SciN Is an Outer Membrane Lipoprotein Required for Type VI Secretion in Enteroaggregative *Escherichia coli*®

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Enteroaggregative *Escherichia coli* (EAEC) is a pathogen implicated in several infant diarrhea or diarrheal outbreaks in areas of endemicity. Although multiple genes involved in EAEC pathogenesis have been identified, the overall mechanism of virulence is not well understood. Recently, a novel secretion system, called type VI secretion (T6S) system (T6SS), has been identified in EAEC and most animal or plant gram-negative pathogens. T6SSs are multicomponent cell envelope machines responsible for the secretion of at least two putative substrates, Hcp and VgrG. In EAEC, two copies of T6S gene clusters, called sci-I and sci-2, are present on the *pheU* pathogenicity island. In this study, we focused our work on the sci-I gene cluster. The Sci-I apparatus is probably composed of all, or a subset of, the 21 gene products encoded on the cluster. Among these subunits, some are shared by all T6SSs identified to date, including a ClpV-type AAA+-ATPase (SciG) and an IcmF (SciS) and an IcmH (SciP) homologue, as well as a putative lipoprotein (SciN). In this study, we demonstrate that sciN is a critical gene necessary for T6S-dependent secretion of the Hcp-like SciD protein and for biofilm formation. We further show that SciN is a lipoprotein, as shown by the inhibition of its processing by globomycin and in vivo labeling with [3H]palmitic acid. SciN is tethered to the outer membrane and exposed in the periplasm. Sequestration of SciN at the inner membrane by targeting the +2 residue responsible for lipoprotein localization (Gly2Asp) fails to complement an *sciN* mutant for SciD secretion and biofilm formation. Together, these results support a model in which *SciN* is an outer membrane lipoprotein exposed in the periplasm and essential for the Sci-I apparatus function.

The virulence of most bacterial pathogens relies on a subset of processes including biofilm formation, interaction with the host cell, and release of toxin proteins (55). This latter mechanism requires dedicated machineries called secretion systems. Seven secretion systems have been described so far, which assemble from 3 to >20 subunits. These secretion systems derive from or coevolved with bacterial organelles, such as ABC transporters (type I), type IV pili (type II), flagella (type III), or conjugative macromolecules (type IV) (12, 23, 43, 44). The newly identified type VI secretion (T6S) system (T6SS) is present in most pathogens that have contact with animals, plants, or humans (8, 10, 21, 33). T6SSs have been identified in several bacteria but are not yet well characterized. These secretion systems are involved in numerous pathogenic processes, including resisting predation by amoebae for *Vibrio cholerae* and *Burkholderia cenocepacia* (4, 56), inhibiting the macrophage phagocytizing activity of macrophages for *Aeromonas hydrophila* (67), favoring persistence within the host for *Salmonella enterica* (54), biofilm formation for *Vibrio parahaemolyticus* (29), rotting of potato stems for *Pectobacterium atrosepticum* (46), or the global virulence of the fish pathogen *Edwardsiella tarda* (57). T6SSs assemble at the bacterial cell envelope and form an apparatus dedicated to the secretion of essentially two proteins into the culture supernatant (8, 10, 33, 75). These proteins, called Hcp (hemolysin coregulated protein) and VgrG (valine glycine repeat), were found in culture supernatants of bacteria carrying T6S gene clusters (28, 52, 55, 56, 67), although whether these proteins are real substrates of the machine or are released in the supernatant following mechanical shearing in liquid culture is still matter of debate (10, 55). However, the presence of Hcp and VgrG in culture supernatants constitutes a good indicator of T6SS function. In a recent systematic deletion approach, 11 of the 13 *evp* genes of the *E. tarda* T6S have been found to be necessary for secretion of Hcp and VgrG into culture supernatant (75). The products of these genes may thus assemble as a secretion apparatus to deliver substrates to the medium or the host cell. Among these genes, several are shared by all T6SSs identified so far and are called “core components.” These genes encode an AAA+-ATPase of the ClpV family, a putative lipoprotein, several putative cytoplasmic proteins of unknown function, and inner membrane (IM) proteins sharing similarities with the type IVb secretion IcmF and IcmH proteins (8, 10, 33). The ClpV-like ATPases are thought to assemble as a ring structure capable of hydrolyzing nucleoside triphosphate to energize machine assembly or substrate secretion (59). In the *Legionella pneumophila* Dot/Icm T4bSS, the IcmF and IcmH (or DotU) proteins interact and stabilize the transport apparatus (62). Interestingly, most T6S-associated IcmH proteins carry C-terminal extensions resembling the peptidoglycan binding domain of the OmpA/Pal/MotB family and may thus anchor the apparatus to the cell wall.

Two gene clusters encoding T6S machines, namely *sci-I* and *sci-2*
sci-2, are present on the pheU pathogenicity island of enteropathogenic Escherichia coli (EAEC) strains (28). EAEC strains define an emerging E. coli pathotype responsible for severe and persistent diarrheaa of infants, young children, or immunocompromised individuals (39, 53). Although not well understood, the EAEC virulence mechanism is based on colonization of the intestinal mucosa involving both the flagella and adhesives structures called fimbrae, formation of a thick biofilm at the surface of the colon epithelia, and secretion of enterotoxins and cytotoxins (45). These processes involve plasmidborne and chromosomal virulence factors that had not been identified yet. However, several pathogenic determinants are encoded within the pheU pathogenicity island, and the two sci T6SS gene clusters are interesting candidates. Besides the presence of genes encoding homologues of Hcp and VgrG, these clusters possess characteristic features of functional T6SS machines, including core components that are conserved in all clusters identified so far. The sci-2 gene cluster expression has been shown to be controlled by the AraC family transcriptional activator AggR, which is a master regulator of EAEC virulence (28). It assembles a T6SS machine responsible for the secretion of the Hcp homologue AaiC. However, mutagenesis studies showed that no virulence defects are linked to sci-2 mutations. The sci-1 gene cluster has not been characterized, but the Sci-1 T6S proteins assemble and function under the laboratory conditions (28). The sci-1 cluster encodes typical T6S-associated proteins, including the SciD Hcp-like protein, a VgrG homologue, the SciG AAA-ATPase, the SciP IcmH-like protein, the SciS IcmF-like protein, and SciN, a probable lipoprotein.

In the present work, we initiated studies on the EAEC sci-1 gene cluster by targeting the sciN gene. We showed that an sciN mutant is unable to secrete the Hcp-like SciD substrate in the supernatant, which is accompanied by a decrease in biofilm formation, and is thus an essential component of the T6SS machine. Although SciN is predicted to be a lipoprotein based on its consensus N-terminal sequence for recognition by the signal peptidase II and lipid modification, its localization and acylation have not been determined experimentally. Herein, we performed experiments that demonstrated that SciN is a lipoprotein localized in the outer membrane (OM). We further showed that SciN is exposed in the periplasmic space. Muta-
tional analyses showed that proper localization at the OM is required for SciN function.

MATERIALS AND METHODS

Bacteria, plasmids, chemicals, and growth conditions. E. coli K-12 DH5α was used for cloning procedures. The lpp deletion strain JES505 (68) was used for copurification experiments. The EAEC strain 17-2 (kindly provided by Arlette DarfeuilleMichaud, University of Clermont-Ferrand, France) was used for this study. Unless otherwise indicated, strains were routinely grown in LB broth at 37°C, with aeration. Plasmids were maintained by the addition of ampicillin (100 mg/ml for K-12 and 200 mg/ml for EAEC), kanamycin (50 mg/ml for K-12, 50 mg/ml for chromosomal insertion on EAEC, and 100 mg/ml for plasmid-bearing EAEC), and chloramphenicol (40 mg/ml). Plasmid pAMR43L, allowing production of L-octa with the mutation R43L and a six-His tag [His6-LolA(R43L); kindly provided by Hajime Tokuda, University of Tokyo, Japan] has been described.

The SciS IcmF-like protein, and SciN, a probable lipoprotein. We further performed experiments that demonstrated that SciN is a T6S protein, including the SciD Hcp-like protein, a VgrG homologous protein, and SciN, a probable lipoprotein. These processes involve plasmidborne and chromosomal virulence factors that had not been identified yet. However, several pathogenic determinants are encoded within the pheU pathogenicity island, and the two sci T6SS gene clusters are interesting candidates. Besides the presence of genes encoding homologues of Hcp and VgrG, these clusters possess characteristic features of functional T6SS machines, including core components that are conserved in all clusters identified so far. The sci-2 gene cluster expression has been shown to be controlled by the AraC family transcriptional activator AggR, which is a master regulator of EAEC virulence (28). It assembles a T6SS machine responsible for the secretion of the Hcp homologue AaiC. However, mutagenesis studies showed that no virulence defects are linked to sci-2 mutations. The sci-1 gene cluster has not been characterized, but the Sci-1 T6S proteins assemble and function under the laboratory conditions (28). The sci-1 cluster encodes typical T6S-associated proteins, including the SciD Hcp-like protein, a VgrG homologue, the SciG AAA-ATPase, the SciP IcmH-like protein, the SciS IcmF-like protein, and SciN, a probable lipoprotein.

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tional analyses showed that proper localization at the OM is required for SciN function.

Double PCR technique (70), allowing amplification of the sciN gene, carrying a FLAG epitope coding sequence at the 3' end, flanked by extensions annealing to the target vector (pUC12) (74). The product of the first PCR was then used as a template for oligonucleotides for the second PCR using the target vector as a template. The oligonucleotides used were forward: 5'-ggaaagcttacacggctgtaaagccggtcggg (TsoI restriction site is underlined) and 5'-CTGGTGAAGGCGCCGCAAAAGAA (XhoI restriction site is underlined). The PCR product was digested by EcoRI and XhoI and cloned into the same sites of the pMS600 vector (a derivative of the pOK12 cloning vector [72] allowing in-frame fusion with a C-terminal HA coding sequence) (M. S. Aschtgen, unpublished data). Plasmid pSciN(G2D) with a G2D mutation in SciN was constructed by site-directed mutagenesis using plasmid pSciN-HA as the template and oligonucleotides 5'-TCTGTTAATACGACGCTACTACAG and 5'-TTTGCTAGATGCTACCG (Genome Express).

Construction of EAEC isogenic mutants. Isogenic mutants were generated using a modified protocol of a one-step inactivation procedure (22). The kanamycin resistance cassette was generated by PCR using the pKD4 plasmid as a template and a pair of oligonucleotides carrying 50-nucleotide extensions homologous to regions adjoining the target gene and downstream of the target gene (in the case of oligonucleotide sequences). Oligonucleotides used in this study were 5'-AATACTAGATGCTACCG (where HA is hemagglutinin) was constructed by amplification of the sciN gene and 5'-GAAGATACGCTACTACAG and 5'-TTTGCTAGATGCTACCG (Genome Express).

In vivo acylation assay. Lipoproteins from 3 x 10^9 cells grown in 2 ml of LB broth for 18 h were subjected to a second low-speed centrifugation and then resuspended in loading buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Globomycin treatment. EAEC cells producing SciN-HA from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter were grown to an optical density at 600 nm of ~0.6 before addition of 50 µM of globomycin. After 10 min of incubation, IPTG was then added at a final concentration of 100 µM, and cells were further incubated for 30 min at 37°C. Cells were harvested, and samples were analyzed by SDS-PAGE and immunoblotting.

In vitro acylation assay. Lipoproteins from 3 x 10^9 exponentially growing EAEC cells producing SciN-HA were labeled with 100 µCi of [9,10(α)-3H]palmitate from 1 h at 37°C. Proteins of interest were solubilized by a procedure using SDS-Triton X-100 and immunoprecipitated with specific antibodies coupled to protein A-Sepharose CL-4B beads (GE Healthcare) as previously described (15).
fractions were recovered by ultracentrifugation at 100,000 g for 40 min at 100°C. Incubation on a rotating wheel for 1 h at room temperature. Insoluble and soluble proteins were analyzed by an NADH oxidase enzymatic test using anti-FLAG monoclonal antibody (lower panel) and the anti-TolB polyclonal antibodies (upper panel). (C) Effect of the sciN mutation on biofilm formation. Biofilms formed in static cultures of WT, sciN, and complemented sciN cells (none, LB medium) were visualized in glass tubes by crystal violet staining (upper panel) and quantified using the ethanol-solubilization procedure, relative to the WT EAEC strain (lower graph).

Immunoprecipitated samples were analyzed by SDS-PAGE and autoradiography for 4 to 6 days at ~80°C.

Spheroplast formation and fractionation. Cells were converted to spheroplasts as previously described (14). Briefly, a pellet of 2 × 10⁹ exponentially growing cells was resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0)–20% sucrose and incubated for 10 min on ice. After the 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. Spheroplasts were washed with 10 mM Tris-HCl (pH 8.0)–20% sucrose, resuspended in 1 ml of 10 mM Tris-HCl–20% sucrose, and then subjected to five cycles of freezing and thawing before centrifugation to remove unbroken cells and to ultracentrifugation (40 min at 100,000 × g) to separate cytoplasm and membrane fractions. Membranes were washed with 10 mM Tris-HCl (pH 8.0)–2 mM MgCl₂. Periplasmic and cytoplasmic fractions were precipitated with 15% TCA and resuspended in loading buffer prior to analysis by SDS-PAGE and immunoblotting.

Proteinase & accessibility. A total of 10⁹ spheroplasts or whole cells resuspended in 1 ml of 20 mM Tris-HCl (pH 8.0)–10 mM MgCl₂ were treated with proteinase K at a final concentration of 100 μg/ml for 20 min on ice, collected by centrifugation, and then analyzed by SDS-PAGE and immunoblotting.

Sarkozyl differential extraction. Membranes prepared from 10⁹ cells using the fractionation protocol were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0) supplemented with 2% sodium N-lauroyl sarcosinate (SLS; Sigma-Aldrich), and incubated on a rotating wheel for 1 h at room temperature. Insoluble and soluble fractions were recovered by ultracentrifugation at 100,000 × g for 40 min before analysis by SDS-PAGE and immunoblotting.

Membrane sedimentation analyses. Inner and outer membranes were separated using discontinuous sedimentation sucrose gradients. A total of 2 × 10⁹ EAEc cells producing SciN-HA or SciN(G2D)-HA were harvested, suspended in 1.5 ml of 10 mM Tris-HCl (pH 7.4), 20% sucrose and 10 μg/ml RNase, lysed by French press treatment. 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 100 h, and 500-μl fractions were collected from the top. The composition of these fractions was analyzed by an NADH oxidase enzymatic test and by SDS-PAGE and immunoblotting with the anti-AcrA and anti-OmpA polyclonal antibodies. The NADH oxidase activity was measured in 96-well polystyrene microtiter dishes, using 20 μl of each fraction diluted in 180 μl of 50 mM Tris-HCl (pH 7.5), 0.2 mM dithiothreitol, and 0.5 mM NADH. The decrease in 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. Each fraction was tested in triplicate.

Six-histidine pull-down experiments. A total of 5 × 10⁹ EAEC cells producing ScnN-HA (from pSciN-HA plasmid) or both SciN-HA and His6-LolA(R43L) (from plasmid pAMR43L) were harvested and processed for spheroplast formation. The supernatant (periplasm fraction) was collected, and NaCl (100 mM final), imidazole (10 mM final), and protease cocktail inhibitors were added; the suspension was mixed with 100 μl of cobalt-coupled agarose beads (Talon; Clontech) and further incubated on a rotating wheel for 4 h at 25°C. The unbound fraction was collected, and beads were washed twice with 1 ml of buffer A containing 10 mM imidazole before elution with 1 ml of buffer A supplemented with 500 mM imidazole. Eluted material was then precipitated with TCA at a final concentration of 15% and resuspended in loading buffer before analysis by SDS-PAGE and immunoblotting.

Miscellaneous. Caenorhabditis elegans killing assays were performed as follows. A total of 10⁶ bacteria to be tested were spotted on the center of nematode growth medium plates and were incubated overnight at 37°C. Twenty-five worms were then transferred to each plate and kept at 25°C. The number of living versus dead or paralyzed worms was scored every 24 h for 12 days. Worms were considered dead when they were nonmotile and did not respond to a stimulus such as probing with a wire pick. Each strain was tested in triplicate. Proteins suspended in loading buffer were subjected to SDS-PAGE and immunoblotting 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 the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Anti-OmpA, -Pal, -TolB, -TolR, -TolA, -EF-Tu, -AcrA, and -MalE antibodies were from our laboratory collection. Anti-FLAG (Sigma-Aldrich), anti-His5, (Qiagen), and alkaline phosphatase-conjugated goat anti-rabbit, mouse, or rat antibodies (Millipore) were purchased as indicated.

RESULTS

SciN is required for T6S. In all T6SSs described so far, two proteins were found in culture supernatants: (i) the Hcp or Hcp-like proteins, which form hexameric rings; and (ii) the
VgrG proteins, which resemble the tail spike of bacteriophage T4, An Hcp-like protein and a VgrG homologue are encoded within the EAEC sci-I gene cluster (Fig. 1A). We first tested whether the Hcp-like protein SciD is released from EAEC 17-2 wild-type (WT) cells under our conditions, as previously reported (28). We thus constructed a plasmid allowing IPTG-inducible expression of sciD. This SciD protein carries a FLAG epitope at the C terminus. As expected, SciD-FLAG was found in culture supernatants of EAEC cells but was not released from E. coli K-12 DH5α cells (Fig. 1B and data not shown). Immunodetection of the periplasmic TolB protein (47) further demonstrated that no lysis or periplasmic leakage occurred during the experiment. To verify that this was not a nontypic release, we constructed an EAEC mutant with a deletion of the sciG (clpV) gene. ClpV proteins are ATPases of the AAA+ family linked to T6SS and have been shown to be essential for T6S in V. cholerae, P. aeruginosa, and E. tarda (52, 56, 75). SciD-FLAG was not recovered in culture supernatants of EAEC sciG cells, demonstrating the specificity of secretion (data not shown).

Among the T6SS core component genes, one encodes a putative lipoprotein (COG3521 family) (8, 10). In the EAEC sci-1 gene cluster, this gene, called sciN, is the last open reading frame of the locus (Fig. 1A). To test whether this conserved gene is required for the T6SS, we constructed an sciN deletion mutant and a plasmid allowing the IPTG-inducible production of a C-terminally HA-tagged SciN protein. Fractionation experiments further showed that SciD-FLAG was not secreted in the sciN strain carrying the empty vector, but SciD-FLAG secretion was restored when sciN-HA was supplied in trans (Fig. 1B). This result demonstrates that SciN is required for T6S.

No role for the sci-1 T6S gene cluster had been assigned for EAEC virulence. Using the worm C. elegans, previously shown to be a model for testing virulence of pathogenic E. coli (3), we showed that WT EAEC cells killed the worms at a 50% lethal dose of 7 days (data not shown). In contrast, the E. coli K-12 strain DH5α had no effect on C. elegans during the 12 days of the experiments. The EAEC sciN strain displayed a 50% lethal dose similar to that of the WT EAEC on C. elegans (data not shown). Because some T6S gene clusters have been shown to be necessary for biofilm formation or to be expressed during biofilm development (29, 65), we tested bacteria aggregation on polyethylene plastic wells and glass tubes. Crystal violet staining showed that the sciN mutant forms reproducibly lower amounts of biofilm than the WT cells or sciN cells complemented by the HA-tagged version of SciN (Fig. 1C).

SciN is a lipoprotein. The N terminus of SciN shares characteristic features of classical lipoproteins, which include a predicted lipoprotein signal sequence consensus (lipobox: L-A/S-G/A) followed by a cysteine residue (69) (Fig. 2A). This SciD protein carries a FLAG epitope at the C terminus. A total of 2 x 10^8 EAEC cells producing the HA-tagged SciN protein treated (+) or not (−) by the SPII inhibitor antibiotic globomycin (globom) were loaded on a 15% acrylamide SDS-PAGE gel and subjected to immunodetection with antibodies directed against the periplasmic MalE, the cytoplasmic EtTu, the membrane TolR, and the EAEC protein (from top to bottom panel). Molecular weight markers are indicated on the left. (C) SciN is not processed in the presence of globomycin. A total of 2 x 10^6 EAEC cells producing the HA-tagged SciN protein treated (+) or not (−) by the SPII inhibitor antibody globomycin (globom) were loaded on a 15% acrylamide SDS-PAGE gel and subjected to immunodetection with antibodies directed against OmpA, anti-Pal, or anti-HA antibody (from top to bottom panel). The unprocessed species of the Pal lipoprotein and SciN-HA are indicated by asterisks. Molecular weight markers are shown on the left. (D) SciN is labeled by [3H]palmitate. A total of 3 x 10^6 cells of EAEC producing SciN or SciN-HA were labeled in vivo by [3H]palmitate, SDS-Trition X-100 solubilized, and immunoprecipitated using anti-OmpA, anti-Pal, and anti-HA antibodies, as indicated. Samples were loaded on 15% acrylamide SDS-PAGE gels and subjected to autoradiography (upper panel) or immunodetection by corresponding antibodies (lower panels). Molecular weight markers are indicated on the left. α, anti; IgG, immunoglobulin G.
SciN-HA associated with the membrane fraction in EAEC (Fig. 2B). As controls, the TolR protein fractionated with the membrane fraction, and MalE localized in the periplasm whereas EfTu was found in the cytoplasmic fraction. To test whether SciN is a lipoprotein, we used (i) inhibition of SPII with the specific inhibitor globomycin (41) and (ii) [3H]palmitate labeling. The unprocessed forms of SciN-HA and of the Pal lipoprotein were detected when globomycin was added to growing EAEC cells expressing sciN-HA, whereas the OM OmpA protein was correctly processed (Fig. 2C). These results suggest that SciN is processed by SPII. Similarly, both SciN-HA and Pal were labeled in vivo with [3H]palmitate, whereas OmpA was not (Fig. 2D), demonstrating that SciN is fatty acylated. Overall, these results demonstrate that SciN is a lipoprotein.

SciN fractionates with the OM. To test whether SciN associates with the IM or OM, we performed (i) selective detergent solubilization using sodium lauroyl sarcosinate (SLS) and (ii) isopycnic sucrose sedimentation gradients. SLS has been previously shown to selectively disrupt the IM (32). Under the conditions used in this study (2% SLS, no Mg\textsuperscript{2+}, and low-ionic-strength buffer), the OM is resistant to solubilization. As shown in Fig. 3A, SciN, as well as the OM OmpA and the OM-associated Pal and Lpp lipoproteins, was found in the SLS-insoluble fraction whereas the IM TolR and TolA proteins were extracted with SLS. The OM localization of SciN

FIG. 3. SciN is an OM protein. (A) SciN is not solubilized by SLS. Total (T) membranes from EAEC cells producing SciN-HA were treated with SLS, and solubilized (S) and insolubilized (I) membranes were separated. Samples from 5 × 10\textsuperscript{6} cells were loaded on 15% acrylamide SDS-PAGE gels and subjected to immunodetection with antibodies directed against TolA, TolR (upper panel), and Pal (which recognizes OmpA and Lpp; middle panel) (11) and against the HA epitope (lower panel). Molecular weight markers are indicated on the left. (B) SciN cofractionates with the OM. Total (T) membranes from EAEC cells producing SciN-HA were separated on a discontinuous sedimentation sucrose gradient. Collected fractions were analyzed for contents using the anti-AcrA, anti-OmpA, and anti-HA antibodies (from top to bottom panel) and with an NADH oxidase activity test (upper graph). NADH oxidase activity is represented relative to the fraction having the highest activity. The positions of the IM- and OM-containing fractions are indicated. (C) SciN coprecipitates with LolA(R43L). Periplasm fractions (lanes T) of EAEC cells producing pSciN-HA and carrying (+) or not (–) plasmid pAMR43L, encoding the His\textsubscript{6}-LolA(R43L) mutant protein, were subjected to pull-down on cobalt beads. His\textsubscript{6}-LolA(R43L) was eluted with imidazole. Unbound (U) and eluted (E) materials were loaded on 15% acrylamide SDS-PAGE gels and subjected to immunodetection using the anti-His\textsubscript{6} and anti-HA antibodies. Molecular weight markers are indicated on the left.
was then confirmed using sedimentation density gradient centrifugation of membrane fractions, using the OM OmpA and the IM AcrA proteins as controls (Fig. 3B). Because AcrA interacts with the OM TolC proteins and a fraction of AcrA remains associated with the OM (40), we confirmed IM fractions of the gradient using the NADH oxidase enzymatic test (Fig. 3B). Overall, our results clearly demonstrate that SciN associates with the OM in E. coli. However, a portion of SciN is retained in the IM fractions or in fractions with intermediate densities (see Discussion).

In contrast to IM lipoproteins, OM lipoproteins are conveyed through the periplasm by the lipoprotein-specific periplasmic carrier LolA protein and are released to the OM LolB protein for assembly (69). The LolA(R43L) mutant has been shown to interact with OM lipoproteins but is unable to release them to LolB (51). As a consequence, OM lipoproteins are sequestered in the periplasm, in complex with LolA(R43L) (51). The periplasm fraction of the lpp strain JES505 (a strain devoid of the most abundant OM lipoprotein) (68) coproducing His6-LolA(R43L) and SciN-HA was subjected to pull-down experiments using affinity chromatography. Figure 3C shows that SciN-HA was efficiently co-precipitated with His6-LolA(R43L), further adding evidence for SciN OM localization.

**FIG. 4.** SciN is exposed in the periplasm. Whole cells (WC) or spheroplasts (Sph) of EAEC cells producing SciN-HA were treated (+) or not (−) with proteinase K (Prot K). Samples from 2 × 10⁸ cells were loaded on 15% acrylamide SDS-PAGE gels and subjected to immunodetection with anti-OmpA, anti-Pal, and anti-HA antibodies (from top to bottom panel). The degradation product of OmpA, corresponding of the cleavage of the external loop, is indicated by an asterisk. Molecular weight markers are indicated on the left.

**FIG. 5.** OM localization of SciN is required for T6SS function. (A) The SciN(G2D) mutant protein cofractionates with the IM. Total (T) membranes from EAEC cells producing SciN-HA were separated on a discontinuous sedimentation sucrose gradient. Collected fractions were analyzed for contents using anti-AcrA, anti-OmpA, and anti-HA antibodies (from top to bottom panel) and with an NADH oxidase activity test (upper graph). NADH oxidase activity is represented relative to the fraction having the highest activity. The positions of the IM- and OM-containing fractions are indicated. (B) SciD-FLAG secretion was assessed by separating cells (WC) and supernatant (Sn) fractions from WT, and sciN cells complemented with the HA-tagged SciN (sciNWT) or the G2D mutant, sciN(G2D). A total of 2 × 10⁸ cells and the TCA-precipitated material of the supernatant from 5 × 10⁸ cells were loaded on a 15% acrylamide SDS-PAGE gel and subjected to immunodetection using the anti-Flag monoclonal antibody (lower panel) and the anti-TolB polyclonal antibodies (upper panel). (C) Biofilms formed in static cultures of the WT and sciN cells complemented with the HA-tagged SciN (sciNWT) or the G2D mutant, sciN(G2D), were visualized in glass tubes by crystal violet staining (upper panel) and quantified using an ethanol-solubilization procedure, relative to the WT EAEC strain (lower graph).
SciN is exposed in the periplasm. To test whether the OM SciN lipoprotein is exposed at the cell surface or protrudes in the periplasm, we performed proteinase K accessibility experiments. Similar to the Pal lipoprotein, SciN-HA was resistant to proteinase K degradation in whole cells but was degraded upon cell permeabilization (Fig. 4). In contrast, the OmpA protein, which possesses surface-exposed loops, was partly degraded to a discrete fragment upon treatment of whole or permeabilized cells with proteinase K. This result suggests that SciN is exposed at the periplasmic side of the OM.

SciN proper localization is required for function. SciN is an OM lipoprotein exposed in the periplasm. To test whether the OM localization of SciN is required for function, we constructed a point mutation targeting the +2 glycine residue, which is responsible for OM localization. E. coli lipoproteins that possess an aspartate residue at position +2 are usually retained in the IM (34, 63, 73). The plasmid producing the Gly2Asp SciN mutant protein, SciN(G2D), was constructed by site-directed mutagenesis using pSciN-HA as a template (see Materials and Methods). Pilot studies showed that SciN(G2D) was solubilized upon SLS treatment (data not shown) and cofractionated with IM fractions in a sedimentation sucrose gradient (Fig. 5A). The SciN(G2D) lipoprotein thus mislocalizes at the IM. We then tested whether this mislocalization alters SciN function. Figure 5B shows that SciD-FLAG was not secreted in sciN cells producing SciN(G2D). Furthermore, sciN cells producing SciN(G2D) formed small amounts of biofilm (Fig. 5C). Overall, the results displayed in Fig. 5 demonstrate that mislocalization of the SciN OM lipoprotein in the IM prevents correct functioning of the EAEC Sci-1 T6SS.

DISCUSSION

Two gene clusters of the pheU pathogenicity island of EAEC strain 17-2, sci-1 and sci-2, encode subunits of two distinct T6SSs (28). Herein, we showed that the Sci-1 T6S machine is responsible for the specific secretion of the SciD protein, an Hcp-like protein. SciD is released from EAEC cells but not from E. coli K-12 DH5α cells. Further experiments demonstrated that at least two genes of the sci-1 gene cluster are required for secretion of SciD: sciG, which encodes a putative AAA+ ATPase of the ClpV family, and sciN. The homologues of these genes have been found to be necessary for the secretion of the Hcp-like EvpC and the VgrG-like EvpI proteins in E. tarda in a systematic deletion approach (75). As expected, the sci-1 gene cluster is not required for the secretion of the Hcp-like AaiC protein encoded within the second T6SS gene cluster, sci-2. Further experiments showed that in the absence of the sci-2 gene cluster, the sci-1 cannot substitute for secretion of AaiC (M. S. Aschtgen and E. Cascales, unpublished observations), suggesting a tightly regulated mechanism of substrate specificity between the two T6S machines encoded within the EAEC pheU pathogenicity island. It is noteworthy that, under our experimental conditions, only a portion of the produced SciD-FLAG was found in the culture supernatant of WT EAEC cells, suggesting either that the expression level of the plasmid-borne sciD is far more than the T6S machine can secrete or that the C-terminal FLAG epitope somehow alters the secretion mechanism.

Our results also showed that the sci-1 gene cluster is involved in biofilm formation. T6SSs have been shown to be involved in numerous processes linked to the virulence of bacterial pathogens, including cytotoxicity, invasion, intracellular growth, survival, and persistence within the host (10). Our results demonstrating a probable role of the Sci-1 T6S machine for adherence on abiotic surfaces, although preliminary, do not constitute a novel phenotype of T6SS mutations. Mutations in the T6S gene cluster have been found in a screen to identify genes of V. para-haemolyticus involved in biofilm formation (29). Furthermore, proteome studies showed that the Pseudomonas aeruginosa Hcp-like proteins PA0085 and PA0263 and the HSI-1 ClpV-type ATPase PA0090 are produced more abundantly during biofilm formation (58, 65). However, whether the SciN lipoprotein only, or both T6SS apparatus, or secreted effectors are necessary for biofilm formation remains to be answered.

One of the genes present on all T6S gene clusters encodes a probable lipoprotein. These conserved proteins share a characteristic N-terminal sequence, called the lipobox (69). This signal sequence is specifically processed by LspA, the SPII, releasing an N-terminal cysteine residue which is then acylated to give the mature lipoprotein. In this study, using the EAEC sci-1 gene cluster as a model, we showed experimentally that sciN encodes an acylated protein processed by SPII. We concluded that SciN is a lipoprotein. Because all SciN homologues share the characteristic features of lipoproteins, one may suggest that these proteins are all lipoproteins. After processing, E. coli lipoproteins are distributed to the IMs or OMs, depending of the nature of the amino acid immediately following the acylated cysteine residue (73). Lipoproteins with an aspartate at position +2 are sequestered in the IM, whereas lipoproteins carrying any other amino acid at this position are conveyed to the OM by the LolA protein and inserted through the action of the OM LolB lipoprotein (69). Using sedimentation sucrose gradients and selective solubilization procedures, we demonstrated that SciN is a lipoprotein anchored to the OM. Further indirect evidence has been provided by the coprecipitation of SciN with a His8-tagged LolA protein carrying the R43L mutation, which prevents release of OM lipoproteins from LolA to LolB. Sucrose density gradient analysis also showed that a portion of SciN colocalized with IM fractions or fractions with intermediate densities. The aberrant localization in intermediate density fractions was also observed for the SciN(G2D) protein and has been previously shown for proteins involved in formation of trans-envelope complexes, such as the Tol-Pal system (47). One may suggest that SciN is retained in these fractions through contacts with T6SS components from both membranes. As an argument for this hypothesis, SciN is found only in OM fractions upon production in the E. coli K-12 DH5α strain (data not shown), suggesting that SciN may interact with an IM T6SS component in EAEC. Using a yeast two-hybrid assay, Zheng and Leung showed that the SciN homologue of the E. tarda Evp T6SS, EvpL, interacts with the IcmF-like IM component ExpO (75). Further experiments demonstrated that mislocalization of the SciN lipoprotein at the IM through modification of its glycine +2 residue to an aspartate led to a defect in SciD secretion and the capacity of the strain to produce WT levels of biofilm.

Because homologues of sciN exist in all T6S gene clusters and because of the critical role of this gene in T6SS function, one may suggest that T6SS-linked lipoproteins play an important role in machine assembly or substrate secretion. Lipoproteins thus mislocalizes at the IM. We then tested whether this mislocalization alters SciN function. Figure 5B shows that SciD-FLAG was not secreted in sciN cells producing SciN(G2D). Furthermore, sciN cells producing SciN(G2D) formed small amounts of biofilm (Fig. 5C). Overall, the results displayed in Fig. 5 demonstrate that mislocalization of the SciN OM lipoprotein in the IM prevents correct functioning of the EAEC Sci-1 T6SS.
teins have been identified in all secretion systems having a large number of subunits, including T2SS, T3SS, and T4SS (1, 20, 24, 30, 61, 64). In all these secretion systems, lipoproteins are essential components and have been shown to be involved in machine assembly or stabilization of the secretion apparatus. In the prototypic Klebsiella oxytoca Pul T2SS, the OM PulS lipoprotein has been shown to be necessary for proper localization of the homomultimeric OM secretin PulD (37, 38). Further experiments suggested that PulS may convey PulD through the periplasm to the OM, and it has therefore been called “pilot” (36). In the S. enterica serovar Typhimurium and Yersinia enterocolitica T3SS, the OM lipoproteins InvH and YscW are required for proper localization and stabilization of the OM secretin InvG and YscC, respectively (9, 19, 20). In the Agrobacterium tumefaciens T4SS, the OM VirB7 lipoprotein forms an intermolecular disulfide bridge with the C-terminal domain of the VirB9 protein (2, 6, 66). Whether VirB9 is an OM component has yet to be determined, but this has been suggested by the observation of numerous β-strand transmembrane segments in its central domain (17, 42) and by immunolocalization studies (7). Whether VirB7 is necessary for VirB9 localization is not known, but it has been shown that VirB7 stabilizes most VirB proteins and may thus participate in the early stages of T4S machine morphogenesis, perhaps as a nucleation factor (13, 31). Other examples of the stabilization or assembly of transport apparatus have been demonstrated for several OM lipoproteins including the PilP lipoprotein of the gonococcal type IV pilus (5, 26), the CsgG lipoprotein of the curli apparatus (48), the FlgH lipoprotein of the OM ring of the flagellar basal body (60), or the Rhizobium leguminosarum PssN and P. aeruginosa PscC lipoproteins in the exopolysaccharide export systems (50, 71). The function of the T6SS-associated lipoproteins in the morphogenesis or stabilization of the apparatus has yet to be determined. One additional common feature of secretion system-associated lipoproteins is the interaction with OM components. Bioinformatics analysis of the proteins encoded within the sci-1 gene cluster does not give any clue about putative OM proteins. However, an OM component has been identified recently in the Franciscella tularensis T6S gene cluster (49). One alternative may be that the SciN lipoprotein is anchored to the OM through a lipid moiety and is also an acylated OM integral protein, as is the Wza lipoprotein required for group 1 capsular polysaccharide translocation (25, 27). Our results from proteinase K accessibility cannot discriminate between these possibilities since we detected the SciN lipoprotein through a C-terminal epitope tag, which may be degraded by the protease, leaving the integral part of the protein integrated in the OM. Studies aiming at defining whether SciN is a periplasmic OM anchored lipoprotein and whether it associates with any OM partner are currently under way in our laboratory. Future studies of the T6S protein characteristics should provide important information to understand how T6SSs assemble and function.

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