For submission to *Water Research*

Revised Manuscript WR34138R2

**Membrane fouling potentials and cellular properties of bacteria
isolated from fouled membranes in a MBR treating municipal
wastewater**

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23   **Abstract**

24   Membrane fouling remains a major challenge for wider application of
25   membrane bioreactors (MBRs) to wastewater treatment. Membrane fouling is mainly
26   caused by microorganisms and their excreted microbial products. For development of
27   more effective control strategies, it is important to identify and characterize the
28   microorganisms that are responsible for membrane fouling. In this study, 41 bacterial
29   strains were isolated from fouled microfiltration membranes in a pilot-scale MBR
30   treating real municipal wastewater, and their membrane fouling potentials were directly
31   measured using bench-scale cross-flow membrane filtration systems (CFMFSs) and
32   related to their cellular properties. It was found that the fouling potential was highly
33   strain dependent, suggesting that bacterial identification at the strain level is essential to
34   identify key fouling-causing bacteria (FCB). The FCB showed some common cellular
35   properties. The most prominent feature of FCB was that they formed convex colonies
36   having swollen podgy shape and smooth lustrous surfaces with high water, hydrophilic
37   organic matter and carbohydrate content. However, general and rigid biofilm formation
38   potential as determined by microtiter plates and cell surface properties (i.e.,
39   hydrophobicity and surface charge) did not correlate with the fouling potential in this
40   study. These results suggest that the fouling potential should be directly evaluated under
41   filtration conditions, and the colony water content could be a useful indicator to identify
42   the FCB.

43   **Keywords:** Membrane fouling potential; cross-flow filtration system; fouling-causing
44   bacteria (FCB); colony water content; hydrophilic organic matter.

45   **1. Introduction**

46   It is widely accepted that membrane fouling is a main obstacle for wider
47   application of membrane bioreactors (MBRs). Although extensive studies have been
48   conducted to understand the detailed mechanisms of membrane fouling in MBRs and to
49   develop its effective mitigation strategies (Huang and Lee 2015; Lin et al. 2014; Malaeb
50   et al. 2013), improvement of fouling control and management is still remarkably slow in
51   practice. One of research approaches is to identify key players (microbial strains) of
52   membrane fouling and subsequently to control and inhibit their activity by any means
53   (Calderón et al. 2011; Ma et al. 2013b; Miura et al. 2007). Although extensive studies
54   have been conducted to reveal microbial communities in pilot-scale MBRs treating real
55   municipal wastewater using several molecular biological techniques, our understanding
56   is still largely limited to identify key bacteria responsible for membrane fouling (Kim et
57   al. 2013; Lim et al. 2012; Miura et al. 2007; Pang and Liu 2007; Wu et al. 2011; Xia et
58   al. 2010). The dominant species in MBRs (Calderón et al. 2011) and in biocake
59   (biomass accumulated on the membranes) (Miura et al. 2007; Pang and Liu 2007), or
60   the increasing species as membrane fouling (Lim et al. 2012) have been regarded as key
61   bacterial groups responsible for membrane fouling. For example, *Proteobacteria* has
62   been determined as a dominant phylum in MBRs and thus regarded as fouling causing
63   bacteria (Lim et al. 2012; Ma et al. 2013a). It was also reported that *Proteobacteria*
64   plays a pioneering role in biocake formation on membrane (Gao et al. 2014; Miura et al.
65   2007; Vanysacker et al. 2014a). This is, however, not always true because membrane
66   fouling is known to be mainly caused by metabolites (i.e., extracellular polymeric

67 substances (EPS) and soluble microbial products (SMP)) produced by or released from
68 microorganisms and cellular materials (debris), which are highly dependent on
69 microbial species and operational conditions (Guo et al. 2012; Lin et al. 2014). For
70 examples, changes in operational conditions (i.e., DO and temperature) and
71 environmental stress (i.e. salinity concentration) induced the changes in microbial
72 community structures and microbial metabolisms (production rates and compositions of
73 EPS and SMP) and consequently influenced membrane fouling (Gao et al. 2011; Gao et
74 al. 2013; Guo et al. 2015; Ng and Ng 2010).

75   Several papers have highlighted the relevance of cellular properties such as
76 bacterial motility and cell surface properties of environmental isolates in biofilm
77 formation, which was often overinterpreted to membrane fouling potential. For
78 examples, a strain belonging to *Arthrobacter* sp. having a relatively small cell size in
79 mixed liquor of an aerobic granular membrane bioreactor (AGMBR) can penetrate the
80 microfiltration (MF) membrane and develop an internal biofilm that induce membrane
81 fouling (Juang et al. 2010). Furthermore, biofilm formation potentials of strains isolated
82 from reverse osmosis (RO) membranes treating potable water were measured in
83 microtiter plates and related to their cellular properties such as hydrophobicity, surface
84 charge, and motility (Pang et al. 2005). However, membrane fouling potentials of these
85 isolates were not directly measured under filtration conditions in their studies.

86   Biofilm formation potentials determined in microtiter plates sometimes do not
87 reflect membrane fouling potentials, because convective permeate flow is known to be a
88 major mechanism that transports bacterial cells and EPS to membrane surfaces during

89 filtration (Habimana et al. 2014). Therefore, biofilm formation and/or biomass
90 deposition on membrane surfaces, furthermore membrane fouling, are more likely to be
91 independent of bacterial cell surface properties and motility (Habimana et al. 2014;
92 Juang et al. 2010). Thus, membrane fouling potential must be directly evaluated for
93 each bacterial strain under defined filtration conditions. However, membrane fouling
94 potentials have been evaluated for only a few isolates and model bacteria (e.g.,
95 *Escherichia coli* and *Pseudomonas*) using dead-end or cross-flow filtration systems
96 (Choi et al. 2006; Feng et al. 2009; Juang et al. 2008; Vanysacker et al. 2014a).
97 Therefore, there is very little information on fouling potentials of environmental isolates
98 and the relevance to their cellular characteristics (Malaeb et al. 2013).

99   The objectives of this study are to identify key fouling-causing bacteria (FCB)
100 for microfiltration (MF) membranes in MBRs treating real municipal wastewater and
101 characterize their cellular properties. To achieve these objectives, 41 bacterial strains
102 were isolated from fouled MF membrane gel layers (biofilms) in the MBRs, and their
103 fouling potentials were directly evaluated using bench-scale cross-flow membrane
104 filtration systems (CFMFSs). FCB strains were phylogenetically identified, and their
105 cellular properties such as cell surface properties (hydrophobicity and surface charge),
106 and bacterial motility, biofilm formation potential and colony properties were analyzed
107 in details to evaluate the relevance of these cellular properties in their membrane fouling
108 potentials.

109

110 **2. Materials and methods**

111   **2.1.   Isolation of bacterial strains**

112   The firmly attached biomass (gel layer) on fouled microfiltration (MF)
113 hollow-fiber membranes was collected from a pilot-scale MBR treating municipal
114 wastewater located at Soseigawa Municipal Wastewater Treatment Plant, Sapporo,
115 Japan (**Fig. S1**). The hollow-fiber polytetrafluoroethylene (PTFE) membrane with 0.3
116 μm nominal pore size was submerged in a reaction tank (175 L) of the MBR. The MBR
117 was operated at a permeate flux of ca. 0.2 m/d and sludge retention time of 35 day.
118 Average MLSS concentration in the reaction tank was ca. 50 g/l. Characteristics of the
119 raw wastewater of this plant can be found elsewhere (Kimura et al., 2005).

120   The biomass was collected after washing loosely attached biomass with tap
121 water in April, 2013. The biomass was serially diluted with sterilized MilliQ water,
122 spread on R2A agar plates, and incubated at 30°C. The colonies grown on the plates
123 were transferred to new R2A agar plates and the plates were incubated at 30°C again.
124 Forty-one strains were obtained by repeating this process more than 3 times. These
125 isolates were analyzed by 16S rRNA gene sequencing and repetitive sequence-based
126 PCR (rep-PCR) as described below. All 41 strains were subjected to the analyses of
127 membrane fouling potential and cellular properties (**Table 1**). According to 16S rRNA
128 gene sequencing data, OTUs were generated using identity cut-offs of 97%. When the
129 isolates shear >97% 16S rRNA gene sequence identity, rep-PCRs were performed to
130 further differentiate the isolated strains. Based on the results of these analyses,
131 representative strains of each unique strain group were subjected to further analyses of
132 colony water content and hydrophilic organic matter content. All isolated strains were

133 cultivated on R2A agar plates in this study.

134 DNA was extracted from each isolate as described previously (Ashida et al.
135 2010). Briefly, the isolated bacterial colonies grown on R2A agar plates were suspended
136 in 100 µl of 0.05 M NaOH, and then the suspension was heated at 95°C for 15 min.
137 After centrifugation (1,000 rpm, 5 min), the supernatant was diluted 10-fold, which was
138 used as template DNA for PCR amplification (Ashida et al. 2010). A primer set of 27F
139 and 1492R was used to amplify 16S rRNA gene fragments for 16S rRNA gene sequence
140 analysis (Goodfellow and Stackebrandt 1991). The 16S rRNA gene sequences (ca.
141 1,352 bp) were compared to sequences available in the BLAST database (Altschul et al.
142 1997). For the phylogenetic analysis, a neighbor-joining tree (Saitou and Nei 1987) was
143 constructed (1,000 replicate bootstraps) by using MEGA 5.2. software. Rep-PCR
144 fingerprinting was performed as described elsewhere (Ishii and Sadowsky 2009).

145

146 **2.2. Microbial community analysis**

147 The microbial communities of the firmly attached biomass on fouled MF
148 membranes (gel layer; Gel) and biomass in the mixed liquor (ML) were analyzed by
149 applying next-generation sequencing (Illumina MiSeq) analysis (Illumina; Hayward,
150 CA, USA). Total community DNA was extracted from the gel layer and the pellet of the
151 mixed liquor by using a PowerSoil DNA Isolation Kit (MoBio Laboratories; CA, USA).
152 The partial 16S rRNA gene including v3 and v4 regions were amplified with Bakt_341F
153 and Bakt_805R primers (Herlemann et al. 2011). Next-generation sequencing (Illumina
154 MiSeq) 16S rRNA gene analysis was performed as described elsewhere (Rathnayake et

155 al. 2015). In total, 58,134 and 76,354 sequence reads were analyzed for the ML and Gel
156 samples by using QIIME 1.8.0 with the Silva 119 database, respectively (Caporaso et al.
157 2012).

158

159 **2.3. Membrane fouling potential**

160 Membrane fouling potential of each isolated strain was determined using a
161 bench-scale cross-flow membrane filtration system (CFMFS). The CFMFS consists of a
162 reactor (75 mL), which was operated as chemostat, and a cross-flow membrane
163 filtration cell (CFMFC) (**Fig. S2**). The dimension of the CFMFC was 0.5 mm (height)
164 by 20 mm (width) by 100 mm (length), corresponding to a volume of 1,000 mm³ and
165 effective filtration surface area of 2,000 mm², respectively. Flat membrane filter (0.2 µm,
166 hydrophilic PTFE; Advantec Toyo; Tokyo, Japan. The detail information was shown in
167 **Fig. S3**) was used for all filtration experiments in this study. Cross-flow velocity was set
168 at 1 cm/s. Hydraulic retention time (HRT) and solid retention time (SRT) were adjusted
169 at 2.5 hr and 12.5 day, respectively.

170 Overnight grown cultures (75 ml, OD₆₀₀ was adjusted to approximately 0.3
171 with fresh R2A broth medium (3.2 g/L)) of each isolated strain were prepared and
172 inoculated into the reactor. The fresh R2A broth medium was then continuously fed to
173 the reactor at a flow rate of 0.71 L/d. Growth rates of isolates can be controlled by
174 changing the flow rate (i.e., dilution rate). Feeding substrate to the reactor was
175 accomplished with a peristaltic pump (MP-1100; EYELA; Tokyo, Japan). The feed cell
176 suspension was circulated through the reactor and CFMFC by a peristaltic pump

177 (MasterFlex EW-07553-80; Cole-Parmer Instrument Co.; IL, USA) at a flow rate of 6.0
178 L/h. All filtration experiments were carried out at room temperature ($25 \pm 2^\circ\text{C}$) with a
179 constant permeate flux of 0.3 m/d, which was controlled by a digital peristaltic pump
180 (ISM 936; IDEX Health & Science; Wertheim, Germany) mounted on the permeate line.
181 The trans-membrane pressure (TMP) was continuously monitored and recorded with a
182 data logger (midi logger GL220; Graphtec; Yokohama, Japan). Trans-membrane
183 pressure after 4 hr (TMP_4) and 24 hr (TMP_{24}) of filtration were measured to
184 characterize membrane fouling potential of isolated strains. After 24 hr of operation, the
185 biomass concentration (OD_{600}) in the reactor and the biomass accumulated on the
186 membrane surface were measured with an optical absorbance meter (Smart Spec Plus;
187 Bio-Rad; CA, USA). The biomass accumulated on the membrane was removed and
188 suspended in 25 ml of 0.05% NaCl solution by vortexing for 1 min, and then OD_{600} was
189 measured (described as OD_{600} of suspended BC). All apparatus except for the CFMFC
190 and membrane were autoclaved, and the CFMFC and membrane were sterilized with
191 sodium hypochlorite (3,000 ppm for 30 min) and rinsed well with sterilized MilliQ
192 water.

193

194 **2.4. Cellular characterization**

195 Maximum specific growth rate (MGR) was determined by monitoring OD_{600}
196 increase in walls of a 96-well microtiter plate. Each isolated strain (initial $\text{OD}_{600} = 0.1$)
197 was incubated at 30°C , the OD_{600} was monitored every 1 hr for 24 hrs, and then the
198 MGR was determined based on the OD_{600} growth curves.

199 Cell surface hydrophobicity was measured as described previously with minor
200 changes (Pang et al. 2005). Overnight grown cultures of each isolated strain in R2A
201 broth (5 ml) were prepared, and then the cells were harvested by centrifuged at 4°C,
202 6,000 ×g for 15 min and washed twice with 0.85% NaCl solution. The washed cells
203 were resuspended in 0.85% NaCl solution to OD₆₀₀ of approximately 0.7. The washed
204 cell culture (3.0 ml) was mixed with 1.0 ml of xylene, vortexed for 1 min, and incubated
205 for 10 min in a water bath at 37°C. Zeta potentials of the washed cells (OD₆₀₀ = 0.1)
206 were measured by using an electrophoretic light-scattering spectrophotometer (Zetasizer
207 Nano ZS; Malvern Instruments; Malvern, Worcestershire, UK).

208 Biofilm formation potential was determined by quantifying the amount of
209 biomass attached on walls of a 96-well microtiter plate as described previously
210 (Burmølle et al. 2006). Briefly, 200 µl of each washed cell culture (OD₆₀₀ = 0.1) was
211 inoculated into each well and incubated at 30°C for 24 hr. After 24 hr of incubation,
212 OD₆₀₀ in the suspended culture was measured, and attached biomass (biofilm) was also
213 measured after staining with 1% crystal violet (CV) solution for 30 min and
214 resuspending into 220 µl of a mixed solution of ethanol and acetone (ethanol : acetone =
215 8:2). The biofilm formation potential was normalized by the growth of suspended cells
216 and expressed as the ratio of CV value to OD₆₀₀ value.

217 The biofilm firmly adhered on the walls of a 96-well microtiter plate was
218 measured, which is defined as “rigid biofilm formation potential” in this study. Loosely
219 attached biofilms were removed by ultrasonic treatment (400W for 30 min) followed by
220 rigorous washing with MilliQ water, and thereafter the remaining biofilm was

221 determined after staining with 1% crystal violet (CV) as described above.

222   Swimming and swarming motilities were measured as described previously
223 with minor modification (Déziel et al. 2001; Rashid and Kornberg 2000). For
224 swimming and swarming motility, the strains were inoculated on the center of 0.3% and
225 0.5% (w/v) R2A agar plate, respectively. These plates were incubated at 30°C for 24 hr,
226 and the diameter of each colony was measured.

227   In order to evaluate the degree of bacterial cell aggregation, particle size
228 distributions of bacterial cells were measured by using Nano Sight NS500
229 (SALD-7100; Shimadzu Co., Kyoto, Japan). Overnight grown cultures of each isolated
230 strain in R2A broth (10 ml) were prepared, and then the cells were harvested by
231 centrifuged at 4°C, 6,000 ×g for 15 min and washed with 0.85% NaCl solution to
232 eliminate the influence of small particles associated with fresh R2A medium. Five ml of
233 the diluted washed bacterial cells was gently vortexed and loaded to the Nano Sight
234 NS500.

235   All the statistical analyses were carried out with R 3.0.2 (R Development
236 Core Team; Vienna, Austria). *P* values less than 0.05 were considered statistically
237 significant in all analyses.

238

239 **2.5. Colony water content**

240   Colony water contents were quantified as follows. The representative strains
241 were inoculated on R2A agar plates (1.5% (w/v)) and incubated for 3 days. Colonies
242 formed on R2A agar plate were collected carefully with spatula in a 1.5 ml tube, and its

weight was measured. After drying at 100°C for 24 hr, the weight was measured again.

The colony water content was calculated as following:

$$\text{Water content (\%)} = (W_{\text{initial}} - W_{\text{dried}}) / W_{\text{initial}}$$

where, W_{initial} (mg) and W_{dried} (mg) were the colony weight before and after dried, respectively. Photographs of each colony were taken after 3 days of incubation (**Fig. S4**).

2.6. Soluble microbial products and extracellular polymeric substance

Soluble microbial product (SMP) and extracellular polymeric substance (EPS) in colonies of isolated strains were extracted as described previously with minor modifications (Ramesh et al. 2007; Wang et al. 2009). Briefly, colonies were collected from R2A agar plate (1.5% (w/v)), suspended in 0.05% NaCl solution, dispersed well by vortexing, and then diluted to $OD_{600} = 0.3$ to eliminate the concentration effect. The colony suspension was centrifuged at 4°C and $6,000 \times g$ for 15 min. The supernatant was regarded as SMP. The remained pellet was suspended with 0.05% NaCl solution and then was subjected to heat treatment at 80°C for 1 hr. After dispersed well by vortexing, the suspension was centrifuged again at 4°C and $6,000 \times g$ for 15 min. The supernatant was regarded as EPS.

Fractions of hydrophilic, hydrophobic, and neutral organic matter in SMP were obtained by using DAX-8 (Supelite™ DAX-8; Sigma-Aldrich Co.; MO, USA) and XAD-4 resin (Amberlite® XAD-4; Sigma-Aldrich Co.; MO, USA) as described previously with minor modifications (Aiken et al. 1992; Yamamura et al. 2014). The

265 SMP was diluted to TOC concentration < 20 mg/L and acidified to pH 2 with 11.2 M
 266 HCl, and 5 ml of each solution was passed through DAX-8 and XAD-4 at a flow rate of
 267 200 mL/min in series. The fraction passed through both DAX-8 and XAD-4 resins was
 268 regarded as hydrophilic fraction, the fraction that was retained on the DAX-8 resin was
 269 designated as the hydrophobic fraction, and the fraction that was retained on the XAD-4
 270 resin was designated as the neutral fraction, respectively. Hydrophobic and neutral
 271 fractions were collected by eluting each resin with 5 ml of NaOH (pH 12) at a flow rate
 272 of 120 mL/min. The amounts of each fraction were determined based on TOC
 273 concentration as follows:

$$274 \quad \text{Hydrophilic organic matter content} = a \times \text{TOC}_{\text{hydrophilic}} / \text{OD}_{600}$$

$$275 \quad \text{Hydrophobic organic matter content} = a \times \text{TOC}_{\text{hydrophobic}} / \text{OD}_{600}$$

$$276 \quad \text{Neutral organic matter content} = a \times \text{TOC}_{\text{neutral}} / \text{OD}_{600}$$

277 where, a is dilution factor, OD_{600} is the value of OD_{600} of the colony suspension, and
 278 $\text{TOC}_{\text{hydrophilic}}$, $\text{TOC}_{\text{hydrophobic}}$, and $\text{TOC}_{\text{neutral}}$ are TOC concentration (mg/l) of hydrophilic,
 279 hydrophobic, and neutral fraction, respectively. The concentration of TOC was
 280 measured by using a TOC analyzer (TOC-V CSH; Shimadzu; Kyoto, Japan).

281 Carbohydrate and protein concentrations of the supernatant were measured
 282 with the phenol-sulfonic acid method with glucose as the standard and the Lowry
 283 method using BSA as the standard, respectively. The contents of carbohydrate and
 284 protein were determined as follows:

$$285 \quad \text{Carbohydrate} = a \times C / \text{OD}_{600}$$

$$286 \quad \text{Protein} = a \times P / \text{OD}_{600}$$

287 where, C and P are the carbohydrate and protein concentration of SMP and EPS (mg/l),
288 respectively.

289

290 **2.7. Dead-end filtration test**

291 Dead-end filtration test was performed to measure the fouling potential of
292 SMP and EPS of isolated strains as described previously with minor modification
293 (Ramesh et al. 2007; Kimura et al. 2012). Five ml of SMP or EPS was transferred to a
294 stirred filtration unit (UHP-25K; Advantec Toyo; Tokyo, Japan) with a flat membrane
295 filter (0.2 μm , hydrophilic PTFE; Advantec Toyo; Tokyo, Japan), and filtered under 50
296 kPa of ambient air. MilliQ water (approximately 10 mL) was added in the filtration unit
297 and filtered again under the same pressure. The permeate flow rate of MilliQ water was
298 measured, and the membrane resistance of the fouled membrane was calculated as
299 follows;

$$300 \text{ Membrane resistance (m}^{-1}\text{)} = PA/\mu Q$$

301 where, P is the pressure (Pa), A is the filtration area of membrane (m^2), μ is the dynamic
302 viscosity of MilliQ water ($\text{Pa} \cdot \text{s}$), and Q is the permeate flow rate of MilliQ water (m^3/s).

303

304 **3. Results**

305 **3.1. Microbial community analysis**

306 Bacterial diversity and composition of the gel layer (biofilm) formed on
307 fouled MF hollow fiber membranes of a pilot-scale MBR treating real municipal

308 wastewater was determined based on 16S rRNA gene sequences using next-generation
309 sequencing (Illumina MiSeq) and compared with those of the mixed liquor (ML) (**Fig.**
310 **S1**). Microbial community structure of the gel layer was basically similar to that of the
311 mixed liquor. *Proteobacteria* and *Bacteroidetes* are dominant phyla in both ML and Gel,
312 which was agreement with the previous report (Duan et al. 2009) (**Fig. S1**). The
313 *Xanthomonadaceae* and *Comamonadaceae*, which were frequently detected in MBRs
314 treating municipal wastewater (Mulina-Munoz et al. 2009), were found to be dominant
315 families in the gel layer and ML (approximately 2 - 5% of total reads) (**Table 1** and **Fig.**
316 **S1D** and **S1H**). Although many isolated strains were affiliated with the
317 *Enterobacteriaceae* and *Microbacteriaceae* families (see below), which also often
318 detected as dominant species in MBRs (Lim et al. 2012; Xia et al. 2010), both the
319 families accounted for less than 0.17 % in both the gel layer and mixed liquor samples
320 (**Table 1**).

321

322 **3.2. Membrane fouling potential**

323 Forty-one strains were isolated from the fouled MF membranes and identified
324 based on ca. 1,352 bp of 16S rRNA gene sequences and rep-PCR DNA fingerprinting
325 analyses (**Fig. 1**). These isolates were then tested for membrane fouling potentials using
326 bench-scale cross-flow membrane filtration systems (CFMFSs). During filtration, the
327 trans-membrane pressure after 4 hr (TMP₄) and 24 hr (TMP₂₄) of filtration were
328 measured to characterize the fouling potentials of these isolated strains.

329   The isolated stains formed distinct phylogenetic clusters (families) based on
330    $\geq 97\%$ 16S rRNA gene sequence similarity; 12 isolates (4 OTUs) in *Enterobacteriaceae*,
331   1 isolate in *Moraxellaceae*, 4 isolates (2 OTUs) in *Pseudomonadaceae*, 1 isolate in
332   *Comamonadaceae*, 6 isolates (3 OTUs) in *Xanthomonadaceae*, 1 isolate in
333   *Phyllobacteriaceae*, 5 isolates (2 OTUs) in *Bacillaceae*, 2 isolates (1 OTU) in
334   *Paenibacillaceae*, and 8 isolates (1 OTU) in *Microbacteriaceae* (**Table 1**). Strain S25
335   was affiliated with the order *Rhizobiales*, but did not have $\geq 97\%$ 16S rRNA gene
336   sequence similarity to any families. These families were frequently detected in various
337   MBRs (Gao et al. 2014; Guo et al. 2015; Lim et al. 2004; Lim et al. 2012;
338   Mulina-Munoz et al. 2009; Oh et al. 2012; Shapiro et al. 2010).

339   Although 4 *Enterobacteriaceae* isolates (OTU 1), strains S01, S02, S03, and
340   S04, had $\geq 99\%$ 16S rRNA gene sequence similarity (**Table S1**), only strain S04 showed
341   a significantly high fouling potential as compared with others (**Fig. 1**). A dendrogram
342   generated from rep-PCR banding profiles revealed that the strain S04 differed from
343   other three strains with the relative similarity of 20%, indicating a different strain
344   (Healy et al. 2004). Similarly, although the *Microbacteriaceae* strains S36 and S37
345   shared $\geq 99\%$ 16S rRNA gene sequence identity, rep-PCR analysis could discriminate
346   the two strains which showed different fouling behavior. The similar results were
347   obtained for the *Xanthomonadaceae* strains S20 and S21 and the *Pseudomonadaceae*
348   strains S16 and S17.

349   Overall, the isolated strains were grouped into three groups based on
350   development of membrane fouling; **I.** membrane fouling is quickly developed and

351 reached TMP > 20 kPa within 4 hr, **II**. Membrane fouling is gradually developed but
352 reached TMP > 20 kPa within 24 hr, and **III**. Membrane fouling is gradually developed
353 and reached TMP < 20 kPa after 24 hr (**Fig. 2**). The isolated strains classified into the
354 group **I** were defined as fouling-causing bacteria (FCB) in this study, which includes the
355 *Enterobacteriaceae* strains S04, S05, and S06, *Pseudomonadaceae* strain S16,
356 *Xanthomonadaceae* strains S23 and S24, *Phyllobacteriaceae* strain S26,
357 *Paenibacillaceae* strains S32 and S33, and *Microbacteriaceae* strains S34, S35, S38,
358 S39, S40, and S41 (**Table 1** and **Table S1**).

359   The different fouling patterns could be attributed to biomass concentrations
360 (OD_{600}) in the mixed liquor during filtration, i.e., the growth rates of the isolated strains.
361 However, there was no clear correlation between TMP₂₄ and the biomass accumulated
362 on the membrane after 24 hr of filtration (suspended BC: suspended biocake) (**Fig. 3A**)
363 and biomass concentrations in the mixed liquor (ML) (**Fig. 3B**). These results indicate
364 that membrane fouling was not mainly caused by accumulation of bacterial cells,
365 instead probably caused by soluble microbial products (SMP) and/or extracellular
366 polymeric substances (EPS) as previously reported (Guo et al. 2012; Lin et al. 2014).
367 This also suggests that dominant species or faster-growing species in MBRs may not be
368 necessarily FCB.

369

370 **3.3. Cellular properties**

371   Maximum specific growth rate, motility (swimming and swarming), zeta
372 potential, cell surface hydrophobicity, particle size distributions of bacterial cultures,

and biofilm formation potential (CV_{570}/OD_{600}) of the isolated strains were determined in details to evaluate the relevance of these cellular properties in their membrane fouling potentials (**Table 1**). The biofilm formation potential was normalized by the growth of suspended cells and expressed as CV_{570}/OD_{600} . Multiple linear regression analysis was performed to determine the most influential cellular property on membrane fouling (**Table S2**). As a result, FCB (group **I**) generally showed lower swimming motility than other strains (group **II** and **III**) ($p<0.05$, **Fig. S5B**). There is, however, no clear explanation of the relationship between the lower swimming motility and higher fouling potential. Furthermore, biofilm formation potential was not related to the membrane fouling potential. Firmly attached (rigid) biofilm was also quantified (**Table 1**). However, the difference in rigid biofilm formation potential was not statistically significant among the isolated strains (**Fig. S5G**).

3.4. Colony characteristics

The most prominent feature of FCB was that FCB formed convex colonies having swollen podgy shape and smooth lustrous surfaces (swollen podgy colony) (**Fig. S4**). In this study, the water contents of colonies were directly measured (**Table 1**). The FCB in the group **I** showed significantly higher ($>93\%$, **Fig. 4A**) colony water contents than isolated strains in other groups ($p<0.001$, **Fig. S5I**). In addition, FCB produced more soluble hydrophilic organic matter ($p<0.01$) and carbohydrate ($p<0.01$) in SMP than the other strains (**Fig. 4C**, **Fig. S5J** and **S5K**). On the other hand, there was no significant difference between the amounts of carbohydrate in SMP and protein in EPS

395 of FCB and the other strain colonies (**Fig. 4D**, and **Fig. S5L to S5N**). Since hydrophilic
396 material is known to have higher water-retention property than hydrophobic material
397 (Fontani et al. 2013; Pandey et al. 2014), the higher water content of FCB colonies
398 could be attributed to the higher content of soluble hydrophilic organic matter (**Fig. 4B**
399 and **Fig. S6**). Fouling potentials of SMP and EPS produced in colonies of isolated
400 strains were measured using dead-end filtration test to ensure the fouling behavior of
401 colonies is similar to ones measured in the CFMFS (**Fig. S7**). The SMP produced by
402 FCB resulted in significant membrane resistance as compared with the other strains
403 ($p<0.01$, **Fig. S7A**), which was in good agreement with TMP_4 . However, there was no
404 clear correlation between the fouling potential of EPS produced by FCB and TMP_4 .
405 These results indicated that the colony water content and SMP are more closely related
406 to fouling potentials of isolated strains.

407

408 **4. Discussion**

409 The membrane fouling potentials of 41 bacterial strains isolated from fouled
410 MF membranes in a MBR treating real municipal wastewater were determined and
411 related to their cellular properties; cell surface properties, motility, biofilm formation
412 potential, cell size, and colony characteristics. It was found that fouling causing bacteria
413 (FCB) were affiliated with diverse families and the fouling potential was highly strain
414 dependent; bacterial strains in the same family displayed different fouling potentials.
415 This suggests that bacterial identification at the strain level is essential to identify key
416 FCB. These FCB showed several common cellular properties; formation of swollen

417 podgy colonies with high water, hydrophilic organic matter, and carbohydrate contents.

418   Major microbial species in MBRs have been frequently considered to be key
419 bacteria responsible for membrane fouling. Although *Proteobacteria* have been
420 considered to be one of dominant species and key FCB in MBRs (Lim et al. 2012;
421 Miura et al. 2007; Vanysacker et al. 2014b; Xia et al. 2010), only a few *Proteobacteria*
422 strains were isolated and classified as FCB in our study. Similarly, *Xanthomonadaceae*
423 constituted 2.73% and 4.21% in ML and Gel samples in this study, but less than half of
424 the *Xanthomonadaceae* isolates (strains S23 and S24) were classified as FCB. In
425 contrast, although *Microbacteriaceae* was one of minor groups in the MBR (0.17% and
426 0.02% in ML and Gel, respectively), 6 out of 8 strains (strains S34, S35, S38, S39, S40,
427 and S41) caused severe membrane fouling. Based on these experimental results,
428 dominant bacterial species are not always necessary to be FCB.

429   In this study, colony water content was clearly correlated to fouling potential,
430 whereas general and rigid biofilm formation potential as determined with microtiter
431 plates and cell surface properties (i.e., hydrophobicity and surface charge) did not
432 reflect the fouling potential. Colony water content is easy to measure and thus could be
433 a useful parameter to identify the potential FCB even for a large number of isolates.
434 Colony morphology has been used to characterize bacterial properties (Yildiz and
435 Visick 2009). For example, bacteria that form wrinkled colonies were capable of
436 producing more polysaccharides and more biofilm (Enos-Berlage and McCarter 2000;
437 Yildiz and Schoolnik 1999). Based on such colony characteristics, mutant screening
438 was also performed to identify biofilm-related genes (Park et al. 2015).

439   In addition, the bacteria that form high water content colonies secrete more
440 soluble hydrophilic organic matters and carbohydrate. These characteristics were
441 consistent with the previous findings in which hydrophilic organic matters and
442 carbohydrate caused severe membrane fouling, respectively (Kimura et al. 2012; Tran et
443 al. 2015). However, the hydrophilic organic matter and carbohydrate content could not
444 explain all the variation of membrane fouling (**Fig. S5J** and **S5K**), which may suggest
445 that specific types and structures, but not amount (concentration), of hydrophilic organic
446 matter are important for membrane fouling. This result is consistent with the previous
447 report showing that specific polysaccharide fractions were responsible for significant
448 membrane fouling of a pilot-scale MBR treating real municipal wastewater (Kimura et
449 al. 2012). Further study is obviously required to investigate the compositions and
450 properties of soluble hydrophilic organic matter excreted by the FCB in details and to
451 relate to the degree of membrane fouling. In addition, thermodynamic analysis could
452 provide plausible explanations for the mechanism of membrane fouling by the soluble
453 hydrophilic organic matter based on the XDLVO theory (Hong et al. 2013).
454 Furthermore, since membrane fouling is developed by multispecies of bacteria, not by a
455 single of bacteria, the symbiotic interactions of bacteria should be investigated for better
456 understanding about fouling mechanisms.

457   It should be noted that since the difference in experimental conditions (e.g., a
458 cross flow velocity, permeate flux, substrates (medium) and so on) between the
459 lab-scale CFMFS used in this study and pilot-scale MBR is large, species identified as
460 FCB might not behave in the same way in the MBR. In particular, the nutrient rich R2A

medium was used to isolate and cultivate the strains and evaluate the membrane fouling in this study, which might lead to diverse isolated strains and membrane fouling behaviors (McAdam et al. 2007; Villain and Marrot 2013; Villain et al. 2014). However, the same synthetic medium was successfully used to investigate the contributions of biofilm formation (Pang et al. 2005), membrane surface colonization (Choi et al. 2006), growth in ML and on membrane (Chao et al. 2011), and specific polysaccharide production (Yoshida et al. 2015) on membrane fouling. Furthermore, since it has been recognized that most of the bacteria present in wastewater could not be cultured and the number of isolates is limited in this study, the isolated strains might not reflect the microbial community of fouled membrane of MBR. However, the objective of this study was to identify FCB and to evaluate the relevance of the cellular properties in their membrane fouling potentials. The common characteristics (i.e. high water, hydrophilic organic matter, and carbohydrate contents in their colonies) of FCB found in this study must be analyzed for FCB that will be isolated from other MBRs to justify as useful universal parameters.

5. Conclusions

In summary, 41 bacterial strains were isolated from a pilot-scale MBR treating real municipal wastewater and tested for membrane fouling potentials. In addition, their cellular properties were characterized to evaluate the relevance of these cellular properties in their membrane fouling potentials. The fouling potential was highly strain dependent, suggesting that bacterial identification at the strain level is

483 essential to identify key fouling-causing bacteria (FCB). The FCB showed some
484 common cellular properties; they formed convex colonies having swollen podgy shape
485 and smooth lustrous surfaces with high water, hydrophilic organic matter, and
486 carbohydrate contents. Colony water content is easy to measure and therefore could be a
487 useful parameter to identify the potential FCB even for a large number of samples.

488

489 **Acknowledgements**

490 The authors would like to thank to Mr. Yusuke Ikawa and Mr. Takatomo Ohta
491 for technical assistance. This work has been supported by Core Research of Evolutional
492 Science and Technology (CREST) project of Japan Sciences and Technology Agency
493 (JST), which was granted to S. Okabe and Grant-in Aid for JSPS Fellows (2600217604)
494 of the Japan Society for the Promotion of Science (JSPS). The authors acknowledge
495 Mitsubishi Rayon Co., Ltd., Toyohashi, Aichi 440-8601, Japan for kindly constructing
496 and providing the CFMFS.

497

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699 **Figure 1.** Phylogenetic relationships and membrane fouling potentials of the strains
700 isolated fouled MF membranes. The fouling potential was determined using bench-scale
701 cross-flow membrane filtration system (CFMFS) that was continuously operated for 24
702 hr. White and gray dots represent trans-membrane pressure at 4 hr (TMP₄) and 24 hr
703 (TMP₂₄), respectively. The membrane filtration experiments were performed in
704 duplicate and the error bars indicate the standard deviations. Isolated strains were
705 identified based on 16S rRNA gene sequencing analysis (phylogenetic tree, left) and
706 rep-PCR fingerprint analysis (dendrogram, right). OTUs were generated using 16S

707 rRNA gene identity cut-offs of 97%. When isolates sheared >97% 16S rRNA gene
708 sequence identity, rep-PCRs were performed to further differentiate the isolated strains.

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711 **Figure 2.** Classification of the isolated strains based on development of membrane
 712 fouling. (A) Relationship between TMP₄ and TMP₂₄ shown in Figure 1. The fouling
 713 patterns could be classified into three groups; **I.** membrane fouling is quickly developed
 714 and reached TMP > 20 kPa within 24 hr (black) (**B**), **II.** membrane fouling is gradually
 715 developed but reached TMP > 20 kPa within 24 hr (light gray) (**D**), and **III.** membrane
 716 fouling is gradually developed and reached TMP < 20 kPa after 24 hr (white) (**C**). The
 717 region of each group was enlarged as **Fig. 2B**, **2D** and **2C**, respectively.

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721 **Figure 3.** Relationships between fouling potentials (TMP₂₄) and the biomass
 722 accumulated on membrane (BC) (A) and the mixed liquor (B) in CFMFS. Biomass
 723 accumulated on membrane surfaces during 24 hr filtration was resuspended in NaCl
 724 solution, and its OD₆₀₀ was measured (OD₆₀₀ of BC). The biomass concentrations in the
 725 mixed liquor were measured after 24 hr filtration (OD₆₀₀ of ML). Black, gray, and white
 726 dots represent the isolated strains in group I, II and III, respectively, as shown in
 727 **Figure 2.**

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731 **Figure 4.** Relationships between fouling potentials (TMP₄) and colony water content
 732 (A) and between colony water content and hydrophilic organic matter content (B),
 733 carbohydrate content (C), and protein content (D) in the colonies of representative
 734 strains, respectively (Table 1). Black, gray, and white dots represent the isolated strains
 735 in group I, II and III, respectively (Figure 2).

736 **Table 1.** Summary of the fouling potentials and cellular characteristics of isolated strains.

Family	^a OTU	^b Strain	^c Rep-PCR	Maximum growth rate (h)	Swimming (mm)	Swarming (mm)	Zeta potential (mV)	Hydrophobicity (-)	Biofilm formation potential (-)	Rigid biofilm formation potential (-)	Median of particle size distribution (µm)	Water content (%)	Hydrophilic secretion in colony (mg/OD0.3)	Carbohydrate in SMP in colony (µg/OD0.3)	Protein in SMP in colony (µg/OD0.3)	Carbohydrate in EPS in colony (µg/OD0.3)	Protein in EPS in colony (µg/OD0.3)	^d Fouling-potential of SMP (m ²)	^d Fouling-potential of EPS (m ²)	Fouling-potential group	^e ML (%)	^f Gel (%)
Enterobacteriaceae	1	S01	+	0.107	80	3	-9.3	33.6	0.49	0.51	1.52	81.9	18.2	15.8	26.7	23.5	32.4	5.E+11	3.E+11	III	0.17	0.15
		S02	+	0.104	70	2	-5.7	46.1	1.26	0.81										III		
		S03	+	0.111	70	2	0.0	5.3	0.33	0.31										III		
		S04	+	0.052	2	1	-12.1	26.1	1.09	0.63	1.17	94.0	15.1	54.3	0.0	59.1	30.3	1.E+12	3.E+11	I		
	2	S05	+	0.084	3.5	8	-11.6	45.2	0.66	0.41	1.08	96.7	68.8	224.7	44.9	11.3	32.4	2.E+13	5.E+11	I		
		S06	+	0.068	3	8	-8.3	65.8	0.53	0.25										I		
		S07	+	0.082	7	2	-14.2	50.2	0.29	0.28	1.04	88.2	5.2	0.0	0.0	13.1	13.7	1.E+12	4.E+11	II		
		S08	+	0.113	5	2	-16.1	69.4	0.31	0.34										II		
		S10	+	0.100	25	3	-11.0	62.5	0.28	0.18	1.13	88.1	9.5	0.0	114.5	15.8	24.1	3.E+11	5.E+11	III		
		S11	+	0.119	55	0.5	-7.3	37.0	0.20	0.22	1.04	81.0	4.2	16.5	41.8	14.0	24.1	9.E+11	3.E+11	III		
Moraxellaceae	3	S09	+	0.088	70	2	-16.3	42.6	0.32	0.29	1.17	83.6	17.8	85.8	23.9	28.4	19.9	1.E+11	3.E+12	III		
	4	S12	+	0.099	4	2	-14.6	30.6	0.26	0.24	0.89	85.1	16.6	29.2	24.5	13.5	36.6	2.E+11	2.E+10	II		
	5	S13	+	0.101	1	2	-8.2	42.0	0.72	0.25	1.05	85.9	12.7	8.6	0.0	7.2	17.8	3.E+11	9.E+11	III	1.61	0.50
Pseudomonadaceae	6	S14	+	0.150	23	1	-13.0	35.0	2.18	0.55	2.01	85.1	17.7	22.5	69.1	55.5	17.8	1.E+11	3.E+11	III	0.08	0.30
	7	S15	+	0.121	55	2	-17.9	9.5	1.45	0.66	1.25	85.6	19.1	52.5	14.3	29.8	42.8	7.E+10	2.E+12	II		
		S16	+	0.091	15	5	-6.1	14.2	0.89	0.22										I		
Comamonadaceae	8	S17	+	0.209	23	40	-6.6	22.2	1.25	0.49	1.02	84.0	4.8	0.0	38.5	64.1	30.3	2.E+11	1.E+12	III	4.69	4.52
		S18	+	0.131	3	2	-5.3	36.7	1.10	0.29	1.14	89.8	23.4	85.8	90.8	106.0	49.0	9.E+10	9.E+11	III		
Xanthomonadaceae	9	S19	+	0.098	1	1	-5.9	25.0	0.63	0.22	8.97	83.3	32.3	0.0	114.0	135.8	74.0	5.E+11	5.E+12	III	2.73	4.21
	10	S20	+	0.129	2	3	-4.4	62.5	0.25	0.28	0.75	83.6	15.9	74.7	78.7	14.4	40.7	1.E+11	3.E+12	II		
		S21	+	0.127	8	2	-4.0	-1.4	0.27	0.20	4.10	82.0	13.0	13.1	1.9	36.1	15.8	2.E+11	4.E+11	III		
	11	S22	-	0.063	35	4	-24.0	42.4	0.93	0.16	1.79	89.8	23.4	58.0	15.5	8.6	15.8	9.E+10	8.E+10	II		
		S23	-	0.087	1	3	-12.8	-4.8	1.32	0.72										I		
Undetermined	12	S24	-	0.091	0	1	-10.6	30.8	1.15	0.30										I		
Phyllobacteriaceae	13	S25	+	0.059	2	1	-5.8	6.4	0.49	0.30	2.12	82.5	51.2	24.4	0.0	22.6	15.8	1.E+12	5.E+11	III	0.00	0.00
		S26	+	0.098	6	1	-10.9	21.6	1.02	0.97	1.89	97.4	109.2	2347.4	55.2	15.8	24.1	6.E+13	3.E+11	I	0.01	0.03
Bacillaceae	14	S27	+	0.106	14	10	-6.0	37.6	0.60	0.21	2.99	85.0	5.2	15.9	40.4	49.6	59.4	4.E+11	6.E+11	III	0.05	0.04
		S28	+	0.081	11	10	-7.4	-33.0	0.25	0.26										III		
		S29	-	0.071	20	5	-11.5	30.7	0.26	0.15	2.11	91.6	70.9	0.0	1.0	5.0	22.0	4.E+11	9.E+12	II		
		S30	-	0.104	40	5	-12.5	1.7	0.23	0.18	1.96	90.3	4.2	0.0	0.0	14.9	19.9	4.E+11	4.E+11	II		
Paenibacillaceae	15	S31	+	0.119	10	10	-17.3	40.9	1.09	0.14	2.45	88.7	21.8	76.9	13.9	123.2	17.8	7.E+10	4.E+12	III	0.00	0.01
		S32	+	0.140	17	5	-18.4	4.0	0.27	0.18	1.30	95.3	43.3	984.8	57.4	39.7	28.2	6.E+12	5.E+11	I		
		S33	+	0.084	12	3	-10.2	35.8	0.30	0.25										I		
Microbacteriaceae	16	S34	+	0.116	1.5	1	-12.2	12.9	0.25	0.23	5.61	95.0	16.1	10.4	0.0	18.5	11.6	2.E+12	4.E+11	I	0.17	0.02
		S35	+	0.132	1	1	-12.6	12.4	0.26	0.21										I		
		S36	+	0.031	1	2	-29.5	-2.3	0.92	0.71	1.21	92.1	23.3	0.0	0.0	19.8	11.6	1.E+12	4.E+11	II		
		S37	+	0.072	1	1	-4.1	24.7	2.20	1.71	1.45	80.1	28.7	2.1	4.3	11.7	22.0	7.E+10	7.E+10	III		
		S38	+	0.122	1.5	2	-10.6	41.5	2.41	2.18	0.63	94.4	71.2	18.8	7.6	27.5	40.7	1.E+12	2.E+12	I		
		S39	+	0.119	3	3	-10.3	22.2	2.46	1.20	1.11	95.2	28.6	58.0	18.1	36.5	15.8	4.E+11	2.E+11	I		
		S40	+	0.124	2	2	-11.0	33.8	3.50	3.29										I		
		S41	+	0.122	3	2	-11.3	40.2	2.46	2.87										I		

738 ^aStrains filled with dark gray boxes: representative strains of each OTU (differentiated with light gray and white colors, respectively),
739 which were discriminated based on 60% DNA similarity of rep-PCR analysis. The colony properties (i.e., water content and
740 hydrophilic organic matter content) were analyzed only for these representative strains.

741 ^bRep-PCR: “+” and “-” indicated whether any amplicon was obtained by rep-PCR.

742 ^cFouling-potential group: classification based on development of membrane fouling as shown in **Fig. 2**.

743 ^dFouling potential of SMP and EPS in colony were measured by dead-end filtration.

744 ^eML (%) and ^fGel (%): the percentage of the 16S rRNA gene sequences affiliating with specific family in total sequence reads of
745 Miseq analysis for the mixed liquor (ML) and gel layer (Gel) samples.

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752   Graphical Abstract

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