Diarrhoea is the second leading cause of death in children under the age of five. The bacterial species, *Vibrio cholerae* and enteropathogenic *Escherichia coli* (EPEC), are among the main pathogens that cause diarrhoeal diseases, which are associated with high mortality rates. These two pathogens have a common infection site—the small intestine. While it is known that both pathogens utilize quorum sensing (QS) to determine their population size, it is not yet clear whether potential bacterial competitors can also use this information. In this study, we examined the ability of EPEC to determine *V. cholerae* population sizes and to modulate its own virulence mechanisms accordingly. We found that EPEC virulence is enhanced in response to elevated concentrations of cholera autoinducer-1 (CAI-1), even though neither a CAI-1 synthase nor CAI-1 receptors have been reported in *E. coli*. This CAI-1 sensing and virulence upregulation response may facilitate the ability of EPEC to coordinate successful colonization of a host co-infected with *V. cholerae*. To the best of our knowledge, this is the first observed example of 'eavesdropping' between two bacterial pathogens that is based on interspecies sensing of a QS molecule.
environment and LuxO is active. HapR levels are accordingly low, and the expression of virulence and biofilm formation genes is high. At high bacterial population densities, the concentration of the AIs increases, resulting in activation of the QS receptors and repression of LuxO. The HapR expression level is raised, resulting in the repression of virulence and biofilm formation genes. This multi-factor coordination is critical to the ability of V. cholerae to colonize its host when the bacterial population density is low and to adapt bacteria for transmission once it becomes critically high.

A pathogen that shares a common infection site with V. cholerae is the bacterial species enteropathogenic E. coli (EPEC). This bacterial pathogen, similarly to V. cholerae, colonizes the small intestine and causes severe diarrhoea, mostly in infants. EPEC utilizes several QS systems to monitor cell density. One such system is a variant of the LuxI/R system, which is used by most Gram-negative bacteria. This system was first discovered in V. fischeri, although, notably, it is not present in V. cholerae. LuxI is responsible for the synthesis of N-acyl-homoserine-lactones (AHLs), while LuxR is the AHL receptor. E. coli strains, including EPEC, lack the luxI gene and therefore cannot synthesize AHLs. However, they encode the SdiA protein, a LuxR homolog that recognizes a wide range of AHLs synthesized by other bacterial species and respond to it by activating acid resistance genes and repressing virulence genes.

An additional QS system found in EPEC is the AI-2 system. While some studies have reported that the AI-2 system acts on virulence and motility genes, more recent studies have suggested that virulence and motility are controlled by a different signalling molecule, designated AI-3. Indeed, the AI-3 pathway is associated with virulence and flagellar genes. The AI-3 system acts on virulence and biofilm formation genes, and hence to upregulate its virulence when V. cholerae virulence is downregulated, thereby optimizing the timing for successful colonization of the small intestine.

**Results**

EPEC alters its T3SS activity when grown in co-culture with V. cholerae. To reveal the cross-talk between V. cholerae and EPEC, both of which colonize the small intestine, we cultured wild-type (WT) EPEC in the presence of V. cholerae and then examined the EPEC T3SS activity. To support both V. cholerae growth and the induction of EPEC T3SS, which requires growth conditions that simulate those in the human gastrointestinal tract, the bacterial strains were grown statically in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM) and Luria-Bertani (LB) medium in a tissue culture incubator (with 5% CO2). We evaluated EPEC T3SS activity by determining the levels of two EPEC translocators, EspA and EspB, found in the bacterial supernatants. We also determined the expression levels of EscJ, which is a structural component of the T3SS apparatus, and of the effector protein Tir, which is expected to be retained within the bacterial cells in the absence of host cells. Plain LB, without a bacterial inoculum, and co-culture of EPEC with E. coli DH10B strain were used as controls.

The supernatant fraction of the EPEC and V. cholerae co-culture showed elevated levels of EspA and EspB compared to their levels in the supernatant of an EPEC pure culture (Fig. 1). As expected, the supernatant of the V. cholerae pure culture was negative for both EspA and EspB, thereby excluding the possibility that the anti-EspA and anti-EspB antibodies react with a V. cholerae component. Examination of the expression levels of EscJ and Tir within the bacterial pellets showed higher expression of the two T3SS proteins in the co-culture sample of EPEC and V. cholerae relative to the pure culture of EPEC. The bacterial pellet of the V. cholerae pure culture was negative for anti-EscJ and anti-Tir, thereby confirming that these antibodies are specific to EPEC T3SS components. DNAK levels within the bacterial pellets demonstrated equal loading (Fig. 1). To determine whether the T3SS-related virulence of EPEC is specifically upregulated in response to V. cholerae, we co-cultured WT EPEC with E. coli DH10B and examined EPEC T3SS activity. The supernatant of the EPEC and E. coli DH10B co-culture showed levels of T3SS translocators similar to those in the supernatant of the EPEC pure culture, while no signal was observed for the E. coli DH10B pure culture (Fig. 1). In addition, the bacterial pellet of the EPEC and E. coli DH10B co-culture showed expression levels of EscJ and Tir similar to those of the EPEC pure culture. These findings suggest that the T3SS activity of EPEC is upregulated specifically in response to V. cholerae and not simply in response to the presence of another bacterial strain.

Finally, to show that the upregulated T3SS activity observed in the co-culture of EPEC and V. cholerae was not due to a higher bacterial count of EPEC, we determined the number of colony forming units (CFUs) in both co-cultures and pure cultures. To facilitate separation between EPEC and V. cholerae or E. coli DH10B, we grew
the bacteria in Transwells (Merck MultiScreen filter plate 0.22 μm), which allow the passage of molecules but not bacterial cells. After 6 h growth in 1:1 (v/v) DMEM:plain LB mixture, EPEC was collected and spotted at 10-fold serial dilutions on LB plates containing streptomycin. We observed a ~100-fold decrease in EPEC CFUs when EPEC was co-cultured with either *V. cholerae* or *E. coli* DH10B relative to the number of CFUs in an EPEC pure culture (Fig. S1). These results confirmed that the upregulation of the T3SS activity observed in the EPEC and *V. cholerae* co-culture sample was not due to higher EPEC counts.

**Altered T3SS activity of EPEC in response to exposure to a *V. cholerae* supernatant.** To determine whether the T3SS activity of EPEC is upregulated in response to *V. cholerae* signalling molecules secreted into the *V. cholerae* growth medium, we cultured *V. cholerae* in LB medium overnight and separated the supernatant from the bacterial cells by centrifugation and filtration. To assess whether EPEC can detect the signalling molecules of *V. cholerae* and respond to them by alteration of its T3SS activity, we added a purified *V. cholerae* supernatant to the growth medium of WT EPEC in a 1:1 ratio (Fig. 2A). Plain LB and the supernatant of an *E. coli* DH10B culture were used as controls.

WT EPEC grown in a 1:1 ratio of DMEM:*V. cholerae* culture supernatant showed elevated levels of translocator secretion (EspA and EspB) relative to EPEC grown in a 1:1 (v/v) DMEM:plain LB mixture or in a DMEM:*E. coli* DH10B culture supernatant (Fig. 2B). A similar upregulation pattern was observed for the expression of the T3SS proteins, EscJ and Tir, when WT EPEC was grown in DMEM:*V. cholerae* culture supernatant relative to growth in DMEM:plain LB or DMEM:*E. coli* DH10B culture supernatant, all in a 1:1 ratio (Fig. 2C). DnaK levels demonstrated equal loading of bacterial lysates. These findings suggested that the T3SS activity of EPEC is upregulated in response to secreted components present in the supernatant of *V. cholerae* cultures.

**Effect of synthetic CAI-1 on EPEC virulence and growth.** Since CAI-1 is the primary QS molecule of *V. cholerae*, we examined whether the addition of synthetic CAI-1 (for chemical structure, see Fig. 3A) would induce an effect on the T3SS activity of EPEC similar to that of the *V. cholerae* supernatant or to co-culture with *V. cholerae* (the relative levels of CAI-1 in the supernatants of *V. cholerae* and *E. coli* DH10B are presented in Fig. S2). To this end, we synthesized CAI-1 and examined its effect on the T3SS activity of WT EPEC. In this set of experiments, we grew WT EPEC strain in DMEM alone, as is commonly done when examining the T3SS activity of EPEC. We added to these WT cultures micromolar concentrations of CAI-1, based on the concentrations of CAI-1 under natural conditions, which vary between ≤1 μM in planktonic cultures of *V. cholerae* and 32 μM in *V. cholerae* biofilms. The secreted proteins were analyzed by loading the concentrated supernatants onto SDS-PAGE and visualizing all the T3SS translocators (EspA, EspB, and EspD) by Coomassie staining. The ΔescN EPEC strain, a T3SS ATPase mutant that is devoid of T3SS activity, was used as a negative control. When CAI-1 was added to the WT EPEC culture at concentrations of 5 μM or higher, we observed elevated secretion of T3SS components into the extracellular medium compared to a sample supplemented with DMSO alone (the solvent for the stock solution of CAI-1, 1% (v/v); Fig. 3B). This result suggests that the elevated EPEC T3SS activity that...
we observed for EPEC and \textit{V. cholerae} co-culture and for the EPEC strain grown in the presence of \textit{V. cholerae} supernatant are most probably related to the ability of EPEC to detect and respond to CAI-1—whether natural or synthetic. To better observe the effect of synthetic CAI-1 on EPEC, we added 2, 5, 10 and 25 μM CAI-1 to WT EPEC grown in 1:1 DMEM:plain LB. These conditions do not induce a maximal T3SS response and therefore allow better detection of T3SS upregulation activity. Analysis of the supernatants of WT EPEC in the presence of synthetic CAI-1 showed dose-dependent elevation of EspA and EspB relative to WT EPEC supplemented only with 0.25% (v/v) DMSO (Fig. 3C). A similar dose-dependent pattern was observed for the expression of Tir and EscJ within the bacterial pellets of WT EPEC grown in the presence of CAI-1 (Fig. 3D). We estimate that the CAI-1 concentration in the \textit{V. cholerae} supernatant (Fig. 2B,C) was 10–25 μM, based on the EspA, EspB, Tir and EscJ levels observed when WT EPEC was incubated with known concentrations of CAI-1 (Fig. 3C,D).

To examine whether the effect of CAI-1 on EPEC T3SS activity is related to the effect of the AI on bacterial growth, we monitored the optical density of WT EPEC samples grown under T3SS inducing conditions [static growth in 1:1 (v/v) DMEM:plain LB; 5% CO₂; Fig. 4] and non-T3SS inducing conditions (LB medium; shaking; data not shown) in the presence of 25 μM CAI-1 or 0.25% (v/v) DMSO. Regardless of the growth conditions, we did not observe any effect of CAI-1 on the growth rate of EPEC (Fig. 4 and data not shown). This result contradicts the findings of other studies showing that bacterial AIs, such as CAI-1, play a role in slowing down the growth rate as the bacterial culture enters the stationary phase\textsuperscript{27,36,37}. Here, we observed a specific case in which the bacteria did indeed sense the presence of the AI in the medium, but reacted to it with an induction of virulence mechanisms but not with a retardation in growth rate. Examination of the effect of CAI-1 on EPEC motility and biofilm formation, which were previously shown to be affected by QS\textsuperscript{34}, revealed no effect of CAI-1 on these factors (Fig. S3).

**Effect of synthetic CAI-1 on the transcription of T3SS genes.** Since CAI-1 is known to act through gene regulation in \textit{V. cholerae}\textsuperscript{11,17,18,38,39}, we examined whether addition of CAI-1 to a WT EPEC culture would enhance EPEC virulence through upregulation of T3SS genes. To this end, we cultured WT EPEC under semi-optimal T3SS-inducing conditions [static growth in 1:1 (v/v) DMEM:plain LB; 5% CO₂] in the presence (25μM) or the absence [0.25% (v/v) DMSO supplement] of CAI-1 and evaluated the transcription levels of four...
representative T3SS genes that are encoded on different operons within the LEE, namely, \(\text{tir}\), which encodes the first translocated effector of the T3SS (LEE5), \(\text{espA}\) and \(\text{espB}\), the genes that encode the T3SS translocators \(\text{EspA}\), \(\text{EspB}\) and \(\text{EspD}\) are indicated to the right of the gel. Also indicated is the location of \(\text{EspC}\), which is an autotransporter that is also secreted during EPEC infection. We observed that addition of CAI-1 at a concentration of 5 μM or higher enhanced EPEC T3SS activity. (C) and (D) WT EPEC was grown in 1:1 (v/v) DMEM:plain LB mixture for 6 h in the presence or the absence of synthetic CAI-1 (2, 5, 10 and 25 μM). The secreted proteins were concentrated as described above and analyzed by SDS-PAGE and western blot analysis using anti-\(\text{EspB}\) and anti-\(\text{EspA}\) antibodies (C) while the bacterial pellets were analyzed by SDS-PAGE and western blot analysis using anti-\(\text{Tir}\), anti-\(\text{EscJ}\) and anti-DnaK antibodies (D).

**Effect of synthetic CAI-1 on the ability of EPEC to translocate proteins into host cells.** To evaluate the ability of CAI-1 to enhance EPEC virulence, we examined the level of the effectors’ translocation from WT EPEC in host cells in the presence or absence of CAI-1. For this purpose, we used the translocation assay developed by Charpentier and Oswald [40]. In brief, EPEC strains carrying an EspZ-TEM-1 chimeric protein were grown under T3SS-inducing conditions with or without CAI-1 and then used to infect HeLa cells for 90 min. Thereafter, the HeLa cells were washed and stained with CCF2/AM, which is a TEM-1 β-lactamase substrate. When excited at wavelength of 409 nm, uninfected HeLa cells exhibited green fluorescence, indicating
the absence of β-lactamase activity, while HeLa cells infected with the WT EPEC exhibited blue fluorescence, with the green to blue shift (530 → 460 nm) being caused by CCF2 cleavage by the EspZ-TEM-1 chimeric protein translocated into the host cells by the T3SS of WT EPEC (Fig. 6A). Similarly, we observed that WT EPEC grown in the presence of 25 μM CAI-1 exhibited a significantly elevated level of effector translocation compared to the same strain grown without CAI-1 [with 0.25% (v/v) DMSO supplement alone] (Fig. 6B). As expected, the escN null mutant, which is a T3SS ATPase mutant, exhibited very low translocation ability, due to its inability to secrete and translocate effector proteins (Fig. 6B). These results suggest that high levels of CAI-1 are detected by WT EPEC and prime the bacteria for virulence.

To confirm the effect we observed for CAI-1 on the translocation activity of EPEC, we performed an additional set of experiments using an alternative assay. For these experiments, we modified the method developed by Baruch et al., which monitors the activity of the natural EPEC effector known as NleD upon its translocation into the host cells, where it cleaves the host protein JNK41. To determine the effect of CAI-1 on the translocation activity of EPEC, we infected HeLa cells with WT EPEC in the presence of 25 μM CAI-1 or with 0.25% (v/v) DMSO and examined the cleavage pattern of JNK. To allow detection of enhanced translocation activity, we used a short infection time, which prevented full degradation of cellular JNK by WT EPEC. The HeLa culture infected with WT EPEC showed a clear degradation of JNK, in contrast to the uninfected HeLa sample and the sample infected with the ΔescN mutant strain (Fig. 6C). However, when we infected HeLa cells with WT EPEC that had been incubated with 25 μM CAI-1, we observed higher level of JNK degradation compared to infection of WT EPEC without CAI-1 (Fig. 6C), thereby providing further support for the notion that CAI-1 enhances EPEC virulence.
Effect of synthetic CAI-1 on EHEC and *Salmonella* type III secretion. To determine whether the CAI-1 detection and response mechanism is found in other gastrointestinal bacterial pathogens, we examined the effect of CAI-1 on the T3SS activity of EHEC O157:H7 and *Salmonella enterica* serovar Typhimurium (SL1344). For that purpose, we cultured the bacterial strains under T3SS-inducing conditions in the presence of CAI-1 (25 μM) or 0.25% (v/v) DMSO, and collected and analyzed their supernatants by Coomassie blue staining or by western blot analysis using an anti-EspA antibody for EHEC or anti-SigD for *Salmonella*. No obvious effect was observed on the EHEC or *Salmonella* T3SS secretion levels (Fig. 7). To examine whether the addition of CAI-1 to EHEC or *Salmonella* cultures upregulated their T3SS genes, we extracted RNA from the bacteria grown in the presence or absence of CAI-1 and analyzed the transcription levels of three representative T3SS genes (*espA*, *espB* and *tir* in EHEC and *sipB*, *sipC*, and *avrA* in *Salmonella*). A twofold increase of transcription was detected.
only for the espA gene of EHEC in the presence of CAI-1 (Fig. 7A). These results suggest that the ability to sense and respond to the V. cholerae QS molecule, CAI-1, is not ubiquitously distributed among all human intestinal bacterial pathogens.

Discussion
CAI-1 is the primary AI of V. cholerae, and together with AI-2, it regulates the transcription levels of virulence and biofilm formation genes. This process ensures the coordinated behavior of V. cholerae populations that optimizes the ability of the pathogen to infect the small intestine or move on to another host when conditions are optimal. However, the small intestine does not play host to V. cholerae alone: in addition to the commensal microbiota, other pathogens, such as EPEC, may also be present in the small intestine. Therefore, in order to successfully colonize their host, intestinal pathogens need to monitor both the environmental conditions, which promote bacterial adaptations to the specific niches, and the presence of other – competing or cooperating – pathogens, which will affect their chances to successfully colonize the host.

In this study, we examined whether EPEC can sense and respond to the presence of V. cholerae. We hypothesized that if such a sensing and responding mechanism does exist, it would promote upregulation of virulence genes in the presence of the competing bacterial species so as to enable successful competition. Surprisingly, we found that EPEC virulence is upregulated when the concentration of CAI-1 indicates that V. cholerae’s virulence is actually downregulated. This mechanism probably allows optimization of the ability of EPEC to colonize its host just as V. cholerae prepares to leave it. Recently – most probably due to improved technologies for bacterial diagnosis in stool samples – it has indeed become evident that in patients with diarrheal disease there is high prevalence of mixed infections with two or more agents44–46. Similarly, it has been reported that 15–30% of patients...
hospitalized with diarrhoeal diseases are concomitantly infected with *V. cholerae* and EPEC or enterotoxigenic *E. coli*, a related *E. coli* strain that also infects the small intestine.47,48

We observed a mild increase of EPEC virulence at CAI-1 concentrations representative of those in planktonic cultures (∼2.5–5 μM) and a more pronounced effect when concentrations were similar to the concentration measured in *V. cholerae* biofilms (∼30 μM).23,28 Although microscopic examination of *V. cholerae* in the intestinal mucosa of rabbits and two-photon microscopy studies of mouse intestines revealed in-vivo formation of biofilm aggregates of *V. cholerae*, the exact CAI-1 concentration within the small intestine biofilm is unknown.

A recent study that examined the cross-talk between human gut microbiota and *V. cholerae* found that *Ruminococcus obeum*, which is a member of the healthy individual’s microbiota, represses *V. cholerae* colonization by causing *V. cholerae* virulence genes to be downregulated.51 That study suggested that *R. obeum* synthesizes AI-2 molecules that repress virulence mechanisms and colonization factors of *V. cholerae*. Although this result was to be expected, since AI-2 is synthesized and detected by *V. cholerae* through its LuxP/Q system, the researchers suggested that AI-2 acts through an as-yet uncharacterized mechanism, since the colonization levels of ΔluxP and WT *V. cholerae* were not significantly different in the presence and absence of *R. obeum*.31 Other studies have reported that AI-2 produced by host microbiota species upregulates several major virulence genes of *Pseudomonas aeruginosa*, which does not produce AI-2 itself.24,25 Those studies therefore demonstrated that the QS communication between host microbiota and bacterial pathogens is inhibitory in nature, whereas the communication between bacterial pathogens revealed by our results was designed to promote bacterial infection.

While it has been suggested that AI-2 functions as an interspecies communication signal, CAI-1 is commonly considered to function mainly as an intra-genus communication signal, since the CqsA/CqsS system is almost exclusively restricted to Vibrio species and is highly conserved in this genus.13–15 CqsA/CqsS displays low signal discrimination, and multiple CAI-1 variants can activate these circuits at different intensities. Here, we found that CAI-1 can detect and respond to both endogenic and synthetic CAI-1. Nevertheless, since homologues of the CAI-1 synthase (CqsA) or the CAI-1 receptor (CqsS) have never been reported in EPEC, further investigation is required to identify the proteins that are involved in the response to CAI-1 in EPEC. It is possible that EPEC does have a receptor to CAI-1, but not the synthase enzyme, similarly to the LuxR/I system in *E. coli*. Such a receptor would allow EPEC to respond to CAI-1 without being able to produce it.

QS in different strains of *E. coli*, which has mostly been studied in EHEC, evokes a wide range of responses to AIs. These include upregulation of virulence genes by the self-produced AI-3 and the host hormones epinephrine or norepinephrine, while the *E. coli* AI-1 has the reverse effect on virulence and represses gene expression.19 This wide range of responses allows the bacterial cells to respond to complex environments and integrate large amounts of information regarding the density of the *E. coli* bacterial population and the composition of bacterial and host cells. In this study, we found that this complexity is not merely a function of the composition of QS molecules in the extracellular medium, as the same molecule, CAI-1, can induce contradictory responses in different bacterial species (e.g., *V. cholerae* versus EPEC). This observation suggests that bacterial communication is dependent not only on the nature of the AIs but also on the particular responding bacteria.

Our findings that CAI-1 upregulates virulence in EPEC but not in the closely related *E. coli* strain, EHEC, or the gastroenteritis pathogen, *Salmonella*, suggest that the ability to detect and respond to CAI-1 has evolved in EPEC due to its common niche – the small intestine – with *V. cholerae*. The ability of EPEC to detect both the presence and the virulence status of *V. cholerae* has possibly improved the fitness of EPEC, as this ability reduces its competition with *V. cholerae* and enables it to time its infection so as to increase its chances to successfully colonize its host. This strategy of a QS-based mechanism that allows coordination between potentially competing bacterial pathogens may well be a broader phenomenon that occurs in other pathogens that infect common environmental niches, and presents enticing new research avenues.

**Materials and Methods**

**The bacterial species, deletion mutant and plasmid.** The bacterial species and the plasmid used in this study are listed in Table 1. The bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics [streptomycin (50 μg/mL) or tetracycline (12.5 μg/mL)] at 37 °C, with shaking. For infection assays, WT and ΔescN EPEC strains were transformed with a previously described pCX341 plasmid containing EspZ fused to the mature form of TEM-1 β-lactamase.52 The ΔescN EPEC strain is a T3SS ATPase mutant that is devoid of T3SS activity.

**Bacterial co-culture.** WT EPEC, *V. cholerae*, and *E. coli* DH10B cultures were grown separately overnight at 37 °C (EPEC and DH10B) or at 30 °C (*V. cholerae*) in LB broth. The overnight cultures were then each diluted 1:40 into mixture of 1:1 (v/v) DMEM:plain LB medium and grown together in a tissue culture incubator (with 5% CO2) statically for 6 h, and their optical density at 600 nm (OD600) was measured. The cultures were centrifuged at 18000 × g for 10 min to collect the bacteria, the pellets were dissolved in SDS-PAGE sample buffer, and the supernatants were separated out and then filtered through a 0.22-μm low protein binding filter. The volumes of the supernatants and the bacterial pellets were normalized according to the OD600 of the bacterial cultures to ensure equal loading of the samples. To precipitate the proteins secreted into the culture medium, the supernatants were treated with 10% (v/v) trichloroacetic acid overnight at 4 °C.

**T3SS activity assay.** EPEC strains were grown overnight at 37 °C in LB broth with appropriate antibiotics. The cultures were diluted 1:40 in either DMEM or in 1:1 (v/v) DMEM:plain LB supplemented with either 0.25–1% (v/v) DMSO or various concentrations of CAI-1 and grown in a tissue culture incubator (with 5% CO2) statically for 6 h; the optical density at 600 nm (OD600) of the cultures was measured. The cultures were centrifuged at 18000 × g for 10 min to collect the bacteria, the pellets were dissolved in SDS-PAGE sample buffer, and the supernatants were separated out and then filtered through a 0.22-μm low protein binding filter. The volumes
of the supernatants and the bacterial pellets were normalized according to the OD_{600} of the bacterial cultures to ensure equal loading of the samples. To precipitate the proteins secreted into the culture medium, the supernatants were treated with 10% (v/v) trichloroacetic acid overnight at 4 °C. The secreted proteins were analyzed on 12% SDS-PAGE gels and stained with InstantBlue (Expedeon) or analyzed by western blotting.

To test the effect of bacterial supernatant fractions on EPEC T3SS activity, *V. cholerae* and *E. coli* DH10B were grown separately in LB broth overnight at 30 °C and 37 °C, respectively. The cultures were then centrifuged at 10000 × g for 5 min; supernatants were collected, centrifuged at 18000 × g for 10 min to remove the remaining cell debris and filtered through 0.22-μm filters. WT EPEC was inoculated 1:40 into 1:1 mixtures of either DMEM:plain LB or into DMEM:filtered bacterial supernatants (*V. cholerae* or *E. coli* DH10B) and continued as described above for evaluating T3SS activity in the presence of CAI-1.

The T3SS activity of EHEC was evaluated in a similar manner to that described for EPEC. We typically cultured 4-mL cultures for EPEC strains and 8-mL cultures for EHEC, which secreted much smaller amounts of proteins than EPEC.

T3SS activity of the *Salmonella* pathogenicity island 1 (SPI-1) was determined as described previously. Briefly, the bacteria were grown in LB broth overnight at 37 °C. The cultures were diluted 1:40 into fresh LB, which approximates SPI-1-inducing conditions, supplemented with either 0.25% (v/v) DMSO or 25 μM CAI-1 and grown for 4 h. Then, the culture supernatants were collected and filtered, and their protein content was precipitated with 10% (v/v) trichloroacetic acid overnight at 4 °C. The samples were then centrifuged at 16000 × g for 30 min and washed with cold acetone, and the precipitated proteins were then dissolved in SDS-PAGE sample buffer.

**Western blotting.** Samples were subjected to SDS-PAGE and transferred to nitrocellulose or PVDF membranes. Blots were blocked for 1 h in 5% (w/v) skim milk-PBST (0.1% Tween 20 in PBS) and then incubated with the primary antibody diluted in 5% skim milk-PBST for 1 h at room temperature. The optimal dilution for each antibody was calibrated individually. The secondary antibodies were diluted in 5% skim milk-PBST, incubated with the blots for 1 h at room temperature and detected with ECL reagents. The following commercial antibodies were used: mouse anti-DnaK (Abcam), mouse anti-JNK (BD Pharmingen), and mouse anti-actin (MPBio). Antibodies directed against T3SS components were a generous gift from Prof. B. Brett Finlay (University of British Columbia, Canada) and included mouse anti-EspA, mouse anti-EspB, rat anti-EscJ, mouse anti-Tir, and rabbit anti-SigD. Secondary antibodies were HRP-goat anti-mouse (Abcam) and HRP-goat anti-rat (Jackson ImmunoResearch).

**Chemical synthesis.** Full synthetic methodologies to obtain racemic CAI-1, based on the synthesis of deuterated CAI-1, are described in the Supplementary Methods.

**EPEC cultures.** EPEC was grown overnight in LB in a shaking incubator at 37 °C. Bacterial growth was then measured under semi-optimal T3SS-inducing conditions by inoculating WT EPEC into pre-warmed 1:1 (v/v) DMEM:plain LB containing 0.25% (v/v) DMSO or 25 μM CAI-1. Bacterial growth was measured by following the OD_{600} of the cultures growing statically in a tissue culture incubator (with 5% CO₂). At least three independent experiments were conducted, and average values are presented.

**RNA extraction and qPCR analysis.** Bacteria were grown overnight in LB broth in a shaking incubator at 37 °C. WT EPEC was diluted 1:50 into pre-warmed 1:1 (v/v) DMEM:plain LB, while WT EHEC was diluted 1:50 into pre-warmed DMEM supplemented with either 0.25% (v/v) DMSO or 25 μM CAI-1 and grown statically in a tissue culture incubator (with 5% CO₂) for 2 h (early exponential growth phase). *Salmonella* was diluted 1:50 into fresh LB medium supplemented with either 0.25% (v/v) DMSO or 25 μM CAI-1 and grown in a shaking incubator at 37 °C. Bacteria (5 × 10⁸) were collected and subjected to RNA extraction with the NucleoSpin Bacterial RNA isolation kit according to the manufacturer’s guidelines (Macherey-Nagel). From each sample, 200 ng of RNA were taken for cDNA synthesis by ProtoScript II First Strand cDNA Synthesis Kit (NEB) using random primer mix. cDNA was examined for genomic DNA contaminations, subjected to additional DNase I treatment, and extracted using TRIzol reagent when needed. The sequences of the primers used for the qPCR experiments are presented in Table 2. Melting curve analysis was used to ensure the specificity of each primer pair. RT-qPCR

| Species | Description | Reference |
|---------|-------------|-----------|
| Wild-type enteropathogenic *E. coli* (EPEC) | EPEC strain E2348/69, streptomycin resistant | 38 |
| EPEC, ΔescN | Non-polar deletion of escN | 39 |
| *Vibrio cholerae* | *Vibrio cholerae* biotype El-Tor serotype Inaba O1 In ET-122 (+) | 40 |
| *Citrobacter rodentium* DBS100 | *C. rodentium* ATCC 51459 | 41 |
| *Salmonella enterica* | Serovar Typhimurium (SL1344) | 42 |
| Enteroohaemorrhagic *E. coli* (EHEC) | O157:H7 strain 86–24, nalidixic acid resistant | 42 |

**Table 1.** Species and plasmid used in this study.
The obtained data was analyzed by LightCycler 480 software to extract the critical threshold (CT) value. Translocation efficiency was expressed as the emission ratio of the cleaved CCF2 (blue; 460 nm) to original CCF2 (green; 530 nm) according to the manufacturer's instructions. Three independent experiments were performed.

Emissions at 460 nm and 530 nm were recorded (SpectraMax Paradigm; Molecular Devices). Relative TEM-1 gene expression was quantified using the relative quantification method. Real-time data are presented as the fold change in expression.

Translocation assay. Translocation of TEM-1 fusions into HeLa cells was carried out as previously described, with some modifications. Briefly, a day before the experiment, HeLa cells were seeded in black 96-well plates at a concentration of 2 × 10^4 cells/well, and WT EPEC and EPECΔescN strain expressing the EspZ-TEM-1 fusion protein were grown overnight at 37 °C in LB. The bacteria were diluted 1:50 into DMEM containing 0.25% (v/v) DMSO or 25 μM CAI-1 for 2.5 h to OD600 of 0.1 under T3SS-inducing conditions before being used to infect HeLa cells at a multiplicity of infection of 1:100. Thirty minutes after the addition of the bacteria to the HeLa cells, EspZ-TEM-1 expression was induced by 1 mM of isopropyl-β-d-1-thiogalactopyranoside (IPTG) for an additional hour. Cell monolayers were then washed twice with HBSS (Biological Industries) and incubated for an additional hour. Cell monolayers were then washed twice with PBS, collected, and lysed with RIPA buffer. The samples were centrifuged at maximum speed for 5 min to remove unlysed cells, and the supernatants were collected, mixed with SDS-PAGE sample buffer, and subjected to western blot analysis with anti-JNK and anti-actin antibodies (loading control). Uninfected samples and the ΔescN mutant strain infected samples were used as negative controls.

To confirm the translocation ability of EPEC, by using an additional translocation assay, we modified the protocol previously described. Briefly, HeLa cells were infected for 60 min with EPEC strains and the ΔescN strain that had been pre-induced for 2 h for semi-optimal T3SS activity (pre-heated 1:1 DMEM:plain LB mixture, statically, in a CO2 tissue culture incubator). HeLa cells were then washed with PBS, collected, and lysed with RIPA buffer. The samples were centrifuged at maximum speed for 5 min to remove unlysed cells, and the supernatants were collected, mixed with SDS-PAGE sample buffer, and subjected to western blot analysis with anti-JNK and anti-actin antibodies (loading control). Uninfected samples and the ΔescN mutant strain infected samples were used as negative controls.

Data Availability
No datasets were generated or analyzed during the current study.

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| Gene     | Primer name | Primer sequence | Reference              |
|----------|-------------|-----------------|------------------------|
| rpoA     | qPCR_rpoAF  | GGCGGCTACCTCTCTCTGGAT | Modified based on ref.54 |
|          | qPCR_rpoAR  | GGCGGCTACCTCTCTGGTG |                        |
| espB EPEC| qPCR_EspBF  | GGCTTCTTTGCTGCCATTAATACC |                        |
|          | qPCR_EspBR  | TCTGCTGCATCTGCAATACC |                        |
| espA EPEC| qPCR_EspAF  | GTCGGGAATGCGAGACTTTGCAGC | This study             |
|          | qPCR_EspAR  | TTGAGCGCCCTGAAACACCGG |                        |
| tir EPEC | qPCR_TirF   | GGACTCTTGTATTTCTGGTGT | This study             |
|          | qPCR_TirR   | GTCGCCGGTAAACAAATAACCT |                        |
| escF EPEC| qPCR_EsclF  | GCAAGCAGCTGTTCTATCCA | This study             |
|          | qPCR_EsclR  | GCTTGGGTGGGAAATAAACTC |                        |
| espB EHEC| qPCR_EspBH_F| GCTTCGGAGAGTACGAGCCG | This study             |
|          | qPCR_EspBH_R| CGCGCCTGCGAATCTGAGTCG |                        |
| espA EHEC| qPCR_EspAH_F| CCAAGCGCAAAAGATGGCC | This study             |
|          | qPCR_EspAH_R| CCGCCCTTCAGCTTTGCGAC |                        |
| tir EHEC | qPCR_TirH_F  | GGCCTGTAAAGAATTCTATGGC | This study             |
|          | qPCR_TirH_R  | GCCCTGTAAAGATCTCGAGCGG |                        |
| sipB     | qPCR_SipBF  | GGAAACAAAGTCCGGCGGAGG | 64                     |
|          | qPCR_SipBR  | TGAGACAGCGGAAACATCGCG |                        |
| sipC     | qPCR_SipCF  | AACCCCATTTAGCGGAGCAG | 64                     |
|          | qPCR_SipCR  | TGCCGTCGTATTAGCGCAT |                        |
| avrA     | qPCR_AvrAF  | TTGTAGGCCCTGCGAAAGTG | 65                     |
|          | qPCR_AvrAR  | CAGATTCACGCGCTTCCATT |                        |

Table 2. Primers used for real-time-PCR.
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Author Contributions

O.G., L.S. and K.Y. designed and performed the biological experiments, N.L. synthesized CAI-1, O.G., L.S., N.L., M.M.M. and N.S. analyzed the data and wrote the manuscript. All authors reviewed and approved the manuscript for publication.

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