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NOTE

Involvement of Curli Fimbriae in the Biofilm Formation of *Enterobacter cloacae*

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In this study, we examined the biofilm forming ability, the mRNA expression of curli genes and the morphologies of curli fimbriae and biofilms in clinical isolates of *Enterobacter cloacae*. The *csgBA* operon was found in 11 (78.6%) of the 14 isolates. The ability of *E. cloacae* isolates to form biofilms was significantly correlated with the mRNA expression level of the *csgA* and *csgD* genes. The curli protein fimbriae appeared as tangled fibers and the curli-proficient strain formed mature biofilms. Our data suggest that the expression of the curli fimbriae play an important role in biofilm formation in *E. cloacae*.

Keywords: curli, biofilm, *Enterobacter cloacae*

A biofilm is a complex aggregation of microorganisms marked by the excretion of a protective and adhesive matrix. The ubiquitous habitats of biofilms include the human body; moreover, biofilm-related infections are increasing. It has been estimated that more than 60% of hospital-acquired infections may be biofilm-related (Raad, 1998; Crump and Collignon, 2000; Donlan and Costerton, 2002; Vinh and Embil, 2005). Due to the resistance of biofilms to numerous antimicrobial agents and to products of the immune system, biofilm-related infections are known to be extremely difficult to treat (Costerton *et al.*, 1999).

Biofilms are generally characterized by an extracellular matrix that helps to form three-dimensional structures. Curli belong to a class of highly aggregated, proteinaceous extracellular fibers expressed by *Escherichia* and *Salmonella* spp. that are involved in biofilm formation and the colonization of inert surfaces (Austin *et al.*, 1998; Romling *et al.*, 1998; Vidal *et al.*, 1998). These fibers mediate binding to a variety of host proteins and also mediate host-cell adhesion and invasion (Olsen *et al.*, 1993; Sjobring *et al.*, 1994; Ben Nasr *et al.*, 1996). Curli-encoding genes are clustered in two operons: *csgBA*(*C*) and *csgDEFG* (Hammer *et al.*, 1995; Loferer *et al.*, 1997). CsgA encodes the curlin subunit; CsgB is thought to nucleate CsgA curlin fibers (Hammer *et al.*, 1996), CsgD is a positive transcriptional activator of the *csgBA* operon, and CsgE, CsgF, and CsgG are three putative curli assembly factors (Brombacher *et al.*, 2006).

Recently, we investigated clinical isolates of *Enterobacteriaceae* for biofilm forming ability and the presence of *csgA* gene (Choi *et al.*, 2011). Among the 22 clinical isolates of *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens*, and *Pantoea* spp., biofilm forming ability was highest in *E. cloacae*. The *csgA* gene was detected in 1 strain of *C. freundii* and in 4 strains of *E. cloacae*. Moreover, in *E. cloacae*, a good correlation between the phenotypic detection of curli fimbriae by congo-red staining and the genotypic detection of the curli gene was observed. Therefore it is suggested that the curli fimbriae may be an important extracellular matrix component of *E. cloacae* biofilms. To discover the role of the curli fimbriae in biofilm formation of *E. cloacae*, we investigated the biofilm forming ability, the presence of curli genes, the correlation between biofilm forming ability and the mRNA expression level of curli genes, and conducted a morphological analysis of the curli fimbriae and biofilm structure with additional clinical isolates of *E. cloacae*.

Fourteen clinical isolates of *E. cloacae* collected from Kyungpook National University Hospital were used. The biofilm assay was performed by procedures outlined by Heilmann *et al.* (1997). *E. coli* strain DH5α was used as the negative control for the biofilm assay. As shown in Table 1, four strains (*E. cloacae* 40, 41, 48, and 50) showed a much higher production of biofilms compared to the other strains whereas three strains (*E. cloacae* 24, 47, and 51) produced very low levels of biofilms ranging from OD_{570} values of 0.19±0.09 to 0.25±0.02.

The presence of a *csgBA* operon was determined by PCR using *csgBA(C)*- and *csgA*-specific primers listed in Table 2. Chromosomal DNA was prepared by the method provided by the manufacturer of theWizard genomic DNA preparation kit (Promega, USA). PCR amplifications were performed in a DNA Thermal Cycler with the following parameters:

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Table 1. Biofilm forming abilities and mRNA expression levels of the curli genes in *E. cloacae* isolates[a]

Strains	Biofilm forming ability	Detection of curli genes	mRNA expression level of *csgA*	mRNA expression level of *csgD*	Source
E. cloacae 22	0.51 ± 0.04	Positive	13 ± 2	46 ± 5	Choi *et al.* (2011)
E. cloacae 23	0.81 ± 0.09	Positive	31 ± 3	83 ± 9	Choi *et al.* (2011)
E. cloacae 39	0.68 ± 0.07	Positive	29 ± 3	28 ± 9	Choi *et al.* (2011)
E. cloacae 40	1.51 ± 0.58	Positive	60 ± 5	133 ± 9	This study
E. cloacae 41	1.55 ± 0.13	Positive	65 ± 4	128 ± 10	Choi *et al.* (2011)
E. cloacae 42	1.11 ± 0.17	Positive	44 ± 4	56 ± 6	This study
E. cloacae 44	0.79 ± 0.27	Positive	42 ± 2	94 ± 7	This study
E. cloacae 46	0.66 ± 0.27	Positive	41 ± 4	22 ± 4	This study
E. cloacae 48	1.38 ± 0.45	Positive	61 ± 4	102 ± 4	This study
E. cloacae 50	1.45 ± 0.39	Positive	61± 5	120 ± 4	This study
E. cloacae 52	0.52 ± 0.15	Positive	14 ± 2	50 ± 2	This study
E. cloacae 24	0.25 ± 0.02	Negative	ND[b]	ND	Choi *et al.* (2011)
E. cloacae 47	0.25 ± 0.02	Negative	ND	ND	This study
E. cloacae 51	0.19 ± 0.09	Negative	ND	ND	This study
E. coli DH5α	0.15 ± 0.09	ND	ND	ND	Choi *et al.* (2011)

[a] Each experiment was repeated three times. Data are expressed as means±SD.
[b] ND, not determined.

denaturation for 5 min at 94°C; 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; extension for 10 min at 72°C. The *csgBA* operon was detected in 11 of 14 isolates but not detected in three strains showing very low levels of biofilm production (Table 1).

The mRNA expression levels of *csgA* and *csgD* were also examined by comparative RT-PCR analysis (Fig. 1), and the results were expressed as ratios of each mRNA normalized to the 16S rRNA amplified from the same cDNA sample (Table 1). Total RNA was prepared from each sample using the Invisorb Spin Cell RNA Mini Kit (Invitek, Germany) following the manufacturer's instructions. First-strand cDNA was obtained using a reverse transcription kit (Fermentas, USA) with 2 μg of total RNA, and the resulting cDNA was used as a template for the next PCR step. PCR primers for the detection of *csgA* and *csgD* were *csgA*-specific primers and *csgD*-specific primers respectively (Table 2). PCR amplification was comprised of 22 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Band intensities of the PCR products were measured using an image analysis program (MetaMorph; Universal Imaging Corp., USA), and data were expressed as ratios of each mRNA normalized to 16S rRNA amplified from the same cDNA sample. Statistical analysis was performed with the software package SPSS 12.0K for Windows (SPSS Inc., USA). Correlations between biofilm formation, *csgA* expression, and *csgD* expression were evaluated using Pearson's correlation coefficient and Spearman's rank correlation. The mRNA expression level of *csgA* was highest in strain 41 and lowest in strain 22. The four strains with higher levels (values of OD_{570}, 1.38±0.45 to 1.55±0.13) of biofilm formation had higher expression levels (ranging from 60±5 to 65±4) of *csgA* than of the other strains whereas two strains with lower levels (values of OD_{570}, 0.51±0.04 and 0.52±0.15) of biofilm formation had much lower expression levels (13±2 and 14±2) of *csgA*. The mRNA expression levels of *csgA* was significantly correlated with the biofilm forming abilities (n=11, r=0.928, p<0.001). The mRNA expression level of *csgD* was also higher in the strains with higher levels of biofilm formation (Table 1). The mRNA expression level of *csgD* was also significantly correlated with the biofilm forming abilities (n=11, r=0.854, p<0.002). In addition, there was a positive correlation between the mRNA expression levels of *csgA* and *csgD* (n=11, r=0.785, p<0.001).

Table 2. Primers used in this study

Target gene	Sequences of primer	Product size
csgBA(C)	S : 5′-ATGATGTTAACAATACTGGGTGC-3′ A : 5′-CGGCCATTGTTGTGATAAAG-3′	1.3 kb
csgA	S : 5′-TTCAAAGTGGCAGTTATTGCAG-3′ A : 5′-TTTTTGCAGCAGATCGATAGAA-3′	0.276 kb
csgD	S : 5′-GAAATTGCATAATATTCAACGTTC-3′ A : 5′-TTTGTTCAGGATCTCTTTTTCAC-3′	0.384 kb
16S rRNA	S : 5′-TGGCTCAGATTGAACGCTGGCGGC-3′ A : 5′-TACCTTGTTACGACTTCACCCCA-3′	1.5 kb

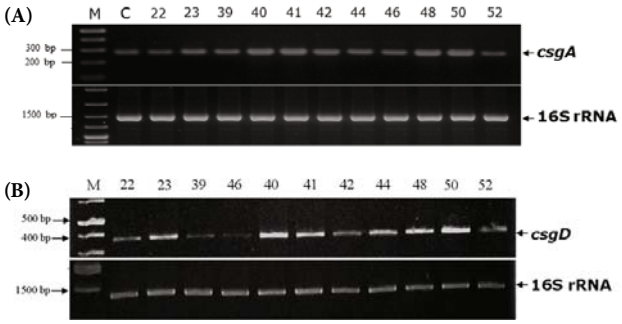

Fig. 1. Comparative RT-PCR analysis of *csgA* mRNA and *csgD* mRNA in clinical isolates of *E. cloacae*. (A) Agarose gel electrophoresis showing a representative RT-PCR result of amplified *csgA* and 16S rRNA. (B) Agarose gel electrophoresis showing a representative RT-PCR result of amplified *csgD* and 16S rRNA. The density of each band was measured by scanning densitometry and then expressed as the ratio of the *csgA* mRNA normalized to the 16S rRNA (Table 2). M, DNA size marker; C, positive control (*C. freundii* strain 8); *E. cloacae* strains 22, 23, 39, 40, 41, 42, 44, 46, 48, 50, and 52.

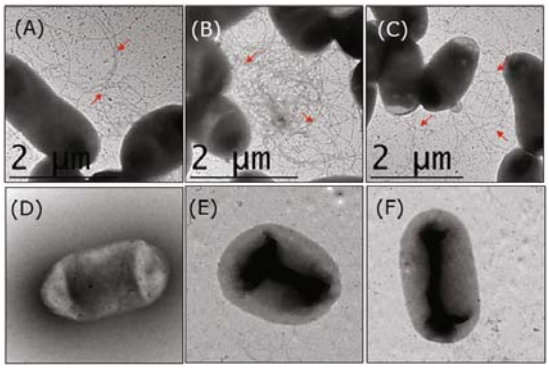

Fig. 2. Transmisson electron microscope (TEM) analysis of the curli fimbriae in clinical isolates of *E. cloacae*. Isolates of *E. cloacae* were incubated at 28°C for 24 h and negatively stained with 2% uranyl-acetate. The curli fimbriae were observed in the representative, biofilm-forming isolate, *E. cloacae* strain 41, and marked with red-colored arrows (A–C). Curli fimbriae were not observed in *E. cloacae* strain 24 in which the *csgA* gene was not found (D–F).

To observe the curli fimbriae produced by *E. cloacae*, we did negative staining and transmission electron microscopy (TEM) analysis (Fig. 2). For the analysis, samples were prepared as described previously (Martinez *et al.*, 1999). The samples were negatively stained with 1% uranyl-acetate for 30s, rinsed again with distilled water and viewed using an H-7600 transmission electron microscope (HITACHI, Japan). Images were acquired using a Slow Scan CCD Camera (V533, AMT, USA). The Curli fimbriae appeared as aggregated fibers in *E. cloacae* strain 41 (Figs. 2A–C). The curli fimbriae, however, were not observed in *E. cloacae* strain 24 in which the *csgA* gene was not detected (Figs. 2D–F).

The structures of the biofilms formed by the isolates of *E. cloacae* were observed by scanning electron microscopy (SEM) analysis (Fig. 3). For the analysis, biofilms formed on the plastic coverslips were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. Then they were sequentially dehydrated for 5 min in 50%, 70%, 90%, and 100% ethanol, fixed to aluminum stubs using carbon tape, coated with a layer of gold (7 nm thick), and examined with an S-4300 scanning electron microscope (Hitachi, Japan) at 15 kV. The morphology of the biofilms formed by the curli-proficient *E. cloacae* strain 41 differed from that of the biofilms formed by curli-deficient *E. cloacae* strain 24. At 24 h of incubation, the image revealed well adhered cells with aggregate formation in the curli-proficient strain 41 (Fig. 3A) compared with randomly distributed cells without aggregate formation in the curli-deficient strain 24 (Fig. 3B). At 48 h of incubation, mature biofilms with clusters of bacteria surrounded by water channels were revealed in the curli-proficient strain 41 (Fig. 3C).

E. cloacae is one of the important nosocomial enteric pathogens that cause biofilm-related infections such as catheter-associated urinary tract infections and biliary tract infections (Fernandez-Baca *et al.*, 2001; Harbarth *et al.*, 1999). To date, little is known about curli fimbriae of *E. cloacae*. In this study, we examined the role of curli fimbriae in the formation of biofilms in clinical isolates of *E. cloacae*. The *csgBA* operon was found in 11 (78.6%) of the 14 isolates of *E. cloacae* and the ability of *E. cloacae* isolates to form biofilms was positively correlated with the mRNA expression of *csgA*. In addition, *csgA*-positive strains expressed aggregated curli fibers, which were not found in the *csgA*-negative strain. The results indicate that the curli fimbriae may play a role in the biofilm formation of *E. cloacae*.

It has been shown that CsgD positively regulates the

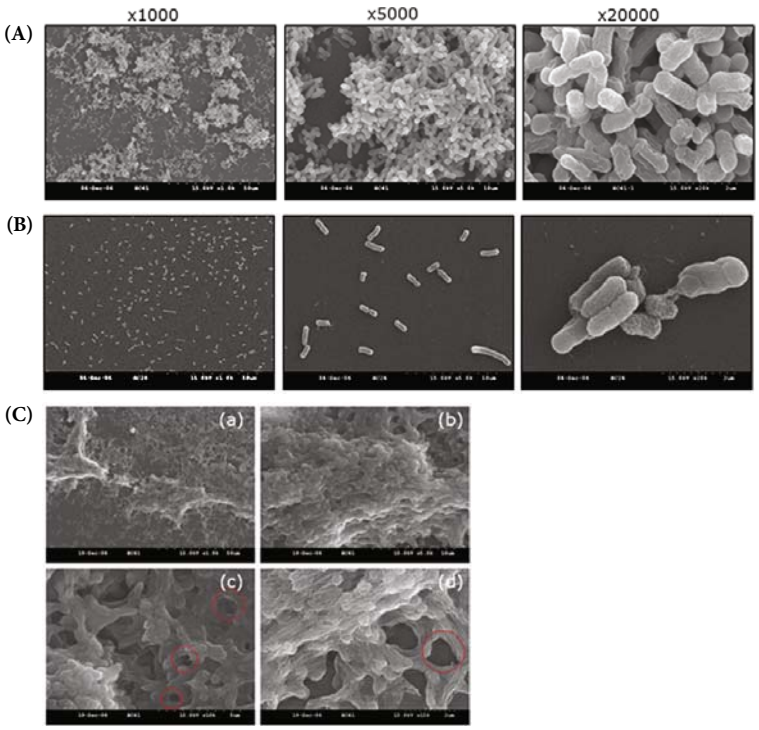

Fig. 3. Scanning electron microscope (SEM) analysis of biofilms produced by clinical isolates of *E. cloacae*. *E. cloacae* strain 41 (A) and 24 (B) were incubated at 37°C for 24 h and viewed at magnification of 1,000×, 5,00×, and 20,000×. (C) *E. cloacae* strain 41 was incubated at 37°C for 48 h and viewed at magnification of 1,000× (a), 5,000× (b), 10,000× (c), and 15,000× (d).

csgBA operon and that the expression of curli depends on the CsgD protein, a putative transcription regulator in the FixJ/UhpA family (Hammar *et al.*, 1995). This study also showed a positive correlation between the mRNA expression levels of *csgA* and *csgD*. With the positive correlation between *csgA* expression and biofilm forming ability, this finding suggests that the upregulation of CsgA expression through CsgD might be crucial to the abilities of *E. cloacae* isolates to enhance biofilm formation. Jonas *et al.* (2007) also showed that a curli mutant and a strain mutated in the CsgD of *Salmonella* Typhimurium were severely impaired in biofilm formation, and overexpression of CsgD led to a much thicker and more rapidly growing biofilm.

Negative staining with TEM analysis in this study showed the shape of curli expressed in *E. cloacae* for the first time. Curli expressed by *E. cloacae* were indistinguishable from those presented naturally on the bacterial surface, appearing as aggregated fibers of varying lengths and widths. Jonas *et al.* (2007) demonstrated that the curli fibers and cellulose but not BapA expression have a major impact on the formation and morphology of the biofilm in *Salmonella typhimurium*, wherein the curli fibers seem to be more important for the formation of cell aggregates than cellulose. Similarly, Kikuchi *et al.* (2005) showed that the curli-expressing *E. coli* K12 strain formed more biofilms on polyurethane than the isogenic curli-deficient strain. This study also showed the importance of the curli fibers for biofilm formation in *E. cloacae* within contrast to the flat biofilms of scattering bacteria formed by the curli-deficient strain, the curli-proficient *E. cloacae* strain formed mature biofilms of pillar-shaped bacterial clusters, surrounded by water channels, on polystyrene surfaces.

In conclusion, this study showed that most of the *E. cloacae* clinical isolates were able to form biofilms and express curli proteins. Moreover, a strong association between the biofilm forming ability and the expression of curli genes, as well as a different morphology between the biofilms formed in the curli-proficient strain and curli-deficient strain, suggest that the curli fimbriae play an important role in the formation and morphology of biofilms in *E. cloacae*.

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