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**Impacts of hydrophilic colanic acid on bacterial attachment to microfiltration
membranes and subsequent membrane biofouling**

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ABSTRACT

In order to examine the interactions between physicochemical properties of specific extracellular polymeric substances (EPS) and membrane biofouling, we investigated the impacts of hydrophilic colanic acid, as a model extracellular polysaccharide component, on initial bacterial attachment to different microfiltration (MF) membranes and membrane biofouling by using *Escherichia coli* strains producing different amounts of colanic acid. In a newly designed microtiter plate assay, the bacterial attachment by an *E. coli* strain RcsF⁺, which produces massive amounts of colanic acid, decreased only to a hydrophobic membrane because the colanic acid made cell surfaces more hydrophilic, resulting in low cell attachment to hydrophobic membranes. The bench-scale cross-flow filtration tests followed by filtration resistance measurement revealed that RcsF⁺ caused severe irreversible membrane fouling (i.e., pore-clogging), whereas less extracellular polysaccharide-producing strains caused moderate but reversible fouling to all membranes used in this study. Further cross-flow filtration tests indicated that colanic acid liberated in the bulk phase could rapidly penetrate pre-accumulated biomass layers (i.e., biofilms) and then directly clogged membrane pores. These results indicate that colanic acid, a hydrophilic extracellular polysaccharide, and possible polysaccharides with similar characteristics with colanic acid are considered as a major cause of severe irreversible membrane fouling (i.e., pore-clogging) regardless of biofilm formation (dynamic membrane).

Impacts of hydrophilic colanic acid on MF membrane fouling □

Construction of bacterial strains □	Non-producing bacteria □	Hydrophilic colanic acid-producing bacteria □
	Hydrophobic cell surface □	Colanic acid □ Hydrophilic cell surface □
Membrane fouling potential □ (Cross-flow filtration test) □	Moderate reversible fouling □	Severe membrane pore-clogging □

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INTRODUCTION

Membrane bioreactors (MBRs) have been applied in wastewater treatment and reclamation. However, membrane fouling needs to be addressed to reduce the treatment costs. Membrane fouling in MBR systems is complex and occurs as a result of accumulation of bacterial cells, extracellular polymeric substances (EPS) and other soluble microbial products (SMP) on membrane surfaces and membrane pores (Chang et al., 2002; Le-Clech et al., 2006; Meng et al., 2009; Drews, 2010; Ni et al., 2011). The EPS and SMP are either deposited from the bulk liquid or produced in biofilms formed on membrane surfaces. The soluble fraction of EPS or SMP, particularly polysaccharides, has been considered as a major cause of membrane fouling because soluble polysaccharides are predominant in EPS in the mixed liquor of MBRs (Lesjean et al., 2005; Fan et al., 2006; Ng et al., 2006; Kimura et al., 2012). An extensive effort has been made to reveal the interactions between EPS and membrane fouling. However, most studies have simply dealt with the impacts of the EPS concentration in the mixed liquor (Chang et al., 2002; Le-Clech et al., 2006; Meng et al., 2009; Drews, 2010) and their characteristics (i.e., biodegradability and size) (Miura et al., 2007a, 2008; Ni et al., 2011; Zhou et al., 2012) on membrane fouling. It should be also noted that some contradictory results could be found in the literatures due to the complex nature of the interactions (Chang et al., 2002; Le-Clech et al., 2006; Meng et al., 2009; Drews, 2010; Ni et al., 2011). Therefore, further studies are obviously required to better understand the interactions between EPS and membranes.

The composition and characteristics of EPS are dependent on growth conditions such as the reactor feed (wastewater) characteristics and growth phase. Numerous studies have suggested the importance of EPS production in biofilm formation (Davey and O'Toole, 2000). Biofilm formation involves complex developmental processes, including initial attachment of

microorganisms, formation of microcolonies, and maturation of the microcolonies (Davey and O'Toole, 2000). Cellular characteristics (motility, cell surface charge and hydrophobicity) are considered to play important roles in the initial bacterial attachment to a membrane surface (Pang et al., 2005; Jinhua et al., 2006). Production of certain EPS has been reported to promote the distinct stages of biofilm development (Davey and O'Toole, 2000). For example, during biofilm formation of *Escherichia coli*, poly- β -1,6-GlcNAc (PGA) (Wang et al., 2004) and curli (Pringent-Combaret et al., 2000) are required for both primary surface colonization and subsequent biofilm formation. Interestingly, while colanic acid reduces initial cell attachment (Hanna et al., 2003; Chao and Zhang, 2011), its production is important for development of voluminous three-dimensional biofilms (Pringent-Combaret et al., 2000; Danese et al., 2000; May and Okabe, 2008).

Bacterial attachment rate is strongly related to biofilm formation rate. Bacterial characteristics involved in cell attachment to membrane surfaces and subsequent biofilm formation have been studied by using pure-culture of bacterial strains but the key characteristics are still not well understood. The relevance of cellular characteristics (motility and cell surface properties) to biofilm formation on reverse osmosis (RO) membranes was investigated using bacterial strains isolated from a fouled RO membrane system (Pang et al., 2005). However, membrane fouling potential was not measured in this study. Since the convective transport of bacteria to the membrane by filtration remains the main cause of biofilm formation, biofilm formation was highly enhanced under filtration conditions (Eshed et al., 2008). Thus, bacterial attachment to membranes and subsequent biofouling potential must be evaluated under filtration conditions. Even under implementation of continuous physical cleaning (air-scrubbing) of membranes, specific microbial populations attached and formed biofilms on the microfiltration (MF)

membrane, resulted in severe membrane fouling in MBRs treating real domestic wastewater (Miura et al., 2007b,c).

The impacts of specific EPS components required for biofilm formation on initial bacterial attachment to MF membranes and subsequent membrane fouling are not well understood. The role of bacterial exopolysaccharides in bacterial attachment and biofouling potential of an RO membrane was evaluated using an alginate overproducing (mucoid) *Pseudomonas aeruginosa* under cross-flow conditions (Herzberg et al., 2009). Alginate overproduction increased the hydrophilicity of the mucoid strain, which resulted in the lower cell attachment and consequently decelerated biofouling of the RO membrane (Herzberg et al., 2009). EPS produced by microorganisms are composed of many different components, which can interact with membranes in different ways (Kimura et al., 2012). The effect of other EPS components on membrane fouling potential need to be studied for better understanding of the complex interactions between EPS and membranes.

Although EPSs have been identified as the principal foulants in MBR (Ng et al., 2006; Kimura et al., 2012), little is understood about the effect of each EPS component on membrane biofouling. In this study, we focused on one of EPS, colanic acid, which is a hydrophilic, highly viscous capsular polysaccharide and common antigen produced by many enterobacteria (Rättö et al., 2006), and investigated the effect of colanic acid on membrane biofouling using *Escherichia coli* as a model organism. Because colanic acid reduces initial cell attachment (Hanna et al., 2003; Chao and Zhang, 2011), its production by the primary colonizers may influence the biofouling propensity of MF membranes. We constructed genetically modified *E. coli* K-12 strains producing different amounts of colanic acid to investigate the impacts of colanic acid production on initial cell attachment to different MF membranes and consequent fouling

potential. The degree of cell attachment was measured by a newly designed microtiter plate assay. The membrane fouling potential was assessed by bench-scale cross-flow filtration tests. The results of cell attachment and biofouling potential were discussed in correlation with the physicochemical characteristics of bacterial strains and MF membranes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 ($F^+ \lambda^+$) was used as a model bacterium for cell attachment assay and filtration experiments in this study. Four strains including *E. coli* K-12 harboring an empty cloning plasmid pCA24N (WT), K-12/pCA24N-*rcsF* ($RcsF^+$), K-12 $\Delta wcaA::kan$ /pCA24N ($WcaA^-$), and K-12 $\Delta wcaA::kan$ /pCA24N-*rcsF* ($WcaA^- RcsF^+$) were used as test strains. The *rcsF* gene is a positive regulator of colanic acid synthesis (Gervais and Drapeau, 1992), and this gene was overexpressed by isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible P_{T5-lac} promoter localized in pCA24N (Kitagawa et al., 2005). The *wcaA* gene is one of the genes for colanic acid synthesis (Stevenson et al., 1996), and this deletion mutant was used as a colanic acid-lacking strain. *E. coli* K-12 $\Delta wcaA$ was constructed as follows. *E. coli* K-12 MG1655 ($F^- \lambda^- ilvG rfb-50 rph-1$) $\Delta wcaA::kan$ was constructed according to the method described by Datsenko and Wanner (2000). Then, a bacteriophage P1 lysate was made from MG1655 $\Delta wcaA::kan$ and the *kan* allele was transduced into *E. coli* K-12. Unless otherwise described, the strains were routinely grown in Luria-Bertani (LB) medium at 30°C with 25 μ g/mL of chloramphenicol (for maintenance of the plasmid) and 100 μ M IPTG.

Quantification of EPS production. Purification and quantification of EPS were carried out

based on the methods previously described (Dische and Shettles, 1948; Obadia et al, 2007).

Soluble extracellular polysaccharide was purified from bacterial colonies grown for 24 hr at

30°C on LB agar plates. EPS production was quantified by measuring fucose which is one of the

major components of colanic acid (Garegg et al., 1971). The procedures are found in the

Supplementary Information.

Scanning electron microscopy. Scanning electron microscopy (SEM) analysis was performed

as previously described (May et al., 2009). The colonies were obtained from LB agar plate after

24 hr of incubation at 30°C, washed with phosphate buffer saline (PBS) two times and fixed with

2.5% glutaraldehyde in PBS overnight at 4°C. The samples were washed three times for 10 min

with PBS. The fixed samples were dehydrated in an acetone series (sequentially 50, 70, 80, 90

and 95% for 15 min each and 100% for 15 min three times) and substituted with isoamyl acetate.

The samples were dried with a critical-point drier using liquid CO₂ and coated with platinum and

palladium for 2 min. The coated samples were examined with a SEM (S-400; Hitachi, Tokyo,

Japan).

Characteristics of bacterial cell surface. Relative hydrophobicity of bacterial cells was

measured by the bacterial attachment to hydrocarbons (BATH) assay (Rosenberg, 1984). The

procedure is found in the Supplementary Information. Cell surface charge was evaluated by zeta

potential. Bacterial cells were collected from LB agar plates after 24 hr of incubation at 30°C,

washed three times with 0.85% NaCl solution to remove any interfering solutes and suspended in

the solution. The cells were then adjusted to OD₆₀₀ = 0.1 in 15 mM NaCl solution.

150 Electrophoretic mobility of the cells was measured by a zeta potential analyzer (Zetasizer Nano
151 ZS; Malvern, Worcestershire, UK).

152 **Characteristics of membrane materials.** Relative hydrophobicity of membrane surface was
153 evaluated by measuring the contact angle between a water droplet and the membrane surface. A
154 10 μ L water droplet was deposited on membranes and the forward contact angle was measured
155 from the picture taken within 5 sec. At least five different measurements were carried out for
156 each membrane. Zeta potential of membrane surface was determined by measuring
157 electrophoretic mobility of the standard particles (Otsuka Electronics, Osaka, Japan) on the
158 membranes based on laser Doppler electrophoresis with a zeta potential analyzer (Delsa Nano
159 HC; Beckman Coulter, Brea, CA).

160 **Microtiter plate assay.** Bacterial attachment potential of the strains on membranes was
161 evaluated by a newly designed microtiter plate assay. Twenty four-well polypropylene microtiter
162 plates, of which a membrane specimen (15 $\times$ 10 mm) was placed in each well, were prepared and
163 used in this assay (see supplemental **Figure S1**). Overnight grown cultures were diluted in LB
164 medium to an initial cell density of OD₆₀₀ = 0.05, and 1.5 mL of them was added to each well.
165 After 72 hr of incubation at 30°C, the OD₆₀₀ of the suspended culture was measured as the
166 growth rate. Loosely attached cells on membranes were gently rinsed twice with 0.85% NaCl
167 solution and resuspended into the solution by sonication. The cells were stained with 1 μ g/mL of
168 FilmTracer FM1-43 (Invitrogen, Carlsbad, CA). After 30 min of incubation in the dark, the
169 fluorescence was measured at Ex/Em = 485/580 nm by a plate reader (ARVO 1420 multilabel
170 counter; Perkin Elmer, Waltham, MA). Attached cells were normalized by the growth of
171 suspended cells and expressed as A₅₈₀/OD₆₀₀. Membranes used in this study were commercially

available flat-sheet polyvinylidene difluoride (PVDF; Millipore, Billerica, MA), mixed cellulose ester (MCE; ADVANTEC, Tokyo, Japan) and polytetrafluoroethylene (PTFE; ADVANTEC) with pore size of 0.2 μm . Both PVDF and PTFE membranes were hydrophilized by manufacturers. Borosilicate glass (MATSUNAMI GLASS, Osaka, Japan) was used as a control sample.

Bench-scale cross-flow filtration experiments. Biofouling potential was evaluated with bench-scale cross-flow filtration units (**Fig. 1**). Pure cultures were fed to the membranes, and the retentates were recirculated back to the feed bottles by using peristaltic pumps (MP-1100; EYELA, Tokyo, Japan). Filtration was carried out at 25°C with a constant flux of 1.1 m/d, and the transmembrane pressure (TMP) was measured every 10 min during the filtration. Effective membrane surface area was 3.5 cm². Cross-flow velocity was 0.015 cm/s, and the culture volume was 1 L. Prior to the cultivation, LB medium was filtered by 0.2 μm polyethersulfone membrane (CORNING, Corning, NY) in order to remove pre-existing foulants. For disinfection, the test unit was autoclaved before insertion of membranes, and the membranes were exposed to ultraviolet light for 3 min.

The overnight cultures were washed three times with 0.85% NaCl solution to avoid carrying over EPS into the feeding cultures. The washed cultures were inoculated to the feed bottles at an initial density of OD₆₀₀ = 0.05, and membrane filtration was performed with PVDF, MCE and PTFE membranes for 10 hr in triplicate. After the filtration, biofilms formed on the membranes were removed by pipetting and resuspended in 0.85% NaCl solution. Cell density of the resuspended biofilms was measured by OD₆₀₀. Filtration resistance of the washed membrane was

measured by dead-end filtration of distilled water under pressure of 30 kPa with a test cell (UHP-25K, ADVANTEC) (Kimura et al., 2012).

Fouling potential of biofilms and hydrophilic EPS was further compared by using the cross-flow filtration units under the same operating conditions. For the following experiments, membrane filtration was performed with only PVDF membrane in duplicate. First, the fouling potential of supernatants of the WcaA⁻ and RcsF⁺ were compared. The supernatants were prepared from the exponentially growing cultures (OD₆₀₀ = 1.0) by centrifugation at 10,000 × g for 30 min after vortexing them for 3 min. The remaining cells in the supernatants were checked to be less than 10⁶ CFU/mL for both strains. Prior to feeding, the supernatants were diluted twice with the fresh medium. Re-growth of the remaining cells was inhibited by the addition of a bacteriostatic antibiotic, tetracycline (10 µg/mL). Second, the fouling potential of supernatants was evaluated in the presence of biofilms formed on membranes. The washed cultures of the WcaA⁻ were inoculated to the feed bottles at an initial density of OD₆₀₀ = 0.05, and membrane filtration was performed. At 9 hr, the WcaA⁻ cultures were replaced to a fresh medium containing tetracycline and the inhibition of biofilm growth was checked by monitoring TMP. At 13 hr, the supernatants of the RcsF⁺ and WcaA⁻ were added to the feed bottles, which were diluted twice with the remaining fresh medium.

RESULTS AND DISCUSSION

EPS production and physicochemical properties of *E. coli* strains. EPS production of tested *E. coli* strains were investigated by colony morphology (**Fig. 2A**), fucose concentration (**Fig. 2B**) and SEM observation (**Fig. 2C**). Fucose is one of the components in a repeat-unit of colanic acid

(Garegg et al., 1971). RcsF⁺ showed mucoid colony phenotype and produced 6.9-fold higher amounts ($9.7 \pm 1.9 \mu\text{g}/\text{OD} = 1$) of extracellular fucose-based polysaccharide than the WT ($1.4 \pm 0.4 \mu\text{g}/\text{OD} = 1$), indicating that RcsF⁺ excessively produced colanic acid. The fucose concentration of a colanic acid defective strain WcaA⁻ was lower ($0.2 \pm 0.2 \mu\text{g}/\text{OD} = 1$) than the WT. The non-mucoid strain WcaA⁻ RcsF⁺ produced 2.4-fold higher amounts ($3.4 \pm 0.8 \mu\text{g}/\text{OD} = 1$) of extracellular fucose-based polysaccharide than the WT.

Cell surface characteristics were evaluated by measuring the surface potential (zeta potential) and hydrophobicity (**Table 1**). The cell hydrophobicity of RcsF⁺ was significantly lower (32 ± 10 and $19 \pm 6\%$ with xylene and n-hexadecane, respectively) as compared with the other strains (70 to 81 and 35 to 52%), indicating that the presence of colanic acid made cell surface more hydrophilic. The zeta potential of RcsF⁺ ($-49 \pm 0.7 \text{ mV}$) was not significantly different from those of WT ($-45 \pm 0.4 \text{ mV}$) and WcaA⁻ ($-48 \pm 0.5 \text{ mV}$) in our experimental condition while it has been reported that colanic acid production induced negative charge on the cell surface (Hanna et al., 2003; Chao and Zhang, 2011). On the other hand, WcaA⁻ RcsF⁺ ($-7 \pm 0.8 \text{ mV}$) was less negatively charged than the other strains (**Fig. 2C**).

Production of unknown EPS was observed due to the overexpression of the *rscF* gene in RcsF⁺ and WcaA⁻ RcsF⁺ (**Fig. 2B** and **2C**). The larger agglomerate-like EPS produced by WcaA⁻ RcsF⁺ is apparently different from the tiny ball-like EPS dominantly produced by RcsF⁺ (**Fig. 2C**), which appears similar to a previous observation of colanic acid reported by Prigent-Combaret et al. (2000). The difference in their major EPS is reflected by their colony morphology (**Fig. 2A**), cell hydrophobicity and zeta potential of RcsF⁺ and WcaA⁻ RcsF⁺ (**Table 1**). These results suggest that the hydrophilic colanic acid would be the major EPS in RcsF⁺.

Bacterial attachment to MF membranes. We used glass as a control and three membrane materials including MCE, PVDF and PTFE for testing the bacterial attachment. The contact angle was 36 ± 7 , 25 ± 3 and $75 \pm 3^\circ$ and the zeta potential was -14 ± 3 , -8 ± 3 and -10 ± 3 mV for PVDF, MCE and PTFE, respectively. PTFE was the most hydrophobic and the surface charge was not significantly different among the membranes applied in this study (**Table 2**). To investigate the impacts of the hydrophilic colanic acid production on bacterial attachment to MF membranes, the microtiter plate assay with the membrane specimens was performed (**Fig. 3**). All strains attached on MCE 3-fold more than PVDF and PTFE, suggesting that the attachment potential is different depending on membrane materials. The attachment of the colanic acid overproducing strain RcsF⁺ was lower than WT to hydrophobic materials, including glass (30 ± 30 and 250 ± 90 RFU/OD₆₀₀ for WT and RcsF⁺, respectively) and PTFE (90 ± 100 and 400 ± 100 RFU/OD₆₀₀). On the other hand, the attachment of WcaA⁻ (4900 ± 600 RFU/OD₆₀₀) was higher than that of WT to the hydrophilic membrane MCE (2300 ± 1300 RFU/OD₆₀₀). These results suggest that hydrophilic colanic acid reduces the cell attachment to hydrophobic materials.

The lower cell attachment of RcsF⁺ to hydrophobic materials is likely due to the increased cell surface hydrophilicity by colanic acid production (**Table 1**). In addition, the electrostatic repulsion force might also have contributed to the low cell attachment to more negatively charged glass because the effect of electrokinetic potential has been reported to increase with decreasing hydrophobic interactions (van Loosdrecht et al., 1978). The reduction of *E. coli* attachment by colanic acid production was previously reported (Hanna et al., 2003; Chao and Zhang, 2011), and the increase in cell surface hydrophilicity was one of the significant factors for the low attachment (Chao and Zhang, 2011). However, since there was no clear correlation between the amounts of attached cells on membranes and these surface properties, further study

on other properties including the roughness of membranes and the motility of bacterial strains is necessary to study the complex interactions.

Membrane biofouling. To examine the impacts of the hydrophilic extracellular polysaccharide production on membrane biofouling, the biofouling potential was evaluated by feeding the *E. coli* cultures to a cross-flow filtration unit operated at a constant flux. RcsF⁺ and WcaA⁻ RcsF⁺ caused TMP increase within 2 hr whereas the WT and WcaA⁻ caused moderate TMP increase at 8 hr for all membranes tested (**Fig. 4A**). It was confirmed that their growth was not different (**Fig. S2**). The accumulated biomass was quantified after 10 hr of filtration, and then the filtration resistance was measured after the accumulated biomass was removed (**Fig. 4B** and **4C**). The amounts of accumulated biomass of RcsF⁺ (OD₆₀₀ = 6 ± 4, 6 ± 1 and 7 ± 2 for PVDF, MCE and PTFE, respectively) and WcaA⁻ RcsF⁺ (OD₆₀₀ = 7 ± 1, 5 ± 1 and 8 ± 4) on the membranes were one third of those of the WT (OD₆₀₀ = 34 ± 4, 34 ± 3 and 25 ± 3) and WcaA⁻ (OD₆₀₀ = 33 ± 4, 33 ± 6 and 29 ± 8) (**Fig. 4B** and **Fig. S3**). However, the membrane resistance of the membranes fouled by RcsF⁺ (23 ± 7, 23 ± 9 and 13 ± 1 × 10¹⁰ m⁻¹ for PVDF, MCE and PTFE, respectively) and WcaA⁻ RcsF⁺ (17 ± 6, 27 ± 4 and 9 ± 3 × 10¹⁰ m⁻¹) remained 3-fold higher than those of the WT (6 ± 1, 4 ± 0 and 3 ± 0 × 10¹⁰ m⁻¹) and WcaA⁻ (6 ± 1, 4 ± 0 and 3 ± 0 × 10¹⁰ m⁻¹) which were almost fully recovered to that of the virgin membrane (5 ± 0, 3 ± 0 and 3 ± 0 × 10¹⁰ m⁻¹) by removing the biomass (**Fig. 4C**). These results clearly indicate that extracellular fucose-based polysaccharides cause severe membrane pore-clogging whereas the accumulation of biomass on membranes causes moderate reversible membrane fouling.

It should be noted that the results of cell attachment potential (i.e., microtiter plate assay) were not correlated to their biofouling potentials (i.e., amounts of accumulated biomass and TMP increase). The difference may be attributed to the presence of permeate drag force because the

permeate flow enhances EPS and bacterial cell transport to membranes (Eshed et al., 2008). Thus, membrane fouling potential must be evaluated by using cross-flow filtration units.

Fouling potential of biofilms and hydrophilic extracellular polysaccharides. To corroborate the fouling potential of the hydrophilic extracellular polysaccharide liberated in the bulk phase, the supernatants of the RcsF⁺ and WcaA⁻ cultures were fed to membranes in the absence and presence of pre-accumulated biomass (i.e., biofilms) on the membranes. In the absence of pre-formed biofilms, the supernatant of RcsF⁺ caused severe TMP increase within 5 hr while that of WcaA⁻ slightly increased TMP at 10 hr (**Fig. 5A**). The result suggests that the hydrophilic extracellular polysaccharide present in the RcsF⁺ supernatant caused rapid TMP increase by membrane pore-clogging. The supernatants were also fed to membranes after the biofilms were formed on the membranes. The WcaA⁻ biofilm formation was initiated until the bacterial culture was replaced to the fresh medium containing tetracycline. The TMP was unchanged during feeding the fresh medium with tetracycline (from 9 hr to 13 hr). The addition of the RcsF⁺ supernatant caused abrupt TMP increase whereas the WcaA⁻ supernatant moderately increased TMP. These results indicate that the hydrophilic extracellular polysaccharide liberated in the bulk phase penetrates the pre-formed biofilm layer and directly caused irreversible membrane fouling.

Colanic acid is produced by many enterobacteria (Rättö et al., 2006) and also involved in the development of voluminous biofilms (Pringent-Combaret et al., 2000; Danese et al., 2000; May and Okabe, 2008). We focused on this hydrophilic extracellular polysaccharide as a model fouling component and showed that colanic acid penetrated biofilms on membranes and caused

severe irreversible membrane fouling. Although heterogeneous EPS/SMP are present in MBRs, the presence and putative bacterial source of polysaccharides with high fouling potential have been reported (Kimura et al., 2012). Further study on polysaccharides with similar characteristics would help identify important properties of polysaccharides as foulants and understand mechanisms of membrane fouling.

Biofilm formation has been thought to be a major contributor to membrane biofouling in terms of the resistance-in-series model (Chang et al., 2002; Meng et al., 2009). Factors affecting the membrane filterability are the structure and porosity of biofilms rather than its biovolume.³⁶ For the dense structure of biofilms, biopolymers are essential because they fill the void space between microbial cells and membranes (Chu and Li, 2005; Hwang et al., 2008; Dreszer et al., 2013). Metzger et al. (2007) have reported that significant amounts of SMP localized on membrane pores underneath biofilms, which made the bottom part of the biofilm the densest after membrane filtration. Our results suggest that extracellular polysaccharides with similar characteristics with colanic acid liberated in the bulk phase easily penetrate biofilms and clog membrane pores. In addition, the direct deposition of biopolymers negates the putative role of biofilms as a self-forming dynamic membrane, which works as a barrier by capturing biopolymers and alleviates membrane fouling (Lee et al., 2001).

CONCLUSIONS

We investigated the impacts of hydrophilic colanic acid, as a model component of extracellular polysaccharides (EPS), on initial cell attachment to different MF membranes and subsequent membrane biofouling. The following conclusions can be drawn:

● Colanic acid, a hydrophilic EPS, made cell surfaces hydrophilic and reduced cell attachment to hydrophobic MF membranes in both static and cross-flow conditions.

● Colanic acid could penetrate the pre-formed biofilms and directly clogged membrane pores, which caused severe irreversible membrane fouling during cross-flow filtration tests.

Further studies are needed to investigate the impacts of other EPS and/or SMP components on the initial cell attachment and membrane fouling, which would lead to the development of effective fouling mitigation strategies.

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Figure 1. Schematic diagrams of (A) a bench-scale cross-flow filtration unit and (B) a membrane cell. Permeate was withdrawn by a peristaltic pump at a constant flux of 1.1 m/d. TMP was automatically measured in every 10 min. All dimensions are given in mm.

Figure 2. EPS production by WT, RcsF⁺, WcaA⁻ and WcaA⁻ RcsF⁺. (A) Colony morphology on LB agar after 24 hr of incubation at 30°C, (B) fucose concentration in soluble polysaccharides purified from the colonies and (C) SEM images of the four strains grown on LB agar plates. White and black arrows indicate EPS predominantly observed in each strain. Bar represents 1 μm. Error bars indicate the standard deviations from three samples.

Figure 3. Bacterial attachment on different membranes. Amount of attached cells on membranes was measured by fluorometry and normalized by the growth of suspended cells (OD₆₀₀) after 72 hr of incubation at 30°C. Measurement was conducted with 24-well microtiter plates, of which a membrane specimen was placed in each well (see Supplemental **Figure S1**). Error bars indicate the standard deviations from three samples.

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487 **Figure 4.** Membrane biofouling; (A) TMP increase, (B) amounts of accumulated biomass on
 488 membranes and (C) filtration resistance after removing the biomass. Cross-flow filtration units
 489 were operated for 10 hr at 25°C at a constant permeate flux of 1.1 m/d and cross-flow velocity of
 490 0.015 cm/s. Pure cultures were fed to the membranes at an initial cell density of $OD_{600} = 0.05$.
 491 The TMP data are representative results of triplicate experiments. Error bars in **B** and **C** indicate
 492 standard deviations from triplicate experiments.

Figure 5. Fouling potential of biofilms and hydrophilic extracellular polysaccharides liberated in the bulk phase. Fouling potential of the supernatants of WcaA⁻ and RcsF⁺ cultures was compared (A) in the absence and (B) the presence of biofilms on membranes. For biofilm formation, the WcaA⁻ cultures were inoculated to the feed bottles at an initial density of OD₆₀₀ = 0.05 and membrane filtration was performed for 9 hr. Then, the WcaA⁻ cultures were replaced to a fresh medium containing tetracycline, and the inhibition of biofilm growth was checked by monitoring TMP. At 13 hr, the supernatants of RcsF⁺ and WcaA⁻ were added to the feed bottles. Experiments were performed with only PVDF membrane under the same operating condition with **Fig. 4**. Representative results of duplicate experiments were shown.

TABLES.

Table 1. Surface characteristics of bacterial strains. Hydrophobicity was measured by BATH assay with xylene and n-hexadecane. Zeta potential was measured in 15 mM NaCl solution at cell density of $OD_{600} = 0.1$. The results are shown as means $\pm$ standard deviations from three samples.

Strains	WT	RcsF ⁺	WcaA ⁻	WcaA ⁻ RcsF ⁺
Hydrophobicity (%)				
Xylene	81 $\pm$ 6	32 $\pm$ 10	76 $\pm$ 13	70 $\pm$ 4
n-Hexadecane	52 $\pm$ 1	19 $\pm$ 6	35 $\pm$ 5	43 $\pm$ 2
Zeta potential (mV)	- 45 $\pm$ 0.4	- 49 $\pm$ 0.7	- 48 $\pm$ 0.5	- 7 $\pm$ 0.8

Table 2. Surface characteristics of glass and membrane materials. The results are shown as means $\pm$ standard deviations from at least three samples.

Membranes	Borosilicate glass	PVDF*	MCE	PTFE*
Pore size (μ m)		0.2	0.2	0.2
Contact angle ($^{\circ}$)	51 $\pm$ 4	36 $\pm$ 7	25 $\pm$ 3	75 $\pm$ 3
Zeta potential (mV)	- 40 $\pm$ 6	- 14 $\pm$ 3	- 8 $\pm$ 3	- 10 $\pm$ 3

*: Membranes were hydrophilized by manufacturers.

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