* is also shown diagrammatically here as a green ring in the cytoplasmic part of the T3SS injectisome. *S. flexneri* T3SS proteins that hypothetically build the cytoplasmic platform in analogy to *S. enterica* T3SS are Spa33, MxiK and MxiN. Central to the platform should probably located proteins related to the T3SS ATPase of the A0-A1 type, like Spa47 (A/B subunits), Spa13 (D subunit) while a MxiA carboxyterminal subdomain is reminiscent of the peripheral stalk of the archaeal A0-A1 ATPases. Homologs of MxiA along with Spa40 have been characterized as T3SS gate proteins. These are represented in pink colors, while the pink central part that represents the ATPase (A/B subunits) is an actual cut through view of the HrcN negative stain electron microscopy reconstraction map (EMDB_1160). HrcN is a homolog of Spa47 from the T3SS of *Pseudomonas syringae*. IM: Inner Membrane, OM: Outer Membrane. (B) Genetic organization of the *S. flexneri* T3SS gene cluster. Open reading frames (ORFs) are indicated by arrow-shaped boxes, depicted in the sense orientation. ORFs coding for specific protein subunits that build the T3SS injectisome are colored according to A. Rest of the genes that are not thought to participate in the injectisome structure or are not represented in A in a diagram are left white. Gene symbols on the right and on the left of the ORFs (A, B, C, etc.) correspond to the main four nominal gene categories: mxi, spa, ipa, ipg. Diagrams are based on annotated information available in Virulence Factor DataBase (VFDB) for *S. flexneri*.

interaction of Spa40^{SctU} with Spa32^{SctP} is required for the control of the needle length.[78]

Subsequently, the needle tip forming protein (IpaD) and the translocation pore (IpaB and IpaC) are inserted into the host membrane. IpaD polymerizes at the tip of the needle to form the needle tip structure (light blue part of the diagram in **Fig. 2A**). Different kinds of chaperones (see below) are recruited to prevent a premature oligomerization of these T3SS substrates in the bacterial cytoplasm before secretion. IpaD, in comparison to the equivalent protein from Yersinia (BipD), possesses an intramolecular chaperone domain.[79] IpaC and IpaB are chaperoned by IpgC, a protein that possesses some TPR repeats found to homodimerize. The IpaB chaperone-binding domain (CBD) was found to be in an extended conformation when bound to the IpgC chaperone.[80]

With the second signal certifying that the T3SS needle is docked to the translocation pore, the T3SS effector proteins are the last to be released.[76] In Shigella MxiC^{SctW,} it is thought to block their secretion prior to docking to the host membrane. It has been characterized as the "T3SS gatekeeper" in various T3SS. The absence of MxiC^{SctW} leads to a constitutive secretion of T3SS effectors and impaired the secretion of the translocators.

T3SS chaperones. Some specific chaperones are associated with several T3S substrates as targeted to the secretion apparatus, thus keeping them in a secretion competent state and preventing their premature polymerization in the bacterial cytoplasm or their premature association with other partners. Three classes of chaperones have been identified to date.[81]

The first class involves proteins that bind the T3SS effector proteins. These chaperones were identified first since they possess some characteristics common across various T3S systems. Although, they are difficult to identify by primary structure comparison, they possess a common topology of α-β-β-β-α-β-β-α secondary structure elements with minor exceptions that fold in a two-layer sandwich. The two layers are formed by an antiparallel β-sheet with the clustering of α-helices on one side of the β-sheet. The second α-helix of each monomer contributes to their dimerization interface as class I chaperones are only found to be dimeric in solution (homodimers or heterodimers). These chaperones bind their cognate effector/effectors through a β-strand extension mechanism.[82] Effectors bind chaperones through their Chaperone Binding Domain (CBD) which is located in their N-terminal region, downstream of the T3SS secretion signal which is usually attributed to the first 15 residues of the effectors.[82] The CBD is usually disordered and a portion of it gains structure by forming a small β-strand that interacts with the β-sheet layer of the chaperone.

In the case of specialized T3SS effector chaperones (class IA), the corresponding gene is usually located in close proximity to the T3SS associated effector gene in an operonic organization. Recently, Button and Galan[83] identified a mechanism that coordinates the expression of the *S. typhimurium* class IA chaperone SicP and its cognate effector, as in an inhibitory RNA structure the absence of their translational coupling prevents the translation of the effector. So far, in *S. flexneri*, two IA chaperones have been identified (IpgA and IpgE) which bind the IcsB and IpgD effectors respectively.[84,85]

Some cases of T3SS effector chaperones that bind multiple effectors have also been reported (class IB). These chaperones are usually encoded from a T3SS gene cluster. In *S. flexneri*, Spa15 is a class IB chaperone which was found to be homodimeric in crystals and in solution possessing the classical 2-layer sandwich-fold of class I chaperones.[86] However, the main difference with other class I chaperones is that the second α-helix of the dimer interface adopt a different orientation between the monomers that leads to a 30° rotation with respect to each other.[87] Spa15 binds the effectors IpaA, IpgB1, IpgB2, OspC3, OspC2, OspD1 and OspC1.[88] Another class 1B chaperone, OspD1, binds the activator MxiE and acts as an anti-activator before the secretion activation.[89]

The class II T3SS chaperones bind the translocator proteins. These are usually all α-helical proteins that possess tetratricopeptide repeats (TPR). In *S. flexneri,* IpgC binds the translocator proteins IpaC and IpaB. Two chaperone-binding domains in tandem have been identified in the N-terminal of IpaB while there is only one in the IpaC sequence.[90] IpgC captures the respective chaperone-binding domains of IpaB in an extended conformation.[80]

The class III T3SS chaperones associate with the α-helical subunits of the T3SS needle and the needle tip components (MxiH and IpaD). This class of chaperones is also extended beyond the non-flagellar T3SS to the flagellar ones as it involves known chaperones of the flagellum secretion substrates. This class is structurally the most variable. The common tendency of these proteins is to form coiled-coil interactions with the secretion substrates in order to prevent the premature polymerization of the substrates in the bacterial cytoplasm prior to their secretion. In *S. flexneri* chaperones for the MxiH have not been identified. In another hand the IpaD needle tip protein of *S. flexneri* possess an N-terminal domain that prevents premature oligomerization and acts as intramolecular class III chaperone.[79]

Host-T3SS components/effectors interaction. *Inv-Mxi-Spa T3SS translocators and the entry locus.* Bacterial uptake by non-phagocytic cells is triggered by manipulating the host cell actin. In Shigella the genes responsible for bacterial entry are organized in an operon (IpaBCDA).[91]

The C-terminal domain of IpaC is located inside the host cell cytoplasm after the pore is formed. It was shown to induce the Src-dependent actin nucleation and ruffle formation during *S. flexneri* invasion.[92,93] IpaC also activates Cdc42, which in turn activates Rac, two of the most important GTPases involved in *S. flexneri* entry.[94] IpaB was shown to interact with the host CD44 hyaluronan receptor and participates in the invasion of epithelial cells[95] while also inducing pyroptosis in macrophages through caspase-1.[96] The T3SS effector IpaA appears to promote the disassembly of the filamentous actin by forming a complex with vinculin that caps the actin filaments. The disassembly of the filamentous actin may provide actin monomers for the biogenesis of new filaments on behalf of the bacterial uptake.[97]

VirB-dependent T3SS effector expression. Operons encoding the main structural components and early effector proteins of the *S. flexneri* T3SS are repressed (as are many other principal virulence genes) by the H-NS system. De-repression of these genes is achieved by VirB, a protein that is similar to the ParB family of plasmid partitioning proteins. Bioinformatic analysis suggest that VirB has been co-opted into its current role as an H-NS antagonist in *S. flexneri*.[98] The *virB* gene transcription is activated by VirF and is temperature-dependent. When bacteria are grown at 37°C, the transcription of *virB* is highly activated, while at 30°C the level of the transcription of *virB* decreases significantly.[58] The *icsB* gene is immediately downstream of some VirB binding sites in the T3SS gene cluster (**Fig. 2B**). It encodes for a T3SS effector that helps Shigella to evade the host autophagy system by binding to the Atg5 host protein. At the same time, it possesses a domain between residues 288–351 that is capable of binding cholesterol.[77] *IpgA, ipgB* and *ipgC* are located downstream of the *icsB* gene. *ipgA* encodes for an IcsB chaperone.[85] T3S effectors IpgB1 and IpgB2 directly manipulate Rho GTPase-dependent actin polymerization. *ipgB* (or *ipgB1*) encodes for an effector with a pivotal role in producing membrane ruffles by exploiting the RhoG-ELMO-Dock180 pathway to stimulate Rac1 activity.[99] IpgC is the chaperone of translocator proteins, as previously described.[80]

The *ipgD* gene is immediately downstream of the VirB binding site in the T3SS gene cluster facing the opposite direction of the *icsB* gene (**Fig. 2B**). IpgD is a phosphoinositide 4-phosphatase responsible for the inhibition of chemokine-induced T cell migration. This effect is achieved by reducing the phosphatidylinositol 4,5-bisphosphate (PIP^2) pool at the host cell membrane resulting in the formation of the polarized edge required for cell migration.[17]

T3SS effectors controlled by VirB and MxiE. OspF and OspC1 are T3SS effectors required for post-invasion and both of them have been found to localize in the host cell nucleus. They are both implicated in the mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase pathway activation.[100] OspF harbors a phosphothreonine lyase activity that irreversibly dephosphorylates MAPKs p38 and ERK to dampen innate immunity activation. It was unexpectedly found to potentiate the activation of JNK and NFκB by disruption of a negative feedback loop between p38 and TAK1, while inhibiting the transcription of c-Jun.[101] OspB, as well as OspF, facilitate the remodelling of chromatin via interactions with retinoblastoma protein, resulting in diminished inflammatory cytokine production.[100,102]

Besides manipulating host cell signaling pathways after invasion, some T3SS effectors are dedicated to promoting the intracellular motility of Shigella. The *virA* gene is controlled by VirB as well as the adjacent *icsA* autotransporter gene which faces the opposite direction. It is suspected that Shigella movement within the host cytoplasm is hindered by microtubules. In spite of the initial assumption that VirA possesses a cysteine-like activity that is α-tubulin specific,[72] the crystal structure determination of VirA did not result in the identification of any putative protease-like active site while it highlights the homology to the EspG virulence factor of pathogenic *E. coli*.[103]

MxiE-dependent T3SS effectors expression. MxiE belongs to the AraC family of transcription activators and together with IpgC regulate the expression of the late T3SS effectors. OspD1 negatively controls secretion of these proteins as it binds the Spa15 class IB T3SS chaperone (i.e., a chaperone for a great number of early T3SS effectors) and the activator MxiE until its secretion.[52] The OspE T3SS effector was found to reinforce host cell adherence to the cell membrane of the basal epithelium by interacting with integrin-linked kinase (ILK). This interaction is responsible for suppressing epithelial detachment, increasing bacterial cell-to-cell spreading and promoting bacterial colonization.[104] OspG and IpaH(9.8) T3SS effectors downregulate the host innate immune response. OspG is a protein kinase that binds various ubiquitinylated ubiquitin-conjugating enzymes, including UbcH5, which belongs to the stem cell factor SCF(β-TrCP) complex promoting ubiquitination of phosphorylated inhibitor of NFκB type α (phospho-IκBα).[105] The IpaH protein family comprises a variable leucine-rich repeat-containing N-terminal domain responsible for substrate specificity and a conserved E3 ubiquitin ligase C-terminal domain harboring an invariant cysteine residue that is crucial for its activity.[106] OspZ T3SS effector from *S. flexneri* 6 and *Shigella boydii* blocks translocation of the p65 subunit of the transcription factor NFκB to the host cell nucleus. This activity is not found in the truncated OspZ from *S. flexneri* 2a.[107]

Environmental modifications of the T3SS. Oxygen availability has been reported to be crucial for the T3SS activity through FNR (fumarate and nitrate reduction regulator, a regulator of the anaerobic metabolism) sensing. *S. flexneri* and *S. enterica,* by occupying the same niche, possibly detect similar environmental signals allowing for the switching of their Type III secretion. Recently, it has been shown that a small zone of oxygenation exists immediately above the epithelial cell layer and oxygen abundance may be the essential signal for switching the T3SS from early to late substrates.[14] The *S. flexneri* Δ*fnr* mutant failed to colonize the gut while $spa32^{sctP}$ and $spa33^{sctQ}$ genes are upregulated in this mutant. It may be hypothesized that late substrates of the Shigella T3SS, such as translocators and effectors, are stored inside the bacterial cytoplasm bound to their specific chaperones until the signal that informs the proximity of the target host cells has arrived. Interestingly, $Spa33^{SctQ}$ from Shigella, that was found to be regulated by oxygen, is homologous to $SpaO^{SctQ}$ which, with $OrgA^{SctK}$ and $OrgB^{SctL}$, are thought to form a T3SS cytoplasmic sorting platform in *S. enterica* sv Typhimurium.[73] $orgA^{sctK}$ and $orgB^{sctL}$ genes were initially identified during an oxygen regulated gene screen required for *S. enterica* sv Typhimurium invasion.[108] However, the oxygen availability during the infectious process still has to be elicited in vivo. After the formation of the translocation pore, a signal should also arrive at the T3SS gate to inform the appropriate docking of the T3SS needle to the host cell membrane and release the "gatekeeper". The nature of this signal is under investigation. Small molecular weight molecules such as ROS and NO, which are secreted as a host defense mechanism,[34,3536,37] are probably sensed by the microorganism and may dictate its secretory competency. On the other hand, the assembly of the T3SS tip

components, including IpaD, have been shown to interact in vitro with deoxycholate (a bile salt), stimulating IpaB recruitment.[109] ROS sensing mediated by DsbA in Shigella has been shown to play a major role in T3SS Ipa protein release—even though the molecular mechanism is still unclear[58,59] (**Fig. 1**). As Salmonella T3SS has been shown to be sensitive to pH to allow for effector translocation, it may be speculated that similar observations can be made for the function of the T3SS of Shigella.[110]

Despite the fact that a huge amount of experimental data (structural, biochemical, functional) are available for T3SS across bacterial species, little is still known about its cytoplasmic soluble part structure, assembly and function. The exact mechanism of secretion, the energy source, and reorganization of the cytoplasmic subunits for the different secretion substrates to be accessible to the machinery in the three secretion modes (secretion of the needle/inner rod subunits, secretion of the translocators and secretion of effector proteins) remain to be elucidated. Technically, the harsh purification conditions used to isolate the T3SS from the membranes are probably enough to disrupt a loosely attached C-ring evolved to serve the T3SS transient nature. The question of environmental modulation of T3SS assembly is still pending. Development of electron-microscopy techniques should help decipher these fine molecular tunings in relation with the T3SS function regulation.

The autotransporters (T5SS). T5SS mechanism is a terminal branch of the GSP (General Secretion Pathway) and can be further subdivided in three categories: the autotransporters (type Va), the two-partner secretion system (type Vb) and the oligomeric autotransporters (type Vc).[111]

Autotransporters are usually large multidomain proteins with a cleavable N-terminal secretion signal in order to be targeted to the SecYEG complex. This N-terminal secretion signal is atypically long in some cases, such as in members of the Ser protease autotransporters of the Enterobacteriaceae (SPATE) family.[112] The C-terminal domain of an autotransporter is the actual translocation unit needed to bypass the outer membrane. This domain will form a β-barrel structure in the outer membrane to assist the translocation of the effector or passenger domain of the autotransporter to the extracellular milieu (see **Fig. 3** for details). The passenger domains are the actual effector molecules and as such they are very diverse in sequence and in function. In most cases these are tightly linked to bacterial virulence. The most salient feature is their long right handed β-helix (**Fig. 3**), which is capped at the C-terminus by the autochaperone domain. The autochaperone domain is required to trigger and ensure correct folding of the passenger domain to the bacterial surface.[113]

The best-studied autotransporters in Shigella are IcsA, SepA, Pic and SigA. SepA, Pic and SigA belong to the SPATEs family. IcsA is cleaved when localized on the bacterium surface by the outer membrane protease IcsP but remains attached to the bacterial surface. *icsP* is also regulated by the H-NS antagonist VirB.[114] *icsA* is expressed upon invasion of the host through its regulators H-NS, VirF and a 450 nt antisense RNA (RnaG) acting as transcriptional attenuator. H-NS was shown to repress *icsA* transcription at 30°C but not at 37°C.[115]

Host-autotransporters interaction. IcsA is involved in cell-to-cell spreading process, manipulating actin-forming actin tails of Shigella when the bacterium enters the cytoplasm[32] and allows Shigella to escape cell autophagy.[31] Pic seems to target a broad range of human leukocyte O-linked glycans-rich adhesion proteins[116] and possesses a serine protease activity.[117] SepA still has an unknown function even though it has sequence homologies with IgA1 proteases and the corresponding *sepA* mutant is attenuated in vivo.[118] The enterotoxin SigA degrades the cytoskeleton components in epithelial Hep-2 cells in vitro, including the fodrin (spectrin analog).[119]

Autotransporters environmental modifications. The IcsA disulfide bonds formation, required for its function, has been shown to be regulated by environmental ROS sensed by DsbA[58,59] (**Fig. 1**). This statement might be of interest for investigating the cell-to-cell spreading of Shigella in infected tissues, infiltrated by PMNs, releasing large amount of ROS through MPO activity. No report of Pic, SepA and SigA expression modulation in vivo have been made so far.

Beside T3SS and T5SS, PAMPS play a crucial role in host cell response to Shigella. We describe here the main structural components of the lipopolysaccharide and the peptidoglycan, the two most-studied PAMPS, their specific interaction with host cells and their environmental modulations.

Lipopolysaccharide and peptidoglycan. *The lipopolysaccharide (LPS).* The LPS of Shigella consists of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating "core" oligosaccharide and a distal polysaccharide (or O-antigen). It has been shown that Shigella rough mutants lacking LPS are avirulent in vivo.[120,121] As described above, tetrasaccharide O-antigen variability defines multiple Shigella serotypes. Common O-antigen backbone consists of a repetition of 3 rhamnoses (Rha) and one N-acetylglucosamine (N-Glu) (**Fig. 4**). The LPS biosynthesis is a complex process involving a large set of enzymes. Corresponding genes are mainly located on the chromosome.

The Lipid A is a glucosamine-based phospholipid. Its synthesis (reviewed in ref. 122) is necessary for the survival of Gram-negative bacteria. The LPS core sugar is common to all Shigella serotypes and is divided into an inner and an outer core. It contains 2 KDO (3-deoxy-D-manno-oct-2-ulosonic acid) and a polysaccharide composed of heptose, glucose, galactose and N-acetylglucosamine (**Fig. 4**). LPS O-antigen synthesis is reviewed in reference 123. Several families of genes determine the O-antigen length, including *cld* (chain length determinant),[124] *rol* (regulation of O-antigen length) and wzz, involved in the controlled modal chain length (mode A and mode B).[125,126] Serotype differences stand in an O-antigen glucosylation (catalyzed by the glucosidases GtrA, GtrB and GtrX[127]) and/or an acetylation (catalyzed by acetylase Oac[128,129]) and their corresponding localizations (**Fig. 4**).

The Host-LPS interaction. The LPS is one of the main inducers of the innate immune response. Its recognition in mammals is mediated by the TRL4 cell-surface receptor.[130] It has been demonstrated that MyD88/TLR4 activation leads to translocation of cytosolic NFκB into the nucleus promoting expression of pro-inflammatory genes (e.g., IL6, IL8, IL1-β, TNFα and β) in

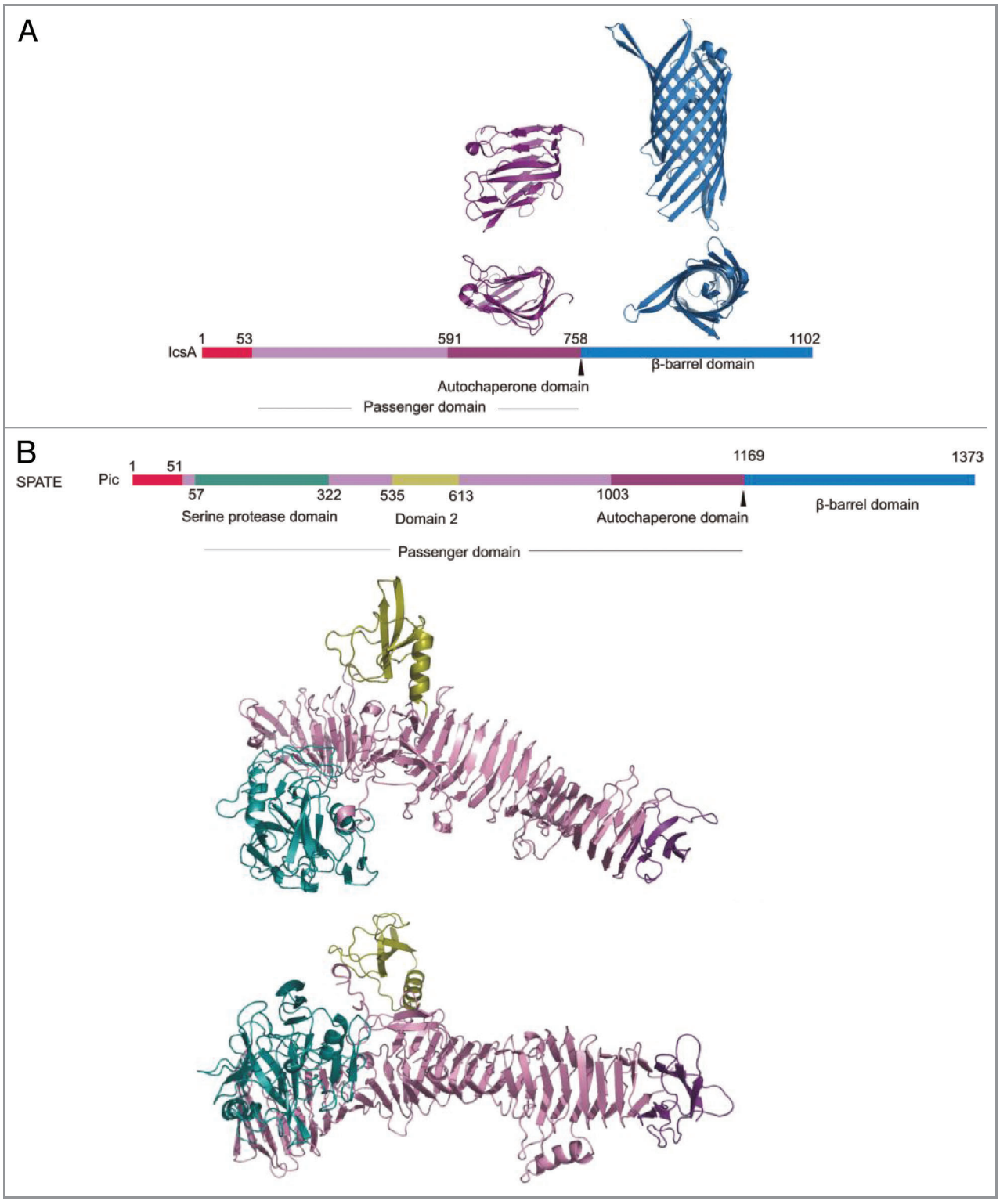

Figure 3. For figure legend, see page 114.

addition to a Myd88-independent pathway through IRF3. The O-antigen tetrassacharide is also involved in the resistance of Shigella to the complement-mediated lysis.[131] The Lipid-A endotoxin is the main LPS component responsible for the inflammation activation through MsbB1 and MsbB2 Lipid A acylation.[132,133]

Environmental modifications of the LPS. A periplasmic disulfide catalyst, YihE, has recently been showed to be involved in the

Figure 3 (See previous page). Diagrammatic representation of various domains of autotransporters. The edges of each domain are indicated by residue numbers. Above in A (IcsA) or below in B (Pic) are shown ribbon models of these domains in two different views of a 90 degree rotation along the horizontal axis. Arrows indicate the points of processing after the export of the passenger domain to the extracellular milieu. (A) *S. flexneri* IcsA possesses a typical domain distribution for Va autotransporters of an N-terminal signal sequence (in red) followed by the passenger domain (here in pink) and the C-terminal transmembrane β-domain (in blue). The C-terminal part of the passenger domain is thought to act as an autochaperone critical for the secretion from periplasm to bacterial surface and for the folding of the rest of the domain after its export (here in violet). The autochaperone domain of IcsA was experimentally determined (pdb_id: 3ML3). The β-barrel domain of IcsA is here modeled through the fold recognition server PHYRE[148] (based on the crystal structure of the *E. coli* EspP autotransporter β-domain, pdb_id: 2QOM with an E-value of 2.689e-25). (B) *S. flexneri* 2a Pic (GenBank: AAK00464.1) belongs to the SPATEs family of autotransporters as SepA (GenBank: CAA88252.1) and SigA (GenBank: AAF67320.1) from *S. flexneri*. All of them are homologs of the pathogenic *E. coli* Heme Binding Protein, Hbp, autotransporter. Although the fold recognition server PHYRE[148] was used here to build the entire passenger domain of Pic (based on the Hbp crystal structure (pdb_id: 1WXR with a zero E-value), homology modeling can take place as well. Pic seems to possess, as Hbp, a long right handed β-helix core (in pink) with the autochaperone cap (in violet) and two domains protruding out of this β-helix; the Serine protease domain is in green and a domain of unknown function is in yellow.

LPS assembly.[134] YihE is a bacterial kinase targeted by the Cpx pathway,[135] itself activated by various stresses (ROS, pH, osmolarity) (**Fig. 1**). Although molecular mechanisms are still unclear, these discoveries may be of great importance in understanding LPS O-antigen modifications during infection in vivo. This could lead to modulation of Shigella recognition by the host immune system, but also to having a key role in T3SS cell accessibility with respect to LPS O-antigen length modulation.[21]

Additionally, it has been shown that under limited magnesium conditions MsbB2 is overexpressed in a PhoP/PhoQ-dependent manner, leading to lipid A acylation[136] (**Fig. 1**). This lipid A modification could confer increased resistance of Shigella to stressful environments and antimicrobial peptides found in PMNs vacuole.

The peptidoglycan (PGN). As a Gram-negative bacterium, Shigella possesses a peptidoglycan layer between the inner and outer membranes. This component is essential for the cell integrity and shape, but also for its survival, contributing to the anchoring of other cell envelope components, including proteins.[137] The PGN layer is continuously produced and recycled by bacteria through multi-enzymatic pathways[138] to ensure the PGN layer integrity, especially during cell division cycles.

In *E. coli*, used as a model organism for Gram-negative bacteria, the PGN is a polymer of murein, consisting in heteropolymer of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNac) linked in β.[1-4] The terminal residues of glycan strands are GlcNac and 1–6 anydroMurNac, as oligomerization of glycan strand average stands between 25 and 40 disaccharide units. Glycan strands are cross-linked by short peptides containing L- and rare D-aminoacids (**Fig. 5**). The stem peptide attached to the lactyl group of muramic acid consists in L-Ala-γ-D-Glu-(L)-meso-diaminopimelic acid (DAP)-D-Ala, although it may lack D-Ala or more rarely terminates with D-Ala-D-Ala. In addition, an interpeptide bridge may be observed between a D-Ala at position 4 and a diaminoacid at position 3.[139]

Based on the sequence homology of enzymes involved in its biosynthesis, it is speculated that the Shigella PGN architecture is similar to *E. coli*. Murein synthesis is a two-stage dynamic process (synthesis and hydrolysis) required during the elongation and the division of bacteria, adapting the cell wall to the bacteria shape. The sacculus enlargement is catalyzed by a set of murein synthases, anchored in the innermembrane, having catalytic transglycosylation (TG) and transpeptidation (TP) domains located in the periplasm. Pre-existing PGN is simultaneously cleaved by murein hydrolases, releasing small and soluble fragments. *E. coli* possesses 3 classes of murein hydolases.[140,141]

If the chemical composition and the 3-dimensional structure of the peptidoglycan subunit structure have long been known, the gobal assembly of the sacculus has only recently been solved by electron cryotomography in *C. crescentus* which shows a thin single disorganized layer.[142] This important advancement requires further species comparison studies, including in Shigella.

To date, the architecture and the synthesis of the PGN of Shigella are largely unexplored, as only a single study has described the PGN recycling mechanisms involving AmpG and MppA. A mutation of corresponding encoding genes leads to abundant extracellular release of a large diversity of PGN fragments in which GlcNAc-MurNAc-L-Ala-D-Glu-mesoDap-D-Ala and L-Ala-D-Glu-mesoDap- D-Ala, respectively, are the most abundant[143] (**Fig. 5**).

The host-PGN interaction. A critical aspect in the host-PGN interaction is the periplasmic localization of the PGN layer in Gram-negative bacteria. It has been shown that extracellular release of PGN fragments (meso-DAP) is occurring, even though the transport molecular mechanism is still unknown. Shigella PGN-released products recognition in mammals is mediated intracellularly by Nod-like receptors (NLR) Nod1 (expressed ubiquitously) and Nod2 (expressed mostly in monocytes, macrophages and dentritic cells).[12] Soluble PGN is recognized by several recognition proteins belonging to the PGRPs family.[144]

NLR possess a N-terminal caspase recruitment domain (CARD), a nucleotide-binding domain and a C-terminal leucine-rich domain similarl to TLRs. PGN minimum motifs sensed by Nod1 and Nod2 are D-Glu-mesoDap (mostly produced by Gram-negative bacteria) and MurNAc-L-Ala-D-Glu (common to Gram-negative and Gram-positive bacteria), respectively.[145] Regarding their specific cell-type localization, the activation of the NOD pathways succeed bacteria cell-entry (invasion of epithelial cells or phagocytosis by myeloid cells) and consists of Nod oligomerization leading to RICK and IKK recruitment which phosphorylates IκBα, stabilizing NFκB activation.[12] Nod1 activation has been demonstrated in epithelial cells infected by *S. flexneri*,[143] but further investigations are required to elicit the role of Nod2 in challenged immune cells.

Environmental modifications of the PGN. To date, no study has reported Shigella PGN modifications in vivo; however, it has been

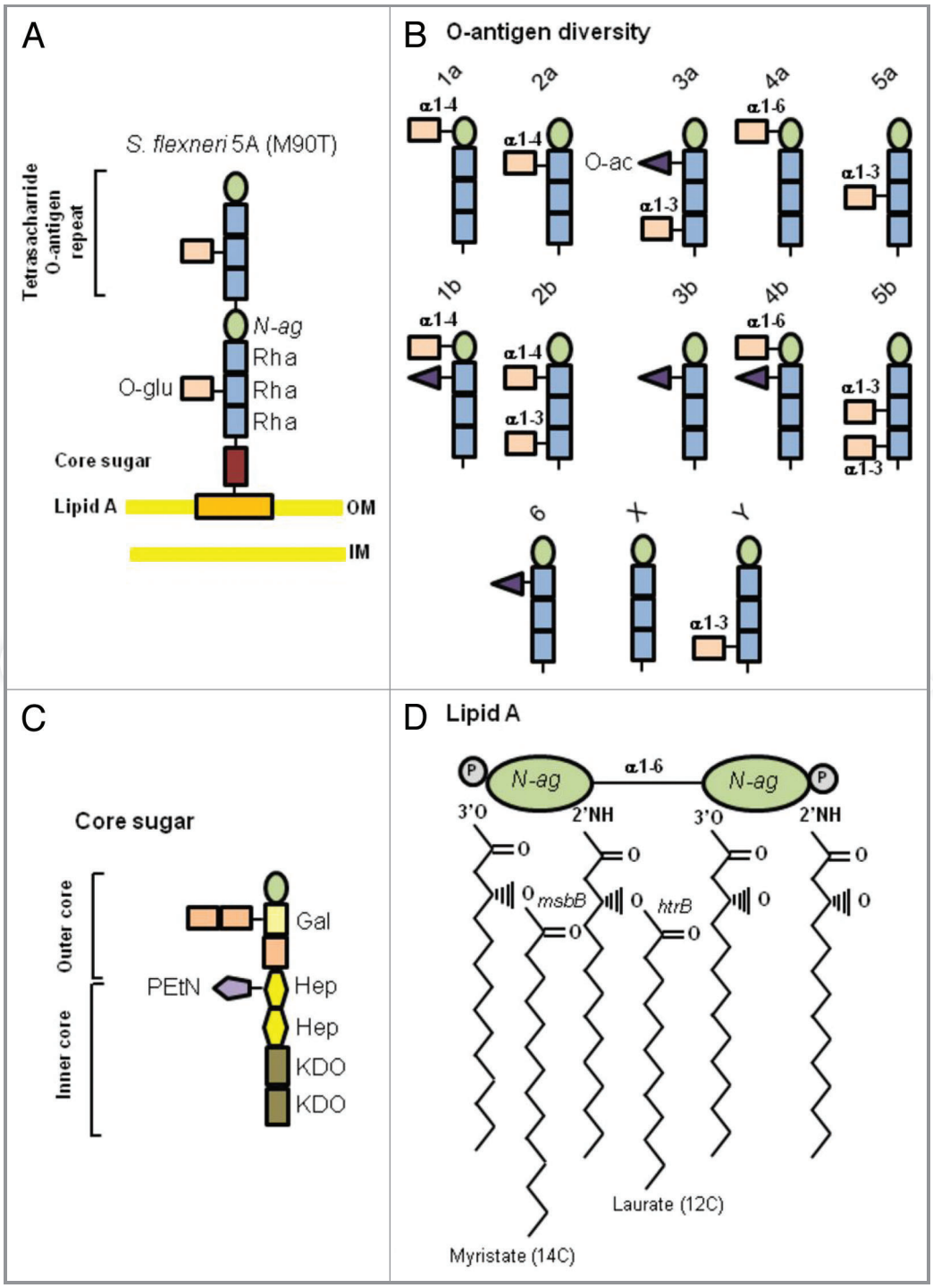

Figure 4. Shigella lipopolysaccharide (LPS) structure. (A) General schematic representation of Shigella flexneri 5A LPS structure. Rha: rhamnose, N-ag: N-acetylglucosamine, O-glu: O-glucose. (B)Shigella flexneri 5A (M90T) O-antigen diversity description based on O-antigen repeats modifications. O-ac: O-acetyl. Sugar links are indicated on each ramification. (C) Core sugar structure. Gal: galactose, Hep: heptose, PetN: phosphoryl-ethanolamine, KDO: 3-deoxy-D-manno-oct-2-ulosonic acid. (D) Lipid A structure. Laureate (12C) and myristate (^{14}C) are linked to N-ag through acyl-oxy-acyl bounds.

clearly described in other bacterial species such as *Helicobacter pylori, Neisseria gonorrhoeae* and *Salmonella typhimurium* that the thickness and the structure of the sacculus are finely tuned during infections (reviewed in ref. 139). This aspect requires further investigation in Shigella.

Perspectives and Future Directions

Studying the adaptation of Shigella to the gut microenvironments faced by the bacteria in the mucus layer, the epithelium surface and the mucosa during an infection process, requires the

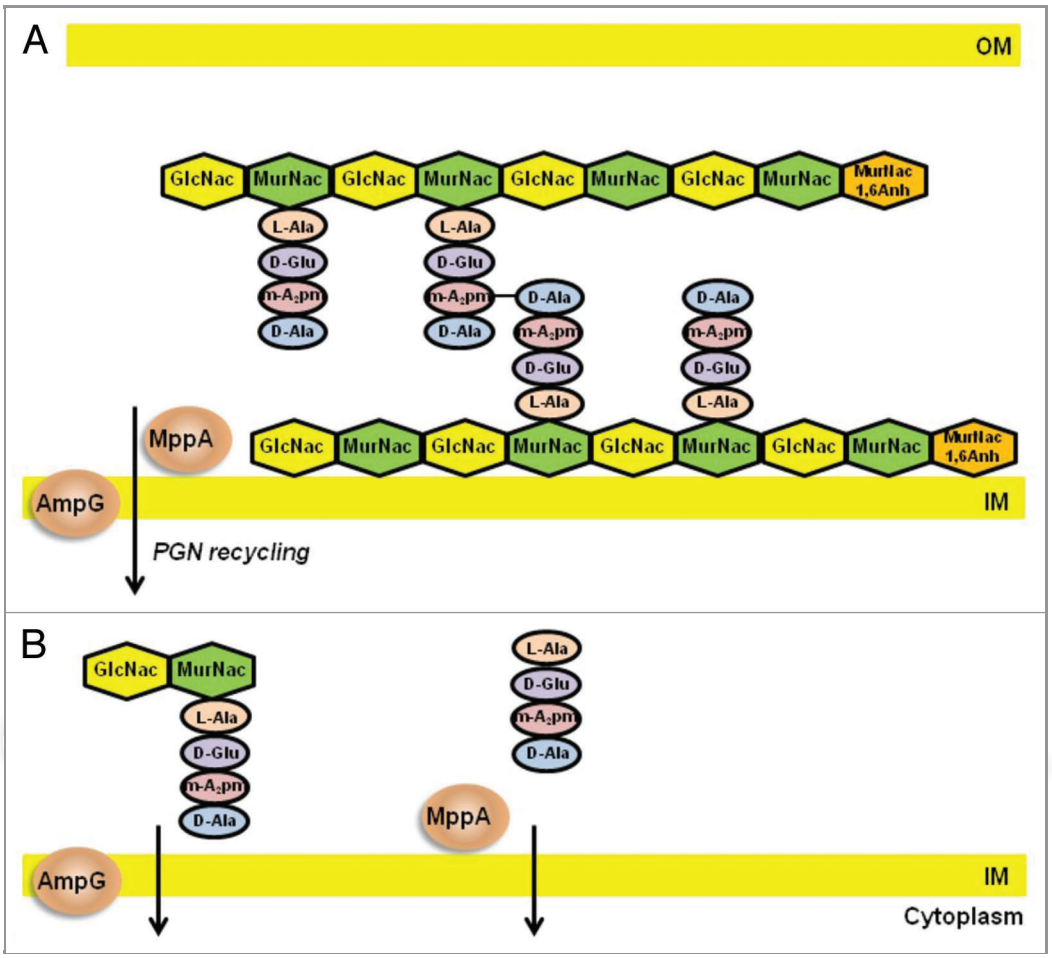

Figure 5. Shigella Peptidoglycan (PGN) structure. (A) General representation of PGN structure showing glycan heteropolymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNac) linked in β1-4 Stem peptide attached to the lactyl group of muramic acid consists in L-Ala-γ-D-Glu-(L)-meso-diaminopimelic acid (DAP)-D-Ala allows glycan association. (B) *Shigella flexneri* PGN recycling. AmpG and Mppa specificity is highlited in substrate recognition.

understanding of the physiology of these environments. The choice of the adapted animal model is a key aspect of such analysis. The recent validation of the Guinea pig colonic model of shigellosis will probably lead to new insights. To date, only a limited number of reports are aimed at characterizing the modulation of virulence elements (T3SS, T5SS and PAMPS) with respect to the physiological characterization of environmental cues during infection. A similar statement has to be made concerning outer-membrane proteins (OMPs, including lipoproteins, belonging to the PAMPS) for which expression modulation under in vivo conditions is particularly important regarding innovative vaccine design through the identification of novel specific antigens.

To date, in vivo studies have been initiated to validate in vitro observations (including validation of Shigella mutants attenuation). A new paradigm has emerged, beginning with in vivo observations (including screening STM mutants in vivo[21]) and followed by molecular mechanisms analysis performed in vitro. This approach is certainly more reliable regarding Shigella virulence analysis in the context of the host inflammatory and immunological response. The complexity of this approach will probably lead researchers in this field to new insights, including the effect of the microbiota, such as Lactobacilli strains, on Shigella pathogenicity.[146] A special emphasis will have to be made on the description of these new environmental condition modifications created by Shigella proliferation in the host gut, considered as an organ, tissue (formation of abscesses) or at the single cell level (intracellular replication analysis[147]).

Recent live microscopy developments (e.g., two-photons) are applied to new in vivo Shigella studies, which will certainly allow a better characterization and comprehension of the infectious process.

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