Inhibitory Effects of *Escherichia coli* on the Formation and Development of *Staphylococcus epidermidis* Biofilm

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In the present study, we examined whether a commensal gut bacterium *Escherichia coli* might prevent the formation and development of the biofilm of *Staphylococcus epidermidis*, a nosocomial extraintestinal pathogen but not a gut microorganism. When co-cultured with *S. epidermidis, E. coli* strain ATCC 35218, a non-pathogenic strain, was found to be dominant in the biofilm formed on the surface of wells of a microtiter plate. In addition, *E. coli* significantly incorporated and grew in a niche preoccupied by *S. epidermidis* biofilm. Two other *E. coli* strains (strain K-12 and B) also showed to interfere the biofilm formation by *S. epidermidis*. In contrast, *S. epidermidis* could not grow in a niche preoccupied by *E. coli* biofilm. These results suggest that, through inhibition of the formation and development of the biofilm, *E. coli* may eliminate *S. epidermidis* from the gastrointestinal tract.

**Key words**: Biofilm / *Escherichia coli* / *Staphylococcus epidermidis*.

About 60 tonnes of food pass through human gastrointestinal (GI) tract through an average lifetime together with many environmental microorganisms contaminating the food. These microorganisms might pose threat on GI tract integrity (Bengmark, 1998) or may colonize the GI tract and serve as a source for spreading perilous microorganisms, such as antibiotic resistance bacteria, to other individuals or to the environment (Boyee et al., 2007). The gut microbiota is the term used to refer to all bacteria, archaea and eukarya living in the GI tract. Balance between the GI tract microbiota is important for maintaining host health, so that, distortion of this balance has been hypothesized to be involved in various diseases such as inflammatory bowel diseases, diabetes and others (Gu et al., 2013).

Colonization resistance is the mechanism whereby the intestinal microbiota protects itself against invasion by new and potentially harmful microorganisms. Colonization resistance can occur through direct or indirect manner (Buffie and Pamer, 2013). In the first form, the commensals compete with invading microorganisms for available nutrient and niche establishment or through production of antimicrobial peptides and/or toxins against the invaders. Indirect colonization resistance is derived from stimulation of host innate and adaptive immune response by commensals against the invaders (Sassone-Corsi and Raffatellu, 2015). *Escherichia coli* is a gram-negative motile bacterium and a commensal bacterium found commonly in the large intestine of human and animals (Poulsen et al., 1994). *Staphylococcus epidermidis* is a gram-positive non-motile bacterium belonging to the coagulase negative staphylococci. This bacterium is widely present in the cutaneous ecosystem but linked to medical device associated nosocomial infections (von Eiff et al., 2002). However, *S. epidermidis* is also a prominent coagulase negative bacteria recovered from the GI tract of children (Domínguez et al., 2002). Namely, Akikumi and Lamikanra (2010) reported the recovery of drug resistant *S. epidermidis* strains from the GI tract of children, and Ohno et al. (2007) documented that 22.3 % of hospitalized pediatric children were carriers of methicillin resistant *S. epidermidis* strains. These studies suggest...
that the GI tract of children may act as the reservoir of the drug resistant bacteria.

We recently found that, in the GI tract, \textit{E. coli} might prevent the biofilm formation by \textit{Vibrio vulnificus}, a pathogen causing systemic septicemia after ingestion of the contaminated seafood (Ohn et al., 2020). On the other hand, \textit{V. vulnificus} was found to reduce the amount of the preformed \textit{E. coli} biofilm (Ohn et al., 2020). In the present study, we tested whether the co-presence of \textit{E. coli} and \textit{S. epidermidis} cause to interfere the formation and/or development of biofilm each other. The results showed that \textit{E. coli} might disturb the formation and development of \textit{S. epidermidis} biofilm.

In the present study, non-pathogenic \textit{E. coli} strain ATCC 35218 (from canine), K-12 (from human feces) (Kuhnert et al., 1995) and B (from human feces) (Muhldorfer and Haker, 1994), and \textit{S. epidermidis} strain ATCC 35984 (a methicillin-resistant isolate from a patient with catheter sepsis) were used. For bacterial cultivation, trypticase soy broth (TSB) from Becton, Dickinson and Company (Franklin Lakes, NJ, USA), mannitol salt agar from Nissui (Tokyo, Japan), or MacConkey agar from Nissui were used. To prepare trypticase soy agar (TSA) plates, 1.8 % of agar from Fujifilm Wako (Osaka, Japan) was added to TSB. Bacterial strains were cultivated overnight into TSB at 37 °C, and the cell density was adjusted to 0.5 McFarland Standard for \textit{E. coli} strain ATCC 35218 and K-12, and for \textit{S. epidermidis} strain ATCC 35984 and to 1.0 McFarland Standard for \textit{E. coli} strain B with fresh TSB by using a McFarland Densitometer DEN-1B (Biosan, Riga, Latvia). The cell numbers in 1.0 mL of the suspension of \textit{E. coli} strain ATCC 35218, K-12 and B were 8.3 ± 2.1 x 10^7 CFU, 5.5 ± 0.9 x 10^7 CFU, 2.1 ± 1.0 x 10^7 CFU, respectively, and those of \textit{S. epidermidis} was 7.1 ± 4.3 x 10^6 CFU.

All experiments were repeated at least three times in the present study. Additionally, in the crystal violet assays, each experiment was carried out with three samples. The data was presented as the mean ± S.D. and was analyzed by Student’s \textit{t} test (two-tailed analysis). The \textit{p} value less than 0.05 was determined to be significantly different.

First, we examined the total amount of biofilm formed by the single or mixed culture of \textit{E. coli} and \textit{S. epidermidis}. For biofilm formation by the single culture, 0.2 mL of the cell suspension of \textit{E. coli} or \textit{S. epidermidis} was inoculated into a well of a Nunclon™ Delta Surface 96-welled microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA). In the case of the mixed-culture, 0.1 mL of each of the bacterial suspension was mixed and inoculated into the well of the microtiter plate. Thereafter, the microtiter plate was incubated at 37 °C for 24 ± 2 h in static condition. The biofilm amounts were measured according to the method of Baldassarri et al. (2002) and Extremina et al. (2011) by using 0.1 % crystal violet. After incubation at 37 °C for 24 ± 2 h, the microtiter plate was gently washed three times with 10 mM phosphate-buffered saline (pH 7.4) and dried at 60 °C for 1 h. Thereafter, the biofilm was fixed with 95 % methanol and stained with 0.1 % crystal violet for 5 min, free crystal violet was removed by washing the wells with distilled water, and then, crystal violet bound to the biofilm was extracted with 80 % ethanol-20 % acetone for 5 min and quantified by measuring the absorbance at 570 nm with iMark™ Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). In the case of the mixed culture of \textit{E. coli} strain ATCC 35218 and \textit{S. epidermidis} strain ATCC 35984, the amount of the biofilm formed (A570) was reduced to 28 % of that of \textit{S. epidermidis} single culture but was comparable with \textit{E. coli} single culture (Table 1), suggesting that the \textit{E. coli} strain showed negative impact on the biofilm formation by \textit{S. epidermidis}.

| Culture | Biofilm amount (A570) | Biofilm-associated (CFU/mL) | Planktonic (CFU/mL) |
|---------|----------------------|-----------------------------|---------------------|
| Single culture \textit{S. epidermidis} | 2.64 ± 0.55 | 1.4 ± 0.5 x 10^8 | 9.5 ± 4.2 x 10^8 |
| Single culture \textit{E. coli} | 0.47 ± 0.17 | 1.1 ± 0.6 x 10^8 | 1.8 ± 0.5 x 10^9 |
| Mixed culture | 0.73 ± 0.20 | 6.1 ± 3.3 x 10^5 \textit{S. epidermidis} | 1.5 ± 1.7 x 10^6 \textit{E. coli} |
| | | 1.1 ± 0.9 x 10^8 | 1.1 ± 0.7 x 10^9 |

\textbf{TABLE 1.} Biofilm formation by the single and mixed culture of \textit{S. epidermidis} strain ATCC 35984 and \textit{E. coli} strain ATCC 35218

\textit{A570} is the absorbance at 570 nm with iMark™ Microplate Reader.
S. epidermidis cell suspension (0.2 mL) was inoculated into the well of the microtiter plate and incubated at 37 ºC for 24 ± 2 h. Then, the culture fluid was removed, and the well was washed three times with 0.5 % NaCl. After that, the cell suspension of S. epidermidis or E. coli (0.2 mL) was added to the well, and the microtiter plate was incubated again at 37 ºC for 24 ± 2 h. When TSB or S. epidermidis cells was added to the well precoated with S. epidermidis biofilm, amounts of the biofilms were around 2.5 after 24 h incubation (Fig. 1). In contrast, when the cell suspension of E. coli strain ATCC 35218 was applied over the preformed S. epidermidis biofilm, the amount of S. epidermidis biofilm was reduced to 1.01 ± 0.37 (Fig. 1). Additionally, the numbers of S. epidermidis cells associating with the biofilm was found to be 3.4 ± 2.7 x 10^4 CFU/mL in the wells to which E. coli cell suspension was added (in the experiments Fig. 1C). On the other hand, those were estimated to be 5.5 ± 3.7 x 10^4 CFU/mL in the wells to which S. epidermidis cell suspension was added (in the experiments Fig. 1B).

The findings described above may indicate that E. coli strain ATCC 35218 produces the substance(s) inhibiting the growth of S. epidermidis. Therefore, the cross-streak plate assay was carried out to check if the E. coli strain produced the bactericidal or bacteriostatic substance against S. epidermidis or not. S. epidermidis was cultivated overnight at 37 ºC in TSB and horizontally streaked on a TSA plate with intervals at least 1 cm. Then, the overnight culture of E. coli was streaked vertically across the middle of S. epidermidis streaks. Thereafter, the plate was cultivated again at 37 ºC for 24 h, and the presence of the growth-inhibiting zone at the intersection points was observed. However, negligible production of the antibacterial substance was observed because no growth inhibition was observed (Fig. 2).

As shown in Table 2, in the presence of E. coli strain K-12 or B, the amount of biofilm formed by S. epidermidis was also reduced to 30 % or 20 % of that formed by S. epidermidis single culture. Like strain ATCC 35218, both strain K-12 and B significantly decreased the amount of the preformed S. epidermidis biofilm (Table 3). Namely, by the addition of the cell suspension of strain K-12 or B, amounts of the biofilms (A570) were reduced to 1.41 ± 0.15 or 1.49 ± 0.54, respectively. These findings suggest that the ability to inhibit the formation and development of S. epidermidis biofilm is common to E. coli strains.

In contrast to the E. coli strains, S. epidermidis strain ATCC 35984 showed no effect on the preformed biofilm of E. coli strain ATCC 35218. The E. coli cell suspension

We also determined the numbers of the biofilm-associated or planktonic cells. For counting of the biofilm-associated bacterial cells, the biofilm formed on the surface of the well of the microtiter plate was washed three times with 0.5 % NaCl, scraped free several times from the well and resuspended into a total of 1.0 mL of 0.5 % NaCl by using a pipette tip as described by Leuck et al. (2014) and Lopes et al. (2018). Thereafter, the bacterial cell suspension was serially 10-fold diluted with 0.5 % NaCl, and 0.1 mL of each diluted sample was inoculated on three agar plates. Mannitol salt agar plate or TSA plate was used for S. epidermidis, and MacConkey agar plate or TSA plate was used for E. coli. For counting of the planktonic bacterial cells in the well of the microtiter plate, an aliquot of the bacterial culture fluid was withdrawn and serially 10-fold diluted with 0.5 % NaCl, and 0.1 mL of each diluted sample was inoculated on three agar plates. Then, the plates were incubated at 37 ºC for 24-48 h, and the colonies formed were counted. As shown in Table 1, in both biofilm-associated and planktonic cells, the numbers of E. coli strain ATCC 35218 in the mixed culture were not different from those of the single culture. However, in the case of S. epidermidis, the numbers of either cells were significantly decreased in the mixed culture with E. coli (p < 0.05). Namely, in the mixed culture, the percentages of the biofilm-associated and planktonic S. epidermidis cells were only 0.55 % and 0.14 %, respectively. In other words, more than 99 % of the bacterial cells in the biofilm or culture fluid was E. coli.

Next, we examined the effect of addition of E. coli cells on the preformed S. epidermidis biofilm. The
(0.2 mL) was inoculated into the well of the microtiter plate and incubated at 37 ºC for 24 ± 2 h. Then, the culture fluid was removed, and the well was washed three times with 0.5 % NaCl. After that, the cell suspension of *S. epidermidis* or *E. coli* (0.2 mL) was added to the well, and the microtiter plate was incubated again at 37 ºC for 24 ± 2 h. As shown in Fig. 3B, no increase in the biofilm amounts was observed even when *S. epidermidis* cells were added. To kill *E. coli* cells in the preformed biofilm, the well coated with *E. coli* biofilm was treated with 0.3 mL of 70 % ethanol for 15 min at room temperature. Then, the well was washed with 0.5 % NaCl and air dried for 30 min. When *S. epidermidis* cells were applied over the ethanol-treated *E. coli* biofilm and incubated at 37 ºC for 24 ± 2 h, the amounts of the biofilm (A570) was increased to 2.16 ± 0.36. This amount was close to that of the biofilm formed by the single culture of *S. epidermidis* (Table 1 and 2). Therefore, it is thought that the *S. epidermidis* cells can disrupt the preformed *E. coli* biofilm only when *E. coli* cells were not alive.

The present study indicated that three non-pathogenic *E. coli* strains could suppress commonly the biofilm formation by *S. epidermidis* in the mixed culture. Similarly, Millezi et al. (2012) reported that the total biofilm amounts of the mixed culture of *Staphylococcus aureus* and *E. coli* were lower than either of the single culture. Makovcova et al. (2017) also documented significant reduction in the cell numbers of *S. aureus* in the biofilm via mixed cultivation with *E. coli*.

Both colicins and microcins are bacteriocins produced by *E. coli* and other species in the *Enterobacteriaceae*, but their action spectrum is limited to closely related bacterial species. The result of our cross-streak plate assay showed that *E. coli* strain ATCC 35218 did not produce

### Table 2. Effects of *E. coli* strain K-12 and B on the biofilm formation by *S. epidermidis* ATCC 35984

| Addition                | *E. coli* strain K-12 (A570) | *E. coli* strain B (A570) |
|-------------------------|-----------------------------|--------------------------|
| Single culture          | 2.73 ± 0.55                 | 2.68 ± 0.32              |
| *S. epidermidis*        | 0.59 ± 0.14                 | 0.24 ± 0.05              |
| Mixed culture           | 0.82 ± 0.30                 | 0.53 ± 0.14              |

### Table 3. Negative impact of *E. coli* cells on the development of *S. epidermidis* biofilm

| Addition                | *E. coli* strain K-12 (A570) | *E. coli* strain B (A570) |
|-------------------------|-----------------------------|--------------------------|
| TSB                     | 2.49 ± 0.45                 | 3.01 ± 0.62              |
| *S. epidermidis* cells  | 2.14 ± 0.23                 | 2.63 ± 0.55              |
| *E. coli* cells         | 1.41 ± 0.15*                | 1.49 ± 0.54*             |

* p < 0.05

FIG. 2. The cross-streak plate assay to detect the bactericidal or bacteriostatic substance. *S. epidermidis* strain ATCC 35984 (SE) was cultivated overnight in TSB and streaked horizontally on a TSA plate with intervals at least 1 cm. Then, the overnight culture of *E. coli* strain ATCC 35218 (EC) was streaked vertically across the middle of *S. epidermidis* streaks. Thereafter, the plate was cultivated again at 37º C for 24 h, and the presence of the growth-inhibiting zone was observed.

FIG. 3. No effect of the cells of *S. epidermidis* strain ATCC 35984 on the amount of the preformed biofilm of *E. coli* strain ATCC 35218. The wells of the microtiter plate were coated with biofilm of *E. coli* strain ATCC 35218 by cultivation at 37º C for 24 ± 2 h. Thereafter, TSB (A), *S. epidermidis* strain ATCC 35984 (B) or *E. coli* strain ATCC 35218 (C) was added to the wells, and the microtiter plate was cultivated again at 37º C for 24 ± 2 h. Then, the amount of biofilm was measured (n = 3).
the active bacteriocin to *S. epidermidis*. On the other hand, the culture supernatant of uropathogenic *E. coli* is known to inhibit the biofilm formation in a wide variety of microorganisms including *S. epidermidis* without affecting the cell viability (Valle et al., 2006). Therefore, it is possible that *E. coli* strains utilized in the present study may also produce the antibiofilm substance(s) against *S. epidermidis*. Chen et al. (2019) reported that the amount of the biofilm formed by *Vibrio parahaemolyticus* was reduced when mixed with *Listeria monocytogenes*, and they also mentioned that this reduction might be due to downregulation of the biofilm-regulated genes. Induction of the similar phenomenon is possible by the interaction between *E. coli* and *S. epidermidis*, which results in reduced biofilm formation by *S. epidermidis* in mixed culture.

*E. coli* may grow faster than *S. epidermidis*. For example, the generation times of *E. coli* (17.9 ± 0.9 min) was shorter than that of *S. epidermidis* (27.5 ± 10.5 min) when grown in TSB at 36-37 °C (Gottenbos et al., 2000; Guerini et al., 2006). So, it is possible that, due to the faster growth rate, *E. coli* consumes most available nutrient in the culture medium and supplies little nutrients to *S. epidermidis*. This may lead to the reduced cell growth of and biofilm formation by *S. epidermidis* in mixed culture.

Hourya et al. (2012) reported that *Bacillus thuringiensis* could infiltrate and form pores in the biofilm matrices due to its motile nature. Therefore, the motility of the *E. coli* strains may play a role in reduction of the amount of the preformed *S. epidermidis* biofilm. However, in contrast to other strains, *E. coli* strain B is known to lacks flagella mediating the bacterial motility (Yoon et al., 2012). Therefore, the reduction of the amount of the preformed *S. epidermidis* biofilm by *E. coli* strain B may not be due to the ability to infiltrate the biofilm. The preformed *S. aureus* biofilm was recently documented to be eradicated by *Streptococcus pneumoniae* in the contact-dependent manner (Khan et al., 2016). Similar mechanisms may be employed by *E. coli* strain B to block the development of the preformed *S. epidermidis* biofilm.

In conclusion, although the experimental conditions utilized in the present study might vary greatly from human intestinal circumstance, the results obtained herein suggest that *E. coli* can prevent the formation and development of *S. epidermidis* biofilm in the GI tract. This may result in elimination of *S. epidermidis*, a non-intestinal commensal bacterium, from the GI tract.

**CONFLICT OF INTEREST**

The authors declare that we have no conflict of interest.

**ETHICAL STATEMENT**

This study did not contain any human or animal subject.

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