

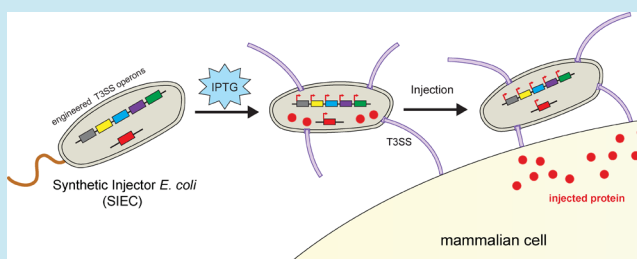
# Engineering the Controlled Assembly of Filamentous Injectisomes in *E. coli* K-12 for Protein Translocation into Mammalian Cells

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## Supporting Information

**ABSTRACT:** Bacterial pathogens containing type III protein secretion systems (T3SS) assemble large needle-like protein complexes in the bacterial envelope, called injectisomes, for translocation of protein effectors into host cells. The application of these “molecular syringes” for the injection of proteins into mammalian cells is hindered by their structural and genomic complexity, requiring multiple polypeptides encoded along with effectors in various transcriptional units (TUs) with intricate regulation. In this work, we have rationally designed the controlled expression of the filamentous injectisomes found in enteropathogenic *Escherichia coli* (EPEC) in the nonpathogenic strain *E. coli* K-12. All structural components of EPEC injectisomes, encoded in a genomic island called the locus of enterocyte effacement (LEE), were engineered in five TUs (eLEEs) excluding effectors, promoters and transcriptional regulators. These eLEEs were placed under the control of the IPTG-inducible promoter Ptac and integrated into specific chromosomal sites of *E. coli* K-12 using a markerless strategy. The resulting strain, named synthetic injector *E. coli* (SIEC), assembles filamentous injectisomes similar to those in EPEC. SIEC injectisomes form pores in the host plasma membrane and are able to translocate T3-substrate proteins (e.g., translocated intimin receptor, Tir) into the cytoplasm of HeLa cells reproducing the phenotypes of intimate attachment and polymerization of actin-pedestals elicited by EPEC bacteria. Hence, SIEC strain allows the controlled expression of functional filamentous injectisomes for efficient translocation of proteins with T3S-signals into mammalian cells.



**KEYWORDS:** *E. coli*, EPEC, genomic engineering, injectisome, type III secretion system

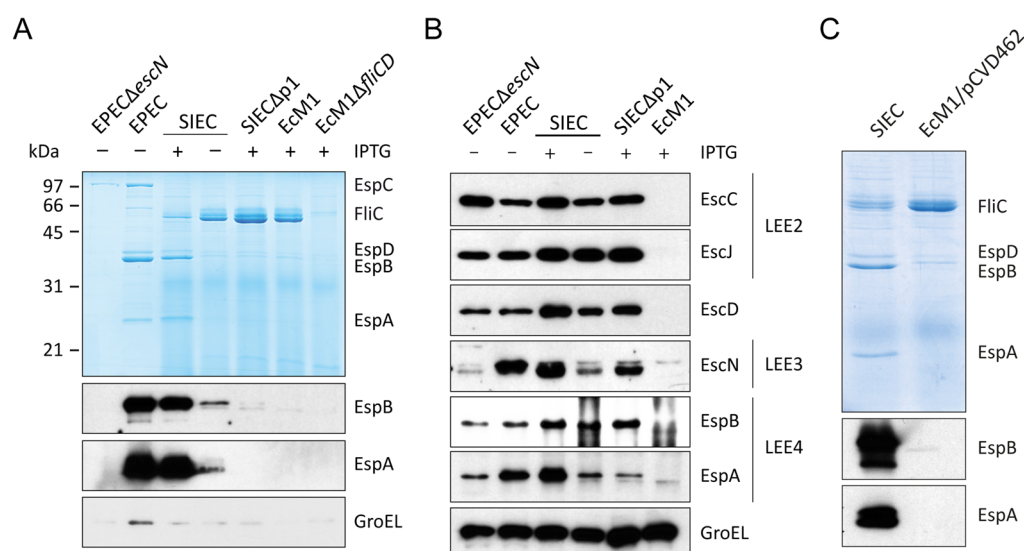
Type III protein secretion systems (T3SS) are “molecular syringes” found in many Gram-negative bacterial pathogens specialized in the translocation of specific cytotoxic proteins, called collectively as effectors, into the cytoplasm of host cells during infection.<sup>1,2</sup> T3SSs assemble large multiring protein complexes, referred to as injectisomes, comprising a core cylindrical basal body that spans the bacterial inner membrane (IM), the peptidoglycan (PG) and the outer membrane (OM), and a hollow needle-like structure on the extracellular surface of the bacterium.<sup>3</sup> In some T3SS the needle is extended by a long filament that allows plant pathogens and some animal pathogens (e.g., enteropathogenic *Escherichia coli*, EPEC) to access the plasma membrane of host cells “at-a-distance”, translocating effectors across the plant cell wall or the mucus layer covering the apical surface of intestinal epithelial cells.<sup>4,5</sup> In the cytosolic entrance of the injectisomes, a number of cytoplasmic and IM proteins, including a dedicated ATPase, recognize effectors bound to specialized T3SS chaperones and energize dissociation of the chaperones and secretion of effectors through the needle complex.<sup>6–8</sup> Some injectisome proteins associated with the IM have structural and sequence similarity with components of the flagellar basal body, indicating a common origin of both protein-export machineries.<sup>2,9</sup>

Injectisomes have shown a great potential as nanomachines for the delivery into eukaryotic cells of vaccine polypeptides and proteins of therapeutic potential (e.g., transcription factors, enzymes, antibody fragments) fused to short N-terminal noncleavable signal sequences of effectors.<sup>10–15</sup> However, in these studies the use of attenuated strains of T3SS-positive pathogens was required for protein delivery. Engineered expression of injectisomes in nonpathogenic bacteria has been hindered by the intricate organization of different genes and operons needed for injectisome assembly<sup>16</sup> and their complex transcriptional regulation, which responds to various environmental signals (e.g., temperature, pH, CO<sub>2</sub>, presence of divalent ions, quorum sensing molecules).<sup>2</sup> Moreover, the attenuated strains of pathogens frequently express multiple effectors that are translocated into the host cell along with the protein of interest. Hence, engineering nonpathogenic bacteria with a controlled assembly of functional injectisomes would be instrumental for the development of designed bacteria able to deliver specific protein(s) into human cells for therapeutic applications.

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**Figure 2.** SIEC bacteria express filamentous injectisomes upon induction. (A) Coomassie staining and Western blot of proteins in culture media of the indicated strains (EPEC, EPEC $\Delta$ escN, SIEC, SIEC $\Delta$ p1, EcM1 and EcM1 $\Delta$ fliCD) induced (+) or not (-) with IPTG. Anti-EspA, -EspB and -GroEL antibodies were used in Western blot. Detection of cytoplasmic GroEL was used to control for the absence of bacterial lysis. The protein bands of T3SS translocators EspA, EspB, EspD, of autotransporter EspC, and flagellin (FliC) are labeled on the right. Molecular weight markers are indicated on the left (in kDa). (B) Western blot of the bacterial whole-cell protein extracts from the indicated bacterial strains, induced (+) or not (-) with IPTG, detecting proteins of the T3SS (EscC, EscJ, EscD, EscN, EspB, EspA) and GroEL as a loading control. Identity of each protein band is indicated on the right along with the encoding operon. (C) The proteins found in culture media of induced SIEC and EcM1/pCVD462 bacteria (carrying cosmid with wt LEE) were analyzed by Coomassie staining and Western blot with anti-EspA and anti-EspB antibodies. The protein bands of T3SS translocators EspA, EspB, EspD, and flagellin (FliC) are labeled on the right.

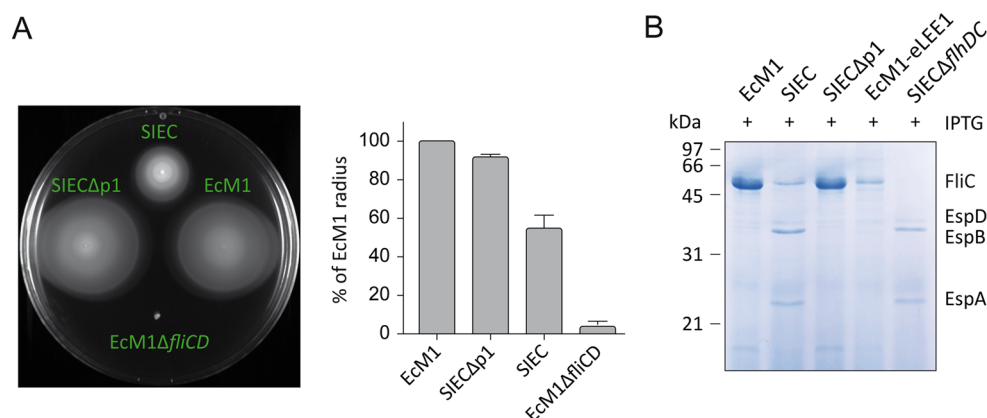
proteins EspA, EspB and EspD, which are secreted through the needle of a functional injectisome basal body. EspA polymerizes a long extracellular hollow filament that extends the needle and allows insertion of pore-forming proteins EspB and EspD in the host plasma membrane.<sup>5,30–32</sup>

A model of EPEC injectisomes is shown in Figure 1B, indicating the proposed location of the structural components and identifying the TUs/operon encoding each subunit. The LEE also encodes T3SS chaperones (CesT, CesF, CesAB, CesA2, CesD, CesD2, EscE, EscG) that bind effectors, translocators and the needle protein in the cytoplasm prior to their export through the injectisome. Finally, the LEE harbors the genes encoding six effectors (Tir, EspF, Map, EspH, EspG, and EspZ),<sup>17</sup> intimin (*eae*),<sup>24</sup> a transglycosylase (*etgA*),<sup>33</sup> two ORFs of unknown function (*orf4*, *rorf1*) and three transcriptional regulators (Ler, GlrA, and GlrR).<sup>34,35</sup> Transcription of LEE operons and genes is highly regulated by external cues and the physiological state of the bacterium.<sup>36,37</sup> The first gene encoded in LEE1, *ler*, is the master transcriptional regulator that activates the transcription of other LEE operons, counteracting the inhibitory effect of the global regulator H-NS.<sup>34,38</sup> High levels of Ler function as a repressor of LEE1, creating a negative feedback loop.<sup>39</sup> Other transcriptional regulators found in the LEE (*e.g.*, GlrA/GlrR) and outside the LEE (*e.g.*, PerC) also control the expression of Ler.<sup>35,40</sup> These regulatory genes also act outside the LEE. Ler activates the autotransporter protease EspC<sup>41</sup> whereas GlrA inhibits transcription of the flagellar master operon *flhCD* under conditions of T3SS expression.<sup>42</sup> Under *in vitro* culture conditions, expression of EPEC T3SS is induced when bacteria are grown under conditions mimicking host environment (*e.g.*, 37 °C and 5% CO<sub>2</sub> in DMEM). Lack of proper regulation may explain the weak expression of the T3SS of EPEC in *E. coli* K-12 harboring a cosmid with the entire LEE.<sup>26</sup>

In this work, we have engineered the controlled expression of the filamentous injectisomes of EPEC in the nonpathogenic *E. coli* K-12 strain MG1655 by constructing engineered LEE operons encoding solely the components needed for the assembly of functional injectisome and placed them under the control of the heterologous inducible promoter Ptac. These constructs were integrated into the chromosome of *E. coli* K-12 by a marker-less strategy, generating an engineered strain named synthetic injector *E. coli* (SIEC), which upon induction expresses injectisomes capable of efficient protein translocation into mammalian cells.

## RESULTS AND DISCUSSION

**Generation of the Synthetic Injector *E. coli* (SIEC) Strain.** Our aim was to integrate the genes needed for the assembly of EPEC injectisomes in the chromosome of *E. coli* K-12. We selected the genes encoding structural proteins (*orf4*, *escL*, *escR*, *escS*, *escT*, *escU*, *escC*, *escJ*, *escI*, *escV*, *escN*, *escO*, *escP*, *escQ*, *escC* and *escF*), the switch complex (*sepD*, *sepL*, *cesL*), chaperones of injectisome components (*escE*, *cesAB*, *cesD*, *cesD2*, *escG*), and the translocators (*espA*, *espB*, and *espD*) (Figure 1A), and ruled out genes encoding effectors (*tir*, *map*, *espF*, *espG*, *espZ*, *espH*) and chaperones of effectors (*cesT*, *cesF*), intimin (*eae*), *etgA* muramidase (*etgA*), and transcriptional regulators (*glrA*, *glrR*, *ler*). The total genes selected summed up 27. We designed 5 TUs, referred to as operons eLEE1, eLEE2, eLEE3, eLEE4 and eEscD (Figure 1A). The corresponding DNA segments were amplified from the chromosome of EPEC strain 2348/69,<sup>19</sup> from the start codon of the first selected gene to the stop codon of the last one. The strategy implied the exclusion of native promoters, terminators, and the first ribosome-binding site (RBS) found in the native LEE operons. The parental strain for integration of eLEEs was *E. coli* K-12 MG1655 lacking type 1 fimbriae operon  $\Delta$ *fimA-H*, named



**Figure 3.** Interference between the assembly of injectisomes and flagella. (A) Swimming motility assay of SIEC, SIEC $\Delta$ p1, EcM1 and EcM1 $\Delta$ fliCD bacteria on soft LB-agar plates with IPTG (left) and graph representing the mean radius (with SEM) of the colonies of each strain (relative to the values of EcM1 strain) in three independent experiments (right). (B) Coomassie staining of proteins in culture media of the indicated *E. coli* strains (EcM1, SIEC, SIEC $\Delta$ p1, EcM1-eLEE1, and SIEC $\Delta$ flhDC) grown in LB with IPTG (+). The protein bands and molecular weight markers (in kDa) are labeled as in Figure 2

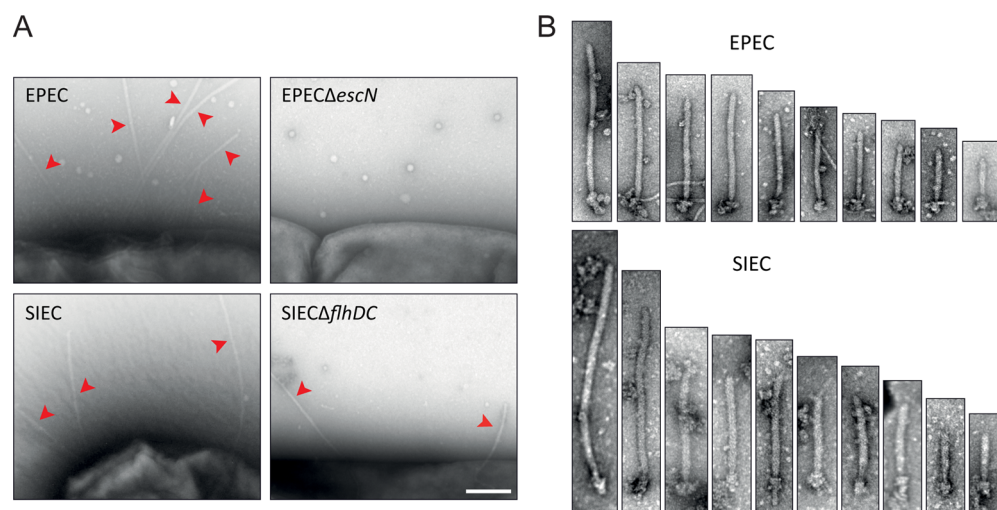
EcM1.<sup>43</sup> For selection of the integration sites for eLEE operons, we tested eight *loci* encoding adhesins of *E. coli* K-12 (*yeeJ*, *yebT*, *yra*, *yfaL*, *yfc*, *sfm*, *flu*, *mat*; Supporting Information Table S1) because these are nonessential genes/operons whose deletion would reduce the nonspecific adhesion of our engineered bacteria.<sup>44–46</sup> Gene expression in these sites was analyzed integrating a reporter Ptac-*gfp* fusion (Supporting Information Figure S1). Since LEE2 and LEE4 appear to be expressed at higher levels than other LEE operons,<sup>38,47</sup> we matched the integration sites producing higher expression GFP with eLEE2 (*yeeJ*) and eLEE4 (*yebT*), using other sites for integration eLEE3 (*yraA*), eEscD (*yfc*) and eLEE1 (*yfaL*) (Figure 1C). Transcriptional terminators of Ptac-eLEE constructs are provided by the 3'-regions of the selected chromosomal sites. The Ptac promoter and a strong RBS from T7 phage was introduced upstream of the eLEE coding region. Integration of Ptac-eLEE fusions was carried out with suicide plasmids bearing homology regions (HRs) flanking the targeted gene. Co-integrants obtained after transformation were resolved by homologous recombination after expression of I-SceI *in vivo*, a strategy that allowed a marker-less integration of the constructs leaving no scars nor antibiotic resistance genes in the chromosome (Supporting Information Figure S2).<sup>48,49</sup> Fusions of Ptac with eLEE2, eLEE3, eEscD, and eLEE4 were sequentially integrated in the corresponding sites of EcM1. The Ptac-eLEE1 fusion was integrated in the strain carrying the other eLEEs generating the SIEC strain. We also attempted initially to obtain a SIEC strain expressing Ler, but fusions between Ptac and a longer version of eLEE1 containing *ler* (eLEE1\*) accumulated mutations in *ler* (data not shown). Ler-containing constructs were only obtained in the absence of the Ptac promoter, suggesting that Ler expression is toxic for *E. coli* K-12. A promoter-less eLEE1\* was integrated in *yfaL* in the strain carrying the other eLEEs (except eLEE1), generating SIEC $\Delta$ p1, which was later used as a control strain (see below).

**Expression of T3SS Injectisomes in SIEC Strain.** To demonstrate the ability of SIEC to express functional injectisomes we analyzed by SDS-PAGE the secreted proteins of bacteria grown for 6 h in LB at 37 °C, with and without the inducer IPTG, and compared it with those found in EPEC grown at 37 °C in DMEM with 5% CO<sub>2</sub> (Figure 2). Coomassie staining revealed the presence of protein bands corresponding

to T3SS translocators EspA, EspB, EspD in the culture media of wt EPEC and IPTG-induced SIEC, but not in negative control strains SIEC $\Delta$ p1, EPEC $\Delta$ escN, and the parental EcM1 (Figure 2A). As expected, a protein band corresponding to the protease domain of EspC autotransporter (not secreted through the T3SS) was only found in EPEC strains (Figure 2A). This result strongly suggested the assembly of injectisomes in SIEC at levels close to those found in EPEC. Importantly, the growth curves and viability of SIEC and the parental strain EcM1 grown in LB with IPTG were identical (Supporting Information Figure S3), indicating that expression of the T3SS injectisomes in SIEC was not toxic. Western blots with anti-EspA and anti-EspB antibodies confirmed the presence of secreted EspA and EspB in cultures of wt EPEC and IPTG-induced SIEC, but not in SIEC $\Delta$ p1 (Figure 2A). Small amounts of secreted EspA and EspB were detected in SIEC culture without IPTG, which indicate some leakiness of the Ptac promoter. Detection of minor amounts of cytosolic GroEL, which is conserved in all *E. coli* strains, was used to control the absence of bacterial lysis in the induced cultures.

The level of injectisome proteins in bacteria harvested from the above cultures was analyzed by Western blot with antibodies against EspA, EspB, EscC, EscJ, EscD, and EscN (Figure 2B). Detection of cytosolic GroEL was used in this case as a loading control. This experiment revealed that the levels of EscC and EscJ (eLEE2), EscD (eEscD) and EspA and EspB (eLEE4), were slightly higher in the induced SIEC than in EPEC. In the case of the ATPase EscN (eLEE3) we found identical expression levels in the induced SIEC and EPEC strains, although a small shift in their electrophoretic mobility suggested that EscN could have a differential post-translational processing in these strains. Although higher levels of EscC, EscJ and EscD were found in the induced SIEC, significant expression of these proteins was detected in the absence of IPTG, indicating leakiness of Ptac-eLEE2 and Ptac-eEscD fusions. In contrast, the levels of EscN and EspA proteins were low in the uninduced SIEC bacteria and were strongly induced by IPTG.

Lastly, we compared the expression of injectisomes in SIEC and the parental EcM1 strain harboring the natural LEE cloned in the cosmid pCVD462.<sup>26</sup> Cultures were grown at 37 °C in LB with IPTG (adding chloramphenicol for selection in the culture



**Figure 4.** Visualization of the injectisomes by electron microscopy. (A) TEM micrographs of negatively stained bacteria of the indicated strains (EPEC, EPEC $\Delta$ escN, SIEC and SIEC $\Delta$ flhDC), grown under conditions for injectisome expression. EspA-like filaments are labeled with red arrowheads. The scale bar is 200 nm. (B) TEM micrographs showing representative injectisome-like particles visualized in negatively stained protein samples after injectisome purification from induced EPEC and SIEC bacteria, as indicated. Injectisome particles are aligned at their basal bodies to show the different lengths of their filaments. Scale bars represent 50 nm.

of EcM1/pCVD462) and proteins in culture media were analyzed by Coomassie staining of SDS-PAGE and Western blot with antibodies against EspA and EspB. This experiment clearly showed high levels of EspA, EspB and EspD proteins secreted by SIEC but not by EcM1/pCVD462 strain, in which only a faint band of secreted EspB was detectable by Western blot (Figure 2C). This result demonstrates that the engineering of SIEC produces higher injectisome levels than those elicited by the cloning of natural LEE in *E. coli* K-12, having the additional benefits of a controlled inducible expression, lack of effectors and absence of antibiotic resistance genes.

#### Injectisome Assembly Interferes with Flagella in SIEC.

Coomassie staining of proteins found in culture media also revealed that the protein band corresponding to flagellin (FliC) of *E. coli* K-12 (absent in EcM1 $\Delta$ fliCD; Figure 2A) was strongly reduced upon induction of SIEC, but not in the induced SIEC $\Delta$ p1 or EcM1/pCVD462 strains (Figure 2A and 2C). This result suggested that expression of T3SS injectisomes was interfering with the correct expression of flagella. In this regard, we found that SIEC has a significant reduction on its motility (ca. 55% of the parental EcM1 strain) on LB soft-agar plates supplemented with IPTG (Figure 3A). This reduction in flagellar motility was not observed in SIEC $\Delta$ p1. As expected, the EcM1 $\Delta$ fliCD mutant strain lacking flagellin was not motile in this assay. Albeit in EPEC the GlrA regulator inhibits transcription of the flagellar master operon *flhDC*,<sup>42</sup> SIEC bacteria do not express GlrA arguing for a different mechanism of interference. Given the structural similarities between components of the basal bodies of T3SS injectisomes and flagella,<sup>50</sup> we speculated that this interference could be in the assembly or the activity of these nanomachines.

We wanted to further investigate this phenomenon to elucidate whether this interference could be bidirectional, that is, whether expression of flagellar basal body components could also be affecting the assembly of the injectisomes in SIEC. Since FlhDC are the master regulators required for the expression of all flagellar operons,<sup>51</sup> we deleted *flhDC* in the genome of SIEC generating SIEC $\Delta$ flhDC strain. In the absence of FlhDC the flagellar basal body is not expressed. Analysis of secreted

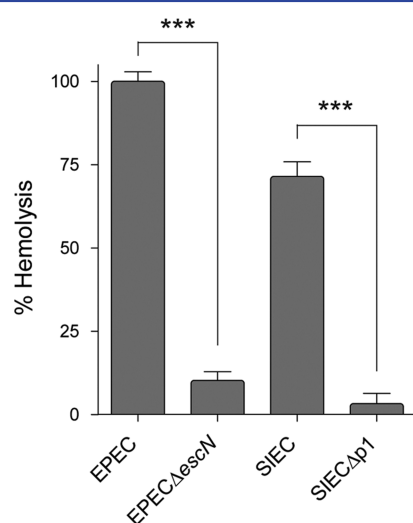
proteins in culture media did not show a higher level of EspA, EspB and EspD translocators in SIEC $\Delta$ flhDC than in SIEC strain (Figure 3B). As expected, SIEC $\Delta$ flhDC did not produce flagellin (FliC). This result indicates that expression of flagellar body does not interfere with assembly of T3SS injectisomes. In contrast, induction of Ptae-eLEE1 alone, in a strain having this operon (EcM1-eLEE1), was sufficient to reduce the levels of secreted flagellin (Figure 3B) and the motility of EcM1 strain (Supporting Information Figure S4) like the induced SIEC, whereas induction of all other eLEEs (SIEC $\Delta$ p1) had no effect on flagellin levels and bacterial motility. Hence, protein(s) expressed from eLEE1 specifically interfered with the activity/assembly of the flagellar apparatus. The eLEE1 encodes EscE, CesAB, Orf4, EscL, EscR, EscS, EscT, and EscU proteins, some of them sharing high homology to flagellar components. For instance, EscRST are homologues of FliPQR integral IM proteins essential for the flagellar function and are believed to assemble within the IM ring of the basal body. EscL is the regulator of the EscN ATPase and is homologue to FliH, which also regulates the flagellar ATPase. These subunits form a conserved IM export apparatus in these two nanomachines.<sup>50</sup>

#### Electron Microscopy of the Injectisomes Assembled in SIEC.

We wanted to visualize the injectisomes assembled by SIEC and compared them with those of EPEC. EspA filaments up to 700 nm long can be observed on the surface of EPEC bacteria expressing functional T3SS.<sup>31</sup> Thus, EPEC, EPEC $\Delta$ escN, SIEC and SIEC $\Delta$ flhDC bacteria were grown under T3SS-inducing conditions, negatively stained and visualized by transmission electron microscopy (TEM). Filamentous organelles structurally compatible with EspA filaments were observed in EPEC, SIEC, and SIEC $\Delta$ flhDC bacteria (Figure 4A), but not in EPEC $\Delta$ escN (negative control). The presence of EspA-like filaments in SIEC $\Delta$ flhDC ruled out that they could be flagellar filaments. Next, we attempted to isolate injectisomes from SIEC bacteria following a purification procedure reported for EPEC.<sup>52</sup> SIEC and EPEC bacteria grown under T3SS-inducing conditions were lysed with zwitterionic detergent LDAO and the lysate subjected to ultracentrifugation in a CsCl density gradient. Fractions containing injectisomes were identified by

Western blot with anti-EspA antibodies (data not shown), negatively stained and inspected by TEM (Figure 4B). Both EPEC and SIEC samples presented similar sheath-like structures with a basal body at one end, compatible with the injectisomes reported in EPEC.<sup>5</sup> Figure 4B shows 10 aligned injectisome particles of each preparation to compare their overall structure. The needle and basal body structures were connected with filaments of different lengths, having sizes ranging between 50 and 400 nm in both SIEC and EPEC samples. Hence, these results demonstrate that SIEC is capable of assembling filamentous injectisomes that share an overall structure highly similar to those assembled by EPEC.

**Injection of Proteins into Mammalian Cells Using SIEC.** Next, we aimed to determine whether SIEC was capable of forming protein translocation pores on the plasma cell membranes of mammalian cells and inject proteins to the cytoplasm. As a first approach, we measured the induction of lysis caused in red blood cells by the EspB/D translocation pore upon contact with their membrane. This assay has been previously used to determine the formation of functional injectisomes by measuring the liberation of hemoglobin derived from the lysis of erythrocytes.<sup>33</sup> We induced the expression of the T3SS in liquid cultures of EPEC, EPEC $\Delta$ escN, SIEC and SIEC $\Delta$ p1 strains, and incubated harvested bacteria with a solution of erythrocytes in DMEM at 37 °C with 5% CO<sub>2</sub>. The hemoglobin release from erythrocytes was determined at OD 490 nm, and the activity determined for each strain was represented relative to that of wt EPEC. As it can be observed (Figure 5), bacterial strains lacking a functional T3SS displayed weak hemolytic activity, albeit the background activity (non-T3SS dependent) of EPEC $\Delta$ escN was higher than that of SIEC $\Delta$ p1. In contrast, SIEC retained ca. 70% of the hemolysis



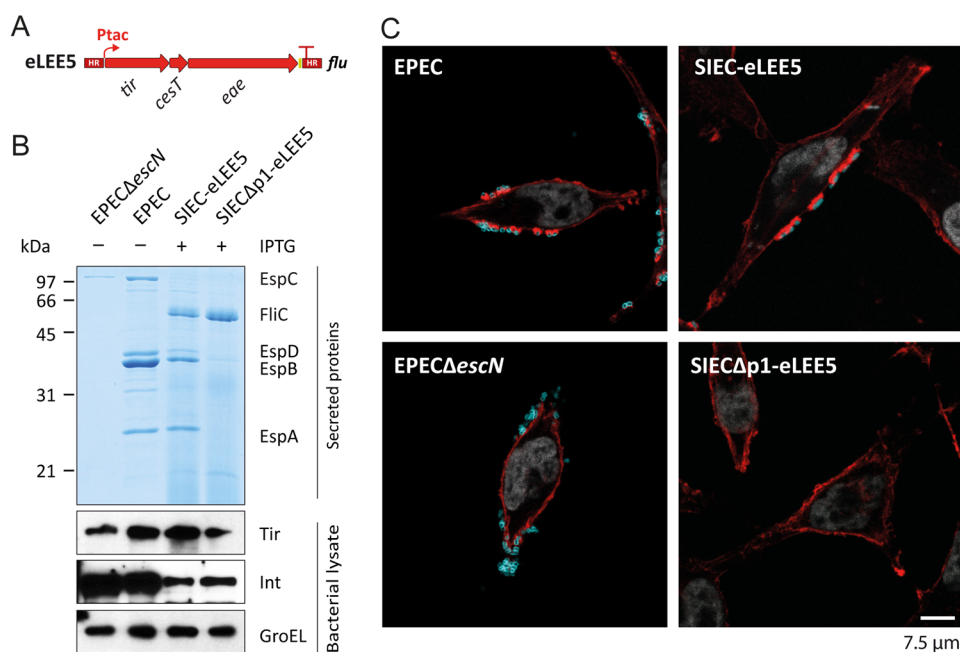
**Figure 5.** Hemolytic activity of bacteria expressing injectisomes. The ability of injectisome-expressing bacteria to form pores in the plasma membrane of mammalian cells was evaluated by the release of hemoglobin from red blood cells measured at OD 450 nm after incubation with the indicated strains (EPEC, EPEC $\Delta$ escN, SIEC, SIEC $\Delta$ p1). The mean data and the standard error (SEM) of three independent experiments are represented. The hemolytic activity obtained by wt EPEC was considered 100%. In all cases, background hemolytic activity obtained with EcM1 bacteria was subtracted from the represented values. The statistical analysis was performed using Student's *t* test comparing the indicated groups (\*\*\*) indicates *p*-values <0.001).

of EPEC, a value that could be underestimated given the lower background activity of SIEC $\Delta$ p1. Therefore, these data indicate that EspB/D translocators of SIEC can assemble pores on the plasma membrane of the mammalian cells.

We investigated the ability of SIEC injectisomes to recognize a specific protein substrate and translocate it in a biologically active form into human cells (*e.g.*, HeLa). We selected Tir as a model protein to test the translocation capacity of SIEC injectisomes since, when injected through the T3SS, it triggers bacterial intimate attachment to the mammalian cell and formation of actin-rich pedestals, a phenotype that can be easily visualized by fluorescence microscopy.<sup>24</sup> In EPEC this phenotype requires expression of intimin on the bacterial surface, which interacts with a domain of Tir exposed on the host plasma membrane. Intimin-Tir interaction induces polymerization of F-actin underneath the bound bacterium mediated by the clustering of phosphorylated cytoplasmic domains of Tir that recruit Nck and other cellular proteins.<sup>25</sup> A simplified model of this process is shown in Supporting Information Figure S5. In EPEC, the operon LEE5 encodes Tir, its chaperone CesT, and intimin (Figure 1A). Following the strategy used for eLEEs, we generated an eLEE5 operon under the control of Ptac (Figure 6A) that was integrated in the chromosome of SIEC, replacing the adhesin *flu* gene (Antigen 43),<sup>53</sup> obtaining the strain SIEC-eLEE5. The Ptac-eLEE5 was also integrated in the same site of the chromosome of SIEC $\Delta$ p1, obtaining strain SIEC $\Delta$ p1-eLEE5, which is defective in the assembly of injectisomes.

The expression of intimin and Tir in IPTG-induced SIEC-eLEE5 and SIEC $\Delta$ p1-eLEE5 bacteria was evaluated by Western blot of whole-cell protein extracts and compared with their expression in EPEC and EPEC $\Delta$ escN strains (Figure 6B). Tir was detected in all of the strains, though EPEC and SIEC-eLEE5 appeared to express slightly higher levels than T3SS-defective strains. Intimin was also well expressed in all of the strains, albeit its expression levels were reduced ~3-fold in SIEC strains compared to EPEC. Analysis of proteins found in culture media (Figure 6B) showed that translocators EspA, EspB and EspD were secreted by SIEC-eLEE5 normally, indicating that induction of the eLEE5 proteins does not affect the assembly of SIEC injectisomes.

Subsequently, we analyzed whether SIEC-eLEE5 was able to translocate Tir into HeLa cells. To visualize SIEC bacteria in fluorescence microscopy, we transformed SIEC-eLEE5 and SIEC $\Delta$ p1-eLEE5 with the stable plasmid pGEN22, which expresses GFP constitutively.<sup>54</sup> GFP-labeled SIEC-eLEE5 and SIEC $\Delta$ p1-eLEE5 bacteria were grown in LB with IPTG to mid log phase and then incubated with HeLa cells for 3 h with IPTG (MOI = 100). Infections of HeLa cells with EPEC and EPEC $\Delta$ escN bacteria were conducted in parallel as positive and negative controls, respectively, albeit for 90 min to reduce the cytotoxic effects of EPEC. After infection, samples were washed, fixed, and stained for fluorescence microscopy to visualize bacteria, F-actin, and DNA. This experiment showed SIEC-eLEE5 bacteria adhered to the surface of HeLa cells with intense F-actin accumulation beneath the bound bacteria, highly similar to the actin-pedestals triggered by EPEC (Figure 6C). On the contrary, SIEC $\Delta$ p1-eLEE5 bacteria were unable to attach to HeLa cells, being washed out of the preparation and not inducing any alteration in the actin cytoskeleton. SIEC $\Delta$ p1-eLEE5 has less adhesive properties than EPEC $\Delta$ escN bacteria, albeit both strains do not trigger actin-pedestal formation. EPEC bacteria express different adhesins independent of the



**Figure 6.** Translocation of Tir protein into HeLa cells by the injectisomes of SIEC. (A) Scheme of the engineered operon (eLEES) encoding Tir, its chaperone CesT, and intimin (*eae*), under Ptac regulation and integrated at the *flu* locus of SIEC-eLEES and SIEC $\Delta$ p1-eLEES strains. (B) Proteins in the culture media of the indicated strains (EPEC, EPEC $\Delta$ escN, SIEC-eLEES, SIEC $\Delta$ p1-eLEES) grown under conditions for injectisome expression and analyzed by SDS-PAGE and Coomassie staining (top panel). The expression of intimin (Int) and Tir was detected in the bacterial whole-cell extract by Western blot with specific antibodies (bottom panels). GroEL was used as a loading control. Protein bands and molecular weight markers indicated as in Figure 2. (C) Confocal fluorescence microscopy images of HeLa cells infected with the indicated bacterial strains (EPEC, EPEC $\Delta$ escN, SIEC-eLEES, SIEC $\Delta$ p1-eLEES) and stained with TRITC-conjugated phalloidin (red) to visualize F-actin and DAPI (gray) to visualize DNA and nuclei. EPEC bacteria are labeled with anti-intimin280 and ALEXA488-conjugated antirabbit antibodies (cyan). Fluorescence signals of SIEC bacteria expressing GFP are also colored in cyan. Strong red fluorescence signals are clearly visible beneath adhered EPEC and SIEC-eLEES bacteria indicating F-actin pedestal formation triggered by translocated Tir protein.

filamentous T3SS (e.g., the bundle forming pili, BFP, and *E. coli* common pili, ECP)<sup>55</sup> that are not expressed by SIEC. Hence, this experiment demonstrates that SIEC-eLEES, similar to EPEC, is able to inject functional Tir into HeLa cells, which inserts in the plasma membrane and interacts with intimin on the bacterial surface to mediate intimate attachment and the formation of actin pedestals beneath the adhered extracellular bacterium.

**Concluding Remarks.** The controlled expression of complex protein nanomachines in microorganisms designed for biomedical or environmental applications represents an important challenge for synthetic biology. T3SS are attractive protein delivery systems for the injection of proteins into mammalian cells whose potential have been assessed using different attenuated strains of pathogens, including EPEC, *Pseudomonas aeruginosa*, and *Salmonella* strains.<sup>10–15</sup> Despite their interest, it is difficult to transfer these protein delivery devices to nonpathogenic bacteria due to their structural and regulatory complexity. In this work we have engineered the controlled assembly of functional injectisomes from the filamentous T3SS of EPEC in the nonpathogenic strain *E. coli* K-12 demonstrating the injection of proteins recognized by this system into mammalian cells in a biologically active form. We followed a synthetic biology approach to tackle the expression of T3SS injectisomes of EPEC in a bacterium of choice. We generated five engineered TUs (eLEE1, eLEE2, eLEE3, eLEE4, eEscD) that encode all the structural subunits, chaperones, energizing ATPase, and translocators known to be required for assembly of these injectisomes, but not the transcriptional regulators, native promoters and effectors also

encoded within the LEE. Control expression of these eLEES was achieved with heterologous inducible Ptac promoters, including signals for translation initiation and transcriptional termination. These genetic fusions were integrated by homologous recombination at distinct sites of *E. coli* K-12 chromosome encoding nonessential adhesins.<sup>44–46</sup> By modifying the flanking HRs of the suicide vectors, the designed eLEES could be inserted at different *loci* and other bacteria. Likewise, the modular architecture of eLEES allows the exchange the Ptac for alternative promoters appropriated for specific applications (e.g., P<sub>BAD</sub> and P<sub>tet</sub> promoters for *in vivo* induction).<sup>36</sup>

Integration of the eLEES under Ptac control generated the SIEC strain, which upon IPTG-induction was shown to assemble filamentous injectisomes similar to those found in EPEC bacteria.<sup>5</sup> The long EspA-filaments of these injectisomes are known to have adhesin properties,<sup>57</sup> thus assisting the binding of extracellular bacteria to mammalian cells and allowing translocation of proteins “at-a-distance” from the plasma membrane of the host, even when shielded by extracellular mucins.<sup>5,30,58</sup> In addition, the adhesion of SIEC to specific target cells could be implemented based on the expression on the bacterial surface of synthetic adhesins that can bind to antigens expressed on the surface of mammalian cells.<sup>49</sup> Importantly, we found that expression of EPEC injectisomes does not affect the growth of SIEC bacteria. Nonetheless, expression of eLEE1 (alone or in combination with other eLEES) reduces the assembly of flagella and the motility of SIEC bacteria, likely due to the similarities between components of eLEE1 and the flagellar basal body.<sup>50</sup> Hence, in those instances in which bacterial motility may be required for

efficient colonization of tissues,<sup>59</sup> induction of the injectisomes should be triggered at a later stage.

Lastly, we demonstrated the ability of SIEC to form pores on the membrane of erythrocytes and to inject a protein recognized by this T3SS into HeLa cells.<sup>60</sup> We selected the natural effector Tir as a model to test functionality of SIEC injectisome because of the evident phenotypic effect caused by its translocation, the formation of actin-rich pedestals beneath attached bacteria.<sup>25</sup> Tir could be easily replaced by other protein(s) of interest, either natural T3SS effectors inducing a specific biological activity in the cell (e.g., apoptosis, anti-inflammation responses),<sup>21,22</sup> or heterologous proteins such as single-domain antibody fragments,<sup>13,61</sup> enzymes (e.g.,  $\beta$ -lactamase),<sup>62</sup> transcription activator-like effector nucleases (TALENs),<sup>10</sup> or eukaryotic transcription factors (e.g., MyoD),<sup>63</sup> all of which have been translocated with T3SS of attenuated pathogens. In order to be recognized by SIEC injectisomes, these heterologous polypeptides need to be fused to signals located in the N-terminus of natural effectors (e.g., first 20 amino acids of EspF)<sup>62</sup> and translocators.<sup>64</sup> The use of longer N-terminal signals (ca. 100 amino acids) including the binding domains of effector chaperones (e.g., CesF, CesT) may increase translocation efficiency<sup>65–67</sup> but they could incorporate some effector functions and subcellular localization signals (e.g., mitochondrial targeting signals).<sup>68</sup> These longer T3 signals may interfere with the activity of the heterologous polypeptide within the cell and should be tested on a one-to-one basis. In summary, the SIEC strain developed in this work can be considered a nonpathogenic bacterial chassis that can be further modified to direct the injection of diverse proteins into mammalian cells using the filamentous injectisomes of EPEC.

## METHODS

**Bacterial Strains and Growth Conditions.** *E. coli* strains used in this work are listed in Supporting Information Table S2. Bacteria were grown at 37 °C in Lysogeny broth (LB) agar plates (1.5% w/v),<sup>69</sup> in liquid LB medium or in Dulbecco's Modified Eagle Medium (DMEM), unless otherwise indicated. When needed, antibiotics were added at the following concentrations for plasmid or strain selection: chloramphenicol (Cm) at 30  $\mu$ g/mL; kanamycin (Km) at 50  $\mu$ g/mL; tetracycline (Tc) at 10  $\mu$ g/mL; spectinomycin (Sp) at 50  $\mu$ g/mL; ampicillin (Ap) at 150  $\mu$ g/mL, except for strains expressing the Ap resistance gene from their chromosome, which were selected at 75  $\mu$ g/mL. EPEC strains were grown overnight (o/n) with agitation (200 rpm) at 37 °C in a flask with 10 mL of liquid LB. Next day, cultures were inoculated in capped Falcon tubes (BD Biosciences) with 5 mL DMEM and incubated in static conditions for 2.5 h for the expression of the T3SS. These cultures were used at this point to infect cell cultures. Alternatively, for the analysis of the T3-secreted proteins, the cultures were grown for additional 3.5 h in DMEM. For the analysis of T3-secreted proteins by SIEC strains, bacteria were grown in capped Falcon tubes with 5 mL of liquid LB and 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) with agitation (160 rpm) for 6 h. For the infection of cell cultures with SIEC strains, bacteria were grown as above for 2.5 h and then added to mammalian cell cultures in DMEM with 0.1 mM IPTG for 3 h.

**Plasmids, DNA Constructs, and Primers.** Plasmids used in this study are listed in Supporting Information Table S3. *E. coli* DH10B-T1<sup>R</sup> strain was used as host for the cloning and propagation of plasmids with pBR/pUC- and pSC101-ts origins

of replication. In the case of suicide pGE-plasmid derivatives—harboring the conditional pi-dependent R6K origin of replication—*E. coli* strains BW25141 or CC118- $\lambda$ pir were used (Supporting Information Table S2). The proofreading DNA polymerase Herculase II Fusion (Agilent Technologies) was used to amplify DNA fragments for cloning purposes.<sup>70</sup> PCR products longer than 3 kb were inserted in the pCR-BluntII-TOPO plasmid (Zero Blunt TOPO PCR Cloning Kit, Life Technologies) prior to the cloning in the final vector. All plasmid constructs were fully sequenced (Secugen SL, Madrid, Spain). Details of plasmid constructions and oligonucleotide primers are described in the Supporting Information and Table S4.

***E. coli* Genome Modification and Strain Construction.** Site-specific deletions and insertions in the chromosome of *E. coli* were originated using a marker-less genome edition strategy based on the generation of double-strand breaks *in vivo* with I-SceI endonuclease.<sup>48,71</sup> Construction details of individual *E. coli* strains are described in the Supporting Information (Table S5). The *E. coli* strains to be modified were initially transformed with a Sp<sup>R</sup>-derivative of plasmid pACBSR<sup>72</sup> (expressing the I-SceI and  $\lambda$  Red proteins under the control of the P<sub>BAD</sub> promoter (inducible with L-arabinose). Subsequently, these bacteria were electroporated with the corresponding pGE-based suicide vector (Km<sup>R</sup>) carrying the indicated HRs, gene construct, and I-SceI restriction sites.<sup>49</sup> Individual Km<sup>R</sup>-colonies of the initial integration of the plasmid were grown for 6 h in LB-Sp liquid medium containing L-arabinose 0.4% (w/v) with agitation (200 rpm) to induce the expression of I-SceI and the cleavage of the chromosome at the integration site, promoting a second step of homologous recombination that elicits deletion of vector sequences and integration of gene constructs (Supporting Information Figure S2). A sample of these cultures was streaked on LB agar plates and incubated o/n to isolate individual colonies, which were replicated in LB and LB-Km agar plates to identify Km-sensitive colonies. Using specific primers, the modified strains were identified by PCR screening. Plasmid pACBSR-Sp was cured from the final strains.

Those *E. coli* strains to be modified using the pGETS plasmids were transformed with plasmid pACBSR-Sp as above, and the corresponding pGETS plasmid (Km<sup>R</sup>) - which contains the thermosensitive origin of replication pSC101-ts. The Ap<sup>R</sup>-marker flanked by Flippase Recognition Target (FRT) sites was incorporated in these vectors downstream of eLEEs. Individual colonies were grown at 30 °C in liquid LB medium with agitation (200 rpm) and at OD<sub>600</sub> 0.5, L-arabinose 0.4% (w/v) was added and the temperature was shifted to 37 °C to avoid plasmid replication. The cultures were further grown during 4 h and streaked in LB-Km-Sp plates to select the initial cointegrants. Individual cointegrant colonies were resolved by I-SceI expression and bacteria with insertion of the gene construct were selected in LB-Ap plates and tested for sensitivity to Km by replica-plating as above. Lastly, the Ap<sup>R</sup> gene was excised by the recombination of the FRT sites with Flippase expressed from plasmid pCP20 (Cm<sup>R</sup>).<sup>73</sup> Individual Ap-sensitive colonies were selected and integration of the gene construct confirmed by PCR and DNA sequencing (Secugen SL, Madrid, Spain).

**SDS-PAGE and Western Blot.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed following standard methods<sup>74</sup> using the Miniprotean III system (Bio-Rad). Proteins separated by SDS-PAGE were either stained with Coomassie Blue R-250 (Bio-



Rad) or transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) for Western blot using semidry transfer (Bio-Rad). Electrophoresis and transfer conditions, including buffers for blocking, washing and incubation of PVDF-membranes, were reported previously.<sup>75</sup> These membranes were incubated with primary rabbit antibodies anti-EscC (1:1000), anti-EscD (1:1000), anti-EscN (1:500), anti-EscJ (1:5000), anti-EspA (1:2000), anti-EspB (1:5000), anti-Tir (1:5000) and anti-intimin280 (1:5000) to detect T3SS components. Polyclonal sera against EscC, EscN and EscD were obtained by immunization of New Zealand White rabbits (Granja San Bernardo, Navarra, Spain) as described in the Supporting Information. Rabbit polyclonal sera against EspA, Tir and intimin-280 were a kind gift of Prof. Gad Frankel (Imperial College London). Rabbit polyclonal serum against EspJ was a kind gift of Prof. Bertha González-Pedrajo (UNAM, México). In all cases, bound rabbit antibodies were detected with secondary Protein A-peroxidase (POD) conjugate (Life Technologies, 1:5000). GroEL was detected with anti-GroEL-POD conjugate (1:5000; Sigma). Membranes were developed by chemiluminescence using the Clarity Western ECL Substrate kit (Bio-Rad) and exposed to X-ray films (Agfa) and to a ChemiDoc XRS system (Bio-Rad) for quantification of light emission in the protein bands (Quantity One Software, Bio-Rad).

To analyze the secretion of T3SS components in SIEC and EPEC strains, cells were harvested from 1 mL aliquots of the induced cultures by centrifugation (5000g, 5 min, RT). To obtain whole-cell protein extracts, bacteria were resuspended in 400  $\mu$ L of phosphate-buffered saline (PBS), mixed with 100  $\mu$ L of 5X SDS-PAGE sample buffer<sup>76</sup> and boiled for 10 min. Culture supernatants obtained after centrifugation were chilled on ice and incubated 60 min with trichloroacetic acid (TCA 20% w/v; Merck) for precipitation. After centrifugation (20000g, 15 min, 4 °C), TCA-precipitated protein pellets were rinsed with cold acetone (-20 °C), air-dried and resuspended in 30  $\mu$ L of SDS-PAGE sample buffer for Coomassie staining or Western blot.

**T3SS-Mediated Erythrocyte Hemolysis.** The protocol was performed as previously described.<sup>33</sup> Briefly, erythrocytes were obtained from a 5 mL blood sample obtained from New Zealand White rabbits (Granja San Bernardo, Navarra, Spain). The blood was treated adding 500  $\mu$ L of EDTA 1% (w/v) pH 7.5 (100  $\mu$ L for each ml) to avoid coagulation and was centrifuged (3500g, 15 min, RT) to concentrate the erythrocytes. The solution was washed 3 times with one volume of NaCl 0.9% (w/v) followed by centrifugation (1000g, 10 min, RT). The assembly of the T3SS was induced in the indicated cultures as described above until they reached OD<sub>600</sub> 0.4. Then, 0.5 mL of the cultures and 0.5 mL of the erythrocytes suspension (previously diluted to a 4% in DMEM) were mixed in 1.5 mL tubes. The final mixture was centrifuged (2500g, 1 min, RT) to induce the contact of the bacteria with the erythrocytes, incubated at 37 °C with 5% CO<sub>2</sub> during 4 h, and then the erythrocyte pellets were softly resuspended. The samples were centrifuged (12000g, 1 min, RT) and the hemoglobin release to the supernatant was measured at OD<sub>450</sub> in a spectrophotometer (Ultraspec 3100 pro, Amersham Biosciences). The hemolysis induced by the wt EPEC strain was considered 100%. Background hemolysis induced by *E. coli* K-12 strain EcM1 was subtracted from the values obtained in all samples. The experiment was repeated three times, each one with triplicates.

**Infection of Cell Cultures and Fluorescence Confocal Microscopy.** The human cell line HeLa (CCL-2, ATCC) was grown as monolayer in DMEM, supplemented with 10% fetal bovine serum (FBS; Sigma) and 2 mM L-glutamine, at 37 °C with 5% CO<sub>2</sub>. Induced EPEC was used for infection of HeLa cell cultures (10<sup>5</sup> cells/well in 24-well tissue culture plates; Falcon) at a multiplicity of infection (MOI) of 100:1 and infection continued at 37 °C with 5% CO<sub>2</sub> for 90 min. SIEC strains (carrying pGEN22 for GFP expression) were induced for 2.5 h with 0.1 mM IPTG (as described above) and used to infect HeLa cells at a MOI of 100:1 for 3 h. Infections were stopped by three washes of sterile PBS, and fixed with 4% (w/v) paraformaldehyde (in PBS, 20 min, RT). Samples were then washed with PBS three times and permeabilized by incubation with 0.1% (v/v) saponin (Sigma) in PBS for 10 min. To stain EPEC strains, bacteria were incubated with polyclonal rabbit Ab anti-intimin280 (1:500) in PBS with 10% (v/v) goat serum (Sigma) and incubated for 60 min at RT. Coverslips were washed three times with PBS and were then incubated for 45 min with goat antirabbit secondary antibodies conjugated to ALEXA488 (1:500; Life Technologies) in PBS with 10% goat serum, along with Phalloidin-Tetramethylrhodamine (TRITC) (1:500; Sigma) and 4',6-diamidino-2-phenylindole (DAPI) (1:500; Sigma) to label F-actin and DNA, respectively. Coverslips were washed 3 times with PBS after incubation and 4  $\mu$ L of ProLong Gold antifade reagent (Life Technologies) was added. They were then observed at the SP5 confocal microscope (Leica) using the 100 $\times$  objective and an additional 2.5-fold magnification. The images were processed using the ImageJ software.<sup>77</sup>

**Injectisome Purification.** The protocol was adapted from a previously reported method.<sup>52</sup> Cultures of EPEC and SIEC derived strains were grown in 20 mL of LB o/n with agitation (200 rpm). Next day, these cultures were used to inoculate 950 mL of prewarmed DMEM in the case of EPEC or LB in the case of SIEC. Bacteria were grown with gentle agitation (100 rpm) until reaching OD<sub>600</sub> of 0.8 and then centrifuged to harvest bacteria (6000g, 15 min). The supernatant was discarded and the pellet was resuspended in 80 mL of sucrose solution (150 mM Tris-HCl pH 8.0, 0.5 M sucrose). The suspension was agitated for homogenization at 4 °C and 8 mL of freshly prepared lysozyme solution (10 mg/mL) were added drop by drop. Then, EDTA was added at a final concentration of 2 mM and incubated for 60 min. Bacteria were then lysed with 0.3% (w/v) de lauryldimethylamine oxide (LDAO; Sigma). The lysate was supplemented with 12 mM of MgSO<sub>4</sub> and 450 mM of NaCl (final concentrations) and centrifuged (25000 g, 20 min, 4 °C). The supernatant of previous centrifugation was collected and then ultracentrifuged (3 h, 70000g, 4 °C) and the viscous pellet was resuspended in 2 mL of buffer F (10 mM Tris-HCl pH 8.0, 0.1% LDAO, 0.3 M NaCl, 5 mM EDTA). This solution was loaded onto a 30% (w/v) CsCl "cushion" reaching a final volume of 12 mL, which was ultracentrifuged (50000g, 16 h, 20 °C). Fractions of 0.5 mL from the CsCl gradient were collected, diluted with 3.5 mL of buffer F and ultracentrifuged (130000g, 30 min, 4 °C). The protein pellets were resuspended in 100  $\mu$ L of buffer F and kept on ice for Western blot analysis with anti-EspA antibodies. Injectisomes were visualized by transmission electron microscopy (TEM) in the protein fractions having higher levels of EspA.

**Electron Microscopy.** A sample (5  $\mu$ L) of the purified protein fractions with high level of EspA (see above) was

applied to collodion-coated copper grids and incubated for 2 min at RT. After that, grids were washed 3 times with deionized water (Milli-Q, Millipore). Next, samples were incubated with uranyl acetate in a 2% (w/v) solution in deionized water for 1 min. Excess of uranyl acetate solution was removed and the grids were further washed with deionized water. Images were taken with a JEOL 1200EX-II electron microscope operated at 100 kV and recorded at a nominal magnification of 100000 $\times$ . For the visualization of injectisomes on the bacterial surface, cultures of induced of EPEC or SIEC strains (1 mL) were centrifuged softly (100g, 10 s, RT) in 1.5 mL tubes to concentrate bacteria. Then, 5  $\mu$ L taken from the bottom of the tube, were applied to collodion-coated copper grids and incubated for 2 min at RT. The negative staining was performed as above, but using a 1% (w/v) solution of uranyl acetate and a magnification of 50000 $\times$  at the electron microscope.

**Statistics.** Mean and standard errors of experimental values calculated with using Prism 5.0 (GraphPad software Inc.). Statistical analyses comparing the mean of paired experimental groups were conducted with Student's *t* test using Prism 5.0 (GraphPad software Inc.). Data were considered significantly different when *p*-values <0.05.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Supplementary methods, with details of plasmid and strain constructions, and supplementary figures and tables. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00080.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

DMEM, Dulbecco's Modified Eagle Medium; EPEC, enteropathogenic *E. coli*; HRs, homology regions; LB, Lysogeny broth; LEE, Locus of enterocyte effacement; SIEC, Synthetic

Injector *E. coli*; IM, inner membrane; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; OM, outer membrane; PG, peptidoglycan; T3SS, type III secretion system; Tir, translocated intimin receptor; TUs, transcriptional units

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