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보 문

Growth kinetics and chlorine resistance of heterotrophic bacteria isolated from young biofilms formed on a model drinking water distribution system

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모델 상수관망에 형성된 초기 생물막에서 분리한 종속영양세균의 생장 동역학 및 염소 내성

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ABSTRACT: The present work quantified the growth of young biofilm in a model distribution system that was fed with chlorinated drinking water at a hydraulic retention time of 2 h. Bacterial biofilms grew on the surface of polyvinyl chloride (PVC) slides at a specific growth rate of 0.14 ± 0.09 day^{-1} for total bacteria and 0.16 ± 0.08 day^{-1} for heterotrophic bacteria, reaching 3.1×10^4 cells/cm^2 and 6.6×10^3 CFU/cm^2 after 10 days, respectively. The specific growth rates of biofilm-forming bacteria were found to be much higher than those of bulk-phase bacteria, suggesting that biofilm bacteria account for a major part of the bacterial production in this model system. Biofilm isolates exhibited characteristic kinetic properties, as determined by μ_{max} and K_S values using the Monod model, in a defined growth medium containing various amounts of acetate. The lowest μ_{max} value was observed in bacterial species belonging to the genus *Methylobacterium*, and their slow growth seemed to confer high resistance to chlorine treatment (0.5 mg/L for 10 min). K_S values (inversely related to substrate affinity) of *Sphingomonas* were two orders of magnitude lower for acetate carbon than those of other isolates. The *Sphingomonas* isolates may have obligate-oligotrophic characteristics, since the lower K_S values allow them to thrive under nutrient-deficient conditions. These results provide a better understanding and control of multi-species bacterial biofilms that develop within days in a drinking water distribution system.

Key words: *Methylobacterium*, *Sphingomonas*, biofilm, chlorine resistance, drinking water, growth kinetics

Biofilm is an assemblage of surface-associated microbial cells that are frequently embedded in a matrix of extracellular polymeric substances (Donlan, 2002). In drinking water distribution systems or in household plumbing, biofilm formation is a ubiquitous phenomenon, despite the presence of disinfectant in the oligotrophic environment of drinking water. Drinking water biofilms develop on the inner surfaces of pipes, which provide a suitable habitat for the accumulation of cells and organic matter as well as for bacterial replication (Batté *et al.*, 2003a). Their occurrence is of operational concern, as they can lead to pipe corrosion, black water, malodor, and increased bacterial contamination of water (Flemming *et al.*, 2002). In addition, biofilms are a public health concern, since pathogens can attach to biofilms, which then act as an environmental reservoir (Wingender and Flemming, 2011; Simões and Simões, 2013). Operational and public health concerns associated with

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drinking water biofilms have brought the issue of biofilm formation to the forefront of water supply research.

The attachment of microorganisms to surfaces and subsequent biofilm development are very complex processes, generally known to occur through a multi-stage process that includes transport of cells to the surface, initial attachment, cell cluster formation (aggregation), and maturation of biofilm matrix (Dang and Lovell, 2000; Rickard *et al.*, 2003). The primary (early) colonizers are bacteria that can colonize pristine surfaces. Their initial attachment is mediated through a chemotactic interaction with conditioning film components. Once attached, the early colonizers can then multiply on the surface to form microcolonies and provide additional attachment sites for later colonizers (Rickard *et al.*, 2003). The growth and aggregation of cells or cell clusters are eventually followed by the maturation of biofilm matrix. Biofilm maturation results in a more complex architecture, channels, and redistribution of microorganisms. As the biofilm matures, cells on the surface can also detach and return to being free-living cells (Stoodley *et al.*, 2002).

Past studies showed that the composition and morphology of drinking water biofilms change during the growth and maturation phase (Batté *et al.*, 2003b; Keinänen *et al.*, 2004). This implies that the properties of young biofilms may be different from mature biofilms. Moreover, the growth rate of young biofilms has been known to be higher than that of mature biofilms due to lower competition between bacteria (Boe-Hansen *et al.*, 2002b). The importance of young biofilms lies in the fact that it involves pioneering colonizers, which are able to initiate biofilm formation and influence the subsequent growth and maturation of the biofilm. Therefore, research on young drinking water biofilms will contribute to a better understanding of the dynamics of biofilm formation.

This study was performed to quantify the growth of young biofilm on the surfaces of pipe material exposed to chlorinated drinking water and to identify the heterotrophic bacteria that grow within the young biofilm. In addition, the kinetic properties and chlorine resistance of biofilm isolates were characterized for a better understanding and control of early-stage biofilm formation in drinking water distribution systems.

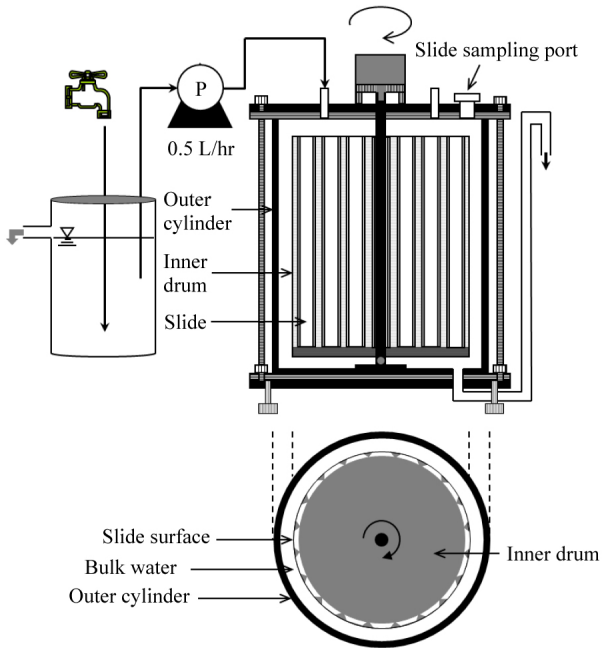

Fig. 1. Schematic diagram of the annular reactor (AR) system used in this study.

Materials and Methods

Model distribution system

An annular reactor (AR) (model 1120 LS; Biosurface Technologies Corporation), consisting of a stationary outer cylinder and a rotating inner drum, was used as a model distribution system (Fig. 1). Rotation of the inner drum was controlled by a motor up to 380 rpm. The AR accommodated 20 removable slides, which are flush-mounted to the surface of the inner drum. Water moves in the region between the outer cylinder and inner drum. In this study, a total of 20 polyvinyl chloride (PVC) slides (BST 503-11; Biosurface Technologies Corp.), each of which have an exposed surface area of 19.5 cm^2, were analyzed for biofilm accumulation in the AR. Prior to use, the disassembled AR system, slides, and related accessories were cleaned thoroughly and autoclaved at 121°C and 15 psi for 15 min and then reassembled carefully on a clean bench. Finally, sterile distilled water was used to confirm that there was no leakage.

Experimental setup

The lab-scale experimental setup for the AR system was fed tap water at a flow rate of 500 ml/h, providing a hydraulic

Table 1. Characteristics of the tap water sample

Parameter	Average	Range
pH	7.02	6.93–7.05
Free chlorine residual (mg/L)	0.46	0.27–0.76
Alkalinity (mg/L as $CaCO_3$)	17	10–22
Hardness (mg/L as $CaCO_3$)	30.5	26–32
Turbidity (NTU)	0.34	0.28–0.43
NH_3-N (mg/L)	0.024	0.007–0.060
PO_4^{3-}-P (mg/L)	0.027	0.021–0.031
Total organic carbon (mg/L)	0.80	0.63–0.91
Assimilable organic carbon (μg/L)	37.3	29.5–57.0
Heterotrophic plate counts (CFU/ml)	8	6–13

retention time (HRT) of 2 h. The rotating speed of the inner drum was 50 rpm. The tap water, the characteristics of which are shown in Table 1, was produced at a nearby water treatment plant that had a process train of coagulation-flocculation, sedimentation, sand filtration, and chlorination. During the course of experimentation, the AR system was operated without further addition of disinfectant, so that a free chlorine residual of ~0.5 mg/L was present in the tap water (Table 1). The AR system, including tubing, was wrapped with aluminum foil to prevent phototrophic growth in the system.

Biofilm sampling and analysis

Biofilm specimens were sampled by removing PVC slides and placing them in a glass tube containing 30 ml of sterile phosphate-buffered saline (PBS, pH 7.2). The slides were scraped manually with a sterile disposable cell lifter (Fisherbrand). The tube was sonicated at 40 kHz and 25°C for 3 min in an ultrasonic cleaning bath (model 8510; Branson Ultrasonics) and scraped again. Subsequently, the slides were removed from the biofilm suspension.

The total cell count (TCC) of the biofilm suspension sample was filtered through a 0.22-μm black polycarbonate membrane (GTBP 02500; Millipore) and enumerated using an epifluorescence microscope (Olympus BX51; Olympus America) following 4',6-diamidino-2-phenylindole (DAPI) staining. Biofilm TCC values were expressed as cells/cm^2.

Heterotrophic plate counts (HPC) of the biofilm samples (CFU/cm) were analyzed by the spread plate method (9215 C) or the membrane filter method (9215 D) of Standard Methods (Eaton *et al.*, 2005) on R2A agar (Difco Laboratories) and incubation at 25°C for 7 days.

Growth kinetics of biofilm isolates

For growth kinetics and disinfection experiments of the biofilm-isolated heterotrophic bacteria, 11 isolates (B1–B11) were inoculated individually into 100 ml of R2A broth and allowed to grow at 25°C for 10 days in a shaking incubator (150 rpm). After incubation, cells were harvested by centrifugation (20 min at 10,000 × *g* and 4°C). The resulting pellet was washed three times with sterile 0.1 M PBS (pH 7.2) and then resuspended in 20 ml PBS. For growth kinetic experiments, cells were inoculated and cultivated on growth medium containing acetate (CH_3COONa) at 0 to 10 g/L. The growth medium was prepared by adding the following components to 1 L of Milli-Q water: 1.0 g $(NH_4)_2SO_4$, 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4$, 50.0 mg $CaCl_2 \cdot 2H_2O$, 3.0 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.05 mg $CoCl_2 \cdot 6H_2O$, and varying amounts of sodium acetate (0–10 g). The pH was adjusted to 7.2 ± 0.2, and the medium was sterilized by autoclaving at 121ºC for 15 min. Individual isolates were inoculated in flasks containing 100 ml of growth medium so that the optical density at 600 nm (OD_{600}) was ~0.2. The flasks were incubated in a shaking incubator at 25°C and 150 rpm. Since the biofilm-isolated heterotrophic bacteria were believed to be capable of forming biofilm on the inner walls of flasks, the culture flasks were sonicated for 5 min using an ultrasonic cleaning bath to disperse attached or aggregated cells before the cell density measurements. Cell growth was monitored by OD_{600} measurements until it reached the stationary phase. Kinetic parameters as a function of carbon concentration were determined by the Monod equation:

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S} \quad (1)$$

, where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the substrate (acetate carbon) concentration, and K_S is the half-saturation constant, which is defined as the substrate concentration at which μ is equal to the half of μ_{max}.

Isolation and identification of biofilm bacteria

Eleven phenotypic colonies grown on a randomly selected

R2A plate that was inoculated with biofilm sample were isolated and purified for further genotypic identification. Genomic DNA extractions were made from pure cultures using a commercially available DNA extraction kit (SolGent™ Genomic DNA Prep Kit; SolGent Co., Ltd.) according to the manufacturer's instructions. Purified DNA was stored at -20°C in the buffer provided with the extraction kit. The 16S rDNA fragments were amplified with 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1518R (5′-AAG GAG GTG ATC CAN CCR CA-3′) primers. Amplified DNA was purified using the AccuPrep® PCR Purification Kit (Bioneer Co.) as described by the manufacturer. The purified templates were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and were analyzed on an ABI Prism® 3730xl DNA Analyzer (Applied Biosystems). The sequences were compared to sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using BLAST programs of the National Center for Biotechnology Information (NCBI) to identify their closest phylogenetic relatives. Three representative bacterial strains (B2, B5, and B10) were selected on the basis of their kinetic properties (i.e., μ_{max} and K_S values as determined by the Monod equation) and deposited in the GenBank database under accession number KT380687–380689, respectively.

Effect of chlorine on representative biofilm isolates

Chlorine exposure experiments with the representative biofilm isolates were performed to determine their behavior in the presence of disinfectant. An aliquot of NaOCl stock solution (0.5% as Cl_2) was added to give a free chlorine residual of 0.5 mg/L, which was measured with the *N*,*N*-diethyl-*p*-phenylene diamine (DPD) colorimetric method using a spectrophotometer (model DR850; Hach Co.). Subsequently, an aliquot of the cell culture was added to a test flask containing 100 ml of chlorinated tap water so that it provided a cell density of ~10^5 CFU/ml. Chlorine exposure tests were conducted at 25°C for 10 min under agitation (150 rpm). After 10 min, biocidal action was stopped by adding 100 μl of 30% (w/v) sodium thiosulfate. Thereafter, the flasks were sonicated for 5 min in an ultrasonic cleaning bath to disperse attached or aggregated cells. Viable counts before and after chlorine exposures were determined with duplicate samples based on serial dilutions and plating on R2A agar.

Water quality analysis

The pH and turbidity values were measured using a pH meter (model 710A; Thermo Fisher Scientific Inc.) and a turbidimeter (model 2100N; Hach Co.), respectively. Free chlorine residual was measured via the DPD colorimetric method using a spectrophotometer (model DR850; Hach Co.). Total organic carbon (TOC) was measured with a TOC analyzer (Phoenix 8000; Tekmar-Dohrmann). Assimilable organic carbon (AOC) was determined by Standard Methods (Eaton *et al.*, 2005) utilizing *Pseudomonas fluorescence* strain P17 and *Spirillum* sp. strain NOX. Ammonia and phosphorus were analyzed with the phenate method (4500-NH_3 F) and ascorbic acid method (4500-P E) of Standard Methods (Eaton *et al.*, 2005), respectively. TCC and HPC levels present in water samples were determined by the same method used for biofilm TCC and HPC enumeration. Results were expressed as cells/ml and CFU/ml for TCC and HPC, respectively.

Results and Discussion

Dynamics of young biofilm formation

Figure 2A shows biofilm formation, as measured by TCC and HPC values, on PVC surfaces in contact with tap water. Biofilm development reached a quasi-steady state after 5 days of operation of the AR system. The time biofilms take to reach steady state varies depending on several factors; e.g., residual disinfectant (chlorine or chloramine), easily biodegradable organic matter, flow velocity (shear stress), pipe material, and temperature (Ollos *et al.*, 2003). In a quasi-steady state, biofilm TCC and HPC densities averaged $3.1 \pm 0.8\times10^4$ cells/cm^2 and $5.8 \pm 1.0\times10^3$ CFU/cm^2, respectively (Fig. 2A). The ratio of HPC to TCC, which is principally related to the culturability of the bacterial population, was in the range 15.4–22.1%. Bacterial culturability has been reported to be higher in young compared to mature biofilm (Boe-Hansen *et al.*, 2002b), where a high fraction (24%) of culturable bacteria was observed in the young biofilm.

Over the period of 5 to 10 days, the TCC and HPC values of bulk water ranged from 3.9×10^3 to 4.8×10^3 cells/ml and from 1.7×10^2 to 1.9×10^2 CFU/ml, respectively, which were higher

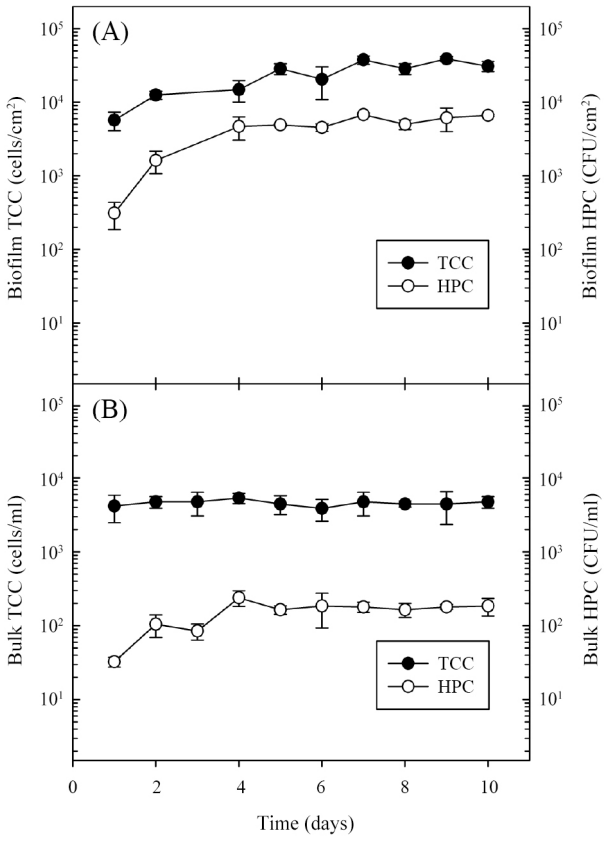

Fig. 2. Total cell count (TCC) and heterotrophic plate count (HPC) levels of the biofilm (A) and bulk water (B) within the AR system fed with tap water.

than those found in tap water (Fig. 2B and Table 1). At a quasi-steady state, bulk HPC accounted for 4.0 ± 0.4% of bulk TCC. The bacterial culturability observed in bulk water was considerably lower than that in the biofilm (19.0%). Bulk HPC seemed to be more susceptible to free chlorine residual present in the tap water compared to biofilm HPC.

Drinking water biofilms are composed of bacterial cells embedded in a heteropolymeric matrix containing humic substances, glycoproteins, polysaccharides, and nucleic acids (Wingender and Flemming, 2011). This exopolymer matrix confers resistant properties to the whole system via the limitation of the effectiveness of disinfection by consuming the oxidants used, such as chlorine or chloramines. The inactivation mechanism includes the reductive power of the biofilm itself (Wang *et al.*, 2012) and the slow diffusion of oxidants in the biofilm (Lee *et al.*, 2011). In addition, the resilience of biofilm bacteria exposed to oxidant stress (Mathieu *et al.*, 2009) makes chlorination appear to be a relatively ineffective disinfection procedure against biofilms compared to bulk water bacteria.

Predicting biofilm growth rates is of great interest, and we determined rates using TCC and HPC values and the method proposed by Manuel *et al.* (2007). A biofilm model can be expressed by the following equation, which is based on the assumption that biofilm development reaches a steady state through a balance between growth and detachment (Manuel *et al.*, 2007):

$$\frac{dX_{\text{biofilm}}}{dt} = P - \frac{X_{\text{biofilm}}}{\beta} \quad (2)$$

, where X_{biofilm} is the number of biofilm bacteria per unit surface area, P is the biofilm production rate per unit surface area, which includes the attachment of new cells and cell growth within the biofilm, and β^1 represents the average residence time of cells within the biofilm.

Assuming that P is constant, as demonstrated by Melo and Vieira (1999) as well as Pereira *et al.* (2002), the integration of Equation (2) then leads to

$$X_{\text{biofilm}} = X_{\text{biofilm, max}}(1 - e^{-\beta t}) \quad (3)$$

and

$$X_{\text{biofilm, max}} = \frac{P}{\beta} \quad (4)$$

, where $X_{\text{biofilm, max}}$ is the maximum biofilm density at steady state.

At steady state, the specific biofilm growth rate (μ_{biofilm}) can be expressed as a function of the maximum biofilm density ($X_{\text{biofilm, max}}$) and the biofilm production rate (P) (Manuel *et al.*, 2007):

$$\mu_{\text{biofilm}} = \frac{P}{X_{\text{biofilm, max}}} \quad (5)$$

, which means that at steady state, biofilm growth is equal to biofilm detachment.

Table 2. Growth parameters of biofilm and bulk bacterial population

Parameter	TCC[a]	HPC[b]
$X_{biofilm,\,max}$ (cells/cm^2)	4.7 ± 1.8×10^4	8.2 ± 2.0×10^3
P (cells/cm^2/day)	6.6 ± 1.7×10^3	1.3 ± 0.2×10^3
$\mu_{biofilm}$ (day^{-1})	0.14 ± 0.09	0.16 ± 0.08
μ_{bulk} (day^{-1})	0.06 ± 0.03	0.04 ± 0.05

[a] Total cell counts
[b] Heterotrophic plate counts

The specific bulk cell growth rate (μ_{bulk}) can be calculated by the following equation (Manuel *et al.*, 2007):

$$\mu_{bulk} = \frac{Q(X_{bulk} - X_{in}) - \mu_{biofilm} X_{biofilm,\,max} A}{X_{bulk} V} \quad (6)$$

, where Q is the inlet flow rate, V is the volume of the system, X_{bulk} is the bulk cell number at steady state, X_{in} is the cell number in the inlet water, and A is the internal surface area.

Based on quasi-steady state TCC and HPC values, the growth parameters of the biofilm and bulk bacteria were estimated and are listed in Table 2. The TCC-based $X_{biofilm,\,max}$ value was about 5.8-fold higher than the HPC-based value (Table 2). This may be explained by the fact that the drinking water biofilm harbored a large number of viable but non-culturable cells (Wingender and Flemming, 2011). The HPC-based P value was similarly lower (4.9-fold) than the TCC-based value (Table 2). The estimated $\mu_{biofilm}$ values were 0.14±0.09 day^{-1} and 0.16±0.08 day^{-1}, which corresponded to doubling times of 5.0 and 4.3 days for TCC and HPC values, respectively.

Several studies have determined the specific growth rates of drinking water biofilms under different conditions (van der Kooij *et al.*, 1995; Boe-Hansen *et al.*, 2002a, 2002b; Kasahara *et al.*, 2004; Codony *et al.*, 2005; Manuel *et al.*, 2007). The $\mu_{biofilm}$ has been reported to range from 0.006 to 0.96 day^{-1} for TCC and from 1.13 to 6.48 day^{-1} for HPC. Previous studies have shown that the $\mu_{biofilm}$ of young, immature biofilms tends to be higher than that of old, mature biofilms (Boe-Hansen *et al.*, 2002b; Kasahara *et al.*, 2004; Manuel *et al.*, 2007). According to Boe-Hansen *et al.* (2002a), the higher $\mu_{biofilm}$ of young, immature biofilms is due to bacterial utilization of organic compounds adsorbed on the pipe wall and lower competition for space and nutrients among the bacteria. Compared to the young biofilm, the $\mu_{biofilm}$ of the mature biofilm may vary significantly due to changes in density, community, and nutritional state (Boe-Hansen *et al.*, 2002a).

As summarized in Table 2, the μ_{bulk} values (0.06±0.03 day^{-1} for TCC and 0.04±0.05 day^{-1} for HPC) were much lower than $\mu_{biofilm}$ values, suggesting that biofilm bacteria account for a major part of the bacterial population. A specific set of operating conditions, such as high surface-to-volume ratio, short HRT, and the presence of residual chlorine, would provide an environment that is more favorable to the growth of biofilm bacteria compared to bulk-phase bacteria. The surface-to-volume ratio of the reactor (1.6 cm^{-1}) used in this study was close to that of a 25-mm diameter pipe. It is noteworthy that the larger surface-to-volume ratio in smaller diameter pipes (compared to larger pipes) tends to favor biofilm bacteria growth over bulk bacterial growth (Kasahara *et al.*, 2004). It should also be noted that the reactor was operated at a HRT of 2 h, which was much shorter than the time required for doubling the number of bacterial cells in bulk water (about 13 days for bulk TCC and 18 days for bulk HPC). In general, bulk water bacteria may dominate in distribution systems with long HRT (Srinivasan *et al.*, 2008). In our case, it seemed that detachment of biofilm bacteria accounted for the increase in HPC observed in the bulk phase (Table 2 and Fig. 2), while the growth of bulk-phase bacteria was negligible. In addition, the lower μ_{bulk} of bulk-phase bacteria may be explained by the fact that they are more vulnerable to free chlorine residual compared to biofilm bacteria (Mathieu *et al.*, 2009).

Kinetic properties of biofilm isolates

Eleven phenotypic colonies of biofilm bacteria (denoted by the symbol B, namely B1, B2, B3, etc.) grown on a randomly selected R2A plate were isolated. Figure 3 shows the kinetic properties (μ_{max} and K_S as determined by the Monod equation) of the heterotrophic biofilm-isolated bacterial strains (B1–B11) that were cultured in defined growth media containing various concentrations of acetate. Acetate carbon is a representative AOC compound and is most readily utilized by bacteria for replication. In the Monod model (Equation 1), μ_{max} can be biologically defined as the highest growth rate that a microorganism can obtain using a particular substrate, whereas

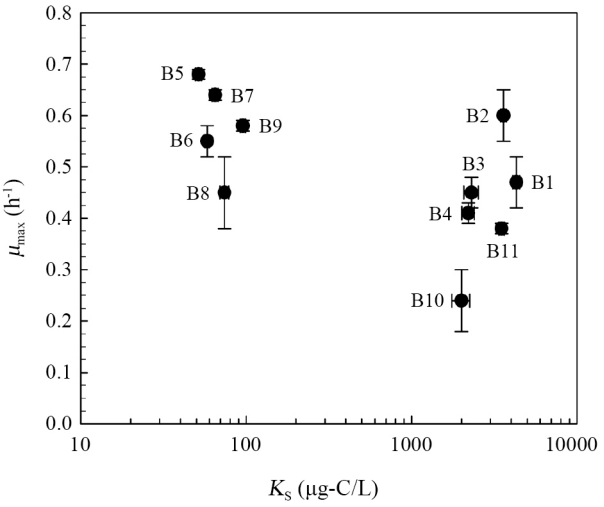

Fig. 3. Kinetic properties of heterotrophic biofilm-isolated bacteria (B1–B11) as determined by growth in the presence of varying amounts of acetate carbon.

K_S represents the microorganism's affinity for the limiting substrate. The observed μ_{max} and K_S values for the biofilm isolates indicated that they exhibit different kinetic properties (Fig. 3). Individual isolates could be distinguished from one another based on their μ_{max} values. Isolate B5, which belongs to the genus *Sphingomonas*, had the highest μ_{max}, whereas B10, belonging to the genus *Methylobacterium*, the lowest. The K_S values of the isolates (B5–B9) that belong to the genus *Sphingomonas* were two orders of magnitude lower for acetate carbon than the K_S values of other isolates. A lower K_S value indicates a higher affinity for a given substrate and, thus, the capability to thrive under nutrient-deficient conditions.

Members of the genus *Sphingomonas* have been commonly isolated from drinking water distribution systems (Koskinen *et al.*, 2000; Srinivasan *et al.*, 2008; McCoy and VanBriesen, 2012). Their presence is known to be associated both with resistance to chlorine disinfectant and with the ability to form biofilms (Vaz-Moreira *et al.*, 2011). The genus *Sphingomonas* has physiological and biochemical traits, such as exopolysaccharide production and antibiotic resistance, which confer an ecological advantage for growth and formation of stable biofilms in oligotrophic water environments (Liu *et al.*, 2012). In this study, based on K_S values (not just presence in a low carbon environment), the five strains affiliated with the genus *Sphingomonas* can be considered as obligate-oligotrophic bacteria. In low-nutrient environments, obligate-oligotrophic bacteria might be the first colonizers, followed by copiotrophs as second colonizers. Therefore any strategy that intends to retard a formation of early biofilm in distribution system should target the obligate-oligotrophic bacteria such as *Sphingomonas*.

Response of biofilm isolates to chlorine

Three isolates (B2, B5, and B10) were chosen for chlorine exposure experiments. This selection was based on variable kinetic properties: high μ_{max} and K_S values (B2), high μ_{max} and low K_S values (B5), and low μ_{max} and high K_S values (B10) (Fig. 3). The responses of these strains to free chlorine disinfectant (0.5 mg/L, 10 min) are presented in Table 3. Isolate B10 showed high resistance to chlorine; only 0.4±0.1 log inactivation was obtained. Isolates B2 and B5 were found to be less resistant to chlorine (log inactivation of 1.9 ± 0.1 and 1.3 ± 0.2, respectively) than B10. These results revealed that even young biofilm was composed of bacterial species with different responses to biocide. A biocide-resistant phenotype allows bacterial cells to form biofilms even in harsh environments, and establishment of a resistant phenotype might be induced by nutrient deficiency, certain types of stress, high cell density, or a combination of these phenomena (Mah and O'Toole, 2001).

In the present study, isolate B10, closely related to the genus *Methylobacterium*, was highly resistant to chlorine (Table 3). Hiraishi *et al.* (1995) reported that 28 strains of *Methylobacterium* out of 30 isolated from potable water tanks were resistant to treatment with chlorine (0.1 mg/L for 5 min). The occurrence of such a large fraction (93%) of resistant strains was attributed to their capacity for generating energy by photophosphorylation, allowing them to thrive under oligotrophic environments such

Table 3. Response of biofilm-isolated heterotrophic bacteria to free chlorine

Isolate[a]	Kinetic property[b]		Survival (CFU/ml)[c]		Log inactivation
	μ_{max}	K_S	Before	After	
B2	High	High	$6.3 \pm 1.1 \times 10^5$	$7.6 \pm 1.1 \times 10^3$	1.9 ± 0.1
B5	High	Low	$5.8 \pm 1.8 \times 10^5$	$3.1 \pm 0.4 \times 10^4$	1.3 ± 0.2
B10	Low	High	$6.5 \pm 2.1 \times 10^5$	$2.6 \pm 0.4 \times 10^5$	0.4 ± 0.1

[a] Closest match of each isolates were *Pseudomonas* (B2), *Sphingomonas* (B5), and *Methylobacterium* (B10) with highest similarities of 99.0%, 94.5%, and 96.4%, respectively
[b] Kinetic properties were based on the results shown in Fig. 3
[c] Exposed to 0.5 mg/L of free chlorine for 10 min

as potable water. However, the mechanism for acquiring chlorine resistance by this genus has yet to be answered. We propose that the high resistance of strain B10 (*Methylobacterium*) to chlorine (0.5 mg/L for 10 min) was due to their slow growth rate (Fig. 3). Such a property would result in the observed resistance to chlorine, as supported by the finding that slow-growing cells have been documented to be less susceptible to a variety of antimicrobial agents compared to fast-growing cells (Lewis, 2001; Morató *et al*., 2003).

In summary, we identified biofilm bacteria play a greater role in bacterial production than bulk-phase bacteria in a model system based on the comparison of growth parameters. Culture-based kinetic studies of biofilm bacteria revealed that *Sphingomonas* (low K_S) and *Methylobacterium* (low μ_{max}) were well adapted to the oligotrophic environment of drinking water and highly resistant to chlorine treatment, respectively. Our results provide a better understanding of multi-species bacterial biofilms that develop within a few days and identify target species for the development of prevention strategies in drinking water systems.

적 요

본 연구에서는 염소 소독제를 함유한 수돗물을 수리학적 체류시간 2시간 수준으로 공급한 모델 상수관망에서 형성된 초기 생물막의 생장에 대해 연구하였다. PVC slide 표면에 형성된 세균 생물막의 비생장률(specific growth rate, μ)은 총세균수와 종속영양세균수 기준으로 각각 0.14 ± 0.09 day^{-1}와 0.16 ± 0.08 day^{-1}로 측정되었으며, 생물막 형성 정도는 실험 개시 10일 후에 각각 3.1×10^4 $cells/cm^2$와 6.6×10^3 CFU/cm^2에 이르렀다. Bulk-phase 세균에 비해 훨씬 높은 생물막 형성 세균의 비생장률(μ)은 관망내에서 생물막 세균의 증식이 세균 재생장의 주된 요인으로 작용함을 의미하였다. 분리 배양된 생물막 균주들은 acetate 농도를 달리한 생장배지에서 얻어진 Monod 모델에서 특징적인 μ_{max}와 K_S값을 보여주었다. 가장 낮은 μ_{max}값을 보여준 *Methylobacterium* 균주는 느린 생장을 통해 염소 소독제 처리(0.5 mg/L, 10분간)에 대해 높은 내성을 나타내었다. 반포화상수(half-saturation constant) K_S값은 *Sphingomonas* 균주에서 다른 분리 균주들에 비해 100배 정도 낮게 측정되어 기질친화도가 매우 높게 나타났다. 이는 수돗물과 같이 영양물질의 농도가 매우 낮은 조건에서 생존할 수 있도록 적응된 절대 빈영양성 세균의 특징으로 판단된다. 비록 특징적인 μ_{max}와 K_S값을 보이는 균주 만을 대상으로 수행되었지만, 이상의 결과는 상수관망에서 초기에 형성되는 복합 세균종으로 구성된 생물막에 대한 이해와 조절에 도움을 줄 수 있을 것으로 기대된다.

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