Regulation of Type III Secretion Hierarchy of Translocators and Effectors in Attaching and Effacing Bacterial Pathogens


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Human enteropathogenic Escherichia coli (EPEC), enterohemorrhagic E. coli (EHEC), and the mouse pathogen Citrobacter rodentium (CR) belong to the family of attaching and effacing (A/E) bacterial pathogens. They possess the locus of enterocyte effacement (LEE) pathogenicity island, which encodes a type III secretion system. These pathogens secrete a number of proteins into culture media, including type III effector proteins and translocators that are required for the translocation of effectors into host cells. Preliminary evidence indicated that the LEE-encoded SepL and Rorf6/SepD may form a molecular switch that controls the secretion of translocators and effectors in CR. Here, we show that SepL and SepD indeed perform this function in A/E pathogens such as EHEC and EPEC. Their sepL and sepD mutants do not secrete translocators but exhibit enhanced secretion of effectors. We demonstrate that SepL and SepD interact with each other and that both SepL and SepD are localized to the bacterial membranes. Furthermore, we demonstrate that culture media influence the type III secretion profile of EHEC, EPEC, and CR and that low-calcium concentrations inhibit secretion of translocators but promote the secretion of effectors, similar to effects on type III secretion by mutations in sepL and sepD. However, the secretion profile of the sepD and sepL mutants is not affected by these culture conditions. Collectively, our results suggest that SepL and SepD not only are necessary for efficient translocator secretion in A/E pathogens but also control a switch from translocator to effector secretion by sensing certain environmental signals such as low calcium.

One of the emerging themes in bacterial pathogenesis is that diverse gram-negative pathogenic bacteria employ a conserved protein secretion machinery termed the type III secretion system (TTSS) as a major virulence mechanism to cause disease in their hosts (11). These bacteria include some of the most important and devastating human and plant pathogens in the genera Yersinia, Salmonella, Escherichia, Shigella, Pseudomonas, and Xanthomonas. The TTSS is a multiprotein complex evolutionarily related to the flagellar apparatus, and it consists of more than 20 proteins that form a so-called needle complex spanning both the inner and outer membranes of the bacterial envelope. It is postulated that the TTSS apparatus acts as a molecular syringe, injecting effector proteins (effectors) from the bacterial cytosol directly into the host cell cytoplasm, where the effectors act to facilitate bacterial proliferation and disease development (9, 11).

Although the exact numbers can vary, many pathogens secrete a large number of proteins via the TTSS, most of which fall into two categories, effectors and translocators (7, 9, 21). The effectors modulate host cellular functions and signal transduction pathways and subvert host defense mechanisms upon injection into host cells. The translocators are not needed for type III secretion (TTS) but are required for translocating effectors into host cells by assembling a translocation conduit in the host cell membrane. While each pathogen may possess a unique assortment of effectors to suit its own pathogenesis strategy and specific host, translocators are generally conserved among the pathogens. Because translocators are required for the injection of effectors into host cells, it is assumed that the pathogens have evolved mechanisms to ensure that translocators are secreted prior to effectors, so that effectors will be exported directly into the targeted host cells instead of the extracellular milieu. However, the control of this process is poorly understood (9).

TTS is thought to be triggered by direct bacterial contact with the host cell, but the nature of this contact remains controversial (9). Many environmental factors modulate TTS, inducing the secretion of effectors into culture media. For example, low-calcium conditions induce TTS of effector Yops in Yersinia spp. (29), and the dye Congo red triggers TTS of IpA proteins in Shigella flexneri (2, 39). However, whether these environmental cues serve as authentic signals during infections is unknown. Understanding the molecular mechanism of how these signals trigger TTS should lead to better appreciation of the control and regulation of TTS in bacterial pathogenesis.

Human diarrheagenic enteropathogenic Escherichia coli (EPEC), enterohemorrhagic E. coli (EHEC), the mouse pathogen Citrobacter rodentium (CR), and many EPEC and EHEC isolates from pets and farm animals belong to the family of attaching and effacing (A/E) pathogens. These pathogens cause much morbidity and mortality worldwide and represent significant threats to human and animal health (6, 36).
A/E pathogens share a pathogenicity island called the locus of enterocyte effacement (LEE) that encodes a TTSS. A needle complex similar to that found for *Salmonella* and *Shigella* spp. has been visualized in EPEC (43, 48). The needle complex of EPEC displays an extracellular filamentous extension made of EspA, one of the secreted proteins (26, 43). The LEE-encoded TTSS is essential for virulence of these pathogens (6, 15, 37). A/E pathogens secrete several LEE-encoded proteins into culture media using the LEE-encoded TTSS, including EspA, EspB, EspD, EspF, EspG, EspH, Tir, and Map (46). The secreted proteins are divided into effectors (EspF, EspG, EspH, Tir, and Map) and translocators (EspA, EspB, and EspD). In addition, A/E pathogens secrete a number of non-LEE-encoded effectors (5, 15, 23, 31, 32, 34, 37). One of them is NleA/EspI, which is targeted to the host cell Golgi and is critical for bacterial virulence (23, 35).

As for many TTSSs in other bacteria, the molecular mechanism for the hierarchy control of translocator and effector secretion in A/E pathogens is poorly understood. It has been reported that calcium modulates TTS in A/E pathogens as well (24, 25). Calcium chelation of growth medium reduces TTS of the translocators and increases the secretion of effector Tir in EPEC and diffusely adhering EPEC strains by an unknown mechanism (24, 25). We have recently shown that LEE-encoded SepL and Rorf6 differentially regulate the secretion of translocators and effectors in CR and may represent a TTS hierarchy switch (15). Rorf6 has been renamed SepD (38); based on our results, we follow this nomenclature in this report. However, there are conflicting reports with regard to the function of SepL and especially SepD in EPEC and EHEC (27, 38). While needed for translocator but not effector secretion in CR (15), SepD has been reported to be an essential element of the TTSS in EPEC, required for both translocator and effector secretion (38). In this report, we demonstrate that both SepL and SepD are essential for the secretion of translocators but not effectors and that the SepL/SepD switch from translocator secretion to effector secretion is a conserved feature in all A/E pathogens, including CR, EHEC, and EPEC. In addition, we show that calcium limitation suppresses secretion of translocators but enhances general secretion of effectors in EPEC, EHEC, and CR, suggesting a link between calcium sensing and the SepL/SepD switch.

**MATERIALS AND METHODS**

**CR strains and LEE gene deletion mutants.** The CR wild-type (WT) strain and its deletion mutants of a single LEE gene, including espA, espD, espB, *sepL*, and *sepD*, and *escN*, were described previously (14, 15). To create a triple-deletion mutant of CR *espA*, *espD*, and *espB*, two PCR fragments that covered the upstream region of *espA* and the downstream region of *espB* were amplified with PCR primer pairs (Table 1). The two PCR fragments were ligated into the KpnI/SacI-digested *sacB* gene-based suicide vector pKm8 (26, 43). The resulting plasmid was introduced into CR by electroporation, and in-frame triple-deletion mutants of *EspA*, *EspB*, and *EspD* were generated by sucrose selection as described previously (14, 18).

**Generation of *sepL* and *sepD* nonpolar deletion mutants in EHEC and EPEC.** Both *sepL* and *sepD* genes are located within polycistronic operons, at least in EPEC (34). In CR, EPEC, and EHEC O157:H7, the predicted stop codon of *sepL* overlaps in the nucleotide sequence TAAATG with the start codon of the downstream *escC* that encodes an essential component of the TTS apparatus (22), suggesting possible translational coupling. To avoid any polar effects on the expression of genes downstream of *sepL* and *sepD*, we employed the *sacB* gene-based allelic exchange method to generate in-frame *sepL* and *sepD* internal deletion mutants in both EPEC and EHEC. The suicide vector pRE118 (18) was used to generate deletion mutants in the nalidixic acid-resistant (Nal') derivative
of EHEC O157:H7 strain 86-24 and the streptomycin-resistant (Sm r) derivative of EPEC O127:H6 strain E2348/69.

Construction of plasmids for complementation of CR, EPEC, and EHEC mutants. The coding regions and the immediate upstream regions of CR, EPEC, and EHEC were amplified by PCR with the following pairs of primers: CR-espF-Nco and CR-espF-Xho (Table 1). Similarly, EPEC espF or espA gene promoter in the 2HA tagging vector (15). The primers used for PCR amplification of espF were CR-espF-Nco and CR-espF-Xho (Table 1). The resulting PCR fragments were gel purified and cloned in a three-way ligation into the restriction site of E. coli SM10Δpir by electroporation and introduced into EHEC strain 86-24 Nalr by conjugation. After sucrose selection, EHEC colonies resistant to sucrose and ampicillin were diluted 1:100 into 50 ml of LB with both antibiotics and grown at 37°C with shaking until the optical density value at 600 nm (OD600) reached approximately 0.8. Cultures were then moved to room temperature and induced with 0.5 mM isopropylthio-L-galactoside (IPTG). After overnight shaking incubation, the cells were pelleted and sonicated in 0.1 ml of 50 mM Tris (pH 8) and 150 mM NaCl. The soluble fraction after spinning at 14,000 rpm was removed and discarded. The cell suspension (0.1 ml of 50 mM Tris buffer, pH 8) was added to 5,000 rpm, the unbound fraction was removed, and four washing steps (each step, 1 ml and 10 min of mixing at 4°C) were performed with 50 ml Tris (pH 8) and 150 mM NaCl. A further series of five washing steps was done with 50 ml Tris (pH 8), 150 mM NaCl, and 20 mM imidazole used in each step. A sample was removed from the first imidazole wash. Finally the proteins were eluted from the beads with 50 mM Tris (pH 8), 150 mM NaCl, and 300 mM imidazole. All samples were analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE).

TTS assays. Secreted proteins of CR, EPEC, and EHEC were analyzed as described previously (14, 30). Briefly, bacterial strains were grown overnight in LB plus appropriate antibiotics and then subcultured at a dilution of 1:50 into prewarmed Dulbecco's modified Eagle's medium (DMEM) or modified M9 minimal medium (30). We typically grew 4 ml of culture from a single colony in calcium-free DMEM for 6 h in a 5% CO2 tissue culture incubator, the bacterial culture incubator without shaking for 6 h. Secreted proteins were precipitated with 10% trichloroacetic acid from the culture supernatant and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

Analysis of TTS of EspF in CR and EPEC sepL and sepD mutants. The coding regions and their immediate upstream regions of CR and EPEC espF genes were amplified by PCR, cloned into pCR2.1-TOPo vector, verified by DNA sequencing, and then subcloned into pACYC184 (New England Biolabs) as a BamHΙ/Sall fragment.

Bacterial fractionation and protein localization. CR SepL and SepD were tagged with a double hemagglutinin (2HA) epitope at the carboxyl termini with the vector pTOPO-2HA (15). The 2HA-tagged SepL and SepD were expressed in both CR and EPEC WT strains. Bacterial cell fractionation was carried out as described for EPEC (22). Mouse monoclonal antibodies (mAbs) against the HA tag (Covance, Princeton, N.J.) were used for Western blot detection of 2HA-epitope. Mouse polyclonal antibodies (IgG) against the EspF 2HA-epitope were used for Western blot detection of EspF-2HA.

Reverse transcription-PCR and ELISA analysis of expression of tir, espA, and espF genes. To monitor the expression of tir, espA, and espF genes, various CR, EPEC, and EHEC strains were treated similarly to the growth conditions as described above for TTS assays. After induction in DMEM or reconstructed calcium-free DMEM for 6 h in a 5% CO2 tissue culture incubator, the bacterial
RESULTS

SepL and SepD control the TTS hierarchy of translocators and effectors in CR. During our systematic mutagenesis studies of all 41 LEE genes in CR, we found that mutations in either sepL or sepD abolished TTS of translocators EspA, EspB, and EspD but significantly enhanced TTS of effectors such as Tir and NleA (15) (Fig. 1A). A double mutant of sepL and sepD exhibited the same secretion phenotype as their single mutants. However, the translocators were expressed in the sepL and sepD mutants. Not surprisingly, the sepL and sepD mutants produced and secreted, but failed to translocate, Tir into host cells as assayed by immunofluorescence microscopy (data not shown), consistent with the essential role of the translocators in translocating effectors into host cells (6, 20). Our results suggest that SepL and SepD not only are essential for translocator secretion, but also control the TTS hierarchy of translocators and effectors. As expected, both sepL and sepD were critical for CR virulence in mice (15).

SepL and SepD perform the same function in human A/E pathogens EPEC and EHEC. To extend our observations from CR to prototypical human EPEC and EHEC strains and to determine whether SepL and SepD perform the same roles in different A/E pathogens, we constructed nonpolar sepL and sepD deletion mutants in both EPEC O127:H6 strain E2348/69 and EHEC O157:H7 strain 86-24 and examined TTS of the mutants. When grown in DMEM, both EHEC and EPEC WT strains secreted the three major proteins EspA, EspB, and EspD (the translocators) (Fig. 1B and C). Under these same growth conditions, EHEC secreted the same profile, but a smaller amount, of these proteins (approximately fivefold less) than EPEC and CR (data not shown). In contrast, the sepL and sepD mutants of both EHEC and EPEC showed no secretion of the translocators but significantly increased secretion of effectors Tir and NleA that were easily detectable by SDS-PAGE and Coomassie staining (Fig. 1B and C). More-sensitive methods, such as Western blotting, were usually needed to detect these effectors secreted by EHEC and EPEC WT strains. Indeed, WT EPEC secreted at least 500-fold more EspA and EspB proteins than its sepL and sepD mutants by ELISA. The amount of EspA and EspB secreted by the EPEC sepL and sepD mutants was barely detectable even by Western blotting and was only slightly more than that secreted by the negative control type III mutant ΔescN, as estimated by ELISA (data not shown). On the other hand, the EPEC sepL and sepD mutants secreted at least 300-fold more Tir than WT EPEC (Fig. 1C; ELISA data not shown). These TTS phenotypes of the EPEC and EHEC sepL and sepD mutants were identical to those of the corresponding CR mutants (Fig. 1A) (15), indicating that SepL and SepD perform the same function in these three A/E pathogens.

We also analyzed the secretion of another LEE-encoded effector, EspF, in CR and EPEC sepL and sepD mutants, since the role of SepL and especially SepD in EspF secretion was controversial (see the introduction). By doing proteomic analysis of secreted proteins, we previously observed that the sepL and sepD mutants of both CR and EHEC were able to secrete EspF, Map, Tir, EspG, and EspH along with other non-LEE-encoded effectors such as NleA (15; S. Gruenheid, W. Deng, and B. B. Finlay, unpublished results). However, a recent report showed that an EPEC sepD mutant behaved similarly to a TTS mutant, unable to secrete either EspF or the translocator EspB (38). To further support our proteomic data, we generated constructs containing CR or EPEC espF with a C-terminal 2HA tag under the control of CR espG gene promoter and expressed CR EspF-2HA and EPEC EspF-2HA in CR and EPEC WT strains as well as their ΔescN, ΔsepL, and ΔsepD mutants. As shown in Fig. 1D, both CR EspF-2HA and EPEC EspF-2HA, as well as their ΔsepL and ΔsepD mutants, were type III secreted by WT but not the type III mutant ΔescN. In addition, enhanced secretion of CR EspF-2HA was observed in CR ΔsepL and ΔsepD mutants (Fig. 1D), although increased secretion of EspF-2HA was not as obvious in EPEC, likely due to the use of the heterologous CR espG promoter in driving the EPEC EspF-2HA expression. These results indicated that SepD, similar to SepL, is required for TTS of translocators but not effectors (including EspF) in A/E pathogens.

Complementation of CR, EHEC, and EPEC sepL and sepD deletion mutants. The sepL and sepD genes are part of the polycistronic operons LEE4 and LEE2, respectively, as shown in EPEC (34). We attempted to complement their deletion
mutants with the respective WT genes to rule out the possibility that the translocator secretion defect in the mutants was due to polar effects of the mutations on downstream genes involved in TTS. As shown in Fig. 1E to G, the secretion of translocators EspA, EspD, and EspB was restored, while the secretion of Tir and NleA was suppressed in the complemented strains of all three pathogens when grown in DMEM, resembling the secretion profiles of the respective WT strains. Thus, the mutations in the sepL and sepD mutants of CR, EHEC, and EPEC were all nonpolar. These data indicated that the hypersecretion of effectors and abolished secretion of translocators in the mutants result directly from the loss of SepL and SepD, suggesting that SepL and SepD control a switching mechanism from secretion of translocators to secretion of effectors.

A triple mutant of espA, espD, and espB in CR does not exhibit enhanced secretion of effectors. It was possible that SepL and SepD are required only for the secretion of translocators and that the significantly enhanced secretion of effectors seen in the CR, EPEC, and EHEC sepL and sepD mutants was
the result of reduced competition for the secretion channel, due to the abolished translocator secretion. To test this hypothesis, we created a triple espA, espD, and espB deletion mutant (ΔH9004espADB) in CR and analyzed its TTS of effectors in DMEM. As shown in Fig. 1A, enhanced secretion of effectors Tir and NleA was observed only in the sepL and sepD mutants but not in the single mutants of espA, espB, and espD or their triple mutant ΔH9004espADB. This demonstrated that the translocators EspA, EspB, and EspD are not required for SepL and SepD to control effector secretion, suggesting that SepL and SepD play an active role in repressing the secretion of effectors and facilitating the secretion of translocators, thereby determining the substrate hierarchy.

Both SepL and SepD modulate secretion, but not transcription, of translocators and effectors. Abolished secretion of EspA, EspB, and EspD and increased secretion of Tir and NleA by the sepL and sepD mutants could be due to differential expression or differential secretion of the proteins. To determine where SepL and SepD exert their control over the TTS of translocators and effectors, we undertook RT-PCR analysis of tir, espA, and espB transcription in WT, escN, sepL, and sepD mutants of CR, EPEC, and EHEC. No significant difference in transcription of tir, espB, or espA was found between the WT strains and their various mutants, including the type III mutant ΔescN (Fig. 2A; data not shown). This suggests that SepL and SepD control TTS of translocators and effectors at a posttranscriptional level. However, although they were expressed within the bacteria, EspB and Tir did not accumulate to greater amounts in the escN mutant when not secreted; similarly, EspB did not accumulate in the sepL and sepD mutants (15; data not shown). This suggests a possible posttranscriptional control at the level of translational regulation or an equilibrium between protein synthesis and degradation when the proteins are not secreted.

Both SepL and SepD are localized to the bacterial membranes. Our previous results have shown that neither SepL nor SepD was type III secreted in CR (15), suggesting that they function inside the bacteria. We therefore analyzed the cellular localization of both SepL and SepD in CR and EPEC. For this purpose, we tagged CR SepL and SepD at the carboxyl terminus with a 2HA tag (15) and expressed the tagged proteins in both CR and EPEC. Both constructs could complement their respective mutants (data not shown), suggesting that the 2HA tag did not affect the function and cellular localization of SepL.

FIG. 2. Transcription of effector Tir and translocator EspB in EHEC, EPEC, and CR is not affected by mutations in escN, sepD, and sepL or by calcium concentrations in the culture medium. (A) CR, EPEC, and EHEC WT and escN, sepD and sepL mutants were grown in LB and subcultured in DMEM to induce LEE gene expression. Total RNA was isolated and treated with DNase I to remove contaminating DNA. Equal amounts of RNA were used to perform RT-PCR, and PCR products were analyzed by agarose gel electrophoresis. There was no PCR product when the RNA samples were directly used for PCR without RT first, suggesting no contamination of chromosomal DNA in the RNA samples (data not shown). (B) WT CR, EPEC, and EHEC strains were grown in LB and subcultured into regular DMEM, reconstituted calcium-free DMEM, and calcium-free DMEM supplemented with 2 mM CaCl2 to induce LEE gene expression. Transcription of tir and espB was then analyzed by RT-PCR, as described above for panel A.
and SepD. Bacterial fractionation and Western blot analysis using antibodies against the HA tag showed that both SepL and SepD were present in the cytoplasmic, inner, and outer membrane fractions, with the majority localized to the inner membrane (Fig. 3). The integrity of the fractions was verified by localizing proteins known to be present in a particular bacterial fraction: MBP for the periplasm, DnaK for the cytosol, Etk for the inner membrane, and intimin for the inner and outer membranes (Fig. 3). Our results indicated that SepL and SepD have similar localization profiles in both CR and EPEC.

**SepL and SepD interact with each other.** Both sepL and sepD mutants in CR, EPEC, and EHEC display the same secretion phenotype (this study), and their double mutant has the same secretion phenotype as the single mutants in CR (Fig. 1) (15). This suggests that SepL and SepD act in concert and may interact directly. Indeed, three independent methods (bacterial two-hybrid assay, GST pulldown, and coexpression and copurification) showed that SepL and SepD interact with each other (Fig. 4). In the bacterial two-hybrid assay (data not shown), the cotransformants containing the sepL and sepD constructs conferred resistance to carbencillin at concentrations of up to 350 μg/ml, and either SepL or SepD could be expressed in either the bait or target vectors. Some carbencillin resistance was conferred with SepD expressed from both bait and target vectors, suggesting that SepD functions in a multimeric state. To verify the SepL-SepD interaction, we performed GST pulldown assays by generating N-terminal GST fusions of CR SepL and SepD to GST and expressed the fusion proteins in E. coli. As shown in Fig. 4A, GST-SepL and GST-SepD pulled down N-terminally His-tagged CR SepD and SepL, respectively, from crude bacterial lysates, whereas GST alone did not. The SepL-SepD interaction was further confirmed by a coexpression and copurification method. N-terminally His-tagged EPEC SepL was coexpressed with untagged EPEC SepD in the same E. coli host. Purification of His-SepL with nickel agarose beads also purified untagged SepD. When the reverse experiment coexpressing C-terminally His-tagged EPEC SepD and untagged SepL was performed, untagged SepL was copurified with SepD-His as well during nickel affinity purification (Fig. 4B and C). The nickel agarose beads failed to bind either untagged SepD or untagged SepL when they were expressed alone in the same host (data not shown). Collectively, these results showed convincingly that SepL and SepD interact with each other and form a protein complex, consistent with our data showing that they had similar cellular localization within the bacteria (Fig. 3).

**Culture media influence TTS profiles of A/E pathogens, partially mimicking the effects of sepL and sepD mutations.** We observed that EHEC O157:H7 strains exhibit drastically different secretion profiles in different culture media such as DMEM and modified M9. As shown in Fig. 5, EHEC predominantly secreted translocators EspA, EspD, and EspB in DMEM. However, the secretion of the translocators was much reduced in M9, while greatly enhanced secretion of effectors (Tir and NleA) was observed. EPEC (Fig. 5) and CR (data not shown) WT strains showed similar changes in profiles of TSS proteins when grown in DMEM and M9. On the other hand, the sepL and sepD mutants of EHEC, EPEC, and CR exhibited the same secreted protein profile in both DMEM and M9 (Fig. 5; data not shown for CR). Interestingly, the secretion profile of WT EHEC, EPEC, and CR in M9 showed significant similarities to that of their sepL and sepD mutants in both DMEM and M9. The only detectable difference was that the WT
strains secreted some EspA, EspB, and EspD in M9, while the sepL and sepD mutants did not (Fig. 5). These data showed clearly that DMEM promotes translocator secretion and suppresses effector secretion, while M9 has the opposite effect. In addition, our results indicated that induction in M9 mimics the effects of the loss of SepD and SepP function on TTS, suggesting that SepL and SepD regulate TTS hierarchy of translocators and effectors in response to environmental cues and growth conditions.

Calcium concentrations differentially modulate secretion, but not transcription, of translocators and effectors in CR, EPEC, and EHEC. We used several approaches to investigate what chemical components in DMEM and M9 media accounted for the differential effects on secretion of translocators and effectors. The two most obvious differences in chemical compositions between M9 and DMEM are CaCl2 and NH4Cl concentrations. While DMEM contains ~1.8 mM of Ca2+ and no NH4+, M9 has ~18 mM NH4+ and no Ca2+. Addition of NH4+ into DMEM had a small effect on TTS, but did not change the overall secretion profile of EHEC (data not shown). To analyze the effect of calcium concentration on TTS, various concentrations (1 to 4 mM) of EGTA and the more calcium-specific chelator BAPTA were added to DMEM. As shown in Fig. 6, EHEC, EPEC, and CR all displayed similar secretion profiles in calcium-free DMEM and DMEM containing 1.5 to 2 mM of calcium chelators. Adding 1 to 2 mM of CaCl2 into calcium-free DMEM restored translocator secretion to the levels seen in regular DMEM and suppressed secretion of Tir and NleA. This switch from effector secretion to translocator secretion could also be seen when 1 to 2 mM of CaCl2 was added into the modified, calcium-free M9 medium (data not shown). Taken together, these results indicated that calcium concentrations in culture media influence the levels of secreted translocators and effectors differentially and may act as a substrate switch signal for TTS of translocators and effectors.

The differential effect of calcium concentrations on TTS of translocators and effectors could be exerted during either gene expression or protein secretion. To distinguish between these
two possibilities, we analyzed by RT-PCR the mRNA levels of \( \text{tir} \) and \( \text{espB} \) isolated from CR, EPEC, and EHEC grown in regular DMEM, calcium-free DMEM, and calcium-free DMEM supplemented with 2 mM \( \text{CaCl}_2 \). No significant difference in transcription of \( \text{tir} \) and \( \text{espB} \) was detected (Fig. 2B), suggesting that calcium concentrations in growth media affect specifically secretion but not transcription of translocators and effectors.

\( \text{SepL} \) and \( \text{SepD} \) do not need each other for stability, and calcium concentrations do not affect \( \text{SepL} \) and \( \text{SepD} \) interactions. Because \( \text{SepL} \) and \( \text{SepD} \) interacted with each other (Fig. 4), it was possible that one of them served as a stabilizing chaperone for the other. Indeed, \( \text{SepD} \) exhibits certain characteristics of type III chaperones (9, 10; data not shown). To determine whether \( \text{SepL} \) and \( \text{SepD} \) need each other for stability inside bacteria, the 2HA-tagged CR \( \text{sepL} \) and \( \text{sepD} \) were introduced and expressed in various mutants of CR. As mentioned before, these constructs complemented their respective mutants, indicating that the C-terminal 2HA tag did not affect the normal function of \( \text{SepL} \) and \( \text{SepD} \). It was observed that both \( \text{SepL-2HA} \) and \( \text{SepD-2HA} \) were present at similar levels in all the mutants tested (data not shown), including \( \Delta \text{escN} \), \( \Delta \text{sepL} \), \( \Delta \text{sepD} \), and the \( \Delta \text{sepL}\Delta \text{sepD} \) double mutant, suggesting that \( \text{SepL} \) and \( \text{SepD} \) did not need each other for stability. It also suggests that turnover of \( \text{SepD} \) or \( \text{SepL} \) may not be required for substrate switch during translocator and effector secretion.

We demonstrated that TTS by \( \text{sepL} \) or \( \text{sepD} \) mutants did not respond to calcium concentrations in growth media (Fig. 5 and data not shown). We also showed that the effect of chelating calcium in growth media partially mimicked that of mutations in \( \text{sepL} \) or \( \text{sepD} \) (Fig. 6). These results suggest that there may be a link between \( \text{SepL}/\text{SepD} \) and sensing of calcium concentrations. We therefore examined whether calcium concentrations affect \( \text{SepL} \) and \( \text{SepD} \) stability and their interactions. Similar levels of functional \( \text{SepL-2HA} \) and \( \text{SepD-2HA} \) were detected in WT CR as well as its \( \Delta \text{escN} \), \( \Delta \text{sepL} \), \( \Delta \text{sepD} \), and \( \Delta \text{sepL}\Delta \text{sepD} \) mutants in DMEM both in the presence and in the absence of \( \text{CaCl}_2 \) (data not shown). In addition, \( \text{SepL-2HA} \) and \( \text{SepD-2HA} \) were not type III secreted in either regular DMEM or calcium-free DMEM (data not shown). However, small amounts of \( \text{SepL-2HA} \) and \( \text{SepD-2HA} \) could be detected by Western blotting in culture supernatants of both WT CR and its \( \Delta \text{escN} \) (type III) mutant grown in either regular DMEM or calcium-free DMEM (data not shown). This was consistent with our data that both \( \text{SepL-2HA} \) and \( \text{SepD-2HA} \) could be detected in bacterial outer membrane fractions (Fig. 3). We also examined whether calcium concentrations affected the interaction and complex formation between \( \text{SepL} \) and \( \text{SepD} \). \( \text{SepL} \) and \( \text{SepD} \) bound to each other in vitro and formed a

![FIG. 6. Calcium concentrations in culture media affect TTS of translocators and effectors differentially in EHEC (A), CR (B), and EPEC (C).](http://iai.asm.org/...)

WT strains of EHEC, EPEC, and CR were grown in LB and then subcultured into regular DMEM, DMEM supplemented with various concentrations of EGTA and BAPTA, calcium-free DMEM, or calcium-free DMEM supplemented with 1 to 2 mM \( \text{CaCl}_2 \). Secreted proteins were concentrated from the bacterial culture supernatant and analyzed by SDS-PAGE and Coomassie blue G250 staining. Proteins secreted by an equal number of bacteria, as estimated by \( \text{OD}_{600} \) values, were loaded in each lane of the same gel. The locations of translocators (EspA, EspB, and EspD) and effectors (Tir and NleA), as well as EspC (EPEC) and EspP (EHEC), are indicated on the right. Predicted Ca\(^{2+}\) concentrations (in millimoles) in growth media are indicated at the bottom of each lane.
stable protein complex (Fig. 4; data not shown). The presence or absence of various concentrations of CaCl₂ did not have any effect on the SepL-SepD interaction or their complex formation (data not shown).

**DISCUSSION**

Bacterial pathogens employing a TTSS often secrete a large number of proteins (4, 7, 9, 21). Due to the large number of proteins trafficking through the TTS apparatus, a secretion hierarchy is presumably needed for their orderly exodus. The secreted proteins consist of both effectors and translocators, with the latter needed for translocating effectors into host cells. It is therefore pivotal that pathogens secrete translocators before effectors. In addition, different effectors secreted may function at different stages of infection, and some of them may even have opposing functions, suggesting a need for hierarchical and temporal controls over their secretion. Although some progress has been made, TTS hierarchy remains poorly understood (1, 9, 45).

In this study, we analyzed the function of *sepL* and *sepD* genes in three prototypical strains of A/E pathogens, EPEC strain E2348/69, EHEC strain 86-24, and CR. We showed that mutations in *sepL* and *sepD* resulted in abolished secretion of translocators but significantly enhanced secretion of two effectors Tir and NleA. Furthermore, the *sepL* and *sepD* mutants exhibit highly similar secretion phenotypes in all three A/E pathogens. This strong conservation of the SepL and SepD function is consistent with the fact that SepL and SepD are among the most conserved proteins encoded by the LEE from different A/E pathogens, with SepL and SepD showing more than 90 and 88% identity, respectively (13, 19, 40, 44, 50).

The function of SepL and SepD is a matter of controversy in the literature. It has been reported that a *sepL* mutant of EHEC strain EDL933 secretes no translocators but increased p54/NleA (27), similar to our CR, EPEC, and EHEC *sepL* mutants (15; this study). While the manuscript was in preparation, O’Connell et al. reported that an EPEC *sepL* mutant secretes significantly reduced amount of translocators, but increased secretion of effectors by the mutant was not reproducibly seen (38). These phenotypes are different from those of our EHEC, EPEC, and CR *sepL* mutants (15, 27; this study). These discrepancies could be due to the different growth conditions used for TTS assays. O’Connell et al. grew EPEC strains in DMEM in an air incubator with shaking (33, 38). In our assays, however, EPEC strains were grown in DMEM in a CO₂ (5%) tissue culture incubator without shaking, conditions that promote TTS in EPEC and EHEC and have been used by many laboratories (24, 27, 31). Since our CR, EPEC, and EHEC *sepL* mutants all consistently secreted larger amounts of effectors than their respective WT strains, we believe that SepL plays the same role in all A/E pathogens.

We have shown that SepL and SepD regulate secretion hierarchy of translocators and effectors in A/E pathogens. Many other TTSSs regulate the secretion of translocators and effectors differentially, although the mechanisms used can be variable. There are no strong homologues of SepL and SepD encoded by other TTSSs, except for SsaL of *Salmonella* pathogenicity island 2 (SPI-2). SsaL has more than 40% sequence similarity to SepL, and there is evidence that SsaL plays a role in regulating secretion of *Salmonella* SPI-2-encoded translocators (8). Two additional SPI-2-encoded proteins, SsaM and SpiC, appear to regulate the secretion of translocators and effectors similar to SepL and SepD (49). InvE of the *Salmonella* SPI-1-encoded TTS may also play a role similar to that of SepL or SepD. The invE mutation significantly reduces, but does not abolish, TTS of translocators; it slightly increases TTS of some effectors (28). These phenotypes are different from the *sepL* and *sepD* mutants that have abolished secretion of translocators and significantly enhanced secretion of effectors. It has been shown that InvE interacts with a protein complex formed by translocators and their cognate chaperones, but it does not interact efficiently with the individual components of this complex (28). We are investigating the potential interactions of SepL and SepD with the translocators EspA, EspB, and EspD and their chaperones.

In *S. flexneri*, certain translocators such as IpaB can outcompete effectors for the secretion channel, as mutants of these translocator genes exhibit deregulated and increased secretion of a number of proteins, many of which are effectors (4, 39). Our results show that SepL and SepD are required for the secretion of translocators by A/E pathogens. The significantly enhanced secretion of effectors seen in the *sepL* and *sepD* mutants could therefore be the result of reduced competition for the secretion channel, due to the blocked translocator secretion. However, this is not the case, since a triple deletion mutant (ΔespADB) in CR of all the translocator genes secretes normal, not increased, levels of effectors (Fig. 1A); this suggests that SepL and SepD also play a role in suppressing effector secretion under conditions favorable for translocator secretion.

In addition to the bacterial genetic elements discussed above, environmental factors also influence the secretion hierarchy of translocators and effectors. This has been intensively studied in *Yersinia* spp., especially the role of calcium (10). TTS of *Yersinia* Yops occurs in a sequential manner, secreting first the translocators YopBD and followed by the effectors YopEHIJMOT (29). Secretion of the translocators is activated by albumin and other serum proteins in the presence of calcium, whereas secretion of the effectors is triggered by a low-calcium environment (10, 29). How calcium chelation triggers secretion of effector Yops is still not understood. It has been proposed that *Yersinia* species may use the TTS apparatus, which forms a hollow conduit connecting the bacteria to the host cell cytoplasm, as a tool to sense...
and measure host intracellular calcium concentration to signal the transport of Yops (29, 41). According to this hypothesis, the relatively high calcium concentration (about 2.5 mM) in the mammalian host extracellular fluid (10) suppresses TTS of the Yops. On the other hand, the calcium concentration in both the eukaryotic host cell cytoplasm and the bacterial cytosol is estimated to be low, ranging from 100 and 300 nM, and most of the calcium in eukaryotic cells is sequestered in the endoplasmic reticulum (3, 16). Upon contact with host cells, the needle complexes of the Yersinia TTSS, which are assembled prior to host cell contact, are inserted into eukaryotic cells and allow the bacteria to sense the change in calcium concentrations, thereby activating Yop secretion.

In this report, we have confirmed the observation that calcium chelation reduces TTS of the translocators and increases the secretion of effector Tir in EPEC strains (24, 25) and analyzed the effect of calcium on TTS in EPEC in greater detail. In addition, we extended our observations to other important A/E pathogens, namely EHEC and CR. Our results suggest that regulation of secretion by calcium is a conserved mechanism for TTSSs. In calcium-rich media, A/E pathogens secrete mostly translocators and very small amounts of effectors. When calcium is limited, translocator secretion is significantly reduced, while effector secretion is greatly enhanced (Fig. 5 and 6). While calcium concentrations in culture media have opposite effects on the secretion of translocators and effectors in WT A/E pathogens, their sepL and sepD mutants are calcium blind and secrete no translocators but significantly increased effector levels both in the presence and in the absence of calcium (Fig. 1 and 5). This suggests that SepL and SepD may be linked to calcium sensing. It is worth noting that in Yersinia, secretion of translocators occurs regardless of calcium concentrations and that calcium depletion triggers secretion of all Yops, both translocators and effectors. In addition, calcium-blind mutants in Yersinia, such as a yopN mutant, secrete translocators as well as effectors (10, 29, 41), unlike the sepL and sepD mutants of A/E pathogens that secrete greatly increased effectors but no translocators. Our results suggest that A/E pathogens use calcium concentrations not only to regulate TTS of effectors, but also to control the secretion hierarchy of translocators and effectors.

Based on these observations, we have modified the calcium-signaling model in Yersinia suggested by Schneewind and colleagues (29, 41) and propose that TTS in A/E pathogens occurs in two phases during infection. In the first phase, when the A/E pathogens enter their host via ingestion, host gastrointestinal conditions activate LEE gene expression, leading to the assembly of TTS apparatus in the bacterial membranes. In the presence of calcium in the extracellular fluid of the intestinal lumen, the bacteria first secrete the translocators EspA, EspB, and EspD, allowing the assembly of a translocation pore (translocon) on the host cell membrane. The translocon and the TTS apparatus form a conduit connecting the bacterial cytosol and the host cytoplasm, where calcium is limited. In the second phase of TTS, the bacteria detect the low-calcium environment in the host cell via the translocon and the TTS apparatus, which signals the docking of the bacteria onto a host cell. Low calcium suppresses secretion of the translocators, and activates secretion of the effectors. This differential effect of calcium on translocator secretion and effector secretion ensures that effectors are secreted before effectors and that effectors are efficiently translocated into host cells. The regulation of secretion of translocators and effectors is likely to occur at the posttranscriptional level, since we cannot detect significant differences in expression of effectors and translocators by RT-PCR in WT A/E pathogens and their sepD, sepL, and escN mutants or when the bacteria are grown in calcium-rich or calcium-free media (Fig. 2). Our results are consistent with published reports showing that secretion of translocators in EHEC and EPEC is regulated by growth media at the posttranscriptional level (12, 24, 42).

It is not yet clear what senses calcium in A/E pathogens. SepL and SepD are good candidates for this role, since loss of either protein renders the TTS of effectors insensitive to calcium concentration (Fig. 5). As single mutations in sepD or sepL have the same effect on TTS as their double mutant (15), SepD and SepL may perform their function as a complex. Indeed, we and others have shown that SepL and SepD interact with each other by several independent methods (Fig. 4) (38). It does not appear that either SepL or SepD acts as a chaperone, since the stability of either protein is not affected by the absence of the other (data not shown). Both SepL and SepD have been shown to associate with the bacterial membranes (Fig. 3) (27). We propose that SepL and SepD together serve as gatekeepers, instead of as plugs, of the TTSS, since mutations in either sepL or sepD still selectively allow the secretion of effectors to occur. However, we have so far failed to establish a direct link between SepL/SepD and calcium sensing. Calcium does not affect the interaction between SepL and SepD or their secretion and stability in bacteria (data not shown). It is still possible that calcium concentrations affect interaction or engagement of SepL and SepD with an as-yet-unidentified component(s) of the TTS apparatus. It should be pointed out that low calcium may not be the only signal for secretion substrate switch from translocators to effectors, as calcium-free media significantly reduce, but do not abolish, TTS of translocators, unlike mutations in sepL or sepD. These data suggest that, in addition to calcium, other environmental signals also play a role in regulating TTS. We are currently exploring these possibilities. We believe that understanding the molecular mechanism of how SepL and SepD control the secretion of translocators and regulate the secretion hierarchy of translocators and effectors should have broad implications for other TTSSs.

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