**Enhanced Colonization of rpoS-Deficient Escherichia coli Cells on Solid Surfaces by Reinforced csgA Gene Expression**

MINH HONG NGUYEN, YOSHIHIRO OJIMA, AND MASAHITO TAYA *

Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan

Received 10 January, 2014/Accepted 27 February, 2014

Scanning electron microscopy revealed that the rpoS-deficient cells of E. coli K-12 BW25113 (ΔrpoS) increased the number of flagella on the cell surfaces. However, the quantitative analysis of cell colonization showed that the increased number of flagella on ΔrpoS cell surfaces did not cause the enhancement of cell colonization on the surfaces of polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS) after 24 h of incubation at 37°C. To facilitate the enhanced expression of curli, the csgA gene was introduced into the ΔrpoS cells. The transformed cells rich in flagella and curli on the cell surfaces were found to make colonies 2-3 times larger than both the wild type and ΔrpoS cells on the PVC, PP and PS surfaces at 37°C. It was thus verified that the reinforcement of csgA gene in the ΔrpoS cells induced the enhanced colonization on the solid surfaces with the increased flagellum and curli expressions.

Key words: E. coli K-12 BW25113 / csgA / rpoS deficiency / colonization.

In Escherichia coli cells, the biosynthesis of flagellar filaments has been believed to be regulated by the flhC gene encoding a master regulator, which governs the expressions of all other genes of flagellar regulons. Pratt and Kolter (1998) demonstrated that the non-flagellated E. coli strain obtained by flhC deficiency was negligible in motility and biofilm formation. On the other hand, curli is a component of the cell surface strengthening the connection among cells in biofilm colonies. In a previous report, it was shown that curli assembly was inhibited by the mutation of csgA, which encodes a main curli subunit, resulting in the depression of E. coli biofilm formation (Van Houdt and Michiels 2005). It is known that some genes relating to flagellum biosynthesis are regulated by rpoS, a stationary-phase sigma factor, and rpoS mutation releases E. coli cells from the suppression of flagellum formation (Van Houdt and Michiels 2005). On the other hand, Wood et al. (2006) examined the cell colonization of various E. coli derivants, and showed that the E. coli K-12 BW25113 strain had relatively low ability to colonize on a solid surface, compared to other strains, due to the lower expression levels of genes involved in flagellum assembly. Therefore, this BW25113 strain is considered to be a candidate for use to examine the impact of rpoS deletion on the cell colonization of E. coli cells.

In the current study, we investigated the contributions of flagellum and curli assemblies to the cell colonization of E. coli K-12 BW25113 on a solid surface by introducing flhC or csgA gene into the wild-type strain and its rpoS-deficient mutant (ΔrpoS). The E. coli BW25113 and ΔrpoS strains used in this study were obtained from the Keio collection, the National BioResource Project (National Institute of Genetics (NIG), Mishima, Japan). The plasmids, pNTR-SD-csgA (pcsgA) and pNTR-SD-flhC (pfthC), were also provided by NIG. All E. coli strains were preserved at −80°C in 15% glycerol stocks to maintain their phenotypic stability. For each experiment, frozen cultures were warmed up with Lysogeny broth (LB) medium (10 g Polypeptone, 5 g Bacto-yeast extract and 10 g NaCl per liter) for 14 h at 37°C. Ampicillin (50 mg/L) was added to the medium for culturing the strains harboring the plasmids. The warmed-up culture was diluted in fresh LB medium to the optical density at

*Corresponding author. Tel: +81-6-6850-6251, Fax: +81-6-6850-6254, E-mail: taya(at)chng.es.osaka-u.ac.jp*
660 nm (OD_{660}) = 0.01.

Cell colonization assay was carried out according to the method noted in our previous paper (Nguyen et al. 2013) with some modifications. Briefly, the diluted cell suspension in fresh LB medium (175 µL) was put into a 96-well microtitre plate, which was made from polyvinyl chloride (PVC) (Corning Inc., Corning, NY 14831, USA), polypropylene (PP) (Nalge Nunc International, Roskilde, Denmark) or polystyrene (PS) (Nalge Nunc International). After being cultured at 37°C for 24 h, the culture broth containing planktonic cells was removed from each well, and remaining cells on the well surface were stained by incubation with 175 µL of 50 mg/L safranin solution for 20 min at room temperature, followed by 5 washings with deionized water. The stained cells on the well surface were solubilized by adding 175 µL of 20 % (v/v) acetone in ethanol. An index of colonized cells was represented as an absorbance of dye solution measured at 492 nm (A_{492}) with a microtitre plate reader (Chromate-4300, Awareness Technology, Palm City, FL, USA).

The quantitative assay for curli detection was conducted by using the Congo red (CR) binding method described by Ambalam et al. (2012) with some modifications. Briefly, 40 ml of diluted cell suspension in fresh LB medium (OD_{660} = 0.01) was cultured at 37°C. The cells were collected from 3 ml of culture by centrifugation at 13,200 rpm for 1 min at room temperature. Then, precipitated cells were suspended at 46 mg dry cells/mL in phosphate-buffered saline containing 50 mg/L CR and incubated at 37°C for 15 min. After centrifugation, the concentration of remaining dye in supernatant was estimated by measuring the absorbance at 480 nm. The percentage of CR bound to curli was defined by the formula, \((A_n-A_i)/A_i \times 100\), where \(A_o\) and \(A_i\) are absorbance values before and after incubation, respectively.

For morphological observation, E. coli cells of each strain were incubated at 37°C for 16 h in a PVC 96-well microtitre plate containing LB medium with a flat piece of PVC. The attached cells on the piece were fixed with 2.5% (w/v) glutaraldehyde for 1 h, followed by dehydration with serial gradients of aqueous ethanol solutions of 40, 70, 90, and 100% (v/v). Then, the specimen was dried under vacuum for 40 min, and subjected to sputtering osmium coating with a thickness of about 2 nm. Finally, the prepared specimen was observed under a scanning electron microscope (SEM; Model S-4800, Hitachi, Tokyo, Japan).

The gene expression was analyzed by real time PCR, as described in our previous work (Nguyen et al., 2013).

In the present work, E. coli K-12 BW25113, which was poor in flagellum assembly, was used to study the effect of rpoS on the flagellum formation in relation to the cell colonization on a solid surface. The close-up SEM image confirmed that the BW25113 cells synthesized a negligible number of flagella on the cell surfaces (Fig. 1). In a priori experiments, it was found that the expressions of flhC and cheW were enhanced approximately 31 and 9 times, respectively, when the flhC gene was introduced into the BW25113 strain. The flhC and cheW genes encode a sigma 28 factor and chemotaxis-relating element, respectively (Terashima et al. 2008). Similarly, the deficiency of rpoS led to, about 22.6 and 6.8 times, the up-regulated expressions of flhC and cheW genes, respectively, as compared to those in the parent BW25113 strain (data not shown). These results agreed with the observation that the number of flagella significantly increased on the surfaces of ΔrpoS or BW25113/flhC cells (Fig. 1).

It is well known that flagella greatly contribute to cell motility. Ojima et al. (2012) reported that the fraction of cells with high motility in a ΔrpoS cell population was larger than that in a parent BW25113 cell population, resulting in the development of larger colonies of ΔrpoS cells on a soft agar plate. A similar phenomenon was confirmed as well in the culture of BW25113/flhC cells on a soft agar plate (data not shown). It is thus expected that the high motility of ΔrpoS cells offers...
dynamic potential to overcome the repulsive force existing at a liquid and solid interface, thereby leading to the increased number of initially attached cells on a solid surface. It was reported that the formation of

E. coli  K-12 biofilm was enhanced by the mutation of

rpoS, but the efficiency of biofilm formation was dependent on

E. coli  K-12 derivants. For example, the deficiency of

rpoS induced an appreciable enhancement of biofilm formation as influenced by an E. coli  MC4100 derivant while a mutant of

rpoS slightly enhanced the biofilm formation in the case of MM4100 derivant at

37°C (Corona-Izquierdo et al. 2002; White-Ziegler et al. 2008).

In the present study, however, the enhancement of colonization was not achieved in the experiments with the BW25113/pflhC and ΔrpoS strains at 37°C on the examined plastic surfaces of PVC, PP and PS, as shown in Fig. 2. Similarly to these findings, the sparse habitation of the BW25113, ΔrpoS or BW25113/pflhC strain was also observed on a glass surface (data not shown). These results suggest that at 37°C, the enrichment of flagella caused by rpoS deficiency or fhlC introduction is not enough to maintain the BW25113 cell colonization. In other words, E. coli  BW25113 cells require not only flagella but also curli, to maintain the colonized cells on the solid surface. As stated by Van Houdt and Michiels (2005), the development of cell colonization on a solid surface comprises the stages of initial cell attachment and clustering of attached cells. In the first step, flagellum can be important in facilitating the cell access onto a solid surface by means of cell

movement. In the latter step, these attached cells generate newborn cells which establish firm cell colonization. In this stage, curli might be an element responsible for gathering of cells through tangled interactions of cells to cells and cells to the surface.

In order to improve the curli assembly, the csgA gene was introduced into the BW25113 and ΔrpoS cells in the current study. The percentages of CR bound onto both csgA-transformed cells were appreciably higher than those onto the respective parent strains (Fig. 3), and this was attributed to the curli formation encouraged by the overexpression of the csgA gene in the cells. These results led us to think that the ΔrpoS strain could be a tool to help us understand the contributions of both flagella and curli to the maintenance of cell colonization on the solid surfaces. As seen in Fig. 2, it was found that the increase in the curli production, caused by introducing csgA in the ΔrpoS cells, notably enhanced the amount of colonized cells on the PVC, PP and PS surfaces at 37°C, as compared to the BW25113 and ΔrpoS strains. These results demonstrated that the impact of deleting rpoS and introducing csgA on the colonization could be material-independent among the examined solid surfaces.

Our results also indicated that the presence of curli on the E. coli  cell surfaces by itself was not sufficient to enhance the cell colonization, because the csgA overexpression did not encourage the flagellum production on the cell surfaces (Fig. 1). Thus, the efficiency of

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BW25113/pcsgA cell colonization on the solid surfaces was never improved (Fig. 2). In liquid cultures with LB medium, we confirmed that no significant differences in growth rates were detected among all the strains examined in the present study (data not shown). These findings support the idea that the cells rich in both flagella and curli on their surfaces can maintain stable colonization on the solid surfaces through creating firm connections of cells to cells, as reported by Saldana et al. (2009).

In conclusion, we found that the BW25113/pfthC and ΔrpoS strains enhanced flagellum production but these strains did not improve the cell colonization at 37°C on the PVC, PP and PS surfaces. On the other hand, the introduction of csgA into ΔrpoS cells enhanced the cell colonization on the examined solid surfaces, associated with the enrichment of curli on the cell surfaces. Thus, it was demonstrated that the reinforced csgA gene expression brought the rpoS-deficient cells to enhanced colonization on the solid surfaces at 37°C in association with the increased flagellum and curli formation.

ACKNOWLEDGEMENTS

One of authors (M.H. Nguyen) would like to gratefully acknowledge the financial support provided by Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science (No. 2425, 2011). We also thank Dr. Muranaka of the Research Center for Ultra-High Voltage Electron Microscopy, Osaka University, for help in taking the SEM images.

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