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Functional cloning of *Vibrio parahaemolyticus* type III secretion system 1 in *Escherichia coli* K-12 strain as a molecular syringe

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ABSTRACT

The type III secretion system (T3SS) of gram-negative bacteria involves dedicated protein translocation machinery that directly injects proteins into target cells. Pathogenic bacteria already benefit from this unique system. The successful functional cloning of this useful tool into non-pathogenic bacteria would help establish novel clinical and basic biotechnology strategies in areas such as vaccine administration, the development of screening systems for anti-T3SS drugs and the target-specific delivery of bioactive compounds. In this study, we successfully cloned the *Vibrio parahaemolyticus* T3SS1 genetic locus into a non-pathogenic *Escherichia coli* K-12 strain. Assays performed here revealed that the T3SS1 cloned into the *E. coli* K-12 strain has the ability to translocate *V. parahaemolyticus* T3SS1 secreted proteins. Importantly, we also observed this system to allow the *E. coli* K-12 strain to inject foreign protein, as well as the *V. parahaemolyticus* T3SS effector, into cultured cells. These results demonstrate a prospective useful tool with experimental and therapeutic applications.

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1. Introduction

Targeting bioactive molecules to specific human cells or organs is quite fascinating in many ways [1]. Vaccine development, anticancer therapy and other clinical treatments are all amenable to such systems. Various targeting systems have been developed. However, no perfect systems exist that are applicable to all conditions, and the development of novel technology has been anticipated. One of the potential systems for this purpose is a bacterial type III secretion system (T3SS) [2]. The T3SS is composed of the sophisticated protein translocation machinery found in gram-negative bacteria that allows them to inject virulence factors, or so-called 'effectors', directly into host cells. The T3SS thus contributes substantially to the establishment of a bacterial niche in the host. The details of the protein secretion mechanism and structures have been extensively studied, and at present, the minimal components required to establish the functional T3SS are known. The T3SS consists of two components: the needle and the basal body. The needle is the conduit for proteins secreted from the inside to the outside of the bacteria, whereas the basal body is the platform required to prepare the proteins for the secretion [3]. The establishment of this complicated system requires the expression of more than 20 genes. However, successful functional cloning of this useful tool, especially in non-pathogenic bacteria, is beneficial for many researchers seeking to develop novel clinical and basic application strategies in areas such as vaccine administration, the development of a screening system for anti-T3SS drugs and the target-specific delivery of bioactive compounds [4–6]. In this study, we successfully constructed the cloned *Vibrio parahaemolyticus* T3SS1 genetic locus into a non-pathogenic *E. coli* K-12 strain. A translocation assay revealed that the cloned T3SS had the ability to inject foreign proteins tagged with the region essential for secretion of the T3SS1 effector, VepA, as well as that of the proteins secreted by *V. parahaemolyticus* T3SS1, providing a prospective molecular syringe.

2. Materials and methods

2.1. Bacterial strains and growth conditions

V. parahaemolyticus RIMD2210633 [7] and its derivative strains were employed as genomic DNA sources of the T3SS1-related

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gene-coding region (Fig. 1) [8,9]. A non-pathogenic *E. coli* K12 derivative strain, VCS257 (Agilent Technologies, Santa Clara, CA) was used for the cosmid transduction and as a host strain of the cloned T3SS. Bacterial cultures were grown at 37 °C in 3% LB medium (1% bacto tryptone, 0.5% yeast extract, 3% NaCl) for *V. parahaemolyticus* and LB medium (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl) for *E. coli*.

2.2. Cell culture

The human epithelial cancer cell line derived from the cervical adenocarcinoma cells, HeLa cells, and the human colon cancer epithelial cell line Caco-2 were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

2.3. DNA manipulation

Cloning of the T3SS1-related gene-encoding locus into the SuperCos 1 cosmid (Agilent) was carried out as follows. Genomic DNA extracted from the *V. parahaemolyticus* strains was digested with the restriction enzymes *Pac*I and *Bam*HI and was then treated with Antarctic Phosphatase (New England Biolab, Ipswich, MA). SuperCos 1 was digested with *Xba*I, then treated with Antarctic Phosphatase, and finally digested again with *Bam*HI. The resultant genomic DNA and SuperCos 1 were ligated and packaged in a lambda phage using GigaPack III XL packaging extract (Agilent). After their transduction into the *E. coli* VCS257, cosmids with the correct insertion were confirmed with colony PCR using primers to amplify the genes located in the T3SS1 locus (*vepA* and *vcrD1*). The 5′- and 3′-regions of the inserted DNA were also sequenced.

For the translocation assay, two reporter plasmids were constructed as follows. PCR-amplified $vepA_{1-300}$ was ligated into the *Eco*RV-site on pACYC184 (Cm^r), followed by the insertion of β-lactamase TEM-1 gene (*blaM*) from pCX340 [10], to produce the reporter plasmid expressing TEM-1 tagged with $VepA_{1-100}$ (pTEM-1). The PCR-amplified gene for VecA, which is the VepA specific chaperone of T3SS1, was introduced upstream of the TEM-1 fusion on the plasmid pTEM-1 using the In-Fusion Advantage PCR cloning kit (Clontech, Mountain View, CA). As a result, the reporter plasmid bicistronically expressed TEM-1 tagged with $VepA_{1-100}$ and VecA (pTEM-1/VecA).

2.4. Secretion assay

The bacterial culture was grown in LB medium at 37 °C for 18 h and subcultured for 6 h. The bacterial culture was separated by centrifugation into the culture supernatant and the bacterial pellet. The supernatant was filtered with a 0.22-μm pore filter; 10% sodium deoxycholate and ice-cold 100% trichloroacetate were then added to final concentrations of 0.1% and 10%, respectively. The samples were kept on ice for 1 h and then centrifuged at 14,000 rpm for 20 min at 4 °C. The precipitates were washed with ice-cold acetone followed by centrifugation at 14,000 rpm for 10 min at 4 °C. The resulting precipitates from the supernatants and bacterial pellets were applied to SDS–PAGE, followed by Western blotting using antisera against VopD1 and VepA immunized in rabbits [11] or an anti-GroEL antibody (MBL, Nagoya, Japan).

2.5. Cytotoxicity assay

The CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) was used for the cytotoxicity assay. In brief, culture cells were grown in 96-well microplates with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 in air. Bacterial strains were then added at a multiplicity of infection (MOI) of 100. At 10 h of infection, the supernatants were collected and the amount of LDH (lactose dehydrogenase) was quantified using the kit. Phase contrast microscopy (TE2000-U, Nikon) was also performed on cells used in the cytotoxicity assay to examine the cell morphology.

2.6. Translocation assay

Reporter plasmids expressing TEM-1 tagged with $VepA_{1-100}$ (with/without VecA (VepA chaperone)) were introduced into *E. coli* VCS257 strains harboring a series of cloned T3SS1 cosmids. Transformants were grown in LB medium for 18 h and added to the medium-growing HeLa cells on collagen-coated glass cover slips in 24-well microplates at an MOI of 100. After 10 h of incubation, the cells were washed with PBS and loaded by CCF2-AM (LiveBLAzer FRET-B/G loading kit; Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The cells were washed with PBS and fixed with 3% paraformaldehyde. The translocation of the reporter fusion was observed under fluorescent microscopy (TE2000-U, Nikon, Tokyo, Japan) equipped with a UV-1A filter (360–370 nm excitation, 420 nm long-pass emission) and a FITC filter (460–500 nm excitation, 510–560 nm emission). Effector-translocation percentages were calculated as the numbers of cells with blue fluorescence under the UV-1A filter (translocated) divided by the numbers of cells with green fluorescence under the FITC filter (all cells). Experiments were performed at least three times.

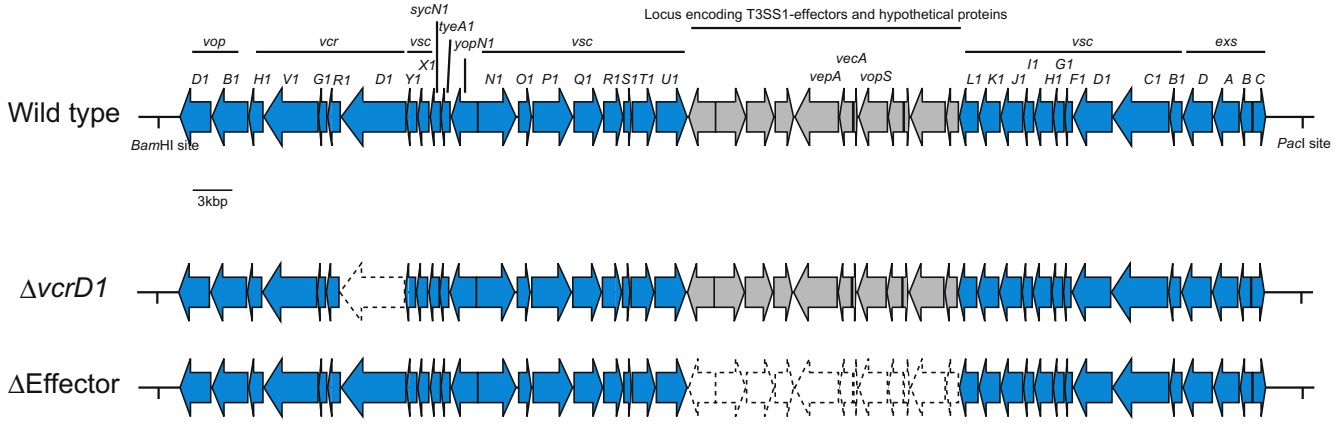

Fig. 1. T3SS1 genetic locus of *Vibrio parahaemolyticus* RIMD2210633. Genes are named after previous reports and the T3SSs of other pathogenic bacteria. This locus is similar to the T3SSs of *Yersinia* spp., except for the region depicted with gray. Gray-colored genes encode T3SS effectors, their chaperones or hypothetical proteins.

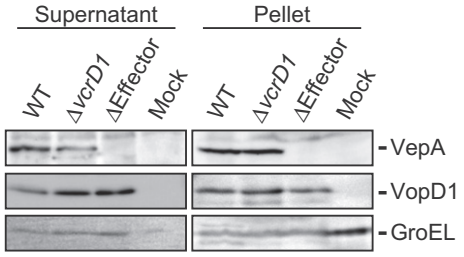

Fig. 2. Secretion assay of *E. coli* K-12 strains carrying cloned T3SS1. *E. coli* strains carrying cosmids encoding cloned wild type T3SS1 (WT), secretion-deficient T3SS1 (Δ*vcrD1*) or Effector-less T3SS1 (ΔEffector) were grown in LB medium. Culture supernatants and bacterial pellets were applied to Western blotting using antisera against VepA (T3SS1 effector), VopD1 (T3SS1 translocon) and GroEL (marker for the leakage of cytoplasmic proteins and equal loading in each bacterial pellet).

2.7. Statistical analysis

All data are expressed as the means ± standard deviations. A one-way ANOVA and Tukey's multiple comparison test were used to analyze the data. P values < 0.05 are considered significant.

3. Results

3.1. Cloning of the T3SS1 genetic locus of V. parahaemolyticus

To construct non-pathogenic *E. coli* expressing T3SS, there are many candidates as a source for T3SS genetic locus. This locus can be constructed using any T3SS from a variety of pathogenic bacteria. However, the locus should be harmless and have no adverse effects in target cells when this molecular syringe is employed as a tool. From this point of view, the T3SS genetic locus must at the minimum establish a protein secretory apparatus. Our previous studies revealed that the genome of food-borne pathogen *V. parahaemolyticus* contains two sets of T3SS-coding loci (T3SS1 and T3SS2) [7], and these systems contribute the pathogenicity of this organism [8,12]. The T3SS2 locus is responsible for enterotoxicity and contains genes for a number of T3SS effectors, as well as components of the T3SS secretory apparatus. This region shares a mosaic structure of the genes encoding the T3SS components, effectors and hypothetical genes. Therefore, it was difficult to clone only the genes necessary to build the T3SS apparatus without involving the T3SS effectors or other hypothetical proteins [7,13]. Another T3SS of *V. parahaemolyticus*, T3SS1, is encoded in the genetic cluster on chromosome 1 of *V. parahaemolyticus*, and it appeared more compact and ordered than the genetic locus encoding T3SS2 [7]. In T3SS1 genetic locus, there is one specific region (hypothetical region) encoding T3SS effectors (VepA (aka VopQ) [11,14], VepB (aka VopS) [11,15] and hypothetical effector VP1683) possessing pathogenic properties [9], whereas no effectors were identified outside of this hypothetical region on chromosome I despite our extensive screening for T3SS1 effectors. This finding suggests that deleting only this region from the T3SS1 genetic locus generates a sufficient gene-set of T3SS components that constitute a secretory apparatus able to secrete and inject proteins into target cells (Fig. 1). The locus encoding T3SS1 was located between the *Pac*I and *Bam*HI sites on chromosome I of *V. parahaemolyticus* RIMD2210633. Therefore, genomic DNA extracted from wild type, the strain with the deletion of the T3SS-effector encoding region (ΔEffector (Δh-1)) [9], and the T3SS-deficient strain (Δ*vcrD1*) were digested with *Pac*I and *Bam*HI and then ligated with the cosmid vector SuperCos 1. After transduction of the resultant DNA into the *E. coli* K-12 derivative strain VCS257, successful constructs of the T3SS1 locus into SuperCos 1 were confirmed by PCR and DNA sequencing and then used for further experiments.

3.2. Protein secretion by E. coli carrying the cloned T3SS1 genetic locus

A series of cosmids carrying the T3SS1 genetic locus were examined for their capability to secrete T3SS1 effectors. As shown in Fig. 2, the strain with the wild type T3SS1 demonstrated VepA (T3SS1 effector) and VopD1 (T3SS1 translocon) in the culture supernatant, and the strain with the effector-less T3SS1 (ΔEffector) locus showed VopD1 in the culture supernatant, as expected. However, the T3SS-deficient strain (Δ*vcrD1*) also demonstrated VopD1 in the supernatant. This finding might suggest that this cloned T3SS was built improperly and lost its T3SS-specific secretion capability. To explore this possibility, an endogenous cytoplasmic protein, GroEL, was detected using an anti-GroEL antibody on same samples from the secretion assay. GroEL was used as a leakage marker of cytoplasmic proteins usually not detected in the supernatants of bacterial cultures of *V. parahaemolyticus* under T3SS-inducing conditions. Supernatants showed a GroEL band except the one from the strain carrying the empty cosmid (Mock). This result indicated that the expression of cloned T3SS1 might influence

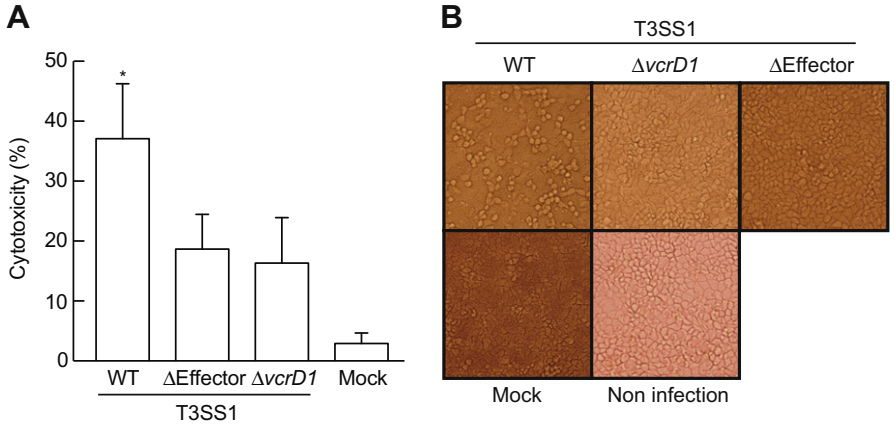

Fig. 3. Cytotoxicity of cloned T3SS1. (A) Cytotoxicity of *E. coli* K-12 strains carrying cosmids encoding cloned wild type T3SS1 (WT), secretion-deficient T3SS1 (Δ*vcrD1*) or Effector-less T3SS1 (ΔEffector). *E. coli* strains were incubated with Caco-2 cells in 5% CO_2 at 37 °C for 10 h, and the cytotoxicity was measured by the release of LDH from Caco-2 cells. Caco-2 cells lysed with lysis buffer were considered to represent 100% cytotoxicity. *P < 0.05. (B) Phase-contrast micrographs of infected Caco-2 cells with *E. coli* K-12 strains carrying cosmids encoding cloned wild type T3SS1 (WT), secretion-deficient T3SS1 (Δ*vcrD1*) or Effector-less T3SS1 (ΔEffector). Caco-2 cells were incubated with *E. coli* strains under the conditions shown in (A) and examined under phase-contrast microscopy (magnification x100).

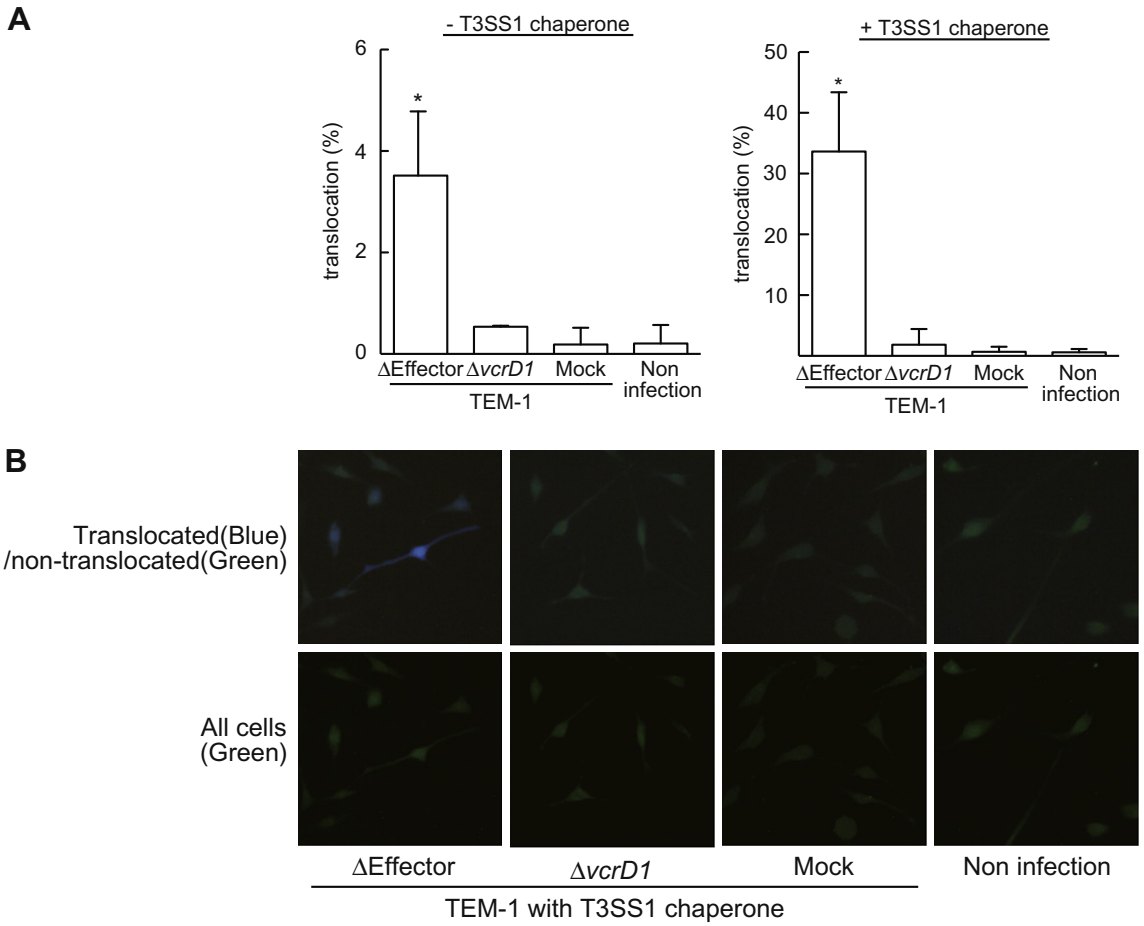

Fig. 4. Translocation of TEM-1 into HeLa cells by cloned T3SS1. (A) Translocation of TEM-1 by cloned T3SS1 with T3SS1-specific chaperones. *E. coli* strains carrying cosmids encoding cloned Effector-less T3SS1 (ΔEffector) or secretion-deficient T3SS1 (Δ*vcrD1*) with the reporter plasmids encoding TEM-1 tagged with $VepA_{1-100}$, with/without the VepA-chaperone VecA. *E. coli* strains were incubated with HeLa cells for 3 h at 37 °C, followed by CCF2-AM staining. Cleavage of CCF2-AM by translocated TEM-1 in the culture cells changed the original green fluorescent emission (520 nm) to blue (447 nm) under the UV-1A filter of fluorescent microscopy. All cells with CCF2-AM were observed in green under FITC filter. The translocation percentages were calculated by the number of translocated cells per the number of whole cells. $^{*}P < 0.05$. (B) Fluorescent micrographs of TEM-1-translocated cells. *E. coli* strains carrying cosmids encoding cloned Effector-less T3SS1 (ΔEffector) or secretion-deficient T3SS1 (Δ*vcrD1*) with the reporter plasmids encoding TEM-1 tagged with $VepA_{1-100}$, with the VepA-chaperone VecA. *E. coli* strains were incubated with HeLa cells for 3 h at 37 °C, and cells were observed under fluorescent microscopy. Upper row: images under the UV-1A filter (translocated cells; blue, non-translocated cells; green), bottom row: images under the FITC filter (All cells treated with CCF2-AM; green).

the integrity of the bacterial outer/inner membranes and cause the leakage of intracellular proteins into the bacterial supernatant.

3.3. Infection of non-pathogenic E. coli expressing cloned T3SS1 with culture cells

Protein translocation is the most distinct characteristic on T3SSs and constitutes a distinct event from protein secretion into the culture medium [16]. In considering the possible usage of this molecular syringe, it is essential that protein translocation by this cloned T3SS be examined. One of the effectors on T3SS1, VepA, has potent cytotoxic activity against various culture cells [9,11,17]. To assess the protein translocation by cloned T3SS1, a cytotoxicity assay against culture cells was performed for this purpose. Caco-2 cells were infected with *E. coli* strains with cloned wild type, secretion-deficient or effector-less T3SS1, and the cytotoxicity was determined for each strain. The strain carrying wild type T3SS1 showed significant cytotoxicity, whereas the other strains (Δ*vcrD1* and ΔEffector) did not (Fig. 3). A similar result was obtained by the phase-contrast micrography of infected Caco-2 cells, as cell-rounding was observed with *E. coli* carrying wild type T3SS1. VepA can be cytotoxic when it exists inside host cells. Even a certain amount of purified VepA added to the medium showed no cytotoxic effect on cultured cells (data not shown). From these results, *E. coli* expressing wild type T3SS1 has a functional T3SS capable of injecting effectors into target cells along with some leakage of cytoplasmic proteins.

3.4. Translocation of foreign protein fused with an amino terminal T3SS1 effector

As shown above, we successfully constructed a functional T3SS1 in *E. coli* K-12. When imagining this T3SS used as a molecular syringe, this new tool must be tested for its ability to foreign proteins other than T3SS effectors into culture cells. To examine this possibility, the reporter plasmid expressing TEM-1 containing the essential region (1–100 amino acids) of VepA for its secretion through T3SS1 was constructed [11]. TEM-1 is one of the β-lactamases that can cleave penicillins and cephalosporins. This enzyme can be used as a translocation marker along with the addition of CCF2-AM. Once this fluorescent substrate was cleaved by translocated TEM-1 inside host cells, its original fluorescence (green at 520 nm (emission) by FRET) was shifted to blue (at 450 nm) by the disruption of FRET under fluorescent microscopy. The

translocation of TEM-1 was examined using HeLa cells with CCF2-AM. A reporter plasmid was introduced into the strains carrying cosmids of cloned effector-less T3SS1 (ΔEffector) and secretion-deficient T3SS1 (Δ*vcrD1*). As shown in Fig. 4, the ΔEffector strain demonstrated a significantly higher rate of TEM-1-translocated cells in comparison with other strains tested. Even without T3SS-specific chaperones, an amino terminal secretion signal can, to some degree, transport foreign proteins via T3SSs in pathogenic bacteria. However, the secretion of T3SS effectors generally becomes much more efficient with T3SS effector-specific chaperones if effectors have their cognate chaperones [11,18]. To examine this detail in *E. coli* K-12 expressing T3SS, the gene encoding the VepA-specific chaperone VecA was added to the reporter plasmid (pTEM-1/VecA) [11]. Translocation of TEM-1 into HeLa cells via T3SS1 increased dramatically with the addition of the T3SS chaperone. This result indicated that the domain for effector secretion, as well as its cognate chaperone, should accompany the proteins to be delivered into the target cells. Taken together, the cloned effector-less T3SS1 (ΔEffector) has the capability to inject foreign proteins containing the region for the amino terminal T3SS1 secretion signal and T3SS-specific chaperone-binding.

4. Discussion

T3SS is a sophisticated system to deliver proteins directly into host cells. A number of studies about T3SS have been reported over the recent couple of decades [19]. T3SS has been found in pathogenic bacteria and has been utilized to establish a niche of infection in host cells. This approach implies that much potential exists for the usage of T3SS as a molecular tool. Previous studies have shown some promising results for the establishment of a "molecular syringe" [20–23]. However, no successful studies have used non-pathogenic bacteria, such as the laboratory strain *E. coli* K-12, as a vehicle to inject foreign proteins into host cells. In this study, we constructed non-pathogenic *E. coli* strains carrying a series of cosmids encoding the T3SS1 genetic locus and then examined these strains' capabilities to secrete and translocate proteins into target cells. As a beneficial molecular tool, foreign protein has to be examined as a substrate for T3SS-dependent translocation into target cells. In this experiment, TEM-1 observed to contain the essential region for VepA secretion via T3SS1 was translocated into culture cells. This result further highlights the possibility of using the cloned T3SS. Previously, similar results were reported as mentioned. However, our *E. coli* strain expressing functional T3SS1 has an advantage in the usage of non-pathogenic *E. coli* as a host and foreign protein-injection. Attenuated pathogenic bacteria equipped with T3SSs by deletion/loss-of-function mutations on genes required for T3SSs from their genome may have unknown harmful effects when administrating to mammalian culture cells or the living body, as their pathogenicity characteristics have not been fully characterized. To reduce this risk, our non-pathogenic *E. coli* strain should be a better candidate for this purpose. Moreover, this *E. coli* strain is easy to handle in terms of DNA manipulation in the laboratory and is suitable for the introduction of additional functions or molecules, such as ligands to target specific sites (e.g., tissues, organs, cancer cells, etc.). Cloned T3SS1 is also used for a study to explore the pathogenicity of *V. parahaemolyticus* and the mechanism of T3SS protein secretion. Using deletion mutant strains of pathogenic bacteria is the best way to determine the contribution of gene products to their virulence, whereas the unexpected combination of several genes may affect the results of the experiment. An individual biological system cloned from the original organisms is one of the solutions to rule out this possibility. Therefore, cloned T3SS1 will serve as a useful platform to examine how each component of the T3SS1, as well as general T3SSs from various pathogenic bacteria, interacts and translocates its specific effectors into host cells.

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