Supplementary material.

Supplementary Experimental Procedures.

**Accessibility to protease (supplementary Fig. S2).**

2 x 10^9 WT EAEC cells producing the N-terminally His-tagged SciZ proteins were resuspended in 1 ml of 10 mM Tris-HCl pH8.0, Sucrose 20%. After incubation 10 min on ice, cells were converted to spheroplasts by addition of 100 µg/ml of lysozyme and 0.5 mM EDTA. After 30 min of incubation on ice, MgCl₂ (2 mM) was added, and half of the mixture was treated by five cycles of freeze and thaw to lyse the cells. Membranes were recovered by centrifugation and resuspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0), sucrose 20%, EDTA 0.5 mM, MgCl₂ 2 mM. Spheroplasts and membranes were treated by 100 µg/ml of proteinase K, and samples were harvested at different times by boiling in Laemmli buffer prior to SDS-PAGE on 16%-acrylamide Tricine gels, and immunoblotting with anti-5His antibody.

**Fractionation (supplementary Fig. S3).**

Cells were converted to spheroplasts to isolate the periplasm fraction. Briefly, a pellet of 2 x 10^9 exponentially growing cells was 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 of lysozyme and 0.5 mM EDTA and further incubation for 25 min on ice, the periplasm and spheroplast fractions were separated by centrifugation. The periplasm fraction was precipitated with trichloroacetic acid (15%), and resuspended in loading buffer prior to analyze by SDS-PAGE and immunoblotting.
**Outer membrane stability assays (supplementary Fig. S4).**

Outer membrane stability assays have been done as previously described (Cascales et al., 2002; 2004). Outer membrane permeability was assessed by measuring the level of detergent susceptibility and periplasmic leakage. Detergent susceptibility was estimated on deoxycholate (DOC)-containing plates (1% final concentration), by spotting 2 µl of normalized 10-fold serial dilutions of the strain to be tested. For lethal dose 50% (LD$_{50}$) measurements, bacteria grown on LB media were plated on LB Petri dishes containing various concentrations of DOC. After overnight incubation, colony forming units were counted, and the value plotted against the DOC concentration. The LD$_{50}$ was then calculated from the graph as the DOC concentration necessary to kill 50% of the bacteria. For periplasmic RNaseI leakage cultures were spotted on LB plates supplemented with 1.5% *Torula* Yeast RNA (Sigma), and leakage was estimated by the formation of an RNA hydrolysis halo after overnight incubation by addition of 10% trichloroacetic acid. For outer membrane stability assays, a *pal* mutant strain was used as control.

**Motility assays (supplementary Fig. S4).**

Swarming was measured as follows. Two microliter of culture of the strain to be tested were spotted on LB plates containing 0.28% agar and incubated at 30°C. Swarm diameters were measured at regular time intervals, and plots of diameter versus time were fitted to a line. The value obtained was corrected by the generation time, and the rates reported are relative to the control strain (WT strain carrying the empty vector). Swimming motility was also scored by phase contrast microscopy. For swarming and swimming assays, a *motA* mutant strain was used as control.
**PG-binding competition experiments (supplementary Fig. S6).**

To carry out the competition experiments for PG binding, two micrograms of each protein (TonBp, Palp, and SciZp) were incubated with 10 micrograms of purified peptidoglycan and increasing amount of a peptide corresponding to the PG-binding region of Pal (DERGTPEYNISLGERRANAVKMYLQGKVSGADQISIVSYGKE) or of a control peptide corresponding to a portion of the periplasmic domain of TolR (CKDVPYEIIKALNLLHSAGVKSGLMTQP) (Bouveret et al., 2009). The mixture was incubated for 30 min at room temperature and centrifuged for 30 min at 400,000 x 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 analyzed by SDS-PAGE and immunoblotting.
Supplementary Legends to Figures.

**Figure S1. Effect of serine and cysteine substitutions on SciZ function.** The consequence of the production of SciZ variants on Hcp release was estimated in *sciZ* cells producing Hcp<sub>HA</sub>. Release was assessed by separating cells (WC) and supernatant (Sn) fractions from EAEC *sciZ* pOK-Hcp<sub>HA</sub> cells carrying the empty vector (vector) or encoding SciZ or its substitution variants (C519S, C557S, Δ2C [C519S, C557S], Δ2C-S5C, Δ2C-S28C, Δ2C-S355C, Δ2C-S445C). 2x10^8 cells and the TCA-precipitated material of the supernatant from 5x10^8 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 (control for cell lysis; upper panel). Molecular weight markers are indicated on the left.

**Figure S2. SciZ membrane topology assessed by protease accessibility.** Spheroplasts (Spheropl., left panel) or membranes (Membr., right panel) from 10^9 EAEC WT cells producing the N-terminally His-tagged variant of SciZ were treated with Proteinase K. Samples were harvested prior to proteinase K treatment (time “0”) and at time 2, 5, or 10 min and subjected to SDS-PAGE and western-blot using anti-5His antibody. Molecular weight markers (in kDa) are indicated on the left. Probable degraded forms of SciZ (based on their apparent molecular weights) are indicated on the right with their theoretical molecular weights (the hexa-histidine tag is indicated by the red ball).

**Figure S3. SciZp is exported to the periplasm.** 2 x 10^9 EAEC WT cells bearing the empty vector (vector) or the vector encoding the periplasmic domain of SciZ (SciZp) downstream of
a signal sequence were induced (+) or not (-) with AHT (0.02% final concentration) for 2 hours prior to centrifugation. The Total cells (T) and the spheroplasts (S) and periplasm (P) fractions are shown. Samples were loaded on 12.5%-acrylamide SDS-PAGE and immunodetected with antibodies directed against the LexA (cytoplasm) and MalE (periplasm) proteins, and against the StreptagII epitope (SciZp). Molecular weight markers are indicated.

**Figure S4. Phenotypic consequences of the production of SciZp.** The consequence of the production of SciZp and its variants in EAEC WT cells was estimated by measuring the level of susceptibility to the detergent deoxycholate (DOC), the level of periplasmic release (RNA) or the effect on swarming (motility) in absence (- AHT) or presence (+ AHT) of the inducer. To minimize the effect of SciZp production on the generation time, the concentration of inducer used in these experiments (0.005%) was determined in pilot experiments to be the highest concentration that does not affect cell growth (as shown on plain agar plates). For cell envelope stability assays, strains are indicated on the left. For swarming motility assays, the strains spotted are indicated on the middle schema.

**Figure S5. Effect of SciZp production on Hcp release.** The consequence of the production of SciZp and its variants on Hcp release was estimated in WT cells producing Hcp_{HA}. Release was assessed by separating cells (WC) and supernatant (Sn) fractions from EAEC WT cells carrying the empty vector (vector) or encoding SciZp and its PG-binding variants. 2x10^8 cells and the TCA-precipitated material of the supernatant from 5x10^8 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 (control for cell lysis; upper panel). The asterisk showed the non-specific release of the periplasmic TolB protein due to the
periplasmic leakage induced by the production of SciZp (see suppl. Fig. S4). Molecular weight markers are indicated on the left.

**Figure S6. Competition experiments for PG-binding with synthetic peptides.** 2 µg of purified TonBp, Palp, and SciZp were incubated 30 minutes with purified PG in absence (-) or increasing amount of Pal or TolR (control) peptide. The ratio (mol prot:mol peptide) were 1:0.5 (lanes 2 and 6), 1:1 (lanes 3 and 7), 1:2 (lanes 4 and 8), and 1:10 (lanes 5 and 9). Soluble (S) and PG-associated fractions were collected by centrifugation and loaded on a 12.5% SDS-PAGE and analyzed by Western blot using anti-5His antibody. Molecular weight markers are indicated on the left.
### Supplementary Table S1. Strains, plasmids, and oligonucleotides used in this study.

#### Strains

<table>
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<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-2</td>
<td>Wild-type enteroaggregative <em>Escherichia coli</em></td>
<td>A. Darfeuille-Michaud</td>
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<tr>
<td>ΔsciZ</td>
<td>17-2 deleted of gene sciZ</td>
<td>This study</td>
</tr>
<tr>
<td>ΔsciN</td>
<td>17-2 deleted of gene sciN</td>
<td>Aschtgen <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>ΔsciP</td>
<td>17-2 deleted of gene sciP</td>
<td>This study</td>
</tr>
<tr>
<td>ΔsciS</td>
<td>17-2 deleted of gene sciS</td>
<td>This study</td>
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#### Plasmids

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<th>Description</th>
<th>Reference</th>
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<td>pASK-IBA37+</td>
<td>Expression vector, CoE1 ori, Tet promoter, N-terminal 6His-tag</td>
<td>IBA</td>
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<td>pIBA-28</td>
<td>sciZ cloned into pASK-IBA37+</td>
<td>This study</td>
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<td>pIBA-28-C519S</td>
<td>Introduction of the SciZ Cys519 to Ser substitution into pIBA-28</td>
<td>This study</td>
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<td>pIBA-28-C557S</td>
<td>Introduction of the SciZ Cys557 to Ser substitution into pIBA-28</td>
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<td>This study</td>
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<tr>
<td>pIBA-28-ΔPGB</td>
<td>Deletion of SciZ residues Asn494 to Ala502 into pIBA-28</td>
<td>This study</td>
</tr>
<tr>
<td>pIBA-28-PGB*</td>
<td>Deletion of SciZ residues Asn494 to Ala502 into pIBA-28</td>
<td>This study</td>
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<td>pASK-IBA4</td>
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<td>pIBA-28p</td>
<td>sciZ (amino-acids 348 to 576) cloned into pASK-IBA4</td>
<td>This study</td>
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<td>pIBA-28p-ΔPGB</td>
<td>Deletion of SciZ residues Asn494 to Ala502 into pIBA-28p</td>
<td>This study</td>
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<tr>
<td>pIBA-28p-PGB*</td>
<td>Introduction of the SciZ Asn494 to Leu, Leu497 to Asn, Ser498 to Ala, Arg501 to Gln, and Ala502 to Asp substitutions into pIBA-28p</td>
<td>This study</td>
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</table>
pET19b    Production plasmid, N-terminal 10His tag, TEV cleavage site   Novagen
pET-28p  sciZ (amino-acids 348 to 576) cloned into pET19b   This study
pET-28p-ΔPGB   Deletion of SciZ residues Asn494 to Ala502 into pET-28p   This study
pET19b-SciZp-PGB*   Introduction of the SciZ Asn494 to Leu, Leu497 to Asn, Ser498 to Ala, Arg501 to Gln, and Ala502 to Asp substitutions into pET-28p   This study
pOK12   Expression vector, P15A ori   Viera and Messing, 1991
pOK-HcpHA   hcp carrying C-terminal HA tag cloned into pOK12   This study
pOK-SciC-HA   sciC carrying C-terminal HA tag cloned into pOK12   This study
pOK-SciN-HA   sciN carrying C-terminal HA tag cloned into pOK12   Aschtgen et al., 2008
pOK-SciP-HA   sciP carrying C-terminal HA tag cloned into pOK12   This study
pOK-SciS-HA   sciS carrying C-terminal HA tag cloned into pOK12   This study

Oligonucleotides

Strain construction a.

ΔsciZ       TCTTCTGTGCTTTGCAAGACCAGGTGCGACCGGCTTACCCGAGGATAACCTGTGATGGCTGGAGCTGCTTCCG
             ATATCTGCACCCACCATCATCTTTTCAGCCACAGATAAAACTGGAATTGCCATCG
ΔsciP       CATCGCTGGGAGATGTGAACTGGAATTTTTTGCGTGTGCGGACATGAAATGTGATGGCTGGAGCTGCTTCCG
             AGGAACAGCCTCGATATTGTCAGCGAGACGGGAACGTACGTGCATGGCATG
ΔsciS       GAGAAGAACATTTTTATCAGTACTGTTACATCAGAAACCAGAAATGAAATGTGATGGCTGGAGCTGCTTCCG
             CCCGGGGTGTGCTGTGGCATGCACTGAGGAGCGAATGAATGGAAGCCATCG

Plasmid construction by regular cloning b.

pOK-SciP-HA   GTCGGGAATTCAATAAACCTGTATTATCTCCCGGGCTGAAC
               CTGCCCTCGAGTCCCTGCCCCGTAAGCCG
pOK-SciS-HA  
GTCGGAATTCATCAAATATTGCGGCTGTCAGTCTGGTCG
CTGCTCGAGTGCAGTCTCCTCCACGGATTC

pOK-SciC-HA  
GTCGGAATTCTGAATTTAAACCTTCGCGTTATTTTCA
CTGCTCGAGATCCGGGAGCAGCGCGATTC

**Plasmid construction by double PCR.**

pOK-Hcp-HA  
GGATAACAATTTTCACACACAGGAAACTGCATGACGTATGCTCTGGGC
CTGCTCGAGACTAGTGGTCTCAGTCTGCTCTGGTCTCTGGGTCTCCCTCCGTTATGTTTTCTGATGCGCC

pIBA-28  
CATCACCATCATCACCATCGAGGCGCCACGTACGTTCCCGTCTCTGGCTGAC
GCTCGAATTCGGGACCGCGGTCTCGCTTTTGTTCTGATGCGCCCTC

pIBA-28pp  
CCACCCCGAGTTCGAAAAAGGCCCTTGGTTCAGCAGCGTGGGGAAC
GAGCTCGAATTCGGGACCGCGGTCTCTACCTCCTGATGTTTTTCTGATGCGCCTC

pET-28  
CGCGCCCATATCGAGCAGCAGACGACGCAACGCTGTTTCCAGCAGCGTGGGGAAC
GTTAGCAGCCGGATCTCGAGCAGTATGATTCTCCGGTATGTTTTTCTGATGCGCCTC

**Substitution mutagenesis by PCR.**

ΔPG  
CAGCGGGTGCGCGCCGCTATTATCCGCTGTGACCCACTGACC
GATAATACCGGCTACCGCGCTGCGGAGCGGCGTTCGGCAGCTGACC

PG*  
CAGCGGGTGCGCGCCGCTATTATCCGCTGTGACCCACTGACC
GATAATACCGGCTACCGCGCTGCGGAGCGGCGTTCGGCAGCTGACC

C517S  
GATAACCGGCGGATATCCCGCAGAGCTTTTTCGCGTACACGGGATAC
GGGAATATCGGCGGATATCCCGCATCCAGTCGCGGAC
C559S   CTGGTACGCAGCCATGCCTCTGCTCTCCGGAGCTTTTC
          ATCGGCTCGGTTACACAGACTGATTTCACACGCAGGT
S5C     GTATTTGTCAGCCAGACGTACGAGCCCACTTGCTGATGTGATG
          CGAAGGGCCACGTACGCTGACTCTGGCTGACACTACT
S28C    CTGTTTTACTGCACACAGCAGGCAGGAATCCCGAAATCAGGCACAGTG
          GATTTCGGGATTCCTGCCGCTGTGTGCAGTAAACCAGATAATCTG
S355C   GTGTCCCCACGCAGCAACACGCGCTGTTATTCATAAAAG
          GAATAACCCAGCGCGCTGCTGCTGCTGCTGCTGGGAGAACACCTGACG
S445C   CAAACAGTGCCAGGCAATTGAGGCGCAACCAGCTCTGCTGAGTAAC
          GACGCCGAACACGCTCGCCTCAAATGCGCTGGAACATGGTTGAGTGCACG

\(^a\) sequence adjacent to the target gene underlined.
\(^b\) \textit{EcoRI} and \textit{XhoI} sites used for restriction underlined.
\(^c\) sequence complementary to target vector underlined.
\(^d\) HA tag coding sequence italicized.

**References.**
**Figure S1**

The figure shows a gel analysis for the proteins `sciZ pHcp_{HA}`. The gel displays samples labeled with different constructs: vector, `SciZ`, `SciZ_{C519S}`, `SciZ_{C557S}`, `SciZ_{Δ2C}`, `SciZ_{S5C}`, `SciZ_{S28C}`, `SciZ_{S335C}`, and `SciZ_{S445C}`. The gel is labeled with markers at 50, 36, 30, and 16 molecular weight units. The bands corresponding to `TolB` and `Hcp_{HA}` are indicated.
Figure S2
Figure S3
Figure S4
Figure S5
Figure S6