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The SciZ protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the cell wall

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Summary

Type VI secretion systems (T6SS) are multi-component machines encoded within the genomes of most Gram-negative bacteria that associate with plant, animal and/or human cells, and therefore are considered as potential virulence factors. We recently launched a study on the Sci-1 T6SS of enteroaggregative *Escherichia coli* (EAEC). The Sci-1 T6SS is composed of all or a subset of the 21 gene products encoded within the cluster, 13 of which are shared by all T6SS identified so far. In the present work, we focussed our attention on the SciZ protein. We first showed that SciZ is required for the release of the Hcp protein in the culture supernatant and for efficient biofilm formation, demonstrating that SciZ is necessary for EAEC T6SS function. Indeed, SciZ forms a complex with SciP, SciS and SciN, three core components of the transport apparatus. Fractionation and topology studies showed that SciZ is a polytopic inner membrane protein with three *trans*-membrane segments. Computer analyses identified a motif shared by peptidoglycan binding proteins of the OmpA family in the SciZ periplasmic domain. Using *in vivo* and *in vitro* binding assays, we showed that this motif anchors the SciZ protein to the cell wall and is required for T6SS function.

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Introduction

Bacterial secretion systems are dedicated machines responsible for the release of virulence factors in the environmental milieu or directly within the eukaryotic host cell cytosol. Seven secretion systems have been described so far, which assemble from three to > 20 subunits. Type VI secretion systems (T6SS) are present in most Gram-negative bacteria, including pathogens being in contact with animals, plants or humans, or environmental isolates (Bingle *et al.*, 2008; Cascales, 2008; Filloux *et al.*, 2008; Pukatzki *et al.*, 2009). In pathogenic bacteria, these secretion systems are involved in various virulence processes, including resisting predation by amoebae for *Vibrio cholerae* and *Burkholderia cenocepacia* (Pukatzki *et al.*, 2006; Aubert *et al.*, 2008), inhibiting the phagocyte activity of macrophages for *Aeromonas hydrophila* (Suarez *et al.*, 2008), rotting of potato stems for *Pectobacterium atrosepticum* (Mattinen *et al.*, 2007), favouring persistence within the host for *Salmonella enterica* (Parsons and Heffron, 2005), or for the global virulence of the fish pathogen *Edwardsiella tarda* (Rao *et al.*, 2004). The presence of T6SS gene clusters in many environmental and marine bacteria (Persson *et al.*, 2009) suggests that T6SS might be involved in more general processes such as cell–cell interactions or biofilm formation. Indeed, a role of T6SS in biofilm formation has been shown in *Vibrio parahaemolyticus* and enteroaggregative *Escherichia coli* (EAEC, Enos-berlage *et al.*, 2005; Aschtgen *et al.*, 2008), and the *Pseudomonas aeruginosa* HSI-2 and HSI-3 T6SS gene clusters have been shown to be upregulated during biofilm formation (Sauer *et al.*, 2002: Southey-pillig *et al.* 2005). Recent studies on quorum-sensing regulation have shown that several T6SS gene clusters such as *P. aeruginosa* HSI-2 and HSI-3, and *A. hydrophila vas* are co-regulated with biofilm formation determinants (Khajanchi *et al.*, 2009; Lesic *et al.*, 2009). A recent study suggested that T6SS might be involved in stress response in *Vibrio anguillarum* (Weber *et al.*, 2009).

Type VI secretion system assemble from at least 13 proteins, which are called 'core components' (Bingle *et al.*, 2008; Cascales, 2008) and may form the minimal machine necessary to function; however, most T6SS gene clusters encode additional proteins for which the roles for the

function of the transport apparatus are unknown. Among the conserved components are the ClpV ATPase that has been shown to regulate subunit sub-assembly (Bönemann *et al.*, 2009), the IcmF- and IcmH-like proteins, that resemble Type IVb secretion proteins (Christie *et al.*, 2005; Ma *et al.*, 2009a), an outer membrane (OM) lipoprotein (Aschtgen *et al.*, 2008), and a subset of cytoplasmic proteins. Two proteins, Hcp (haemolysin co-regulated protein) and VgrG (valine glycine repeat), are found in culture supernatants of bacteria expressing T6SS. Recently, the crystal structures of these proteins have been obtained (Mougous *et al.*, 2006; Leiman *et al.*, 2009) and display similar folds to proteins of bacteriophage T4. The hexameric Hcp resembles the gp19 protein, which constitutes the tail protein (Mougous *et al.*, 2006; Pell *et al.*, 2009), whereas VgrG resembles the syringe of the bacteriophage baseplate, comprised of protein gp27 and the C-terminal domain of gp5 (Kanamaru *et al.*, 2002; Leiman *et al.*, 2009). VgrG proteins sometimes carry a C-terminal extension that may possess effector-like activities, such as actin cross-linking in the case of the *V. cholerae* VgrG1 protein (Pukatzki *et al.*, 2007; Ma *et al.*, 2009b) or actin ADP-ribosylation in *A. hydrophila* (Suarez *et al.*, 2009). The current model for assembly of T6S machines is that the tail-like structure formed by the assembly of the Hcp proteins across the cell envelope delivers the VgrG protein in the culture medium or directly into the host cell (Cascales, 2008; Pukatzki *et al.*, 2009). In *E. tarda*, it has been shown that 11 of the 13 genes of the *evp* cluster are necessary for Hcp and VgrG release into culture supernatants (Zheng and Leung, 2007). The 11 proteins may thus interact to form a machine dedicated to the assembly of the Hcp structure.

Recently, we began to characterize the different genes encoded within the EAEC T6SS *sci-1* gene cluster. The *sci-1* gene cluster is encoded within the *pheU* pathogenicity island of EAEC strains (Dudley *et al.*, 2006), an emerging *E. coli* pathotype responsible for severe and persistent diarrhoea of infants, young children, or immunocompromised individuals (Nataro, 2005; Harrington *et al.*, 2006). We have recently shown that the *sciN* gene encodes an OM lipoprotein (Aschtgen *et al.*, 2008). In this study, we focused our attention on the function of the *sciZ* gene product. We first tested whether SciZ is necessary for Type VI secretion in EAEC. Fractionation, topological and co-precipitation analyses showed that SciZ is an inner membrane (IM) polytopic protein with a large periplasmic domain that forms a complex with the SciP, –S, and –N subunits of the transport apparatus. *In vivo* and *in vitro* experiments showed that the SciZ periplasmic domain interacts with the cell wall through a peptidoglycan (PG)-binding motif, and further demonstrated that this anchorage is required for the function of the T6S apparatus.

Results

The SciZ protein is required for Type VI secretion in EAEC

Among the 21 genes of the *sci-1* gene cluster, 13 are shared by all T6S gene clusters identified so far and thus belong to the 'core components' of the secretion apparatus. One of the current hypotheses for the remaining eight genes is that they form an accessory complex required for efficient secretion or are involved in a process specific to the Sci-1 T6SS (Aschtgen *et al.*, 2008). In this study, we asked whether one of these non-conserved proteins, SciZ, is required for Type VI secretion. To define its role, we first constructed a strain deleted for the *sciZ* gene and tested it for the presence of the Hcp protein in the culture supernatant. As shown in Fig. 1A, Hcp was released from wild-type (WT) EAEC

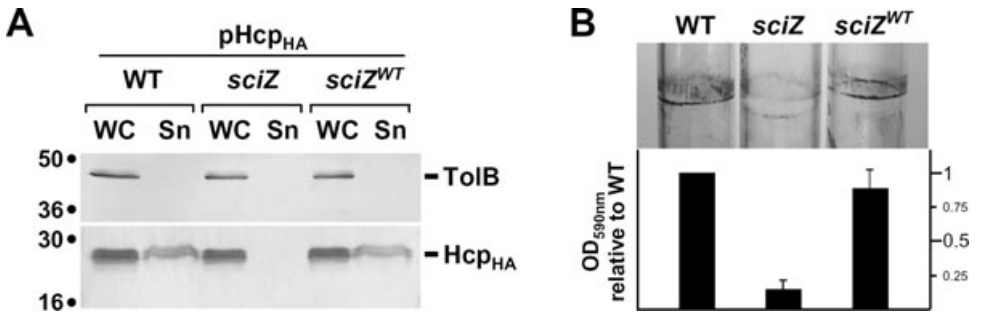

Fig. 1. The SciZ protein is required for Type VI secretion.
A. Effect of the *sciZ* mutation on Hcp protein release. Hcp$_{HA}$ release was assessed by separating whole cells (WC) and supernatant (Sn) fractions from WT, *sciZ*, and complemented *sciZ* (*sciZ*^{WT}) cultures. A total of 2×10^8 cells and the TCA-precipitated material of the supernatant from 5×10^8 cells were loaded on a 12.5%-acrylamide SDS-PAGE and immunodetected using the anti-HA monoclonal antibody (lower panel) and the anti-TolB polyclonal antibodies (lysis control; upper panel).
B. Effect of the *sciZ* mutation on biofilm formation. Biofilms formed in static cultures of WT, *sciZ* and complemented *sciZ* cells were visualized in glass tubes by crystal violet staining (upper panel) and quantified using the ethanol-solubilization procedure (OD_{590}), relative to the WT EAEC strain (lower graph).

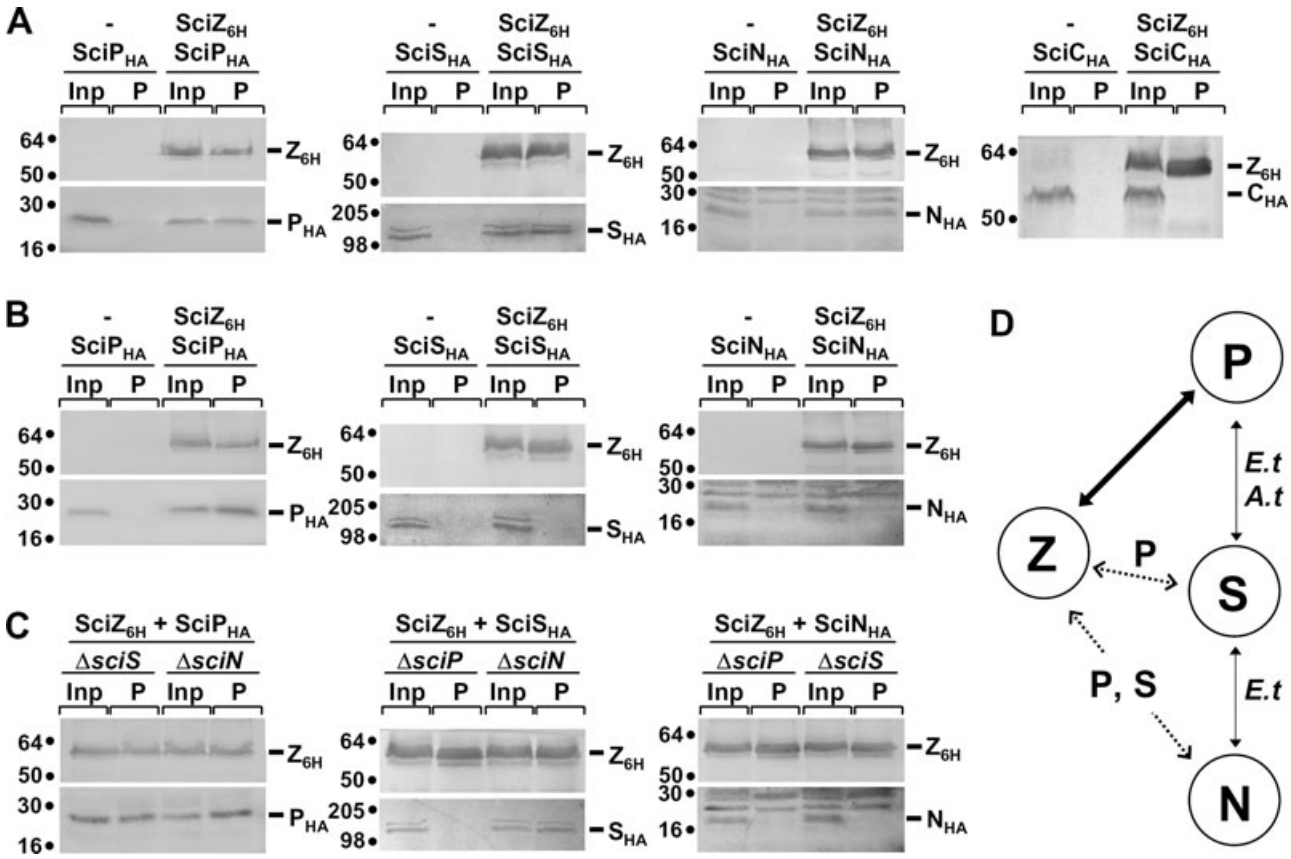

Fig. 2. SciZ interacts with core components of the secretion apparatus. Nonidet P40-solubilized extracts of EAEC (A), *E. coli* K12 W3110 (B) or EAEC Δ*sciN*, Δ*sciP* or Δ*sciS* mutant (C) strains carrying plasmid producing HA-tagged SciP, -S, -N, or -C proteins and His-tagged SciZ [or the parental empty vector (–)] were subjected to metal affinity chromatography. The total solubilized material (Input, Inp) and the precipitated material (P) were TCA-precipitated and loaded on a 10% (for SciS and SciC experiments) or 15% (for SciN and SciP experiments) acrylamide SDS-PAGE, and immunodetected with anti-HA and anti-5His monoclonal antibodies (additional bands may correspond to degradation products or cross-reactive species). Immunodetected proteins are indicated on the right. Molecular weight markers are indicated on the left.
D. Schematic representation of the interactions between the SciZ (Z), SciP (P), SciS (S) and SciN (N) proteins deduced from A, B and C. The large plain arrow shows the direct interaction between the SciZ and SciP subunits. Dotted arrows indicate indirect interactions and the protein requirements. The interactions between the SciS subunit and the SciP and SciN proteins demonstrated in other studies are indicated by thin plain arrows [*E.t. Edwarsiella tarda* (Zheng and Leung, 2007); *A.t. Agrobacterium tumefaciens* (Ma *et al.*, 2009a)].

cells, but not from *sciZ* cells. Hcp release was restored upon production of an N-terminally hexahistidine-tagged SciZ protein, showing that the effect of the *sciZ* mutation on Hcp release is not due to polar effects on the downstream genes. The EAEC *sci-1* T6SS gene cluster has recently been shown to be involved in *in vitro* biofilm formation (Aschtgen *et al.*, 2008). Although the underlying mechanism of T6SS contribution to biofilm formation is actually unknown, we used the biofilm assay as a reporter of Sci-1 T6SS function. Biofilm formation of the WT and its *sciZ* derivative strain was assessed on glass tubes and polyethylene plastic wells. As shown in Fig. 1B, the *sciZ* strain formed lower amount of biofilm than the WT strain or *sciZ* cells complemented with the His-tag SciZ variant. All together, these data show that SciZ is necessary for the function of the EAEC Sci-1 T6SS.

SciZ interacts with core components of the T6SS apparatus

To test whether SciZ interacts with other subunits of the secretion apparatus, we performed co-precipitation experiments. Because SciZ is predicted to be an IM protein, we tested for pair wise interactions with the SciN lipoprotein, the putative membrane proteins encoded within the *sci-1* gene cluster (the IcmH/DotU- and IcmF-like homologues SciP and SciS), and with the putative cytoplasmic subunit SciC. As shown in Fig. 2A, SciZ co-precipitated SciP, SciS, and the SciN lipoprotein from EAEC cells producing His-tagged SciZ and the HA-tagged partners. By contrast, no interaction was found between SciZ and SciC. To test whether these interactions represent direct protein–protein contacts, we repeated the experiments in *E. coli* K12 (i.e. devoid of the

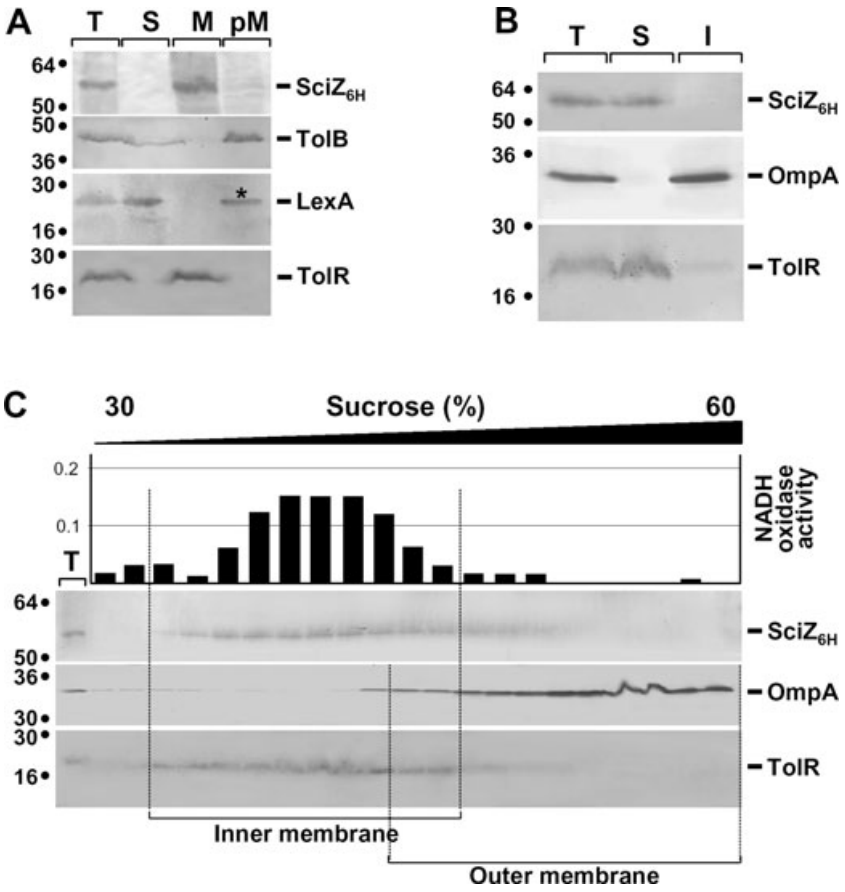

Fig. 3. SciZ is an integral inner membrane protein.
A. SciZ co-fractionates with membranes. A fractionation procedure was applied to EAEC cells producing $SciZ_{6his}$ (T, Total fraction), allowing separation between the soluble (S), and membrane fractions. Membranes were then treated with sodium carbonate to separate integral membrane (M) and peripheral membrane proteins (pM). Samples were loaded on a 12.5%-acrylamide SDS-PAGE and immunodetected with antibodies directed against the LexA (soluble), TolR (integral membrane), and TolB (peripheral membrane) proteins, and the 6-Histidine epitope of SciZ. Note that a portion of LexA is associated with membranes (asterisk) due to its ability to interact with residual DNA. Molecular weight markers are indicated on the left.
B. SciZ is solubilized by sodium lauryl sarcosinate (SLS). Total membranes from EAEC cells producing $SciZ_{6His}$ (T) were treated with SLS, and solubilized (S) and insolubilized (I) membranes were separated. Samples from 10^9 cells were loaded on 12.5%-acrylamide SDS-PAGE and immunodetected with antibodies directed against the TolR (inner membrane) and OmpA (outer membrane) proteins, and against the 6-Histidine epitope of SciZ. Molecular weight markers are indicated on the left.
C. SciZ co-fractionates with the inner membrane. Total membranes (T) from EAEC cells producing $SciZ_{6His}$ were separated on a discontinuous sedimentation sucrose gradient. Collected fractions were analysed for contents using the anti-TolR, anti-OmpA and anti-5His antibodies, and with a NADH oxidase activity test (upper graph). NADH oxidase activity is represented relative to the total activity. The positions of the inner and outer membrane-containing fractions are indicated.

T6SS gene cluster). When co-produced, only the SciZ and SciP proteins interacted (Fig. 2B, left panel), suggesting that these two proteins interact directly. By contrast, SciZ did not co-precipitate SciS and SciN in the *E. coli* K12 background (Fig. 2B, centre and right panels), suggesting that SciZ indirectly interacts with SciN and SciS. To better understand this protein network, these interactions were tested in the Δ*sciN*, Δ*sciP* and Δ*sciS* backgrounds. Figure 2C shows that SciZ co-precipitated SciP independently of the mutant background (Fig. 2C, left panel). SciZ co-precipitated SciS from Δ*sciN* but not from Δ*sciP* cell extracts (Fig. 2C, centre panel), but did not co-precipitated SciN from Δ*sciP* or Δ*sciS* cell extracts (Fig. 2C, right panel). Taken together, these data suggest that SciZ interacts with a SciNPS complex through direct interactions with SciP (Fig. 2D), and is therefore in close contact with the Type VI secretion core apparatus.

The SciZ protein localizes in the IM

To gain insights on the role of the SciZ protein, we first performed experiments aimed at defining its sub-cellular localization. Upon cell fractionation, the SciZ protein localized in the membrane fraction, and was not released from the membranes with sodium carbonate (Fig. 3A), suggesting that SciZ is an integral membrane protein. To

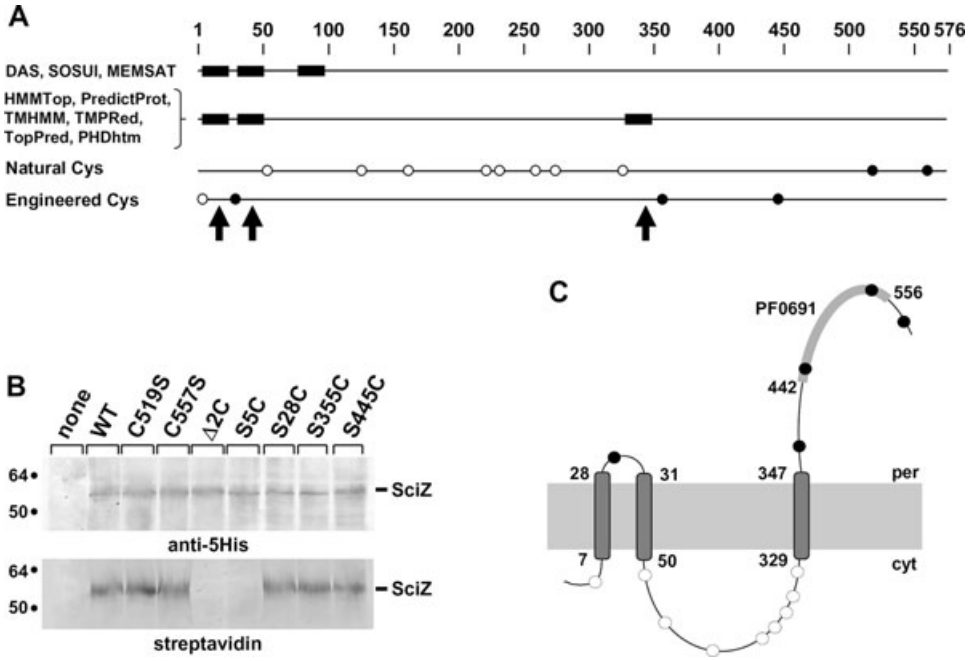

Fig. 4. SciZ has three transmembrane-spanning segments.
A. The computer programs listed at left [available at the ExPASy Proteomics server (http://www.expasy.ch)] predict the transmembrane segments (TMS) shown as filled rectangles. The natural and engineered cysteines are indicated, as well as the results of the accessibility studies. Filled circles indicate cysteine residues labelled with the 3-(*N*-maleimidylpropionyl) biocytin (MPB) probe, whereas open circles indicated unlabelled cysteine residues. Arrows identify TMS supported by the results of the accessibility studies.
B. Accessibility of cysteine residues. Whole EAEC *sciZ* cells carrying the empty vector (none) of producing WT SciZ or mutant proteins were labelled with the MPB probe, solubilized, and SciZ and mutant proteins were isolated by immobilized metal ion affinity chromatography using Cobalt beads. Purified material was subjected to SDS-PAGE and Western blot analysis using anti-5His antibody (SciZ detection, upper panel) and streptavidin coupled to alkaline phosphatase (MPB-labelled SciZ detection, lower panel). Molecular weight markers are indicated on the left.
C. Topology model for the SciZ protein at the inner membrane. The localizations of the labelled and unlabelled cysteine residues are indicated by filled and open circles respectively. The three TMS identified by the accessibility studies are shown, with their membrane boundaries. The putative PG-binding motif of the OmpA/Pal/MotB family (PF0691), located between amino acids 442 and 556, is indicated.

determine whether SciZ is anchored to the inner or to the OM, we performed selective detergent solubilization procedures using sodium lauroyl sarcosinate (SLS), a detergent that selectively disrupts the IM. SciZ, and the IM protein TolR, were extracted by SLS whereas the OM OmpA protein was found in the insoluble fraction (Fig. 3B). The membrane localization of SciZ was then further tested using sedimentation density gradients. As shown in Fig. 3C, SciZ was found in the lower-density fractions, and co-fractionated with the IM protein TolR, and the NADH oxidase activity. Overall, these results demonstrate that SciZ is an integral IM protein.

SciZ is a polytopic IM protein

To define the topology of the SciZ protein in the IM, we first performed *in silico* analyses. Based on hydrophobicity plots, most widely used computer tools predicted the presence of two or three *trans*-membrane segments (TMS), although the positions and the orientations of these TMS differ from one algorithm to another (Fig. 4A).

To test these predictions, we performed topology mapping using cysteine accessibility. This method is now widely used to define the topology of IM proteins, and is based on the accessibility of cysteine residues to 3-(*N*-maleimidylpropionyl) biocytin (MPB), a sulfhydryl reagent which readily passes the OM but only inefficiently through the IM of Gram-negative bacteria including EAEC (Bogdanov *et al*., 2005; data not shown). The EAEC SciZ protein possesses several cysteines, with two (C519 and C557) predicted to locate in the periplasm by most algorithms. Hence, the WT SciZ protein is biotinylated with MPB *in vivo* (Fig. 4B, lower panel). We first constructed single cysteine to serine mutants at positions 519 and 557 (C519S and C557S). These two SciZ proteins were shown to be functional in an Hcp secretion assay (Fig. S1). Accessibility experiments of the individual substitutions of the two cysteine residues showed that the $SciZ_{C519S}$ and $SciZ_{C557S}$ mutant proteins were still labelled with MPB whereas the double substituted protein $SciZ_{\Delta 2C}$ was not biotinylated (Fig. 4B, lower panel). These data show that C519 and C557 locate in the periplasm

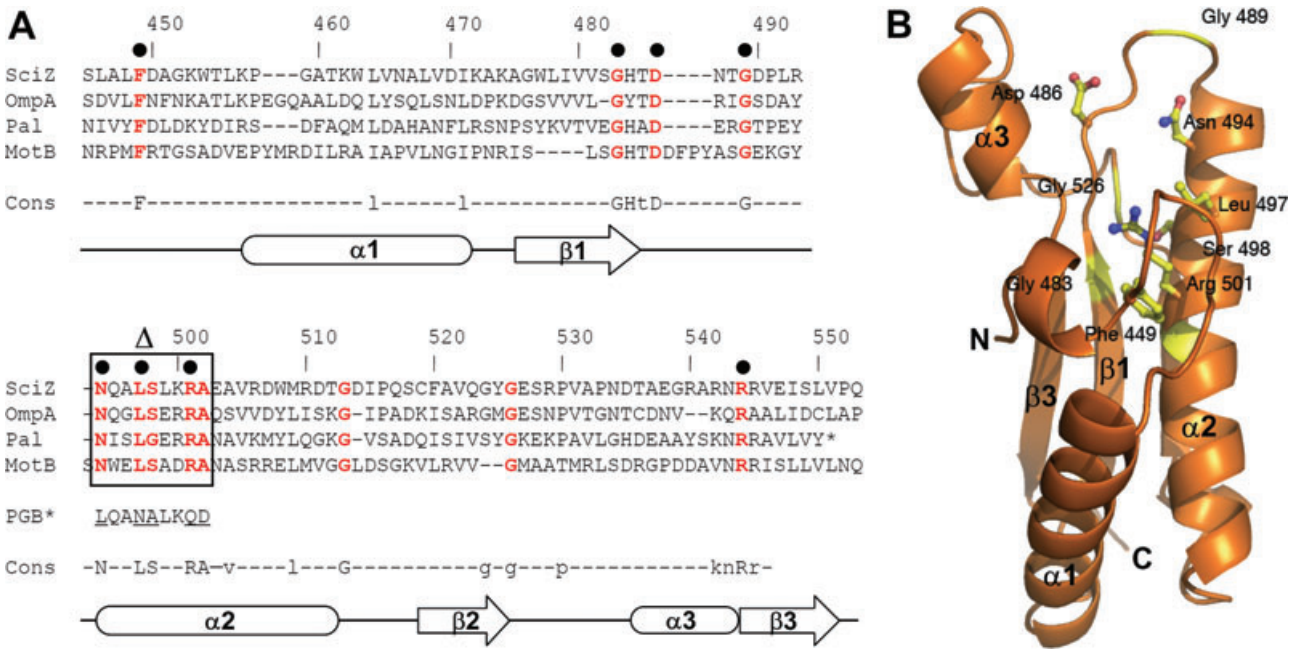

Fig. 5. The C-terminal periplasmic domain of SciZ carries a putative peptidoglycan-binding domain.
A. Sequence alignments of the PG-binding motifs of the OmpA, Pal, MotB and SciZ proteins, numbered following the amino acids of SciZ. Conserved residues (see consensus) are indicated in red letters. Black dots indicate residues in interaction with the PG in the Pal and MotB structures (Parsons *et al*., 2006; Roujeinikova, 2008). The localization of the secondary structures is shown. The region deleted in construction SciZΔPGB is indicated by the frame. The substitutions within the PG-binding motif (SciZPGB*) are indicated below the alignment (modified residues underlined).
B. Structural ribbon model of the PG-binding motif of SciZ. This model was generated by employing the SWISS-MODEL server (http://swissmodel.expasy.org; Arnold *et al*., 2006) based on template 2k1sA, with which SciZ shares 35.6% sequence identity. The modelled domain range from residues 442 to 556. α-Helices and β-strands are numbered following A (please note that strand β2 is hided by helix α2). The conserved residues which interact with the *N*-acetyl-muramyl pentapeptide in the Pal and MotB structures (indicated by black dots in A) are indicated in backbones.

whereas all other cysteine residues (C53, C127, C161, C223, C231, C256, C271, C327) locate in the cytoplasm. These results suggest the existence of a TMS between residues 327 and 519. To further define the SciZ topology, we introduced cysteine substitutions in the Δ2C variant at several positions along the length of SciZ and assayed for accessibility to MPB. All these mutated proteins were produced at similar levels (see Fig. 4B, upper panel) and were functional in an Hcp secretion assay (Fig. S1). Labelling of the Δ2C-S28C, Δ2C-S355C and Δ2C-S445C variants showed that the S28, S355 and S445 residues locate in the periplasm (Fig. 4B). These data confirm the existence of a TMS between residues 28 and 53, and further restrict the C-terminal TMS between residues 327 and 355. By contrast, the Δ2C-S5C variant was not labelled by treatment of whole cells with MPB (Fig. 4B), suggesting that S5 resides in the cytoplasm, and confirming the existence of an N-terminal TMS between residues 6 and 27. The results of the cysteine accessibility studies support a proposal that SciZ is composed of three TMS, with the N-terminus in the cytoplasm and the C-terminus in the periplasm (Fig. 4C). SciZ spans the IM through two TMS oriented in-to-out (residues 7–28, and 329–347) and one TMS oriented out-to-in (residues 31–50) (Fig. 4C). This topology is supported by protease accessibility experiments: a ~40 kDa protease-resistant N-terminal species (probably composed of the three TMS) is retained upon treatment of spheroplast with proteinase K, while an additional smaller N-terminal protease-resistant species of ~8 kDa is retained by treatment of isolated membranes with proteinase K (probably composed of the one or two N-terminal TMS) (Fig. S2).

The periplasmic domain of SciZ binds to the PG

Computer analysis of the large periplasmic domain (residues 347–576) suggested the existence of a putative PG-binding motif of the OmpA/Pal/MotB family (Pfam PF00691) located between residues 442 and 556 (de Mot and Vanderleyden, 1994; Koebnik, 1995; see Fig. 5A). Using the Pal, MotB and RmrM structures, and the PG-binding domain of *E. coli* YiaD as templates, the region 442–556 of SciZ was modelled (Fig. 5B). The structural model suggests that this domain folds as a four-stranded β-sheet floor that faces three α-helices, identical to the Pal, MotB and RmrM structures (Grizot

and Buchanan, 2004; Parsons *et al.*, 2006; Roujeinikova, 2008). A number of residues, essentially located on a face of helix α2 and on connecting loops β1-α2, and β2-α3, are highly conserved and delimitate a groove (Fig. 5B). In the Pal and MotB structures, this groove directly contacts the *N*-acetyl-muramyl pentapeptide of the PG, with the sugar moiety interacting with the exterior, and the peptide moiety inserted within the cleft and making contact with the protein essentially through the *meso*-diaminopimelate (*m*-Dap) residue (Parsons *et al.*, 2006; Roujeinikova, 2008). We therefore asked whether this domain is functional, and anchors the SciZ protein to the cell wall. We used *in vivo* and *in vitro* PG-binding experiments with the WT periplasmic domain. As controls, we used SciZ variants mutated within, or deleted for the putative PG-binding motif (see Fig. 5A). The five substitutions of the SciZ-PGB* variant were chosen to affect residues in the groove that are in contact with the PG, without affecting the overall domain structure in our model (data not shown).

For *in vivo* PG-binding experiments, we cloned the sequence corresponding to the periplasmic domain of SciZ (SciZp) downstream of a Sec-dependent signal sequence, allowing its secretion to the periplasm (see Fig. S3). During pilot experiments aimed at defining the best conditions of expression, we noted that upon induction of *sciZp*, cells stopped growing, suggesting that the periplasmic production of this domain at high levels is toxic for the cell. Using detergent susceptibility and periplasmic leakage assays (Cascales and Lloubès, 2004), we observed that production of SciZp induced a susceptibility to the anionic detergent deoxycholate, as well as a non-specific release of periplasm content (Fig. S4; Table 1). These phenotypes are reminiscent of phenotypes of *lpp*, *pal* or *ompA* cells (Lloubès *et al.*, 2001), suggesting that this domain may compete with Pal and OmpA for PG binding. Indeed, the production of the variant deleted for the putative PG-binding motif (SciZpΔPGB) did not affect the cell envelope integrity whereas production of the SciZpPGB* variant slightly affected cell integrity (Fig. S4; Table 1). Because the flagella MotB protein interacts similarly to Pal and OmpA with the cell wall, we asked whether the production of SciZp may compete with MotB for PG binding. Using motility assays on low-agar plates, as well as phase-contrast microscopy, we observed that the production of SciZp induced a motility defect, whereas the production of SciZpΔPGB or SciZpPGB* did not (Fig. S4; Table 1). Overall, these data indirectly suggest that SciZp interacts with the cell wall.

To gain direct evidence for PG binding, we performed *in vivo* PG-binding experiments. Cells producing SciZp or its PGB variants were chemically cross-linked with dithio-bis-succinimidyl-propionate (DSP) (Leduc *et al.*, 1992). PG was then purified by the SDS-boiling method (Leduc *et al.*, 1992). Figure 6A shows that the Pal and SciZp proteins co-precipitated with the PG, whereas the IM TolR protein did not. By contrast, the PGB variants did not cross-link to the PG and remained soluble (Fig. 6A).

Table 1. Phenotypes associated with the production of the periplasmic domain of SciZ into WT EAEC cells.[a]

	DOC[b]	RNase I[c]	Hcp release[e]	Swarming rate[g]
Vector	– (1.2%)	–	+	1.0
SciZp	+ (< 0.1%)	+	+[f]	0.22
$SciZp^{\Delta PGB}$	– (1.1%)	–	–	0.94
$SciZp^{PGB*}$	–/+ (0.9%)	–/+[d]	–	0.89

a. See Figs S4 and S5. Data are reported as average of triplicate experiments.
b. Growth on 1% deoxycholate-containing plates (–, no effect; +, growth defect; –/+, intermediate growth defects). The LD_{50} (lethal dose 50%: DOC concentration for which 50% of the cells survive) is indicated in brackets.
c. Release of periplasmic RNase I, estimated on RNA-containing plates (–, no leakage; +, leakage; –/+, intermediate leakage).
d. The leakage was estimated to be < 5% relative to WT cells producing SciZp.
e. Release of Hcp_{HA} in culture supernatant (–, no release; +, release).
f. Non conclusive due to non-specific release of the TolB protein in culture supernatant.
g. Swarming rates (speed of swarming on 0.28% agar plates) relative to the control strain (empty). The values varied from an experiment to another by < 15%.

To confirm the *in vivo* data, we purified a recombinant His-tagged SciZp fragment corresponding to the periplasmic domain, and its PGB variants and performed *in vitro* PG-binding experiments. PG was purified from *lpp* cells by the SDS-boiling procedure. In absence of chemical cross-linking, this PG is devoid of the major PG-binding proteins Lpp, Pal, OmpA and MotB proteins (data not shown). Purified PG and the proteins were mixed and PG was pelleted by centrifugation. Figure 6B shows that Pal and SciZ co-precipitated with the PG whereas the periplasmic domain of TonB (TonBp) did not (upper panel). As controls, none of these proteins pelleted in absence of PG (Fig. 6B, upper panel), nor when the PG was digested with lysozyme prior to centrifugation (Fig. 6B, lower panel). Finally, competition experiments using increasing amount of peptides corresponding to the PG-binding motif of Pal or to a fragment of the TolR protein further demonstrated the specificity of the SciZ-PG interaction (Fig. S6).

Taken together, results displayed in Figs 5 and 6 show that the periplasmic domain of SciZ interacts with the PG.

PG-bound SciZ is required for Type VI secretion

To test whether PG binding is required for SciZ function and Type VI secretion, we introduced the PGB deletion

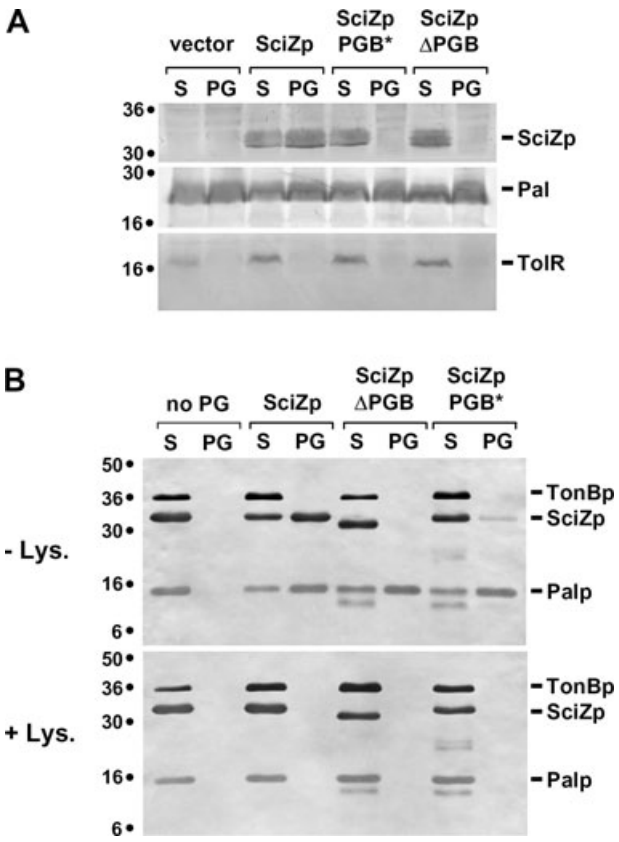

Fig. 6. SciZ interacts with the peptidoglycan.
A. SciZ interacts with the peptidoglycan *in vivo*. A total of 5×10^8 EAEC WT cells bearing the empty vector (vector) or producing the periplasmic domain of SciZ (SciZp), or PG-binding variants of SciZp (SciZpΔPGB and SciZpPGB*) treated with the chemical cross-linker dithio-bis-succinimidyl propionate were solubilized by the SDS-boiling method (see *Experimental procedures*) to isolate peptidoglycan. Soluble (S) and peptidoglycan (PG) fractions were loaded onto a 12.5% SDS-PAGE and analysed by Western blot using Pal (positive control), TolR (negative control) or anti-Streptag II (SciZp) antibodies. Molecular weight markers are indicated on the left.
B. SciZ interacts with the peptidoglycan *in vitro*. Two micrograms of purified TonBp (periplasmic domain of the TonB protein), Palp (soluble Pal protein) and SciZp (and PG variants SciZpΔPGB and SciZpPGB*) was incubated 30 min with purified PG [or without purified PG (lanes no PG)] in absence (–Lys, upper panel) or presence (+Lys, lower panel) of lysozyme. Soluble (S) and PG-associated fractions were collected by centrifugation and loaded on a 12.5% SDS-PAGE and analysed by Western blot using anti-5His antibody. Molecular weight markers are indicated on the left.

and mutations on the SciZ full-length sequence. Western blot analyses showed that WT SciZ and its PGB derivatives were produced at similar levels (data not shown). Figure 7 shows that the deletion of, or mutations within, the PG-binding sequence abolished Hcp release (Fig. 7A) and affected biofilm formation (Fig. 7B). Hence, the PG-binding motif of SciZ and the anchorage of the T6SS to the cell wall are necessary for the function of the transport apparatus.

Discussion

In this manuscript, we report the characterization of one of the non-conserved protein of the EAEC *sci-1* T6SS, SciZ. We have shown that SciZ is required for secretion of Hcp and biofilm formation. How the Sci-1 T6SS contributes to biofilm formation and whether Hcp secretion is necessary for adhesion is still an important question to address; however, a Δ*hcp* strain displays altered biofilm phenotype (data not shown). The requirement for SciZ in T6SS function suggests that SciZ is a component of the secretion apparatus. Indeed, co-precipitation experiments showed that SciZ interacts with the SciNPS complex through contacts with the SciP subunit. We next characterized SciZ in terms of topology and function. SciZ is a polytopic IM protein, and further topology experiments demonstrated that SciZ has three TMS. The large C-terminal periplasmic domain of SciZ shares an OmpA-like PG-binding motif with the Pal, RmrM, OmpA or MotB proteins. This domain is functional, as shown by *in vivo* and *in vitro* PG-binding experiments. Further, deletion of a large part of this motif, or substitution of the conserved residues, abolishes PG binding both *in vivo* and *in vitro*. The deficiency of PG anchorage leads to a non-functional apparatus, as shown by the effects of these mutations on Hcp release and biofilm formation. This is the first report of a PG-binding protein within T6SS, and the first demonstration that the anchorage to the cell wall is necessary for the function of this secretion apparatus. A model summarizing the findings reported in this study is depicted in Fig. 8.

Most of the Type VI secretion gene clusters encode a protein that possesses a putative PG-binding motif, although this motif might be carried by various Type VI secretion components. For example, in some clusters such as in most pathogenic *E. coli* strains, in *Enterobacter*, *Salmonella enterica*, *Shigella dysenteriae*, *Klebsiella pneumoniae* or *Photorhabdus luminescens*, the PG-binding motif is carried by SciZ homologues. By contrast, in most of the other clusters, in which SciZ homologues are not conserved, including *P. aeruginosa* HSI-1, *Rhizobium leguminosarum*, *Agrobacterium leguminosarum*, PG-binding motifs are carried by the IcmH-like proteins. The topology of the *Agrobacterium tumefaciens* IcmH-like ImpK protein has been defined recently (Ma *et al.*, 2009a), and the putative PG-binding motif is present within the C-terminal periplasmic domain. Interestingly, in the Type VI secretion gene clusters which possess *sciZ* genes, the IcmH-like proteins are truncated. Conversely, with the notable exception of *V. cholerae*, in T6S gene clusters in which *sciZ* is not present, IcmH-like proteins carry C-terminal extensions with a putative PG-binding motif (Bingle *et al.*, 2008; Cascales, 2008; Shrivastava and Mande, 2008; Boyer *et al.*, 2009). This PG-binding motif might be of the OmpA/Pal/MotB family,

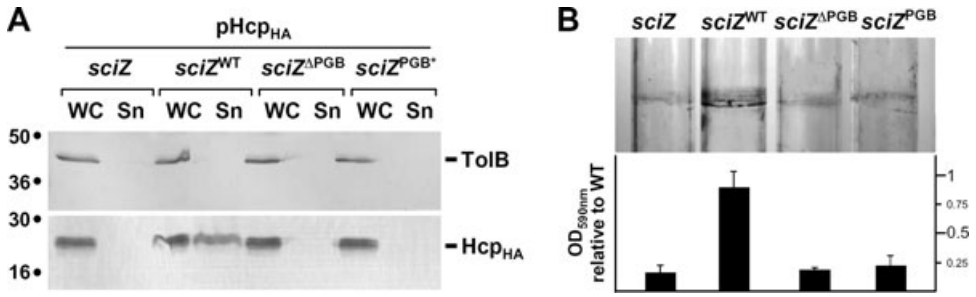

Fig. 7. The PG-binding motif of SciZ is required for Type VI secretion.
A. Effect of the PG-binding mutations of SciZ mutation on Hcp release. Hcp$_{HA}$ release was assessed by separating cells (WC) and supernatant (Sn) fractions from *sciZ* cells, and *sciZ* cells complemented with the WT allele (*sciZ*^{WT}) or its PG-binding variants (*sciZ*$^{\Delta PGB}$ and *sciZ*^{PGB*}). A total of 2×10^8 cells and the TCA-precipitated material of the supernatant from 5×10^8 cells were loaded on a 12.5%-acrylamide SDS-PAGE and immunodetected using the anti-HA monoclonal antibody (lower panel) and the anti-TolB polyclonal antibodies (upper panel).
B. Effect of the *sciZ* mutation on biofilm formation. Biofilms formed in static cultures of *sciZ* and *sciZ* cells complemented with the WT allele (*sciZ*^{WT}) or its PG-binding variants (*sciZ*$^{\Delta PGB}$ and *sciZ*^{PGB*}) were visualized in glass tubes by crystal violet staining (upper panel) and quantified using the ethanol-solubilization procedure (OD_{590}), relative to the WT EAEC strain (lower graph).

or of the PF05036 (SPOR) family such as in *Marinomonas* sp. or *Vibrio harveyi*. One striking example is *Yersinia pseudotuberculosis*, the genome sequence of which showed the existence of four Type VI secretion gene clusters, all of them encoding components with PG-binding domains, two carried by a SciZ homologue, two carried by an IcmH-like homologue. This observation suggests that the presence of the *sciZ* gene within T6SS gene clusters is correlated to the absence of the PG-binding motif carried by the IcmH-like protein. Indeed, the SciZ interaction with the IcmH-like SciP protein in EAEC further emphasizes the intimate link between these two proteins.

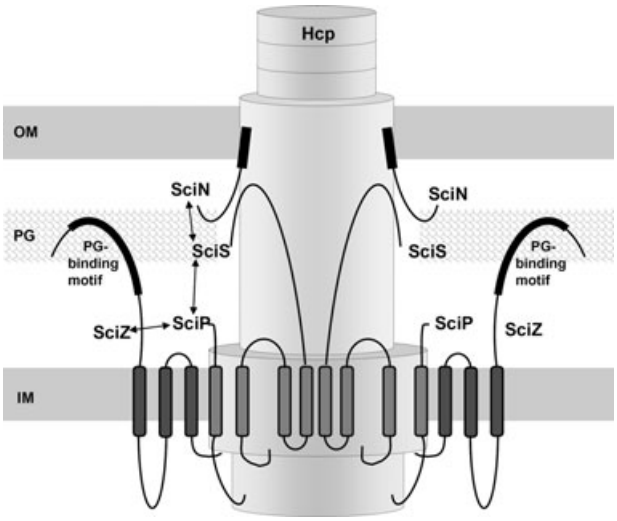

Fig. 8. Schematic representation of the EAEC Sci-1 Type VI secretion system. The secretion system is represented as a putative *trans*-envelope spanning structure (grey tubes and rings) which emphasizes the localizations and topologies of the SciZ, SciN, SciP and SciS proteins. Arrows indicate the interactions shown or suggested in this study (see text for details).

Type VI secretion systems are not the only secretion systems or macromolecular machines of the cell envelope anchored to the cell wall. The *A. hydrophila* Type II secretion apparatus has been shown to interact with the PG through the ExeA subunit (Howard *et al.*, 2006). By contrast to SciZ, ExeA carries a PG-binding motif shared by PG-degradating enzymes (Pfam family PF01471), and it has been suggested that it may remodel the PG layer, allowing the transport of the ExeD secretin through the cell wall, and the correct assembly of the secretion system (Howard *et al.*, 2006). In *Salmonella typhimurium*, the Type III secretion apparatus is anchored to the cell wall through the InvH subunit, and this interaction is important for the efficient assembly of the secretion system (Pucciarelli and García-del Portillo, 2003). However, InvH is not anchored to the cell wall through specific PG-binding motifs such as the PF05036, PF0691 or PF01471 domains. This is reflected by the low level of InvH protein associated with the PG upon cross-linking. Other macromolecular systems, such as flagella or the Tol complex, are anchored to the cell wall (de Mot and Vanderleyden, 1994; Lloubès *et al.*, 2001; Kojima *et al.*, 2008; Hizukuri *et al.*, 2009). In all these case, PG-binding involves a motif of the OmpA family, and has been shown to be critical for the function of these apparatuses.

To anchor the Type VI secretion apparatus to the cell wall, PG-binding proteins such as SciZ or IcmH-like proteins should interact with other components of the machine. In *A. tumefaciens*, the IcmH-like ImpK protein has been shown to interact with ImpL, an IcmF-like protein (Ma *et al.*, 2009a). The production of the periplasmic domains of the SciZ PGB variants into the periplasm induced a defect in Hcp release (Fig. S5; Table 1), suggesting that they compete with the WT SciZ protein for protein–protein interactions. Indeed, we have shown that SciZp forms dimers upon *in vivo* chemical cross-linking,

whereas a dimeric form of the periplasmic domain of SciZ has been obtained upon purification (data not shown). Similarly, PG-binding domains have been suggested to dimerize: the periplasmic domains of Pal and MotB form stable dimers as shown on the tridimensional structures, or by *in vivo* cross-linking (Cascales *et al*., 2002; Parsons *et al*., 2006; Roujeinikova, 2008). Further co-precipitation aimed at identifying SciZ partners showed that SciZ is part of a more global protein network comprising at least three membrane-associated core components of the T6S apparatus: the SciP and SciS (respectively the IcmH- and IcmF-like proteins) IM proteins, and the SciN OM lipoprotein (see Fig. 8). Using various strain backgrounds, we were able to delineate this interaction network, and have shown that while SciZ directly contacts SciP, the interaction between SciZ and SciS and SciN are indirect. We further showed that the interaction between SciZ and SciS requires SciP, whereas the SciZ-SciN interaction requires both SciP and SciS. The simplest tentative model is that (i) SciZ interacts with SciP; (ii) SciP interacts with SciS; and (iii) SciS interacts with SciN (see model in Fig. 8). This sequence is supported by our data and by several reports. An interaction between the SciP and SciS homologues has been demonstrated in both *A. tumefaciens* by yeast two-hybrid and co-precipitation experiments (Ma *et al*., 2009a) and in *E. tarda* by yeast two-hybrid (Zheng and Leung, 2007). Similarly, an interaction between the SciS and SciN homologues in *E. tarda* has been reported (Zheng and Leung, 2007). Overall, the SciZ protein is part of a *trans*-envelope complex of which the exact function in T6S is actually unknown. Using fractionation procedures, we have shown that this complex is not required for SciN OM targeting (data not shown). Experiments currently underway in our laboratory aim at understanding how this protein complex and how the interaction of SciZ with the PG contribute to T6SS assembly and biogenesis.

Experimental procedures

Bacterial strains, growth conditions and chemicals

Escherichia coli K12 DH5α was used for cloning procedures. The EAEC strain 17-2 (kindly provided by Arlette Darfeuille-Michaud, University of Clermont-Ferrand, France) was used for this study. Strains were routinely grown in LB broth at 37°C, with aeration. Plasmids were maintained by the addition of ampicillin (100 μg ml^{-1} for K12, 200 μg ml^{-1} for EAEC), kanamycin (50 μg ml^{-1} for K12, 50 μg ml^{-1} for chromosomal insertion on EAEC, 100 μg ml^{-1} for plasmid-bearing EAEC), chloramphenicol (40 μg ml^{-1}). Sodium deoxycholate, *Torula* yeast RNA, Imidazole, SLS and *N*-ethyl-maleimide were purchased from Sigma-Aldrich, DSP was purchased from Pierce, anhydrotetracyclin (AHT – used at 0.2 μg ml^{-1} throughout the study) from IBA, and MPB was purchased from Molecular probes.

Strain construction

Construction of the Δ*sciN* deletion strain has been previously described (Aschtgen *et al*., 2008). Deletion mutant strains Δ*sciZ*, Δ*sciP* and Δ*sciS* were constructed similarly using the modified one-step inactivation procedure using oligonucleotides carrying 50-nucleotide extensions homologous to regions adjacent to the target gene (strains and oligonucleotides are listed in Table S1). The kanamycin cassette was then excised using plasmid pCP20.

Plasmid construction

Polymerase chain reactions (PCR) were performed with a Biometra thermocycler, using the Pfu Turbo DNA polymerase (Stratagene; La Jolla, CA). Custom oligonucleotides (listed in Table S1) were synthesized by Eurogentec. The construction of pOK-SciN-HA has been previously described (Aschtgen *et al*., 2008). pOK-SciP-HA, pOK-SciS-HA, pOK-SciC-HA were constructed similarly by insertion of the EcoRI-XhoI PCR fragment into pMS600 digested by the same enzymes. Other plasmids have been constructed by a double PCR technique, allowing amplification of the gene of interest flanked by extensions annealing to the target vector. The product of the first PCR has then been used as oligonucleotides for a second PCR using the target vector as template (oligonucleotides are listed in Table S1). pOK-Hcp-HA encodes Hcp carrying an HA-epitope tag at the C-terminus cloned into the pOK12 vector. pIBA-28 encodes full-length SciZ carrying a 6-His tag at the N-terminus cloned into the pASK-IBA37plus vector (IBA). pIBA-28p encodes the periplasmic domain of SciZ cloned in frame with the OmpA signal sequence cloned into the pASK-IBA4 vector (IBA). Plasmid pET-28p allows the T7-dependent production of the periplasmic domain of SciZ fused to a 10-His at the N-terminus cloned into the pET19b vector (Novagen). Site-directed substitutions and deletion were introduced by quick change mutagenesis: complementary pairs of mutagenic oligonucleotides (listed in Table S1) were used to amplify the whole plasmid template, and to introduce the mutation at the desired site. Substitutions and deletion of the PG-binding motif were introduced into the pIBA-28, pIBA-28p and pET-28p plasmids. Cysteine substitutions were introduced into the pIBA-28 plasmid. All constructs have been verified by restriction analyses and DNA sequencing (Genome Express). Plasmids used in this study are listed in Table S1.

Hcp release assay

Supernatant and cell fractions have been separated as previously described (Aschtgen *et al*., 2008). Briefly, 2 × 10^9 cells producing HA epitope-tagged Hcp were harvested and collected by centrifugation at 2000 *g* for 5 min. The supernatant fraction was then subjected to a second low-speed centrifugation and then at 16 000 *g* for 15 min. The supernatant was then filtered on sterile polyester membranes with a pore size of 0.2 μm (membrex 25 PET, membraPure GmbH) before precipitation with trichloroacetic acid (TCA) 15%. Cells and precipitated supernatant were then resuspended in loading buffer and analysed by sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with the anti-HA antibody. As control for lysis, Western blot was immunodetected with antibodies raised against the periplasmic TolB protein.

Biofilm formation assay

The adherence assay was performed in 24 well polystyrene microtitre dishes and glass tubes, after incubation at 30°C without agitation for 20 h. Attached bacteria were stained with 1 % crystal violet for 15 min and washed four times with water. For quantification, the ring of stained bacteria was collected with 500 µl of 95% ethanol and diluted in the same volume of water. The absorbance of the suspension was then measured at 590 nm.

Fractionation

A total of 2×10^9 exponentially growing cells were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0), sucrose 20% and incubated for 10 min on ice. After addition of 100 µg ml^{-1} of lysozyme and 0.5 mM EDTA and further incubation for 25 min on ice, DNase (100 µg ml^{-1}) and $MgCl_2$ (2 mM) were added and cells were lysed by five cycles of freeze and thaw. Unbroken cells were removed by centrifugation, and soluble and membrane fractions were separated by ultracentrifugation for 40 min at 100 000 *g*. Membranes were washed with 10 mM Tris-HCl pH 8.0, $MgCl_2$ 2 mM and then resuspended in 1 ml of 1 M sodium carbonate (Na_2CO_3), incubated on a wheel for 1 h, and then ultracentrifuged for 40 min at 100 000 *g* to separate integral membrane and peripherally membrane-associated proteins. Soluble and membrane-associated fractions were precipitated with TCA (15%), and resuspended in loading buffer prior to analyse by SDS-PAGE and immunoblotting.

Sodium lauroyl sarcosinate extraction

Sodium lauroyl sarcosinate is an anionic detergent that selectively disrupts the IM and solubilizes IM proteins (Filip *et al.*, 1973). Membranes prepared from 10^{10} cells using the fractionation protocol were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0) supplemented with 2% of sodium *N*-lauroyl sarcosinate (SLS; Sigma Aldrich) and incubated on a wheel for 1 h at room temperature. Insoluble and soluble fractions were recovered by ultracentrifugation at 100 000 *g* for 40 min before analyses by SDS-PAGE and immunoblotting.

Sedimentation sucrose gradient

Inner and outer membranes were separated using discontinuous sedimentation sucrose gradients. A total of 2×10^{11} cells were harvested, resuspended in 1.5 ml of Tris-HCl 10 mM pH 7.4, sucrose 20% and RNase 100 µg ml^{-1}, lysed by French-press treatment (four passages at 1200 psi), and total membranes were recovered by centrifugation at 100 000 *g* for 40 min and resuspended in 0.5 ml of 20% sucrose containing a protease inhibitor cocktail (Complete EDTA-free, Roche). The membrane fraction was then loaded on the top of a discontinuous sucrose gradient composed of the superposition of 1.5 ml of 30%, 35%, 40%, 45%, 50%, 55% and 60% sucrose solutions (from top to bottom). Gradients were centrifuged at 90 000 *g* for 90 h and 500 µl fractions were collected from the top. The fractions were analysed by a NADH oxidase enzymatic test, and by SDS-PAGE and immunoblotting with the anti-TolR and anti-OmpA antibodies. The NADH oxidase activity was measured in 96-well polystyrene microtitre dishes, using 20 µl of each fraction diluted in 180 µl of Tris-HCl 50 mM pH 7.5, dithiothreitol 0.2 mM and NADH 0.5 mM. The decrease of absorbance of the NADH at 340 nm, which reflects the activity of the NADH oxidase, was measured after 15 min of incubation at 25°C using a TECAN M200 microplate reader. Each fraction has been tested in duplicate.

Co-precipitation

A 10 ml culture of cells producing various combinations of T6SS subunits were harvested, washed once in 20 mM Tris-HCl pH 8.0, NaCl 0.2 M (buffer TN), and resuspended in the same buffer supplemented with DNase (100 µg ml^{-1}), lysozyme (50 µg ml^{-1}) and protease inhibitors cocktail (Complete EDTA-free, Roche). Bacteria were lysed by French Press (four passages at 1200 psi), and 0.2% of Nonidet P-40 were added in the lysate for membrane protein solubilization, and further incubated for 16 h at 4°C with gentle rotation. Unsolubilized material was discarded by centrifugation 15 min at 14 000 *g*, and the supernatant was supplemented with imidazole 10 mM before incubation with Nickel magnetic beads (PureProteome, Millipore). After overnight incubation at room temperature on a wheel, beads were washed in buffer TN supplemented with imidazole 10 mM, twice with buffer TN supplemented with imidazole 75 mM. 6His-tagged proteins and co-precipitated partner proteins were eluted by buffer TN supplemented with Nonidet P-40 0.06%, imidazole 400 mM and analysed by SDS-PAGE and Western blotting with anti-His and anti-HA antibodies.

Cysteine accessibility

Cysteine accessibility experiments were carried out as described by Jakubowski *et al.* (2004) and Goemaere *et al.* (2007) with modifications. A 40 ml culture of strain Δ*sciZ* producing cysteine-substituted SciZ derivatives was induced for *sciZ* gene expression with 0.02% AHT for 2 h. Cells were harvested, resuspended in buffer A (100 mM HEPES (pH 7.5), 250 mM sucrose, 25 mM $MgCl_2$, 0.1 mM KCl) to a final OD_{600} of 12 in 500 µl of buffer A. MPB (Molecular Probes) was added to a final concentration of 100 µM (from a 20 mM stock freshly dissolved in DMSO) and the cells were incubated for 30 min at 25°C. β-Mercaptoethanol (20 mM final concentration) was added to quench the biotinylation reaction, and cells were washed twice in buffer A, and resuspended in buffer A containing *N*-ethyl maleimide (final concentration 5 mM) to block all free sulfhydryl residues. After incubation 20 min at 25°C, cells were disrupted by four passages at the French press at 1200 psi. Membranes recovered by ultracentrifugation 40 min at 100 000 *g* were resuspended in 1 ml of buffer B [10 mM Tris (pH 8.0), 100 mM NaCl, 5 mM imidazole, 1%

(w/v) Triton X-100, protease inhibitor cocktail (Complete, Roche)]. After incubation on a wheel for 1 h, unsolubilized material was removed by centrifugation 15 min at 20 000 *g*, and solubilized proteins were subjected to immobilized metal ion affinity chromatography (IMAC) using cobalt beads (Talon, Clontech). After 3 h of incubation on a wheel, cobalt beads were washed twice with 1 ml buffer B, once with buffer B supplemented with 10 mM imidazole, and twice with buffer C [10 mM Tris (pH 8.0), 100 mM NaCl, 0.1% (w/v) Triton X-100] supplemented with 25 mM imidazole. Beads were air-dried, and resuspended in Laemli buffer prior to SDS-PAGE analysis and immunodetection with anti-His antibodies, and streptavidin coupled to alkaline phosphatase.

Peptidoglycan purification

Peptidoglycan was prepared according to the method of Leduc *et al.* (1992). Briefly, cell envelopes from 2×10^{10} cells were suspended in 1 ml of 9% NaCl, mixed with an equal volume of 8% SDS, and incubated for 30 min at 100°C. After standing at room temperature overnight, the sample was centrifuged at 30°C for 30 min at 400 000 *g* in a Beckman TLA-100.4 rotor. The pellet was washed by four cycles of resuspension and centrifugation in water, and finally resuspended in 0.5 ml of 10 mM sodium phosphate buffer, pH 8. The final concentration of the PG preparation was estimated as ~1 mg ml^{-1}.

In vivo *DSP cross-linking and purification of PG-bound proteins*

In vivo chemical cross-linking with DSP was done as previously described (Leduc *et al.*, 1992). Briefly, 10^{10} cells were washed in 20 mM sodium phosphate (pH 7.4) buffer, and then resuspended in 0.9 ml of 20 mM sodium phosphate (pH 7.4) containing 20% sucrose. A 100 µl volume of cross-linking reagent (DSP; 20 mM) was then added, and the suspension was allowed to stand for 30 min at room temperature. Cells were washed twice in 20 mM sodium phosphate (pH 7.4) buffer, and PG and soluble fractions were separated as described above.

Protein purification

Five hundred millilitres of BL21(DE3) bearing the pET19b derivatives encoding the periplasmic domain of SciZ or its mutant derivatives was grown to an OD_{600} of 0.5 and induced for 16 h with 100 µM isopropyl-thio-galactopyrannoside at 22°C. The cell pellet was resuspended in Tris-HCl pH 8.0, NaCl 100 mM, lysozyme 100 µg ml^{-1} and lysed by sonication using a Brandon sonifer. Membrane and unsoluble material were recovered by ultracentrifugation 40 min at 100 000 *g*, and resuspended in Tris-HCl pH 8.0, NaCl 100 mM buffer supplemented with urea 8 M. Proteins were purified by IMAC using cobalt beads (Talon) and eluted at ~200 mM imidazole. Proteins were then dialysed step-by-step in 20 mM sodium phosphate buffer containing decreasing concentrations of urea (1 h at 6 M, 1 h at 4 M, 1 h at 2 M, 1 h at 1 M, and overnight without urea). The purity of the preparations (> 95%) were estimated by Coomassie blue staining after SDS-PAGE.

In vitro *PG-binding assay*

The *in vitro* PG-binding assay was done as previously described (Bouveret *et al.*, 1999) with modifications. The purified Palp protein (a soluble truncated form of the Pal lipoprotein, devoid of the lipid anchors and of the 43 first amino acids), the TonBp protein (corresponding to the soluble domain of TonB) and the purified SciZp domain or its variants were first centrifuged for 30 min at 400 000 *g*. Two micrograms of each protein was mixed with 25 µl of the purified PG preparation (~25 µg) in sodium phosphate 10 mM (pH 8) NaCl 100 mM buffer (final volume, 100 µl) and incubated for 30 min at room temperature. The mixture was then centrifuged for 30 min at 400 000 *g*. The supernatant was collected and proteins were precipitated with TCA 15%. The pellet was washed with 500 µl of buffer. Precipitated soluble fraction and pellet were resuspended in loading buffer and analysed by SDS-PAGE and immunoblotting.

Miscellaneous

Proteins suspended in loading buffer were subjected to SDS-PAGE. For detection by immunostaining, proteins were transferred onto nitrocellulose membranes, and immunoblots were probed with antibodies, and goat secondary antibodies coupled to alkaline phosphatase, and developed in alkaline buffer in presence of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Anti-OmpA, -TolB, -TolR, -LexA and -MalE are from our laboratory collection. Anti-HA (Roche), anti-5His (Qiagen), anti-Streptag II (IBA) and alkaline phosphatase-conjugated goat anti-rabbit, mouse or rat antibodies (Millipore) have been purchased as indicated. Streptavidin coupled to alkaline phosphatase has been purchased from Pierce.

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