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Escherichia coli O157:H7 adherence to HEp-2 cells is implicated with curli expression and outer membrane integrity

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***Escherichia coli* (*E. coli*) has ability to express thin aggregative fimbriae, known as curli, on the cell surface. Previously, a few example of curli expression in serogroup O157:H7 of enterohemorrhagic *E. coli* (EHEC) were reported, compared to other *E. coli* groups. However, significance of curliation in the EHEC pathobiology has not been described well in the literature. A highly curliated O157:H7 strain was used in this study in order to elucidate role of curliation in EHEC adherence to cultured HEp-2 cells. The expression of curli in the EHEC isolate was consistent with strong positive indication of Congo-red (CR) binding and formation of clumps in the bottom of the tube containing Luria-Bertani (LB) broth when cultured overnight at 37°C. A few CR-binding negative (CR-) colonies occurred spontaneously within the population of CR+ isolate. The CR+ EHEC showed massive aggregative adhesion pattern, whereas the spontaneous CR- strain showed typical localized adherence on HEp-2 cells. Electron microscopy confirmed highly curliated bacteria in the CR+ EHEC sample. Interestingly, the curliation disappeared in a *msbB1* and *msbB2* double mutant derived from the CR+ EHEC. These results suggest that the compromised outer membrane integrity caused by *msbB* mutations may abrogate curli production in the CR+ EHEC harbouring penta-acylated lipid A structure in their outer membrane.**

Key words: *E. coli* O157:H7, Curli, Adhesion, HEp-2 cells

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC), have been emerged as important food- and water-borne pathogens worldwide [1]. STEC causes a wide range of disease in humans, from mild diarrhoea to severe haemolytic uremic syndrome (HUS). A group of STEC that are associated with hemorrhagic colitis or HUS is called enterohemorrhagic *E. coli* (EHEC) which includes limited member of serotypes, such as O157:H7, O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM [1,2]. Typical EHEC has genetic markers composed of virulence-associated genes, such as *stx* for Shiga toxins, *ehx* for enterohemolysin, and *eae* for intimin [2]. Intimin, an outer membrane protein, has been well characterized in roles of formation of attaching and effacing (A/E) lesions and intestinal colonization [3]. A recent report [4] suggests that multiple bacterial elements control the initial adherence to host intestinal epithelium. However, bacterial factors other than intimin that are required for colonization are still poorly defined. It seems that OmpA may act as an adhesin for EHEC O157:H7 [4], and/or a ToxB homologue encoded in pO157 plasmid may promote the bacterial adherence to cultured epithelial cells by unknown mechanism [5].

Production of thin aggregative fimbriae, known as curli, has been noticed in diverse group of *E. coli* [6,7,8,9]. However, the curliation was rarely reported in EHEC O157:H7 [10,11], compared to other pathogenic *E. coli* strains [7,9,12]. Curli production was estimated as only 2 (4%) of 49 isolates among diverse bovine and human *E. coli* O157:H7 strains, while 5 (38.5%) of 13 non-O157 STEC elaborated curli [7] as judged by characteristic red-coloured colonies formed on CR-binding agar plates after 48 h incubation at 28°C. We hypothesized that curliation in EHEC O157:H7 may play a role in adherence to host epithelial cells as an initial step for colonization of host tissues. In order to examine this hypothesis, curliated EHEC were screened from a collection of diverse *E. coli* O157:H7 (33 isolates in Canada) based on their CR-binding phenotype. An EHEC O157:H7 strain 4304 (phage type 14, human isolate) was chosen for this study because strain 4304 showed strong CR+ phenotype at 28~37°C incubations. For comparison of adhesion pattern mediated by curliation, CR+ strain 4304 and its CR- derivative were tested for HEp-2 cell adherence. We conclude that curli production in EHEC O157:H7 confers the bacterium massive aggregative

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adherence to HEp-2 cells and that the adherence of curliated stain 4304 was also affected by alteration of the outer membrane integrity.

Materials and Methods

Culture of cells

A highly curliated O157:H7 EHEC strain 4304 and its CR- variant were used for HEp-2 cell adherence assay. Congo red (CR)-binding agar plates were prepared based on the recipe for the CFA agar [8]. Approximately, 30~40 mg of Congo red dye was added per liter of the CFA agar consisting of 1% Casamino acids, 0.15% Yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, and 2% agar at pH 7.4. Eagles minimum essential medium (EMEM, Gibco-Invitogen, USA) medium supplemented with 10% FCS (fetal calf serum) was used for tissue culture of HEp-2 (human laryngeal epithelial cells).

HEp-2 cell adherence assay

HEp-2 cell (ATCC CCL23) monolayers were prepared according to a method previously described [13]. Briefly, HEp-2 cells were maintained by serial passage in EMEM supplemented with 10% FCS, 20 mM $_L$-glutamine, 100 μg/ml gentamicin(Sigma, USA), 10 units/ml penicillin G, and 10 μg/ml streptomycin (called complete EMEM). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. For subconfluent HEp-2 cell monolayers, 8-well chamber slides (Lab-Tek, USA) were seeded with 6×10^4 cells/well in complete EMEM and incubated for 24 h until cells reached to ~80% confluent stage. The medium in each well was then removed and replaced with complete EMEM that contained 1% FCS. Cells were maintained under these conditions prior to use within 24 h. For bacterial adherence to HEp-2 cell monolayers, the bacteria (CR+ strain 4304 and its CR- variant) were grown overnight at 37°C in LB broth. The bacterial cell density in the inoculum was adjusted same in each test. Overnight bacterial culture (0.5 ml) was transferred to 2.5 ml of EMEM containing no antibiotics but with 1% $_D$-mannose, that is called EMEM(-), for 3 h at 37°C in 5% CO_2 incubator until the optical density reached to 1 at A_{600}. Then, a 20 μl of the preconditioned culture was added to HEp-2 cell monolayer in tissue culture slide chamber containing 250 μl of fresh EMEM(-). The co-culture system was placed in 5% CO_2 incubator(Forma Scientific, USA) for 3 h at 37°C. Then, each chamber was washed 3 times with prewarmed PBS (pH 7.2) in order to remove non-adherent bacteria. Fresh EMEM- was added to each chamber and the co-culture system was incubated further for another 3 h. Then, the tissue culture slides were washed 3 times with PBS, fixed with 70% methanol, and stained with 10% Giemsa prior to observation under light microscope(Nicon, Japan).

CR+/- conversion

The CR+ strain 4304 was used for repeated 3 time passages at overnight incubation intervals in LB broth in order to obtain spontaneous phenotypic conversion of the curliated EHEC to non-curliated, CR- strain. Estimated 30 ~300 CFU (colony forming unit) of bacterial culture was spread onto CR-binding agar plates, then incubated at 28°C up to 2 days. Arising white (CR-) colonies were counted and purified upon majority of the red (CR+) colonies.

Autoaggregation test

The autoaggregation test was prepared by slight modification of a method [14] described as bacterial clump-formation test in culture tubes. Briefly, the bacterial culture in a test tube containing 5 ml LB broth or EMEM(-) was made at 37°C overnight with gentle shaking. The autoaggregation was scored positive (+ ~ +++) when clearly visible clumps formed as ring in the test tube wall at the broth surface and/or the clumps were gravitated to the round bottom of the tubes.

Electron microscopy of curli fimbriae

Negative staining of CR+ and CR- EHEC bacteria were prepared for EM (Philips, XL30S FESEM, Netherlands) observation. Bacteria grown overnight in LB both were concentrated and resuspended in PBS. The bacterial suspension was mixed with equal volume of 2% (v/v) phosphotungstic acid and a drop of the mixture was transferred onto a Formvar-coated copper grid (Canemco, Canada). Excess fluid was removed by touching the grid with filter paper before EM viewing.

Results

HEp-2 cell adherence assay

In order to compare adhesion pattern mediated by curliation, CR+ strain 4304 and its *msbB* mutants [15] and the CR- variant were co-cultured with HEp-2 cells prepared as monolayers in 8-chamber slide tissue culture system. The curliated (CR+) bacteria showed unusual adhesion pattern to HEp-2 cell monolayer (A and B of Fig. 1). The massive adhesion pattern seen in the CR+ bacteria was different from those of localized adherence (LA) (C and D of Fig. 1), the characteristic adhesion pattern of typical EHEC O157:H7, which is CR-. Rather, this unusual adhesion pattern of CR+ cells closely resembles the aggregative adherence (AA) of enteroaggregative *E. coli* (EAEC) pathotype as described elsewhere [16]. However, the double *msbB* (*msbB1*/*msbB2*) mutant [15] of strain 4304 did not show any of AA-like or LA adhesion pattern (E and F of Fig. 1).

Autoaggregation test

We alternatively examined degree of curliation of the CR+

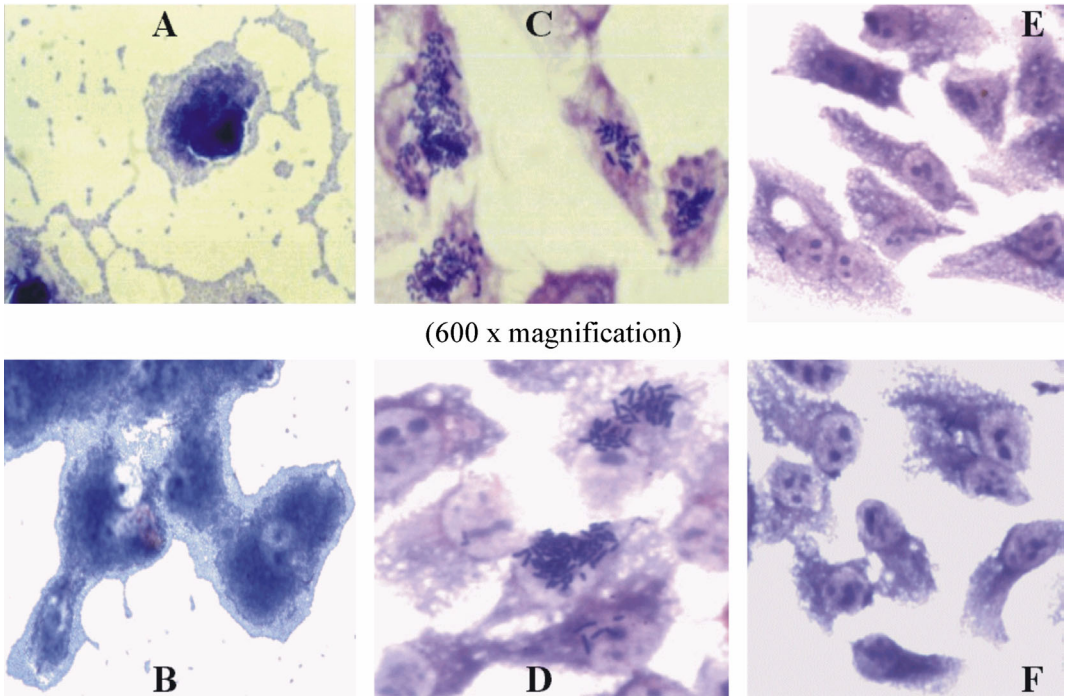

Fig. 1. HEp-2 cell adherence assay with CR+/- wild type EHEC O157:H7 strain 4304 and the double *msbB* mutant derived from CR+ strain. The curliated (CR+) bacteria showed unusual AA-like adhesion pattern to HEp-2 cell monolayer (panels A and B). The massive adhesion pattern seen in panel B was obtained by 6 h coincubation of CR+ bacteria with HEp-2 cells after initial 3 h coincubation in panel A. However, the CR- bacteria showed typical LA pattern (panels C and D) and the double *msbB* (*msbB1*/*msbB2*) mutant [15] of strain 4304 did not showed any of AA-like or LA adhesion pattern (panels E and F).

bacteria by observing the clump-formation [14] in the culture test tubes. Precipitation of bacterial aggregates was dramatically reduced in the double *msbB* mutant whose outer membrane (OM) functions are compromised by alterations of lipid A species [15,17], compared to that of wild type and/or two single *msbB1* and *msbB2* mutants (data not shown). The CR+ bacteria grown in EMEM without containing FCS were slightly more aggregative than those grown in EMEM(-) containing 10% FCS (data not shown). The clump-formation was consistent with massive AA-like adhesion pattern observed in HEp-2 cell monolayer (Fig. 1). Unlike curliation of *E. coli* K-12, the degree of autoaggregation (clumps formation) was not affected by growth temperature and/or osmolarity of culture medium, indicating that curliation of EHEC strain 4304 seemed to be temperature-independent and osmolarity-independent.

Electron microscopy of curli fimbriae

The morphology of curli fimbriae of EHEC strain 4304 was seen by EM in order to observe the characteristic curli hair-like appearance at the cell surface. As shown in Fig. 2, the morphology of curli produced in the EHEC was matched to that of other *E. coli* strain [12,18,19]. The highly curliated CR+ cells were tangled with each other presumably by the curli structure (A, B, and C of Fig. 2). However, non-curliated CR- derivative was observed as singular cell (Fig. 2E). Interestingly, curli production in the double *msbB* mutant [15] of strain 4304 (Fig. 2D) was dramatically diminished, compared to those of two single *msbB1* (Fig. 2B) and *msbB2* (Fig. 2C) mutants, and wild type strain 4304 (Fig. 2A).

Discussion

The genes required for curli production appear to be present in all EHEC O157:H7 isolates. However, only small percent of EHEC O157 can produce the curli structure [7,9]. A point mutation in the *csgD* promoter region was suggested for the explanation in spontaneous phase variation of curli expression [10,11]. Indeed, curli biogenesis seems to be linked tightly to complex regulation [20,21,22]. Curli expression of *E. coli* K-12 has been known to be optimal *in vitro* conditions, at which temperatures below 30°C in medium with low osmolarity and low nutrients, during stationary phase of growth, that are most likely to be met by *E. coli* outside the host intestine [21]. We screened highly curliated EHEC O157:H7 strain 4304 showing routine curliation even at higher growth temperature 37°C in LB broth culture (Fig. 2A). In EHEC strain 4304, growth temperature and culture medium appeared not the factors affecting curli production. One can speculate that the curli expression might be regulated by environmental conditions,

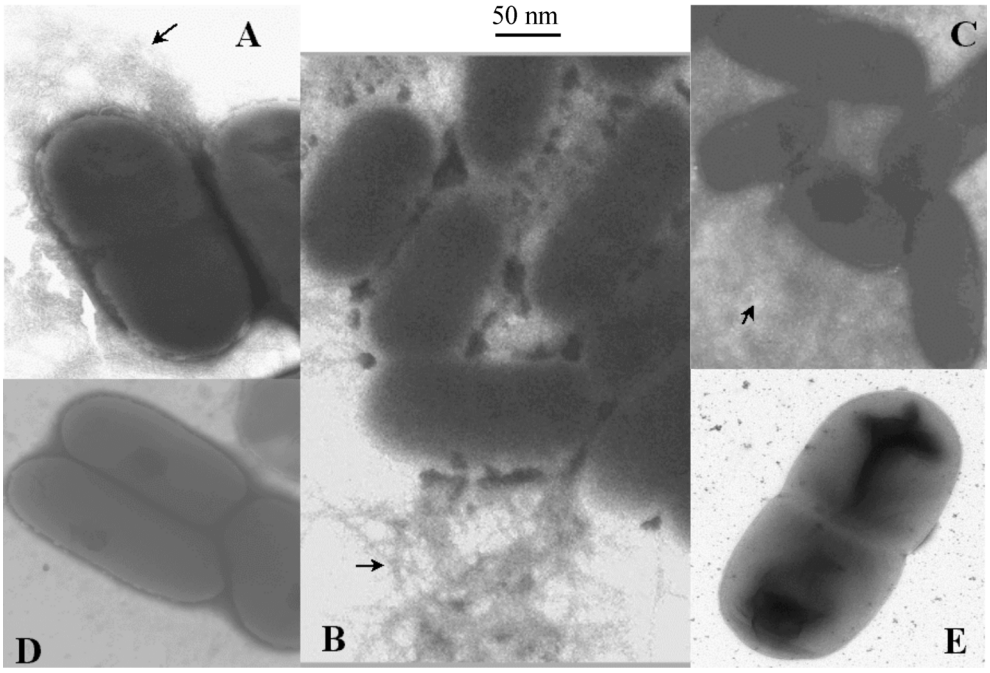

Fig. 2. Electron microscopy of curli fimbriae produced in EHEC O157:H7 strain 4304. The morphology of curli fimbriae produced in EHEC strain 4304 was observed by EM in order to observe the characteristic curli hair-like appearance at the cell surface. The highly curliated (CR+) bacteria were seen in panels A, B, and C, which is CR+ wild type strain 4304, its *msbB1*, and *msbB2* mutant, respectively. However, CR- variant of strain 4304 (panel E) and the double *msbB* mutant (panel D) were defective in curliation.

including that reflected by the host intestine of cattle and/or humans. In this regard, we hypothesized that curliation in EHEC O157:H7 may play a role in adherence to host epithelial cells. The CR- variants of CR+ strain 4304 were obtained from 3 time repeated passages of the strain in LB broth and subsequent spreading of the culture aliquots onto CR-binding agar plates, which generated a few spontaneous CR- (white to pale pink colonies) variants (data not shown). In order to compare adhesion pattern, the CR+ and CR- bacteria were added to HEp-2 cell monolayer in 8-chamber slide tissue culture system. The curliated (CR+) bacteria showed unusual adhesion pattern to HEp-2 cell monolayer (A and B, Fig. 1). The unusual adhesion pattern of CR+ bacteria closely resembled the aggregative adherence (AA) of enteroaggregative *E. coli* (EAEC) pathotype [16]. The AA phenotype of EAEC is conferred by plasmid-encoded locus [16]. However, there is no homologue of the *aaf* gene exist in pO157 plasmid [23], suggesting that such a massive AA-like pattern may be attributed to the curliation of EHEC O157:H7.

In *E. coli* K-12, curli operons (*csgBA* and *csgDEFG*) are placed in opposite transcriptional direction by intergenic region [6]. Curlin, the product of *csgA*, is the major component of curli, while CsgB, an homologue of CsgA, acts as a nucleator, which primes the polymerization of curlin on the cell surface [24]. The first gene of the *csgDEFG* operon encodes the CsgD protein, a putative LuxR-type transcription factor and is required for the transcription of *csgBA* operon [6,10]. The *csgEFG* genes encode three curlin assembly factors, probably involved in export of the curlin subunit [6,24]. We wonder that the mechanism underlying the CR+/- conversion in strain 4304 may also be due to DNA sequence alteration(s) in the intergenic region between the *csgBA* and *csgDEFG* operons, where the promoter of *csgD* located [10,11]. In order to address this question, we cloned the intergenic regions from the CR+ and CR- stain 4304, respectively, for DNA sequence comparison (data not shown). The DNA sequence of the intergenic regions were 100% identical to each other, suggesting that CR- variant of strain 4304 is not related with the proposed phase variation, which occurs due to point mutation in the intergenic promoter region [10]. Considering the complexity of the regulation involved in curli expression [20,21], we are unaware of what mechanism(s) might be involved in the CR+/- conversion at this moment.

We further investigated optimal culture condition for curli production of strain 4304 *in vitro*. The bacteria grown in EMEM without FCS were more aggregative than complete EMEM(-) containing 10% FCS as judged by autoaggregation test (bacterial clumping in the culture tubes). The clump-formation (data not shown) was consistent with massive AA-like pattern observed in HEp-2 cell monolayer (Fig. 1). Unlike curliation of K-12, the curliation of EHEC strain 4304 seemed to be temperature-independent and osmolarity-independent. This result may support that a successful pathogen may have different regulatory

mechanism required for expression of common factors that shared by non-pathogenic counterpart.

In addition, a report [25] suggested that type 1 fimbriae also confer *E. coli* AA-like pattern. However, it has been reported that the genes required for type 1 fimbrial biosynthesis are defective in EHEC O157:H7 [25] due to a deletion mutation in the promoter region of the *fimA* gene. To rule out any possibility that type 1 pili are the case of AA-like pattern in strain 4304, we added $_D$-mannose into the EMEM (final 1% concentration) as control for co-culture system. There was no apparent difference in the AA-like adhesion pattern in EMEM containing $_D$-mannose, indicating that type 1 piliation is not the case for the AA-like adhesion of EHEC strain 4304. Recently, we have shown that pO157-encoded *msbB2* gene could complement loss of *msbB1* function in the single *msbB1* mutant [15]. Either single *msbB1* or *msbB2* mutant showed the same hexa-acyl lipid A structure, whereas the double *msbB* mutant showed mainly penta-acylated lipid As. Consistently, only double *msbB* mutant of strain 4304 was defective in expression of curli and in formation of microcolonies on HEp-2 cell surface in a localized manner. This result suggests that the mainly penta-acylated lipid As generated in the double *msbB* mutant cause alterations of the OM-associated components, which may provide unfavourable OM condition for expression of OM-attached structure, like curli fimbriae.

It seems that curli has diverse biological activities in relation to host cells. For example, curli expression in speticemic *E. coli* contributes to development of sepsis [18,26]. The production of curli fimbriae also plays a role in biofilm formation [27,28]. However, the significance of curliation in EHEC pathobiology has not been clearly understood yet. Research in our laboratory has a focus on the bacterial factor(s) that may be required for the expression of curli fimbriae in EHEC O157:H7. We will continue to investigate the OM-associated structure and function of virulence factors including curli involved in the organisms survival in diverse environments. Future studies will be directed to elucidation of role of curliation in EHEC pathogenesis as well as the bacterial and the environmental conditions linked to curli expression.

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