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Enterotoxigenic *Escherichia coli* CS6 gene products and their roles in CS6 structural protein assembly and cellular adherence

Authors:

Takeaki Wajima[a, b], Subrata Sabui[c], Megumi Fukumoto[a, b], Shigeyuki Kano [a, b], Thandavarayan Ramamurthy[c], Nabendu Sekhar Chatterjee[c], and Takashi Hamabata[a, b*]

[a]Research Institute, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

[b]Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-8577, Japan

[c]National Institute of Cholera and Enteric Diseases, P-33 C.I.T. Road, Scheme-XM, Beliaghata, Kolkata 700 010, India

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*Corresponding author:

Takashi Hamabata, Ph.D.

Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.

TEL: +81-3-3202-7181

FAX: +81-3-3202-7364

E-mail: thama@ri.ncgm.go.jp

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) produces a variety of colonization factors necessary for attachment to the host cell, among which CS6 is one of the most prevalent in ETEC isolates from developing countries. The CS6 operon is composed of 4 genes, CssA, CssB, CssC, and CssD. The molecular mechanism of CS6 assembly and cell surface presentation, and the contribution of each protein to the attachment of the bacterium to intestinal cells remain unclear. In the present study, a series of *css* gene-deletion mutants of the CS6 operon were constructed in the ETEC genetic background, and their effect on adhesion to host cells and CS6 assembly was studied. Each subunit deletion resulted in a reduction in the adhesion to intestinal cells to the same level of laboratory *E. coli* strains, and this effect was restored by complementary plasmids, suggesting that the 4 proteins are necessary for CS6 expression. Bacterial cell fractionation and western blotting of the mutant strains suggested that the formation of a CssA–CssB–CssC complex is necessary for recognition by CssD and transport of CssA–CssB to the outer membrane as a colonization factor.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major causative pathogen of infantile diarrhea in developing countries and traveler's diarrhea in developed countries [1]. Especially in developing countries, ETEC is most frequently isolated from children younger than 5 years old with watery diarrhea. The World Health Organization estimated that there are more than 200 million cases of ETEC infection, resulting in nearly 380,000 deaths each year [2].

ETEC strains produce several virulence factors such as heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), and colonization factors (CFs) for the successful establishment of infections. Among these virulence factors, CFs are essential for adhering to small intestinal epithelial cells [3, 4]. To date, more than 25 CFs have been identified [5]. Epidemiological studies showed that CS6 is one of the most prevalent CFs [6-9], and it has therefore attracted attention as a target molecule for the development of an ETEC vaccine.

The CS6 operon consists of 4 genes, *cssA*, *cssB*, *cssC*, and *cssD* [10]. CssA and CssB are structural subunits, whereas CssC and CssD are estimated to be chaperone and usher, respectively, based on homology with known chaperone and usher proteins [10]. CssA binds to host cell fibronectin [11], while CssB binds to cell surface sulfatide [12]. Tobias *et al*. suggested that CssB is a key factor for binding to host cells and CssA inhibits CssB-mediated binding, and CssD is not involved in assembly or surface expression of CS6 based on findings obtained with laboratory strains in which the complete or deleted CS6 genes had been cloned and

overexpressed [13]. However, the molecular mechanisms of CS6 maturation in ETEC and attachment to intestinal cells are not fully understood, which is partly attributed to differences in the genetic background between laboratory *E. coli* strains and virulent ETEC strains. To gain insight into the mechanisms of CS6 maturation in ETEC and its role in binding to host cells, a series of deletion mutants of each gene in the CS6 operon was constructed within the genetic background of an ETEC clinical isolate in this study.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

E. coli XLI-Blue, Top10 (Invitrogen, Carlsbad, CA) and DH5α were used for plasmid construction and maintenance. *E. coli* BL21 (DE3) was used for expression of recombinant proteins. These laboratory strains were grown in Luria–Bertani (LB) medium alone or with appropriate antibiotics at 37°C. ETEC strain 4266, isolated from a patient with diarrhea from the National Institute of Cholera and Enteric Diseases, Kolkata, India [14], was used as a wild type (WT) control strain as well as a parental strain to construct isogenic CS6 mutant strains in this study. The ETEC strain and its derivatives were grown in CFA medium (1% Casamino acids, 0.15% yeast extract, 0.05% $MgSO_4$, 0.0005% $MnCl_2$, pH 7.4) at 37°C [15].

2.2. Construction of isogenic CS6 nonpolar mutants

Isogenic mutants were obtained by the G-DOC system with modifications [16]. Briefly, the kanamycin (Km)-resistant gene cassette was amplified by PCR using primers containing the flanking regions of the *css* genes (Table 1) and pDOC-K as a template. The PCR product was cloned into the *Eco*RI site of pDOC-C vector. The resulting plasmid was transformed into the WT carrying helper plasmid pACBSce. The resulting colony was cultured for 3 hr in the presence of 0.5% of L-(+)-arabinose to induce lambda Red recombinase and I-SceI. The culture was then supplemented with sucrose to a concentration of 5%, incubated at 30°C for 4 hr, spread on LB agar plates containing 5% sucrose and 30 μg/mL Km, and incubated at 30°C overnight. Mutants were obtained as Km-resistant and sucrose-insensitive colonies. The disruption of target genes was confirmed by PCR and DNA sequencing using the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

2.3. Construction of the CS6 complement vector

The region from 1.5 kb upstream of *cssA* to 1.5 kb downstream of *cssD* was amplified by PCR, using CS6-F and CS6-R as primers (Table 1). The amplified 7.1 kb CS6 gene-containing product was digested with *Sal*I (TaKaRa-Bio, Kyoto, Japan) and cloned into the *Sal*I site of pSTV28 (TaKaRa-Bio) to yield pCS6 (Table 2). The CssA, CssB, CssC, 3×FLAG-tagged CssC (CssC-FLAG), CssD, CssACD, and CssBCD expression plasmids were constructed by inverse PCR using the primers shown in Table 1 and pCS6 as a template. The 5′ end of the primers was

phosphorylated by T4 polynucleotide kinase (TaKaRa-Bio) before being used for amplification. The PCR products were purified by Gene clean kit II (Qbiogenes, Irvine, CA) and ligated by T4 DNA ligase (Invitrogen) at 16°C overnight. The sequence of the resulting plasmids was confirmed by DNA sequencing using the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Each plasmid was electrotransformed into mutants by using Gene Pulser (Bio-Rad Laboratories, Hercules, CA).

2.4. Expression, purification, and preparation of antisera against CssA and CssB

The *cssA* and *cssB* genes were amplified by PCR using the primer set described in Table 1 and whole genomic DNA from WT strains as a template. The amplicons were cloned into the pET151-TOPO vector (Invitrogen) and transformed into BL21(DE3). The DNA sequence of the cloned genes was confirmed using the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Each transformant was grown to $OD_{600} = 0.8$ and protein expression was induced with 0.1 mM IPTG for 3 hr at 25°C. CssA was obtained as an inclusion body, whereas CssB was extracted from the soluble fraction. Recombinant proteins were purified using the His·Bind kit (Merck, Darmstadt, Germany) according to the manufacturer's protocol. Poly histidine-tag removal was achieved by TEV protease (Invitrogen) digestion. The resulting purified proteins were conjugated with Freund's complete adjuvant and injected subcutaneously into rabbits 5 times at 2-week intervals. One week after the last booster, the rabbits were sacrificed by

overanesthetization and the antiserum was isolated from whole blood.

2.5. Reverse transcribed PCR (RT-PCR)

Total RNA extraction was performed as previously described [17] except that the MultiBeads shocker (Yasui Kikai, Osaka, Japan) was used at 1,500 rpm for 20 sec to destroy bacterial cells. Contaminating DNA was removed from total RNAs using the TURBO DNA-free kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. Reverse transcription was performed using the Superscript III one-step RT-PCR kit (Invitrogen) according to the manufacturer's protocol. The primers used to detect gene transcripts are listed in Table 1.

2.6. Cell culture

The human colonic carcinoma cell line Caco-2 and the human embryonic intestinal cell line INT407 were grown in Eagle's Minimum Essential Medium (MEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) at 37^{o}C in 5% CO_2.

2.7. Adhesion assay

Bacteria were grown overnight in CFA alone or with the appropriate antibiotics diluted 1:10 with CFA, and grown at 37°C for 2 hr. After 3 washes with PBS and resuspension in MEM, the

bacteria were added to INT407 or Caco-2 monolayers in 24 well plates for a final concentration of 1×10^7 CFU/well. After 3 hr of incubation at 37°C in 5% CO_2, cells were washed 5 times with PBS and then lysed by 0.1% Triton X-100. Adhered bacteria were counted by serial dilution and plating of the lysate on LB agar.

2.8. Preparation of the bacterial cell fraction

The bacterial culture was prepared using the adhesion assay described above. The bacteria were harvested by centrifugation at 10,000 × *g* for 10 min, resuspended in 0.5 M sucrose, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), and incubated at 22°C for 10 min. After centrifugation at 10,000 × *g* for 10 min, the pellet was suspended in ice-cold 5 mM $MgSO_4$ and incubated on ice for 10 min. After centrifugation at 10,000 × *g* for 10 min, the supernatant was stored as the "periplasmic fraction." The pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 8.0) and sonicated for 5 sec 8 times at 10 sec intervals on ice using the Handy Sonic UR-20P (TOMY SEIKO CO. LTD, Tokyo, Japan). The cell debris was removed by centrifugation at 10,000 × *g* for 10 min and the supernatant was processed at 21,500 × *g* for 2 hr. The supernatant was stored as the "cytosolic fraction," and the pelleted "membrane fraction" was solubilized in 1 × SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2.5% 2-mercaptoethanol, 2% SDS, 0.01% BPB, and 10% glycerol). All steps above were performed at 4°C unless otherwise indicated. Bacterial cell fractions were kept at -30°C until use.

2.9. Immunoprecipitation (IP) assay

pCssC-Flag was transformed into WT and Δ*cssA*::Km strains. Whole cell extracts were collected from the transformants using Bugbuster HT (Merck) and the IP assay was performed using an anti-FLAG M2 affinity gel (Sigma-Aldrich Corp, St. Louis, MO), both according to the manufacturer's protocols. IP complexes were suspended in 2× reductant-free SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% BPB, 10% glycerol), and samples were kept at -30°C until use.

2.10. Western blot analysis

Samples were boiled for 5 min, separated by SDS-PAGE, and blotted to PVDF membranes (ATTO, Tokyo, Japan). CssA, CssB, and CssC-Flag were detected immunologically on PVDF membranes using an anti-CssA antiserum (1:2,500 dilution), anti-CssB (1:5,000 dilution) antiserum, and an anti-Flag M2 (SIGMA) monoclonal antibody (1:1,000 dilution) as primary antibodies. DnaK and RNA polymerase α subunit were detected by anti-DnaK (Assay Designs, Inc., Ann Arbor, MI) and anti-RNA polymerase α subunit (NeoClone Biotechnology International, Madison, WI) monoclonal antibodies, respectively. HRP-conjugated goat anti-rabbit IgG (Nacalai Tesque Inc., Kyoto, Japan) and anti-mouse IgG (Nacalai Tesque Inc.) were used as secondary antibodies in a dilution of 1:5,000. Target bands were visualized using

the Peroxidase Stain Kit for Immunoblotting (Nacalai Tesque Inc.).

3. Results

3.1. Construction of CS6 mutants

To elucidate the role of the CS6 gene, deletion mutants of each *css* gene (*cssABCD*) were generated using the G-DOC system [16] in an ETEC clinical isolate (Fig. 1A). Mutants were generated by replacement of each *css* gene with a Km-resistance gene (Km^r; ca. 1.3 kbp). Primers for PCR were designed from the flanking regions of Km^r insertion sites and used to amplify the *css* genes (Table 1). The PCR products of *cssA*, *cssB*, *cssC*, and *cssD* in WT were 2,273, 989, 663, and 3,344 bp, respectively, whereas those of the mutants were 3,180, 1,859, 1,518, and 2,421 bp, respectively (Fig. 1B). DNA sequencing of these mutant genes further confirmed that each open reading frame was successfully replaced by Km^r.

Because the *css* genes are transcribed from a single promoter [13], the possible presence of a polar effect in the CS6 operon within each mutant was tested. RT-PCR analysis of the mutant strains showed that downstream genes of the Km^r in the CS6 operon were transcribed at the same level as those of the WT strain (Fig. 1C). The mutants therefore did not show polar effects due to the integration of Km^r in the CS6 operon.

3.2. CssA cannot exist alone in ETEC

To analyze the expression of CS6 structural genes in the mutant strains, western blot analysis of total bacterial cell extracts was performed using anti-CssA and anti-CssB antisera. CssB was detected in all mutants except the Δ*cssB*::Km strain, whereas CssA was not detected in the Δ*cssA*::Km or in the Δ*cssB*::Km strains (Fig. 4, whole cell extracts). To test whether this phenomenon was unique to ETEC, CssACD and CssBCD expression vectors were constructed and transformed into laboratory *E. coli* strain TOP10 and confirmed by western blotting. As shown in Fig. 2, CssA and CssB were expressed in TOP10 harboring pcssACD and pcssBCD, respectively. The expression level of CssB was the same in pcssBCD as in TOP10 harboring pCS6, whereas CssA was diminished in pcssACD as compared with TOP10 harboring pCS6 (Fig. 2). The same result was obtained when the *E. coli* XL1-Blue strain was used as a transformation host (data not shown). These data indicated that stable expression of CssA is dependent on CssB but not vice versa, and CssA is more unstable in the ETEC genetic background than in laboratory strains.

3.3. All *css* genes are necessary for maximal adhesion of ETEC to host cells

To analyze the role of the CS6 genes in host cell adhesion, we performed adhesion assays of CS6 mutants using the INT407 and Caco-2 human epithelial cell lines. The adhesion ability of all *css* mutants was significantly decreased in the INT407 and Caco-2 (Fig. 3A) lines. However, the adhesion capacity of the mutants was recovered by introduction of expression plasmids for

each gene (Fig. 3B).

Because the *ΔcssB*::Km strain did not express CssB and CssA as indicated above, this assay was not sufficient to analyze the role of CssB upon adhesion to host cells. Recombinant CssA and native CssA can bind to host fibronectin equivalently [11]. Furthermore, CssA is expressed in the absence of CssB in TOP10 (Fig. 2). Therefore, adhesion assays were performed using TOP10 expressing CssACD, CssBCD, and CssABCD. As shown in Fig. 3C, the adhesion of the CssB-deleted strain was significantly reduced to the same level as that of the CssA-deleted strain and the TOP10 transformed with empty vector. Taken together, these data demonstrate that all *css* genes are required for maximal adhesion to host cells.

3.4. Cellular distribution of CssA and CssB in CS6 mutant bacteria

The adhesion assay suggested that CS6 was not successfully expressed in all mutants. To assess the effect of each gene deletion on CS6 assembly, cell fractionation of all mutants and western blot analysis using anti-CssA and anti-CssB antisera were performed. As shown in Fig. 4, western blot analysis of the membrane-associated and periplasmic fractions showed similar CssA and CssB profiles to those of the whole cell extract. In the cytoplasmic fraction of each mutant, CssA and CssB were significantly reduced compared with the levels in the WT strain. Both CssA and CssB were detected in the supernatant of the WT strain but not in that of any of the mutant strains.

3.5. CssC interacts with CssB

To evaluate the role of CssC in CS6 maturation, strains expressing CssC-FLAG were generated and used for IP assays using whole cell extracts. INT407 cell adhesion assays using *ΔcssC*::Km harboring pcssC-FLAG showed that the CssC-FLAG construct could fully complement the *ΔcssC*::Km strain (Fig. 5A), suggesting that the 3×FLAG did not interfere with the function of CssC. Whole cell lysates of WT, *ΔcssA*::Km, WT pcssC-Flag, and *ΔcssA*::Km pcssC-Flag were used in IP assays. As shown in Fig. 5B, CssA and CssB were precipitated by CssC-FLAG and the amount of precipitated CssB was diminished in the presence of CssA. These data suggested that both CssA–CssB and CssB alone can bind to CssC.

4. Discussion

CS6 consists of 4 subunits and is thought to be assembled through a chaperone–usher pathway [10]. The CS6 subunits are encoded by the *cssABCD* genes as a single operon. To understand the intrinsic role of CS6 in the adherence of ETEC to host cells and the contribution of each subunit, we constructed isogenic CS6 mutants within the ETEC background.

Our results suggested that CssA requires CssB to exist stably in ETEC cells, while in laboratory strains, CssA was expressed alone, although at a lower frequency than in CS6 expressing strains (Fig. 2). CssA has been suggested to be toxic for *E. coli* [13], which was

confirmed by the slow growth of CS6-expressing laboratory strains compared with strains carrying empty vectors, while the growth of ETEC4266 was not affected by the presence or absence of CS6 (data not shown). These results suggest that CssA itself is unstable in *E. coli* and that ETEC may possess a unique system to degrade CssA. Investigation of the potential ETEC-specific CssA-regulating mechanisms is currently taking place in our laboratory.

The mutants constructed in the present study revealed that all CS6 genes were required for maximal adhesion to intestinal cells (Fig. 3). The current results show discrepancies with a previous study using a laboratory *E. coli* strain overexpressing CS6 that reported that CssB and CssC are important for CS6 expression and cell adhesion ability is enhanced in the absence of CssA or of both CssC and CssD [13]. There are at least 2 possible explanations for these discrepancies. First, the genetic background of the ETEC strain differs from that of the laboratory strain with regard to the CS6 expression profile. The current results suggest that the instability of CssA is different in the WT ETEC and the laboratory strain. In addition, native CS6 has been reported to have fatty acid modifications within the CssA subunit, which are not present in recombinant CS6 [11]. Second, the use of the CS6 native promoter and a low copy number plasmid in our CS6 expression analysis with laboratory strains likely resulted in a lower number of *css* gene transcripts compared with those obtained with an IPTG-inducible *tac* promoter.

The adhesion assay performed using complemented strains showed that the adhesion of Δ*cssC*::Km complemented by pcssC and pcssC-Flag was significantly greater than that of the

WT strain (Fig. 3B, 5A), suggesting that complemented strains expressed more CS6 on the outer membrane than the WT strain. CFA/I, which is a well-studied ETEC colonization factor, is reported to contain a stem-loop structure between the *cfaB* major subunit and the *cfaC* usher subunit, and this stem-loop is responsible for both stabilization of *cfaB* mRNA and destabilization of its downstream mRNA [18]. In the present study, the mRNA level of *cssC* was lower than that of the other subunit genes (Fig. 1C), indicating that the stem-loop structure in the CS6 operon might function in a similar manner as that of the CFA/I operon. Moreover, these results also suggested that CssC regulates the level of CS6 expression on the outer membrane.

Cell fractionation analysis and IP assays showed that CssB alone could be targeted to the membrane without other subunits (Fig. 4) and CssB associates with CssC (Fig. 5) in ETEC cells. These results, together with the absence of CssA and CssB from the supernatant of mutant strains (Fig. 4), and the reduction in adhesion by the loss of any subunit (Fig. 2), indicate that CssB alone or the CssB–CssC complex are not transported to the cell surface through the outer membrane via CssD. The formation of a CssA–CssB–CssC complex is therefore assumed to be necessary for recognition by CssD and transport of the CssA–CssB complex through the outer membrane and for its exposure in a functionally active form.

This analysis also showed that the distribution of CS6 subunits is very low in the cytoplasmic fraction of all mutants (Fig. 4). In these mutants, the CS6 subunits are expected to accumulate in the periplasm if they cannot be transported outside, as discussed above. Uropathogenic *E. coli*

(UPEC) has a Cpx 2-component signal transduction system that is activated by misfolding or aggregation of pili subunits in the periplasm, resulting in the upregulation of DegP protease and the negative regulation of genes to alleviate the periplasmic stress [19, 20]. A similar feedback mechanism may operate in ETEC to regulate the expression of CS6.

On the basis of the present results, a CS6 assembly model was generated and is depicted in Fig. 6. First, translated CS6 subunits are transferred to the periplasm, where CssA forms a complex with CssB and CssC. Meanwhile, free CssA is degraded by a system specific to ETEC. The CssA–CssB–CssC complex is recognized by CssD, and CssA–CssB is transported to the cell surface through the outer membrane, while the CssB–CssC complex cannot pass through the outer membrane. The accumulation of free subunits in the periplasm triggers the downregulation of CS6 genes by a feedback system that is still unknown.

In conclusion, the CS6 isogenic mutant strains, as well as CS6 recombinant strains, demonstrated that CssB is required for stabilization of CssA, and all *css* genes in the CS6 operon are necessary for maximal adhesion of ETEC to epithelial cells and CS6 assembly. Although CS6 is the most prevalent ETEC colonization factor isolated in developing countries [8, 9, 21], many aspects of the nature and function of CS6 remain to be elucidated. The present study provides a starting point for the understanding of CS6 maturation and function, particularly in the ETEC background, with the ultimate goal of developing a vaccine to target ETEC colonization factors in the near future.

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Conflicts of interest

The authors declare that there are no conflicts of interest

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')	Description
CssA-F	AGAACAGAAATAGCGACTAA	RT-PCR for *cssA* gene, Confirmation of *cssB*-deleted mutant
CssA-R	TTAGTTTACATAGTAACCAAC	RT-PCR for *cssA* gene, Confirmation of *cssA*-deleted mutant, and Construction of pcssA and pcssB
CssB-F	AGGAAACTGGCAATATAAAT	RT-PCR for *cssB* gene
CssB-R	TTAATTGCTGTAAAATGATA	RT-PCR for *cssB* gene, Confirmation of *cssB*-deleted mutant, and Construction of pcssB
CssC-101F	ATTTACTCTGACAGCACACCATC	RT-PCR for *cssC* gene
CssC-611R	TCTATTTTCTTCTCTGAGTATGG	do.
CssD-392F	ATATCAGCTTTGAGTTTAGCTCC	RT-PCR for *cssD* gene
CssD-3200R	TAAGTTGCCCCCCAGTA	do.
CS6-F	TACCAGCCATCTTAGCTTAC	Construction of pCS6
CS6-R	GACAGAATCTGTCGACCTGAGTCTGAGGATGATCG	do.
CssA-pET151F	CACCAGAACAGAAATAGCGACTAA	Construction of rCssA, rCssB expression plasmid
CssB-pET151F	CACCGGAAACTGGCAATATAAATCTC	do.
CssA-del-F	AAAATGAATTCAGCAATAGTAATGGTGTTATATGAAGAAAACAATTGGTTTGACCGGTCAATTGGCTGGAG	Construction of Km cassette for *cssA*-deletion
CssA-del-R	TTTCAGAATTCTCATACTTCCAGTATTTAGTTTACATAGTAACCAACCATAAATATCCTCCTTAGTTCC	do.
CssB-del-F	AAAATGAATTCATACTGGAAGTATGATTATGTTGAAAAAAATTATTCCGGCGACCGGTCAATTGGCTGGAG	Construction of Km cassette for *cssB*-deletion
CssB-del-R	TTTCAGAATTCATAATGCGGCCTTTTTTTTAATTGCTGTAAAATGATACAGTAATATCCTCCTTAGTTCC	do.
CssC-del-F	GACGAGAATTCCCAGGCAAAAATATGAAATCAAAGTTAATTATATTATTGAGACCGGTCAATTGGCTGGAG	Construction of Km cassette for *cssC*-deletion
CssC-del-R	TCTGAGAATTCAAAAACCTCAGAAAAGCTAATGTAATAGGGTGTATTATTTAATATCCTCCTTAGTTCC	do.
CssD-del-F	TATACGAATTCATCAGATTACAGACTTGCTTTTTTTTCTATTTCTATATCCGACCGGTCAATTGGCTGGAG	Construction of Km cassette for *cssD*-deletion
CssD-del-R	AATTCGAATTCAGATATATCTTTTTCTGAAAAAGGAAATGAACAGACTTTTAATATCCTCCTTAGTTCC	do.
CssA-up-F	ATCACCAGGTATTCTTCCCG	Confirmation of *cssA*-deleted mutant
CssC-F-inverse	ATGAAATCAAAGTTAATTATATTATTG	Confirmation of *cssC*-deleted mutant, Construction of pcssACD and pcssCD
CssC-R2	AAAAGCAAGTCTGTAATCTG	Confirmation of *cssC*-deleted mutant
CssD-F	ATGATGCTGGCGCAAAAAC	Confirmation of *cssD*-deleted mutants
CssD-downR	AGGTGAGCTGAGCTACAGC	do.
CssB-F-inverse	ATGTTGAAAAAAATTATTCCGG	Construction of pcssBCD
Cs6-pro-R-inverse	ATAACACCATTACTATTGCTATA	Construction of pcssBCD, pcssCD and pcssD
CssD-F-inverse	ATGATGCTGGCGCAAAAAC	Construction of pcssD
Css-term F	ACTTCCTGAGAAAGAGGTAAAC	Construction of pcssA, pcssB and pcssC
CssC-R	TTATAAAATTGATTCATAA	Construction of pcssC
CssC-Flag-F	ATGATATCGACTACAAAGATGACGACGATAAATAGTAAACTTCCTGAGAAAGAGGTAAAC	Construction of Flag-tagged CssC expression plasmid
CssC-Flag-R	GATCTTTATAATCACCGTCATGGTCTTTGTAGTCTAAAATTGATTCATAAAGTTTTGTTT	do.

Table 2. Plasmids used in this study

Plasmid name	Description	Source
pET151TOPO	Expression vector, Amp^r, pBR322*ori*	Invitrogen
pETCssA	*cssA* without signal peptide cloned into pET151TOPO	This study
pETCssB	*cssB* without signal peptide cloned into pET151TOPO	This study
pSTV28	Cloning vecter, Cm^r, p15A*ori*	TaKaRa-Bio
pCS6	7 kb fragment containing CS6 cloned into pSTV28	This study
pcssACD	CssACD expression plasmid derived from pCS6	This study
pcssBCD	CssBCD expression plasmid derived from pCS6	This study
pcssCD	CssCD expression plasmid derived from pCS6	This study
pcssA	CssA expression plasmid derived from pcssACD	This study
pcssB	CssB expression plasmid derived from pcssBCD	This study
pcssC	CssC expression plasmid derived from pcssCD	This study
pcssD	CssD expression plasmid derived from pCS6	This study
pcssC-Flag	CssC-3×Flag expression plasmid derived from pcssCD	This study
pACBSce	Red recombinase and I-SceI expression plasmid, Cm^r, p15A*ori*	[16]
pDOC-K	Template plasmid for G-DOC system, Km^r, Amp^r, pMBI*ori*, *ori*T	[16]
pDOC-C	Cloning plasmid for G-DOC system, Km^r, Amp^r, pMBI*ori*, *ori*T	[16]
pDOC-CssA	*cssA* flanking region and Km^r cassette cloned into pDOC-C	This study
pDOC-CssB	*cssB* flanking region and Km^r cassette cloned into pDOC-C	This study
pDOC-CssC	*cssC* flanking region and Km^r cassette cloned into pDOC-C	This study
pDOC-CssD	*cssD* flanking region and Km^r cassette cloned into pDOC-C	This study

References

[1] Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev. 2005;18:465-83.

[2] WHO. Future directions for research on enterotoxigenic *Escherichia coli* vaccines for developing countries. Wkly Epidemio Rec. 2006;81:97-104.

[3] Turner SM, Scott-Tucker A, Cooper LM, Henderson IR. Weapons of mass destruction: virulence factors of the global killer enterotoxigenic *Escherichia coli*. FEMS Microbiol Lett. 2006;263:10-20.

[4] Fleckenstein JM, Hardwidge PR, Munson GP, Rasko DA, Sommerfelt H, Steinsland H. Molecular mechanisms of enterotoxigenic *Escherichia coli* infection. Microbes Infect. 2010;12:89-98.

[5] Walker RI, Steele D, Aguado T. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease. Vaccine. 2007;25:2545-66.

[6] Rockabrand DM, Shaheen HI, Khalil SB, Peruski LF, Jr., Rozmajzl PJ, Savarino SJ, et al. Enterotoxigenic *Escherichia coli* colonization factor types collected from 1997 to 2001 in US military personnel during operation Bright Star in northern Egypt. Diagn Microbiol Infect Dis. 2006;55:9-12.

[7] Vidal RM, Valenzuela P, Baker K, Lagos R, Esparza M, Livio S, et al. Characterization of the most prevalent colonization factor antigens present in Chilean clinical enterotoxigenic *Escherichia coli* strains using a new multiplex polymerase chain reaction. Diagn Microbiol Infect Dis. 2009;65:217-23.

[8] Rivera FP, Ochoa TJ, Maves RC, Bernal M, Medina AM, Meza R, et al. Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. J Clin Microbiol. 2010;48:3198-203.

[9] Sabui S, Ghosal A, Dutta S, Ghosh A, Ramamurthy T, Nataro JP, et al. Allelic variation in colonization factor CS6 of enterotoxigenic *Escherichia coli* isolated from patients with acute diarrhoea and controls. J Med Microbiol. 2010;59:770-9.

[10] Wolf MK, de Haan LA, Cassels FJ, Willshaw GA, Warren R, Boedeker EC, et al. The CS6 colonization factor of human enterotoxigenic *Escherichia coli* contains two heterologous major subunits. FEMS Microbiol Lett. 1997;148:35-42.

[11] Ghosal A, Bhowmick R, Banerjee R, Ganguly S, Yamasaki S, Ramamurthy T, et al. Characterization and studies of the cellular interaction of native colonization factor CS6 purified from a clinical isolate of enterotoxigenic *Escherichia coli*. Infect Immun. 2009;77:2125-35.

[12] Jansson L, Tobias J, Jarefjall C, Lebens M, Svennerholm AM, Teneberg S. Sulfatide recognition by colonization factor antigen CS6 from enterotoxigenic

Escherichia coli. PLoS ONE. 2009;4:e4487.

[13] Tobias J, Lebens M, Kallgard S, Nicklasson M, Svennerholm AM. Role of different genes in the CS6 operon for surface expression of Enterotoxigenic *Escherichia coli* colonization factor CS6. Vaccine. 2008;26:5373-80.

[14] Ghosal A, Bhowmick R, Nandy RK, Ramamurthy T, Chatterjee NS. PCR-based identification of common colonization factor antigens of enterotoxigenic *Escherichia coli*. J Clin Microbiol. 2007;45:3068-71.

[15] Evans DG, Evans DJ, Jr., Clegg S, Pauley JA. Purification and characterization of the CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect Immun. 1979;25:738-48.

[16] Lee DJ, Bingle LE, Heurlier K, Pallen MJ, Penn CW, Busby SJ, et al. Gene doctoring: a method for recombineering in laboratory and pathogenic *Escherichia coli* strains. BMC Microbiol. 2009;9:252.

[17] Matsuda K, Tsuji H, Asahara T, Matsumoto K, Takada T, Nomoto K. Establishment of an analytical system for the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of multicopy rRNA molecules. Appl Environ Microbiol. 2009;75:1961-9.

[18] Jordi BJ, op den Camp IE, de Haan LA, van der Zeijst BA, Gaastra W. Differential decay of RNA of the CFA/I fimbrial operon and control of relative gene

expression. J Bacteriol. 1993;175:7976-81.

[19] Sauer FG, Mulvey MA, Schilling JD, Martinez JJ, Hultgren SJ. Bacterial pili: molecular mechanisms of pathogenesis. Curr Opin Microbiol. 2000;3:65-72.

[20] Hung DL, Raivio TL, Jones CH, Silhavy TJ, Hultgren SJ. Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili. EMBO J. 2001;20:1508-18.

[21] Gaastra W, Svennerholm AM. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). Trends Microbiol. 1996;4:444-52.

Figure captions

Fig. 1.

Construction of CS6 mutants . (A) CS6 genes in the ETEC strain 4266 (WT) and in each mutant. The mutants have a kanamycin-resistance gene (Km^r) insertion within each CS6 gene. (B) Confirmation of gene replacement in mutants by PCR. (C) RT-PCR analysis of CS6 gene transcripts in the mutants. PCR and RT-PCR were performed as described in the Materials and Methods section. A, *cssA*; B, *cssB*; C, *cssC*; D, *cssD*.

Fig. 2.

Western blot analysis of the CS6 structural subunits CssA and CssB in TOP10 and its derivatives. Equal amounts of whole cell extracts estimated by the OD_{600} of the culture were blotted onto PVDF membranes and immunostained with CssA or CssB antisera. Anti-DnaK antibody was used as a loading control.

Fig. 3.

Adhesion assay of the mutant strains in intestinal cells. The adherence of CS6 gene mutants including WT (A), complemented strains (B), and laboratory strain transformants (C) to INT407 and Caco-2 cells is shown. Values indicate the mean ± SE of viable bacteria bound to INT407 cells or Caco-2 cells per well of a 24-well plate. The asterisks indicate significant differences in the number of adhered bacteria ($P < 0.01$ in A and C, $P < 0.05$ in B) from WT (A, B) or TOP10

pSTV28 (C) as judged by the Student's *t* test. Assays were performed in triplicate on 2 different days.

Fig. 4.

Cell fractionation analysis of WT and mutants. WT and all mutants were fractionated as described in the Materials and Methods section. Each fraction was processed by SDS-PAGE and western blotting using anti-CssA and anti-CssB antisera. Fractions are indicated on the left. Anti-RNA polymerase α subunit and anti-DnaK monoclonal antibodies were used as internal controls.

Fig. 5.

Analysis of the function of CssC. (A) Adherence of WT, *ΔcssC*::Km, and *ΔcssC*::Km pcssC-FLAG to INT407. Values represent the mean ± SE of viable bacteria bound to INT407 cells per well of a 24-well plate. The asterisks indicate significant differences in the number of adhered bacteria ($P < 0.01$) from the WT strain as judged by the Student's *t* test. These assays were performed in triplicate on 2 different days. (B) Co-immunoprecipitation between CssC and CssA or CssB. The IP complex and input of each sample were probed with anti-CssA antiserum, anti-CssB antiserum, and anti-FLAG M2 antibody.

Fig. 6.

Schematic model of CS6 maturation. OM, outer membrane; PP, periplasm; IM, inner membrane; CP, cytoplasm; A, CssA; B, CssB; C, CssC; D, CssD; ?, unknown factors.

Response to Reviewers

Thank you very much for your comments. We are very happy and encouraged. Following your suggestion, we revised the manuscript, as follows in detail.

“My primary criticisms are related to the structure of the article as written. I would first refer to the authors to the "Guide for Authors" section of journal website. The sequence of sections in the manuscript is not in keeping with most scientific papers or with the editorial policy of the journal. The current draft follows a sequence of (1) Introduction, (2) Results, (3) Discussion, and (4) Methods. The Methods section should come after the Introduction section, which will make the Results section much clearer for the reader.”

We corrected the sequence of the sections as (1) Introduction, (2) Materials and Methods, (3) Results, and (4) Descussion, as you suggested. We agree with you, this is much better for readers to understand the Result than previous one.

“Similarly, I would move the final sentence of the Introduction (starting on page 5, line 16) to the Discussion or Conclusions section. This is more properly a conclusion based on the results of the study.”

The final sentence of the Introduction part was moved to the Discussion section (in the top of the final paragraph).

Fig.1

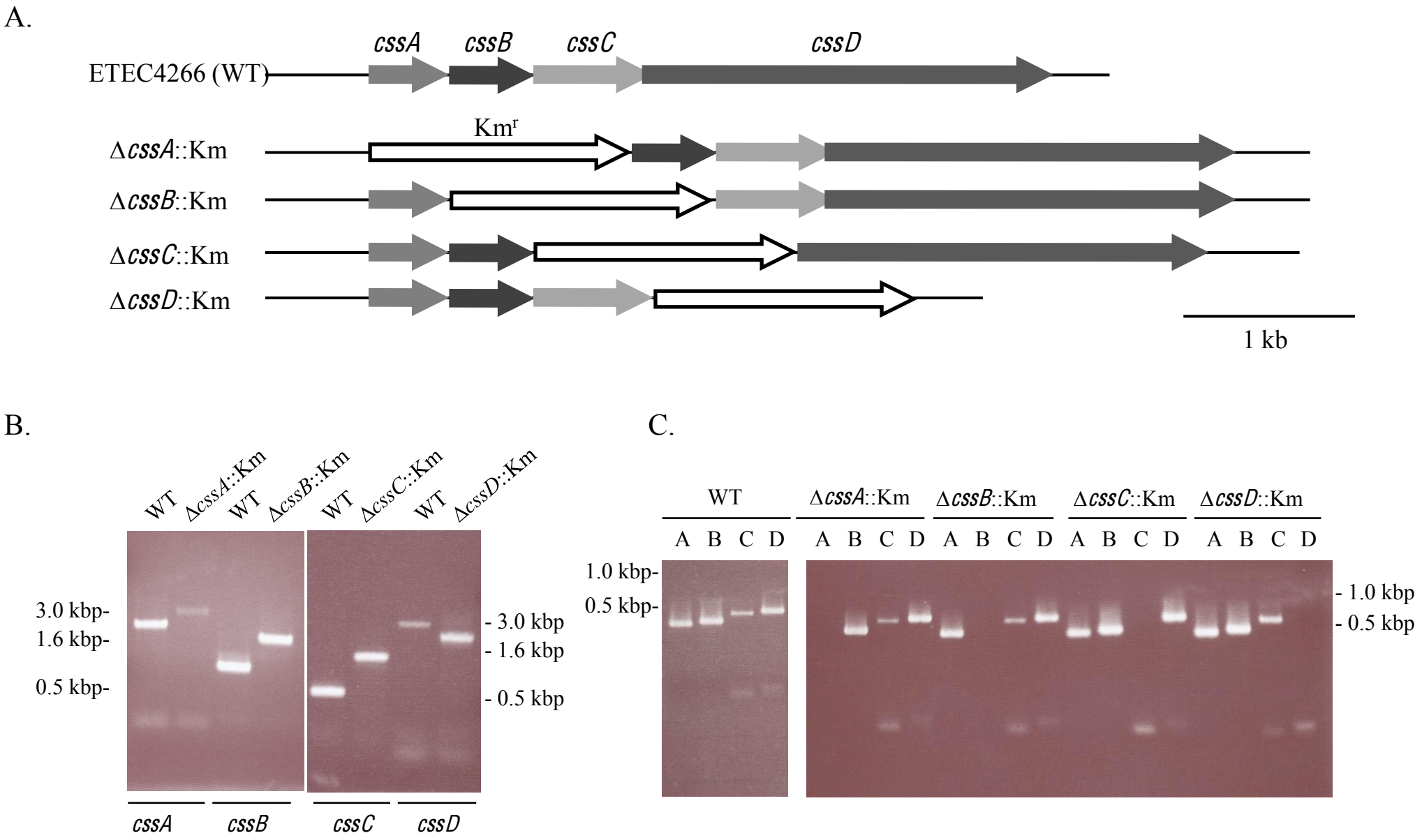

A.
cssA
cssB
cssC
cssD
ETEC4266 (WT)
Km^r
ΔcssA::Km
ΔcssB::Km
ΔcssC::Km
ΔcssD::Km
1 kb
B.
WT
ΔcssA::Km
WT
ΔcssB::Km
WT
ΔcssC::Km
WT
ΔcssD::Km
3.0 kbp-
1.6 kbp-
0.5 kbp-
- 3.0 kbp
- 1.6 kbp
- 0.5 kbp
cssA
cssB
cssC
cssD
C.
WT
ΔcssA::Km
ΔcssB::Km
ΔcssC::Km
ΔcssD::Km
A B C D
1.0 kbp-
0.5 kbp-
- 1.0 kbp
- 0.5 kbp

Fig.1

Fig.2

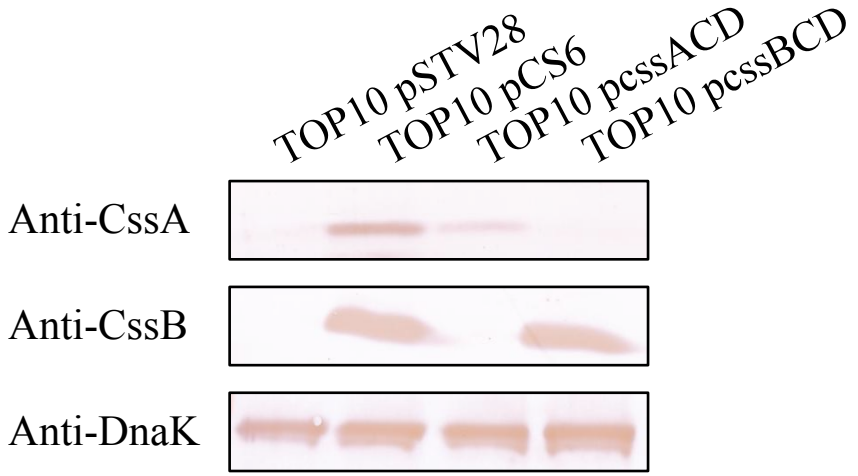

TOP10 pSTV28
TOP10 pCS6
TOP10 pcssACD
TOP10 pcssBCD
Anti-CssA
Anti-CssB
Anti-DnaK

Fig.2

Fig.3

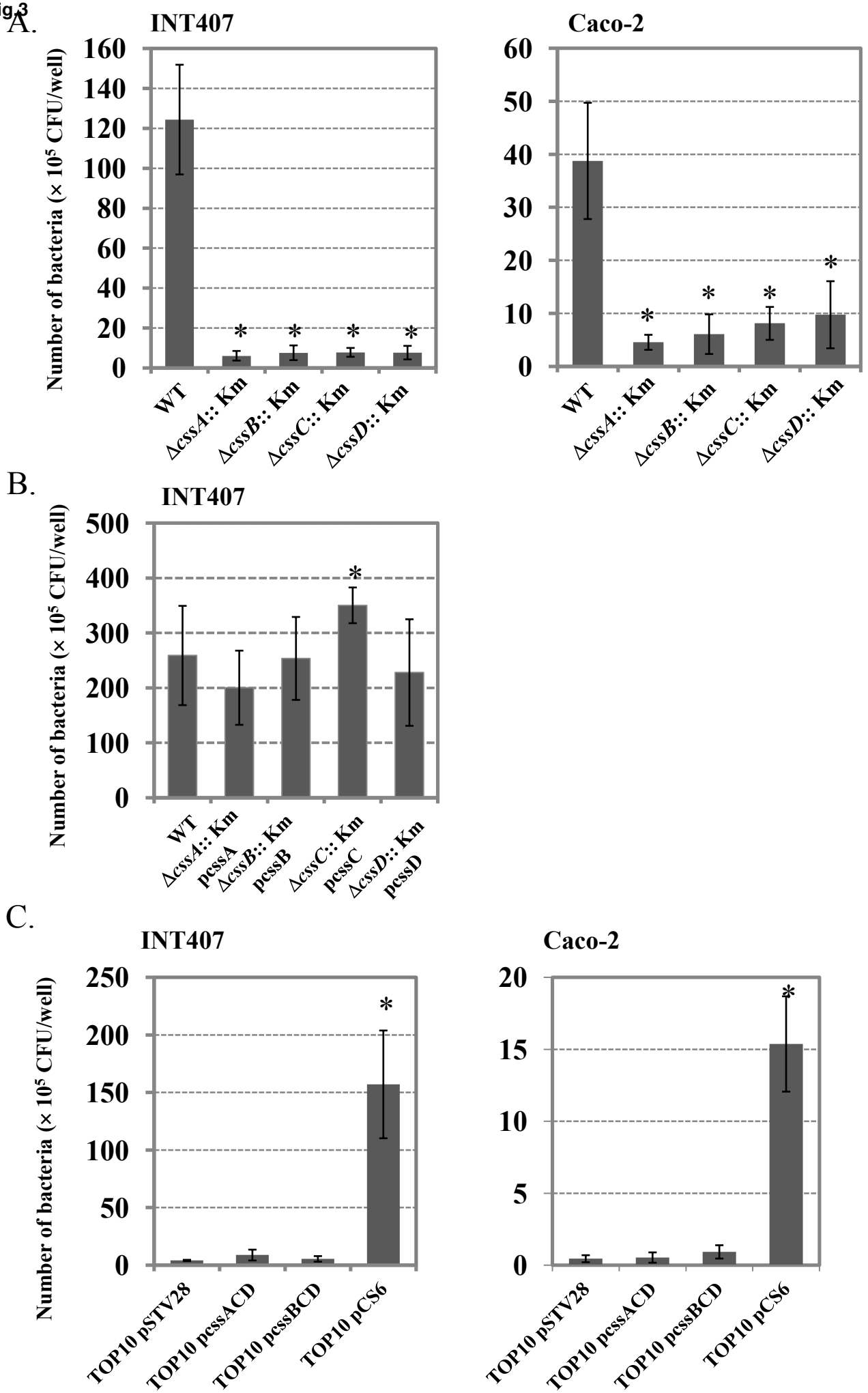

A.
INT407
Caco-2
Number of bacteria (× 10^5 CFU/well)
WT
ΔcssA:: Km
ΔcssB:: Km
ΔcssC:: Km
ΔcssD:: Km
B.
INT407
Number of bacteria (× 10^5 CFU/well)
WT
ΔcssA:: Km
pcssA
ΔcssB:: Km
pcssB
ΔcssC:: Km
pcssC
ΔcssD:: Km
pcssD
C.
INT407
Caco-2
Number of bacteria (× 10^5 CFU/well)
TOP10 pSTV28
TOP10 pcssACD
TOP10 pcssBCD
TOP10 pCS6

Fig.3

Fig.4

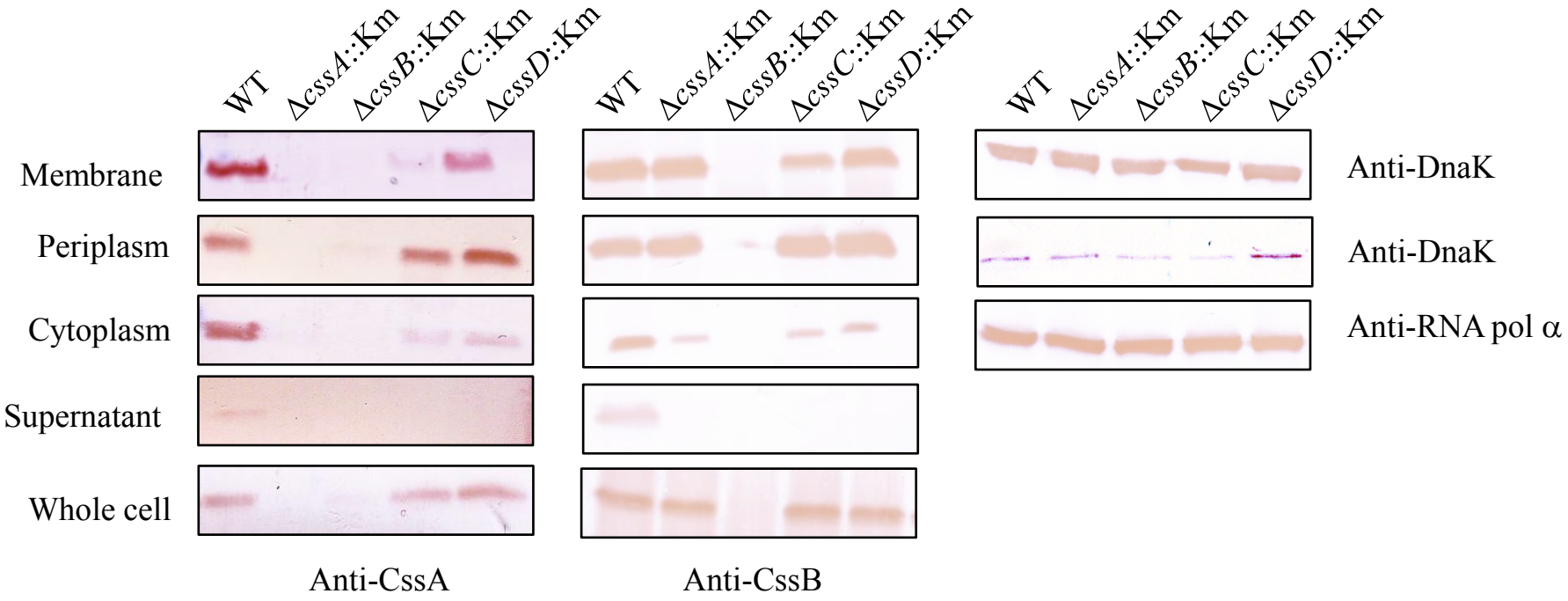
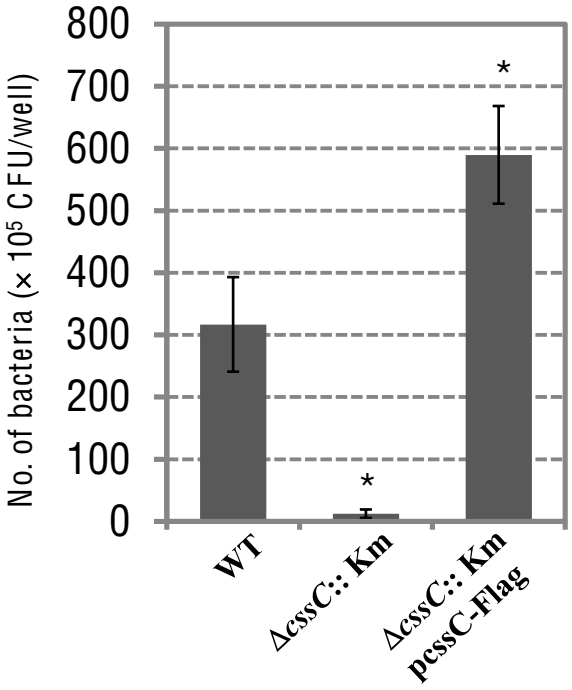

WT
ΔcssA::Km
ΔcssB::Km
ΔcssC::Km
ΔcssD::Km
Membrane
Periplasm
Cytoplasm
Supernatant
Whole cell
Anti-CssA
Anti-CssB
Anti-DnaK
Anti-DnaK
Anti-RNA pol α

Fig.4

Fig.5

A.


800
700
600
500
400
300
200
100
0
No. of bacteria (× 10^5 CFU/well)
*
*
WT
ΔcssC:: Km
ΔcssC:: Km pcssC-Flag

B.

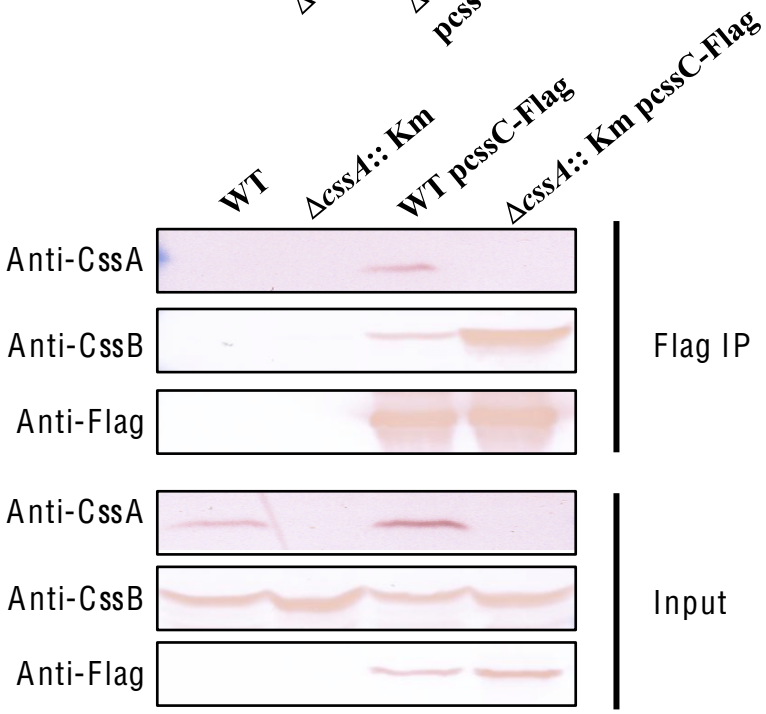

WT
ΔcssA:: Km
WT pcssC-Flag
ΔcssA:: Km pcssC-Flag
Anti-CssA
Anti-CssB
Anti-Flag
Flag IP
Anti-CssA
Anti-CssB
Anti-Flag
Input

Fig. 5

Fig.6

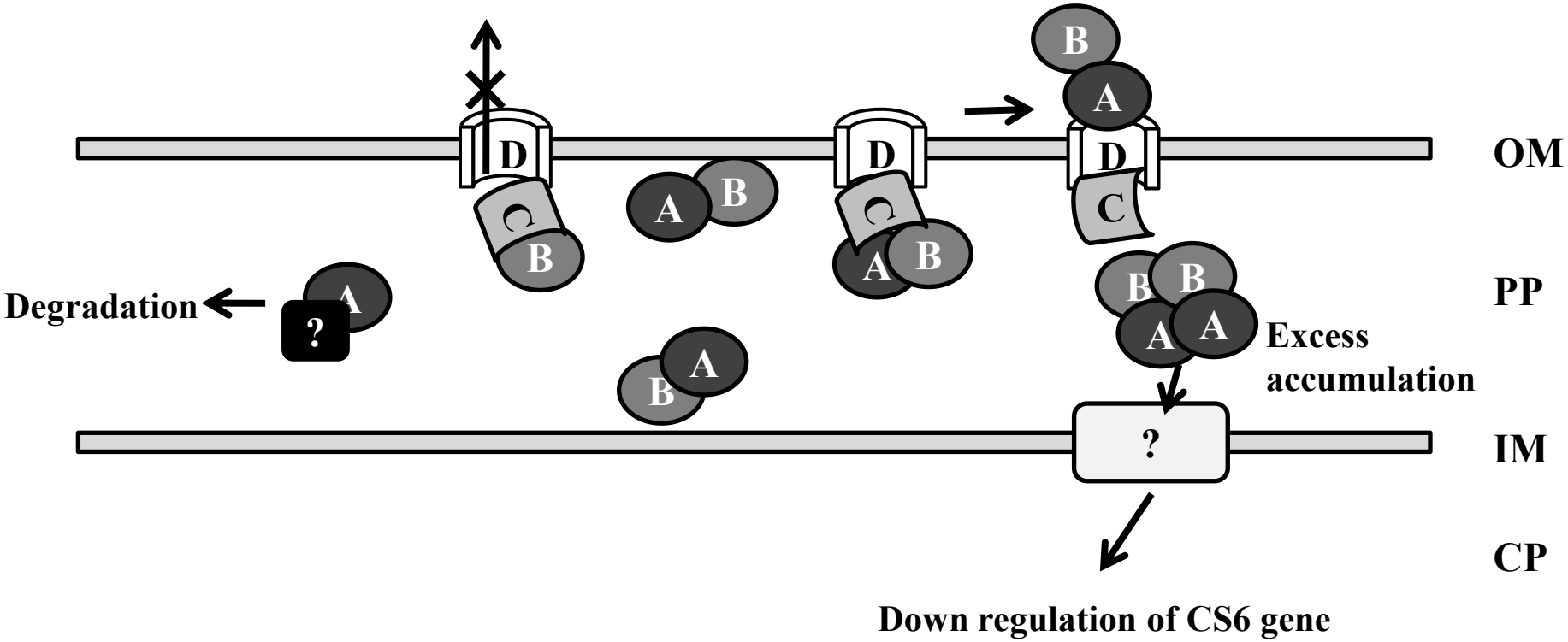

B
A
D
D
D
OM
C
C
C
A
B
A
B
B
B
Degradation
A
?
A
A
Excess
accumulation
B
A
?
IM
CP
Down regulation of CS6 gene

Fig.6