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The Multivalent Adhesion Molecule SSO1327 plays a key role in Shigella sonnei pathogenesis

Running title: SSO1327 is an adhesin required for S. sonnei pathogenesis

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SSO1327 is an adhesin required for *S. sonnei* pathogenesis

**SUMMARY**

Shigella sonnei is a bacterial pathogen and causative agent of bacillary dysentery. It deploys a type III secretion system to inject effector proteins into host epithelial cells and macrophages, an essential step for tissue invasion and immune evasion. Although the arsenal of bacterial effectors and their cellular targets have been studied extensively, little is known about the prerequisites for deployment of type III secreted proteins during infection. Here, we describe a novel *S. sonnei* adhesin, SSO1327 which is a Multivalent Adhesion Molecule (MAM) required for invasion of epithelial cells and macrophages and for infection in vivo. The *S. sonnei* MAM mediates intimate attachment to host cells, which is required for efficient translocation of type III effectors into host cells. SSO1327 is non-redundant to IcsA; its activity is independent of type III secretion. In contrast to the up-regulation of IcsA-dependent and independent attachment and invasion by deoxycholate in *S. flexneri*, deoxycholate negatively regulates IcsA and MAM in *S. sonnei* resulting in reduction in attachment and invasion and virulence attenuation in vivo. A strain deficient for SSO1327 is avirulent in vivo but still elicits a host immune response.

**Keywords:** Shigella, dysentery, adhesin, multivalent adhesion molecule, host-pathogen interaction;
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**INTRODUCTION**

Bacillary dysentery remains a significant threat to public health in the 21st century, with an estimated 160 million episodes worldwide per annum and 1.1 million deaths of which most are children under 5 years old (Kotloff et al., 1999). More recent studies show that *S. sonnei* has indeed become the most prevalent agent in newly industrialized countries such as Korea, Republic of China, Thailand, and Iran and is more associated with dysentery in infants (Seol et al., 2006, Ranjbar et al., 2007). *S. sonnei* originated in Europe and recently disseminated globally. Fast evolving and multi-drug resistant clones of *S. sonnei* are responsible for the current dysentery pandemic (Holt et al., 2012).

Shigella infection is transmitted through contaminated food or water (Leclerc et al., 2002, Chen et al., 2001). Shigella is highly invasive with very low infectious doses (10–100 CFU) (DuPont et al., 1989). Invasion occurs in the recto-colonic mucosa via M cells. Equipped with a type III secretion system (TTSS), the bacteria then invade resident macrophages as well as epithelial cells via the basolateral side. The TTSS injects the effector IpaB into macrophages, which activates caspase I (also known as interleukin I converting enzyme) and in turn triggers apoptosis to kill macrophages. Caspase I activation also results in release of activated Interleukin (IL)-1β and IL-18 which initiates a pro-inflammatory response (Thirumalai et al., 1997). Upon contact with epithelial cells, the TTSS translocates a first wave of effector proteins, which cause actin rearrangements and facilitate bacterial invasion, vacuolar escape and covert intracellular replication. The intracellular bacteria then translocate via TTSS a second wave of effectors that hijack host signalling, inhibit apoptosis and manipulate host innate and adaptive immunity (Schroeder & Hilbi 2008). Via a surface protein, IcsA, the bacteria polymerize host actin that enables bacteria spread intra- and inter-cellularly (Bernardini et al., 1989). The lateral spread of
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the bacteria from cell-to-cell results in infecting and killing of the neighboring epithelial cells, causing ulcers, bleeding and mucosal inflammation (Niyogi, 2005). While initial extracellular translocation of effectors is necessary to trigger invasion, intracellular growth, persistence and spread are required for virulence in vivo; all these events are mediated by TTSS (Schroeder & Hilbi 2008).

Shigella originated from multiple lineages of Escherichia coli, and has evolved mainly through genome reduction through the action of translocation, inversion and frame shifts mediated by insertion sequences (IS) and transposons (Yang et al., 2005). Many genes encoding pili and fimbriae required for host attachment are inactivated in Shigella, and it has long been assumed that the organisms’ capability to invade in a TTSS-dependent fashion has eliminated the need for adhesion (Yang et al., 2005). More recently, it has been demonstrated that the S. flexneri protein IcsA plays a dual role in actin-based motility and adhesion and its role as an adhesin, which is also TTSS-dependent, is required for pathogenesis (Brotcke Zumsteg et al., 2014).

We are studying the molecular pathogenesis of S. sonnei, and found the sequenced S. sonnei strain Ss046 contains an intact gene encoding for a putative Multivalent Adhesion Molecule (SS01327), a protein of 879 amino acids with seven mammalian cell entry (MCE) domains (Fig. S1A). Multivalent adhesion molecules (MAMs) are widely distributed proteins in Gram negative bacteria. The best characterized ortholog, MAM7 from the food-borne pathogen Vibrio parahaemolyticus, consists of an N-terminal hydrophobic region which is required for outer membrane targeting and anchoring of the protein. This region is followed by a stretch of six to seven MCE domains which are responsible for host cell binding. MCE domains mediate binding to fibronectin and phosphatidic acid on the host cell membrane (Krachler et al., 2011, Krachler & Orth, 2011). MAM-mediated binding has been demonstrated to be important for
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pathogenicity in a numbers of pathogens, including *Yersinia* pseudotuberculosis, *Vibrio* cholerae, *Vibrio* parahaemolyticus and enteropathogenic *E. coli* (Krachler et al., 2011, Lim et al., 2014). Prevention of MAM-mediated binding has thus offered a new strategy in treatment of various bacterial infections (Krachler & Orth, 2013, Krachler et al., 2012b, Hawley et al., 2013).

In addition to *S. sonnei* SS01327, intact MAM orthologs are also present in *S. dysenteriae* and *S. boydii*. While function of the only described Shigella adhesin identified to date, IcsA, strictly depends on TTSS activity, MAMs are constitutively active and thus are able to establish contact between host and pathogen during the earliest stages of infection (Krachler et al., 2011). This has intrigued us to investigate whether the MAM ortholog in *S. sonnei* is functional and required for virulence. Here we present evidence that MAM-mediated adherence is non-redundant with IcsA-mediated attachment. It is a prerequisite for TTSS-mediated invasion of host epithelial and phagocytic cells and necessary for Shigella pathogenicity in vivo.

RESULTS

MAM is widely conserved across Shigella species

Using the sequence of the well characterized MAM VP1611 from *Vibrio* parahaemolyticus, we performed a search for MAM orthologs in Shigella. This revealed that *S. sonnei* Ss046 harbors an intact MAM ortholog, SSO1327, a putative protein of 879 amino acids sharing 35% identity and 25% similarity with *V. parahaemolyticus* VP1611. Its topology and domain organization was predicted to be highly similar to that of VP1611, containing an N-terminal hydrophobic stretch of 41 residues and seven putative tandem MCE domains (Fig. S1A). SSO1327 is encoded on the chromosome nearby an *ipaH* pathogenicity island that carries
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ipaH\_3, encoding a type III secreted effector (Fig. S1B). MAM orthologs were also identified in several other Shigella species, including S. boydii (SB01249) and S. dysenteriae (SDY1985), within a similar genetic context (Fig. S1B). In contrast, the S. flexneri MAM ortholog, SF1391, is a pseudogene, encoding for a truncated protein (Fig. S1B). To further investigate the potential biological function of the S. sonnei MAM SSO1327, we constructed a non-polar deletion strain in wild type S. sonnei strain 20071599 (Xu et al., 2014a), ΔSSO1327, (henceforth referred to as ΔMAM), (Fig. S1C, D, E).

Shigella MAM is localized at the bacterial outer membrane and surface exposed

Previous studies have shown that the V. parahaemolyticus MAM, VP1611, possesses an N-terminal hydrophobic region that allows protein targeting and anchoring to the outer membrane (Krachler et al., 2011). We tested whether this was also the case for the S. sonnei MAM protein. Since no MAM-specific antibody is available, we used the ΔMAM strain expressing C-terminally 6xHis-tagged MAM (ΔMAM+pMAM-His) to probe for protein localization. S. sonnei ΔMAM or ΔMAM+pMAM-His strains were grown in rich media, cells subjected to subcellular fractionation and both soluble and membrane fractions probed for the presence of MAM using α-His antibody. Fractionation was successful, as controlled for using an antibody against the outer membrane porin OmpA (Fig. 1A). MAM was exclusively detected in the membrane fraction of the MAM expressing strain, but not the deletion strain (Fig. 1A). To probe for outer membrane localization, we performed microscopy on EGFP-expressing bacteria treated with α-His antibody to detect MAM, followed by TRITC-labelled secondary antibody. As a control, a ΔdsbA strain was also transformed to express DsbA-His, which is a periplasmic protein required for Shigella virulence (Yu, et al., 2000). While MAM-His was detected both in Triton-treated and untreated bacteria...
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(Fig. 1Bii, and 1Cii, respectively), DsbA-His was only detected in Triton-treated but not untreated cells (Fig. 1Eii and 1Dii, respectively). Triton-X100 is able to permeabilize the outer membrane, which renders the periplasmic DsbA-His accessible to the antibody. Since MAM-His was detected in the absence of permeabilization, these data demonstrate that, like its orthologs, *S. sonnei* MAM localizes to the bacterial outer membrane, with the C-terminus oriented towards the extracellular side.

MAM and IcsA are two non-redundant *Shigella* adhesins required for attachment to and invasion of host cells.

*Shigella* attaches to and invades both epithelial cells and macrophages in a TTSS-dependent manner. Although the contribution of TTSS structural components and effector proteins to these processes has been carefully evaluated in the past, until recently nothing was known about factors required to initiate the intimate contact between bacteria and host cells that is necessary to trigger initial TTSS-dependent translocation of effectors which facilitate invasion. Recently, IcsA has been described to be involved in bacteria-host association in *S. flexneri*. Thus, we tested the contribution of MAM and IcsA to *S. sonnei* attachment and invasion, both individually and in combination.

First, we tested both *S. sonnei* wild type and deletion strains for adherence to HeLa epithelial cells, which are a widely used model for assessing bacterial adhesion and invasion. Adherence levels in the ΔMAM and the ΔicsA deletion strains dropped similarly to ~ 25-20% compared to wild type levels (statistically significant, p < 0.05). The wild type strain’s attachment capacity could be restored by complementation of the deletion strains with MAM or IcsA, respectively, expressed in trans (Fig. 2A). A ΔMAM/ΔicsA double mutant showed further
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reduction in adherence to less than 20% compared to the wild type, indicating a synergistic effect between MAM and IcsA. This double mutation could be completely complemented to restore wild type levels of attachment, by expressing both MAM and IcsA from a single plasmid, pMI, in trans. We further tested the growth kinetics of wild type and all mutant strains (Fig. S7), and all strains grown equally well in L-broth, ruling out growth defects as a reason for the different attachment and invasion phenotypes we observed. Thus, we conclude that IcsA and MAM contribute equally to adherence and that there is synergy between the two adhesins. Since the double mutant still retained low levels of adherence, other, as yet unidentified factors may mediate adherence in the ΔMAM/ΔicsA background.

Shigella is well-known for its ability to invade host cells; the significance of attachment in pathogenesis has only been investigated regarding IcsA’s role, which is otherwise known to be required for actin-based cellular motility. Therefore, we investigated whether deletion of the S. sonnei MAM led to reduced invasiveness, using 2 h gentamycin protection assays. Deletion of icsA reduced invasion to 40% of the wild type levels and deletion of MAM caused more profound reduction to less than 10% of the wild type levels (Fig. 2B). Expression of MAM or IcsA in trans, respectively, restored invasiveness in full to these two mutants. Removal of MAM and icsA together caused slightly more reduction than removal of MAM alone, indicating that MAM plays a more important role than IcsA in invasion (Fig. 2B). Expressing both MAM and IcsA from pMI in trans fully complemented the double mutant for invasion (Fig. 2B).

In V. parahaemolyticus, attachment of the MAM VP1611 to host cells is dependent on two host surface receptors; direct, high-affinity binding of MCE domains to phosphatidic acid membrane phospholipids via key conserved basic residues in each domain is necessary for adhesion, while fibronectin acts as a co-receptor and accelerates surface engagement (Krachler &
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Orth, 2011). A comparative analysis of key basic residues reportedly involved in phosphatidic acid binding by VP1611, and the Shigella MAM SSO1327 revealed a high level of conservation of basic residues in key lipid binding positions (Fig. 2C). Thus, we tested the requirement of *S. sonnei* MAM for phosphatidic acid and fibronectin in host cell adherence. Treatment of epithelial cells with phospholipase C (PLC) depletes phosphatidic acid from the membrane (Lim et al., 2014). Following PLC treatment, bacterial adhesion to host cells was decreased to ~10% of wild type levels, and levels comparable to those observed for ΔMAM attachment (Fig. 2D). The requirement for fibronectin was evaluated using a competition assay. Pre-incubation of bacteria with fibronectin from human plasma significantly decreased attachment to epithelial cells, albeit to a lower extent than phosphatidic acid depletion. We conclude that *S. sonnei* MAM has similar binding specificity and host receptor requirements as the *V. parahaemolyticus* MAM VP1611 and engages both phosphatidic acids and fibronectin at the host cell surface. We also noted that blocking or eliminating host cell receptors was more efficient in inhibiting attachment than deleting MAM (Fig. 2A vs. 2D). This may suggest that other, yet unidentified, adhesins also use PA and fibronectin for attachment.

MAM is not involved in production or secretion of type III effectors but enables efficient substrate translocation into host cells

Shigella invasion is dependent on a functional TTSS, and the secreted components IpaB and IpaC are known to form a translocon pore complex in the target cell membrane and facilitate the injection of effector proteins into the host cell cytoplasm to initiate invasion (Menard et al., 1994, Veenendaal et al., 2007). Therefore, we investigated whether removal of MAM reduced secretion of IpaB and IpaC, by use of Congo red as environmental cues for secretion in vitro as described previously (Bahrani et al., 1997). Similar levels of IpaB and IpaC were detected in the
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total cell lysates and supernatants of wild type, ΔMAM mutant as well as the complemented ΔMAM strain (Fig. 3A). Thus, removal of MAM neither affects TTSS protein production nor secretion.

Translocation of TTSS effector proteins into the host cell cytoplasm requires prolonged and intimate contact between bacteria and host cells. Thus, we investigated if the absence of MAM negatively affects the initial translocation of type III secreted effector proteins from adherent bacteria into host cells. HEK293 cells were loaded with the β-lactamase (Bla) cleavable substrate CCF4-AM and infected with Shigella wild type or ΔMAM reporter strains carrying a chromosomal fusion of Bla to the TTSS effector IpgD (ipgD-bla), which is implicated in actin rearrangements which facilitate bacterial invasion of non-phagocytic cells upon host cell contact (Niebuhr et al., 2000). We included a negative control, wild type strain carrying p-GEM-T-Easy, which expresses β-lactamase but won’t be able to translocate it into host cells. Substrate translocation was analyzed by ratiometric analysis of imaged cells and by flow cytometry. While substrate loaded mock infected cells fluoresce green (Fig. 3B and G), wild type infection lead to translocation of IpgD-Bla into the host cell cytoplasm, which was marked by substrate cleavage and a shift towards blue fluorescence within 50 minutes post-infection (Fig. 3C and H). Little translocation and thus, little shift in fluorescence, was observed during infection with the ΔMAM reporter strain and the wild type strain carrying pGEM-T-Easy (Fig. 3D and I, and E and J, respectively), although all strains displayed similar levels of β-lactamase activity (Fig. S2). Taken together, these experiments suggest that although the presence of MAM is not required for TTSS effector secretion, it is necessary to achieve intimate contact between bacteria and host cells required for effector translocation.

MAM is required for S. sonnei pathogenesis and interaction with phagocytes in vivo
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Larvae of the wax moth Galleria mellonella are an abundantly used in vivo infection model, including those with enteric pathogens such as enteropathogenic E. coli (EPEC) and Salmonella (Leuko & Raivio, 2012, Viegas et al., 2013). It is particularly suited to study interactions between pathogens and phagocytic cells in a living organism (Harding et al., 2012). To test the function of S. sonnei MAM during pathogenesis, larvae were infected with doses of either $10^5$ or $10^6$ CFU of S. sonnei wild type, ΔMAM or complemented ΔMAM+pMAM strains by injection into the front, right proleg. Control animals were injected with the same volume of sterile PBS. Larvae infected with $10^6$ CFU of S. sonnei wild type strain all succumbed to infection within one day (Fig. S4). Larvae infected with $10^5$ CFU of wild type S. sonnei showed a pronounced phenotype with rapid melanization indicative of an immune response and loss of mobility, and succumbed to infection between day one and five following infection (Fig. 4A, E). In contrast, larvae infected with $10^5$ CFU ΔMAM showed lower mortality, with 70% of animals alive on day 5 (Fig. 4B, E). Expression of MAM in trans restored mortality rates to wild type levels (Fig. 4C, E), while all control animals lived beyond day 5 (Fig. 4D, E).

S. sonnei virulence in the G. mellonella larval model was strongly correlated with the bacteria’s ability to invade phagocytes. To understand the underlying mechanisms of larval killing by Shigella, hemolymph was collected from larvae infected with EGFP expressing S. sonnei strains, and used to isolate infected phagocytes. Bacterial burden inside phagocytes was evaluated both by flow cytometry and fluorescence microscopy. More than 75% of hemocytes isolated from larvae infected with wild type S. sonnei contained a high bacterial burden of approximately five bacteria per cell on average (Fig. 4F). In contrast, phagocytes isolated from ΔMAM infected larvae essentially lacked intracellular bacteria (average burden of less than one bacterium per cell) and were practically indistinguishable from uninfected phagocytes both by
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flow cytometric and microscopic analysis (Fig. 4G, H, K). Expression of MAM in trans restored wild type levels of bacterial burden and eventual lysis (Fig. 4I, J). G. mellonella hemocytes possess similar properties than macrophages from vertebrates (Harding et al., 2012). Shigella infection typically causes macrophage apoptosis via the action of IpaB (Thirumalai et al., 1997). Therefore, we investigated whether *S. sonnei* causes apoptosis to G. mellonella hemocytes. Apoptotic macrophages typically produce fragmented chromatin, which can be detected by agarose gel electrophoresis (Zychlinsky et al., 1992). We isolated and analyzed total DNA from Shigella infected and uninfected moth larvae. Chromatin fragmentation was apparent in DNA samples isolated from wild type infected, but not ΔMAM or mock-infected larvae (Fig. S3). Thus, *S. sonnei* invaded and caused apoptosis to larvae hemocytes, mirroring the mechanism of killing observed in vertebrates.

MAM-coupled polymer beads mimic bacterial attachment and are protective against Shigella infection

Previous studies showed MAM chemically coupled to polymer beads can mimic bacterial adhesion and competitively exclude pathogens from the host cell surface, thus attenuating infection (Krachler et al., 2012a). We investigated whether MAM-coupled beads would be protective against *S. sonnei* infection in vivo, using the G. mellonella larval model. MAM-displaying beads were co-injected with doses of either $10^5$ or $10^6$ CFU of wild type *S. sonnei* and larvae survival monitored over time. MAM-beads administered at the same time as *S. sonnei* inhibited infection (Fig. 5B) while control beads had no protective effect (Fig. 5C). Dosing experiments revealed that MAM beads inhibited infection with a dose of $10^5$ CFU to concentrations of 6 μM. At $10^6$ CFU, a dose that killed all unprotected larvae within one day, co-
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Injection with MAM beads attenuated infection in a concentration-dependent manner (Fig. S4). An inhibitor concentration of 25 μM protected 90% of larvae beyond day 5 post infection (Fig. S4). The lowest inhibitor concentration used (6 μM) protected 50% of larvae beyond day 5. The protective effect of beads was due to their ability to block host cell invasion in vitro and in vivo, by competitively excluding Shigella from binding to the host surface receptors (Fig. 5E – I). The beads had no visible effect on phagocyte morphology (Fig. 5G) and did not interfere with TTSS effector production or secretion (Fig. S5).

MAM is required for *S. sonnei* induced keratoconjunctivitis and immunization with MAM-deficient Shigella induces limited protective immunity

Although the above data demonstrated the usefulness of *G. mellonella* larvae as a model for Shigella infection, we also exploited the more established guinea pig keratoconjunctivitis model to further characterize the MAM mutant. Shigella strains, wild type and ΔMAM mutant, as well as PBS (mock infection) were administered into the eyes (3 animals in each group) and animals monitored for development of keratoconjunctivitis. *S. sonnei* wild type strain caused all three animals fully developed keratoconjunctivitis by day 3 (Fig. 6A, B), whereas the ΔMAM mutant did not cause any sign of the disease up to 14 days post infection in any of the animals (Fig. 6C, D). At this point, we tested if the attenuated ΔMAM mutant could be used as a vaccination strain. Therefore, we administered the same guinea pigs with ΔMAM mutant at day 14 and day 28 to boost immunization and challenged the animals with wild type strain at day 33. Surprisingly, none of the animals showed any sign of conjunctivitis until day 36. After this, the symptoms progressed slowly for the worse and full keratoconjunctivitis was developed by day 41. To compare the immune responses elicited by the wild type and the ΔMAM mutant, we collected guinea pig spleens for detecting antibody secreting cells (ASC) specific to *S. sonnei*
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lippopolysaccharide. The ΔMAM mutant elicited similar levels of ASC than the wild type strain following ocular administration to guinea pigs (Fig. 6E).

Deoxycholate negatively regulates *S. sonnei* pathogenicity in vitro and in vivo

Brotcke Zumsteg A et al. (Brotcke Zumsteg et al., 2014) have recently described positive regulation of *S. flexneri* by deoxycholate (DOC) in adherence and invasion to host cells, which are partially dependent on IcsA. We therefore investigated whether this is also the case for *S. sonnei*. First, wild type, ΔMAM and ΔicsA strains were tested for their adherence to HeLa cells with or without deoxycholate treatment (Fig. 7A). Adherence was decreased by deoxycholate in wild type and all single deletion strains. Deoxycholate equally decreased invasion of HeLa cells by wild type and all mutant strains (Fig. 7B). Next, transcription levels of MAM and icsA in the presence and absence of DOC treatment were compared using qPCR analysis. Deoxycholate significantly reduced transcription of both MAM and icsA (Fig. 7C), which was consistent with the attachment and invasion data.

Finally, the effect of DOC on *S. sonnei* virulence in vivo was investigated in both the guinea pig keratoconjunctivitis and moth larvae models. DOC treatment significantly delayed the onset of conjunctivitis and reduced disease severity in guinea pigs (Fig. S6A) and also significantly protected moth larvae from killing by wild type as well as all mutant strains of *S. sonnei* (Fig. S6B).

**DISCUSSION**

MAM-mediated attachment is required for translocation of TTSS substrates into host cells
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In this study we have demonstrated that SSO1327 encodes a functional Multivalent Adhesion Molecule, which is expressed on the bacterial surface (Fig. 1) and involved in bacterial attachment to host cells, a key step during molecular pathogenesis (Fig. 2). Furthermore, we show that this initial step is essential for the translocation of a first wave of effectors, including IpgD, into host cells, which facilitates subsequent invasion. It is known that Shigella bacteria can be internalized 15 min after contacting HeLa cell monolayers (Mounier et al., 1997). Thus although the initial attachment via adhesins and TTSS-mediated invasion of non-phagocytic cells are two distinctive events they occur rapidly in a continuing fashion. The graphs presented in Fig. 2A and 2D for attachment are likely to be a total sum of large numbers of extracellular and small numbers of intracellular *S. sonnei*. For scope of this study, it was unnecessary to exhaustively analyze the contribution of each of these two populations of bacteria. Most importantly, we have demonstrated that the deletion of MAM causes a decrease in initial cell-associated bacteria, which has a knock-on effect on invasion as shown in Fig. 2B. We further show that decreased adherence leads to inefficient translocation of early TTSS effectors from extracellular bacteria into the host cells cytoplasm, which implies strongly that both the decrease in attachment as well as the impairment of effector translocation play a role in the observed invasion deficiency. Like its ortholog from the sea-foodborne pathogen *V. parahaemolyticus*, *S. sonnei* MAM specifically binds to fibronectin and phosphatidic acids (Fig. 2D). Most strikingly, failure to adhere to the host cell surface, either by deletion of the MAM gene or by competitive inhibition of MAM-mediated binding using MAM-based, synthetic beads, leads to a significant reduction of cell invasion and host protection in a moth larvae infection model (Fig. 2 and 4). Deletion of MAM does not cause a secretion defect, as in the absence of MAM both IpaB and IpaC were still produced and secreted in response to the environmental cue, Congo red (Fig. 3A). This suggests that MAM does not directly impact on the function of TTSS. Conversely, TTSS function is not
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needed to achieve MAM-mediated binding, as was previously shown in *V. parahaemolyticus* (Krachler et al., 2011). Thus, we conclude that in contrast to IcsA, MAM and TTSS are mutually independent. However, bacteria fail to inject effector proteins into host cells, when MAM-mediated attachment is abrogated by MAM gene deletion (Fig. 3). This finding suggests the following as a likely scenario: MAM-mediated intimate attachment brings the bacteria within close proximity of the cell membrane, allowing TTSS to engage with the cells membrane for activation. It is known that Shigella TTSS activation requires the formation of a translocon on the host cell membrane (Veenendaal et al., 2007). This event can only occur when Shigella is within close proximity of the host cell surface. MAM is relatively small compared to other known adhesion molecules, such as flagella and pili (Jaglic et al., 2014). Therefore intimate adherence by MAM may be required to bring Shigella within close enough proximity to the host cell surface to allow TTSS translocon formation on the host cell membrane. It is known that Shigella TTSS is also responsible for bacterial escape from the phagocytic vacuoles into the cell cytosol and cell-to-cell spread (Page et al., 1999, High et al., 1992). Whether Shigella MAM plays a part in these events as well as in translocation of late effectors during intracellular growth requires further investigations.

Recently, another surface protein, IcsA, has been described to mediate attachment of *S. flexneri* to the host cell surface, in addition to its role in actin based motility inside the host cells (Brotcke Zumsteg et al., 2014). In *S. sonnei*, IcsA’s role is not redundant with MAM so both adhesins may be required during different stages of the infection process. In *S. flexneri*, IcsA-mediated attachment depends on TTSS activity and, similar to TTSS function, is activated by bile salts which leads to IcsA adopting an altered, protease resistant and attachment competent conformation (Brotcke Zumsteg et al., 2014). In *S. sonnei*, we also found IcsA to be necessary for adhesion and invasion (Fig. 2), but deoxycholate caused a reduction in attachment and invasion
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by the wild type as well as the mutant strains (Fig. 7A and B). qPCR analysis confirmed that
deoxycholate caused significant reduction of MAM and IcsA transcription (Fig. 7C). The
attenuation of virulence by DOC was further confirmed by Sereny test and in our newly
established moth larvae model (Fig. S6A and S6B). The differential effect of DOC to S. flexneri
and S. sonnei may explain why S. sonnei is less virulent and requires a much higher infectious
dose to cause disease [7].

As noted above, the MAM gene in S. flexneri strain 301 (Sf1391) is a pseudogene due to
an in frame stop codon (Yang et al., 2005). Thus, S. flexneri may have evolved different
regulatory mechanisms to coordinate IcsA-mediated attachment and TTSS. According to
available genome sequences, a number of S. flexneri strains have the same in frame stop codon;
they are Shi06HN006, 2003036, 2002017, 8401, and 2457T. We have also confirmed by PCR-
sequencing that strain M90T, used by (Brotcke Zumsteg et al., 2014) has the same mutation (data
not shown). On the other hand, three S. flexneri genomes appear to have intact MAMs;
AFGY01000021.1, AKMV01000023.1, and AKMY01000025.1. The significance of IcsA
attachment in these strains with intact MAM genes remains to be investigated. Investigations to
compare S. flexneri strains with and without intact MAM genes would help us to gain a more
detailed understanding of the regulation of attachment and cell invasion by deoxycholate.

(Brotcke Zumsteg et al., 2014) also observed IcsA-independent attachment. Although the
molecular basis for IcsA-independent attachment is unknown, MAM is unlikely to be involved
since it is heavily truncated in this strain. Many genes involved in adherence are all lost in
representatives of four groups of Shigella strains, due to genome reduction, including fimbriae,
pili and flagella (Yang et al., 2005). At the time of genome annotation MAM was not
recognized. But, an intact orthologue of YadA from Yersinia pestis was noted in the genome of S.
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sonnei strain Ss046. The YadA orthologs in the other three classes of Shigella all possess significant truncations at the C-termini (Yang et al., 2005). YadA mediates effective attachment of *Y. pestis* to host cells and improves the delivery of the TTSS effector protein YopE (Rosqvist et al., 1990). Whether the truncated YadA is functional and responsible for the observed IcsA-independent attachment in *S. flexneri* remains to be investigated. But, *S. sonnei* YadA is the prime suspect responsible for the residual attachment of the ∆MAM∆icsA double mutant observed (Fig. 2A). YadA mediates effective attachment of *Y. pestis* to host cells through binding to fibronectin and β1 integrin (Eitel & Dersch 2002) and improves the delivery of the TTSS effector protein YopE (Rosqvist et al., 1990). Thus, the significance of the YadA ortholog in *S. sonnei* certainly deserves further investigation.

The Galleria mellonella larvae infection model

Non-human primate rhesus monkeys (Macaca mulatta) are natural hosts of Shigella, with an infection phenotype that mimics human dysentery (Islam et al., 2014). This NHP model is however very costly. Therefore, other animal models such as the murine lung and rabbit ligated ileal loop models have been developed for defining some of the immune and inflammatory components of the disease (Philpott et al., 2000). Recently, the great moth Galleria mellonella has gained popularity as a model for assessing virulence and a number of pathogens have been studied using this model, including enteropathogenic *E. coli* (EPEC), *Salmonella typhimurium* and *Listeria monocytogenes* (Leuko & Raivio, 2012, Viegas et al., 2013, Harding et al., 2013). To our knowledge, this study is the first to report the use of this model for assessing virulence of *Shigella*. Wild type *S. sonnei* kills *G. mellonella* larvae in a dose-dependent manner, and mutations in MAM and other virulence genes greatly increased larvae survival (e.g., Fig. 4).
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More importantly, our results have revealed that larvae killing by wild type S. sonnei is in part due to bacterial invasion of hemocytes, which eventually undergo apoptosis (Fig. 4 and S3). Deletion of the MAM gene or application of MAM-based synthetic beads leads to reduced invasion of hemocytes and apoptosis (Fig. 5). Hemocytes possess characteristics of vertebrate macrophages (Harding et al., 2012). Induction of apoptosis in hemocytes is consistent with induction of apoptosis in human macrophages, due to activation of caspase I by secreted IpaB (Chen et al., 1996). Homologs to human caspases have been identified in G. mellonella and are involved in tissue remodelling during metamorphosis (Khoa et al., 2012). However, their characterization has been limited by the absence of a whole genome sequence and little is known about their role during infection.

The results we obtained in the Galleria larvae model were consistent with those obtained using the well-established guinea pig keratoconjunctivitis model. Wild type S. sonnei induced full keratoconjunctivitis within 3 days, whereas the MAM mutant failed to produce visible signs of the disease (Fig. 6). Our unpublished data show that several other mutations in well-defined virulence genes, including mxiD, ipaB and dsbA, also resulted in decreased mortality in the Galleria larvae, consistent with these strains’ inability to cause conjunctivitis in the Sereny test. Taken together, these data indicate Galleria larvae are a suitable initial model for studying Shigella virulence and encourage its use for future studies. Our study along with others, also calls strongly for sequencing the genome of Galleria mellonella.

The potential of MAM deletion strains as a new strategy for vaccine development

Deletion of icsA attenuates Shigella virulence in the Sereny test (Brotcke Zumsteg et al., 2014). This has been the basis of development of live attenuated vaccines, some of which are
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currently undergoing clinical trials (Rahman et al., 2011, Camacho et al., 2013). Although some of these candidates have shown promising results, their efficacy especially in endemic regions has proven insufficient (WHO, 2006). Our results show that a S. sonnei MAM deletion strain leads to attenuation of virulence in the guinea pig eye infection but still elicited strong immune responses (Fig. 6). Thus, deletion of MAM may be an alternative approach in engineering future live attenuated vaccines for S. sonnei.

The potential of using MAM as a target for therapy or vaccination

The use of MAM-based synthetic beads as an adhesion inhibitor has shown potential in the prevention of a number of bacterial infections, in particular in treating Pseudomonas infection of burns and wounds (Krachler et al., 2012a, Krachler et al., 2012b). Our results show clearly that applying MAM-coupled polymer beads can reduce S. sonnei invasion of cultured host cells and protect Galleria larvae from S. sonnei infection (Fig. 5). These data call for further investigations into the potential of MAM-based adhesion inhibitors for therapy and vaccination. One uncertainty is whether a sufficient amount of beads could reach the intestinal tract following oral dosing. Dosing is easily controlled following topical application of beads to skin. However, it is hard to estimate how much inhibitor would be required via oral administration to ensure a sufficiently high concentration at the site of Shigella infection, the rectocolon. In this regard, expression of MAM on the surface of probiotic bacteria such as Lactobacillus might be more practical. Shigella infection elicits antibodies against invasion plasmid antigens (Ipa proteins) which does not offer protection. Shigella infection or vaccination also elicit serotype specific antibodies which offer limited protection which is not long lasting (Camacho et al., 2013). MAM is anticipated to induce a strong antibody response and again expression of MAM in probiotic bacteria may offer an
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alternative for protection against infection by all Shigella strains with functional MAM. Taken together, the present study has uncovered a novel adhesin contributing to S. sonnei pathogenesis. Due to its essential role during infection and its ability to trigger a strong immune response, Shigella MAM may offer future therapeutic potential in the prevention and treatment of Shigellosis.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial wild type strain used in this study was Shigella sonnei strain 20071599 (Xu et al., 2014) and mutants thereof. Bacteria were routinely grown on Congo red TSA plates or in liquid LB at 37° C. For pMAM-His, 0.2 mM IPTG was added to the media to induce protein expression. To obtain EGFP expressing bacteria, strains were transformed with pGEM T-easy or pET28a containing EGFP. 100 µg ml⁻¹ ampicillin or 50 µg ml⁻¹ kanamycin was added for selection of plasmid containing strains. All primers and strains used in this study are listed in Tables S1 and S2.

Construction of S. sonnei deletion and complementation strains

Gene deletions in the S. sonnei 20071599 background (Xu et al., 2014) were constructed using the phage λ Red recombination system (Datsenko & Wanner, 2000). The plasmid, pKD46, which carries the red lambda recombinase genes, was transformed into strain 20071599. The kanamycin resistance cassette flanked by the first and last 51 base pairs of MAM coding...
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sequences was amplified by PCR using primers a, b (S1 Table) and used to replace the wild type MAM gene via red lambda mediated homologues recombination (Fig. S1B). Plasmid, pCP20, was then introduced into the Kan-resistant mutant strain, allowing a second homologues recombination to loop out the Kan-cassette. The resultant strain, ΔMAM, harbored an in frame deletion with a scar of 102 base pairs (Fig. S1C). icsA and MAM icsA double knockout strains were constructed in the same way using primers g, h (Table S1). For complementation of the MAM deletion strain, the entire coding region of MAM SSO1327 was amplified with primers e and f (Table S1), which incorporated 6X histidine codons at the 3’-end of the MAM coding sequence. The PCR product was cloned into pGEM T-Easy (ampR), with the 5’-end of the MAM coding sequence facing the lacZ promoter. The resultant clone was transformed into the ΔMAM mutant. For complementation of the icsA deletion strain, the promoter and entire coding sequence of SSOP143 icsA/virG gene was amplified with primers k and l (Table S1), which incorporated 5’ EcoRI and 3’ Sall restriction sites. The PCR product was cloned into pET28a (kanR). The resultant clone was transformed into the ΔicsA mutant. Plasmid, pMI, was constructed by cloning PCR-amplified icsA gene with its promoter using primers k_Pst and l_Pst. The PCR product was cut with PstI and cloned into the PstI-digested pMAM-6XHis clone. The resultant plasmid, pMI, was used to transform the ΔMAMΔicsA double mutant for complementation experiments. All steps of strain construction as well as complementation were confirmed by PCR and sequencing, using appropriate primers (Fig. S1C-D and Table S1).

Cell fractionation and Western Blotting to determine MAM localization
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For membrane preparation, bacterial cultures were grown overnight at 18 °C, harvested by centrifugation (3500 rpm, 20 minutes, 4 °C), washed once with PBS and resuspended in fresh lysis buffer TE (20 mM Tris, 10 mM EDTA, pH 8.0). Cells were sonicated for 12 cycles (30 sec on, 30 sec off). Samples were first centrifuged at 15000xg for 45 minutes at 4 °C to separate the supernatant. The supernatant fraction was then ultracentrifuged (60000xg, 1 h at 4 °C). The pellets were subjected to phase separation to obtain the membrane fraction. Pellets were resuspended in 200 µl of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-114 at 0 °C. For membrane protein separation, a cushion (300 µl) of 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 6% (w/v) sucrose, and 0.06% Triton X-114 was placed first in 1.5-ml microfuge tube and then the protein sample was then overlaid. The tube was incubated at 30 °C for 3 min before centrifugation for 3 min at 300xg. After centrifugation, the detergent phase was clearly separated at the bottom of the tube as an oily droplet, while the aqueous phase was on the top. The aqueous layer was removed and received 0.5% fresh Triton X-114, the surfactant dissolution happened at 0 °C and then overlaid on the sucrose cushion used before, incubated at 30 °C for 3 min before centrifugation [42]. Finally, the oily droplets of detergent that assumed to have the amphiphilic integral membrane protein were analyzed by SDS-PAGE and Western Blott.

OmpA was detected using the polyclonal antibody orb6940 (anti-OmpA), followed by incubation with Alexa Fluor 680 F(ab)₂ fragment of goat anti-rabbit IgG. MAM-His was detected using the 6X-His epitope Tag antibody (His-H8), followed by incubation with anti-mouse Alexa 680. Membranes were then viewed and imaged using a multi wavelength scanner at 700 nm.

Analysis of MAM localization by immunostaining
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All strains ΔdsbA, ΔMAM, ΔMAM/pMAM-His, and ΔdsbA/pdsbA-His, were transformed with pET28a containing EGFP. Bacteria were grown in LB to mid-log phase, and collected by centrifugation and washed once with PBS. Bacterial suspensions were then spotted on glass slides, left to dry, fixed by 4% paraformaldehyde for 10 minutes and then washed with PBS. Slides were treated with or without 0.1% Triton-X100 in PBS, and then washed with PBS, and blocked for 30 minutes with 5% BSA in PBS. Primary staining was done on both sets using 6X-His epitope tag antibody (His-H8) (1:10 dilution in PBS) at RT for 30 minutes. Secondary staining was done using TRITC-conjugated goat anti-mouse antibody at RT for 30 minutes. Stained samples were analyzed by imaging on a Nikon Eclipse Ti fluorescence microscope and images were prepared using Image J and Corel Draw X5.

Adherence and invasion assays on cultured cells

HEK293 and HeLa cells were routinely cultured at 37 °C and under 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 110 mg L⁻¹ sodium pyruvate, 10 ml L⁻¹ of 100x non-essential amino acids, 100 units ml⁻¹ penicillin and 20 μg ml⁻¹ streptomycin. For adherence assays, bacteria were adjusted to an MOI of 30 in DMEM without supplements and added to host cells. Plates were centrifuged (1000xg, 22 °C, 5 minutes) prior to incubation for 15 min at 37 °C. Following infection, cells were washed thoroughly with PBS (at least three times), and lysed by addition of 0.1% Triton X-100 for 10 minutes. Lysates were serially diluted, plated on LB plates, incubated at 37 °C overnight and colonies enumerated the next day. For PA depletion experiments, 50 μg ml⁻¹ PLC was added to tissue culture cells for 15 minutes prior to infection, as previously described (Lim et al., 2014). Fibronectin-dependency was also tested as previously described (Krachler et al., 2012b), and bacterial cells were pre-incubated with fibronectin from human plasma (200 μg ml⁻¹ in PBS) for 30 minutes prior to
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Infection. For invasion assays, cells were infected with MOI of 10; bacteria were added to cells and centrifuged (2000 rpm, 22 °C, 10 minutes), and incubated for 40 minutes. Following this, cells were washed with PBS prior to addition of 1ml/well of DMEM containing 50 µg ml\(^{-1}\) gentamycin. The plate was then incubated for a further 2 hours, cells washed three times with PBS, lysed and colony forming units determined using dilution plating, as described above. When testing the effect of MAM beads on invasion, host cells were treated with 5 µM MAM beads or control (GST-coupled) beads for 30 minutes prior to invasion assays. Technical details of bead synthesis and molar ratio calculation have been described previously (Krachler & Orth. 2013, Stones et al., 2015)

Analysis of TTSS effector production and secretion by Western Blotting

Colonies were picked from Congo red plates, and bacteria were grown in TSB to mid-log phase. Cells were pelleted, washed with sterile PBS, resuspended in fresh PBS containing 0.01% Congo red and incubated at 37 °C for 30 minutes. When MAM and control beads were tested for their ability to interfere with TTSS, they were added at a final concentration of 10 µM prior to the 30 minute incubation. Cells were then centrifuged to separate samples into pellet fraction (to give total lysates reflecting protein production) and supernatants (to check for protein secretion). Pellets were washed once with PBS and boiled in sample buffer. Supernatants were used to precipitate proteins by precipitation with 10% TCA, as described previously (Yu et al., 2000). Following TCA addition, samples were incubated on ice for 20 minutes prior to centrifugation to pellet proteins (14000 rpm, 4 °C, 20 minutes). Protein pellets were washed in 70% ethanol, dried, resuspended in SDS-PAGE sample buffer and boiled for 5 minutes. Samples were separated by SDS-PAGE and IpaB and IpaC were detected by Western Blotting using the monoclonal antibodies H16 (anti-IpaB) and J22 (anti-IpaC), respectively (Yu et al., 2000) followed by Alexa
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Fluor 680 goat anti-mouse IgG and membranes were imaged on a multi wavelength scanner at 700 nm.

**Analysis of TTSS effector translocation by FRET**

In order to use red lambda system to create an ipgD-TEM fusion (ipgD encodes a TTSS substrate and TEM encode β-lactamase), we modified Plasmid pkD46 for kanamycin resistance instead of ampicillin resistance. By using inverse PCR the whole pkD46 except bla was amplified using primers w and x (Table S1). The purified inverse PCR product as well as Kan cassette PCR product using pkD4 plasmid as template with primers u and v (Table S1), were digested using BglII restriction enzyme, then ligated and electroporated into DH5α. Colonies were selected on Kan/LB agar and the pKD46/pKD4 plasmid was extracted and verified for the right direction using XhoI and HindIII enzymes. The new pKD46/pKD4 plasmid was introduced into both wild type and ΔMAM. The TEM gene was introduced to these two strains using primers s and t (Table S1), which resulted in an ipgD-TEM in frame fusion. The empty pGEM T-Easy (ampR) plasmid was transformed into wild type and ΔMAM to serve as negative controls. The constructed strains both possessed β-lactamase activity and compared to negative controls (wild type strain), by use of nitrocefin discs according to the manufacturer’s protocol (Oxoid). Briefly, pure colonies of all strains were picked, suspended in PBS and applied directly to the discs and discs were incubated at 22 °C for 30 minutes. Positive reaction was identified by red colored discs (Fig. S2). For FRET assays, 6X CCF4-AM substrate loading solution was prepared, 10^6 cells of HEK293 cells were prepared in 100 µl of EM buffer (120 mM NaCl, 7 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose) (Nothelfer et al.). 20 µl of 6X CCF4-AM were added to each tube of 100 µl of the
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cells and incubated in the dark at 22 °C for 1 hour. FRET reporter strains were added to HEK293 cells at an MOI of 100 in DMEM and centrifuged (300g, 10 minutes, 22 °C) prior to incubation at 37 °C for 50 minutes (Nothelfer et al.). Cells were washed once with PBS, and the infection was stopped by adding EM buffer containing 50 µg ml⁻¹ gentamycin. Cells were fixed with 4% paraformaldehyde and mounted on glass slides for FRET imaging, using a Nikon A1R confocal fluorescence microscope. Ratiometric analysis was done using Image J. Alternatively; cells were subjected to analysis by flow cytometry (excitation 355nm, and emission 457 nm for coumarin and emission 529 nm for fluorescein).

G. mellonella larvae infection model

Bacterial strains were grown to mid-log phase in LB to prepare stocks for infections. For each experimental condition, 10 healthy 2-3 day old larvae of approximately equal weight were infected with S. sonnei strains by injection of 10 µl total volume into the front, right proleg. The control group received the same volume of sterile PBS buffer instead. Two infectious doses (10⁵ CFU and 10⁶ CFU) were tested. Since a dose of 10⁶ CFU S. sonnei wild type strain caused mortality within one day, the lower dose was used for subsequent experiments; exceptions are stated in the figure legend. For MAM bead protection experiments, larvae were co-injected with 10 µl total volume containing a mixture of either 10⁵ CFU or 10⁶ CFU S. sonnei wild type bacteria and 6-25 µM MAM or GST (control) beads. Preparation of MAM beads has been described elsewhere (Lim et al., 2014). To study the effects of DOC treatment on infection, larvae were challenged with S. sonnei strains grown overnight with or without DOC (2.5 mM). Following infection, larvae were incubated at 37 °C and larval survival was scored daily, for five
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days. Larvae were scored as dead when they no longer responded to touch. Survival data were analyzed using the Kaplan-Meier estimator. Survival curves were compared using the Mantel-Cox log-rank test and differences in the mortality of larvae infected with wild type and ∆MAM strains were significantly different (p<0.0001).

G. mellonella hemolymph processing for flow cytometry and microscopy

Following 4 hours of infection, hemolymph of 10 larvae per experimental condition was collected by making an incision between the two segments nearest the larvae tail to avoid gut disruption. Hemolymph was collected in 0.5 ml of sterile PBS and processed within 10 minutes to prevent clotting (Harding et al., 2013). For imaging, hemolymph was transferred to a glass coverslip in a 24-well plate; 0.5 ml of PBS was added and mixed well. The plate was then centrifuged at 2000 rpm for 10 minutes at room temperature using an aerosol-tight centrifuge plate holder. The supernatant was carefully removed and cells were washed with PBS. Cells were fixed in 4% (v/v) paraformaldehyde in PBS for 10 minutes. 0.5 ml of 15 mM NH₄Cl in PBS was used to quench residual paraformaldehyde and the plate was incubated at RT for 15 minutes. Afterwards, cells were permeabilized for 5 minutes at RT using 0.5 ml of 0.1% Triton X-100 in PBS. Blocking was done for 1 hour using 2% (w/v) BSA in PBS, and then cells were stained with Alexa Flour 548-phalloidin and Hoechst. Samples were mounted and cured overnight prior to imaging. For flow cytometry, hemolymph was centrifuged at 2000 rpm for 10 min at RT, and hemocytes were resuspended in 1 ml PBS containing 4% (v/v) paraformaldehyde in PBS for fixation. Cells were vigorously mixed and then were used for flow cytometry. For analysis,
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Lasers emitting at 488 nm were used for detection of EGFP expressing bacteria. Data acquisition and analysis was performed using Kaluza™ software.

qPCR analysis of icsA and MAM transcription

Wild type *S. sonnei* strain was grown in the presence or absence of 2.5 mM DOC overnight in L-broth with shaking (200 rpm). RNA was isolated using a Total RNA kit (Bioline). The house-keeping gene, cycG, was used as an internal control. Primers y,z; y1,z1 and y2,z2 (Table S1) were used to prepare cDNA for icsA, MAM and cycG genes, respectively. To establish standard curves for each gene, a serial dilution (from 100 to $10^8$ molecules/µl) of bacterial genomic DNA was used for PCR using SYBR-Green QRT-PCR kit on Rotor Gene 6000 (Qiagen). Changes in gene expression between untreated and DOC-treated cultures were calculated using the $2^{-\Delta\Deltact}$ method and proprietary software in the Rotor Gene instrument (version 1.7.34). Triplicate RNA samples from triplicate cultures (n = 3) were used to prepare cDNAs, which were quantified by the same PCR procedure. The amplification curves of MAM and icsA were normalized with that of cycG, and quantification was calculated using the standard curves. The levels of transcripts from bacteria grown in the absence of DOC were set as calibrator and levels of transcripts from bacteria grown in the presence of DOC were expressed as $\log_2{2^{-\Delta\Deltact}}$.

Guinea pig keratoconjunctivitis model (Sereny test)

Experimental protocols involving guinea pigs were performed with approval of Xi’an Jiao Tong University ethics committee. Guinea pigs 6-8 weeks old, weighing 200-300 g, were infected with $5 \times 10^8$ CFU per eye of *S. sonnei* wild type or ΔMAM strains, as previously described [40]. For testing deoxycholate regulation, wild type *S. sonnei* was grown overnight in...
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the presence or absence of 2.5 mM DOC. Development of keratoconjunctivitis was monitored and scored daily up to 14 days.

Immunization experiments

Guinea pigs (n = 3) were infected with 5X10^8 of ΔMAM strain per eye at days 1, 14, 28. On day 31, wild type *S. sonnei* (5X10^8 CFU per eye) were used to challenge the animals. Animals were observed daily for development of keratocunjunctivitis. A second group of guinea pigs (n = 3) were immunized with ΔMAM strain at day 1, 14, 28, 32, and 48. On day 51, animals of the immunization group as well as those challenged with wild type *S. sonnei* were sacrificed. Spleen lymphocytes were isolated for detecting specific antibody secreting cells against *S. sonnei* by ELISPOT. All procedures were described previously [40].

Ethics Statement

The animal experiments in this study were approved by the Laboratory Animal Administration Committee of Xi’an Jiaotong University under the licence (No XJTU2014-102), and performed according to the guidelines of Animal Experimentation of Xi’an Jiaotong University, The Guideline on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research, and The Guide for the Care and Use of Laboratory Animal published by the US National Institute of Health (NIH publication No. 85-23, revised 2011). We carry out our best laboratory practice to ensure animals suffer from minimal stress during all experiments.
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AUTHOR CONTRIBUTIONS

Conceived and designed experiments: AMK JY

Performed the experiments: RYM DHS WL DW YW

Analyzed the data: RYM DHS AMK JY

Contributed reagents/materials/analysis tools: DHS AMK JY ME ERA

Wrote the paper: RYM AMK JY

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

FIGURE LEGENDS

Figure 1. The Shigella sonnei MAM is localized at the bacterial outer membrane. (A) S. sonnei ΔMAM (Δ) and complementation strain (ΔMAM+pMAM-His, M) were grown to mid-log
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phase and soluble and membrane fractions separated by SDS-PAGE. MAM and OmpA (OM protein, fractionation control) were detected by Western Blotting using antibodies against His-tagged MAM and OmpA, respectively. (B, C, D, E) Strains ∆MAM/pMAM-His and ∆dsbA/pDsbA-His expressing EGFP were treated with or without Triton X-100, and anti-His antibodies, followed by TRITC-labelled secondary antibodies. EGFP-Shigella (green), MAM-His and DsbA-His (red), and merged channels are shown. Scale bar = 5 μm.

**Figure 2. Functional characterization of *S. sonnei* MAM and IcsA in host cellular adhesion and invasion.** Attachment of *S. sonnei* strains to HeLa cells (A) following a 15 min infection at an MOI of 30 was determined by Triton X-100 lysis and plating. (B) Invasion of HeLa cells was determined by gentamycin protection experiments following 2 hours of infection at an MOI of 10. (C) Conservation (Y) or absence (N) of basic residues (H/K/R) at key positions required for high affinity phosphatidic acid binding within MCE domains 1-7 of *V. parahaemolyticus* and *S. sonnei* MAMs. Sequence based alignment is shown for MCE2 domains, with key conserved PA-binding residues highlighted in pink. (D) Attachment of wild type and complemented MAM deletion strain to HeLa cells, which were left untreated or treated with phospholipase C (+PLC) or preincubated with fibronectin (+Fn). All results are means ± standard deviation (n=3). Significance of results compared to those for the wild type strain was determined using a t-test: (*) p = 0.0163, (**) p = 0.0021, (***) p < 0.0001.

**Figure 3. Analysis of *S. sonnei* MAM role in production, secretion and translocation of type III secretion system substrates.** (A) *S. sonnei* wild type, ∆MAM or ∆MAM/pMAM-His strains were grown to mid-log phase, type III secretion was induced with Congo red and cell lysates and culture supernatants analyzed for the presence of type III system secreted effectors by SDS-PAGE and Western Blotting with antibodies against IpaB and IpaC. HEK293 cells were either
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mock infected (B and G), or infected with *S. sonnei* wild type (C, H) or ΔMAM (D, I) strains carrying chromosomal ipgD-bla fusions or wild type carrying pGEM T-Easy (E, J). Substrate translocation was analyzed by FRET imaging (B-E). Scale bar, 10 μm. Results of ratiometric image analysis are means ± standard deviation (F: n=3). Significance of results compared to those for the control (mock infection) was determined using a t-test and asterisks indicate p-values < 0.001. ns: not significant. Substrate translocation was also analyzed by flow cytometry (G-J). Marked increase of donor (450 nm) and decrease of acceptor fluorescence (535 nm) was observed in cells infected by wild type strain compared to cells infected by ΔMAM strain or cells infected by wild type strain carrying pGEM-T-Easy (H vs. I; H vs. J).

**Figure 4. MAM is required for *S. sonnei* pathogenesis and interaction with phagocytes in vivo.** 2-3 day old *G. mellonella* larvae were injected with a dose of $10^5$ CFU of *S. sonnei* wild type (A), ΔMAM (B), ΔMAM+pMAM-His (C) or an equivalent volume of sterile buffer (D) and imaged on day 2. Larval mortality of animals injected with buffer (control, crosses), or $10^5$ CFU of wild type (black square), ΔMAM (black triangles) or ΔMAM+pMAM-His (empty circles) was followed over five days and data shown as Kaplan-Meier survival curves (E). Hemocytes were isolated from the hemolymph of larvae infected with *S. sonnei* wild type (F), mock infected (G), or infected with ΔMAM (H) or ΔMAM+pMAM-His (I) at $10^5$ CFU for 4 hours. Samples were visualized by microscopy and DIC (grey), Hoechst (blue), GFP Shigella (green), F-actin (red) and merged images are shown. Scale bar, 5 μm. Bacteria per cell were quantified by image analysis (J) and at least 100 hemocytes were analyzed per experimental condition. NA: not analyzed. Alternatively, hemocytes (infected with wild type *S. sonnei*, grey or ΔMAM, green) or mock infected (yellow trace) were analyzed for GFP fluorescence by flow cytometry (K). All results are means ± standard deviation (n=3). Significance of results compared to those for the wild type
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strain was determined using a t-test and asterisks indicate p-values < 0.0001. ns: not significant.

Also see S3 Fig.

**Figure 5. A MAM mimetic adhesion inhibitor can efficiently protect against *S. sonnei* invasion and pathogenesis.** *G. mellonella* larvae were injected with $10^5$ CFU *S. sonnei* wild type (A), a mixture containing $10^5$ CFU *S. sonnei* and 25 μM MAM beads (B) or a mixture containing $10^5$ CFU *S. sonnei* and 25 μM GST (control) beads (C) and imaged on day 2. Mortality rates over 5 days of larvae injected with $10^5$ CFU wild type and either buffer (black squares), or MAM beads at a final concentration of 25 (empty triangles), 19 (black triangles), 12.5 (dotted line) or 6 μm (empty circles) were analyzed using Kaplan-Meier survival curves (D). Hemocytes were isolated from larvae injected with a mixture containing $10^5$ CFU *S. sonnei* and 25 μM GST (control) beads (E), a mixture containing $10^5$ CFU *S. sonnei* and 25 μM MAM beads (F) or 25 μM MAM beads but no bacteria (G). Samples were visualized by microscopy and DIC (grey), Hoechst (blue), GFP Shigella (green), F-actin (red) and merged images are shown. Scale bar, 5 μm. Bacteria per cell (green bars) and beads per cell (black bars) were quantified by image analysis, and at least 100 hemocytes were analyzed per experimental condition (H). NA: not analyzed. The effect of MAM beads or control (GST) beads on bacterial invasion of HEK293 cells was determined using gentamycin protection assays and compared to invasion of untreated (-) cells (I). Results are means ± standard deviation (n=3). Significance of results was determined using a t-test and asterisks indicate p-values < 0.0001. ns: not significant. Also see Figure S3 and S4.

**Figure 6. Role of MAM in a keratoconjunctivitis model of Shigella infection and immunity.** (A-D) Sereny test. Guinea pigs (n = 3) were infected with $5 \times 10^8$ CFU of bacteria per eye. All
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Animals infected with wild type *S. sonnei* developed full keratoconjunctivitis in 3 days. Animals infected with ∆MAM strain did not show any sign of conjunctivitis up to 14 days. Animals before (A) and after (B) infection with wild type and before (C) and after (D) infection with ∆MAM deletion strain for five days are shown. E. ELISPOT data. Animals in group 1 (gp/Wt) were immunized with ∆MAM three times and challenged with wild type *S. sonnei*; animals in group 2 (gp/delMAM) immunized with ∆MAM strain four times; animals in group 3 were mock infected with PBS four times. Both group 1 and group 2 animals produced significantly higher antibody secreting cells (ASC) specific to *S. sonnei* LPS in spleen lymphocytes compared to group 3 mock infected animals (** indicate p < 0.001). The difference between group 1 and group 2 animals are not statistically significant (P = 0.05).

**Figure 7. Deoxycholate negatively regulates MAM and icsA in *S. sonnei*.** (A) Attachment of *S. sonnei* strains either grown in LB or LB containing deoxycholate (+DOC) to HeLa cells following a 15 min infection at an MOI of 30 was determined by Triton X-100 lysis and plating (A). Invasion of HeLa cells was determined by gentamycin protection experiments following 2 hours of infection at an MOI of 10, either following growth in LB or LB containing DOC (+DOC), (B). All results in A, B are means ± standard deviation (n=3). Significance of results compared to those for the wild type strain was determined using a t-test: (*) p < 0.05, (**) p < 0.01, (***) p <0.0001. (C) qPCR analysis of MAM and icsA transcription upon deoxycholate treatment. Data present mean values from triplicate cultures (n=3) for each strain.

**REFERENCES**


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Mounier J, Bahrani FK, Sansonetti PJ. (1997) Secretion of *Shigella flexneri* Ipa invasins on contact with epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent. *Infection and Immunity* **65**: 774-82.


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---

**A**

<table>
<thead>
<tr>
<th>Membrane (mem)</th>
<th>Soluble (sol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAM-His</strong></td>
<td></td>
</tr>
<tr>
<td>OmpA</td>
<td></td>
</tr>
</tbody>
</table>

EGFP: 120 kDa

**B**

- Bi: MAM-His
- Cii: MAM-His + Tx
- Dii: DsbA-His
- Eii: DsbA-His + Tx

**C**

Position of mce2 in Vp and Ss:

<table>
<thead>
<tr>
<th>Position</th>
<th>Vp mce2</th>
<th>Ss mce2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

**D**

- % Attachment
- % Invasion

---

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A lysate

IpaB

IpaC

A supernatant

IpaB

IpaC

B wt control

wild type: *ipgD-bla*

C ΔMAM: *ipgD-bla*

D wt+pGEM-T-Easy

E

F

G

H

I

J

MMI_13255_F3

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![Image of bacterial colonies and fluorescent microscopy images]

**A** DIC, **B** DNA, **C** Shigella, **D** actin, **E** merge.

**F** MMI_13255_F4

**G** wild type, **H** mock, **I** ΔMAM, **J** ΔMAM + ΔMAM.

**K** MMI_13255_F5

**L** Fraction survival

![Graph showing bacterial survival over days]

**M** bacteria/cell, **N** strain, **O** max. fraction, **P** GFP intensity.

**Q** Fraction survival

![Graph showing fraction survival over days]

**R** 0 μM, **S** 25 μM, **T** 19 μM, **U** 12.5 μM, **V** 6 μM.

**W** objects/cell

![Graph showing objects per cell]

**X** bacteria/cell, **Y** beads/cell.

**Z** % HEK invasion

![Bar graph showing HEK invasion]

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A

% attachment

wt | wt+DOC | ΔMAM | ΔMAM+DOC | ΔicaA | ΔicaA+DOC

B

% invasion

wt | wt+DOC | ΔMAM | ΔMAM+DOC | ΔicaA | ΔicaA+DOC

C

log2Δct

MAM exp. | icsA exp.

MMI_13255_F7