Note

Roles of Multidrug Efflux Pumps on the Biofilm Formation of *Escherichia coli* K-12

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We genetically analyzed the roles of multidrug efflux pumps on the biofilm formation of *Escherichia coli* K-12 BW25113. We used 22 mutants missing various genes related to the multidrug efflux pumps and found that the biofilm formation of *emrD*, *emrE*, *emrK*, *acrD*, *acrE* and *mdtE*-deleted mutants was extremely decreased. These results indicate that some multidrug efflux pumps significantly contribute to the biofilm formation of *E. coli*.

Key words : Biofilm / *Escherichia coli* / Multidrug efflux pump.

Biofilm is defined as an adherent matrix-enclosed bacterial population (Aoi 2002; Costerton et al., 1995; Costerton et al., 1999; Davey et al., 2000). In nature, microorganisms usually adhere to solid surfaces (especially on the liquid-solid interface). After attachment, they form microcolonies, produce extracellular polymeric substances (EPS), entrap cell debris and other species of cells, and form biofilm (Aoi 2002; Costerton et al., 1995; Costerton et al., 1999; Gomez et al., 2003; Terada et al., 2003). In general, biofilm is very resistant to antibiotics and some physical treatments (Aoi 2002; Costerton et al., 1995; Costerton et al., 1999; Kumar et al., 1998; Zottola et al., 1994), and biofilm is considered to be a serious problem in food hygiene.

There have been some studies on the roles of efflux pumps in drug resistance and biofilm formation (Ito et al., 2008; Hoffman et al., 2005; May et al., 2009; Zhu et al., 2008). Hoffman et al. (2005) reported that aminoglycoside antibiotics induce bacterial biofilm formation (Hoffman et al., 2005). Ito et al. (2009) showed that *E. coli* biofilm has higher antibiotic resistance, and that the gene expression of some efflux pumps was increased in biofilm. May et al. (2009) showed that antibiotics at sub-inhibitory concentrations induced the gene expression of some ef-

flux pumps in *E. coli* with pBR322, and that the expressed efflux pump genes contributed to the maturation of biofilm. Kvist et al. (2008) showed that the addition of efflux pump inhibitors decreased the biofilm formation of some bacteria including *E. coli*. The results in these previous studies showed that some multidrug efflux pumps seemed to contribute to the biofilm formation.

In this study we investigated the effects of deleting genes coding multidrug efflux pumps on the biofilm formation of *E. coli* K-12. Multidrug efflux pump genes in *E. coli* K-12 have been well studied, and they are categorized into 5 types: the ATP binding cassette (ABC) superfamily, major facilitator superfamily (MFS), small MDR (SMR) family, resistance-nodulation-cell division (RND) family and multidrug and toxic compound extrusion (MATE) family (Lubelski et al., 2007; Nishino et al., 2009). Pumps except of the ABC superfamily are proton motive force (PMF) or sodium ion motive force (MATE family) type pumps. In this study, we used mutants with deleted genes except from the MATE family of *E. coli* K-12 BW25113.

The bacteria used were *Escherichia coli* K-12 BW25113 ($(\Delta araD\text{-}araB)567$, $\Delta lacZ4787(::rrnB\text{-}4)$, *lacI* $p\text{-}4000$ (*lacI*^r)) and gene-related mutants derived from BW25113 were obtained from the National Institute of Genetics (Shizuoka, Japan) supported by National BioResource Project (NIG, Japan): *E. coli*.

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E. coli cells were grown in LB broth (MP Biomedicals, OH, USA) overnight.

Estimation of biofilm formation was performed according to the method described previously (Jackson et al., 2002; Narisawa et al., 2005) with slight modifications. Overnight cultures were inoculated into fresh LB medium or Davis medium (K_2HPO_4 7 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, Sodium citrate 0.5 g, Glucose 2 g) a 1:100 ratio (total 200 μ l) and grown in 96-well microtiter plates at 30°C for 16 to 48 h. In this study, we adopted polystyrene microtiter plates without (Porvair Sciences, U. K.) or with (TPP Techno Plastic Products AG, Switzerland) corona discharge treatment. The hydrophobic polystyrene plates without corona discharge treatment are usually used in bacterial biofilm formation assays and the hydrophilic polystyrene plates with corona discharge treatment are usually used in culturing human and animal cells.

Biofilm formation was estimated by measuring the crystal violet staining of the cells attached to the microtiter plate wells. After discarding the medium in the microtiter plate wells, the wells were rinsed twice with water. Then, 0.1% crystal violet (Merck, Tokyo) was added to the wells for staining the attached cells. After discarding the crystal violet solution and rinsing the wells with water, crystal violet was extracted by 70% ethanol solution, and that solution was transferred to a new microtiter plate, and the absorbance at 590 nm was measured using a plate reader (E-max; Nihon Molecular Devices, Tokyo). For each experiment, correction for background staining was made by subtracting the value for crystal violet bound to un-inoculated controls. At least eight microtiter plate wells were used for each sample, and each experiment was performed at least twice. The standard deviations of crystal violet absorbance for the eight microtiter plate wells were from 1/20 to 1/5 of their average values, and the standard deviations for three or more independent experiments were also from 1/20 to 1/5 of their average.

All experiments were done in duplicate. The data presented are the means of three or more experiments. Significant differences were determined by Student's *t* test ($P < 0.05$).

We used *E. coli* K-12 IAM 1246 ($F^+ \lambda^+$), MG1655 ($F^- \lambda^- rph-1$) and W3110 ($F^- \lambda^- IN(rrnD-rrnE)1 rph-1$) strains in previous studies (Kawarai et al., 2009; Narisawa et al., 2005), and the above strains formed biofilm very well between approx. 0.2 to 0.9 (A_{590}) for 24 h in LB medium by using the hydrophobic polystyrene 96-well plate. However, *E. coli* K-12 BW25113 (($\Delta araD-araB$)567, $\Delta lacZ4787::rrnB-4$), *lac/p-4000*(*lac^r*)) formed less biofilm by approx. 0.05

(A_{590}) in the same conditions. Therefore we investigated the conditions for the improved biofilm formation of *E. coli* K-12 BW25113.

At first, the biofilm formation of *E. coli* K-12 BW25113 was compared on hydrophilic plates (with corona discharge treatment) and hydrophobic plates (without corona discharge treatment) in two types of culture medium (LB and Davis medium) at various culture times (Fig. 1). The biofilm formation on the hydrophilic plates was relatively higher than that on the hydrophobic polystyrene plates, which had been conventionally used in bacterial biofilm formation assays. Especially the biofilm formation of *E. coli* K-12 BW25113 in Davis medium on the hydrophilic plate was higher irrespective of cultivation time. However, the combination of Davis medium and hydrophilic plates was not suitable for the biofilm assay because the amounts of biofilm formed were obviously unstable judging from the large standard deviations (Fig. 1B). On the other hand, *E. coli* K-12 BW25113 formed biofilm in stable and adequate amounts in LB medium on hydrophobic plates after 40h cultivation (Fig. 1A). Hereafter, the biofilm formation of *E. coli* K-12 BW25113 was assayed by cultivation in LB medium on hydrophobic plates for 40 h. These results indicate that hydrophobic or hydrophilic properties of the solid surface significantly affect the biofilm formation of *E. coli*.

The effect of deleting of genes related to multidrug efflux pumps on the biofilm formation of *E. coli* K-12 BW25113 was investigated under the above determined assay conditions. We used 22 mutants that were missing various genes related to multidrug ef-

FIG. 1. Effects of the plate (with or without corona discharge treatment), media and cultivation time on the biofilm formation of *E. coli* K-12 BW25113. LB: LB medium, Davis: Davis medium.

FIG. 2. Effects of MDR-related gene deletion on the biofilm formation of *E. coli* K-12 BW25113.

ABC: ATP binding cassette superfamily, MFS: Major facilitator superfamily, SMR: Small MDR family, RND: Resistance-nodulation-cell division family.

flux pumps. All gene-deleted mutants used in this study showed significantly decreased biofilm formation compared with the parent strain *E. coli* K-12 BW25113. Especially, mutants without *emrD*, *emrE*, *emrK*, *acrD*, *acrE* and *mdtE* showed extremely lower biofilm formation (Fig. 2). These six genes belong to the genes coding the proton motive force (PMF) pumps. From these results, PMF pumps were considered to have important roles in the biofilm formation of *E. coli*.

Previous studies suggested that some efflux pumps related to the efflux of antibiotics contributes to the biofilm formation of *E. coli* (Lucas et al., 2005; May et al., 2009). Our results partly corresponded to the results of these previous studies. Precise mechanisms of the contribution of these efflux pumps on the *E. coli* biofilm formation should be clarified in the future. They might contribute to the biofilm formation via the export or import of some substances that are necessary or harmful for biofilm formation.

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