Journal of Microbiology (2018) Vol. 56, No. 12, pp. 902–909
DOI 10.1007/s12275-018-8336-4
eISSN 1976-3794
pISSN 1225-8873

Antibiofilm effect of biofilm-dispersing agents on clinical isolates of *Pseudomonas aeruginosa* with various biofilm structures

Soo-Kyoung Kim, Xi-Hui Li, Hyeon-Ji Hwang, and Joon-Hee Lee*

Department of Pharmacy, College of Pharmacy, Pusan National University, Busan 46241, Republic of Korea

(Received Jun 20, 2018 / Revised Aug 16, 2018 / Accepted Aug 21, 2018)

***Pseudomonas aeruginosa*, an opportunistic human pathogen, causes many biofilm-mediated chronic infections. In this study, biofilm structures of various clinical strains of *P. aeruginosa* isolated from hospitalized patients were examined and their influence on the biofilm-dispersing effects of chemicals was investigated. The clinical isolates formed structurally distinct biofilms that could be classified into three different groups: 1) mushroom-like, 2) thin flat, and 3) thick flat structures. A dispersion of these differently structured biofilms was induced using two biofilm-dispersing agents, anthranilate and sodium nitroprusside (SNP). Although both SNP and anthranilate could disperse all types of biofilms, the thick flat biofilms were dispersed less efficiently than the biofilms of other structures. This suggests that biofilm-dispersing agents have higher potency on the biofilms of porous structures than on densely packed biofilms.**

***Keywords*:** *Pseudomonas aeruginosa*, clinical isolates, biofilm, biofilm dispersion, sodium nitroprusside, anthranilate, biofilm structure

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic human pathogen that causes a range of infections on the lungs, cornea, and burn wound sites, and the persistent colonization of *P. aeruginosa* via biofilm formation often aggravates many diseases in the human body, such as cystic fibrosis, and causes considerable losses in many industrial facilities (Kumar and Anand, 1998; Costerton *et al.*, 1999; Hancock and Speert, 2000; Willcox *et al.*, 2008).

Most chronic infections by *P. aeruginosa* are closely related to biofilm formation (Costerton *et al.*, 1999). Biofilms are a sessile life mode originating from the attached growth of microbes on the surfaces, and biofilm formation provides microbial cells with many advantages, such as easy uptake of nutrients, protection from a physical force, and strong survivability (Parsek and Greenberg, 2005; Kim and Lee, 2016). In addition, biofilms are extremely resistant to antimicrobial treatment and host immunity because they are embedded in a matrix composed of extracellular polymeric substances (EPS), such as polysaccharides, extracellular DNA (eDNA), and proteins that protect the bacterial cells from environmental stresses. Moreover, biofilm-mediated infections cause persistent and prolonged chronic diseases (Karatan and Watnick, 2009). Many indwelling medical devices have been shown to harbor biofilms, resulting in an increase in device-related nosocomial infections (Donlan, 2001). Therefore, the control of biofilms is a very important issue in medicine, public health, and industry. Thus far, a range of strategies have been attempted to control biofilms, of which the induction of biofilm dispersion is one of the most promising (Li and Lee, 2017).

A biofilm develops gradually from initially surface-attached cells to a mature structured biofilm and the last developmental stage is the dispersion of cells from the biofilm, which enables the cells to move away and restart a new cycle of biofilm development (Sauer *et al.*, 2002; Kim and Lee, 2016). Biofilms are harmful reservoirs of pathogens that threaten public health because they release bacterial cells to environment continuously by repeating this cycle; the shed cells move to distal sites for further colonization. On the other hand, in terms of biofilm elimination, it would be useful to artificially induce biofilm dispersion. In *P. aeruginosa*, biofilm dispersion is regulated by various intracellular or environmental factors, such as nutritional condition, production of biosurfactants, cell-to-cell signaling, iron availability, and intracellular level of cyclic diguanylate (c-di-GMP), which is a critical signal molecule that regulates the transition between biofilm and planktonic life modes (Sauer *et al.*, 2004; Simm *et al.*, 2004; Banin *et al.*, 2006; Schleheck *et al.*, 2009; Kim and Lee, 2016). Recent studies have shown that biofilm dispersion can be induced artificially by a treatment with chemicals, such as matrix-degrading enzymes, nitric oxide (NO)-generators, and anthranilate (Li and Lee, 2017). For example, the treatment of *P. aeruginosa* biofilms with sodium nitroprusside (SNP), a NO generator, decreased the c-di-GMP level and induced biofilm dispersion (Barraud *et al.*, 2009). Anthranilate has also been reported to induce the dispersion of pre-formed bacterial biofilms by reducing c-di-GMP and modifying extracellular polysaccharide production and motility (Kim *et al.*, 2015; Li *et al.*, 2017).

Because these results for biofilm dispersion have mostly been obtained from standard laboratory strains, it is unclear if these biofilm-dispersing agents can actually be applied to diverse biofilms of strains detected in the environment or clinics. Indeed, previous studies have shown that the clinical isolates

*For correspondence. E-mail: joonhee@pusan.ac.kr; Tel.: +82-51-510-2821; Fax: +82-51-513-6754

of *P. aeruginosa* produce different amounts of biofilm despite having identical genomic profiles and highly diverse biofilms in biomass, morphology, and drug-resistance (Head and Yu, 2004; Lee *et al.*, 2005; Lima *et al.*, 2017). In this study, *P. aeruginosa* clinical isolates were also found to form structurally diverse biofilms (Fig. 1). Therefore, this study examined whether two biofilm-dispersing agents, anthranilate and SNP, can effectively inhibit biofilm formation in *P. aeruginosa* clinical isolates that form structurally distinct biofilms. These results showed that although both SNP and anthranilate could disperse all types of biofilm, the thick flat biofilms were dispersed less efficiently, suggesting that biofilm-dispersing agents have better potency on biofilms with a porous structure than on densely packed biofilms.

Materials and Methods

Bacterial strains and culture conditions

The *P. aeruginosa* clinical strains used in this study were isolated from Korean patients in the Pusan Paik Hospital, Busan, South Korea (Jung *et al.*, 2010). Five clinical isolates were selected according to their biofilm structure, and PAO1 (wild type), *Dpel* mutant, and *DwspF* mutant of *P. aeruginosa* were used as the reference strains (Table 1). All *P. aeruginosa* strains were grown in Luria-Bertani broth (LB; yeast 5 g/L, bacto-tryptone 10 g/L, NaCl 5 g/L) at 37°C with vigorous shaking. Growth was measured by the optical density at 600 nm (OD_{600}).

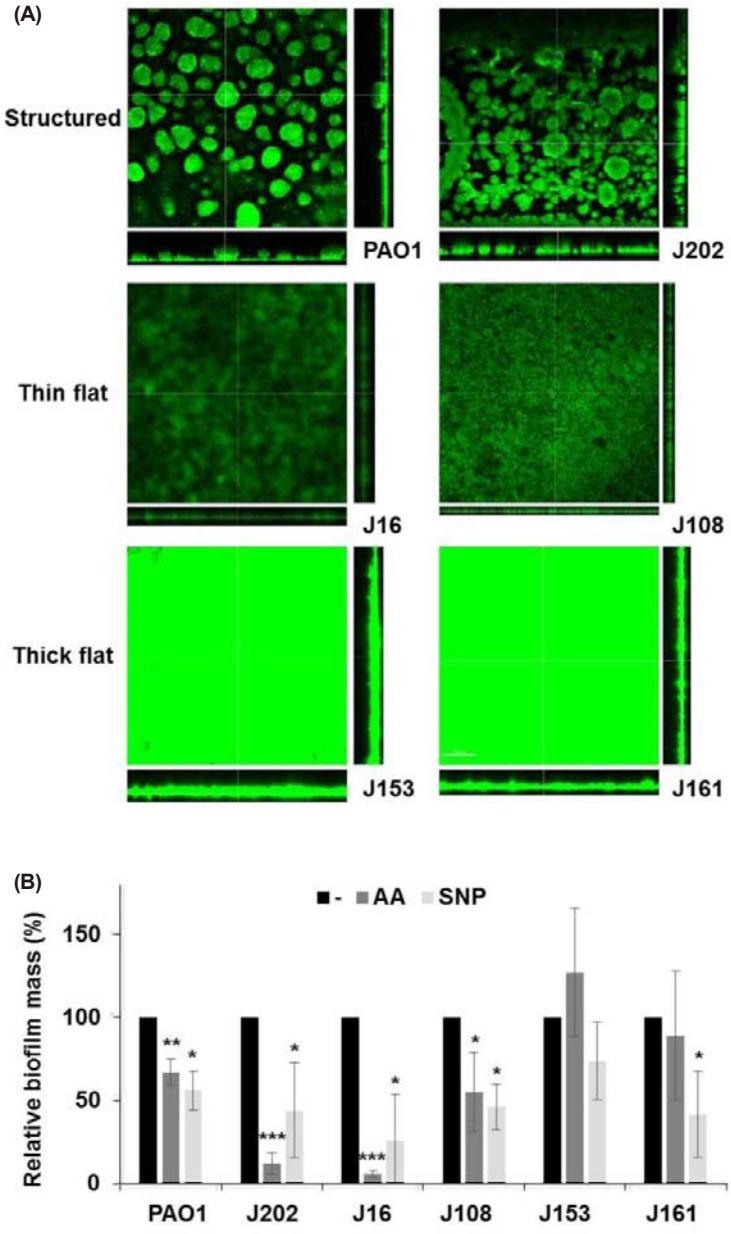

Fig. 1. Different biofilm structures of *P. aeruginosa* clinical isolates. (A) clinical isolates (J202, J16, J108, J153, and J161) of *P. aeruginosa* and a control strain (PAO1) were grown to form a biofilm for 6 days in a flow-cell and then stained with 0.1% SYTO 9 to allow CLSM observations. The biofilm images are presented with views from the top and two cross sections, respectively. The biofilms were classified as mushroom-like structured (structured), thick flat, and thin flat. (B) biofilm formation by the clinical isolates was measured using a static biofilm assay in 96-well plates after a treatment with either 0.1 mM anthranilate (AA) or 5 μM SNP. *P*-values, * < 0.05; ** < 0.005; *** < 0.0005.

Table 1. List of strains

Strains	Characteristics	Source or Reference
PAO1	*P. aeruginosa* wild type	Pearson *et al.* (1997)
J202	*P. aeruginosa* clinical isolate	Jung *et al.* (2010)
J16	*P. aeruginosa* clinical isolate	Jung *et al.* (2010)
J108	*P. aeruginosa* clinical isolate	Jung *et al.* (2010)
J153	*P. aeruginosa* clinical isolate	Jung *et al.* (2010)
J161	*P. aeruginosa* clinical isolate	Jung *et al.* (2010)
Dpel	In-frame deletion of *pelA*	Chung *et al.* (2008)
DwspF	In-frame deletion of *wspF*	Chung *et al.* (2008)

Static biofilm assay

A static biofilm assay was carried out, as described elsewhere (Kim *et al.*, 2015). Briefly, *P. aeruginosa* strains were grown to OD_{600} = 3 in LB broth and diluted to OD_{600} = 0.06 in fresh M63 medium [M63 salt (KH_2PO_4 12 g/L, K_2HPO_4 28 g/L, NH_4SO_4 8 g/L), 1 mM $MgSO_4$, 0.5% casamino acid, 0.2% citrate] on 96-well polystyrene plates. Anthranilate or SNP was added at 0.1 mM or 5 μM, respectively, and then incubated at 37°C for 24 h without shaking. After measuring cell growth by the OD_{600}, the planktonic cells were poured out and the plate was washed with water and dried. Subsequently, 180 μl of crystal violet (0.1%, w/v) was added to each well and incubated for 7 min to stain the biofilms attached to the well surface. After a brief wash, the biofilm-stained crystal violet was dissolved in 200 μl of absolute ethanol. The absorbance was then measured at 600 nm (A_{600}) to determine the amount of crystal violet, which was normalized to cell growth (OD_{600}).

Biofilm formation in flow-cell system

For flow-cell biofilm formation, *P. aeruginosa* cells were grown overnight and diluted to OD_{600} = 0.1 in LB, 200 μl of which was injected into a flow-cell chamber (2 mm × 2 mm × 50 mm). Initially, the cells were incubated for 1 h in the absence of flow to allow attachment and 1% tryptic soy broth (Bacto™ Tryptic Soy Broth, BD) was then flowed through the flow-cell chamber at 200 μl/min. To determine the biofilm structure, biofilms in the flow-cell were grown for 5–6 days at room temperature and visualized by 10 min-staining with 0.1% SYTO 9 (Life Technologies). For biofilm dispersion analysis, the biofilms were formed for 5 days in a flow-cell without an anthranilate or SNP treatment, and the pre-formed biofilms were treated with 0.1 mM anthranilate or 5 μM SNP for 1 day or 90 min, respectively (Barraud *et al.*, 2009; Kim *et al.*, 2015; Li *et al.*, 2017). The biofilm of the *ΔwspF* mutant was grown for two different times, 4–5 days for the intermediate structure and 6 days for the fully grown structures, and anthranilate or SNP was then added. The biofilms were then stained with SYTO 9 (Jung *et al.*, 2015; Li *et al.*, 2017) and observed by confocal microscopy.

Biofilm imaging and quantification

Biofilm images were obtained using confocal laser scanning microscopy (CLSM; Olympus, FV1000) after SYTO 9-staining. The excitation and emission wavelengths were 488 nm and 500 nm, respectively. Three-dimensional images of the biofilms were reconstructed from plane images using Bitplane Imaris 6.3.1 image analysis software. Quantification of the biofilms was performed using AutoQuant software.

Statistical analysis

The data were analyzed statistically using a student's *t*-test (two-sample assuming equal variances) in MS office Excel (Microsoft). A P-value < 0.05 was considered significant.

Results

Clinical isolates formed structurally distinct biofilms

In a previous study, a number of *P. aeruginosa* clinical strains were analyzed isolated; 74 of them were analyzed for virulence-related phenotypes, including biofilm-forming ability (Jung *et al.*, 2010). The results showed that these clinical isolates have different abilities for biofilm formation and their biofilms can be structurally diverse. To better characterize the biofilm architecture, the biofilm structures of the clinical isolates were examined in a flow-cell system, which showed that the biofilm structure was divided largely into three types: mushroom-like structured, thick flat, and thin flat biofilms (Fig. 1A). To determine the efficacy of biofilm dispersing agents on these biofilms with different structures, two strains for each structural type of biofilm were selected: wild type PAO1 and J202 for the structured biofilm, J16 and J108 for the thin flat biofilm, and J161 and J153 for the thick flat biofilm. The biofilm of the J202 strain exhibited a mushroom-like structure and was similar to that of PAO1, whereas the J16 and J108 strains formed flat and thin biofilms (Fig. 1A). The biofilms of the J161 and J153 strains were also flat but much thicker than those of J16 and J108 (Fig. 1A).

Biofilm formation by the *P. aeruginosa* clinical strains was inhibited by biofilm-dispersing agents to a different extent depending on their structure

After confirming the biofilm structures of the clinical isolates, biofilm inhibition was examined using two biofilm-dispersing agents, anthranilate and SNP, in the absence of shear force. When the clinical isolates were incubated in a static biofilm system and treated with either anthranilate or SNP, the structured and thin flat biofilms formed by PAO1, J202, J16, and J108 were well inhibited (Fig. 1B). The anthranilate treatment reduced the J202, J16, and J108 biofilm masses by 88.0%, 94.3%, and 44.9%, respectively, and SNP reduced the corresponding biofilm masses by 55.9%, 74.3%, and 54.0% (Fig. 1B). The biofilm formation of wild type PAO1 was decreased by 33.0% and 43.9% with the anthranilate and SNP treatments, respectively (Fig. 1B). On the other hand, the thick flat biofilms of J153 and J161 were either not inhibited or less inhibited by these dispersing agents. Anthranilate failed to reduce the biofilm mass of J153 and J161 in the static system (Fig. 1B). SNP also failed to reduce the biofilm mass of J153, whereas it could reduce the biofilm mass of J161 by 58.4% (Fig. 1B). This suggests that the biofilm-dispersing agents may work less efficiently in thick flat biofilms.

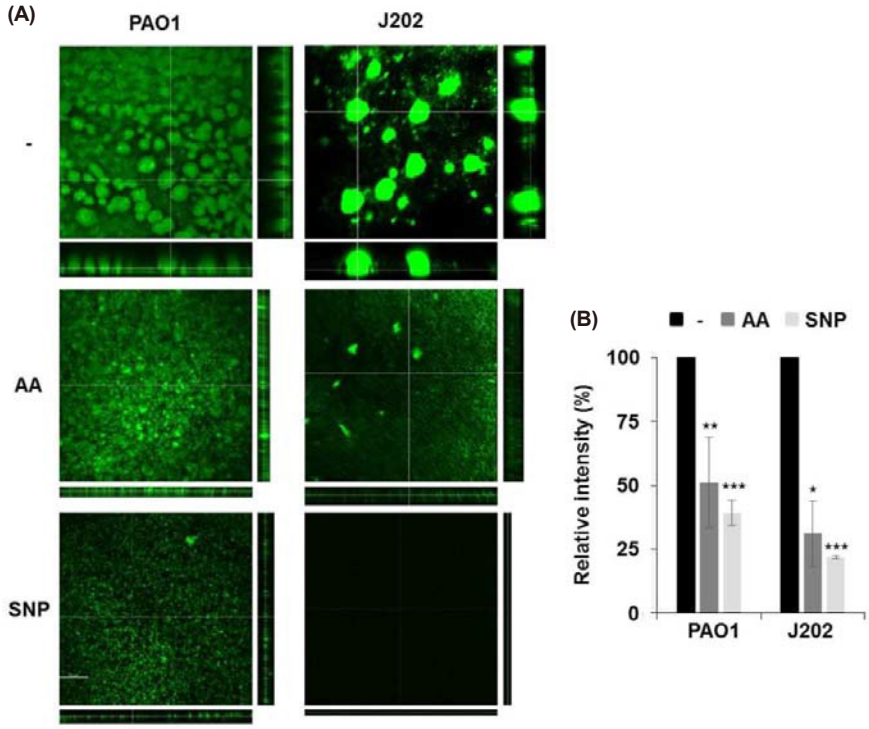

Fig. 2. Dispersion of mushroom-structured biofilms by anthranilate or SNP in a flow cell system. (A) the structured biofilms of PAO1 and J202 were formed for 5 days, and then treated with 0.1 mM anthranilate (AA) or 5 μM SNP for 1 day or 90 min, respectively. The remaining biofilms were stained with 0.1% SYTO 9 and observed by CLSM. (B) the fluorescence intensity was quantified using AutoQuant software and is reported as a relative value (untreated = 100%). *P*-values, * < 0.05; ** < 0.005; *** < 0.0005.

Pre-formed biofilms of clinical isolates were dispersed to a different extent depending on their structure

The application of biofilm-dispersing agents was assessed by testing their ability to remove the pre-formed biofilms of clinical stains by dispersion in the presence of a shear force. Each structural type of biofilm (structured, thin flat, and thick flat) was formed in a flow-cell system for 5 days and the pre-formed biofilms were treated with either anthranilate or SNP. The structured PAO1 and J202 biofilms were well dispersed by both anthranilate and SNP. Anthranilate reduced the PAO1 and J202 biofilm mass by 49.0% and 68.8%, respectively (Fig. 2). SNP dispersed them efficiently with 60.8% and 78.1% of the PAO1 and J202 biofilms

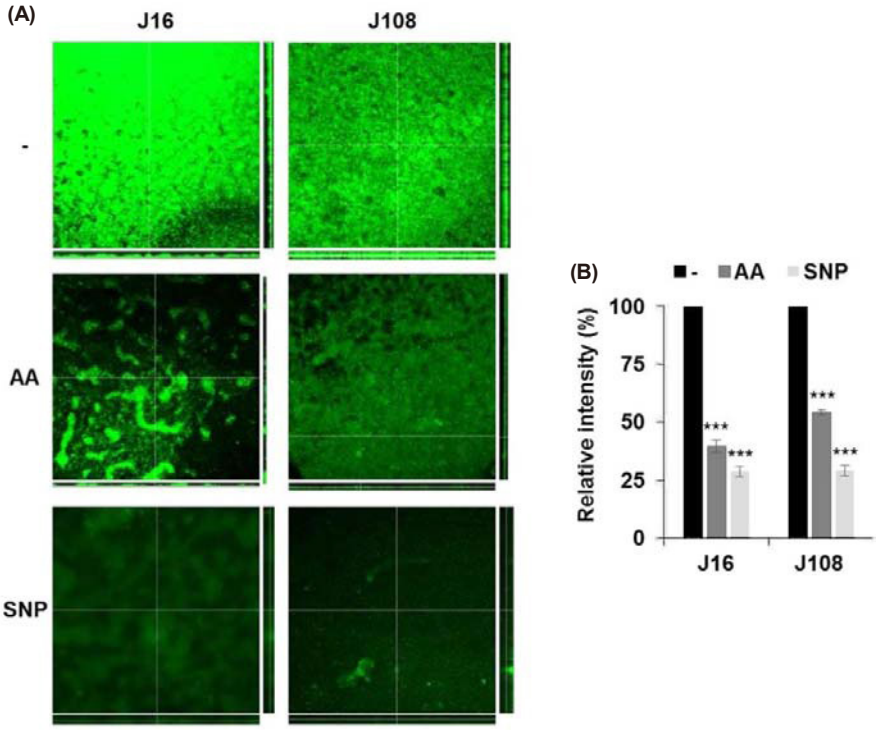

Fig. 3. Dispersion of the thin flat biofilms in a flow cell system. (A) thin flat biofilms of J16 and J108 were formed for 5 days and then treated with either 0.1 mM anthranilate (AA) or 5 μM SNP for 1 day or 90 min, respectively. The remaining biofilms were stained and observed by CLSM. (B) the fluorescence intensity was quantified and is reported as a relative value. *P*-value, *** < 0.0005.

being removed, respectively (Fig. 2). The thin flat biofilms of J16 and J108 were removed more efficiently by dispersion. Anthranilate removed 60.3% and 45.8% of the J16 and J108 biofilm mass, respectively (Fig. 3). SNP removed 71.3% and 70.9% of the pre-formed J16 and J108 biofilms, respectively (Fig. 3).

Unlike the static biofilm system, in a flow-cell system with a shear force, thick flat biofilms were usually dispersed by these agents but to a lesser extent. When the thick flat J153 and J161 biofilms were pre-formed and treated with either anthranilate or SNP, anthranilate decreased the J153 and J161 biofilm mass by 27.2% and 36.8%, respectively, and SNP reduced the J161 biofilm mass by 62.2% but failed to reduce the J153 biofilm mass (Fig. 4). These flow-cell results also suggest that although the shear force can somehow help induce dispersion, the dispersing effect by anthranilate and SNP is still less efficient in the thick flat biofilm structure.

Differential effects due to structure were also observed in the mutant strains of a known structure

P. aeruginosa mutant strains, which form biofilms similar in structure to the structural types above, were prepared to confirm the influence of the biofilm structure on the dispersing effect. A mutation of the *wspF* gene of *P. aeruginosa* resulted in the activation of diguanylate synthase and an increase in intracellular c-d-GMP levels, which causes the *wspF* mutant to form a thick flat biofilm (Hickman *et al.*, 2005). The *pel* gene of *P. aeruginosa* encodes Pel, an extracellular polysaccharide that functions as a mature biofilm, and the *pel* mutant forms a thin flat biofilm (Colvin *et al.*, 2011). The biofilm structures of the *wspF* and *pel* mutants were tested and their thick flat and thin flat biofilm structures, respectively, were confirmed (Fig. 5). On the other hand, while the *wspF* mutant formed a thick flat biofilm when fully grown, until then, the intermediate form of biofilm had a slight mushroom shape (Fig. 5). In the early stages, a valley-like structure is formed temporally between the mushroom stalks, but the valleys are filled with excess extracellular polysaccharide as the biofilm grows because the *wspF* mutant produces a high level of extracellular polysaccharides (Hickman *et al.*, 2005).

Therefore, the *wspF* mutant was grown for different times to form two different biofilm structures and the intermediate mushroom-structured and fully grown thick flat biofilms were prepared. A thin flat biofilm was also prepared with the *pel* mutant and the pre-formed biofilms were treated with anthranilate and SNP. Anthranilate dispersed the pre-formed intermediate biofilm of the *wspF* mutant by 28.4% and the pre-formed thin flat biofilm of the *pel* mutant by 68.3% (Fig. 5A and B). SNP also dispersed the intermediate biofilm of the *wspF* mutant by 37.6% and the thin flat biofilm of the *pel* mutant by 74.2% (Fig. 5A and B). On the other hand, the fully grown thick flat biofilm of *wspF* mutant was less dispersed by anthranilate and SNP, where biofilm reduction was 18.3% and 13.7%, respectively (Fig. 5A and B).

To compare the biofilm dispersion efficiency depending on the biofilm structure, the biofilm mass reduction rate by biofilm dispersion was averaged according to the biofilm structure. When all the biofilm mass reductions by anthranilate and SNP measured in a flow-cell system were combined, the degree of biofilm dispersion was significantly different according to the biofilm structure, as shown in Fig. 6, in which the thick flat biofilms were less dispersed by the biofilm-dispersing agents. Overall, these results suggest that anthranilate and SNP can disperse biofilms of various *P. aeruginosa* clinical strains, but there is a difference in the dispersing effectiveness depending on the structure of the bio-

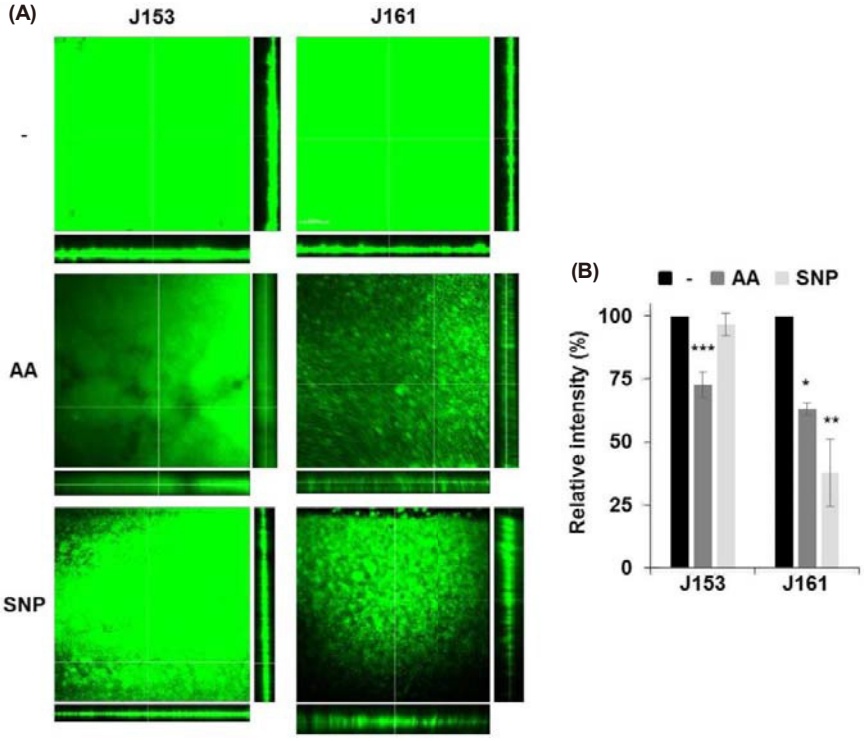

Fig. 4. Dispersion of the thick flat biofilms. (A) thick flat biofilms of J153 and J161 were formed for 5 days and treated with either 0.1 mM anthranilate (AA) or 5 μM SNP for 1 day or 90 min. The remaining biofilms were stained and observed by CLSM. (B) the fluorescence intensity was quantified and is reported as a relative value. *P*-values, * < 0.05; ** < 0.005; *** < 0.0005.

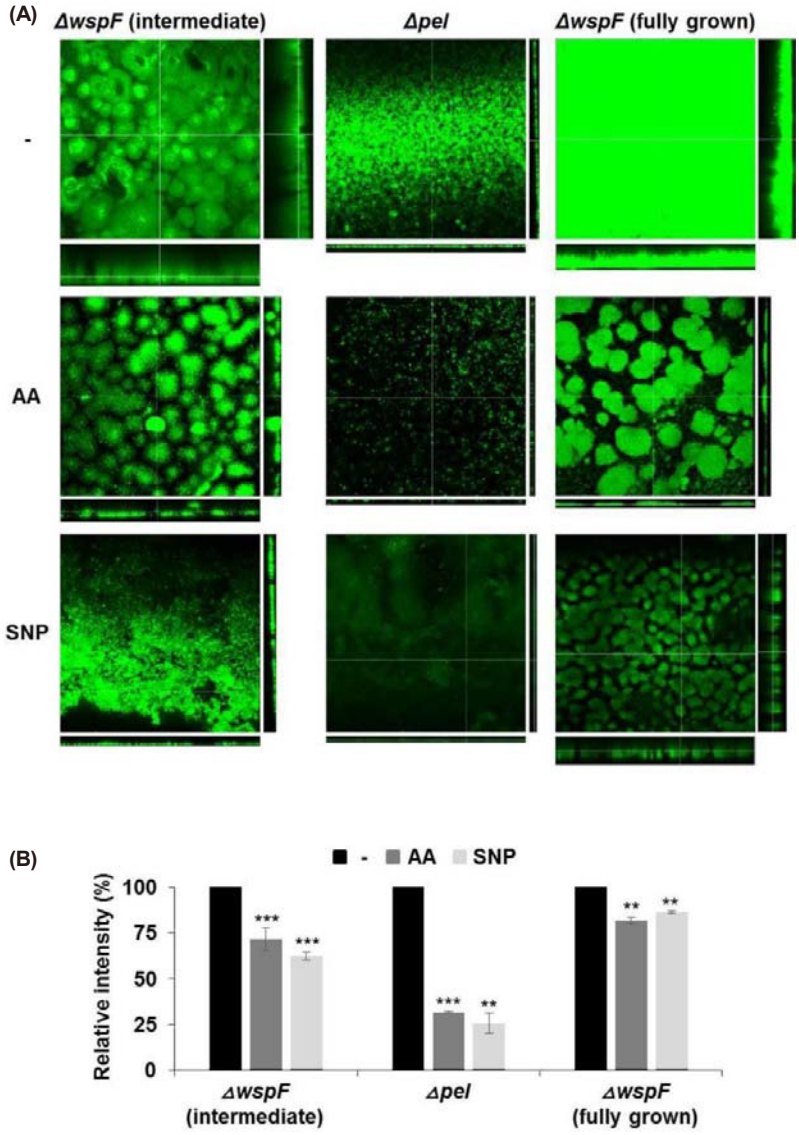

Fig. 5. Dispersion of the mutant strains with known biofilm structures. (A) *pel* and *wspF* mutants (*Δpel* and *ΔwspF*) were grown in a flow-cell system to form biofilms. The *pel* mutant was grown for 5 days and treated with either 0.1 mM anthranilate (AA) or 5 μM SNP for 1 day or 90 min, respectively. The *wspF* mutant was grown for 6 days to form a fully grown biofilm but grown for a shorter time (4–5 days) to form an intermediate biofilm. These biofilms were then treated with 0.1 mM anthranilate or 5 μM SNP for 1 day or 90 min, respectively. After treatment, the biofilms were stained with SYTO 9 and observed by CLSM. (B) the fluorescence intensity was quantified and given as a relative value. *P*-values, ** < 0.005; *** < 0.0005.

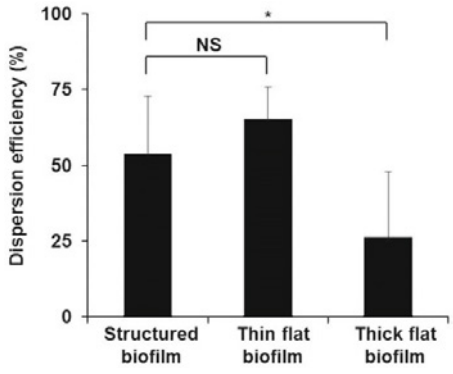

Fig. 6. Dispersion efficiency depending on the biofilm structure. The biofilm mass reduction rate by biofilm dispersion was averaged according to the biofilm structure. All the biofilm mass reductions by anthranilate and SNP measured in a flow-cell system were combined and the dispersion efficiency was calculated as the percentage reduction in biofilm mass (100 × [(non-treated biofilm mass - treated biofilm mass) / non-treated biofilm mass]). *P*-value, * < 0.05

film; they have a better effect on biofilms with a porous structure than densely packed biofilms.

Discussion

The *P. aeruginosa* clinical strains isolated from patients produced several different types of biofilm structures. Although the biofilm of *P. aeruginosa* has a mushroom-like structure and is regarded as a universal biofilm model, the flat biofilm of *P. aeruginosa* has been reported to be formed under some conditions (Klausen *et al.*, 2003; Kirisits and Parsek, 2006; Kim and Lee, 2016). Attention has been paid to this difference in biofilm structures, in which the biofilm structures have been classified into two types, "structured" and "flat", based on laboratory studies, and it has been suggested that there is a link between the structure and physiological properties of biofilms (Kirisits and Parsek, 2006). The flat biofilms were thought to be more susceptible to antimicrobials,

but no studies have examined the physiological differences among thick flat, thin flat, and structured biofilms that the authors are aware of. In this study, an attempt was made to identify the differences in artificially induced biofilm dispersions according to the biofilm structure using clinical strains of *P. aeruginosa*. Why these clinical strains form biofilms with different structures is unclear. The most likely reason is the difference in extracellular polysaccharide production. To determine whether there is a difference in the extracellular polysaccharide production among the clinical strains, the extracellular polysaccharide was stained with Congo red which can bind to a wide range of polysaccharides (Spiers *et al.*, 2003; Ghafoor *et al.*, 2011; Jung *et al.*, 2015; Li *et al.*, 2017). Although this Congo red staining showed some difference in the extracellular polysaccharide production among the clinical strains, it did not show a significant correlation with the biofilm structure (data not shown). We believe that the biofilm structure is the result of comprehensive involvement of various factors as well as extracellular polysaccharides.

Biofilm development is regulated in several stages and dispersion normally takes place at the final stage, in which the bacteria actively escape from the biofilm matrix and return to their free planktonic life mode (Kim and Lee, 2016). On the other hand, the induction of a biofilm dispersion is considered a promising method for eradicating harmful biofilms because it can be induced artificially at any point in time using biofilm-dispersing agents. The problem with using dispersion for biofilm control is that biofilms found in clinics and the environment are quite diverse and have unique structural and physiological properties (Head and Yu, 2004; Lee *et al.*, 2005; Lima *et al.*, 2017). Moreover, it is unclear whether knowledge of the effectiveness of biofilm-dispersing agents obtained from a laboratory strain can actually be applied to clinical biofilms. For this reason, in this study, the biofilm-dispersing abilities of anthranilate and SNP were evaluated using *P. aeruginosa* clinical isolates of diverse biofilm structures. When three distinct structural types of biofilm were treated with anthranilate or SNP, while all types could be dispersed, the thick flat biofilm of the clinical strains was less dispersed than the biofilms with mushroom-like or thin flat structures. Although it is unclear whether there is an intrinsic difference in the biofilm physiology of *P. aeruginosa* clinical isolates, these results show that the dispersion efficiency of biofilm-dispersing agents is affected to some extent by the biofilm structure. Because thick flat biofilms are not as porous as mushroom-like structured biofilms, anthranilate and SNP barely penetrate the thick flat biofilms, which suggests that extracellular polysaccharides in the biofilm block the penetration of antimicrobials and confer resistance to biofilms (Davies, 2003).

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIP) (NRF-2016R1A2B4014963). For Soo-Kyoung Kim, this work was also supported by the NRF grant funded by the Korea Government (MSIP) (2016R1C1B1006671).

References

Banin, E., Brady, K.M., and Greenberg, E.P. 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl. Environ. Microbiol.* **72**, 2064–2069.

Barraud, N., Schleheck, D., Klebensberger, J., Webb, J.S., Hassett, D.J., Rice, S.A., and Kjelleberg, S. 2009. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J. Bacteriol.* **191**, 7333–7342.

Chung, I.Y., Choi, K.B., Heo, Y.J., and Cho, Y.H. 2008. Effect of PEL exopolysaccharide on the *wspF* mutant phenotypes in *Pseudomonas aeruginosa* PA14. *J. Microbiol. Biotechnol.* **18**, 1227–1234.

Colvin, K.M., Gordon, V.D., Murakami, K., Borlee, B.R., Wozniak, D.J., Wong, G.C.L., and Parsek, M.R. 2011. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog.* **7**, e1001264.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. 1999. Bacterial biofilms: A common cause of persistent infections. *Science* **284**, 1318–1322.

Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* **2**, 114–122.

Donlan, R.M. 2001. Biofilms and device-associated infections. *Emerg. Infect. Dis.* **7**, 277–281.

Ghafoor, A., Hay, I.D., and Rehm, B.H. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl. Environ. Microbiol.* **77**, 5238–5246.

Hancock, R.E. and Speert, D.P. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and impact on treatment. *Drug Resist. Updat.* **3**, 247–255.

Head, N.E. and Yu, H.W. 2004. Cross-sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: Biofilm formation, virulence, and genome diversity. *Infect. Immun.* **72**, 133–144.

Hickman, J.W., Tifrea, D.F., and Harwood, C.S. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc. Natl. Acad. Sci. USA* **102**, 14422–14427.

Jung, K.J., Choi, Y., Ha, C., Shin, J.H., and Lee, J.H. 2010. Analysis of quorum sensing-related phenotypes of *Pseudomonas aeruginosa* clinical isolates. *Korean J. Microbiol.* **6**, 240–247.

Jung, Y.G., Choi, J., Kim, S.K., Lee, J.H., and Kwon, S. 2015. Embedded biofilm, a new biofilm model based on the embedded growth of bacteria. *Appl. Environ. Microbiol.* **81**, 211–219.

Karatan, E. and Watnick, P. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol. Mol. Biol. Rev.* **73**, 310–347.

Kim, S.K. and Lee, J.H. 2016. Biofilm dispersion in *Pseudomonas aeruginosa*. *J. Microbiol.* **54**, 71–85.

Kim, S.K., Park, H.Y., and Lee, J.H. 2015. Anthranilate deteriorates the structure of *Pseudomonas aeruginosa* biofilms and antagonizes the biofilm-enhancing indole effect. *Appl. Environ. Microbiol.* **81**, 2328–2338.

Kirisits, M.J. and Parsek, M.R. 2006. Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? *Cell. Microbiol.* **8**, 1841–1849.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S., and Tolker-Nielsen, T. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48**, 1511–1524.

Kumar, C.G. and Anand, S.K. 1998. Significance of microbial biofilms in food industry: A review. *Int. J. Food Microbiol.* **42**, 9–27.

Lee, B., Haagensen, J.A., Ciofu, O., Andersen, J.B., Hoiby, N., and Molin, S. 2005. Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J. Clin. Microbiol.* **43**, 5247–5255.

Li, X.H., Kim, S.K., and Lee, J.H. 2017. Anti-biofilm effects of anthranilate on a broad range of bacteria. *Sci. Rep.* **7**, 8604.

Li, X.H. and Lee, J.H. 2017. Antibiofilm agents: A new perspective for antimicrobial strategy. *J. Microbiol.* **55**, 753–766.

Lima, J., Alves, L.R., Paz, J., Rabelo, M.A., Maciel, M.A.V., and Morais, M.M.C. 2017. Analysis of biofilm production by clinical isolates of *Pseudomonas aeruginosa* from patients with ventilator-associated pneumonia. *Rev. Bras. Ter. Intensiva* **29**, 310–316.

Parsek, M.R. and Greenberg, E.P. 2005. Sociomicrobiology: The connections between quorum sensing and biofilms. *Trends Microbiol.* **13**, 27–33.

Pearson, J.P., Pesci, E.C., and Iglewski, B.H. 1997. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* **179**, 5756–5767.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* **184**, 1140–1154.

Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A., Davies, D.G., and Gilbert, P. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J. Bacteriol.* **186**, 7312–7326.

Schleheck, D., Barraud, N., Klebensberger, J., Webb, J.S., McDougald, D., Rice, S.A., and Kjelleberg, S. 2009. *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. *PLoS One* **4**, e5513.

Simm, R., Morr, M., Kader, A., Nimtz, M., and Romling, U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* **53**, 1123–1134.

Spiers, A.J., Bohannon, J., Gehrig, S.M., and Rainey, P.B. 2003. Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. *Mol. Microbiol.* **50**, 15–27.

Willcox, M.D., Zhu, H., Conibear, T.C., Hume, E.B., Givskov, M., Kjelleberg, S., and Rice, S.A. 2008. Role of quorum sensing by *Pseudomonas aeruginosa* in microbial keratitis and cystic fibrosis. *Microbiology* **154**, 2184–2194.