Biofilm-Forming Activity of Bacteria Isolated from Toilet Bowl Biofilms and the Bactericidal Activity of Disinfectants against The Isolates

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To evaluate the sanitary conditions of toilets, the bacterial counts of the toilet bowl biofilms in 5 Kansai area and 11 Kansai and Kanto area homes in Japan were measured in winter and summer seasons, respectively. Isolates (128 strains) were identified by analyzing 16S rRNA sequences. The number of colonies and bacterial species from biofilms sampled in winter tended to be higher and lower, respectively, than those in summer. Moreover, the composition of bacterial communities in summer and winter samples differed considerably. In summer samples, biofilms in Kansai and Kanto areas were dominated by Blastomonas sp. and Mycobacterium sp., respectively. Methylobacterium sp. was detected in all toilet bowl biofilms except for one sample. Methylobacterium sp. constituted the major presence in biofilms along with Brevundimonas sp., Sphingomonas sp., and/or Pseudomonas sp. The composition ratio of the sum of their genera was 88.0 from 42.9% of the total bacterial flora. The biofilm formation abilities of 128 isolates were investigated, and results suggested that Methylobacterium sp. and Sphingomonas sp. were involved in biofilm formation in toilet bowls. The biofilm formation of a mixed bacteria system that included bacteria with the highest biofilm-forming ability in a winter sample was greater than mixture without such bacteria. This result suggests that isolates possessing a high biofilm-forming activity are involved in the biofilm formation in the actual toilet bowl. A bactericidal test against 25 strains indicated that the bactericidal activities of didecyldimethylammonium chloride (DDAC) tended to be higher than those of polyhexamethylene biguanide (PHMB) and N-benzyl-N,N-dimethyldodecylammonium chloride (ADBAC). In particular, DDAC showed high bactericidal activity against approximately 90% of tested strains under the 5 h treatment.

Key words: Biofilm / Toilet bowl / Bactericidal Activity / Biofilm-forming bacteria.

INTRODUCTION

The concept of comfort has spread along with improved living standards and there is an increasing desire to live comfortably and cleanly in a living space. In connection with higher standards of cleanliness, the problem of biofilms occurring in the bathroom, toilet, and kitchen has come to attract more notice. A biofilm is an aggregate of microorganisms such as bacteria and fungi that are attached to the surface of a solid or liquid (Hall-Stoodley et al., 2004; Watnick and Kolter, 2000). Generally, the developmental process of biofilm formation can be characterized by three steps: (i) adherence of planktonic cells to the surface, (ii) biofilm maturation, and (iii) complete or partial disintegration of the biofilm. Biofilms may cause serious problems not only in a living environment but also in industry (Hall-Stoodley et al., 2004; Simoes et al., 2010) due to sliming, clogging, corrosion, and odors in the drain outlets or pipes of a plant, bathroom, toilet, or kitchen (Furuhata et al., 2010; Hallam et al., 2001). Furthermore, the increased
resistance of biofilm-associated organisms to antimicrobial agents causes infections in patients (Bridier et al., 2011; Stewart et al., 2001).

The toilet environment can be a reservoir for large numbers of microorganisms, and changes in colony counts and biofilm formation in toilet bowls have been investigated (Egert et al., 2010; Ojima et al., 2002; Pitts et al., 1998, 2001; Scott et al., 1982). Bacteria derived from the toilet bowl were detected in an air sample taken after flushing and several pathogenic bacteria have been known to survive on the surfaces of the toilet bowl for extended periods of time (Barker and Bloomfield, 2000; Barker and Jones, 2005). Moreover, because the number of bacteria and viruses in the toilet bowl cannot be removed completely even after repeated flushing or with the use of cleaners, there is a possibility that individuals may become infected with pathogenic bacteria and viruses from the toilet bowl (Barker and Bloomfield, 2000; Barker and Jones, 2005; Gerba et al., 1975). Therefore, in order to prevent the accumulation of harmful bacteria in the toilet bowl, the isolation and characterization of bacteria from toilet bowl biofilms, followed by the establishment of an inhibition method of biofilm formation, are very important from a hygienic viewpoint. However, relatively little is known about microbial diversity in the toilet bowl environment, and there has been no report on the relevance of this diversity and biofilm formation. The present study isolated bacteria from biofilms on toilet bowls and investigated their biofilm-forming abilities. Furthermore, the bactericidal activities of some kinds of disinfectants against the strains that showed a high ability to form biofilms were evaluated.

**MATERIALS AND METHODS**

**Isolation of bacteria from toilet bowl biofilms**

After washing the inside surface of the toilet bowl, biofilm samples were collected for two weeks in winter and one week in summer. Biofilms on the inside of the toilet bowl above the waterline were scraped off with a sterile cotton fabric (Japan Textile Evaluation Technology Council, Tokyo, Japan) of 10 by 10 cm and then placed into 20 ml of LP diluent (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan). The biofilm was removed from the cotton fabric by agitation, and the suspension was transferred to a 50 ml Falcon tube (Becton Dickinson, Lincoln Park, NJ, USA). After mixing with a vortex, the suspension was diluted appropriately with phosphate-buffered saline (PBS; Wako Pure Chemical, Osaka, Japan). A total of 0.1 ml of the diluted solution was spread on an R2A agar (Nihon Pharmaceutical, Co.) plate and cultivated at 25 °C for 1 week. Isolated colonies were stored in 15% glycerol solution at -80 °C.

**16S rRNA gene sequence analysis**

For the classification of isolated bacterial strains, 16S rDNA sequences were determined. A single colony of an isolated strain was picked out and suspended in 20 µl of distilled water. Cell suspensions were heated at 98 °C for 10 min and immediately cooled on ice. Solutions were used directly as a PCR template without isolation of chromosomal DNA. A partial region of 16S rDNA was amplified with the PCR method using the following: 15 µl of a reaction solution containing 1 µl of the cell suspension after heat treatment, 50 pmol of primer set, 0,2 mMs of dNTPs, the buffer desirable for DNA polymerase, and 1 U of Taq polymerase (Takara, Osaka, Japan). The primer set consisted of 5’-GAGTTTTGATCCTGGTCAAG-3’ (9F) and 5’-CCGTCAATTCCTTTGAGTTT-3’ (927R) (Lane, 1991). The reaction conditions were as follows: one cycle of 98 °C for 1 min, 30 cycles of 96 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and one cycle of 72 °C for 1 min. Amplified fragments were checked on agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Purified fragments were sequenced with the primer set consisting of 9F primer and 5’-GTATTACCGCGCTGCTGG-3’ (518R) (Lane, 1991), a BigDye Terminator v3.1 cycle sequencing kit, and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Corp. CA). The nucleotide sequences of both strands were determined. DNA sequence data were analyzed with Aporon software (Techno Suruga Laboratory Co., Shizuoka, Japan) and the GenBank/EMBL/DDJB databases.

**Biofilm formation**

Biofilm formation activity was tested as previously described (Furuhata et al., 2008) with some modifications. Tested strains were inoculated into 200 µl of R2A medium in a 96-well flat-bottom microplate (AGC Techno Glass Co., Chiba, Japan). Biofilm formation in a mixed bacteria system that corresponded to the ratio of bacteria isolated from the toilet bowl was carried out as follows. One platinum-loop amount of each tested strain was transferred from R2A agar to 5 ml of R2A medium. One ml of the bacteria mixture of the tested strain at the ratio found in the isolation studies was prepared after shaking, and 5 µl of the mixed suspension was inoculated into 200 µl of R2A medium in a 96-well flat-bottom microplate. R2A medium only was used as the control sample. After cultivation at 25 °C for 48 h or 7 d, the pellicles and medium were removed from the 96-well flat-bottom microplate, and their turbidities were determined (OD595). Surfaces of the biofilms were rinsed twice with distilled water, dried, and stained with 250 µl of 0.1% crystal violet (CV) solution for 15 min. The CV solution was removed, and the microplate was
washed twice with 250 µl of distilled water. The CV attached to the biofilm was dissolved in 200 µl of 33% acetic acid and the obtained extraction was quantified by measurement of the absorbance at 595 nm after shaking for 20 s. Biofilm-forming activities were calculated as OD<sub>595</sub> - OD<sub>control</sub> (OD<sub>c</sub>).

**Bactericidal test**

Bactericidal activity was determined using a modified version of the preservative-effectiveness test listed in the *Japanese Pharmacopoeia Sixteenth Edition* (2011). Tested strains were pre-incubated on R2A agar at 25 °C for 5-7 d. Cells were suspended in a test tube containing 5.0 ml of R2A medium to give a cell concentration of approximately 3 × 10<sup>8</sup> CFU/ml. Each one-fourth disinfectant solution was prepared using didecylidimethylammonium chloride (DDAC), polyhexamethylene biguanide (PHMB) or N-benzyl-N,N-dimethyldecylammonium chloride (AD Bac). These one-fourth solutions were further diluted to 1/1000 with distilled water, and 98.6 ml of tap water was added to 1.4 ml of the diluent. Finally, the disinfectant solutions were adjusted to 14 ppm. Then, 9.9 ml of disinfectant solutions were added into newly readied test tubes. A control sample was treated with sterilized tap water instead of disinfectant solutions using the same procedure. A bacterial suspension (0.1 ml) was inoculated into each disinfectant solution, and was incubated at 25°C. After incubation for 1 and 5 h, viable bacterial counts were determined by serial dilution and colony counts on R2A agar. Bactericidal activity values were calculated based on the following equation.

Bactericidal activity value = log (viable bacterial count of control) – log (viable bacterial counts of each disinfectant solution)

**RESULTS AND DISCUSSION**

**Isolation of bacteria from toilet bowl biofilms**

There have been four reports analyzing the microbial communities of toilet bowls: the analysis of liquid samples from 251 homes in the United Kingdom by the API 20 system (Scott et al., 1982); the analysis of toilet bowl biofilms from 3 residences in America by the API biochemical test kit (Pitts et al., 1998); the analysis of toilet bowl rim samples from 86 households in Japan by the stamp agar method (Ojima et al., 2002); and the analysis of toilet bowl rim biofilms from 6 households in Germany by non-cultivated 16S rRNA gene-based terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting (Egert et al., 2010). For the first time in this study all cultivated isolates from toilet bowl biofilms were identified by 16S RNA gene-based DNA sequencing analysis, and their percentage of occurrence was then determined. The toilet bowls of 5 homes in winter and 11 homes in summer in Japan were examined. Biofilms sampled in the Kansai area in winter, in summer, and in the Kanto area in summer were named WA-WE (5 samples), SA-SF (6 samples), and SP-ST (5 samples), respectively.

The number of constituent bacteria and relative frequency of bacteria determined by morphological characteristics are shown in TABLE 1. A total of 128 bacterial strains isolated from toilet bowl biofilms were classified with 16S rDNA sequence analysis. Each partial 16S rDNA region in chromosomal DNA was amplified with the PCR method as described in MATERIALS AND METHODS. Using the 16S rDNA sequences analyzed and GenBank/EMBL/DDBJ databases, the names of strains closely related to the isolated strains are listed in TABLE 1.

Although the samples WA-WE and SA-SE were derived from the same home, the number of colonies and bacterial species of biofilms sampled in winter tended to be higher and lower, respectively, than those in summer. Moreover, the composition of bacterial communities differed considerably between summer and winter. In summer samples, most biofilms in Kansai and Kanto areas were dominated by *Blastomonas* sp. and *Mycobacterium* sp., respectively. Interestingly, similarities were observed between the same areas and between the same seasons (TABLE 1), although the family structure and the age and design of the toilet bowl were different (data not shown). *Methylobacterium* sp., present in water environments such as tap water or drinking water, are pink-pigmented bacteria that exhibit resistance to chlorine (Furuhata et al., 2011; Hiraishi et al., 1995). All pink-pigmented bacteria isolated in this study were identified as *Methylobacterium* sp. and these bacteria were detected in all toilet bowl biofilms except the ST sample. In winter samples, *Methylobacterium* sp. constituted the major presence in the biofilms along with *Brevundimonas* sp., *Sphingomonas* sp., and/or *Pseudomonas* sp., and the composition ratio of the sum of their genera was 88.0 from 42.9% of total bacterial flora.

**Biofilm-forming abilities of isolates**

The biofilm formation abilities of 128 isolates were investigated in order to reveal the bacterial strains associated with biofilm formation in toilet bowls (FIG. 1). Biofilm-forming strains were classified under one of four categories: OD<sub>595</sub> ≤ OD<sub>c</sub>; non-adherent, OD<sub>c</sub> < OD<sub>595</sub> ≤ 2 × OD<sub>c</sub>; weakly adherent, 2 × OD<sub>c</sub> < OD<sub>595</sub> ≤ 4 × OD<sub>c</sub>; moderately adherent, and 4 × OD<sub>c</sub> < OD<sub>595</sub>; strongly adherent, as has been previously described (Stepanovic et al., 2000). The numbers of non-adherent, weakly adherent, moderately adherent,
TABLE 1. Bacterial species isolated from toilet bowl biofilms and their percentage frequency occurrence.

| NO. | WA sample (%) | NO. | WB sample (%) | NO. | WC sample (%) | NO. | WD sample (%) |
|-----|---------------|-----|---------------|-----|---------------|-----|---------------|
| 1   | 1.2 x 10^3 CFU/ml | 2   | 1.6 x 10^3 CFU/ml | 3   | 4.3 x 10^3 CFU/ml | 4   | 1.5 x 10^3 CFU/ml |
| 1   | Microbacterium sp. | 2   | Lysobacter sp. | 3   | Enterococcus sp. | 4   | M. neoaurum |
| 2   | Methylobacterium sp. | 3   | M. aquaticum | 4   | Mycobacterium sp. | 5   | Bacillus subtilis |
| 3   | Mycobacterium sp. | 4   | Pseudomonas sp. | 5   | Sphingomonas sp. | 6   | Mycobacterium sp. |
| 4   | Acidovorax sp. | 5   | Total | 6   | Total | 7   | Total |
| 5   | Pseudomonas sp. | 6   | Total | 7   | Total | 8   | Total |

| NO. | WE sample (%) | NO. | SA sample (%) | NO. | SB sample (%) | NO. | SC sample (%) |
|-----|---------------|-----|---------------|-----|---------------|-----|---------------|
| 1   | 1.3 x 10^3 CFU/ml | 2   | 6.1 x 10^3 CFU/ml | 3   | 4.3 x 10^3 CFU/ml | 4   | 2.3 x 10^3 CFU/ml |
| 1   | Methylobacterium sp. | 2   | Lysobacter sp. | 3   | M. aquaticum | 4   | M. neoaurum |
| 2   | Pseudomonas sp. | 3   | M. aquaticum | 4   | Mycobacterium sp. | 5   | Bacillus subtilis |
| 3   | Microbacterium sp. | 4   | Pseudomonas sp. | 5   | Sphingomonas sp. | 6   | Mycobacterium sp. |
| 4   | Acidovorax sp. | 5   | Total | 6   | Total | 7   | Total |
| 5   | Pseudomonas sp. | 6   | Total | 7   | Total | 8   | Total |

| NO. | SD sample (%) | NO. | SE sample (%) | NO. | SF sample (%) | NO. | SP sample (%) |
|-----|---------------|-----|---------------|-----|---------------|-----|---------------|
| 1   | 3.3 x 10^3 CFU/ml | 2   | 4.1 x 10^3 CFU/ml | 3   | 5.8 x 10^3 CFU/ml | 4   | 2.5 x 10^3 CFU/ml |
| 1   | Blastomonas sp. | 2   | Blastomonas sp. | 3   | M. aquaticum | 4   | M. neoaurum |
| 2   | Sphingomonas sp. | 3   | M. aquaticum | 4   | Mycobacterium sp. | 5   | Bacillus subtilis |
| 3   | Methylobacterium sp. | 4   | Total | 5   | Total | 6   | Total |
| 4   | Microbacterium sp. | 5   | Total | 6   | Total | 7   | Total |

| NO. | SQ sample (%) | NO. | SR sample (%) | NO. | SS sample (%) | NO. | ST sample (%) |
|-----|---------------|-----|---------------|-----|---------------|-----|---------------|
| 1   | 4.4 x 10^3 CFU/ml | 2   | 5.5 x 10^3 CFU/ml | 3   | 2.5 x 10^3 CFU/ml | 4   | 7.8 x 10^3 CFU/ml |
| 1   | Mycobacterium sp. | 2   | M. radiotolerans | 3   | M. aquaticum | 4   | M. neoaurum |
| 2   | Leucobacter sp. | 3   | M. radiotolerans | 4   | Mycobacterium sp. | 5   | Mycobacterium sp. |
| 3   | Microbacterium sp. | 4   | Sphingomonas sp. | 5   | M. aquaticum | 6   | M. neoaurum |
| 4   | Mycobacterium sp. | 5   | Total | 6   | Total | 7   | Total |
| 5   | Blastomonas sp. | 6   | Total | 7   | Total | 8   | Total |

* : Values indicate percentage frequency occurrence of isolates.
** : Viable numbers

Samples WA-We, SA-SF, and SP-ST were isolated at Kansai area in winter, at Kansai area in summer, and at Kanto area in summer, respectively.
and strongly adherent isolated strains were 3, 43, 23 and 59, respectively. The sums of moderately adherent and strongly adherent isolates in winter and summer samples were 14 and 45 strains, respectively. 5 Methylobacterium species, 2 Pseudomonas species and 3 Sphingomonas species were in the 14 strains, and 6 Methylobacterium species, 8 Sphingomonas species, 3 Sphingobium species, 6 Blastmonas species and Sphingopyxis sp. were in the 45 strains. These results suggest that Methylobacterium sp., Pseudomonas sp. and Sphingomonas sp. are involved in biofilm formation in winter, and Methylobacterium sp., Sphingomonas sp. and their closely related strains are involved in biofilm formation in summer.

To confirm whether bacteria with the highest biofilm-forming activity are involved in biofilm formation in the actual toilet bowl, biofilm formation was examined with and without the addition of bacteria that showed the

![FIG. 1](image1.png)

**FIG. 1.** Biofilm-forming abilities of isolates from toilet bowl biofilms. Samples WA-WE, SA-SF, and SP-ST were isolated from Kansai area in winter, from Kansai area in summer, and from Kanto area in summer, respectively. Average values and standard deviations of three independent determinations are shown.

and strongly adherent isolated strains were 3, 43, 23 and 59, respectively. The sums of moderately adherent and strongly adherent isolates in winter and summer samples were 14 and 45 strains, respectively. 5 Methylobacterium species, 2 Pseudomonas species and 3 Sphingomonas species were in the 14 strains, and 6 Methylobacterium species, 8 Sphingomonas species, 3 Sphingobium species, 6 Blastmonas species and Sphingopyxis sp. were in the 45 strains. These results suggest that Methylobacterium sp., Pseudomonas sp. and Sphingomonas sp. are involved in biofilm formation in winter, and Methylobacterium sp., Sphingomonas sp. and their closely related strains are involved in biofilm formation in summer.

To confirm whether bacteria with the highest biofilm-forming activity are involved in biofilm formation in the actual toilet bowl, biofilm formation was examined with and without the addition of bacteria that showed the

![FIG. 2](image2.png)

**FIG. 2.** Biofilm formation in a mixed bacteria system that corresponded to the ratio of bacteria isolated from toilet bowl. The strains isolated from biofilms (samples WA-WE in Table 1) in the Kansai area in winter were used. a, the bacterial mixture corresponded to the ratio found in isolation studies; b, the bacterial mixture was determined by subtracting the incubation medium numbers of c from a; c, only bacteria (strains WA2, WB1, WC2, WD2, or WE2 in Figure 1) with a highest biofilm-forming activity.
were also tested under the same conditions. The bactericidal activities of disinfectants (DDAC, PHMB, or ADBAC) against these strains were tested under 1 and 5 h treatments. Bactericidal activity results are shown in TABLE 2. The bactericidal activities of DDAC tended to be higher than those of PHMB and ADBAC. In particular, DDAC showed high bactericidal activity against approximately 90% of tested strains under the 5 h treatment, suggesting that DDAC is the most powerful disinfectant among the three tested. Only DDAC was effective against *Methylobacterium* sp., which showed a high biofilm-forming ability.

Moreover, because DDAC is able to show bactericidal activities even in tap water (the highest biofilm-forming activity. In all tested samples, the values of systems that included the highest biofilm-forming bacteria were higher than those without such bacteria (FIG.2). These results suggest that isolates having a high biofilm-forming activity are involved in biofilm formation in the actual toilet bowl.

**Bactericidal test against strains that showed a high biofilm-forming ability**

Strains (21 strains) that had a high ability to form biofilms in each biofilm sample were selected for this part of the study. Four strains that were found at a high rate despite having a low ability to form biofilms were also tested under the same conditions. The bactericidal activities of disinfectants (DDAC, PHMB, or ADBAC) against these strains were tested under 1 and 5 h treatments. Bactericidal activity results are shown in TABLE 2. The bactericidal activities of DDAC tended to be higher than those of PHMB and ADBAC. In particular, DDAC showed high bactericidal activity against approximately 90% of tested strains under the 5 h treatment, suggesting that DDAC is the most powerful disinfectant among the three tested. Only DDAC was effective against *Methylobacterium* sp., which showed a high biofilm-forming ability. Moreover, because DDAC is able to show bactericidal activities even in tap water (the highest biofilm-forming activity).

**TABLE 2. Bactericidal activity of disinfectants against the strains which showed a high ability to form biofilm.**

(a)

|        | WA2  | WA5  | WC1  | WC3  | WE2  | SA1  | SA2  | SA4  | SA7  | SA10 | SA11 |
|--------|------|------|------|------|------|------|------|------|------|------|------|
| DDAC   | 0.91 | 0.20 | 2.97 | 6.08 | 6.96 | 6.91 | 4.89 | 5.71 | 0.42 | 5.37 | 5.06 |
| PHMB   | 0.00 | 1.04 | 1.43 | 0.14 | 1.70 | 1.21 | 0.16 | 0.11 | -0.14| 4.10 | 0.98 |
| ADBAC  | 0.30 | -0.07| 1.57 | 1.70 | 1.52 | 1.65 | 1.52 | 0.88 | 0.21 | 0.18 | 1.82 |

|        | SB1  | SB2  | SB6  | SB7  | SC2  | SC3  | SC4  | SC11 | SD2  | SF3  | SF7  |
|--------|------|------|------|------|------|------|------|------|------|------|------|
| DDAC   | -0.16| 6.61 | 4.83 | 6.48 | 1.88 | 4.81 | 0.03 | 6.44 | 3.85 | 6.72 | 3.24 |
| PHMB   | -0.12| 3.11 | 2.45 | 0.21 | 0.01 | 3.21 | -0.01| 1.33 | 0.71 | 2.88 | 0.55 |
| ADBAC  | -0.07| 3.22 | 2.78 | 1.23 | 0.14 | 0.25 | 0.05 | 1.01 | 0.74 | 2.19 | 1.50 |

|        | SP7  | SQ1  | SQ4  | SQ9  | SS10 | SS12 | ST6  | ST11 |
|--------|------|------|------|------|------|------|------|------|
| DDAC   | 4.11 | 0.25 | 0.77 | 6.91 | 2.48 | 6.00 | 3.97 | 3.63 |
| PHMB   | 2.48 | 2.77 | 0.02 | 2.94 | 0.02 | 0.53 | 2.46 | 1.68 |
| ADBAC  | -0.11| -0.03| 0.01 | 2.08 | 0.01 | 2.49 | -0.10| 0.25 |

Incubation at 25°C for (a) 1 and (b) 5 h. Data present average of two independent experiments. Shadow box presents the bactericidal effect values above 2.0.

*: Percentage of the bactericidal effect in tested strains.

(b)

|        | WA2  | WA5  | WC1  | WC3  | WE2  | SA1  | SA2  | SA4  | SA7  | SA10 | SA11 |
|--------|------|------|------|------|------|------|------|------|------|------|------|
| DDAC   | 4.86 | 4.81 | 3.33 | 6.81 | 6.89 | 6.75 | 6.26 | 6.74 | 3.00 | 6.71 | 5.18 |
| PHMB   | 0.21 | 1.95 | 3.33 | 2.71 | 4.13 | 1.91 | 0.61 | 0.87 | -0.84| 5.23 | 1.81 |
| ADBAC  | 1.77 | -0.10| 2.03 | 5.11 | 4.62 | 6.75 | 6.26 | 5.20 | 0.87 | 1.16 | 5.18 |

|        | SB1  | SB2  | SB6  | SB7  | SC2  | SC3  | SC4  | SC11 | SD2  | SF3  | SF7  |
|--------|------|------|------|------|------|------|------|------|------|------|------|
| DDAC   | 0.85 | 6.61 | 5.33 | 6.82 | 6.10 | 6.27 | 0.77 | 6.56 | 5.25 | 6.60 | 6.35 |
| PHMB   | 0.04 | 4.68 | 1.33 | 1.15 | 1.13 | 6.27 | 0.07 | 2.09 | 2.56 | 5.30 | 1.89 |
| ADBAC  | 0.17 | 6.61 | 5.33 | 4.70 | 1.36 | 4.97 | 0.29 | 2.37 | 2.58 | 5.60 | 4.57 |

|        | SP7  | SQ1  | SQ4  | SQ9  | SS10 | SS12 | ST6  | ST11 |
|--------|------|------|------|------|------|------|------|------|
| DDAC   | 6.84 | 3.98 | 3.48 | 6.87 | 4.16 | 6.59 | 6.30 | 5.32 |
| PHMB   | 5.23 | 5.28 | 1.82 | 5.69 | 0.78 | 3.85 | 2.98 | 1.23 |
| ADBAC  | 0.23 | 1.86 | 1.32 | 5.17 | 2.61 | 4.39 | 0.08 | 1.33 |

Incubation at 25°C for (a) 1 and (b) 5 h. Data present average of two independent experiments. Shadow box presents the bactericidal effect values above 2.0.

*: Percentage of the bactericidal effect in tested strains.
presence of chlorine: 0.3 ppm), it may be effective in the actual toilet environment. There is a need for further studies, for example, on the development of cleaning agents containing effective disinfectants from this study to inhibit the formation of toilet bowl biofilms.

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