Characterization of the Genus *Methylobacterium* in Bathrooms and the Regulation

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Takehisa YANO

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Takehisa YANO

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Chapter 1

GENERALINTRODUCTION

1-1. Pink biofilms

  Dwelling environments such as bathrooms and kitchens give microorganisms various stresses including rapid flowing water, drying, low nutrients, and occasional exposure to cleaning agents. Still microorganisms thrive (Finch*et al*. 1978, Ojima*et al*. 2002, Williams *et al*. 2009), often by forming biofilms (Costerton*et al*. 1999, McBain*et al*. 2003); microbial communities that attach to biotic or abiotic surfaces (Costerton*et al*. 1999, Stewart *et al*. 2001, Watnick*et al*. 2000). The biofilms in moist places often exhibit pink (Feazel*et al*. 2009, Furuhata*et al*. 1992, Kelley*et al*. 2004) or black (Hamada *et al*. 2009, Hunter *et al*. 1988, Koch *et al*. 2000) pigmentation.

  Notably, pink biofilms in bathrooms (Fig. 1) recur more rapidly than other forms of staining, and are difficult to remove under dry conditions. These characteristics have led pink biofilms to be the second most common microbial stain in bathrooms of Japan following fungal black biofilms according to our survey from 2006 to 2007.

1-2. Microbial composition of pink biofilms

  Several studies have focused on the microbial composition of pink biofilms in bathrooms such as on shower curtains (Kelley*et al*. 2004) or shower heads (Feazel*et al*. 2009). Some microorganisms with pink-red pigmented colonies have been isolated, including pink-pigmented yeasts, the genus *Rhodotorula*, and bacteria, the genera *Methylobacterium*, *Brevundimonas*, and *Rhodobacter*, suggesting that these species affect the colors of the biofilms (Furuhata*et al*. 1992). Notably, the genus *Methylobacterium*, a pink pigmented facultative methylotroph (Hanson *et al*. 1996), has been also isolated from neighboring environments like tap water (Furuhata*et al*. 1992) and human feet (Anesti*et al*. 2004) and mouths (Anesti*et al*. 2005). Still, the predominant species and the reason why biofilms in bathrooms are pink remain unclear. Studying them would help to clarify the mechanisms by which the microorganisms adapt to these severe conditions.

1-3. Genus *Methylobacterium*

  The genus *Methylobacterium* infects immunocompromised patients, causing severe diseases (Lai *et al*. 2011, Lee *et al*. 2004, Sanders*et al*. 2000). Because species of this genus are isolated from various environments including water, soil, plants (Hanson *et al*.

1996), humans (Anesti*et al*. 2004), and households, such as from the water supply (Hiraishi*et al*. 1995) and bathrooms (Feazel*et al*. 2009, Yano *et al*. 2013), their effective regulation is crucial from a public health perspective.In clinical health issues like treatments of infections, aminoglycosides and quinolones were reported to be effective (Brown *et al*. 1992, Sanders *et al*. 2000), while public health issues have been countered with surfactants and sanitizers like quaternary ammonium compounds (Gerba*et al*. 2015).

On the other hand, as described above, the genus *Methylobacterium* is famous as a pink-pigmented facultative methylotroph, which is capable of growth on single-carbon (C1) compounds such as methanol and formate, and multicarbon compounds such as glucose, pyruvate, and succinate. The unique metabolisms have been widely investigated (Hanson *et al*. 1996). Especially, *Methylobacterium*extorquens AM1 has been studied for decades (Chistoserdova*et al*. 2011), and is a model organism for understanding the metabolic components required for methylotrophic growth. Global approaches to study multiple layers in the metabolic hierarchy including transcriptomic, metabolomics (Peyraud*et al*. 2011), and proteomic (Gourion*et al*. 2006)) approaches have showed that the majority of genes responsible for C1 and multicarbon metabolisms are differentially expressed and produced according to their function in these two different modes of growth. The unveiled networks were systematically studied and arranged to use the bacteria for biotechnological production of valued-added chemicals from methanol. The results above would help understand characteristics of the microorganism and the regulations.

1-4. Quaternary ammonium chloride and the antibacterial mechanisms

In clinical health issues like treatments of infections, aminoglycosides and quinolones were reported to be effective against genus *Methylobacterium*(Brown *et al*. 1992, Sanders *et al*. 2000), while public health issues have been countered with surfactants and sanitizers like quaternary ammonium compounds(QACs) (Gerba*et al*. 2015). However, we found that *Methylobacterium* strains isolated from bathrooms were tolerant to various stresses including surfactants like benzalkonium chloride (BAC) (Kovaleva*et al*. 2014, Yano *et al*. 2013). Some isolates even survived in 5.0% BAC after 5.0 min of exposure, indicating that cleaners require more immediacy and versatility.

QACs generally contain one quaternary nitrogen associated with at least one major hydrophobic substituent. Benzalkonium chloride (BAC), a widely used QAC, is mixtures of *n*-alkyldimethylbenzyl ammonium chlorides where

the *n*-alkyl groups can be of variable length within a specified range. The mode of action of BAC involve a disruption of lipid bilayer structure of the bacterial cytoplasmic membrane and the outer-membrane of Gram-negative bacteria, which perturb respiration, solutes transport, and cell wall biosynthesis (Gilbert *et al.* 2005). So it can be easily imagined that BAC exhibits antimicrobial activities with Gram-negative and Gram-positive bacteria, as well as yeasts and fungi.

On the other hands, there are numerous reports of resistance toward BAC. Some reports showed that efflux pumps can actively remove BAC from membrane cores (Hier*et al.* 1999). These reports imply that using BAC in dwelling environments could affect their microbiota.
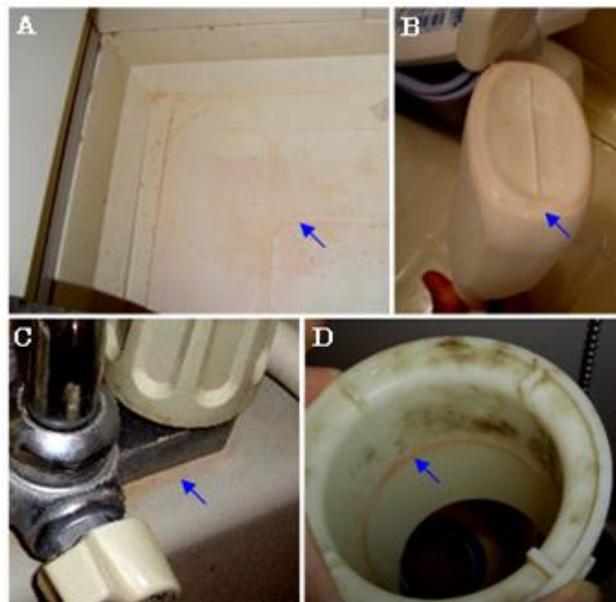
Fig. 1.Pink biofilms in bathrooms. The arrows indicate the biofilms on the floor (A), on the bottom of a bottle (B), around the connection between the faucet feed line and its housing (C), and inside the drain (D).

CHAPTER 2

STRESSTORELANCESOF*METHYLOBACTERIUM*

2-1. Introduction

The predominant species and the reason why biofilms in bathrooms are pink remain unclear. Studying them would help to clarify the mechanisms by which the microorganisms adapt to these severe conditions. Therefore, we focused on pink biofilms in bathrooms and investigated the microbial flora. Using an electron microscope and fluorescence *in situ* hybridization (FISH), we studied whether one or multiple genera dominate the biofilms. Then we searched for the factors responsible for their predominance by examining tolerances to cleaning agents and desiccation stress.

2-2. Materials and methods

Strains and culture conditions

The strains used were bacteria isolated from pink biofilms in bathrooms; *Methylobacteriummesophilicum* KMC10, *Methylobacteriumradiotolerans* KMC5 *Methylobacteriumfujisawaense* KMC4, *Brevundimonasvesicularis* KMC13,*CandidatusChryseobacteriummassiliae*KMC14,*Chryseobacterium*sp. B4P3221,*Flavobacterium*sp. KMC16,*Rhodococcus*sp. KMC17,*Rhodococcus*sp. KMC18,*Roseomonas mucosa* KMC19, *Burkholderiacepacia*KMC20, *Deinococcusgrandis*KMC21,*Microbacteriumarborescens*KMC22. *Roseomonas mucosa* KMC19 and *Microbacteriumarborescens* KMC22 were cultivated with R2A broth (Becton Dickinson, Sparks, MD), *Burkholderiacepacia* KMC20 was cultivated with Soybean Casein Digest broth (Becton Dickinson, Sparks, MD), and the other bacteria were cultivated with Potato Dextrose broth (Becton Dickinson, Sparks, MD) for 3 d at 30°C.

Scanning Electron Microscopy (SEM) observation

The biofilms were sampled from 42 points in the bathrooms of 14 houses in Japan with toothpicks, and fixed for 3 d at room temperature by adding 5 ml of glutaraldehyde, followed by dehydration by successive 50, 70, 90, 99, and 100% (v/v) ethanol washes (3 min. each), dried, sputtered with platinum/ palladium (Pt-Pd) with sputter coater (E-1030 ion spatter; Hitachi, Tokyo, Japan), and stored at room temperature. The specimens were then examined with a scanning electron microscope (S4300SE/N,

Hitachi, Tokyo, Japan) operated at 7 to 15 kV.

Fluorescence in situ Hybridization (FISH) assay

TheFISH assay was performed as described elsewhere (Amann*et al*. 1990, Christensen *et al*. 1999, Moter*et al*. 2000) with some modifications. The biofilms on the surfaces of shampoo bottles and shower baskets for storing soap and sponges were removed from 14 biofilms in Japan by slicing with a box cutter, and fixed for 2 h at 4°C by adding 5 ml of 3% (v/v) paraformaldehyde. Subsequently, the fixatives on the sliced abiotic surfaces were picked up, and washed gently (so as not to disturb the biofilm structures) with phosphate-buffered saline (PBS). Then the biofilms were put into plastic cases, and embedded by gently introducing 20% (w/v) acrylamide. The acrylamide was allowed to polymerize at 30°C for 1 h. The embedded biofilms were carefully lifted from the cases, and cut into 1-in. sections.

 For hybridization, the probe EUB338, specific for the domain bacteria (Amann*et al*. 1990), and probe MB, specific for the genus *Methylobacterium* (Pirttilä*et al*. 2000), were used. The probes are listed in Table 1. Oligonucleotide probes labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) were purchased from Hokkaido System Science (Hokkaido, Japan). Competitor probes for ensuring specificity (Manz*et al*. 1992), and helper probes for enhancing signal intensity (Fuchs*et al*. 2000) were generally used together with the fluorescent probe in cases of low specificity. Because MB alone gave low fluorescence, a competitor probe and helper probe were constructed (Table 1) and used to obtain high fluorescence. Simultaneous hybridization with probes that required different stringency conditions was performed; hybridization with the probe requiring higher stringency was performed first, followed by that with the probe requiring lower stringency. All samples were simultaneously stained with calcofluor white (McLennan *et al*. 2008) for 10 min. in the dark to determine β[1-3] and β[1-4]-linked glucosyl polymers-containing exopolysaccharides and fungal distribution. For microscopy and image analyses, a model LSM510 META confocal laser scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with a diode laser (405 nm), Ar ion laser (458 and 488 nm) and HeNe ion laser (543 nm) was used. All images were combined and processed with Imaris 5 software. The biomasses of three representative biofilms were quantified using Comstat2 (Heydorn *et al*. 2000, Singhal*et al*. 2012) under the Image J shell, and the proportion of MB was calculated as follows; (MB biomass) / (MB biomass + EUB338 biomass + calcofluor white biomass) x 100. The average proportion was determined by using 10 representative microscopic images of each sample.

Isolation of pink microorganisms

The samples were collected from 42 pink biofilms in Tokyo, Wakayama, and Tochigi, Japan. To isolate microorganisms, the pink biofilms in bathrooms were sampled with toothpicks, streaked onto Potato Dextrose Agar (PDA, Becton Dickinson, Sparks, MD), Nutrient Agar (NA, Becton Dickinson, Sparks, MD), and R2A Agar (R2AA, Wako Pure Chemical Ltd, Osaka, Japan), and cultured at 30° C for bacterial colonies and 25° C for fungal colonies.

Sequencing and phylogenic analyses

Template DNA samples for use in PCR were prepared as follows: a single colony of the isolate on solid growth medium was removed with a sterile toothpick and placed in 1 ml of MilliQ water. The cell suspension was heated to 100 °C for 10 min, and the lysate was used in PCR. Approximately 500-bp 16S rRNA sequences were amplified with a Microseq 500 16S rRNA PCR module (PE Applied Biosystems). The reaction mixture (50 µl) contained 25 µl of diluted genomic DNA and 25 µl of the ready reaction mixture. The reaction profile for the amplification was an initial denaturation at 95°C for 10 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, a final extension at 72°C for 10 min, and a 4°C soak. The PCR products were purified with a High Pure PCR Product Purification kit（Roche Diagnostics GmbH, Mannheim, Germany）according to the manufacturer's directions. The purified DNA was recovered in 25 µl of deionized water. The amplified 16S rRNA was subjected to cycle sequencing with the Microseq module. The reaction mixture (20 µl) contained 3 µl of purified PCR product, 4 µl of deionized water, and 13 µl of the sequencing reaction mixture (forward and reverse sequencing mixture in separate reactions). The cycling conditions were 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, followed by a 4°C soak. The cycle-sequenced DNA was precipitated with a DyeEx 2.0 spin kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions, and finally analyzed with an ABI Prism 3130 genetic analyzer (PE Applied Biosystems). The sequence data were compiled with DNASIS-Pro software (Hitachi Software Engineering, Tokyo). The fragments were subjected to homology-based searches of the APORON database (Techno Suruga Laboratory, Shizuoka) and phylogenetic trees were constructed to ascertain the phylogenetic positions of the isolates. In constructing phylogenetic trees, related species to identified species, which were estimated from previous reports (Madhaiyan*et al*. 2007, Raja *et al*. 2008, Sahin*et al*. 2008), were added to the database.

Pink biofilm formation assay

  Although there are various biofilm formation assays (O'Toole *et al.* 1999), we imitated the environments of bathrooms, to construct a novel biofilm model. The isolated bacteria from pink biofilms were grown as indicated in culture conditions, and centrifuged for 5 min at 7,000×g, which was washed and resuspended in water of 3.5° DH (CaCl$_2$; 52.0 mg/ l, MgCl$_2$6H$_2$O; 31.8 mg/ l) to an OD$_{600}$ of 0.5. Circular holes of 1.2 cm in diameter were made on a 5 mm thick silicone sheet (As One Co., Osaka, Japan), and the sheet was attached to a fiber reinforced plastics (FRP) sheet (Engineering Test Service Co., Osaka, Japan), a major material for bath tubs, by pressure bonding. Some 500 µl of prepared microorganisms was inoculated in the pore of the silicone sheet on FRP. After 24 hr. of incubation at 30℃, water was completely removed and incubated again for 24 hr. at 30℃. Finally, the silicone sheet was removed. In observing bacterial viabilities in models of pink biofilms on FRP, a *Bac*Light™ LIVE/DEAD® bacterial viability staining kit (Molecular Probes Inc., Eugene, Oreg.) was used as previously described (Webb *et al.* 2003) with some modifications. A 100 µm thick silicone sheet with circular holes 1.2 cm in diameter was attached on the model without covering the pigmented part. Two stock solutions of stain (SYTO 9 and propidium iodide) were each diluted to 3 µM by water of 3.5° DH, and 5 µl of the mixed solution was added to the samples. Cover glass was attached by instant adhesive, and observed by CLSM.

Cleaning agent-susceptibility assay in test tubes

  The following agents were used: alkyl dimethyl benzyl ammonium chloride (Sanisol C, Kao corporation, Tokyo, Japan), SDS (Wako Pure chemical Ltd, Osaka, Japan), triton X-100 (Sigma Chemical Co., St. Louis, MO), butyl diethylene glycol (BDG, Tokyo Kasei Kogyo, Tokyo). All agents were prepared with water of 3.5 ° DH supplemented with 50 mM HEPES buffer, adjusted pH to 7.4, and diluted to 5.0, 1.0, and 0.1% (w/ v). The bacteria were grown in PDB for 3 d at 30°C, centrifuged for 5 min at 7,000×*g*, washed in water of 3.5° DH, and resuspended in water of 3.5 °DH to an OD$_{600}$ of 0.8. The cultures were added to the assay mixtures at a ratio of 1 to 100 and incubated for 5 min., 120 min., and 24 hr. After the reaction was stopped by the addition of "Diluent with Lecithin & Polysorbate 80" solution (LP, Wako Pure Chemical Ltd, Osaka, Japan) at as much as ten times the volume of the reaction mixture, 3 µl was spotted on PDA for KMC4, KMC5, KMC10, KMC13, KMC14, KMC15, KMC16, KMC17, KMC18, and KMC21, and R2AA for KMC19 and KMC22, and SCDA for KMC20, and incubated

for 3 d at 30°C, to observe their colonies to clarify the minimal concentrations of agents at which the bacteria could not grow.

Cleaning agents susceptibilities assay on FRP

After pink biofilms formed, 500 μl of 5.0% BAC (pH7.4) was added, and incubated for 5 min. After the agents were removed, 500 μl of water of 3.5° DH was added and removed. The biofilms on FRP sheets were then transported into 50 ml centrifuge tubes with 10 ml of LP and 5.0 g of glass beads, 1.5-2.5 mm in diameter, and vortexed vigorously for 1 min. to remove the bacteria from the sheets. LP containing bacteria was diluted, spread on each medium as indicated for the bactericidal assay in tubes, and incubated at 30℃ for 3 days,

Desiccation tolerance assay

Although there are various desiccation assays (Hugenholtz*et al.* 1995), we imitated the environments of bathrooms to construct a novel model. In the pink biofilm formation assay, the microorganisms on FRP were incubated for 10 days at 30℃ after water removed. Their surviving bacteria were quantified as described in the bactericidal activity assay of biofilms.

Accession numbers

The 16S gene sequence data of the isolated *Methylobacterium*were deposited in the DNA Data Bank of Japan (DDBJ) under serial accession numbers AB629723 to AB629736.

2-3. Results

SEM observation

For a detailed characterization by SEM, 42 pink biofilms were investigated. Some 24% biofilms were wet and slimy, the others were dry. Nineteen percent were near drains, 31% biofilms were on walls, on floors, and around doors or windows, and 50% biofilms were on in-bath products including chairs, bottles, and brushes. The all biofilms were mat-like structures, and Fig. 2 shows a typical image. Interestingly, yeast-sized cells were rarely observed (12%), and few in number when they were. Instead, rod–shaped microorganisms, 0.3 to 0.5 by 0.9 to 1.4 µm, were predominant in all the biofilms tested. These biofilms contained round microbial clumps (17%), and networks of fungal filaments (12%), as well as the clumps of rod-shaped microorganisms. In rather wet areas like the drains of the bathrooms, slimy networks which would be extracellular polymeric substances were also found (9.5%). In this investigation, the biofilms were directly fixed with glutaraldehyde without any incubation, because some microorganisms in biofilms could have proliferated, changing the population ratio before the observation through the long complicated processes for sample preparation.

FISH assay

Through SEM assays, yeast-sized cells were rarely observed, and small in number even if detected. *Rhodotorula* were isolated from 17 of the 42 biofilms, while the *Methylobacterium* were isolated from every biofilm. Then we analyzed the microbial communities using FISH, to clarify if the dominant cells are of *Methylobacterium*. The spatial distribution of the genus *Methylobacterium* and other bacteria was visualized and quantified by simultaneous *in situ* hybridization with fluorescence-labeled 16S rRNA targeting probes (Fig. 3). EUB338 was reported to be insufficient for the detection of all bacteria (Daims*et al.* 1999), but the all microorganisms in the viewfields of the pink stains were dyed with these two probes, therefore these two probes were considered to be sufficient for staining pink stains. Calcofluor white, which specifically stains β[1-3] and β[1-4]-linked glucosyl polymers, major polysaccharides in cell walls of yeasts and other fungi, was also used simultaneously to identify yeast cells. Fig. 3A-C are typical images of the samples from Tochigi, Wakayama, and Tokyo in Japan, showing that *Methylobacterium* was the predominant genus in the pink biofilms. Ten photographs of three representative biofilms were quantified and their average biomasses were compared (Fig. 3D-F). It was concluded that *Methylobacterium* was predominant in the pink biofilms.

Isolation of Methylobacterium from pink biofilms

  We isolated 1691 colonies from 42 pink biofilms. To identify microorganisms that could contribute to the pigmentation of the biofilms, approximately 500 bp sequences of 16S rRNA in pink, orange, and red colonies (405 colonies) were compared to sequences in GenBank. The colonies were identified as numerous genera including *Methylobacterium*, *Brevundimonas*, and*Roseomonas* (Table 2), and a phylogenetic tree of representative *Methylobacterium* strains was constructed (Fig. 4). The *Methylobacterium* isolates were divided into 4 groups. *Methylobacteriummesophilicum*KMC10, *Methylobacteriumfujisawaense* KMC4, and *Methylobacteriumradiotolerans* KMC5 were used as typical *Methylobacterium* isolates in subsequent experiments. *Methylobacterium* strains were isolated from the all biofilms.

Pink biofilm formation assay

  Model biofilms of various isolated strains including the *Methylobacterium* were formed to clarify if they could show pink biofilms. After being resuspended in water of 3.5° DH, the general degree of water hardness in Japan, the isolated bacteria were incubated on Fiber reinforced Plastics (FRP), a common material in bath tubs, floors, and walls. Typical biofilms 24 hr. after the water removal are shown in Fig 5. The biofilms of the genus *Methylobacerium* were similar to those observed in bathrooms in their color, but those of other species including *Rhodococcus*sp. KMC17 and *Roseomonas mucosa* KMC19 exhibited similar characteristics.

Susceptibility to cleaning agents in test tubes

  To investigate tolerance to the components of cleaning agents, susceptibility to benzalkonium chloride (BAC), sodium dodecyl sulfate (SDS), polyoxyethylene p-*t*-octylphenyl ether, and diethylene glycol *n*-buthyl ether (BDG) was tested for the *Methylobacterium* and other microorganisms isolated from the biofilms (Table 3). The surviving numbers of the isolated *Methylobacterium* were significantly higher than those of other bacteria.

Susceptibility to cleaning agents on FRP

To investigate the susceptibility of the isolated bacteria in biofilm-like conditions, bactericidal activity assays were conducted against model biofilms by inoculating log 7-8 cells on each FRP sheet (Table 4), because biofilm bacteria are generally more tolerant of stress than planktonic bacteria (Stewart *et al.* 2001). Some biofilms like *Rhodococcus* sp. KMC17 formed were removed only after water was added. Even in biofilms not washed away by water, the surviving numbers of the genus *Methylobacterium* were significantly higher than those of other bacteria in biofilm conditions. In removing bacteria from FRP sheets, vigorous vortexing with beads was adapted. Planktonic bacteria were vortexed vigorously with beads and FRP sheets, and the surviving number was investigated. The loss of surviving number was less than 1 log, meaning that the treatment wouldn't have significantly affected the results. Also the FRP sheets after vortexed were observed by Confocal Laser Scanning Microscope (CLSM). Aggregates that attached to FPR sheets were not observed, meaning the vortexes were enough to remove the microbes from FRP sheets.

Desiccation tolerance of the Methylobacterium

We examined the desiccation tolerances of the isolated strains on FRP by inoculating log 7-8 cells on each FRP sheet. At 10 days after drying, the reduction of the survival in *Methylobacterium* was less than 1 log. The drying of other strains, however, led to values below the detection limit (Table 5). The result indicated *Methylobacterium* to be tolerant against drying in bathrooms. The biofilms were stained with a LIVE/DEAD®*Bac*Light™ kit, and observed with CLSM (Fig. 6). Red cells were

2-4. Discussion

In this study we examined pink biofilms in bathrooms by microscopic and quantitative analyses, and clarified that the genus *Methylobacterium* predominated. To our knowledge, this is the first conclusive investigation about various pink biofilms in bathrooms, and detailed investigation of the reason why the *Methylobacterium* predominated. Pink yeasts including the genus *Rhodotorula* have been also reported to be isolated from pink biofilms (Hamada *et al.* 2009). Moreover, detailed microscopic analyses of various biofilms revealed the number of yeast-sized cells to be far smaller than those of the genus *Methylobacterium*, indicating that the genus *Rhodotorula* would not be the predominant species in the biofilms.

Methylobacterium have been isolated from various environments including soil (Mancinelli*et al.* 1995), river (Boden*et al.* 2008), tap water (Furuhata*et al.* 1992), humans (Anesti*et al.* 2005), and aquatic sediment (McBain*et al.* 2003), suggesting it would not be unimaginable for the bacteria to be found in bathrooms. However, the fact that many other species simultaneously exist in bathrooms raised us the question of why *Methylobacterium* was predominant.

Interestingly, yeasts, bacteria other than the *Methylobacterium*, and fungi were mounted on biofilms of the *Methylobacterium* without exception (Fig. 3). That the isolated *Methylobacterium* tended to easily aggregate in liquid media, and previous reports that some *Methylobacterium* attach to plant roots (Hanson *et al.* 1996, Kaga *et al.* 2009, Lidstrom*et al.* 2002, Lindow*et al.* 2003) and to coaggregate with other species (Rickard *et al.* 2004, Simões *et al.* 2008), led us to speculate that the *Methylobacterium* aggregated, attached to solid surfaces in the bathrooms, and other microorganisms attached to them. It is possible that the ability to aggregate allows the *Methylobacterium* not to be washed away by water flow. Accordingly, this ability might have led the *Methylobacterium* to predominate in the microbial flora.

Also, the *Methylobacterium* might have other characteristics to survive in bathroom environments. One possibility is tolerances of cleaning agents. Tolerance of *Methylobacterium* against cleaning agents has not been reported, but some *Methylobacterium* were reported to tolerate to high concentrations of chlorine (Hiraishi*et al.* 1995). The results showed that the *Methylobacterium* were more tolerant against the agents tested than other isolated strains. In addition, there was a similar tendency on FRP sheets. From a previous report of nosocomial outbreaks from inadequate antiseptics, bacteria were tolerant against at most 0.1-0.2% of BAC (Weber *et al.* 2007). Therefore, the *Methylobacterium* in the microbial flora were considered to be more tolerant by over 10 times than the previously reported strains, though the

mechanism of their tolerance remains unclear.

The bactericidal mechanism of BAC has been reported to be based on disruption of the membrane structure, followed by a proton imbalance and the accumulation of active oxygen species (Gilbert *et al.* 2005, Ioannou *et al.* 2007, Neu *et al.* 1996, Russell *et al.* 2002). Regarding membrane permeability, the lipid composition of methane oxidizers including the genus *Methylobacterium* is unique in several respects. Methyl sterols, which are rarely observed in bacteria, have been shown to be present, and the *Methylobacterium* possess a system of paired peripheral membranes (also called intracytoplasmic membranes) and are predominantly composed of monounsaturated C_{18} fatty acids (Hanson *et al.* 1996, Patt *et al.* 1978). Such systems may affect the delay in the permeation of BAC. Actually, the structure of their peripheral membranes was confirmed by observing the cytoplasm of *Methylobacterium mesophilicum* KMC10 with a transmission electron microscope (data not shown), although the relevance to permeability remains unclear and further study is needed. The quite high anti-oxidizing activities of carotenoids would also contribute to the tolerance against cleaning agents. The carotenoids could scavenge and prevent the formation of free radicals induced by BAC.

The third reason why the *Methylobacterium* were predominant is their desiccation tolerance. There are repeated wet-dry cycles in bathrooms. Dry conditions are not normally suitable for the survival of microorganisms. It is considered that the longer dry conditions last, the fewer microorganisms survive. Therefore, only highly desiccation-tolerant bacteria like the genus *Methylobacterium* could survive in bathrooms. However, the reason why these bacteria are highly tolerant to drying remains unclear. Dry stress generally causes dysfunction in enzymes and /or electron transport chains, and subsequent lipid peroxidation, protein denaturation, and mutation of DNA (Billi *et al.* 2004). The increased van der Waal's interactions between phospholipids, followed by an increase in the phase transition temperature (T_m) of membranes, could be an underlying mechanism (Potts *et al.* 1999). A higher T_m results in the aggregation of proteins, leakage and loss of solutes from cells (Potts *et al.* 1999). The *Methylobacterium* could have certain superior evasion system(s) such as polysaccharide secretion (Choi *et al.* 2008, Penalver *et al.* 2006), because it was reported that exopolysaccharide contributes to the desiccation tolerance of bacteria (Nielsen *et al.* 2011, Roberson *et al.* 1992). Therefore, we searched the pink biofilms by staining them with calcofluor white to detect β[1-3] and β[1-4]-linked glucosyl polymers. Some biofilms were stained, others were not (data not shown), indicating that a large amount of glucan wouldn't play a crucial role in survival in bathrooms. Also, glucans were not

detected in the *Methylobacterium* used in the desiccation tolerance experiments (data not shown).   More studies are needed to clarify whether monosaccharides, proteins, or small amounts of polysaccharide contribute to the desiccation tolerance, as well as antioxidant like carotenoid. Concerned with the localization of the dead cells (Fig. 6), similar phenomena have been reported in various biofilms (Mai-Prochnow*et al.* 2004, Webb *et al.* 2003), and were observed without any desiccation treatments. Therefore, the biofilm development process might have led to cell death, and not the desiccation process.

There remain many other possibilities which might make the *Methylobacterium* predominant. For example, it is possible that greater numbers of the *Methylobacterium* invade bathrooms than those of other bacteria or fungi. This is because the *Methylobacterium* have been reported to frequently occur in humans (Anesti*et al.* 2005, 3) and human-made environments (Feazel*et al.* 2009, Furuhata*et al.* 1992, Kelley*et al.* 2004).

Our results suggest the special characteristics mentioned above could lead the *Methylobacterium* to predominate. On the other hand, bathrooms also provide special characteristic environments; rapid water flow, dry, low nutrients, occasional cleaning agents, and so on. These special characteristics could lead special bacteria, the *Methylobacterium*, to predominate. Further study is expected to clarify the role of *Methylobacterium* in the microbial ecology of bathrooms.

Table 1. Representative strains isolated from pink biofilms

Strain designation	Closest type strains	Accession No.[a]	% Sequence similarity[b]
Methylobacterium species			
KMC10	*Methylobacteriummesophilicum* JCM2829T	AB629729	99.3
KMC4	*Methylobacteriumfujisawaense* DSM5686T	AB629731	99.1
KMC5	*Methylobacteriumradiotolerans* JCM2831T	AB629725	99.3
Other species			
KMC13	*Brevundimonas vesicularis* IAM12105T	AB021414	99.8
KMC14	*Chryseobacterium*sp. AG13	EU336941	100
KMC15	*Rhodococcuscorynebacteroides*DSM20151T	AD430066	99.6
KMC16	*Chryseobacteriumgregarium* DSM19109T	AY230767	96.3
KMC17	*Rhodococcus* sp. DSM20151T	AF430066	99.4
KMC18	*Rhodococcusqingshengii* djl-6T	JF937542	99.8
KMC19	*Roseomonas mucosa* MDA5527T	AF538712	97.8
KMC20	*Burkholderiacepacia*ATCC25416T	AF097530	99.3
KMC21	*Deinococcus grandis* DSM3963T	Y11329	95.3
KMC22	*Microbacterium arborescens* DSM20754T	X77443	100
KMC23	*Brevundimonas nasdae* GTC1043T	AB071954	99.2

a, Accession numbers based on 16S rRNA partial gene sequences.

b, Sequence similarity (16S rRNA, approximately 500 bp.) was searched using Aporon DB-FU 2.0. (Technosuruga Lab., Co., Shizuoka, Japan)

Table 2. Oligonucleotide probes

Probe	Sequence (5' to 3')	FA[a] (%)	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	20	Most Bacteria	mann *et al.*, 1990
MB	AGCGCCGTCGGGTAAGA	30	Genus *Methylobacterium*	Pirttilä *et al.* 2000
Comp MB[b]	AGCGCCGTCTGGTAAGA	-	Competitor for MB	this study
Help MB[c]	CCAACTCCCATGGTGTGACGG	-	Helper for MB	this study

a, FA, formamide concentration in the hybridization buffer.

b, Unlabeled probe MB used as a competitor to enhance specificity.

c, Unlabeled probe MB used as a helper to enhance specificity.

circular holes of
1.2 cm in diameter

silicone sheet
(5 mm thick)

FRP sheet

pressure
bonding

500 µl of Bacteria in water
of 3.5° DH were injected

Incubated at 30℃ for 24 h,
and supernatant removed
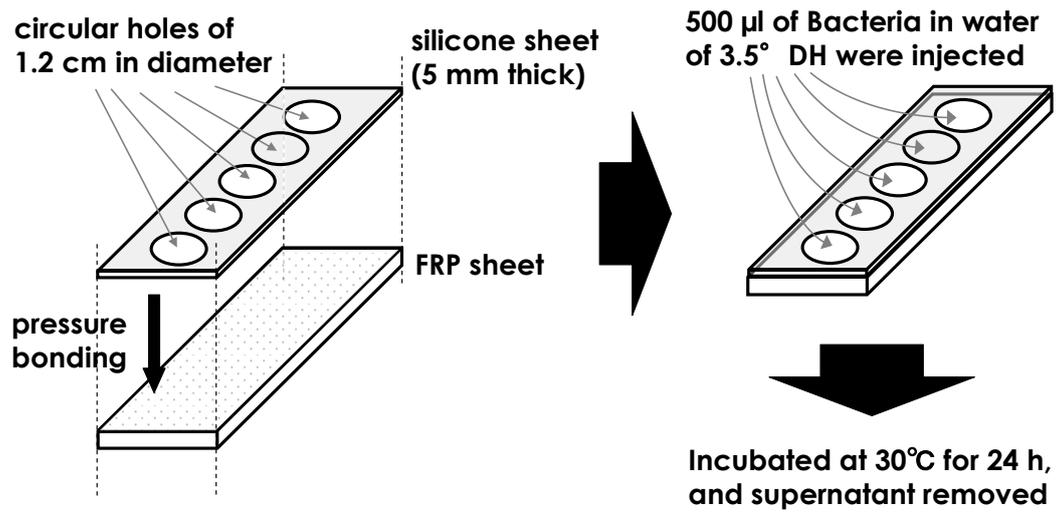
Fig. 2 Biofilm preparation
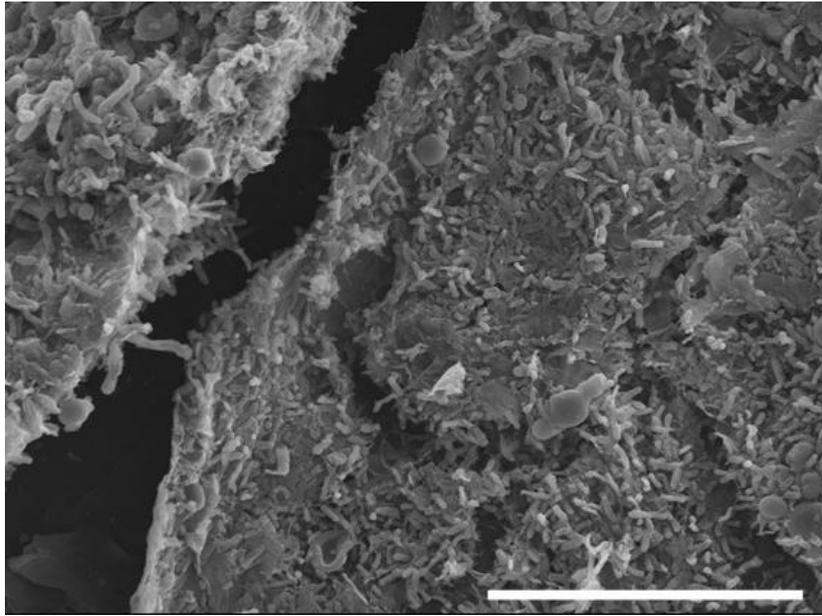
Fig. 3. Representative pink biofilm observed by SEM. Bar, 20 μm.
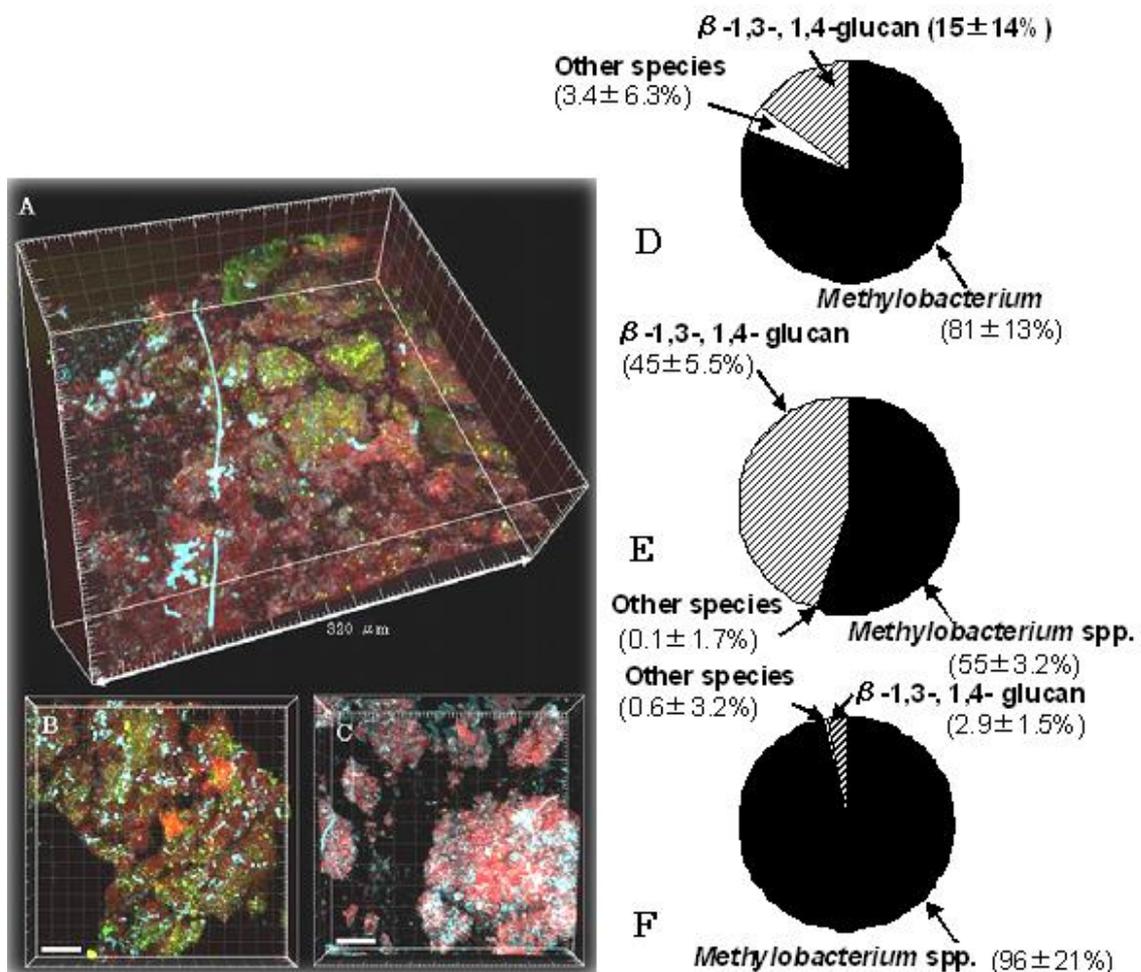
Fig. 4.FISH assay and microbial community composition of pink biofilms. A-C, CLSM photomicrographs showing the spatial distribution of microorganisms in three independent pink biofilms. The organisms were targeted by *in situ* hybridization with the ROX-labeled probe MB and FITC-labeled probe EUB338, and simultaneously stained with calcofluor white. Cells of MB-stained *Methylobacterium* are red; cells of EUB338-stained bacteria are green. Cells containing α-1,3-glucan like fungi are aqua blue. D-F, pie charts of *Methylobacterium*, other bacteria, and β-1,3-, 1,4- glucans. *Methylobacterium* is the bacterial group that hybridized with MC, other bacteria are the bacterial group that hybridized with EUB338, and β[1–3] and β[1–4]-linked glucosyl polymers are the position that hybridized with calcofluor white. The values are the mean ± standard deviation of duplicate samples.
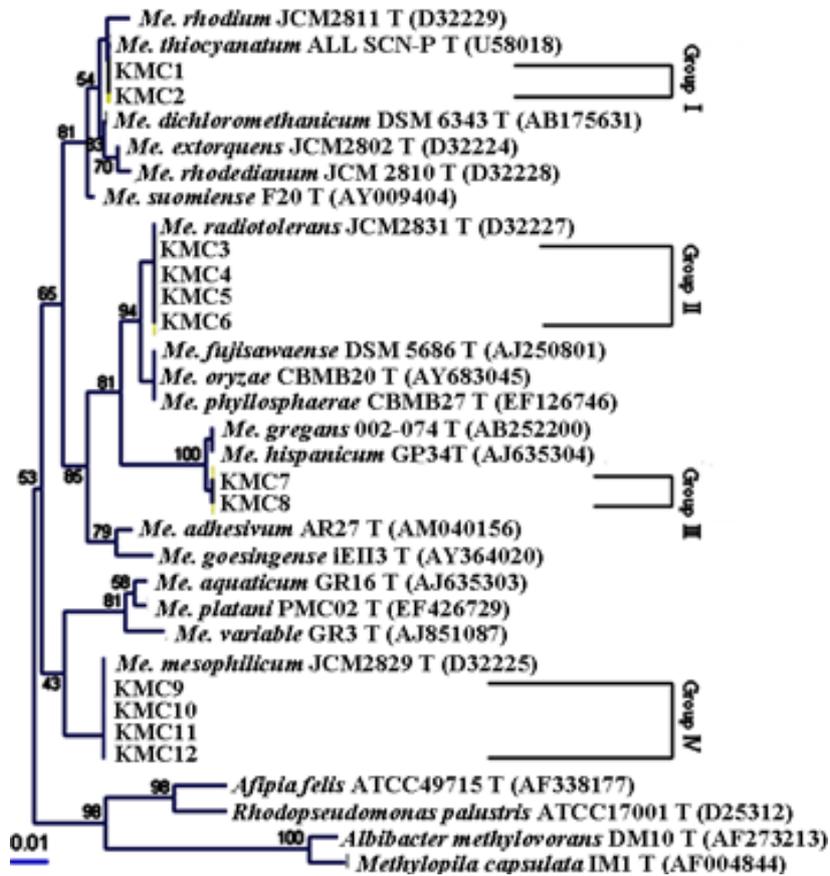
Fig. 5.16SrRNA phylogenetic tree. Phylogenic tree based on approximately 500 bp of the 16S rRNA gene sequences of *Methylobacterium* isolates from bathrooms using the neighbor-joining method. The data for type strains of *Methylobacterium* and other genera were from GenBank. Bootstrap percentages (> 50%) based on 100 replications are given at branch points. Bar, 0.01 changes per nucleotide position.
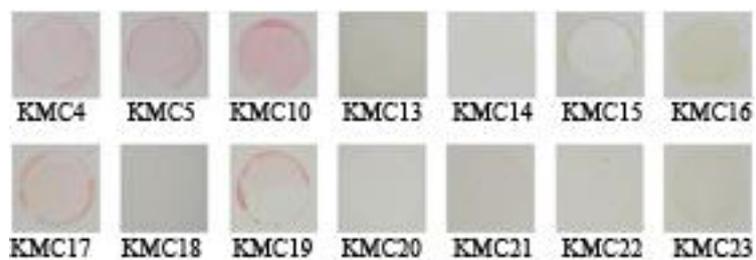
Fig. 6 Model biofilms formed on FRP sheets. Photographs show the model biofilms and names of the strains below the photographs indicate the strains of the upper photographs. KMC10, KMC4, and KMC5 indicate the biofilms of the *Methylobacterium*.
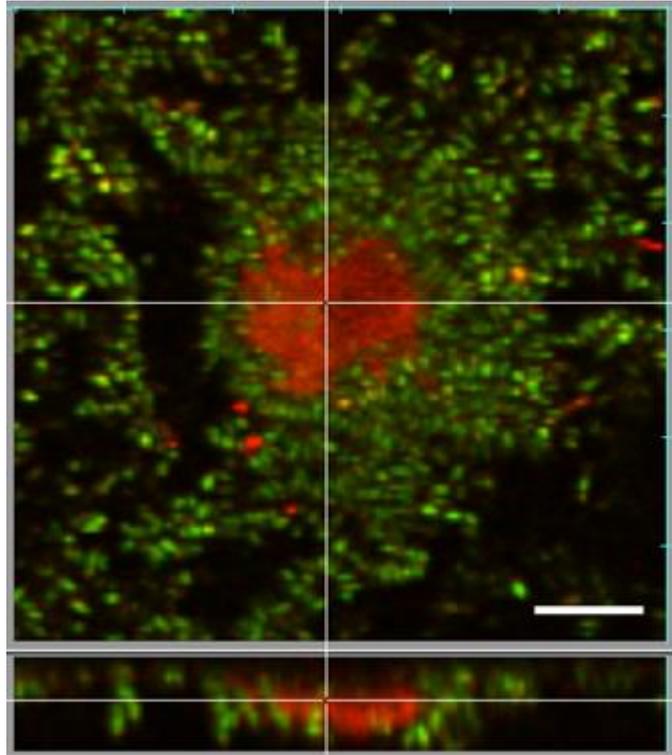
Fig. 7 CLSM photographs showing viability within microcolonies in biofilms containing the Me. mesophilicum KMC10. The cells were visualized by using the LIVE/DEAD®BacLightTM kit. Green fluorescent cells are viable, whereas red fluorescent cells are dead. The results are representative of the figures of three experiments. Bar, 10 µm.

Table 4.Susceptibility to cleaning agent components of isolated bacteria on FRP sheets.

Strains	Log survivals (log CFU/ FRP sheet)	
	Water[a]	BAC[b]
Methylobacterium species		
KMC10	7.15	7.3
KMC4	7.52	7.27
KMC5	7.78	7.68
Other species		
KMC15	<2.00	<2.00
KMC21	6.58	<2.00
KMC20	2.9	<2.00
KMC14	<2.00	<2.00
KMC16	7.18	<2.00
KMC18	7.51	<2.00
KMC17	5.3	<2.00
KMC19	2.78	<2.00
KMC22	2.9	<2.00
KMC23	2.78	<2.00
KMC13	2.6	<2.00

a. The log survival numbers after water was applied for 5 min. on various biofilms formed on FRP.
b. The log survival numbers after 5.0% BAC was applied for 5 min. on various biofilms formed on FRP.

Table 5. Desiccation tolerance

Strains	Log survivals (log CFU/ FRP sheet)
Methylobacterium species	
KMC10	6.15
KMC4	6.26
KMC5	6.38
Other species	
B4P3221	3.4
KMC14	<2.00
KMC16	2.78
KMC18	4.67
KMC17	<2.00
KMC21	<2.00
KMC20	<2.00
KMC19	<2.00
KMC22	3.88
KMC23	<2.00
KMC13	<2.00

Table 3.Susceptibilities to cleaning agent components of isolated bacteria in test tubes.

Strains	Minimal concentrations (%) of agents required for 4 log reduction in three exposure times a											
	Cleaning agents											
	BAC			SDS			BDG			Triton X-100		
	5min.	2hr.	24hr.	5min.	2hr.	24hr.	5min.	2hr.	24hr.	5min.	2hr.	24hr.
Methylobacterium strains												
KMC10	>5.0	>5.0	<0.10	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC4	>5.0	>5.0	1.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC5	0.1	1.0	0.1	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
Other strains												
KMC15	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	>5.0	>5.0	>5.0	1.0
KMC21	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	1.0	<0.10	<0.10	<0.10
KMC14	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	>5.0	>5.0	>5.0	1.0
KMC16	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	>5.0	>5.0	>5.0	1.0
KMC20	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	1.0	>5.0	>5.0	>5.0
KMC18	1.0	<0.10	<0.10	>5.0	>5.0	1.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC17	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	1.0	<0.10	<0.10	<0.10	<0.10	<0.10
KMC19	<0.10	<0.10	<0.10	>5.0	1.0	1.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC22	<0.10	<0.10	<0.10	>5.0	1.0	1.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC23	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
KMC13	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	>5.0	>5.0	>5.0

a. After 0.1, 1.0, and 5.0% agents were mixed with each culture and incubated for 5 min., 2 hr., and 24 hr., surviving cells were detected by subsequent dilution, spotted on agar, and incubated.

CHAPTER 3

REGULATION OF *METHYLOBACTERIUM*

3-1. Introduction

As described in chapter 2, we found that *Methylobacterium* strains isolated from bathrooms were tolerant to various stresses including surfactants like benzalkonium chloride (BAC) (Kovaleva *et al.* 2014, Yano *et al.* 2013). Some isolates even survived in 5.0% BAC after 5.0 min of exposure, indicating that cleaners require more immediacy and versatility.

In contrast, we also found that *Methylobacterium* was rather susceptible after longer exposure (24 hr) to BAC (Yano *et al.* 2013), suggesting that the slow action of BAC against cells would cause their tolerance. Since BAC attacks bacteria by destroying membrane structures (Gilbert *et al.* 2005, Ioannou *et al.* 2007, Russell *et al.* 2002), we considered the acceleration of BAC accumulation or the increase of membrane permeability to be valid for killing in a short contact time. Alcohols are known as penetration enhancers, and help various chemicals penetrate biological membranes (Williams *et al.* 2004). Thus, in the present study, we attempted the simultaneous addition of various alcohols and BAC for killing bacteria, expecting them to act as penetration enhancers.

In fact, combinational use of chlorhexidinediacetate, a cationic agent, with some organic solvents was already shown to be effective against *Pseudomonas aeruginosa* (Fitzgerald *et al.* 1992, Richards *et al.* 1973). The antimicrobial mechanisms of some organic solvents were also intensively investigated (Ramos *et al.* 2002, Segura *et al.* 2012, Sikkema *et al.* 1995). It was suggested that organic solvents accumulate in cellular membranes, disorganize the structures, and the resultant losses of intracellular ions and metabolites lead to cell death (Segura *et al.* 2012, Sikkema *et al.* 1995). Organic solvents also induce various cellular responses including structural changes of membrane (Aono *et al.* 1997, 4), and up-regulation or down-regulation of various other genes, as revealed by transcriptomic analyses (Segura *et al.* 2012, Shimizu *et al.* 2005). Nonetheless, with regard to the simultaneous addition of cationic agents and alcohols, many questions remain, such as how each agent acts individually on microbes. Furthermore, most studies have used agents at above bactericidal concentrations, while

simultaneous use assumes application at non-lethal concentrations. Therefore, we screened the alcohols effective at non-lethal concentrations in combination with BAC, and clarified the underlying mechanisms of killing.

3-2. Materials and methods

Strains and culture conditions. *M. mesophilicum* KMC10 was isolated from a pink biofilm in a bathroom in Japan, and cultivated aerobically in Potato Dextrose broth (Becton Dickinson and Company, Sparks, MD) for 3 d at 30°C and 200 rpm, as previously described (Yano *et al.* 2013). *Escherichia coli* BW25113 (Engelk*et al. 2001)* was cultivated aerobically in Luria-Bertani (LB) medium (Wako Pure Chemical Industries, Japan) at 37°C and 200 rpm.

Bactericidal assay of BAC and/or alcohols. Bactericidal assays of *M. mesophilicum* and *E. coli* were performed as described previously (Yano *et al.* 2013) with modifications. Briefly, cell suspensions were washed and resuspended in saline to OD_{600} of 0.8, and the 10 µl suspensions were mixed with 1000 µl of test fluid. After incubation, 100 µl was mixed with 900 µl of Lecithin &Polysorbate 80 (LP; Wako Pure Chemical Industries), diluted, spread on PDA for *M. mesophilicum* and on LBA for *E. coli* strains, and incubated.

Cellular alcohols and BAC accumulation measurements by confocal Raman microscopy. Dynamic accumulation of alcohols and BAC was measured using a confocal Raman microspectrometer (Nanofinder; Tokyo Instrument Inc., Japan) as previously described (Huang*et al.* 2005) with modifications. *M. mesophilicum* KMC10 cells were washed and resuspended in saline to OD_{600} of 0.8. The resultant 10 µl of cell suspension was added to 100 µl of test fluids in a glass-bottomed dish (glass diameter, 10 mm) on the stage of the microscope. Measurements were performed as follows.

  The 632.8 nm line (3 mW on the sample stage) of a He-Ne laser (MellesGriot 05-LHP-991) was focused on a position in a selected bacterial cell. After passing through a 100 µm pinhole, the scattered light was introduced into the spectrometer and detected by a thermoelectrically cooled (-70°C) CCD detector (Andor DU420-BV). All measurements were carried out at room temperature, and the background was subtracted. The areas of the respective peaks were calculated and compared.

Laurdan generalized polarization measurements. Physical property changes on cellular membranes by BAC and/or alcohols were investigated by generalized polarization (GP) of 6-dodecanoyl-2-dimethylaminonaphthalene

(laurdan; Life Technologies, Grand Island, NY) fluorescence. The measurements were performed as described previously (Gidwani*et al.* 2001) with modifications. This method is based on the bilayer structure-dependent fluorescence spectral shift of laurdan, which can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment (Engelk*et al. 2001*, Parasassi*et al.* 1995). Laurdan was reported to be located at the glycerol backbone of the bilayer with the lauric acid tail anchored in the phospholipid acyl chain region. Water penetration is correlated with lipid packing and membrane fluidity. The emission spectrum of laurdan in a single phospholipid bilayer is centered at 440 nm when the membrane is in the gel phase and at 490 nm when it is in the liquid-crystalline phase (Parasassi*et al.* 1995). Therefore, the extent of water penetration into the bilayer surface as a result of the dipolar relaxation effect could be searched for by using laurdan.

M. mesophilicum KMC10 was cultivated, washed with saline, resuspended, and 3.0 µM laurdan was added. The cells were incubated overnight at 30°C, washed with saline, and resuspended in saline to OD_{600} of 10. The resultant cell suspensions and test fluids were preincubated at 25°C for 20 min, mixed with each other, added in a preheated quartz cuvette, placed in a fluorometer chamber, and laurdan emission spectra were obtained using a spectrofluorometer (Fluorolog-3™; Horiba JobinYvon, Longjumeau, France) at an excitation wavelength of 380 nm and emission wavelengths of 440 nm and 490 nm. The GP was calculated using the following equation.

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

In this equation, I_{440} and I_{490} indicate fluorescence intensities at 440 nm and 490 nm, respectively.

Laurdan generalized anisotropy measurement. A modified version of the method described by Gidwani*et al.* (Gidwani*et al.* 2001) was used for anisotropy measurements. *M. mesophilicum* KMC10 cells were mixed with 3.0 µM laurdan, incubated overnight at 30°C, washed, and resuspended in saline to OD_{600} of 10. The resultant cell suspensions and test fluids were preincubated at 25°C for 20 min, mixed with each other, added in a preheated quartz cuvette (four clear sides), placed in a fluorometer chamber, and the anisotropy was measured.

Anisotropy values were based on the emitted intensity of laurdan,

measured with a spectrofluorometer (Fluorolog-3™; Horiba JobinYvon). Lowered membrane fluidity leads to less mobility of laurdan. This causes less distortion of the emitted signal and higher rigidity values were recorded. The vertically polarized light was set at 380 nm to excite the probe. The emitted light was measured at 440 nm through a vertical polarizer vertically (I_{VV}) and horizontally (I_{VH}) to the excited light. To give a grating factor (G), I_{HV} and I_{HH} through a horizontal polarizer were used. The slit widths for excitation and emission were set at 3.0 nm. Two milliliters of unlabeled cells were measured to establish baseline values. At least 10 readings were performed and averaged, to record change of the anisotropy. The anisotropy (r) was calculated using the following equation.

$$ r = \frac{I_{vv} - G \times I_{vh}}{I_{vv} + 2G \times I_{vh}} \quad G = \frac{I_{HV}}{I_{HH}} $$

DASPEI, ethydium bromide, and HOECHST33342 accumulation assay. All assays were performed as described previously (Coldham *et al.* 2010, Germ *et al.* 1999, Murakami *et al.* 2004) with some modifications. Overnight cultures of *E. coli* were centrifuged at 4000 x *g* for 10 min, washed once with saline, and resuspended in saline to OD_{600} of 10. 2-(4-(Dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI; Molecular Probes, Eugene, OR), ethydium bromide (EtBr; Sigma-Aldrich, St. Louis, MO), and 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloridetrihydrate (HOECHST33342; Dojindo, Japan) were added at 2.0 μM, 0.25 μM, and 2.5 μM, respectively. The resultant suspensions were mixed with 0.5% BA, PeA, BzA, or HEPES buffer with or without BAC in a 96-well plate, and the fluorescence was measured every minute. The excitation and emission wavelengths were 461 nm and 560 nm, 520 nm and 590 nm, and 352 nm and 461 nm, respectively.

Giant vesicle preparation. Giant vesicles were prepared using gentle swelling on agarose, by a modified version of a method described previously (Horger *et al.* 2009). A uniform and thin film of agarose was prepared on a cover glass using a 1.0% (w/v) solution of ultra-low-gelling agarose (Sigma-Aldrich) in double-distilled water at 40°C for 2.0 hr. Lipid mixtures (1.0 mg/ml 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Sigma-Aldrich), 1.0 mg/ml

1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC, Sigma-Aldrich), and 3.0 μM BODIPY FL-C16 (Molecular Probes)) were added to the film and allowed to dry for 1.0 hr at room temperature. The subsequent glass was equilibrated in 300 μl of 50 mM HEPES buffer (pH7.4) for 1.0 hr at room temperature.

Morphological analyses of giant vesicles using confocal laser scanning microscopy (CLSM). Various 2.0% alcohol solutions (described above) were added to the giant vesicle-containing solution on a cover glass, and were immediately observed using CLSM META (Carl Zeiss, Germany).

3-3. Results

Synergetic killing effects of alcohols with BAC on *M. mesophilicum* KMC10

Alcohols with or without BAC were mixed with *M. mesophilicum* KMC10 cell suspension, and the numbers of cells surviving after 5.0 min were counted. We employed BAC and alcohols at 1.0 mg/ml and 10 mg/ml, respectively, which reduced bacterial numbers by less than 1.0 log CFU/ml when they were added alone. Among alcohols with various alkyl chain lengths, harboring ether bonds and/or a benzene ring, pentanol (PeA), phenoxy glycol (PhG), benzyl alcohol (BzA), and benzyl glycol (BzG) successfully reduced the surviving numbers by approximately 4 log in combinational use with BAC (Fig. 1). To clarify the effects of the alcohols specific to BAC or valid with other chemicals, we also tested the synergetic killing effects of BAC with different alkyl chain lengths ranging from C14 to C18, and found that the all tested BAC homologs exhibited similar effects with BzA (data not shown).

In situ **quantitation of alcohols and BAC in cells.** Among the effective alcohols, deuterium-labeled pentanol and benzyl alcohol were commercially available (dPeA and dBzA, respectively), and we used them for the following analyses. In addition, deuterium-labeled butanol (dBA) was used as a negative control. The antimicrobial activities of deuterium-labeled compounds were not significantly different from those of non-labeled compounds (data not shown). After mixing the alcohols with or without BAC and a cell suspension in glass-bottomed dishes, the Raman spectra were detected from a single cell. Because each alcohol and BAC had their original spectra (Fig. S1A), the identical peak areas in the spectra were calculated and compared with those of cells exposed to HEPES buffer (Fig. 2). Interestingly, significantly more BAC accumulated in cells exposed to dPeA and dBzA than those to dBA after 5.0 min of exposure (Fig. 2A), indicating that the two alcohols accelerated BAC accumulation. For the alcohols, however, only dBzA accumulated more upon simultaneous addition with BAC after 5.0 min of exposure (Fig. 2B-D), indicating that BAC accelerated only dBzA accumulation. The dynamic analyses showed that the increases of each compound became significant from after 4.0 min of exposure (Fig. S1B-G).

Effects of alcohols on membrane structures. To investigate the effects on cellular membrane structures, we performed fluorescence analyses with a hydrophobic fluorescent probe, laurdan. Membrane fluidization induces the invasion of polar molecules like water, which leads to a spectral shift of laurdan localized in membrane. We firstly confirmed that laurdan did not increase or decrease the number of surviving cells in BAC and/or alcohols (Fig. S2). Then, cells prestained with laurdan were exposed to alcohols and/or BAC, followed by spectral shift measurements. The results after 5.5 min of contact are shown in Fig. 3A. The vertical axis (GP value) shows membrane hydrophobicity, with a higher value indicating a more hydrophobic state. The BAC and BzA significantly changed the GP value, while PeA rarely changed it. The change in PeA exposure was similar to that in BA, a negative control. To determine the structural changes in membrane fluidity, changes in the anisotropy of the fluorescence were analyzed. However, no significant decreases but increases were detected with PeA (Fig. 3B). Because spectroscopic assays cannot detect regional effects on membrane structures, we directly observed the effects by *in vitro* experiments with giant vesicle (GV). We simply mixed GV with alcohols or BAC, and observed the morphological changes by microscopy. However, no morphological changes were observed upon PeA addition, while BzA and BAC influenced the sizes and shapes (Fig. 3C). Especially for the change in sizes, we calculated and compared them over time. That is, we randomly selected 5 vesicles, analyzed the area sizes at various time points with Image J 1.47 (National Institutes of Health, USA), and divided by the size before adding alcohols. Still, no significant changes were detected with PeA exposure. For BzA, apparently some got bigger at the last time point, suggesting multiple vesicles united. Therefore, we compared the sizes at the earlier time points, to check if vesicles once got smaller before uniting. However, no significant changes were observed (data not shown).

Effects of alcohols on membrane proteins. The results described thus far imply that at least PeA has other targets on bacterial surfaces, such as membrane proteins. However, although CliABC, a RND transporter which is responsible for efflux of many chemotherapeutic agents in *M. extorquens* DM1 was reported (Muller *et al.* 2011), the functions of membrane proteins are poorly understood in the *M. mesophilicum*. We also found similar

synergetic killing effects in *E. coli* (Fig. S3A). After checking that laurdan did not increase or decrease the surviving cells in BAC and/or alcohols (Fig. S3B), we performed laurdan assay with *E. coli*, and found that it showed a similar tendency to *M. mesophilicum* KMS10 (Fig. S3C). Thus, we adopted *E. coli* as a model to analyze the effects on protein functions.

In *E. coli*, AcrAB-TolC efflux pump, which transports various substrates including alcohols and BAC (Murakami *et al.* 2004), expresses when under BAC exposure (Holdsworth*et al.* 2013), and takes an important role for the tolerance (Bore *et al.* 2007,Sulavik*et al.* 2001). Besides, their homologous sequences were found to be conserved among many *Methylobacterium* species by mean of a BLAST search (data not shown). Therefore, we hypothesized that alcohols inactivate various transporters including the pump, to accelerate BAC accumulation. This possibility was assessed by using other fluorescent probes known as substrates for AcrAB-TolC: DASPEI, EtBr, and HOECHST33342 (Bore *et al.* 2007, Germ *et al.* 1999, Murakami *et al.* 2004).

We firstly confirmed that the probes did not increase or decrease the number of surviving cells (Fig. S4). Then, we added alcohols and/or BAC to *E. coli* cells with the fluorescent probes. PeA and BzA caused more accumulation of all of the fluorescent probes than HEPES buffer, the negative control. On the other hand, no significant changes in fluorescence were observed in BA and BAC (Fig. 4A, B, and C).

3-4. Discussion

The present study determined the alcohols that are effective for killing *M. mesophilicum* KMC10 in 5.0 min with BAC. In addition, we also performed various experiments with PeA and BzA in order to unveil the synergetic mechanisms.

Alcohols are known to induce hyperfluidization of bacterial membranes and protein denaturation, following an increase of reactive oxygen species (ROS) (Segura *et al.* 2012, Sikkema *et al.* 1995). Indeed, a recent genomic study showed that BA upregulated genes responsible for envelope stresses, metabolite transportation, and oxidative stresses (Rutherford *et al.* 2010). Furthermore, some mutants that upregulated cell-surface proteins raised their tolerances (Anfelt *et al.* 2013, Oh *et al.* 2012). Physiological effects of PeA and BzA on membranes and proteins were also investigated. They induced interdigitation in DPPC membrane (Löbbecke *et al.* 1995, Rowe *et al.* 1994), causing an increase in proton permeability (Ebihara *et al.* 1979, Zeng *et al.* 1993). In a *Bacillus subtilis* strain, the lethal concentration of BzA increased the membrane fluidity (Konopasek *et al.* 2000). For protein structures, BzA induced the aggregation of proteins such as human interleukin (Tobler *et al.* 2004) and interleukin-1 receptor antagonist (Roy *et al.* 2006) by partially unfolding these proteins (Bis *et al.* 2015, Singh *et al.* 2010). Unfolding actions were also reported for short-chain alcohols (Sashi *et al.* 2012). However, alcohols in this study exhibited synergetic effects in 5.0 min at non-lethal concentrations, when used simultaneously with BAC. Therefore, we explored their antibacterial mechanisms at non-lethal concentrations by focusing on dynamic phenomena within 5.0 min *in vivo*, especially on membrane structures and proteins.

We firstly investigated the effects of a fluorescent probe, laurdan, on membrane structures. Laurdan is an environmentally sensitive probe, and the associated spectral shift reflected a change in membrane polarity (Parasassi *et al.* 1995). For BzA, its addition has been reported to cause a disordered membrane structure (Konopasek *et al.* 2000, Sulavik *et al.* 2001). Our data indicated that a non-lethal concentration of BzA also caused disorder of membrane structures, suggesting that the structural change could increase the permeability of BAC to accelerate BAC accumulation in cells. On the other hand, no effects were detected by PeA (Fig. 3). Because previous *in vitro* experiments at higher concentrations indicate that PeA also

changed membrane polarity (Löbbecke *et al.* 1995), we further assessed the effects by anisotropy change of laurdan, as it could affect membrane fluidity in an undetectable manner by the spectral shift. However, no decreases in anisotropy were detected (Fig. 3B). To investigate the possibility that PeA partially disrupts the membrane structures, another experiment with GV was performed. Still, no effects were observed (Fig. 3). These results indicate surprisingly that 1% PeA did not fluidize the membrane structure at least in this condition.

For membrane protein analyses, we performed assays with other fluorescent probes, DASPEI, EtBr, and HOECHST33342 in *E. coli*. All probes were reported to be substrates of an efflux pump, AcrAB-TolC (Coldham *et al.* 2010, Germ *et al.* 1999, Murakami *et al.* 2004). Also, some *acrAB-tolC* mutants accumulated EtBr and HOECHST33342 more than the wild type (Bohnert *et al.* 2008, Opperman *et al.* 2014). For DASPEI, it accumulated more in adding a proton uncoupler; cccp (carboxy-cyanide *m*-chlorophenylhydrazone) (Murakami *et al.* 2004). AcrAB-TolC requires proton motive force to export the substrates, and the effect of cccp suggests that AcrAB-TolC take a role in exporting DASPEI.

PeA and BzA increased the accumulation of all probes (Fig. 4), indicating that they disrupted AcrAB-TolC. Interestingly, the changes in fluorescent intensities differed among the probes. The reason for this remains obscure, but we speculated that low specificities of the alcohols and variation of transportation paths for the dyes might have contributed to these differences. BzA was reported to partially unfold a pharmaceutically relevant protein, interferon α-2a (IFNA2) (Bis *et al.* 2015). This implied that BzA might unfold multiple proteins, suggesting that PeA and BzA might affect the functions of other surface proteins including porins responsible for the transportation of fluorescent probes in addition to AcrAB-TolC. On the other hand, although the all probes were substrates of AcrAB-TolC, we speculated that the dyes could be also partially exported by other pumps or porins, and even by fluidized membrane structures at the same time, and the partial paths may be variable among the dyes. Therefore, we considered the effects of the alcohols on these surface structures might have increased the influxes or decrease the effluxes and contributed to the differences on dye accumulation.

The results in this study suggest that PeA did not affect the membrane physiology, but mainly interrupted the functions of membrane proteins. Both

effects on proteins might interrupt the efflux of BAC, resulting in its accumulation inside the cell. In contrast, BzA could also increase the membrane permeability. The change in membrane permeability could increase the influx of BAC, suggesting that it further accelerated BAC accumulation. The result that BAC accumulated more in combinational use with BzA than with PeA (Fig. 2) supports this hypothesis. It is noteworthy that PeA at a non-lethal concentration only affected protein functions. The reason why PeA did not influence lipid bilayers remains unclear, but the short and non-branched alkyl chain could be easily dissociated from membrane structures. This speculation is consistent with the data that alcohols with bulky and hydrophobic structures exhibited synergetic effects with BAC (Fig. 1). If so, the benzene ring in BzA may play an important role in membrane impairment.

On the other hand, it has also not been clarified whether a non-lethal concentration of BAC might also change the antimicrobial effects of alcohols. It is expected that these unveiled mechanisms will be explored in order to build up a new theory that facilitates the search for the best compounds in combinational use.
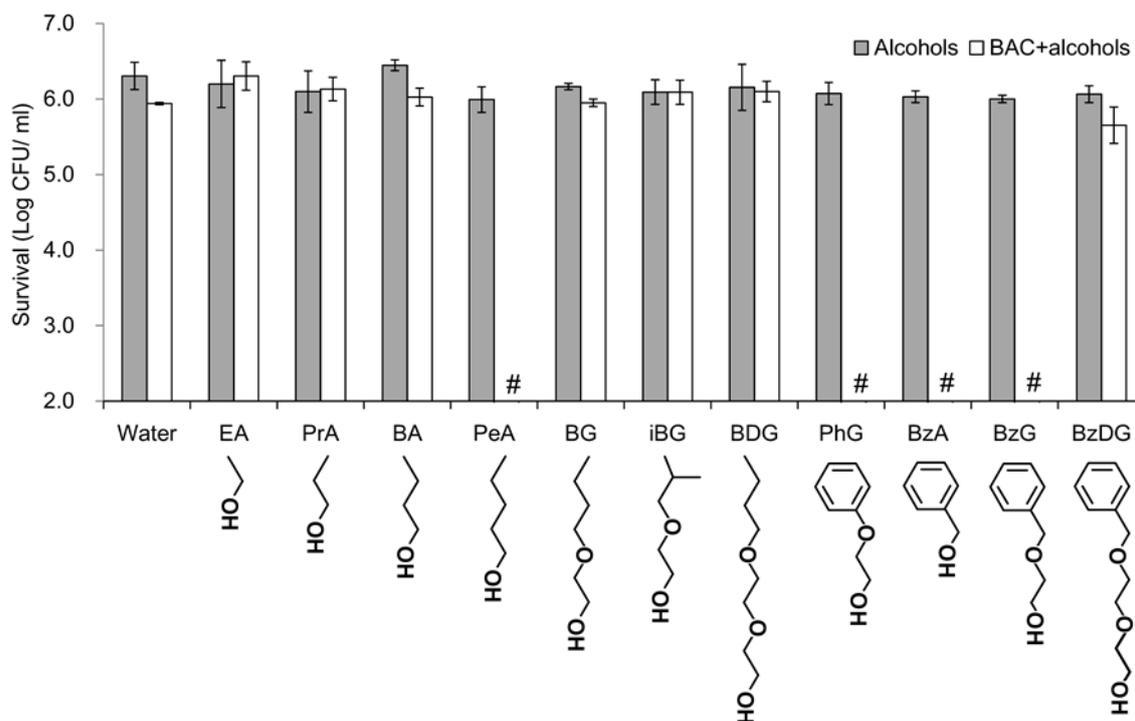
Fig. 8.Bactericidal activities of various alcohols with or without BAC. M. mesophilicum KMC10 cells were mixed with solutes with or without BAC, and the numbers surviving after 5.0 min were measured. Chemical structures indicate the tested alcohols: ethanol (EA), propanol (PrA), butanol (BA), pentanol (PeA), butyl glycol (BG), isobutyl glycol (iBG), buthyldiglycol (BDG), phenoxy glycol (PhG), benzyl alcohol (BzA), benzyl glycol (BzG), and benzyl diglycol (BzDG). All alcohols and BAC were used at 1.0% (v/v) and 0.1% (v/v), respectively. Gray bars are for various alcohols, and white bars are for the combinational use of various alcohols and BAC. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. # indicates below the detection limit.
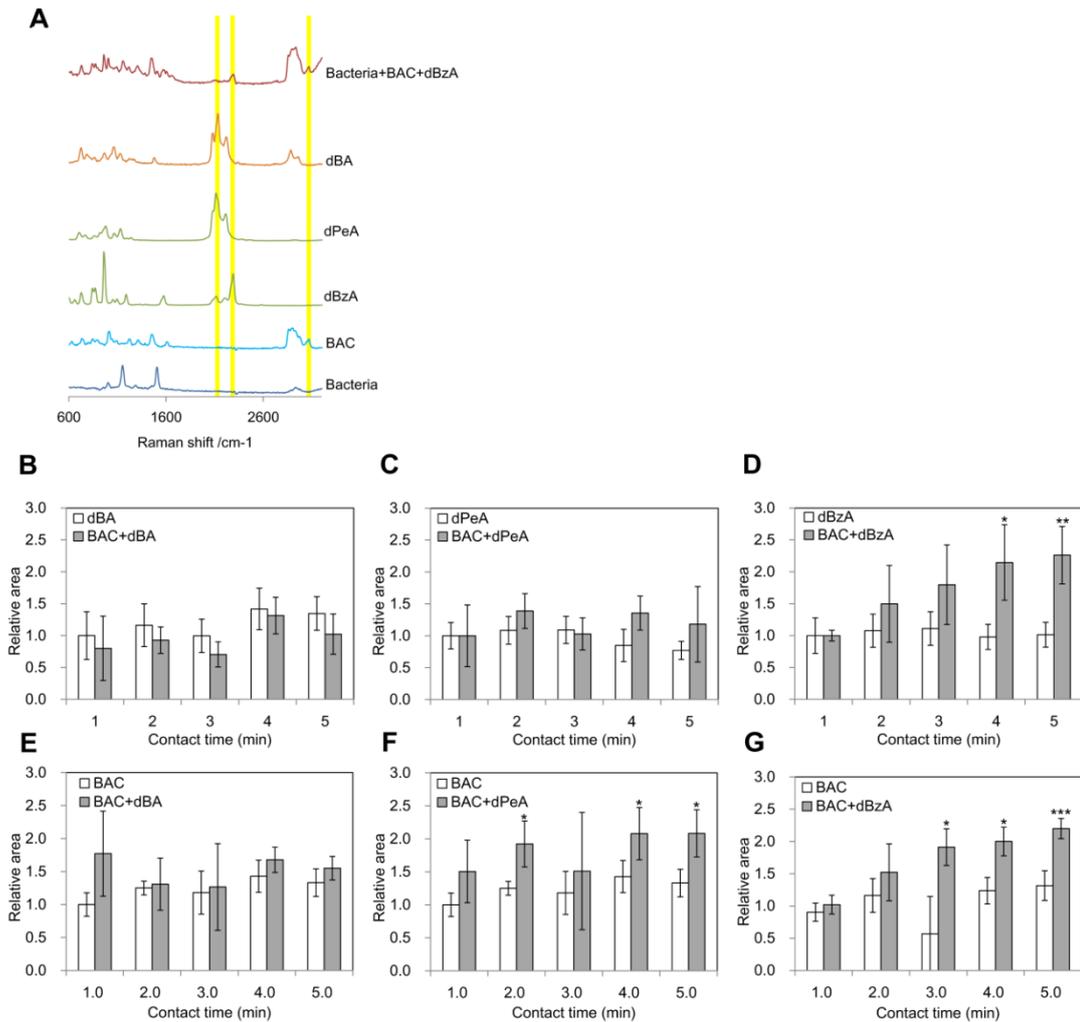
Fig. 9.Dynamic accumulation of alcohols and BAC on *M. mesophilicum* **KMC10.** A) Raman spectra of each alcohol (*d*-butanol, *d*-pentanol, and *d*-benzyl alcohol), BAC, bacteria without any treatments (bacteria), and bacteria after combinational addition of 0.1% BAC with 1.0% dBzA for 5 min (BAC+BAC+dBzA). Yellow areas on each spectrum were used for quantitation. B-D) Accumulation of 1.0% alcohols with (gray) or without (white) 0.1% BAC.E-G) Accumulation of 0.1% BAC with (gray) or without (white) 1.0% alcohols at every minute to 5.0 min. The figures are for dBA, dPeA, and dBzA from left to right. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. * indicates P<0.05.
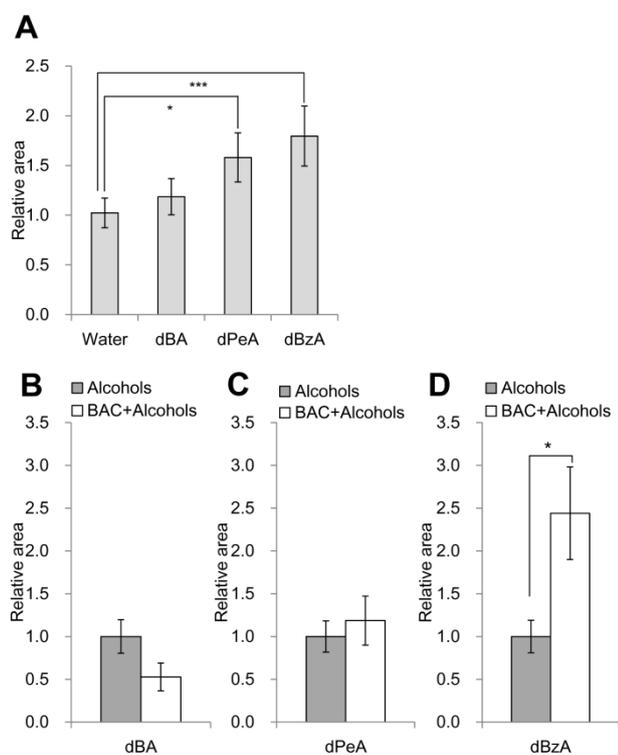
Fig. 10.Accumulation of BAC and alcohols in *M. mesophilicum* KMC10. Cell suspensions were mixed with 1.0% dBA, dBzA, dPeA, or 50 mM HEPES buffer with or without 0.1% BAC, and the Raman spectra of each 5 cells after 5.0 min were measured. The areas of respective peaks were divided by those of HEPES buffer, averaged, and are presented as relative areas. A) BAC, B) dBA, C) dBzA, and D) dPeA accumulation are presented. In B-D), gray bars are for alcohols and white bars are for the combinational use of alcohols with BAC. Each bar is the mean of five independent experiments, and the error bars represent the standard deviations. * indicates $p < 0.05$, and *** indicates $p < 0.001$.
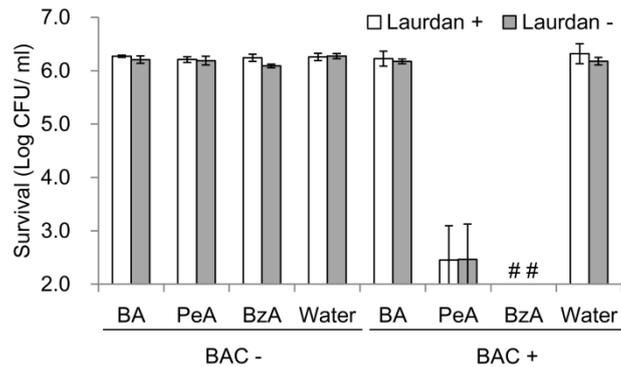
Fig.11. Effect on the surviving numbers by staining with laurdan. *M. mesophilicum* KMC10 cells prestained with laurdan were mixed with 1.0% BA, PeA, BzA, and/or 0.1% BAC, and the numbers surviving after 5.0 min were measured. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. # indicates below the detection limit.
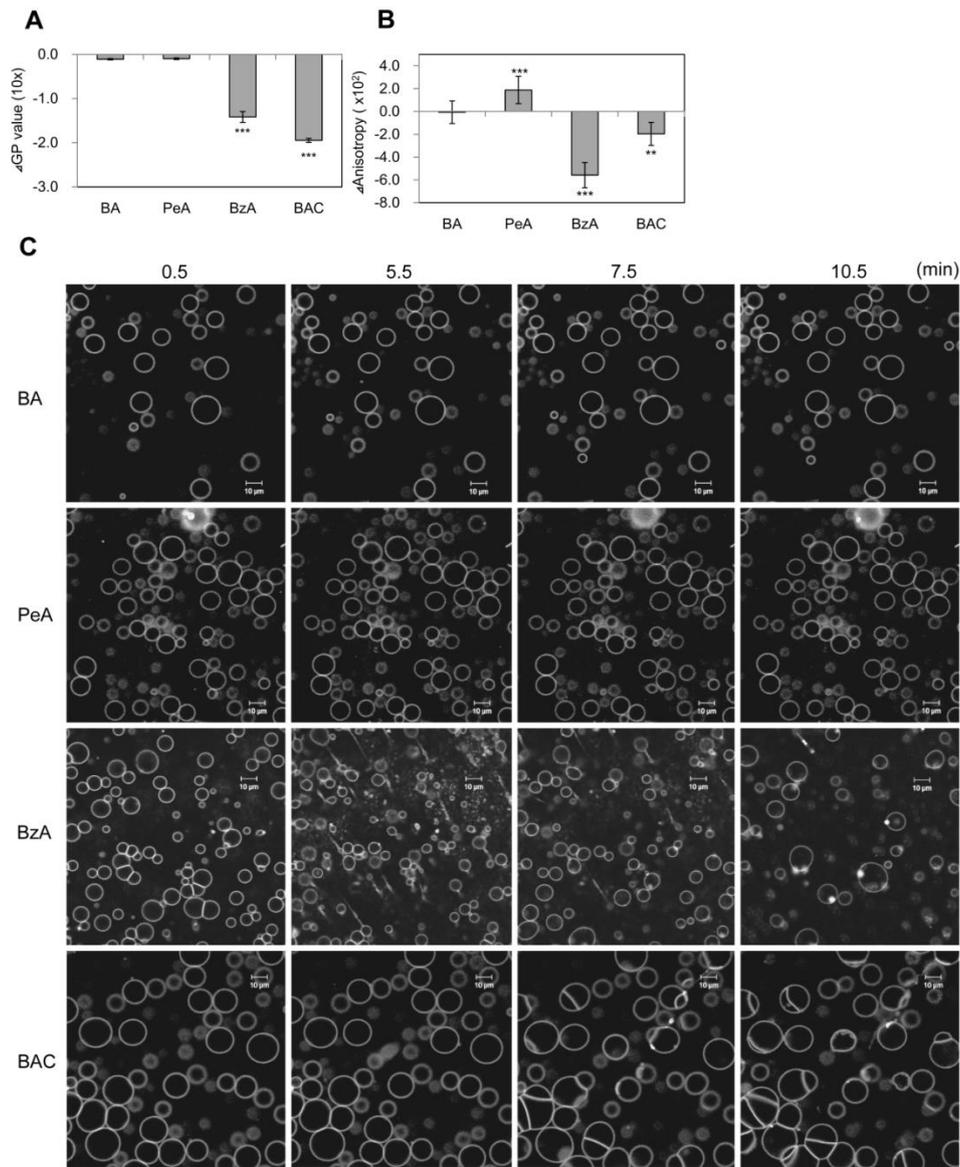
Fig. 12.Effects of alcohols on lipid structures.A) GP value and (B) anisotropy changes after addition of 1.0% alcohols or 0.1% BAC. M. mesophilicum KMC10 cells prestained with laurdan were mixed with alcohols or BAC and the spectral shifts after 5.5 min of contact were measured. The shifts were subtracted by those with HEPES buffer, and are presented as relative intensities. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. ** indicates $p<0.01$, and *** indicates $p<0.001$. C) Dynamic morphological changes of giant vesicles mixed with alcohols or BAC after 0.5, 5.5, 7.5, and 10.5 min from left to right. The results represent BA, PeA, BzA, and BAC from top to bottom.
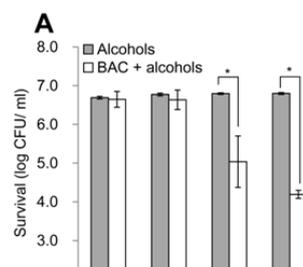
Fig. 13. **Synergetic killing effects and effects of alcohols on lipid structure on** *E. coli.* A) Bactericidal activities of 0.5% alcohols with or without 4.0 mg/l BAC upon 5.0 min of exposure. White bars are for combinational use of various alcohols and BAC, and gray bars are for various alcohols. B) *E. coli* cells prestained with laurdan were mixed with 0.5% BA, PeA, BzA, and/or 4.0 mg/l BAC, and the numbers surviving after 5.0 min were measured. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. C) Changes of GP value after 0.5% alcohols or 4.0 mg/ l BAC additions were measured. *E. coli* cells prestained with laurdan were mixed with alcohols with or without BAC and the spectralshift after 5.5 min contact were measured. The shifts were subtracted by those with HEPES buffer, and are represented as relative intensities. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations. # indicates below the detection limit. * indicates P<0.05.
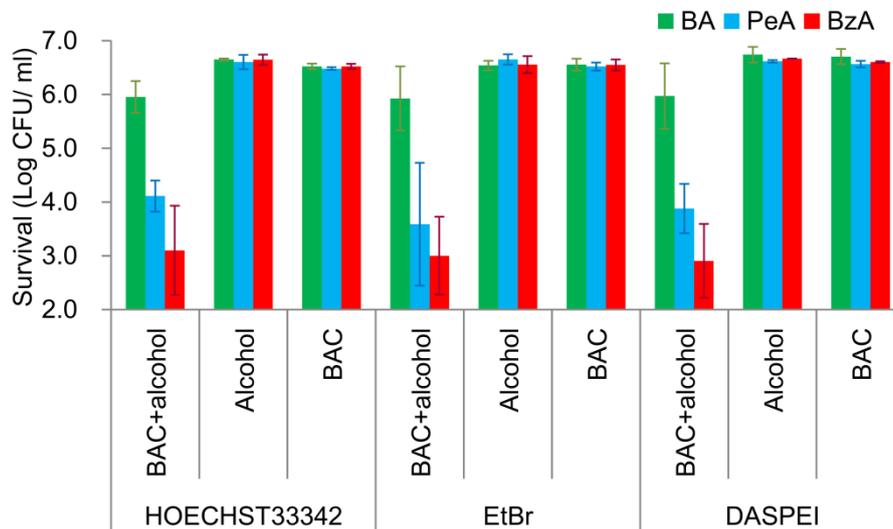
Fig. 14.Effect on the surviving numbers by staining with HOECHST33342, EtBr, and DASPEI. *E. coli* cells prestained with HOECHST33342, EtBr, and DASPEI were mixed with 0.5% alcohols and/or 4.0 mg/l BAC, and the numbers surviving after 5.0 min were measured. Each bar is the mean of three independent experiments, and the error bars represent the standard deviations.
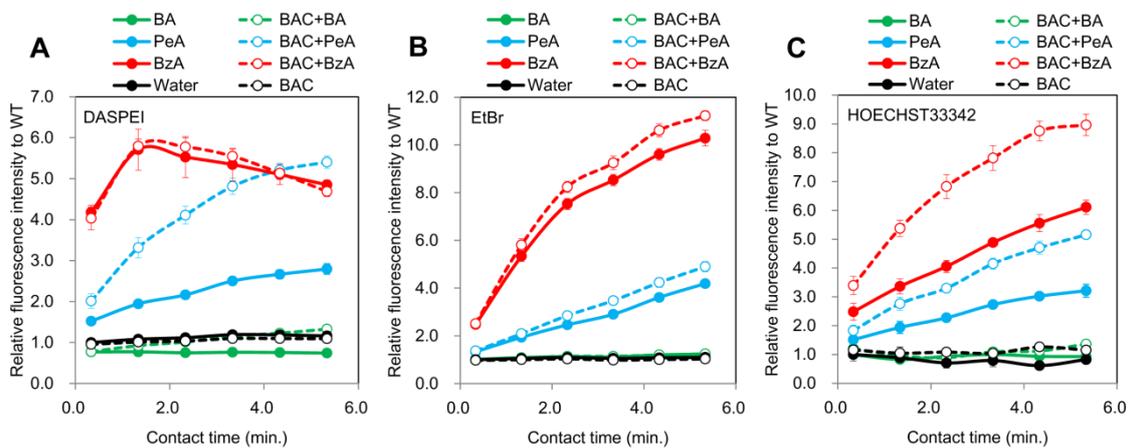
Fig. 15.Effect of alcohols and/or BAC on the accumulation of
HOECHST33342 (A), DASPEI (B), and EtBr (C). Accumulation of probes on
E. coli upon exposure to 0.5% BA (green), PeA (blue), BzA (red), or HEPES
buffer (black) with (dashed lines) or without 4.0 mg/l BAC (solid lines) was
measured every minute, and the relative fluorescent intensities upon
exposure to water are presented. Each bar is the mean of three independent
experiments, and the error bars represent the standard deviations.

CHAPTER 4

GENERAL DISCUSSION

4-1. Bathrooms and characteristics of the genus *Methylobacterium*

Bathrooms are full of stresses for microorganisms to grow. The microorganisms face rapid flowing water, drying, low nutrients, and occasional exposure to cleaning agents. The stresses allow limited species to survive, including genus *Methylobacterium*. It was found that *Methylobacterium* strains isolated from bathrooms were tolerant to drying and cleaning agents. Also, the strains easily aggregate and strongly attach to various abiotic surfaces (data not shown). These results suggest that changes of the environmental stresses could influence the microbiota. For example, if the materials of bathrooms changed from fiber reinforced plastics (FRP) into woods or stones, pools of water would rarely formed, leading *Methylobacterium* strains to be easily flown away. As consequences, *Methylobacterium* strains are known to form less frequency and less amounts of pink biofilms in traditional Japanese bathrooms made of woods or stones, and the needs for the regulation increased as bathrooms made from FRP increased. Therefore, in case with environmental changes in bathrooms, we need to speculate if the changes could *Methylobacterium* strains to survive there. The data in this thesis could suggests a point of view to discuss the possibility.

4-2. Regulation of the genus *Methylobacterium*

In chapter 3, certain alcohols were suggested to influence on membrane proteins and/or membrane structures of *Methylobacterium* strains, leading BAC to accumulate on the cell. Natural habitat of the strains, in contrast, the bacteria would rarely be exposed to just the same chemical compounds. However, the similar structural chemical compounds could affect similar structural changes on the bacterial cell surfaces. These changes could contribute to inter- and intra-species communications driven by some chemical signals.

For example, some *Methylobacterium* strains are known to survive on plant surfaces, and they benefit from methanol produced by plants. It is considerable that the bacterial surface structures could be influenced by

certain chemical compounds produced by plants, and the effects could somehow promote or inhibit accumulations of signals for communications. That is, the regulatory circuits proposed in this thesis could contribute not only to develop artificial regulatory techniques but also to understand the bacterial behavior and physiological roles in natural habitats.

CHAPTER 5

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