Novel insights into the mechanism of SepL-mediated control of effector secretion in enteropathogenic Escherichia coli

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Abstract
Type three secretion systems (T3SSs) are virulence determinants employed by several pathogenic bacteria as molecular syringes to inject effector proteins into host cells. Diarrhea-producing enteropathogenic Escherichia coli (EPEC) uses a T3SS to colonize the intestinal tract. T3S is a highly coordinated process that ensures hierarchical delivery of three classes of substrates: early (inner rod and needle subunits), middle (translocators), and late (effectors). Translocation of effectors is triggered upon host-cell contact in response to different environmental cues, such as calcium levels. The T3S substrate specificity switch from middle to late substrates in EPEC is regulated by the SepL and SepD proteins, which interact with each other and form a trimeric complex with the chaperone CesL. In this study, we investigated the link between calcium concentration and secretion regulation by the gatekeeper SepL. We found that calcium depletion promotes late substrate secretion in a translocon-independent manner. Furthermore, the stability, formation, and subcellular localization of the SepL/SepD/CesL regulatory complex were not affected by the absence of calcium. In addition, we demonstrate that SepL interacts in a calcium-independent manner with the major export gate component EscV, which in turn interacts with both middle and late secretion substrates, providing a docking site for T3S. These results suggest that EscV serves as a binding platform for both the SepL regulatory protein and secreted substrates during the ordered assembly of the T3SS.

KEYWORDS
EPEC, EscV, injectisome, secretion hierarchy, SepL, T3SS

1 | INTRODUCTION

Type III secretion systems (T3SSs) are essential virulence determinants of many animal and plant bacterial pathogens, as they serve to inject virulence proteins, or effectors, directly into the host cell cytoplasm in a process called translocation (Buttner, 2012; Gaytan, Martinez-Santos, Soto, & Gonzalez-Pedrajo, 2016). These effectors modify host cell physiology for the benefit of the bacteria (Dean, 2011; Tosi, Pflug, Discola, Neves, & Dessen, 2013). T3SSs or injectisomes are employed by pathogenic bacteria that threaten human health, including enteropathogenic Escherichia coli (EPEC), which colonizes the small intestine and produces a singular histopathological modification called the attaching and effacing (A/E) lesion. This alteration is characterized by the effacement of epithelial microvilli, the intimate attachment of the bacteria to the host cell, and finally the development of an actin-rich pedestal-like structure beneath the adherence site (Kaper, Nataro, &
The injectisome of EPEC consists of an outer (EscC) and a pair of inner (EscJ and EscD) membrane rings that are interconnected through a periplasmic inner rod (EscI), forming a core structure, the so-called basal body, that spans both bacterial membranes (Ogino et al., 2006; Sal-Man, Deng, & Finlay, 2012; Spreiter et al., 2009; Yip et al., 2005). The export apparatus resides within the inner membrane ring and is formed by five highly conserved proteins, named EscR, EscS, EscT, EscU, and EscV (Moraes, Spreter, & Strynadka, 2008). The cytoplasmic side of the basal body is associated to a ring-shaped oligomeric structure (EscQ and EscK), which functions as a substrate recognition platform, and provides a docking site for the ATPase complex (EscN, EscO, and EscL) (Andrade, Pardo, Espinosa, Perez-Hernandez, & Gonzalez-Pedrajo, 2007; Biemans-Oldehinkel, Sal-Man, Deng, Foster, & Finlay, 2011; Romo-Castillo et al., 2014; Soto et al., 2017; Zarivach et al., 2008). Moreover, the extracellular part of the injectisome comprises a 23 nm in length needle-like structure (EscF), which is extended by a long filament (EspA) (Knutton et al., 1998; Monjaras Feria et al., 2012; Ogino et al., 2006; Sekiya et al., 2001; Wilson, Shaw, Daniell, Knutton, & Frankel, 2001). Upon host cell contact, the filament serves as a scaffold for the assembly of the translocation pore (EspB and EspD) (Chatterjee, Caballero-Franco, Bakker, Totten, & Jardim, 2015; Luo & Donnenberg, 2011).

Notably, although there is a temporal regulation of LEE gene expression in which the LEE1 operon is expressed first, the genes encoding all secreted proteins are expressed simultaneously (Yerushalmi, Litvak, Gur-Arie, & Rosenshine, 2014). Therefore, T3S regulators are involved in setting up a hierarchy of secretion to ensure that the structural proteins that make up the T3SS are secreted prior to effectors (Gaytan et al., 2016; Portaliou, Tsolis, Loos, Zorzini, & Economou, 2016). Depending on the timing of secretion, the T3S-dependent substrates are classified as early (EscI and EscF), middle or translocators (EspA, EspB, and EspD), and late substrates or effectors (Deane, Abruscic, Johnson, & Lea, 2010). Coordinated secretion of middle and late substrates has been suggested to be controlled by the LEE-encoded proteins SepL and SepD (Deng et al., 2004, 2005; O’Connell et al., 2004; Wang, Roe, Mäteer, Shipston, & Gally, 2008). Deletion of sepL or sepD completely abolishes translocator secretion and significantly increases effector secretion (Deng et al., 2004, 2005; Wang et al., 2008). SepL belongs to a family of proteins whose members include MxiC from Shigella (Botteaux, Sory, Biskri, Parsot, & Allaoui, 2009), InvE and SsaL from Salmonella pathogenicity islands 1 and 2, respectively (Coombes, Brown, Valdez, Brumell, & Finlay, 2004; Kubori & Galan, 2002), CopN from Chlamydia (Silva-Herzog et al., 2011), YopN/TyeA from Yersinia (Forsberg, Vlietanen, Skurnik, & Wolf-Watz, 1991; Iriarte et al., 1998), and PopN/Pcr1 from Pseudomonas (Yang et al., 2007). These proteins, known as gatekeepers, prevent premature effector secretion before host cell contact is established. In most systems, the regulatory function of gatekeepers relies on their ability to disengage from the T3SS base once there is an activation signal upon host cell contact, and subsequently either be secreted, as is the case of YopN, PopN, CopN, and MxiC (Archuleta & Spiller, 2014; Botteaux et al., 2009; Cheng, Kay, & Schneewind, 2003; Cherradri et al., 2013; Lee, Zmina, Stopford, Toska, & Rietsch, 2014), or degraded, as is the case of SsaL (Yu, McGourty, Liu, Unsworth, & Holden, 2010). However, although SepL has been shown to contain a T3S-signal at its N-terminal domain, the full-length protein is not secreted (Deng et al., 2004, 2005; Younis et al., 2010).

SepL forms a trimeric complex with SepD and CesL (Younis et al., 2010). SepD has been proposed to be a homolog of SycI from Salmonella SPI-2, and CesL a homolog of SycN from Yersinia, and both SpIC and SycN are known to be involved in regulation of secretion hierarchy (Cheng et al., 2003; Day & Plano, 1998; Schubot et al., 2005; Younis et al., 2010; Yu, Liu, & Holden, 2004; Yu et al., 2010). Moreover, CesL was suggested to function as a SepL chaperone (Younis et al., 2010), and deletion of the cesL (orf12) gene in the A/E pathogen Citrobacter rodentium abolishes secretion of both translocators and effectors (Deng et al., 2004). Thus, the SepL/SepD/CesL complex resembles the YopN-TyeA/YscB/SycN complex from Yersinia and the SsaL/SycI/SsaM complex from Salmonella SPI-2 (Younis et al., 2010). These complexes trigger effector secretion by different mechanisms. In the case of Yersinia, substrate secretion switching occurs upon YopN secretion, whereas in Salmonella it requires the switch protein complex dissociation and the subsequent degradation of its components. In both cases, the switching event occurs in response to environmental cues detected upon host membrane contact, for example, calcium depletion and a pH change, respectively (Cheng et al., 2001; Day & Plano, 1998; Yu et al., 2010). In EPEC, calcium depletion from the bacterial growth media has an effect on substrate secretion similar to that observed in a sepL or sepD null mutants (Deng et al., 2005; Ide, Michgeln, Knappstein, Heusipp, & Schmidt, 2003). Yet, the molecular mechanism by which the switch protein complex participates in the regulation of substrate secretion in the absence of calcium is still poorly understood. In this work, we investigated the effect of calcium depletion in the gatekeeper-dependent triggering of effector secretion and uncovered the existence of novel protein interactions.

### 2 | MATERIALS AND METHODS

#### 2.1 | Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. For overnight (O/N) cultures, bacteria were aerobically grown in Lysogeny Broth (LB) at 37°C with constant shaking at 250 rpm. Bacterial growth conditions required for induction of T3S and overproduction of recombinant proteins are indicated below. When necessary, bacterial cultures were supplemented with the appropriate antibiotics at the following concentrations: streptomycin (Sm, 25 μg/
| Strain/plasmid                  | Description                                                                 | Reference                        |
|--------------------------------|-----------------------------------------------------------------------------|----------------------------------|
| **Escherichia coli**            |                                                                             |                                  |
| EPEC E2348/69                   | WT EPEC O127:H6 strain; Sm’                                                | Levine et al. (1978)             |
| ΔescN mutant                    | E2348/69 carrying an in-frame deletion of escN; Sm’                         | Gauthier et al. (2003)           |
| ΔespA mutant                    | E2348/69 carrying an in-frame deletion of espA; Sm’ Km’                      | Gift of the Navarro F. Lab       |
| ΔespB mutant                    | E2348/69 carrying an in-frame deletion of espB; Sm’ Km’                      | Gift of the Xicohtencatl J. Lab  |
| ΔespD mutant                    | E2348/69 carrying an in-frame deletion of espD; Sm’ Km’                      | Gift of the Xicohtencatl J. Lab  |
| ΔsepL mutant                    | E2348/69 carrying an in-frame deletion of sepL; Sm’ Km’                      | Gift of the Puente JL Lab        |
| ΔescU mutant                    | E2348/69 carrying an in-frame deletion of escU; Sm’ Km’                      | Soto et al. (2017)               |
| JPEP39 (ΔgrlR mutant)           | E2348/69 carrying an in-frame deletion of grlR; Sm’                         | Garcia-Angulo et al. (2012)      |
| EPEC sepL-3FLAG::km             | E2348/69 expressing 3-FLAG-tagged sepL; Sm’ Km’                            | This study                       |
| EPEC cesL-2HA::km               | E2348/69 expressing 2-HA-tagged cesL; Sm’ Km’                               | This study                       |
| EPEC sepL-3FLAG cesL-2HA::km    | E2348/69 expressing 3-FLAG-tagged sepL and 2-HA-tagged cesL; Sm’ Km’        | This study                       |
| BL21(DE3)/pLysS                 | Strain used for expression of pET19b constructs; Cm’                       | Novagen                          |
| XL1-Blue                       | Strain used for cloning; Tc’                                                | Stratagene                       |
| **Salmonella**                  |                                                                             |                                  |
| JR501                           | Strain used to convert plasmids to Salmonella compatibility                 | Ryu & Hartin (1990)              |
| SJW1368                         | Strain used for expression of pTrc99A_FF4 constructs; flagellar master operon mutant, Δ(cheW-flhD) | Ohnishi et al. (1994) |
| **Plasmids**                    |                                                                             |                                  |
| pTrc99A_FF4                     | Modified pTrc99A expression vector under the control of the trc promoter; Ap’ | Ohnishi, Fan, Schoenhals, Kihara, & Macnab (1997) |
| pET19b                          | Expression vector under the control of the T7 promoter; Ap’                 | Novagen                          |
| pACTrc                          | Expression vector under the control of the trc promoter; Cm’               | Gift of the Fraser GM Lab        |
| pKD46                           | Red recombinase system plasmid under the control of the araB promoter; Ap’  | Datsenko & Wanner (2000)         |
| pSUB11                          | Template plasmid for amplification of 3-FLAG-kanamycin-resistance cassette; Ap’, Km’ | Uzzau et al., (2001)             |
| pSU315                          | Template plasmid for amplification of HA-kanamycin-resistance cassette; Ap’, Km’ | Uzzau et al. (2001)             |
| pFLP2                           | Flp recombinase expression plasmid; Ap’                                     | Hoang et al. (1998)              |
| pMTpL                           | sepl cloned into pTrc99A_FF4                                                | This study                       |
| pMTpLΔC75                       | sepl lacking codons 277 to 351 cloned into pTrc99A_FF4                      | This study                       |
| pMTpLΔC11                       | sepl lacking codons 341 to 351 cloned into pTrc99A_FF4                      | This study                       |

(Continues)
ml), kanamycin (Km, 50 μg/ml), ampicillin (Ap, 100 μg/ml), chloramphenicol (Cm, 25 μg/ml), or tetracycline (Tc, 25 μg/ml).

2.2 | Plasmid construction and oligonucleotides

The sequences of the oligonucleotides used in this study are listed in Table 2. The sepL, sepD, and cesL genes were amplified from chromosomal DNA of the wild-type EPEC strain E2348/69 (WT), using the following primer pairs: sepL_Fw/sepL_Rv, sepL_Fw/sepLΔC75_Rv, sepL_Fw/sepLΔC11_Rv, sepD_Fw/sepD_Rv, and cesL_Fw/cesL_Rv. The resulting PCR products containing the Ndel and BamHI restriction sites were cloned into pTrc99A_FF4 to generate plasmids pMTpL, pMTpLΔC75, pMTpLΔC11, and pATpD, or into pET19b to generate plasmid pMEcL. To construct plasmids pMATpL and

| Strain/plasmid | Description | Reference |
|---------------|-------------|-----------|
| pKEEvC        | escV codons 335 to 675 cloned into pET19b | This study |
| pKEDNi        | escD codons 1 to 120 cloned into pTrc99A_FF4 | This study |
| pKEDN         | escD codons 1 to 120 cloned into pET19b | This study |
| pJPhi         | escI cloned into pET19b | Monjaras Feria et al. (2012) |
| pETeI         | escI cloned into pTrc99A_FF4 | This study |
| pSlo4         | escK cloned into pMAL-c2x | Soto et al. (2017) |
| pJHeH         | espH with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHnC         | nleC with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHeI         | escI with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHnH2        | nleH2 with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pATpD         | sepD cloned into pTrc99A_FF4 | This study |
| pMEcL         | cesL cloned into pET19b | This study |
| pMTBiSpDcL    | sepD and his-cesL cloned into pTrc99A_FF4 | This study |
| pMATPL        | sepL cloned into pACTrc | This study |
| pMATLΔC11     | sepL lacking codons 341 to 351 cloned into pACTrc | This study |

| Oligonucleotide | Sequence 5′ – 3′ |
|-----------------|-----------------|
| sepL_Fw         | AGTTTCATATGGCTAATGGTATTG |
| sepL_Rv         | CTATAAAAAAAAGGATCCTCACAT |
| sepLΔC75_Rv     | TAGCATGGATCCTCAAATGACATC |
| sepLΔC11_Rv     | AATCTATGGATCCTCAAATCATTA |
| sepD_Fw         | TAAATCATAATGAAATATATAGT |
| sepD_Rv         | AAAAACTTATTTGATCCATTACAC |
| cesL_Fw         | AGAGCCTGCATATGAATCTTTTAG |
| cesL_Rv         | ATTTAACAGAGATCCATGATGTC |
| escV_CtermFw    | AATAATAAGAGATCATATGGGAGCTGATTG |
| escV_CtermRv    | GTGGGATAGGATCCATACAGAATC |
| escD_Fw         | GGATGAATAAAATTTAGATATCCTCATATAA |
| escD_Nterm_Rv   | CTGACCAGGATCGCAGTTAGTTG |
| sepL-3FLAG_Fw   | ATACATTATTAATGTTGTAAGGTAGATTATAAGGAGATGGATTAGTTGAGCTACAAAGACCATGACGG |
| sepL-FLAG_Rv    | CTCCTTCATAATCTTTTCTGCTAGCATGACAAATCAATACAAAACTGAAAATGATTTCTCCTTACAGT |
| cesL-2HA_Fw     | CTTTTCACACAGCATGTGCGAGATTATGTCAGCGGTCCGGTCGAGGATCACATATCAGGATGATTGCTGAGCATGATTCCTGAT |
| cesL-2HA_Rv     | AAGATCGTGATATGACCTCGCTGTTTTTTTTAATAATATTTAAGAGTTTATCATATGGAATACCTCCTAGTTG |

TABLE 1 (Continued)

| Strain/plasmid | Description | Reference |
|----------------|-------------|-----------|
| pKEdNi        | escD codons 1 to 120 cloned into pET19b | This study |
| pKEdN         | escD codons 1 to 120 cloned into pET19b | This study |
| pJPhi         | escI cloned into pET19b | Monjaras Feria et al. (2012) |
| pETeI         | escI cloned into pTrc99A_FF4 | This study |
| pSlo4         | escK cloned into pMAL-c2x | Soto et al. (2017) |
| pJHeH         | espH with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHnC         | nleC with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHeI         | escI with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pJHnH2        | nleH2 with its native RBS cloned into pTOPO-2HA | Monjaras Feria et al. (2012) |
| pATpD         | sepD cloned into pTrc99A_FF4 | This study |
| pMEcL         | cesL cloned into pET19b | This study |
| pMTBiSpDcL    | sepD and his-cesL cloned into pTrc99A_FF4 | This study |
| pMATPL        | sepL cloned into pACTrc | This study |
| pMATLΔC11     | sepL lacking codons 341 to 351 cloned into pACTrc | This study |

TABLE 2 Oligonucleotides used in this study
pMATpL∆C11, -sepL or -sepL lacking the last 11 codons were subcloned from plasmids pMTpL and pMTpL∆C11, respectively, into the Ndel/BamHI sites of the pACTrc vector. The KEEVc plasmid, encoding the cytoplasmic region of EsCD, was constructed using primers escD_CtermFw and escD_CtermRv, to amplify a PCR fragment that was cloned into the Ndel/BamHI sites of the pET19b vector. To generate the bicistronic plasmid pMTBISpDcL, the pMEcL plasmid was double digested with XbaI and PstI, and the resulting fragment was cloned into the XbaI/PstI sites of the pAPDpL plasmid. The PKTeD4 vector was constructed by amplifying the cytoplasmic region of CesL (codons 1 to 120) using primers escL_Fw and escL-Rv. The obtained PCR fragment was cloned into the Ndel/BamHI sites of the pTrc99A_FF4 vector. To generate plasmid pKDeD4, the PKTeD4 vector was double digested with Ndel and BamHI and the resulting fragment was cloned into pET19b. The pJEel plasmid (Monjaras Feria et al., 2012) was double digested with Ndel and BamHI, and the resulting fragment was cloned into the Ndel/BamHI sites of the pTrc99A_FF4 vector to generate plasmid pETel. DNA fragments cloned from PCR-amplified material were sequenced to verify that no undesired base changes had been introduced.

### 2.3 Epitope tagging of chromosomal genes

A modification of the Red recombinase system was used for chromosomal epitope tagging as described in (Uzzau, Figueroa-Bossi, Rubino, & Bossi, 2001). The EPEC strains expressing a 3xFLAG-tagged version of -sepL (EPEC sepL-3FLAG::km) or a 2xHA version of cesL (EPEC cesL-2HA::km), were constructed by amplifying the kanamycin cassette from either plasmid pSUB11, using the primer pair sepL-3FLAG_Fw and sepL-3FLAG_Rv, or from plasmid pSU315, using the primer pair cesL-2HA_Fw and cesL-2HA_Rv. The resulting PCR products were electroporated into EPEC WT strain carrying plasmid pKD46, which was grown at 30°C in LB medium containing 100 mmol/L of L-arabinose to induce Red recombinase expression. Transformant cells were grown at 37°C to eliminate the pKD46 plasmid. Recombinant EPEC sepL-3FLAG::km and EPEC cesL-2HA::km strains were selected on LB plates supplemented with 300 μg/mL Km and tested for Ap sensitivity. To generate the double-tagged EPEC sepL-3FLAG cesL-2HA::km strain, the kanamycin cassette of the EPEC sepL-3FLAG::km strain was excised using the helper plasmid pLP2 (Hsang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998), and the homologous recombination of cesL-2HA::km was performed as described above.

### 2.4 Type III protein secretion assay

To induce EPEC T3S, prequillibrated regular Dulbecco’s modified Eagle’s medium (DMEM, Gibco, 12100-046) was inoculated with 1/100 of an overnight (O/N) culture of LB and grown under static conditions at 37°C in a 5% CO₂ atmosphere. At an optical density at 600 nm (OD₆₀₀) of 0.8 to 1, the culture was centrifuged (19,800g for 10 min) to separate secreted proteins from the bacterial cells. The resulting pellet was resuspended in SDS-PAGE sample buffer and normalized according to the OD₆₀₀. The supernatant, containing secreted proteins, was precipitated overnight at 4°C with 10% trichloroacetic acid. Precipitated proteins were collected by centrifugation at 19,800g for 30 min, and protein pellets were resuspended in SDS-PAGE sample buffer, normalized according to the OD₆₀₀ and neutralized by adding a saturated Tris solution. Protein secretion profiles were analyzed by 15% SDS-PAGE and immunoblotting. Calcium-free DMEM (Gibco, 21068-028) was used to evaluate the effect of calcium on the EPEC secretion profile. The calcium-free DMEM solution was supplemented with L-Glutamine and HEPES to a final concentration of 4 mmol/L and 100 mmol/L, respectively.

### 2.5 Immunoblotting

Samples subjected to SDS-PAGE were transferred onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes and blocked O/N at 4°C with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk. After washing with TBS-Tween, membranes were probed against rabbit-raised polyclonal anti-Tir, anti-Map, anti-EspA, anti-EscJ, anti-EspD, anti-EsCl, anti-SepD, or anti-SepL antibodies, and monoclonal anti-DnaK (MBL International), anti-FLAG (SIGMA) or HRP-conjugated anti-HA (Roche) antibodies. Secondary antibodies used in this study (horseradish peroxidase (HRP)-conjugated goat antirabbit and goat antimouse), were obtained from Santa Cruz Biotechnology. Protein detection was performed with the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore). Animals were handled and cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals (AS5281-01) and with the approval of the local Animal Use and Care Committee (CICUAL) of the Instituto de Fisiología Celular, UNAM for protocol BGP70-15.

### 2.6 Protein overproduction and pull-down assays

Salmonella SJW1368 strain, which lacks the flagellar master operon and has been extensively used for expression of pTrc99A-based plasmids (Gonzalez-Pedrajo, Minamino, Kihara, & Namba, 2006; Ohnishi, Ohto, Aizawa, Macnab, & Iino, 1994; Okabe, Minamino, Imada, Namba, & Kihara, 2009), was transformed with plasmids pMTpL, pMTpLΔC75, or pMTpLΔC11, and E. coli BL21 (DE3)/plysS (BDP) strain with plasmid pKEEVC, for protein overproduction. O/N cultures of the transformed strains were used to inoculate fresh LB containing appropriate antibiotics, and cultures were grown at 37°C with shaking. At an OD₆₀₀ of 0.8, protein production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mmol/L, and bacterial growth was continued for 4 hr at 30°C. The bacterial cells were harvested by centrifugation at 15,500g for 10 min, and the pellets were resuspended in binding buffer (20 mmol/L Tris-HCl pH 8, 0.5 mol/L NaCl) containing 20 mmol/L imidazole and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication. Cell lysates were centrifuged (15,500g for 30 min) and the supernatants (cleared lysates) containing soluble proteins were recovered. The cleared lysate of the His-EscVc recombinant protein was mixed with the cleared lysate of SepL, SepLΔC75, or SepLΔC11 and incubated...
for 2 hr at 4°C. Mixed lysates were loaded onto a polypropylene column (QIAGEN) containing 100 μl of preequilibrated Ni-nitrilotriacetic acid (Ni-NTA) agarose beads. The resin was washed with 10 column volumes of binding buffer containing increasing concentrations of imidazole and bound proteins were eluted with 500 mmol/L imidazole. To purify the SepL/SepD/CesL protein complex, Salmonella SJW1368 cells were cotransformed with the bicistronic plasmid pMTBISpDcL and the plasmid pMATPl or pMATPlΔCesL. Protein production was induced by the addition of 1 mmol/L IPTG. The cleared lysate was prepared as described above, incubated with 100 μl of Ni-NTA for 2 hr at 4°C and then loaded onto a polypropylene column. After extensive washing, proteins were eluted with 500 mmol/L imidazole. To assess the effect of calcium on the EscVc/SepL and SepL/SepD/CesL interactions, 2 mmol/L CaCl₂ was added to all buffers used in the pulldown assays.

To evaluate the interaction of EscVc and EscD with T3-secreted proteins, a modified version of the method employed by Thomas et al. (2005) to identify cognate substrates of the CesT chaperone was used. Briefly, the cleared lysate of His-EscVc or His-EscD was incubated with Ni-NTA agarose beads for 2 hr and the resin was packed into a polypropylene column. After washing with 20 mmol/L imidazole-binding buffer, the mixed supernatant of mutant strains Saepl and ΔgrlR grown under T3S-inducing conditions was loaded onto the column, washed with five column volumes of binding buffer containing increasing concentrations of imidazole, and eluted with 500 mmol/L imidazole. All resulting samples were subjected to SDS-PAGE and immunoblotting.

### 2.7 | Subcellular fractionation

Subcellular fractionation was carried out as previously reported (Gauthier, Puente, & Finlay, 2003) with slight modifications. The EPEC sepl-3FLAG cesL-2HA::km strain was grown under T3S inducing conditions in regular DMEM or DMEM without calcium until an OD₆₀₀ of 1. Cells were harvested by centrifugation (15,500g for 10 min), the cell pellet was washed once with phosphate-buffered saline and then resuspended in 20 mmol/L Tris-HCl pH 7.5 containing 1 mmol/L PMSF. Cells were disrupted by sonication and unbroken cells were removed by centrifugation at 19,800g for 10 min. The cleared lysate was subjected to ultracentrifugation at 90,000g for 1 hr. The supernatant (containing the cytoplasmic proteins) was collected into a clean tube, and the membrane-containing pellet was washed with 20 mmol/L Tris-HCl pH 7.5. Both fractions were ultracentrifuged once again as previously described, and the cytoplasmic and membrane fractions were collected. Total protein concentration of the samples was quantified using the DC protein assay (Bio-Rad), and proteins were analyzed by immunoblotting as described above.

### 2.8 | Protein stability assay

The EPEC sepl-3FLAG cesL-2HA::km strain was grown in calcium-free DMEM added with 1.8 mmol/L CaCl₂ under T3S inducing conditions. When an OD₆₀₀ of 1 was reached, cells were harvested by centrifugation at 4,600g for 10 min, washed with and resuspended in either calcium-free DMEM or calcium-free DMEM added with 1.8 mmol/L CaCl₂ containing 50 μg/ml Cm in order to stop protein synthesis. Bacterial growth was continued and samples were taken every 30 min during 3 hr. Samples were normalized according to the OD₆₀₀ and proteins were analyzed by immunoblotting as described above.

### 2.9 | Coimmunoprecipitation

50 mL of DMEM without calcium or the same medium added with 1.8 mmol/L CaCl₂ was inoculated with O/N cultures of the EPEC sepl-3FLAG cesL-2HA::km or EPEC cesL-2HA::km strains. Cell cultures were grown under static conditions at 37°C in a 5% CO₂ atmosphere. At an OD₆₀₀ of 1, cultures were harvested by centrifugation, cell pellets were washed with HEPES 20 mmol/L, NaCl 250 mmol/L pH 7.4, resuspended in the same buffer and lysed by sonication. Cell lysates were incubated with Triton X-100 [0.1% (v/v)] for 15 min and then cleared by centrifugation at 19,800g for 30 min. Supernatants were carefully collected and the crosslinker diethio bis succinimidyl proionate was added to a final concentration of 1 mmol/L. After 1 hr incubation at room temperature, the crosslinking reaction was stopped by the addition of 20 mmol/L Tris pH 7.5. Cross-linked samples were mixed with 40 μl of preequilibrated ANTI-FLAG M2 Affinity Gel beads (Sigma) and incubated O/N at 4°C with shaking. SepL-3FLAG coupled beads were centrifuged at 13,500g for 2 min and washed three times with HEPES 20 mmol/L, NaCl 250 mmol/L pH 7.4. Finally, the beads were resuspended in 20 μl of SDS-PAGE sample buffer, containing 2 μl of β-mercaptoethanol and boiled for 5 min. Protein samples were resolved by SDS-PAGE and analyzed by immunoblotting as described above.

### 3 | RESULTS

### 3.1 | Calcium removal from the culture media solely affects effector secretion

Previous studies have implicated calcium as an important player in the regulation of T3 substrate secretion in EPEC (Deng et al., 2005; Ide et al., 2003; Kenny, Abe, Stein, & Finlay, 1997; Shaulov, Gershberg, Deng, Finlay, & Sal-Man, 2017). Therefore, we examined the secretion profile of EPEC wild-type strain E2348/69 grown under T3S inducing conditions in regular DMEM (containing 1.8 mmol/L CaCl₂) or calcium-free DMEM. We confirmed that the secretion of the effector Tir is increased in the absence of calcium (Figure 1a), and extended this finding to other LEE and Nle effectors as judged by the enhanced secretion of Map, EspH-2HA, NleH2-2HA, and NleC-2HA into the supernatant (Figure 1a and b). Addition of 1.8 mmol/L CaCl₂ to calcium-free DMEM restored effector secretion to the levels seen with regular DMEM (Figure 1), corroborating that the observed effect is due to the absence of calcium. For comparison, the secretion profiles of the sepl null mutant, which entirely deregulates effector secretion, and that of the secretion-deficient escU null mutant, are shown
In addition, our results demonstrated that protein production was not affected by the different culture media used or in the mutant strains (Figure 1a lower panel and b right panel). Furthermore, in contrast to previous reports (Deng et al., 2005; Ide et al., 2003), we did not observe a significant decrease in translocator secretion (Figure 1a). Therefore, to provide a more quantitative measure of the
overall change in the secretion of effectors and translocators, we performed a statistical analysis on the secretion data of several independent experiments by quantifying the Coomassie-stained protein band intensities of the translocator EspA and the effector Tir. The values were normalized relative to the corresponding band intensity of the autotransporter EspC, which is secreted through the type V secretion system and thus serves as a loading control (Mellies et al., 2001; Stein, Kenny, Stein, & Finlay, 1996). This analysis showed no significant differences in EspA translocator secretion in the different media used in the assay, whereas there was a clear 11-fold increase in Tir effector secretion in the absence of calcium (Figure 1c). Moreover, we investigated the effect of calcium on the secretion of an early substrate, namely, the inner rod component Escl. As shown in Figure 1b, secretion of Escl-2HA was not altered by different calcium levels in the growth media. It is worth mentioning that although overproduction of Escl-2HA caused a decrease in T3 secretion, EPEC was still responsive to calcium depletion as demonstrated by an increased secretion of Tir (Figure S1A). Besides, overproduction of an untagged version of Escl, which did not interfere with T3 secretion, further demonstrated that calcium levels had no effect on the secretion of this early substrate (Figure S1B). Overall, our results suggest that only late substrate secretion is significantly affected by calcium depletion from the growth media. Thus, we next decided to investigate the mechanism that triggers effector secretion under this condition.

3.2 Calcium sensing is translocon-independent

It has been previously shown that effector secretion is elicited upon host cell contact, and that different environmental cues are involved in the activation of this secretion stage, for example, a pH shift in Salmonella and low-calcium in Yersinia, Pseudomonas, and A/E pathogens (Beuzon, Banks, Devick, Hensel, & Holden, 1999; Deng et al., 2005; Kim et al., 2005; Lee, Mazmanian, & Schneewind, 2001; Mills, Baruch, Charpentier, Kobi, & Rosenshine, 2008; Rosqvist, Magnusson, & Wolf-Watz, 1994; Yu et al., 2010). These in vitro conditions are proposed to mimic those found during the bacterial infection process. Therefore, we reasoned that the translocon proteins in EPEC, which establish contact with host cells, could be involved in sensing calcium-level changes. This hypothesis was evaluated by comparing the secretion profile of the EPEC wild-type strain with that of the isogenic translocator null mutants espA, espB, and espD, when grown in the presence or absence of calcium. Translocator null mutants were still responsive to calcium depletion, as shown by an increased secretion of the effector Tir in calcium-free medium (Figure 2). Tir production was not affected in the different culture media or in the mutant strains (Figure 2 lower panel). Thus, although translocators are the proteins that make direct contact with host cells, they appear not to be required for in vitro calcium sensing.

3.3 The SepL/SepD/CesL complex is insensitive to calcium concentration changes

It has been reported that the SsaL/SpIC/SsaM complex of the S. enterica SPI-2 T3SS prevents premature effector secretion at pH 5.0, a condition found within host cell vacuoles. However, upon T3SS vacuolar membrane contact, the detection of a pH increase to 7.2 in the cytoplasm leads to dissociation of the protein complex and degradation of its components, resulting in effector secretion (Yu et al., 2010). Since the proteins forming the SsaL/SpIC/SsaM complex in Salmonella are homologous to the ones forming the SepL/SepD/CesL complex in EPEC, and both protein complexes participate in the substrate specificity switch from translocators to effectors without secretion of the gatekeeper protein (Coombes et al., 2004; Younis et al., 2010; Yu et al., 2010), we argued that the mechanism by which these complexes respond to environmental cues that induce effector secretion could be similar. Therefore, we examined the effect of calcium depletion on the stability, formation, and subcellular localization of the SepL/SepD/CesL complex. To this end, we constructed a sepl-3FLAG cesL-2HA chromosomally tagged strain and showed that its T3 secretion profile is comparable to that of the wild-type strain (Figure S2). However, we were unable to obtain a functional SepD chromosomally tagged strain, because the C-terminal tag affected the protein secretion profile (data not shown). Then, we evaluated the effect of calcium depletion on the protein stability of SepL-3FLAG and CesL-2HA. Stability assays were performed as described under Materials and Methods, inhibiting de novo protein synthesis with chloramphenicol in cells grown in the presence or absence of calcium. As shown in Figure 3a, the stability
profile of SepL and CesL was similar under both conditions tested, indicating that neither of these proteins is degraded in response to a drop in calcium concentration. Intracellular protein stability of SepL-3FLAG and CesL-2HA was examined every 30 min during 3 hr after the addition of chloramphenicol, by immunodetection using anti-FLAG and anti-HA antibodies. Anti-DnaK was used as a loading control. (b) Immunoprecipitation of SepL-3FLAG from the EPEC sepL-3FLAG cesL-2HA and EPEC cesL-2HA strains grown in the presence or absence of calcium. Coimmunoprecipitation of SepD, CesL-2HA, and DnaK with SepL-3FLAG was corroborated with specific antibodies against SepD, HA-tag, and DnaK. (c) The EPEC sepL-3FLAG cesL-2HA strain grown in the presence or absence of calcium was fractionated into cytoplasmic (C) and membrane (M) fractions. Immunodetection of SepL-3FLAG and CesL-2HA in the cell lysate (Lys) and in the cytoplasmic and membrane fractions was performed using antibodies against the FLAG and HA tags. To confirm proper fractionation, samples were probed with anti-DnaK and anti-EscJ antibodies as cytoplasmic and membrane markers, respectively.
3.4 SepL interacts with EscV, a T3SS component engaged in substrate recognition

The gatekeeper protein SepL and its homologs in different bacteria have been shown to interact with diverse T3SS components (Archuleta & Spiller, 2014; Botteaux et al., 2009; Cherradi et al., 2013; Kubori & Galan, 2002; Lee et al., 2014; Roehrich et al., 2017; Shen & Blocker, 2016; Wang et al., 2008). On the basis of our previous results, we reasoned that T3SS proteins involved in substrate recognition or targeting might be prone to calcium-mediated control.

Homologous proteins of the EPEC export apparatus component EscV have been implicated in the recruitment of T3 secretion substrates and chaperones, in both the flagellar and virulence T3SSs. In Xanthomonas spp., the cytoplasmic domain of HrcV was shown to bind early and late substrates, as well as chaperones (Alegria et al., 2004; Buttner, Lorenz, Weber, & Bonas, 2006; Hartmann & Buttner, 2013), whereas the cytoplasmic domain of the flagellar export gate protein FhA, binds different chaperone/substrate complexes (Bange et al., 2010; Kinoshita, Hara, Imada, Namba, & Minamino, 2013). To investigate whether EscV might play a role in substrate recognition, we tested the ability of the C-terminal cytoplasmic domain of EscV (EscVc) to interact with secreted proteins. To this aim, His-EscVc was coupled to Ni-NTA agarose and incubated with the combined supernatants of the secretion assays of a ΔsepL mutant, which hyper-secretes effectors, and a ΔgrlR mutant, which produces and secretes more effectors and translocators (Deng et al., 2004, 2005; Wang et al., 2008). After extensive washing, proteins were eluted and analyzed by SDS-PAGE and immunoblotting. The results showed that His-EscVc interacts with both middle (EspA and EspD) and late (Tir) substrates (Figure 4 left panel). As a control, substrate binding to EscD, which is also an inner membrane protein with a cytoplasmic domain (EscDc), was evaluated under the same conditions and no interaction was observed (Figure 4 middle panel). In addition, we discarded the possibility of substrate nonspecific binding to the Ni-NTA resin (Figure 4 right panel). These results suggest that the major export gate component of the EPEC injectisome, EscV, provides a docking site for substrates prior to their T3-dependent secretion.

Next, we investigated a possible link between the gatekeeper SepL and the EscV protein, by evaluating a physical interaction between these proteins and its calcium-dependence. For this purpose, we performed a nickel affinity copurification assay by mixing the cleared lysates of cells producing His-EscVc and untagged SepL, in the presence or absence of calcium. Affinity resin-bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting. The result showed that SepL directly interacts with the cytoplasmic domain of EscV and that this interaction is not dependent on the presence of calcium (Figure 5a). SepL binding to the Ni-NTA resin was not detected (Figure 5a lower panel). As an additional control, we showed that under the conditions tested, His-EscVc does not bind to MBP-EscK (data not shown), as previously observed in a yeast two hybrid
screen (Soto et al., 2017), corroborating the specificity of the EscV-SepL interaction.

To further dissect the SepL interacting region with EscVc, we generated SepL truncated protein versions. In P. aeruginosa, the EscV homolog PcrD interacts with the Pcr1 protein, which is homologous to the C-terminal domain of SepL (Lee et al., 2014). Therefore, we generated two C-terminal truncated versions of SepL (SepL\(_{AC75}\) and SepL\(_{AC11}\)) and assessed their ability to interact with His-EscVc by in vitro pull-down assays. We found that in contrast to full-length SepL, neither SepL\(_{AC75}\) nor SepL\(_{AC11}\) interacted with His-EscVc (Figure 5b). In addition, we demonstrated that the structural integrity of the SepL\(_{AC11}\) truncated protein is not affected, since it is able to form the SepL\(_{AC11}\)/SepD/CesL ternary complex (Figure S4). These results indicate that the last 11 amino acids of SepL are crucial for its interaction with EscVc. Moreover, we further evaluated the ability of the SepL truncated versions to complement the phenotype of the ∆sepL null strain. Neither SepL\(_{AC75}\) nor SepL\(_{AC11}\) could restore the secretion of translocators, and only partially reduced the hypersecretion of effectors of the sepL mutant (Figure 5c). Therefore, our results highlight the importance of the extreme C-terminal region of SepL for substrate secretion regulation.

4 DISCUSSION

Injectisome assembly demands orderly secretion of T3 substrates. Secretion of translocators prior to effectors ensures that the latter are translocated directly into the host cell cytoplasm. In EPEC, this secretion hierarchy is proposed to be controlled by the gatekeeper SepL (Deng et al., 2004, 2005; Wang et al., 2008), and to respond to environmental cues such as calcium concentration (Deng et al., 2005; Shaulov et al., 2017). However, the exact mechanism underlying this specificity-switching process is still poorly understood. In this work,
we evaluated the link between calcium sensing and SepL hierarchical regulation.

Previous reports showed that calcium depletion from the growth medium in A/E pathogens has a differential effect on T3 substrate secretion, that is, reduced secretion of translocators and increased secretion of effectors (Deng et al., 2005; Ide et al., 2003). In accordance, we showed that the absence of calcium in the EPEC growth medium considerably increased the secretion of several LEE and Nle effectors. However, although the secretion of translocators appears to be slightly reduced in the absence of calcium, our statistical analysis demonstrated that the secretion of middle substrates is not significantly affected under this condition (Figure 1). The dissimilar results on the effect of calcium on translocator secretion could be attributed to either differences in the experimental setups for the secretion assay or to the use of chelators to eliminate calcium (Deng et al., 2005; Ide et al., 2003), given that EGTA and BAPTA bind in addition to calcium other multivalent cations, which in turn could also affect T3-dependent secretion (Gode-Potratz, Chodor, & McCarter, 2010; Kenny et al., 1997; Sarty et al., 2012). In agreement with our results, a previous report in P. aeruginosa showed that in vitro calcium levels influence effector but not translocator secretion (Cisz, Lee, & Rietsch, 2008). Hence, it is possible that secretion of translocators and effectors can be promoted by different environmental signals as recently suggested (Roehrich et al., 2017). Additionally, we extended our analysis to investigate the effect of calcium absence on the secretion of an early substrate, the inner rod protein Escl, and demonstrated that its secretion is not affected under this condition. This result supports our proposal that the calcium signal exclusively regulates the substrate specificity switch promoting late substrate secretion.

Moreover, it has been proposed that physical contact between the bacteria and host cells along with the assembly of the translocation pore, allows for detection of lowered calcium levels in the eukaryotic cell cytoplasm, triggering effector secretion (Deng et al., 2005; Lee et al., 2001). Accordingly, the translocon components would be expected to be involved in signal sensing as has been previously reported (Armentrout & Rietsch, 2016; Cisz et al., 2008; Roehrich, Guillossou, Blocker, & Martínez-Ardugo, 2013; Urbanowski, Brutinel, & Yahr, 2007). Here we demonstrated that the translocator proteins EspA, EspB, and EspD are not implicated in transducing the signal that triggers effector secretion upon calcium depletion in vitro (Figure 2). This finding is in agreement with previous reports in S. enterica and P. aeruginosa, where mutants lacking the translocators are still able to trigger effector secretion upon changes in chemical cues (Cisz et al., 2008; Yu et al., 2010). The apparent discrepancy regarding the requirement of translocators could be explained by the nature of the triggering signal. While translocators have been shown to be essential for effector translocation upon host cell contact (Armentrout & Rietsch, 2016; Cisz et al., 2008; Urbanowski et al., 2007), they are not required for induction of effector secretion under calcium depletion in vitro (Cisz et al., 2008; Lee, Stopford, Svenson, & Rietsch, 2010). Thus, under in vitro calcium-depleted conditions in EPEC, either the needle plays an active role in detecting and conveying the environmental signal as has been demonstrated for S. flexneri and Y. pestis (Deane et al., 2006; Kenjale et al., 2005; Torruellas, Jackson, Pennock, & Plano, 2005); or as recently suggested, diffusion of chemical signals through the needle channel might alter the local concentration of different ions at the base of the secretion apparatus (Notti & Stebbins, 2016; Portaliou et al., 2016; Shaulov et al., 2017; Yu et al., 2010), resulting in modifications at the T3SS basal machinery that trigger effector secretion. Nonetheless, further studies are required to elucidate the mechanistic basis of physiological signal sensing and transmission.

The deregulation of effector secretion upon calcium depletion somewhat mimics the hypersecretion phenotype seen in the absence of the SepL and SepD proteins (Deng et al., 2005). SepL and SepD form a ternary complex with Cesl that is homologous to the S. enterica SsaL/SpiC/SsaM complex (Younis et al., 2010). Besides, in contrast to other gatekeeper proteins, SepL and SsaL are not secreted, share more than 40% sequence similarity and SepL partially complements an ssaL null mutant (Coombes et al., 2004; Younis et al., 2010). For these reasons, we hypothesized that the SepL/SepD/CesL complex regulates the switch from middle to late substrates in response to environmental calcium in the same manner as the SsaL/SpiC/SsaM complex does in response to pH (Yu et al., 2010). Nevertheless, our results demonstrated that the stability, localization, and association of the SepL/SepD/CesL complex are not affected by calcium changes (Figure 3 and S3), indicating that this gatekeeper complex is not prone to direct or indirect calcium regulation. These data prompted us to investigate whether the interaction with other T3SS components might be involved in the calcium-signaling pathway.

Gatekeepers are known to interact with several T3SS components (Archuleta & Spiller, 2014; Botteaux et al., 2009; Cherradi et al., 2013; Day & Plano, 1998; Kubori & Galan, 2002; Lee et al., 2014; Roehrich et al., 2017; Shen & Blocker, 2016; Silva-Herzog et al., 2011; Stone, Johnson, Bulir, Gilchrist, & Mahony, 2008). In EPEC, besides the SepL/SepD/CesL complex, SepL binds to the effector Tir, the inner membrane ring-forming protein EscD (Wang et al., 2008), and to the switch protein EscP (Shaulov et al., 2017). Among the gatekeepers interacting partners, those involved in substrate recognition are perfect targets for secretion regulation. Substrate docking sites have been found in the cytoplasmic domain of the export apparatus component SctV and its flagellar homolog FlhA (Bange et al., 2010; Hartmann & Buttnner, 2013; Kinoshita et al., 2013; Minamino et al., 2012). Noteworthy, in the flagellar T3SS, the distinct binding affinities of FlhA for different substrate/chaperone complexes seem to coordinate the order of substrate secretion (Kinoshita et al., 2013). In this study, we demonstrated that the cytoplasmic domain of the major export gate component EscV (EscVc) is indeed capable of interacting with both middle and late T3 substrates (Figure 4). In addition, we showed that SepL interacts with EscVc, even though the interplay between these components was not regulated directly by calcium (Figure 5a). In this same regard, the SepL homologs in P. aeruginosa (PopN/Pcr1) and S. flexneri (MxiC) have been found to interact with SctV (PcrD and MxiA, respectively) (Lee et al., 2014; Shen & Blocker, 2016). In agreement with our result, the Pcr1-PcrD interaction was not sensitive to calcium removal from the growth media (Lee et al., 2014). Interestingly, a single point mutation in PcrD and MxiA that interferes with their binding to Pcr1 and MxiC,
respectively, caused a deregulation of effector secretion (Lee et al., 2014; Shen & Blocker, 2016), suggesting that the SctV-gatekeeper protein interaction is relevant for T3S regulation.

The SepL region involved in the interaction with EscV was mapped to the last 11 amino acids (Figure 5b), and we showed that this protein version, SepL\[^{ΔC11}\] is unable to rescue the phenotype of a sepL null mutant (Figure 5c). This result is similar to previous observations in enterohemorrhagic E. coli, where complementation of a sepL mutant with a SepL\[^{ΔC11}\] protein version did not recover the secretion of translocators to wild-type levels, and only marginally reduced the boosted secretion of effectors (Wang et al., 2008). These results are also in agreement with recent observations in Shigella showing that the C-terminal region of the gatekeeper MxiC is essential for substrate secretion regulation (Roehrich et al., 2017). Remarkably, the last 11 residues of SepL are also involved in EscD and Tir binding (Wang et al., 2008), suggesting a plausible model for SepL control of substrate secretion that could involve its dynamic interactions with EscD, Tir, and EscV. The interaction of SepL with Tir was proposed to delay effector secretion (since Tir secretion is required for the secretion of other effectors (Thomas, Deng, Baker, Puente, & Finlay, 2007)), while its interaction with EscD was suggested to release Tir and promote effector secretion (Wang et al., 2008). This is supported by the finding that overexpression of escD leads to hypersecretion of Tir (Ogino et al., 2006). In addition, since SepL resembles a T3 effector but without being secreted (Younis et al., 2010), it could function as a Trojan horse that, after its recognition as a T3 substrate, blocks a substrate acceptor site on EscV, or modifies the affinity of this export gate component for different substrates. Nonetheless, whether the SepL-EscV interaction participates in secretion regulation remains to be investigated.

Furthermore, it was recently shown that SepL interacts with the molecular ruler protein EscP, and that the SepL-EscP complex is stabilized by calcium, preventing effector secretion. The model proposed that once the translocation pore is assembled in the host cell membrane, the calcium flux to the T3SS base is reduced, resulting in dissociation of the SepL/EscP complex and effector secretion (Shaulov et al., 2017). Thus, the EscP switch protein, which has been shown to play a role in effector secretion control (Monjaras Feria et al., 2012), emerges as an important player in the in vitro calcium-dependent SepL secretion regulatory pathway (Shaulov et al., 2017). Besides, EscP was previously shown to bind to the cognate Tir chaperone CtsT, and suggested to act, together with Tir and the SepL-EscD complex, in the restriction of effector secretion (Monjaras Feria et al., 2012). Overall, the intricate interaction network in which SepL participates may reflect the different regulation layers required to fine-tune the timing of substrate secretion (Figure 6). However, based on previous observations and our results (i.e., neither the translocon senses the absence of calcium nor the SepL-SepD/CesL switch complex is affected by calcium changes), we suggest that the molecular mechanism triggering

![Figure 6](image-url)

**Figure 6** SepL protein–protein interaction network involved in middle and late substrate secretion regulation. (a) The SepL protein binds directly to five different T3SS components: SepD, EscP, Tir, EscD, and EscV. Although SepL forms a ternary complex with SepD and CesL (Younis et al., 2010), no direct interaction between CesL (dashed circle) and SepL or SepD has been reported. The effector Tir binds to its cognate chaperone CtsT (Elliott et al., 1999), which in turn has been demonstrated to interact with EscP (Monjaras Feria et al., 2012). Finally, the interaction between EscV and different T3S substrates is also displayed. (b) Schematic representation of the EPEC T3SS base showing the SepL binding partners described so far. SepL [green] interacts with EscD [cyan], Tir [black] (Wang et al., 2008), and EscV [red] (this work). In addition, SepL was reported to form two mutually exclusive complexes: the SepL-EscP [blue] complex, which was proposed to regulate substrate secretion in response to calcium changes in vitro (Shaulov et al., 2017); and the SepL-SepD [yellow]-CesL [orange] complex (Younis et al., 2010), whose role in substrate secretion regulation remains to be determined.
effect secretion upon in vitro calcium removal could be different from the one triggering effector translocation upon host cell contact, as outlined below.

It is noteworthy that in vitro calcium depletion acts as a common environmental cue that promotes effector secretion in phylogenetically distant bacteria (e.g., *Yersinia*, *Pseudomonas*, *Chlamydia*, *Vibrio*, and A/E pathogens) (Cisz et al., 2008; Deng et al., 2005; Gode-Potratz et al., 2010; Ide et al., 2003; Jamison & Hackstadt, 2008; Kim et al., 2005; Michiels, Wattiau, Brasseur, Ruysschaert, & Cornelis, 1990; Sarty et al., 2012; Shaulov et al., 2017), probably by inducing conformational changes in the needle structure that mimic those adopted upon host cell contact (Torruellas et al., 2005). However, by increasing host intracellular calcium levels, Cisz et al. (2008) demonstrated that the in vivo translocation of effectors is not influenced by calcium concentration changes. Therefore, a distinction should be made between induction of effector secretion in vitro and upon host cell contact. While the former can promote effector secretion in a translocator-independent mechanism (Figure 2) that involves the SepL/EscP protein complex in EPEC (Shaulov et al., 2017), the mechanism participating in effector translocation in vivo is still largely unknown. Finally, our data revealed EscV as a new player that serves as a docking site for both SepL and T3 substrates. Nevertheless, whether EscV acts in concert with the SepL/EspD/CesL regulatory switch complex to ensure the proper timing of substrate secretion remains to be determined.

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**CONFLICT OF INTEREST**

None declared.

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