Functional dissection of SiiE, a giant non-fimbrial adhesin of *Salmonella enterica*

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Summary

*Salmonella enterica* deploys the giant non-fimbrial adhesin SiiE to adhere to the apical side of polarized epithelial cells. The establishment of close contact is a prerequisite for subsequent invasion mediated by translocation of effector proteins of the *Salmonella* Pathogenicity Island 1 (SPI1)-encoded type III secretion system (T3SS). Although SiiE is secreted into the culture medium, the adhesin is retained on the bacterial envelope in the phase of highest bacterial invasiveness. To dissect the structural requirements for secretion, retention and adhesive properties, comprehensive deletional and functional analyses of various domains of SiiE were performed. We observed that β-sheet and coiled-coil domains in the N-terminal moiety of SiiE are required for the control of SiiE retention on the surface and co-ordinated release. These results indicate a novel molecular mechanism for the control of surface display of a T1SS-secreted adhesin that acts cooperatively with the SPI1-T3SS.

Introduction

The close contact and tight binding to a host cell surface is an initial event of bacterial pathogenesis that is of crucial importance for subsequent phases of the interaction. *Salmonella enterica* is a Gram-negative gastrointestinal pathogen that frequently causes food-borne diseases ranging from gastroenteritis to a life-threatening systemic infection known as typhoid fever (Haraga *et al*., 2008). *Salmonella* is an invasive and facultative intracellular pathogen that is equipped with a wealth of sophisticated virulence mechanisms, and protein secretion and translocation systems can be found in remarkable abundance (Gerlach and Hensel, 2007).

During intestinal pathogenesis, *Salmonella* is in contact with enterocytes and this interaction can result in the invasion of host cells and/or the triggering of an inflammatory response of the host. Earlier work demonstrated that *Salmonella* deploys a type III secretion system (T3SS) encoded by *Salmonella* Pathogenicity Island 1 (SPI1) to translocate a complex cocktail of effector proteins directly into the cytosol of host cells (Schlumberger and Hardt, 2006). These effectors gain control over the actin cytoskeleton of the host cell, resulting in local F-actin assembly that lead to membrane ruffles and uptake of *Salmonella* by macropinocytosis.

While SPI1-T3SS-mediated translocation is sufficient to trigger invasion of non-polarized epithelial cells, entry into polarized epithelial cells, like cells found in all epithelial tissues, requires additional factors. Within epithelia, cells are highly polarized with distinct compositions of apical and basolateral sides, close cell contacts due to formation of tight junctions, as well as microvilli and highly glycosylated structures on the apical side (Heintzelman and Mooseker, 1992). These properties allow the epithelial layer to control the uptake of nutrients, but also to form an efficient barrier to separate the sterile underlying tissue from the rich intestinal microflora. Consequently, mucosal pathogens have evolved a variety of strategies to overcome this barrier (reviewed in Kazmierczak *et al*., 2001; Sansonetti, 2004). We recently found that invasion of polarized cells from the apical side requires the cooperation of the SPI1-T3SS with a novel adhesin (Gerlach *et al*., 2008). Work by our group and others demonstrated that the *Salmonella* Pathogenicity Island 4 (SPI4)-encoded giant non-fimbrial adhesin SiiE (Gerlach *et al*., 2007a; Kiss *et al*., 2007) is required for the adhesion to polarized epithelial cells as well as for the invasion (Gerlach *et al*., 2008).
non-fimbrial adhesins of the autotransporter family (Gerlach and Hensel, 2007). Due to its size of 595 kDa, the highly repetitive structure with 53 repeats of immunoglobulin (Ig) domains and its secretion mechanism, SiiE is a rather uncommon adhesin (Latasa et al., 2003; Latasa et al., 2005). We have recently characterized the SPI4-T1SS and observed the composition of three canonical subunits, namely, SiiF as the inner membrane transporter ATPase, SiiD as periplasmic adaptor protein (PAP) and SiiC as outer membrane protein (Gerlach et al., 2007a). The function of these three subunits is essential for the secretion of the giant non-fimbrial adhesin SiiE, as well as for virulence functions mediated by SPI4 such as adhesion to polarized epithelial and host cell invasion (Gerlach et al., 2007a; Morgan et al., 2007). The SPI4 locus contains two additional genes, siiA and siiB that are co-regulated with siiCDEF and we proposed that siiABC-DEF form an operon (reviewed in Gerlach et al., 2007b).

SiiE is secreted in a SPI4-T1SS-dependent manner into the culture medium. This mechanism appears counterintuitive of a protein that is required to mediate binding of a bacterial cell to a mammalian host cell. In fact, we found that during adhesion to the apical side of polarized cells, SiiE was detected in surface-bound form on the bacterial envelope (Gerlach et al., 2007a). Due to these observations, we hypothesized that SiiE and/or its cognate T1SS deploy a specific mechanism to achieve retention to the bacterial surface during adhesion.

In this study, we set out to characterize the molecular mechanisms of the SiiE-mediated adhesion of Salmonella to polarized cells. We found a novel mechanism of surface binding of SiiE and identified domains involved in the control of retention and release of the adhesin.

**Results**

**Ultrastructural analyses of SiiE**

In order to understand the molecular characteristics of the giant adhesin SiiE, we applied ultrastructural analyses of Salmonella adhering to the apical side of polarized epithelial cells. Using scanning electron microscopy (SEM), we previously observed Salmonella cells in intimate contact to the microvilli on the apical side of MDCK cells (Gerlach et al., 2008). Here, we performed immunogold labelling of SiiE on Salmonella adhering to the apical side of MDCK cells (Fig. 1A). Micrographs from SEM revealed that the bacterial surface was in close contact with microvilli. Immunogold labelling for SiiE was frequently observed on adherent bacteria and about 5–10 events of labelling per bacterial cells were detected. Salmonella-infected MDCK cells were also subjected to transmission EM (TEM) and closer examination of micrographs indicated filamentous material on the bacterial surface in contact to structures on the tip of the microvilli (Fig. 1B). Due to the low number of these events immunogold labelling of SiiE in ultrathin sections remained ambiguous. The strong reduction of events of adhesion and invasion for siiE-deficient strains prevented the analysis of the adhesion phenotype of mutant strains by TEM (data not shown).

We next performed molecular imaging of secreted SiiE by enrichment of SiiE from culture filtrates, followed by direct visualization by EM. We detected filamentous material in large amounts in the supernatants of Salmonella WT, but not of siiE strains. In addition to SiiE other large filamentous protein structures are synthesized in the late log phase of growth, for example flagella or Fim fimbriae. In order to exclude the misinterpretation by the presence of such structures, a strain defective in fimD (fimbrial usher) and fliI (flagellar export ATPase) was generated. The culture supernatant of this strain showed identical filamentous structures in high abundance. Therefore, we propose that the material shown in Fig. 2A is secreted SiiE. The molecules showed a linear structure with a rather homogenous length of 175 ± 5 nm. The diameter of individual SiiE molecules appeared uniform over the entire length. In part, the molecules showed bended structures and were partially aggregated, but the examination of a larger number of individual molecules did not indicate a repetitive pattern. Although SiiE molecules were occasionally found as dimers with Y-shaped organization, there were no indications for a regular oligomeric form of SiiE. EM analyses did not allow detection of specific structural properties at either terminus of the molecule, and the identification of N- or C-termini was not possible.

We have previously generated mutant strains that synthesized SiiE derivatives with defined deletions of various numbers of Ig domains. These strains were used for the preparation of culture supernatants and molecular imaging by EM (Fig. 2B). No filamentous structures were detected in culture supernatant of a strain deleted for siiE, fimD and fliI. The material observed in the supernatant of a strain that secreted SiiE with a short deletion of aa residues 117–172 was indistinguishable in length from WT SiiE. The molecules observed in supernatants of the other mutant strains showed reduced length, but the same diameter of the filaments. SiiE variants with deletions of the 5, 10 or 20 Ig domains resulted in the presence of shorter filamentous molecules in the supernatant. The length of the variant was determined resulting in 117 ± 15 nm, 144 ± 13 nm and 154 ± 29 nm for strains...
Fig. 1. Interaction of *Salmonella* with the brush border of polarized epithelial cells. The apical side of MDCK cells was infected with *S. Typhimurium* WT at an moi of 10. The cells were fixed 30 min after infection and processed for analyses by SEM (A) and TEM (B). A. SiiE on *Salmonella* WT during adhesion to MDCK cells. The samples were incubated with antiserum against SiiE and labelled with immunogold. Scale bar, 500 nm.

B. Various events of contact of *Salmonella* (S) to microvilli (MV) on the apical side of MDCK cells. Note the presence of filamentous material linking the bacterial envelope to the membrane of microvilli (indicated by arrowhead). The lower panel shows details at higher magnification. Scale bars, 1 µm in upper panel, 100 nm in lower panel.
expressing SiiE ΔIg21–40, ΔIg21–30 and ΔIg21–25 respectively. The observed reduction of length was in close correlation with theoretical values, i.e. truncation of 33.4% for ΔIg21–40 (37.7% expected), 18.2% for ΔIg21–30 (18.9% expected) and 12.2 for ΔIg21–25 (9.4% expected).

Taken together, these observations indicate that SiiE has a linear, filamentous organization with a length determined by the repeat number of Ig domains. In accordance with previous findings, SiiE is present on the bacterial surface during interaction with host cells and as well is secreted into the culture supernatant.

SiiE is temporally retained to the bacterial surface

We speculated that in order to mediate adhesion, SiiE either is retained on the cell envelope during secretion, or binds to the bacterial surface after secretion into the extracellular space. To test the latter possibility, we used cell-free supernatants of the WT strain that contained secreted SiiE as determined by ELISA or Western blot analysis. Aliquots of the SiiE-containing supernatants were added to WT, siiE and siiF strains for 30 min prior to infection of MDCK cells and invasion experiments were performed as described before. The invasion of the WT was not affected by addition of SiiE-containing supernatants and no increase of the invasion of siiE- or siiF-deficient bacteria was observed (data not shown). This result indicates that secreted SiiE is not able to bind to the Salmonella envelope in a form that is functional in mediating adhesion and invasion.

We used an ELISA specific for SiiE to investigate the kinetics of secretion. The quantification indicated that secretion into the culture supernatant started at 3 h of subculture. The maximal amounts of SiiE were detected at 6 h of subculture and the level remained high in an overnight culture (Fig. 3A). SiiE was not detectable in the supernatants of siiE or siiF strains.
Fig. 3. SiiE is temporarily retained on the bacterial surface. *Salmonella* WT and SPI4-deficient strains were subcultured in LB broth with aeration at 37°C. At various time points of subculture, aliquots were collected and subjected to quantification of SiiE in the supernatant (A), microscopic analyses of SiiE-positive cells (B, E), quantification of surface-bound SiiE (C) and invasion of polarized epithelial cells (D).

A. Culture supernatants were filter-sterilized and amounts of SiiE were determined by ELISA.

B. Bacteria were fixed, immunostained for SiiE and analysed by epifluorescence microscopy. The proportions of SiiE-positive cells were determined by counting at least 100 bacteria per time point and scoring of fluorescence signals for SiiE.

C. Bacteria were grown as for (B), spotted onto nitrocellulose membranes and immunostained for SiiE. The dot blots were quantified by scanning with an Odyssey system.

D. *Salmonella* WT and the *siiF*-deficient strains were subcultured for various periods of time as indicated and aliquots of the cultures were used to infect MDCK cells. The amount of invaded bacteria was determined as described above and is expressed as percentage of the inoculum. The invasion of the *siiF* strain at 3.5 h of cultures was 0.03% (±0.02%) of the inoculum.

E. *Salmonella* WT and *siiF* strains constitutively expressing GFP (green) were fixed at various time points of subculture and immunostained for SiiE (red). Micrographs of representative bacterial cells are shown. Scale bar, 2 μm.
Previous observations indicated that SiiE can be detected if *Salmonella* is adherent to the apical side of polarized cell (Gerlach *et al.*, 2007a). However, bacterial cells grown in rich media without cell contact were devoid of SiiE and the protein was only found secreted in the culture medium. In this study, aliquots of the bacterial cultures were diluted in PBS, spotted on glass slides, allowed to dry and fixed. Immunofluorescence analyses of the samples indicated that about 30% of WT bacteria were positive for SiiE surface staining. This observation was in contrast to our previous experiment, where the bacteria were resuspended in distilled water on a glass slide (Gerlach *et al.*, 2007a). For quantification of the surface localization of SiiE, the amount of SiiE-positive bacteria at various time points of the culture was determined (Fig. 3B). Representative micrographs for the various time points are shown in Fig. 3E. To investigate the interaction of SiiE with the bacterial envelope in more detail, we performed kinetic analyses for SiiE surface localization. Cells of *siiE* or *siiF* strains were devoid of SiiE staining at any time point of the observation (data not shown). We observed no SiiE-positive bacteria of the WT strain in overnight cultures or after 1 or 2 h of subculture. SiiE-positive bacteria were apparent at 3 h of subculture and the proportion rapidly increased to 30% SiiE-positive cells at 3.5 h of subculture. With prolonged subculture, the proportion of SiiE-positive cells rapidly decreased and at 6 h of subculture, only very few SiiE-positive bacteria were detected.

Furthermore, a dot blot was established that allowed the quantification of SiiE retention of aliquots of bacterial cultures. For this purpose, culture aliquots containing identical amounts of bacteria as determined by OD\textsubscript{600} were fixed and spotted on nitrocellulose membranes. Surface-bound SiiE was detected using antibody raised against SiiE and signals were normalized by signals from dots blots developed in parallel for the detection of LPS. The results of dot blot analyses (Fig. 3C) indicated the same kinetics for SiiE retention as observed by microscopy (Fig. 3B).

We next investigated if secretion and surface retention of SiiE is correlated to the ability of *Salmonella* to invade polarized cells in a SPI1/SPI4-dependent manner. Bacteria subcultured for various periods of time were used to infect MDCK cells (Fig. 3D). Quantification of internalized *Salmonella* indicated that highest invasiveness occurred after 3.0–3.5 h of subculture correlating to an OD\textsubscript{600} of 3.5–3.8. The rates of invasion were strongly reduced prior and after this time point.

Jointly, these results demonstrate that surface retention of SiiE, but not the amount of secreted SiiE was in close correlation with invasiveness of *Salmonella* in polarized cells. This observation indicates that only the bacteria that retained SiiE at the cell envelope can invade polarized epithelial cells.

### Functional dissection of SiiE

Based on the data reported so far, we propose that a molecular mechanism for the controlled retention and release of SiiE to the bacterial envelope must exist. SiiE shows a remarkable domain organization with 53 highly similar repeats of Ig domains that determine the length of SiiE, a short insertion between Ig52 and Ig53 with unknown function (aa 5361–5411), a C-terminal domain that harbours the signal sequence for secretion by the SPI4-T1SS (Gerlach *et al.*, 2007a) and a short N-terminal moiety of unknown function (aa 1–236). A more detailed bioinformatics analysis revealed that the N-terminal moiety contains a domain with eight repeats of heptads with predicted coiled-coil (cc) structure (aa 117–172). This domain is enclosed by regions with predominantly \(\beta\)-sheet secondary structure, termed \(\beta\)-sheet domain #1 (aa 14–116) and #2 (173–236).

As an experimental approach towards the contribution of the various domains to the function of SiiE, we performed an in-frame scarless deletional mutagenesis for chromosomal *siiE*. The Red-mediated recombinering technique allows the precise deletion of DNA sequences within a chromosomal gene and enabled the rapid analyses of large genes within their chromosomal context (Gerlach *et al.*, 2009). Based on the bioinformatics predictions of domains in SiiE, in-frame deletions of various extend were constructed as depicted in Figs 4A–C and 5A–C. Specific domains, or parts of domains, were deleted. Details for the position of deletions can be found in Table 1. A summary of results of the mutagenesis of SiiE is shown Fig. S1.

### Roles of the central and C-terminal moieties of SiiE

For the central region of SiiE containing the Ig domains repeats, alleles were generated that had deletions of 5, 10 or 20 Ig domains. For the C-terminal part, we generated strains that expressed SiiE alleles with deletions of Ig48–52, Ig49–52, Ig50–52, Ig51–52, Ig52 or Ig53 only, or the C-terminal half of Ig52 or the N-terminal half of Ig53. Other alleles lacked codons encoding the 51 aa insertion between Ig52 and 53 or the insertion and Ig53. Furthermore, codons in the N-terminal part of Ig53 were deleted as indicated in Fig. 4C. The synthesis of all deletion variants of SiiE was confirmed by Western blot analyses of cell lysates of the corresponding strains (data not shown). As functional tests, the secretion of the SiiE variants was compared with WT SiiE secretion (Fig. 4D). The retention to the bacterial surface was determined by dot blot analyses (Fig. 4E) and finally, the invasion of polarized cells was quantified (Fig. 4F).
For deletions in the central part of SiiE we observed that secretion into the culture supernatant was similar to WT SiiE. Surface retention of D\textsuperscript{Ig}21-40 and D\textsuperscript{Ig}21-30 slightly reduced compared to WT and D\textsuperscript{Ig}21-25 showed highly reduced retention. As previously reported, the reduction of Ig repeats correlated with reduced invasion of polarized cells with about 10-, 100- and 1000-fold reduced invasion for strains with D\textsuperscript{Ig}21–25, D\textsuperscript{Ig}21–30 and D\textsuperscript{Ig}21–40 respectively. All mutations affecting Ig53 resulted in entire lack of SiiE secretion and no surface retention was detectable. As expected, the invasion of polarized cells by these mutant strains was as reduced as that of the siiF strain.

In contrast, deletions of Ig52 or the insertion between Ig52 and Ig53 had no significant effects on invasion of polarized cells. The secretion and retention of these SiiE variants was similar to WT SiiE. The deletions of Ig domains in the region D\textsuperscript{Ig}48–52 had variable effects.
The synthesis of ΔIg50–52 was highly reduced, thus this mutant was not further investigated. The synthesis and secretion of SiIE variants ΔIg48–52, ΔIg49–52 and ΔIg51–52 was comparable to that of WT SiIE. All of these variants showed highly reduced surface retention. Invasion of strain siIE ΔIg51–52 was only slightly reduced, but all other deletion variants in this region resulted in about 1000-fold reduced rates of invasion (Fig. 4F).

Role of the N-terminal moiety of SiIE

The design of deletions in the N-terminal moiety of SiIE is indicated in Fig. 5A and B. Deletions comprised the β-sheet domains #1 and #2, the entire heptad repeats of the cc domain or parts of this domain to various extents. In addition, deletions of the N-terminal Ig domain repeats were generated with siIE alleles lacking codons for Ig1–5, Ig1–4, Ig1–3, Ig1–2 or Ig1 only. For the various SiIE variants with deletions in the N-terminal moiety, we detected secretion similar to that of WT SiIE (Fig. 5C). In contrast, the effects of the deletions on retention (Fig. 5D) and invasiveness (Fig. 5E) were highly divergent. Deletion of 2/3 β-sheet #1 domain (aa 77–116) had only little impact on retention of SiIE, but invasiveness was lost. A smaller deletion 1/3 β-sheet #1 (aa 97–116) resulted in highly increased retention of SiIE at 3.5 h of subculture, but was without effect on invasion. The lack of β-sheet region #2 (aa 173–236) resulted in highly increased retention of the SiIE variant and more than 1000-fold reduced invasion. Between the β-sheet regions, a cc domain was predicted consisting of eight imperfect heptad repeats. The deletions of various numbers of heptad repeats did not alter secretion of SiIE, but affected SiIE retention and invasiveness to different degrees. Invasion was highly reduced if heptads 1–8 or...
heptads 5–8 of the cc domain were deleted (Fig. 5E). Lack of heptad 1–4, or only one or two repeats had no major effect on invasion. The effect on invasion partially correlated to retention of SiiE by the deletion mutant strains, and SiiE variants with deletions of heptad 1–8 or heptad 5–8 showed the lowest levels of retention. In contrast, retention of SiiE variants with deletion of heptad 7–8 or heptad 8 was also highly reduced, but invasion was only reduced to about 20% of WT invasion.

Finally, the effect of deletions of Ig domains in the N-terminal portion of SiiE was analysed, and strains secreting various SiiE variants were constructed ranging from deletion of Ig1 to deletion of Ig1–5. Interestingly, there was a clear correlation between the extent of the deletions and the retention of SiiE, with the lowest retention observed for the SiiE with only Ig1 deleted (Fig. 5D). The retention of SiiE with deletion of Ig1–5 was increased to about 800% of that of WT SiiE. With the exception of SiiE ΔIg1–4, the effect on invasion was gradually affected to the extent of the deletion and a strain with SiiE harbouring a deletion of Ig1 only showed lowest rates of invasion. We currently have no explanation of the strong invasion defect of SiiE ΔIg1–4.

The divergent effects of deletions especially in the N-terminal β-sheet regions on retention of SiiE prompted us to investigate the kinetics of SiiE secretion and retention in more detail. For Salmonella WT and various mutant strains, the amount of secreted (Fig. 6A) and surface-bound SiiE (Fig. 6B) was determined at various time points of growth under SPI1/SPI4-inducing conditions. In addition, the presence of SiiE on the surface of individual bacteria was investigated by immunofluores-
ence of bacteria after 3.5 h or 6 h of subculture (Fig. 6C). Compared to secretion of WT SiiE, SiiE variants with deletions in β-sheet domain #1 showed reduced secretion, while Δβ-sheet domain #2 resulted in similar to increased secretion, respectively, at 3.5 h and later time points. In accordance with the results shown in Fig. 5, the retention of SiiE Δ2/3 β-sheet #1 was highly increased at 3.5 h of culture, but secretion kinetics fol-

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slightly reduced at 6 h and still present after cultivation for 16 h. The microscopic analysis of individual bacteria (Fig. 6C) was in line with the quantification by ELISA and dot blot (Fig. 6B). As observed before, only a proportion of the cells showed presence of SiiE on the surface. For the WT strain, SiiE staining was observed after 3.5 h of subculture and was absent after 6 h. In contrast, similar intense staining was observed for the strain with SiiE Δβ-sheet #2 at both time points. The strain with SiiE Δ1/3 β-sheet #1 exhibited very intensive SiiE surface staining after 3.5 h of subculture, while the cells were almost completely lacking SiiE after 6 h of subculture.

We showed that especially domains in the N-terminal moiety control retention and ordered release of SiiE. A summary of phenotypes of all mutant forms of SiiE is shown in Fig. S1 and data on the effects of mutations in this moiety on SiiE retention and invasiveness are collated in Fig. 7. The integrity of the cc domain is required for the proper retention of SiiE and thereby affects invasion of polarized cells, while the β-sheet domains appear to be essential for the control of release of SiiE. Defects in these domains lead of over-retention or highly delayed release of SiiE.

Discussion

In this study, we performed a detailed functional and molecular dissection of the giant non-fimbrial adhesin SiiE of S. enterica. During infection of polarized epithelial cells, Salmonella was preferentially found in contact with the membranes of microvilli and SiiE appears to establish this contact. The ultrastructural studies of SiiE released into the medium showed that SiiE is a linear filamentous molecule of about 175 nm length. We have previously proposed that the large number of 53 repeats of Ig domains is required to provide an extended molecular structure similar to the shaft of fimbrial adhesins (Gerlach et al., 2007a). The ultrastructure of SiiE as revealed by EM is in line with this model. We also proposed that the length of SiiE is required to project protein domains required for binding to host cells beyond the layer of the O-antigen of LPS (Gerlach et al., 2008). This model is based on the assumption that SiiE acts in a fashion of linear, monomeric adhesins. In fact, we were not able so far to detect linear oligomers of SiiE on the bacterial surface or secreted into the culture medium.

Although genome analyses indicate a high abundance of large T1SS-secreted proteins, only few members of this family have been characterized in further detail so far. In Salmonella, a further T1SS-secreted protein with a highly repetitive domain structure is BapA (Latasa et al., 2005). BapA is involved in systemic virulence of Salmonella and under laboratory conditions of artificial induction, a role in biofilm formation and inter-bacterial binding has been shown. In Pseudomonas aeruginosa, LapA is substrate of a cognate T1SS and a role of LapA in biofilm formation was demonstrated (Hinsa et al., 2003). The molecular characterization of the binding properties of BapA or LapA is pending.

SiiE is substrate of the SPI4-T1SS and secreted into the culture supernatant during growth in rich medium. We
previously detected SiiE on the surface of host cells after contact to host cells, but not after growth in media. The fact that SiiE is released into the external space appears counter-intuitive for a bacterial adhesin, and addition of secreted SiiE could not complement the adhesion of a SiiE-deficient strain to polarized cells. In this study, we changed the procedure for analysing SiiE on bacterial surfaces to resuspension in PBS, in contrast to resuspension in water in the previous study. Bacteria prepared by resuspension in buffer were frequently positive for surface-bound SiiE. This observation may indicate that the retention of SiiE to the bacterial surface is dependent on pH, osmolarity and ionic strength of the medium. Interestingly, we observed that the proportion of SiiE-positive cells was closely correlated with the level of invasiveness, in water in the previous study. Bacteria prepared by resuspension in buffer were frequently positive for surface-bound SiiE. This observation may indicate that the retention of SiiE to the bacterial surface is dependent on pH, osmolarity and ionic strength of the medium. Interestingly, we observed that the proportion of SiiE-positive cells was closely correlated with the level of invasiveness.

SiiE is a highly repetitive protein with 53 repeat of Ig domains and only short protein moieties of distinct structure at the very N- and C-terminal parts. Our previous work indicated a role of the last 60 C-terminal residues as secretion signal for the T1SS (Gerlach et al., 2007a). A short insertion of 51 aa is present between Ig52 and Ig53 is not required for SiiE secretion, retention and adhesive functions. In fimbrial adhesins, a lectin-like subunit is often found at the tip of a polymer of shaft subunits. Whether a single domain of SiiE fulfills a lectin-like function was to be revealed by further work, but deletions of individual Ig domains in the N- or C-terminal moieties of SiiE did not suggest such function. Various deletions in Ig53 and the C-terminal region severely affected secretion of SiiE and consequently, SiiE-dependent invasion was fully abolished. Thus, Ig53 and the C-terminal domain may contain only the secretion signal or both, secretion signal and the function of host cell binding. Previous work on the paradigmatic T1SS substrate HlyA also indicated that the integrity of the C-terminal secretion signal is important for proper folding of the secreted protein (Jumpertz et al., 2010). Since our approach of deletions in the chromosomal siiE gene cannot distinguish between these possible functions, we will further characterize SiiE by binding studies with recombinant C-terminal fragments of SiiE.

Structural predictions for the N-terminal 236 aa residues indicated eight heptad repeats with cc structure that are flanked by regions with predominantly β-sheet structure, and this portion of the molecule is crucial for control of retention and release The deletional analysis led to unexpected observations for the functions of the N-terminal moiety (compiled in Fig. 7). Deletions of the β-sheet domains flanking the cc domain (aa 77–116 or aa 173–236) had dramatic effects on retention and release of SiiE (Fig. 7A). The deletion of β-sheet domain #2 resulted in extended retention of SiiE and a complete loss of SiiE-dependent invasion. A deletion of 2/3 of β-sheet #1 also completely abolished invasion, but retention and release of SiiE were only slightly altered. In contrast, deletion of a smaller portion of β-sheet #1 was without effect on invasion, but led to a highly increased retention during the invasive phase of culture growth. The microscopic examination (Fig. 6B and data not shown) suggests that the amount of SiiE per cell was increased rather than the number of SiiE-positive bacteria, but this increase did not result in a further increase in invasiveness. This indicates that the contribution of SiiE to the invasion mechanisms is already optimized.

Deletion of the entire cc domain or heptad repeats #5–8 resulted in highly reduced retention of SiiE, whereas deletions of heptads #1–4, #1–2 or only #8 had a less pronounced effect on retention of SiiE. The level of retention was directly correlated to the invasion of polarized cells (Fig. 7B). Based on these observations we postulate that the cc domain, and especially its central part, plays an essential role in the retention of SiiE (for a model, see Fig. 8). Deletions of the last 2/3 of β-sheet domain #1 showed a phenotype similar to deletion of the entire cc domain, whereas deletion of the last 1/3 of β-sheet #1 resulted in a rather mild phenotype. The interaction of the cc domain with putative counterparts might be stabilized by the central third of the β-sheet #1 domain. We conclude that the central third of this domain is essential to mediate retention of SiiE.

Coiled-coil domains generally tend to establish stable supercoil structures by homo- or hetero-oligomerization with cc domains of other proteins, whereas isolated α-helical structures are instable and unfolded. Thus, cc domains were described as obligate oligomeric structures (reviewed in Cohen and Parry, 1990; Engel and Kammerer, 2000). This stability is due to apolar side-chains from one helix (knobs) that pack into the hydrophobic spaces between the side-chains (holes) of the adjacent helix [knob-into-hole model (Crick, 1953)]. Hence, the cc domain of SiiE might be the portion of the protein that interacts with other proteins in order to mediate retention and release in a temporal controlled fashion. We assume that the retention of SiiE occurs inside the channel of the T1SS and the formation of a supercoil with cc domains of T1SS subunits occurs. Coiled-coil domains are predicted with a high probability...
for SiiD, but not for SiiF, and conflicting results were obtained for SiiC [according to Coils http://www.ch.embnet.org/software/COILS_form.html (Lupas et al., 1991) and Paircoils http://groups.csail.mit.edu/cb/paircoil/cgi-bin/paircoil.cgi (Berger et al., 1995)]. Coiled-coil motifs were commonly found in PAP subunits of various T1SS (Pimenta et al., 1996) and proposed as 'molecular springs' allowing the adjustment of trans-envelope proteins to changes in the volume of the periplasm. Although the interaction of cc motifs of PAP with T1SS substrate proteins was not reported, such interaction and control of SiiE release in response to changes in the cell envelope would be in line with SiiE function. Additionally, SiiA or SiiB may be considered as interaction partners and putative regulators of SiiE retention. However, prediction tools were unable to predict cc domains for SiiA and the probability for SiiB was only 40%. If SiiA, SiiB or proteins of the T1SS interact with SiiE and especially the N-terminal domain needs further investigation. For the establishment of a supercoil structure, also further yet unknown proteins have to be considered (Fig. 8).

Interestingly, recently studies identified a distinct mechanism of retention and release of LapA, a related T1SS-secreted adhesin involved in biofilm formation of Pseudomonas fluorescens (Navarro et al., 2011; Newell et al., 2011). Outer membrane-bound LapA is released by proteolytic processing by periplasmic LapG, and the activity of LapG is controlled by LapD in response to the c-di-GMP level in the bacterial cytoplasm. Currently, we have no indications that a similar mechanism may apply to SiiE release.

Taken together, our data demonstrate that the precise control of the level of SiiE retention and the temporal release is of central importance for the function of this adhesin in invasion of polarized epithelial cells by Salmonella. The retention and release of a non-fimbrial

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**Fig. 8.** Model for the role of domains in SiiE in function of the adhesin. Based on the functional dissection of SiiE described in this study, we propose a model for the role of various domains of SiiE in the retention of the molecule (A). The N-terminal moiety of SiiE is required for the controlled retention and release of the adhesin. This may involve interactions of the coiled-coil domain in SiiE with components of the T1SS, for example SiiD. The Ig domains contribute to the long linear structure of SiiE but may also have a role in interaction with receptors on the host cell surface. The C-terminal moiety contains the secretion signal and it has to be resolved if this region specifically contributes to the adhesive function of SiiE. Details of the domain organization of the N-terminal part of SiiE (B) and their putative interaction with the T1SS (C) are shown. Black arrows indicate effects of environmental factors or unknown regulators, while the yellow arrow indicates the effect of intracellular messengers on the T1SS.

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adhesin is a novel molecular function observed for *P. fluorescens* LapA and *S. enterica* SiiE. Assembly and retraction was observed for fimbrial adhesins of the family of type IV pili, but the mechanism involved is distinct and involves polymerization of subunits of the pilus shaft. Our findings underline the fine-tuned mechanisms of host cell invasion by *Salmonella* and the additional requirement for cooperation of the SPI1-T3SS and the SPI4-T1SS for invasion of polarized epithelial cells.

**Experimental procedures**

**Bacterial strains and culture conditions**

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) NCTC 12023 was used as wild-type strain in this study and all mutant strains are isogenic to this strain. The characteristics of strains used in this study are listed in Table 1. In-frame deletions in chromosomal *siiE* were constructed as described (Gerlach et al., 2009), using oligonucleotides specified in Table S1. Bacterial strains were routinely grown in LB broth or on LB agar containing antibiotics if required for selection of specific features. Carbenicillin and kanamycin were used at 50 μg × ml⁻¹, and tetracycline was added to a final concentration of 20 μg × ml⁻¹.

**Construction of scarless mutations**

For the construction of scarless in-frame deletions in *siiE*, we deployed the approach previously described by Gerlach et al. (2009). Briefly, the tetAR resistance cassette was inserted by Red-mediated recombination within the region where the deletions were aimed. In a second step, the region harbouring the tetAR cassette was replaced by Red-mediated recombination with synthetic double-stranded DNA of 80 bp. The sequence of the flanks of this DNA fragment determined the position of the deletion. The recombination resulted in the loss of the tetAR cassette and Tet⁺ clones were selected on Bochner-Maloy plates as described (Gerlach et al., 2009). Clones obtained by this positive selection were subsequently checked by colony PCR and relevant positive clones were further analysed by DNA sequencing of the mutated region and functional tests. The oligonucleotides used for insertion of the tetAR cassette, deletion mutagenesis and control PCR reactions are listed in Table S1. If required for the combination of mutations, P22 transduction was used according to standard procedures (Maloy et al., 1996).

**Cell culture**

The canine kidney epithelial cell line MDCK and the human colonic epithelial cell line CaCo-2 BB1 (Peterson and Mooseker, 1992) were used for the generation of a polarized epithelial cell layer. For control experiments with non-polarized epithelial cells, the human cervical cancer cell line HeLa was used. MDCK cells were cultured in MEM medium (Earle’s salts, L-glutamine, PAA) supplemented with 10% FCS (Sigma), 2 mM Glutamax (Invitrogen), non-essential amino acids (PAA), 100 U penicillin × 10⁻¹ ml and 100 μg streptomycin × 10 ml⁻¹ (PAA). For invasion and adhesion assays cells were seeded at a density of 1 × 10⁵ per well in 24-well plates (Cellstar bio-one, Greiner). Cells were allowed to differentiate for 5–6 days.

The human colonic epithelial cell line CaCo-2 BB1 also forms polarized monolayers. Cells were grown in DMEM medium (high glucose, L-glutamine, PAA) supplemented with 2 mM Glutamax (Invitrogen), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin (PAA) and 10 μg ml⁻¹ holotransferrin. For invasion assays, cells were seeded at a density of 1 × 10⁴ in 24-well plates (Cellstar bio-one, Greiner) and allowed to differentiate for 10 days. Growth medium was replaced by fresh medium every second day.

For control experiments with non-polarized epithelial cells, the human cervical cancer cell line HeLa was cultured in DMEM medium (high glucose, glutamate, sodium pyruvate, PAA) supplemented with 10% FCS and 2 mM Glutamax (Invitrogen). Cells were seeded at a density of 5 × 10⁴ per well in 24-well plates (Cellstar bio-one, Greiner) 24 h before infection assays.

**Invasion assays**

At least 4 h prior infection, epithelial cell cultures were washed in PBS and fresh medium without antibiotics was added. Bacteria were subcultured from an overnight culture (1:31) in fresh LB medium and grown for 3.5 h at 37°C. Bacteria were adjusted to an OD₆₀₀ of 0.2 in PBS and subsequently further diluted in MEM medium for infection of MDCK cells at an moi of 5. Infection was allowed to proceed for 25 min at 37°C. Non-adherent bacteria were removed by washing thrice with PBS and cells were further incubated in medium containing 100 μg gentamicin × 10⁻² ml for 1 h to kill extracellular bacteria. Cells were washed once with PBS and lysed with addition of 500 μl of pre-warmed 0.5% sodium deoxycholic acid in PBS for 5 min at 37°C. Several dilutions were made in PBS containing 0.05% Tween 80 and plated on Mueller-Hinton agar plates for determination of colony-forming units (cfu). In parallel, the cfu of the inoculum were determined and the percentage of internalized bacteria was calculated. If required for the comparison of invasion rates of independent experiments, the proportion of internalized bacteria was normalized to the invasion of WT.

Invasion assays with CaCo-2 BB1 cells were performed as for MDCK cells. The conditions for infection of HeLa cells were basically identical, except that HeLa cells were infected with an moi of 10 and lysed by addition of 0.1% Triton X-100 in PBS for 5 min at 37°C.

**Microscopic analysis of SiiE retention on the bacterial surface**

Overnight cultures of bacterial strains were diluted 1:31 in LB supplemented with required antibiotics and subcultures were grown for various periods of time with aeration at 37°C. Optical density was measured and adjusted to an OD₆₅₀ of 0.2 in sterile PBS. Aliquots of 30 μl were spotted on coverslips and allowed to dry. Bacteria were fixed for 20 min by the addition of 500 μl of 3% para-formaldehyde (PFA) in PBS. Coverslips were washed thrice using PBS and subsequently subjected to immunostaining of SiiE using a polyclonal antiserum against SiiE.

**Quantification of SiiE on bacterial cell surface**

Bacterial strains were diluted 1:31 in LB from an overnight culture and grown at 37°C for 3.5 or 6 h. At indicated time
points, aliquots of 1 ml of bacterial culture were taken, pelleted and resuspended in 1 ml of sterile PBS. After an additional washing step with sterile PBS, optical density was measured and adjusted to an OD₆₀₀ of 1 in 500 µl of 3% PFA in PBS. After fixation of bacterial cells for 15 min, PFA was removed by centrifugation (10,000 g; 5 min) and replaced with 500 µl of PBS. Five microlitres of bacterial suspensions were spotted on a nitrocellulose membrane which was pre-wetted with PBS and dried again before addition of bacteria. After drying, membranes were blocked with 5% dry milk powder in TBS/T (TBS; 0.1% Tween 20) for at least 30 min. For detection with PBS and dried again before addition of bacteria. After spotting on a nitrocellulose membrane which was pre-wetted of PBS. Five microlitres of bacterial suspensions were quantified using the Odyssey Imaging System (LI-COR Biotechnology).

Quantification of secreted SiiE in culture supernatants by ELISA

Bacterial strains were diluted 1:31 from overnight cultures in fresh LB medium and grown at 37°C for 6 h. At indicated time points, 1 ml of bacterial culture was sampled and bacteria were pelleted. Supernatants were filtered sterilized (0.22 µm Millex filter units, Millipore) and aliquots of 50 µl were applied to 96-well microtitre plates (Multisorp, Nunc) and incubated overnight in a humid chamber at 4°C. Wells were washed three times with 200 µl per well washing buffer (PBS; 0.05% Tween 20). Antiserum against C-terminal moiety of SiiE was diluted 1:10 000 in PBS containing 100 µl per well for 2 h at room temperature. After five washing steps with washing buffer, horseradish peroxidase-coupled anti-rabbit antibody was diluted 1:50 000 in PBS containing 10% FCS and 100 µl was applied per well. For transmission microscopy of isolated SiiE molecules protein samples were mixed with 50% glycerol (final glycerol concentration 37%), sprayed on freshly cleaved mica sheets and rotary-shadowed with carbon-platinum under an angle of 8° and backed with carbon in a Bal-Tec BAF300 vacuum unit. Replicas were mounted on naked 400 mesh grids (Tyler and Branton, 1980) and molecules were imaged in a Philips CM10 electron microscope.

Preparation of SiiE for electron microscopy

Bacterial strains were subcultured 1:100 in 50 ml of LB medium containing required antibiotics at 37°C, 150 r.p.m. After 6 h, bacteria were pelleted at 10 000 g for 10 min, 4°C and culture supernatant was passed through a syringe filter with 0.45 µm pore size. Proteins were concentrated using Amicon Ultra 15 centrifugal filter device (Millipore) with a molecular weight limit at 100 kDa. After 25- to 50-fold concentration, LB medium was exchanged against PBS.

Electron microscopy

MDCK cells grown on coverslips were incubated with bacteria and then were fixed with 4% PFA in PBS for 15 min at 37°C and kept for 24 h at 4°C. For morphological studies, samples were post-fixed with 1% osmium tetroxide in 100 mM PO₄ buffer, pH 7.2 for 1 h on ice, rinsed with H₂O₂, treated with 1% aqueous uranyl acetate for 1 h at 4°C, dehydrated through a graded series of ethanol and embedded in Epon. Ultrathin sections were stained with 1% aqueous uranyl acetate and lead citrate and viewed in a Philips CM10 transmission electron microscope at 60 kV using a 30 µm objective aperture.

For SEM formaldehyde-fixed samples were blocked with 0.5% bovine serum albumin, 0.2% gelatine in PBS, incubated with purified rabbit anti-GST-SiiE antibodies (diluted 1:100), followed by protein A-15 nm gold conjugates. After post-fixation with 1% glutaraldehyde in PBS and 1% osmium tetroxide in 100 mM PO₄ buffer at pH 7.2, labelled specimen were dehydrated in a graded series of ethanol and critical-point-dried from CO₂. Finally the samples were sputter-coated with a layer of 1 nm chromium (Bal-Tec MED 010) and examined at 10 kV accelerating voltage in a Hitachi S-800 field emission scanning electron microscope equipped with a detector for backscattered electrons (BSE) of the YAG type (Autrata et al., 1992).

For transmission microscopy of isolated SiiE molecules protein samples were mixed with 50% glycerol (final glycerol concentration 37%), sprayed on freshly cleaved mica sheets and rotary-shadowed with carbon-platinum under an angle of 8° and backed with carbon in a Bal-Tec BAF300 vacuum unit. Replicas were mounted on naked 400 mesh grids (Tyler and Branton, 1980) and molecules were imaged in a Philips CM10 electron microscope.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Features of SiIE variants analysed in this study. The levels of secretion of SiIE, surface retention of SiIE and MDCK invasion of strains expressing various siIE variants are indicated as percentage of the data obtained for WT SiIE.

**Table S1.** Oligonucleotides used in this study.

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