

# The *shf* Gene of a *Shigella flexneri* Homologue on the Virulent Plasmid pAA2 of Enteroaggregative *Escherichia coli* 042 Is Required for Firm Biofilm Formation

Rika Fujiyama · Junichiro Nishi · Naoko Imuta · Koichi Tokuda · Kunihiro Manago · Yoshifumi Kawano

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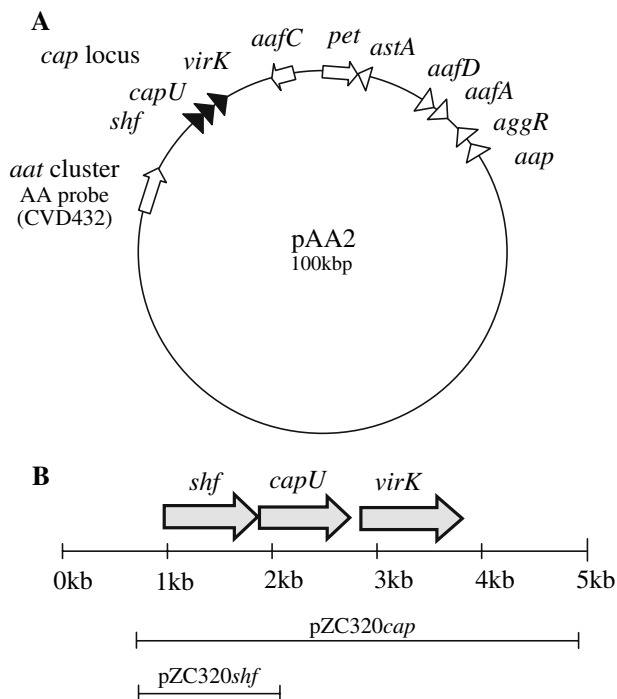
**Abstract** Enteroaggregative *Escherichia coli* (EAEC) is an increasingly important cause of diarrhea in both developing and industrialized countries, and is characterized by strong biofilm formation on the intestinal mucosa. Sequencing of the virulent plasmid pAA2 of the prototype EAEC 042 revealed a cluster of three open reading frames (ORFs; *shf*, *capU*, and *virK*) ca. 93% identical to a similar cluster located in *Shigella flexneri*. The function of the first ORF Shf protein is not known, but the closest well-characterized homologue is the IcaB protein of *Staphylococcus epidermidis*, which plays a crucial role in exopolysaccharide modification in bacterial biofilm formation. To investigate the role of this cluster in the virulence of EAEC, we mutated three genes at this locus. All the mutants maintained the aggregative phenotype in the liquid phase. However, the insertional mutant of *shf* formed a less abundant biofilm in a microtiter plate assay than did the wild type, while the *capU* mutant and the *virK* mutant did not. The complementation of the *shf* mutant with this cluster restored the thick biofilm similar to that of the wild type. The *shf* transcriptional level decreased in the transcriptional regulator *aggR* mutant and was restored when the mutant was complemented with *aggR*. These results suggest that the *shf* gene is required for the firm biofilm formation of EAEC 042, and transcription of the *shf* gene is dependent on *AggR*.

## Introduction

Enteroaggregative *Escherichia coli* (EAEC) is an increasingly important cause of persistent diarrhea in both developing and industrialized countries [12]. Recently, case control studies showed that EAEC is an important cause of childhood diarrhea in the United States [2, 10]. EAEC is defined by its ability to form a “stacked brick” pattern of bacterial cells attached to HEp-2 cells in culture [9]. The pathogenesis of EAEC infection is thought to involve the adherence of the bacterium to the intestinal mucosa, followed by secretion of one or more enterotoxins [5]. Adherence of EAEC to the mucosa is characterized by the presence of a thick, aggregating biofilm [5, 18]. The majority of EAEC strains carry a 100-kb plasmid, which harbors most pathogenic genes: different alleles of aggregative adherence fimbriae (*aafs*), transcriptional regulator (*aggR*), plasmid encoded toxin (*pet*), dispersin (*aap*), and outer membrane transporter (*aata*) [5] (Fig. 1A). Sequencing of pAA2 between *aafC* and *aata* (AA probe) revealed a cluster of three open reading frames (ORFs) transcribed in the same direction with each ca. 93% identical at the amino acid level to a similar cluster located on the large plasmid of *Shigella flexneri* [3, 13]. This locus has been designated “*cap* cluster” (personal communication, Prof. J. P. Nataro, University of Maryland, Baltimore). Three ORFs designated *shf*, *capU*, and *virK* encode secreted 32.8-, 29.0-, and 36.8-kDa proteins, respectively [3] (Fig. 1B). There were no overlapping sequences among the three genes.

The function of the Shf protein is not known, but the closest well-characterized homologue at 25% amino acid identity is the IcaB protein of *Staphylococcus epidermidis*, a protein implicated in intercellular adhesion and deacetylation of the poly-*N*-acetylglucosamine molecule of

R. Fujiyama · J. Nishi (✉) · N. Imuta · K. Tokuda · K. Manago · Y. Kawano  
Department of Pediatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan  
e-mail: nishi1@m2.kufm.kagoshima-u.ac.jp



**Fig. 1** (A) Map of the pathogenic plasmid pAA2 from strain 042, which was modified from that described in a previous report [3]. The black arrows represent the *cap* cluster. (B) Map of the *cap* cluster consisting of three ORFs designated *shf*, *capU*, and *virK*, which are 843, 822, and 951 bp, respectively (GenBank accession no. AF134403). Below the map, the locations of the inserts of *cap* cluster clones are indicated

exopolysaccharide [6, 17]. The second ORF *capU* encodes a protein 50% identical to an *rfbU*-related lipopolysaccharide biosynthetic gene of *E. coli* O157:H7 [3]. The last ORF of this cluster is homologous to *virK*, which has been suggested to be a posttranscriptional regulator of *virG* expression [8]. To investigate the role of the *cap* cluster in the virulence of EAEC, we mutated three genes at this locus and demonstrated that *shf* is required for the firm biofilm formation of EAEC 042, and that *shf* transcription is regulated by the transcriptional regulator AggR.

## Methods

### Bacterial Strains, Plasmids, and Growth Conditions

A prototype strain EAEC 042, the *aggR* mutant 042*aggR*-, and the complement strain 042*aggR*-(pBAD*aggR*) were kindly provided by Prof. J.P. Nataro at the University of Maryland. All *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium or Dulbecco's minimal essential medium with 0.45% glucose (high-glucose DMEM; Invitrogen, Carlsbad, CA). All strains were stored at -80°C in Trypticase soy broth with 15% glycerol. Where appropriate, antibiotics were added at the following

concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and nalidixic acid, 50 µg/ml.

### General Molecular Biology Techniques and Sequencing Procedures

Plasmid DNA purification, restriction, ligation, transformation, and agarose gel electrophoresis were performed using the standard methods. Plasmid DNA was introduced into *E. coli* DH5α competent cells by heat-shock transformation or into 042 and other cells by electroporation using the MicroPulser system (Japan Bio-Rad, Tokyo). DNA sequence was determined by an ABI Prism 310 sequencer (Applied Biosystems Japan, Tokyo).

### Polymerase Chain Reaction (PCR) and Reverse Transcriptase-PCR (RT-PCR)

PCR amplifications were performed using *Taq* DNA polymerase (Takara, Kyoto, Japan) according to the manufacturer's instructions. Amplification reactions were performed in a DNA Thermal Cycler PC 707 (ASTECH, Fukuoka, Japan) for 3 min at 94°C, followed by 30 cycles of 94°C for 30 s, 54°C for 40 s, and 72°C for 1 min per kilobase, concluding with extension at 72°C for 10 min unless otherwise stated. RT-PCR was performed using a method described previously [14]. The primer sequences for PCR or RT-PCR are reported in Table 1.

### Mutagenesis and Complementation

Insertional mutants of *shf*, *capU*, and *virK* genes were constructed by single-crossover insertion of plasmid pJP5603 as previously described [11]. Briefly, an internal portion of the target gene was generated by PCR using the primers described in Table 1, and the product was cloned into *SalI* and *EcoRI* sites of the  $\pi$ -dependent suicide vector pJP5603. The resulting plasmids were propagated into *E. coli* DH5α*λpir* prior to transformation into the donor *E. coli* S17-1*λpir* strain. The mutant strain was then obtained by conjugal mating between the wild-type parent strain 042 (which is nalidixic acid resistant) and the S17-1*λpir* strain (kanamycin resistant). Transconjugants were selected on LB agar supplemented with kanamycin and nalidixic acid. This process resulted in merodiploid integration of the pJP5603 construct into the homologous site in the targeted gene. Integration of pJP5603 constructs resulted in duplication of the predicted codons 127–486 of *shf*, 239–713 of *capU*, and 157–550 of *virK*. To complement the *shf* mutant, the *cap* locus was amplified by PCR using the primers

**Table 1** Primers used in this study

Primer name	Gene(s) amplified	Map coordinates <sup>a</sup>	Sequence (5' to 3')	RE <sup>b</sup>
<i>shf-F</i>	<i>shf</i>	558–576	atgttaaaccgagggggca	
<i>shf-R</i>	<i>shf</i>	1379–1400	ttagtcatccgtaactctggc	
<i>capU-F</i>	<i>capU</i>	1403–1425	atgaataactatttacggaatc	
<i>capU-R</i>	<i>capU</i>	2201–2224	ctacaggcacagaaaatgccgatg	
<i>virK-F</i>	<i>virK</i>	2453–2484	atgtttctataagtaacttatcattatcgg	
<i>virK-R</i>	<i>virK</i>	3379–3403	ttaatttgaattctgatgttctgag	
<i>shf-int-F</i>	<i>shf</i>	705–726	acgtcgaccactgtcatcagatgaactgg	<i>SalI</i>
<i>shf-int-R</i>	<i>shf</i>	1089–1105	acgaattcgcctaataagctctgccg	<i>EcoRI</i>
<i>capU-int-F</i>	<i>capU</i>	1642–1662	acgtcgaccgatgcaatcgtttgtcacag	<i>SalI</i>
<i>capU-int-R</i>	<i>capU</i>	2074–2094	acgaattctcatcatgcatgccatactg	<i>EcoRI</i>
<i>virK-int-F</i>	<i>virK</i>	2610–2629	acgtcgacaactgcgttccttgcctg	<i>SalI</i>
<i>virK-int-R</i>	<i>virK</i>	3002–3021	acggatcccaccgaataacatcaggtcc	<i>BamHI</i>
<i>capW-F</i>	<i>cap</i> cluster	342–362	acggatccttcattggagcacctgaacag	<i>BamHI</i>
<i>capW-R</i>	<i>cap</i> cluster	480408–480427 <sup>c</sup>	acggggccgcgatcggagatccacacatg	<i>NotI</i>
<i>shf-down-R</i>	<i>shf</i> cluster	1504–1525	acggggccgcgctgttctccctgcagacaagc	<i>NotI</i>
<i>cat-F</i>	<i>cat</i>	14–33	tcactggatataccaccggtt	
<i>cat-R</i>	<i>cat</i>	626–645	ccactcatcgcatgactgtt	

<sup>a</sup> Correspond to coordinates in GenBank accession number except for *capW-R*: *cap* cluster, AF134403; *cat*, E15555

<sup>b</sup> Restriction endonuclease cleavage site introduced; shown as underlined sequence in the primer

<sup>c</sup> Corresponds to contig 1 sequence in MAGPIE Automated Genome Project (personal communication, Prof. J. P. Nataro, University of Maryland)

listed in Table 1 and cloned into the single-copy vector pZC320. Amplifications were performed by *EX*-Taq DNA polymerase (Takara) according to the manufacturer's instructions. The PCR products were cloned into the *Bam*HI and *Not*I site of the vector pZC320 (Fig. 1B), and the resultant construct pZC320*cap* was introduced into 042*shf*-. Similarly, the *shf* locus was amplified using the primers *capW-F* and *shf-down-R* in Table 1 and cloned into pZC320 (Fig. 1B). 042*shf*- was also complemented with the resultant construct pZC320*shf*.

#### Microtiter Plate Assay

Microtiter plate assay was performed according to the method described previously [14]. One milliliter of high-glucose DMEM in 24-well flat-bottom microtiter polystyrene plates (Costar, Corning, NY) with or without glass coverslips was inoculated with 10  $\mu$ l of an overnight Luria broth culture grown at 37°C with shaking. The sample was incubated at 37°C for 20 h. The medium was collected and the concentration of the planktonic cells was measured by OD at 600 nm. To evaluate spontaneous settling, precipitated cells on the substratum after aspiration of the medium were collected by scraping into 500  $\mu$ l of PBS and quantified by measuring OD<sub>600</sub>. To assess biofilm formation, adhesive cells on the substratum after washing three times with PBS were collected and quantified as described above.

Biofilm formation was visualized by staining with 0.5% crystal violet for 5 min.

## Results

### Verification of Mutagenesis

RT-PCR of the target gene in each mutant was performed to confirm successful mutagenesis and complementation. The transcription of the target gene was observed in the wild type, but not in each mutant. The transcription of *shf* is restored in the complement strain 042*shf*-(pZC*cap*) and 042*shf*-(pZC*shf*) similar to the wild type. We confirmed the transcription of *capU* and *virK* in the *shf* mutant by RT-PCR (data not shown). The native transcription of *capU* and *virK* genes was not hampered in the *shf* mutant, suggesting that the inactivation of *shf* did not have a polar effect on the downstream.

### The Mutants Maintained the Aggregative Phenotype in the Liquid Phase

There were no differences in growth rate in medium among the strains examined, whether grown in a shaking condition or a static condition. All the mutants showed strong aggregation with shaking in high-glucose DMEM to the same level as the wild type. The aggregative adherence patterns of the mutants

according to the HEp2 cell adherence test for 3-h culture were similar to that of the wild type (data not shown). We evaluated the aggregative phenotype of the mutants in a high-glucose DMEM in a microtiter plate assay. At 20 h of incubation, the concentrations of planktonic mutant cells in the medium were equal to that of the wild type (data not shown). The values of spontaneously precipitated mutant cells on the substratum after aspirating the medium were equal to that of the wild type (Fig. 2A). We considered that the mutants of *shf*, *capU*, and *virK* maintained the aggregative phenotype in the liquid phase similar to that of the wild type.

### The *shf* Mutant Formed Less Abundant Biofilms

We evaluated the biofilm formation of the mutants by means of a microtiter plate assay. At 20 h of incubation, 042*shf*<sup>-</sup> formed less abundant biofilms than did the wild type, 042*capU*<sup>-</sup>, and 042*virK*<sup>-</sup> (Fig. 2B). The biofilm formation of 042*shf*<sup>-</sup> ( $0.230 \pm 0.007$ , mean  $\pm$  SD) was less than that of the wild type ( $0.564 \pm 0.022$ ), 042*capU*<sup>-</sup> ( $0.600 \pm 0.041$ ), and 042*virK*<sup>-</sup> ( $0.585 \pm 0.034$ ). The time courses of biofilm formation are shown in Fig. 1C. The biofilm formation of the wild type increased rapidly after 9 h of incubation, and reached its peak after 12 h of incubation, while that of the *shf* mutant did not.

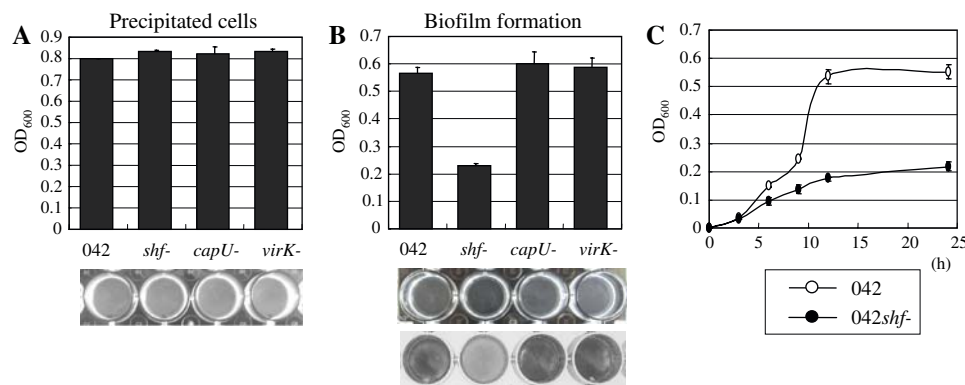
### Complementation of 042*shf*<sup>-</sup> Restored the Thick Biofilm Formation

042*shf*<sup>-</sup> was complemented with the construct including the *cap* locus or the *shf* gene, and the resultant strain 042*shf*<sup>-</sup>

(pZC*cap*) or 042*shf*<sup>-</sup>(pZC*shf*), respectively, was examined for biofilm formation in a microtiter plate assay for 20 h of incubation. 042*shf*<sup>-</sup>(pZC*cap*) and 042*shf*<sup>-</sup>(pZC*shf*) restored thick biofilm formation similar to that of the wild type (Fig. 3A). Quantitatively, biofilm formation in 042*shf*<sup>-</sup> was restored in 042*shf*<sup>-</sup>(pZC*cap*) ( $0.529 \pm 0.011$ ) and 042*shf*<sup>-</sup>(pZC*shf*) ( $0.493 \pm 0.048$ ) to almost the same level as in the wild type (Fig. 3B). The light microscope images of the biofilm formed on the glass coverslip after 20 h of incubation are shown in Fig. 2C. Thick aggregation consisting of multiple layers of bacteria was observed in the wild type, 042*shf*<sup>-</sup>(pZC320*cap*), and 042*shf*<sup>-</sup>(pZC*shf*) but rarely in 042*shf*<sup>-</sup>.

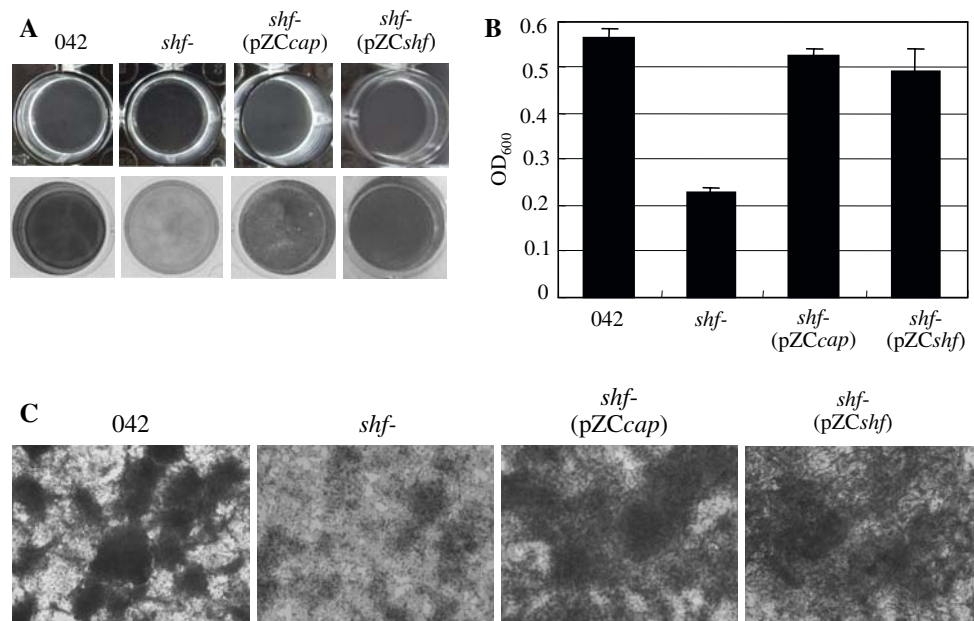
### Transcriptional Dependence of *shf* on AggR

Since the transcriptional regulator AggR activates the transcription of plasmid-born virulence factors of EAEC 042, we hypothesized that the transcription of *shf* is dependent on AggR. To evaluate this hypothesis, we used RT-PCR to investigate the *shf* transcription in the *aggR* mutant 042*aggR*<sup>-</sup> [14]. The transcriptional level of *shf* in 042*aggR*<sup>-</sup> is clearly decreased in comparison with that of the wild type (Fig. 4). To further substantiate the requirement for AggR in *shf* transcription, we evaluated the *shf* transcription in 042*aggR*<sup>-</sup>(pBAD*aggR*) [16], which is the *aggR* complement in *trans* with *aggR* cloned under control of the arabinose promoter in plasmid pBAD30. The *shf* transcription was observed in 042*aggR*<sup>-</sup>(pBAD*aggR*) when cells were grown in the presence of arabinose (*ara*-inducing conditions) but not in glucose (*ara*-repressing conditions) (Fig. 4).



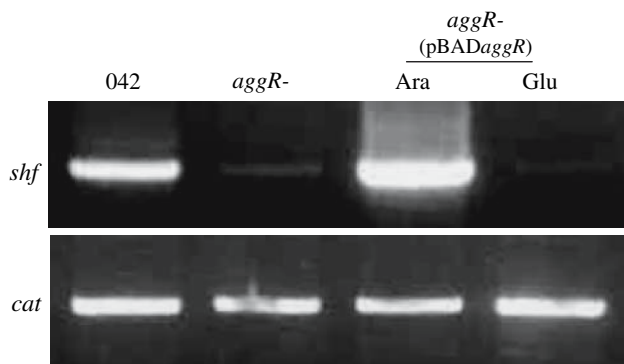
**Fig. 2** Microtiter plate assay of 042 and mutants. Strains were grown in triplicate in high-glucose DMEM in a 24-well microtiter plate for 20 h at 37°C. (A) Quantitative analysis of precipitated cells. Spontaneously precipitated cells on the substratum after aspirating the medium were collected by scraping into 500  $\mu$ l of PBS, and the concentration was measured by OD at 600 nm. (B) Quantitative analysis of biofilm formation. Adhesive cells on the substratum after

washing with PBS were collected and quantified as described above. Biofilm formation was visualized by staining with 0.5% crystal violet for 5 min (bottom panel). (C) Time course of biofilm formation in the microtiter plate assay. Strains were grown for 3, 6, 9, 12, and 24 h as described above. Open circles, 042; filled circles, 042*shf*<sup>-</sup>. Data are presented as means of triplicate experiments, with error bars representing one standard deviation



**Fig. 3** (A) Microtiter plate assay of the wild type 042, 042*shf*-, 042*shf*-(pZCcap), and 042*shf*-(pZCshf). Strains were grown in triplicate in a 24-well microtiter plate in high-glucose DMEM for 20 h at 37°C. Adhesive cells on the substratum after washing three times with PBS (upper panel). Biofilms were visualized by staining with 0.5% crystal violet for 5 min (lower panel). (B) Quantitative analysis of biofilm formation performed as described under Materials

and Methods. Data are presented as means of triplicate experiments, with error bars representing one standard deviation. (C) Light microscope images of biofilms by 042, 042*shf*-, 042*shf*-(pZCcap), and 042*shf*-(pZCshf). Strains were grown on glass coverslips in a 24-well microtiter plate in high-glucose DMEM at 37°C for 20 h. Coverslips were imaged under a light microscope ( $\times 1000$ ) and fields were blindly selected



**Fig. 4** RT-PCR of the *shf* gene in 042, 042*aggR*-, and *aggR*-(pBAD*aggR*). Total RNA was extracted from LB cultures with shaking to midlog phase. The chromosomal chloramphenicol acetyltransferase (*cat*) gene was used as a control. PCR conditions are described under Materials and Methods. Ara, *ara*-inducing conditions (0.1% arabinose); Glu, *ara*-repressing conditions (0.1% glucose)

## Discussion

The insertional mutants of the *shf*, *capU*, and *virK* genes were examined for the characteristic EAEC phenotype in order to reveal the role of the *cap* cluster in the virulence of EAEC infection. No obvious changes were seen in the aggregative phenotype in the liquid phase of shaking culture, in the spontaneous settling in static culture, or in the HEp2 cell adherence test at 3 h of culture. However, the *shf*

mutant formed less abundant biofilms on the polystyrene substratum in a microtiter plate assay than did the wild type and the other mutants. This difference appeared prominent especially after 12 h of culture. The complementation of the *shf* mutant with the *cap* locus or the *shf* gene restored the abundant biofilm formation. These results suggest that the *shf* gene is contributory for firm biofilm formation in EAEC 042.

No function is known for the predicted Shf proteins, but the closest well-characterized homologue is the IcaB protein of *S. epidermidis*, a protein implicated in polysaccharide intercellular adhesin (PIA). Recently, it was reported that the surface-attached protein IcaB is responsible for deacetylation of the poly-*N*-acetylglucosamine molecule of PIA, which plays a crucial role in exopolysaccharide modification in bacterial biofilm formation [17]. Generally, biofilm formation is thought to be a two-step process that requires bacteria to adhere to a substrate surface followed by cell-cell adhesion, forming the multiple layers of the biofilm. Especially in EAEC, strong aggregation in culture medium causes the spontaneous settling of cells onto the substratum and subsequently contributes to thick biofilm formation. Our results suggested that the *shf* gene is not related to aggregation in the liquid phase and adhesion in the early phase but is required to form the multiple layers of biofilm in the maturation phase. Taken

these together, it is speculated that, if expressed, the putative gene product Shf may play a role in intercellular adhesion in biofilm maturation in a way similar to IcaB. Further investigations are needed to verify this hypothesis.

The *shf* mutant maintained the ability to form biofilms on the substratum, although they were less abundant than in the wild type. This fact suggests that the other molecules are also involved in the formation of intercellular adhesins. We performed alignment analysis between EAEC 042 chromosomal sequence ([http://www.sanger.ac.uk/Projects/Escherichia\\_Shigella/](http://www.sanger.ac.uk/Projects/Escherichia_Shigella/)) and intercellular adhesin genes such as *S. epidermidis* *icaABCD* and *E. coli* *pgaABCD* by Blast 2, Version 2.2.17 (NCBI), sequences but did not find other homologues of these genes on the chromosome. Other, unknown genes involved in the intercellular adhesion of EAEC 042 remain to be investigated.

EAEC is known to have genetic heterogeneity [12]. Czczulin et al. reported that the incidence of the *shf* gene among EAEC strains worldwide was 50% [3]. In other studies, the incidence was found to be 65.3% [7] and 52.3% [19]. These reports showed that *shf* is one of the markers frequently detected in EAEC, although the incidence is slightly lower than those of *aggR* and the AA probe (*aatA*). Further investigation on the epidemiology of EAEC strains carrying *shf* is needed to clarify its significance in the virulence of EAEC.

The second ORF *capU* encodes a protein 50% identical to an *rfbU*-related lipopolysaccharide biosynthetic gene of *E. coli* O157:H7 and exhibits motifs specific for hexosyltransferase enzymes [3]. Since the surface polysaccharide of *E. coli* O157:H7 was reported to be involved in organic acid resistance [1], we evaluated the acid resistance of the *capU* mutants but did not find phenotypic change in the organic acid resistance (data not shown). The third gene *virK* has been suggested to be a posttranscriptional regulator of *virG* expression [8], however, Czczulin et al. reported that plasmid pAA2 does not encode *virG* [3]. *capU* and *virK* were considered not to be directly involved in the biofilm formation of EAEC 042 in this study. However, it is possible that *capU* and/or *virK* contribute to biofilm formation in combination with *shf*. Combined mutagenesis of all three genes in this locus would be helpful to verify the hypothesis. The unknown function of both these genes in EAEC 042 remains to be determined.

AggR is a transcriptional activator that controls some plasmid-borne factors including AAF fimbriae, AggR itself, Aap, and Aat. In the chromosome, a *pheU* pathogenicity island is also regulated by AggR [4]. We demonstrated here that *shf* is also regulated by AggR using RT-PCR. These data support the hypothesis that AggR is a global regulator of EAEC virulence determinants. Sheikh et al. reported that EilA, an HilA-like regulator, and *air*,

encoding the predicted outer membrane protein, are associated with biofilm formation [15]. In addition, we found that *E. coli* outer membrane efflux pump TolC promotes the aggregation and adhesion of EAEC 042 [20]. The coordination among biofilm-related genes including *shf* encoded by plasmid or chromosome in EAEC 042 is a target of ongoing research efforts.

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