The Journal of Microbiology (2011) Vol. 49, No. 4, pp. 663-668
DOI 10.1007/s12275-011-1013-5

The Activity of a Small Lytic Peptide PTP-7 on *Staphylococcus aureus* Biofilms

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(Received January 10, 2011 / Accepted March 29, 2011)

One of the most important features of bacterial biofilms is their resistance to antibiotics and to the host immune system. In this study, we have found that a small lytic peptide, PTP-7, is very potent to Gram-positive bacteria and is able to kill antibiotic sensitive and resistant *Staphylococcus aureus* indiscriminately. Further studies have revealed that despite being a cationic peptide, the antibacterial activity of PTP-7 was not affected by the negatively charged extracellular polymeric substance (EPS) of biofilms. PTP-7 could diffuse into the deep layer of *S. aureus* biofilms to kill bacteria inside biofilms efficiently and effectively. Neither the high concentrations of metal ions nor the acidic pH in biofilms affected the activity of peptide PTP-7. It seems that the unique sequence/structure together with the resistant bacteria killing ability of peptide PTP-7 confers its anti-biofilm activity. This study sheds new light on the treatment of bacterial biofilms, especially various biofilm related infections.

***Keywords*:** peptide, biofilm, antimicrobial activity, *Staphylococcus aureus*, bacterial infections

It is estimated that over 95% of bacteria existing in nature are in biofilms (Mack, 2004). The solid-liquid interface between body fluids (e.g., blood) and the surfaces of teeth, tissues, and implanted devices provides an ideal environment for bacterial attachment and colonization. Formation of biofilm by pathogenic bacteria on tissues or indwelling devices often results in various infections. For example, biofilms now account for over 85% of implants associated infections (Costerton *et al*., 2007). Principal implants that can be compromised by biofilm infections include dental implants, central venous catheters, heart valves, ventricular assist devices, fracture-fixation devices, inflatable penile implants, breast implants, and cochlear implants.

One of the most important features of bacterial biofilms is their resistance to antibiotics and to the host immune system (Mack, 2003, 2004; Costerton *et al*., 2007). Now it is known that the resistance of bacteria in biofilms to antibiotics can be attributed to several factors including the expression of resistant genes, low growth rate of bacteria in biofilms, diffusion difficulty of antibiotics in biofilm extracellular matrix, and the inactivation of antibiotics by enriched metal ions and acidic pHs in biofilms (Lewis, 2008). Currently available antibiotics only kill metabolically active bacteria present on biofilm surfaces, but do not affect the cells in the core of biofilms (Mack, 2003, 2004; Costerton *et al*., 2007). Bacteria inside biofilms can exhibit up to 1,000 times higher resistance to antibiotics than planktonic bacteria.

Lytic peptides are a group of antimicrobial peptides which are widely distributed in nature and have been preserved throughout evolution from bacteria to mammals as effective defense mechanisms (Mack, 2004; Xiong *et al*., 2005). The general mode of action of lytic peptides involves binding to negatively charged lipopolysaccharide (LPS) moieties on the microbial membrane. Once adequate aggregates have formed, these peptides destabilize the lipid head groups by formation of multimeric pores, which disturb the cellular membrane (Beckloff *et al*., 2007). Since both the binding and acting sites of lytic peptides are restricted to cell membranes, lytic peptides are active on antibiotic-sensitive and antibiotic-resistant cells (Pascual, 2002; Walters III *et al*., 2003), and bacterial resistance to these peptides can hardly be developed (Zasloff, 2002). In this study, we reported the anti-biofilm ability of a small lytic peptide, PTP-7, a synthetic analogue from Gaegurin 5 (Tu *et al*., 2009).

Materials and Methods

Peptide synthesis

Peptide PTP-7 (FLGALFKALSKLL) was synthesized by GenScript (USA). The purity (>94%) of peptide was analyzed by HPLC and electrospray ionization mass spectrometry. The peptide powder (30 mg) was dissolved in 0.5 ml dimethyl sulfoxide (DMSO) and then diluted with water to form 5.0 mM stock solutions. Peptide stock solution was stored in aliquots at -20°C until used.

Bacterial strains and culture media

S. aureus (ATCC 29213, 25923, and 43300) were purchased from American type culture collection (ATCC, USA). All Staphylococcal bacteria were cultured in tryptic soy broth (Sigma, USA) supplemented with 0.2% Glucose (TSBG). For each experiment, an isolated single bacterial colony was picked from the agar plate and transferred to 5-10 ml of TSBG medium and incubated under orbital agitation (100-150 rpm) for 18-24 h at 37°C. In addition to TSBG, phenol red free DMEM:F12 (50:50) medium (Cellgro, USA) was also used for *S. aureus* in some experiments.

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Reagents and solutions

A buffer containing 0.2% bovine serum albumin (BSA) and 0.01% acetic acid was used in minimal inhibitory concentration (MIC) assay. A 5% MTT (Methylthiazolyldiphenyl-tetrazolium bromide, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) stock solution was prepared in phosphate buffered saline (PBS, pH=7.2) and was diluted to 0.5% (w/v) with water for biofilm susceptibility assay. A LIVE/DEAD staining kit for staining biofilms was purchased from Invitrogen Life Technologies (USA). All other reagents, if not mentioned, were purchased from Sigma Chemical Laboratory (USA).

Determination of MIC and MBC

PTP-7 stock solution was serially diluted and samples of each concentration (10 μl/well) were added in the wells of 96-well tissue-culture-treated plates (Costar, USA), followed by the addition of bacterial cell suspensions (90 μl/well) (final concentrations of PTP-7 were in the range of 1-64 μM). The bacteria was used at a final concentration of $\sim 2\times10^6$ CFU/ml in TSBG or DMEM:F12 medium. The MIC of peptide was determined by measuring the absorbance of each well at 570 nm after 20 h of incubation at 37°C. To determine the minimal bactericidal concentration (MBC), an aliquot of 100 μl from one dilution above and one dilution below the MIC well was obtained, serially diluted (up to 1:10,000) in TSBG, and then placed on tryptic soy agar plates. Formed bacterial colonies were counted after 24 h of incubation at 37°C.

Biofilm growth and biomass assay

Overnight culture of *S. aureus* was diluted in TSBG to 2×10^6 cells/ml. Cell suspensions were then inoculated in either 96-well flat bottom cell culture plates (polystyrene) or LabTek 8-well cover-glass chambers and incubated at 37°C for 4 h. After washed to remove planktonic and loosely adhered cells, the 96-well plates or the LabTek 8-well cover-glass chambers were fed with fresh TSBG and cultured for up to 4 days with medium change every day. Among the three *S. aureus* strains we tested, penicillin resistant *S. aureus* (ATCC 29213) could grow into biofilms (15-20 μm in thickness) with typical biofilm structures and developed antibiotic resistance after 24 h incubation.

The biomass of biofilms was estimated through a well established crystal violet (CV) staining assay (Peeters, 2008). After the removal of culture medium at the end of incubation, formed biofilms were carefully washed with PBS three times to remove planktonic bacteria. Biofilms were fixed with methanol for 15 min at room temperature and then stained with 0.1% (w/v) crystal violet for 5-10 min. The excess of the crystal violet was removed by rinsing the plate with water thoroughly until the control wells became colorless. The crystal violet dye associated with biofilms was then extracted by 33% glacial acetic acid and quantified by measuring the absorbance values at 570 nm using microplate reader.

Biofilm susceptibility assays

Due to the difficulties in harvesting bacteria from formed biofilms and the presence of extracellular matrix, traditional methods such as colony-forming unit (CFU) counting can hardly be applied. A MTT test has been widely used in viability assay of mammalian cells (Pascual, 2002). The principle of MTT assay is based on the reduction of MTT by metabolically active cells to form an insoluble formazan product (dark purple). Our lab has applied MTT method in examining the viability of bacteria in biofilms to evaluate the biofilm susceptibility to antibiotic treatments. Briefly, at the end of antibiotic treatment and after the removal of culture medium and planktonic cells, formed biofilms in 96-well plates were incubated with 0.5 mg/ml MTT at 37°C for 15 min. In order to minimize bacteria/biofilm growth during the incubation and to eliminate the influence of some components existing in TSBG medium, DMEM:F12 but not TSBG medium was used in this assay. After washing, the formazan formed inside biofilms was dissolved by DMSO and measured using a microplate reader by setting the detecting and reference wavelengths at 570 nm and 630 nm, respectively. Unlike crystal violet staining assay, results from MTT assay reflect the viability of bacteria in biofilms.

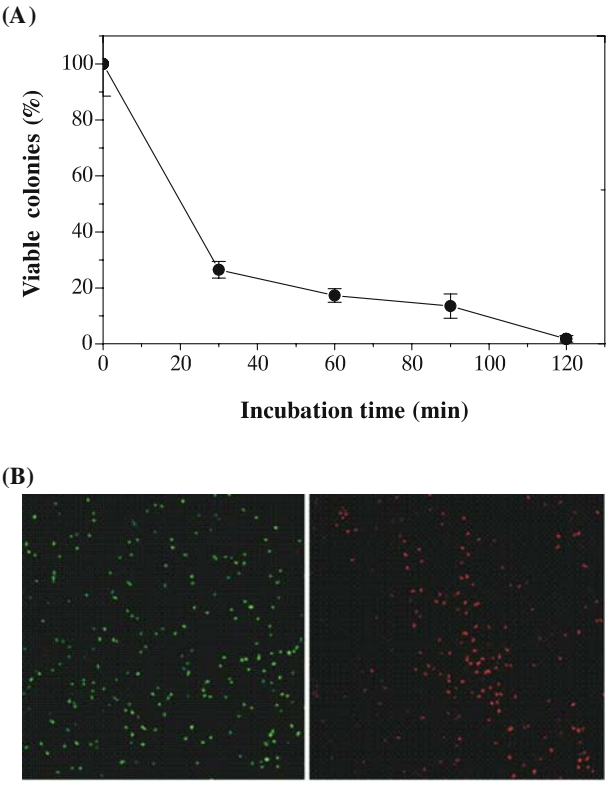

Fig. 1. (A) The killing kinetics of PTP-7 (8.0 μM) tested on planktonic *S. aureus* (ATCC 29213); (B) Confocal images of untreated (left) and 8.0 μM PTP-7 treated (30 min) *S. aureus* cells stained using the LIVE/DEAD staining kit.

Live/dead bacteria distributions in biofilms

Distributions of live and dead bacteria in biofilms were visualized under confocal laser scanning microscopy using a LIVE/DEAD staining kit from Invitrogen (USA). To facilitate this confocal microscopy assay, *S. aureus* biofilms were grown on LabTek 8-well cover-glass chambers for two days in order to obtain biofilms with the same structures as these grown on polystyrene 96-well plates (Fig. 1). After washing, biofilms formed in the glass chambers were treated with peptide PTP-7 or streptomycin at 37°C for 2 h. At the end of incubation, biofilms were washed with saline solution before stained for 15 min using LIVE/DEAD staining kits. A Zeiss Confocal Laser Scanning Microscope (CLSM 510) was used to visualize stained bacteria in biofilms. Images were obtained using a 40× oil immersion objective and analyzed using the Zeiss LSM Image Browser.

Zeta potential measurement

The Zeta potential and the size of peptide PTP-7 in saline solution

(pH=7.2) were measured using a Malvern ZetaSizer (Worcestershire, UK).

Results and Discussion

Lethality of peptide PTP-7 to antibiotic resistant *S. aureus*

The antibacterial activity of peptide PTP-7 was tested on three *S. aureus* strains, antibiotic-sensitive *S. aureus* (ATCC 25923), penicillin-resistant *S. aureus* (ATCC 29213), and methicillin/penicillin resistant *S. aureus* (ATCC 43300) by comparison with two antibiotics, penicillin and streptomycin. As expected, penicillin had much higher (32-64) MIC values on penicillin-resistant and methicillin-resistant *S. aureus* than on penicillin-sensitive *S. aureus* (Table 1). In contrast, peptide PTP-7 and streptomycin were highly active on all tested *S. aureus* strains and were able to kill antibiotic-sensitive and -resistant (both penicillin- and methicillin-resistant) *S. aureus* effectively and indiscriminately.

It is known that antibiotics, especially some antimicrobial peptides, may have their activity nullified in the presence of tryptone, proteins, or high concentration of salts (Sheng and Stewart, 2002; Beckloff *et al*., 2007). To examine the stability of PTP-7 in solutions, the antibacterial activity of peptide PTP-7 was tested in two media, TSBG and DMEM:F:12, containing completely different ingredient (amino acids, proteins, and salts) compositions. Interestingly, although penicillin and streptomycin showed altered antibacterial activities in two different media, neither the MIC nor the MBC values of peptide PTP-7 was affected (Table 1), confirming the stability of PTP-7 in solutions with varied salt and bio-macromolecule concentrations. Results from kinetic studies revealed that there was a bi-exponential decline in viability of *S. aureus* during PTP-7 treatments characterized by a rapid initial phase (0-30 mins) followed by a slow second phase (30 min-2 h) as measured by CFU counting (Fig. 1A). Although it took more than 2 h to achieve complete killing, almost all *S. aureus* cells were stained red within a very short period of time (<30 min) as demonstrated in confocal microscopy assays using LIVE/DEAD staining kit (Fig. 1B). This result suggested that cell membrane damage was an early event in peptide PTP-7 induced *S. aureus* death, and the antibacterial activity of PTP-7 was mainly from PTP-7 caused cell lysis in bacteria. This is consistent with the finding that peptide PTP-7 has almost identical MIC and MBC values under all experimental conditions (Table 1)

The anti-biofilm activity of peptide PTP-7

It is known that bacteria in biofilms live in an extreme environment with enriched bio-macromolecules (proteins, DNA, and polysaccharides) and high salt concentrations (Lewis, 2008). Inactivation of antibiotics by bio-macromolecules inside biofilms and the development of genetical and physiological variants have been considered as two major reasons that lead to the resistance of biofilms to antibiotic treatments. The high stability and resistant bacteria killing ability of PTP-7 (Table 1) imply its possible anti-biofilm capability.

Table 1. The activity of PTP-7 tested on planktonic *S. aureus* cells

	MIC (MBC), μM		
	25923	29213	43300
TSBG medium			
PTP-7	4 (8)	4 (8)	4 (8)
Penicillin G	4 (4)	32 (>64)	>64 (>64)
Streptomycin	4 (4)	4 (4)	8 (16)
DMEM:F-12 medium			
PTP-7	2 (4)	2 (4)	4 (8)
Penicillin G	1 (1)	4 (4)	>64 (>64)
Streptomycin	0.25 (0.5)	0.25 (0.25)	0.5 (0.5)

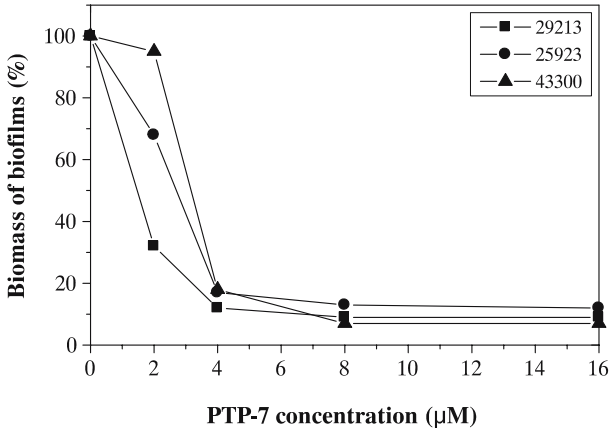

Fig. 2. PTP-7 inhibited biofilm growth from *S. aureus. S. aureus* cells were mixed with PTP-7 of different concentrations and incubated on 96-well plates for 24 h. The biomass of biofilms was determined by crystal violet staining. Data represents the mean of three independent tests.

We first tested PTP-7's ability in inhibiting biofilm formation. *S. aureus* was mixed with PTP-7 of different concentrations and then incubated at 37°C for 24 h. Biofilm formation in wells of 96-well plates was estimated at the end of incubation and after removal of planktonic bacterial cells. As expected, peptide PTP-7 showed strong anti-biofilm activity (Fig. 2). Biofilm formation and growth from all three *S. aureus* stains were completely inhibited when PTP-7 peptide concentrations were kept at MIC or higher.

We further examined the activity of PTP-7 on preformed biofilms. One-day old biofilms from penicillin-resistant *S. aureus* (ATCC 29213) with typical biofilm structures (Fig. 3A) and developed antibiotic resistance were used (Fig. 3C). Based on results from kinetic studies (Fig. 1A), the viability of *S. aureus* in biofilms was examined after the incubation of biofilms with different concentrations of PTP-7 at 37°C for 2 h. As shown in Fig. 3C, PTP-7 demonstrated a dose-dependent killing activity to *S. aureus* in biofilms. At peptide concentration of 10-fold of MIC, the number of live *S. aureus* in biofilms was decreased by more than three-log (>99.9% killing). The anti-biofilm activity of PTP-7 at concentrations higher than 10-fold MIC were not tested because of the formation of peptide aggregates. No aggregation was found if PTP-7 concentrations were lower than 80 μM as demonstrated in a peptide size measurement using ZetaSizer (data not shown).

Since streptomycin had demonstrated the same capability as that of PTP-7 in killing antibiotic resistant *S. aureus* (Table 1), it was used as a control in the anti-biofilm studies. However, unlike PTP-7, streptomycin showed limited activity on pre-

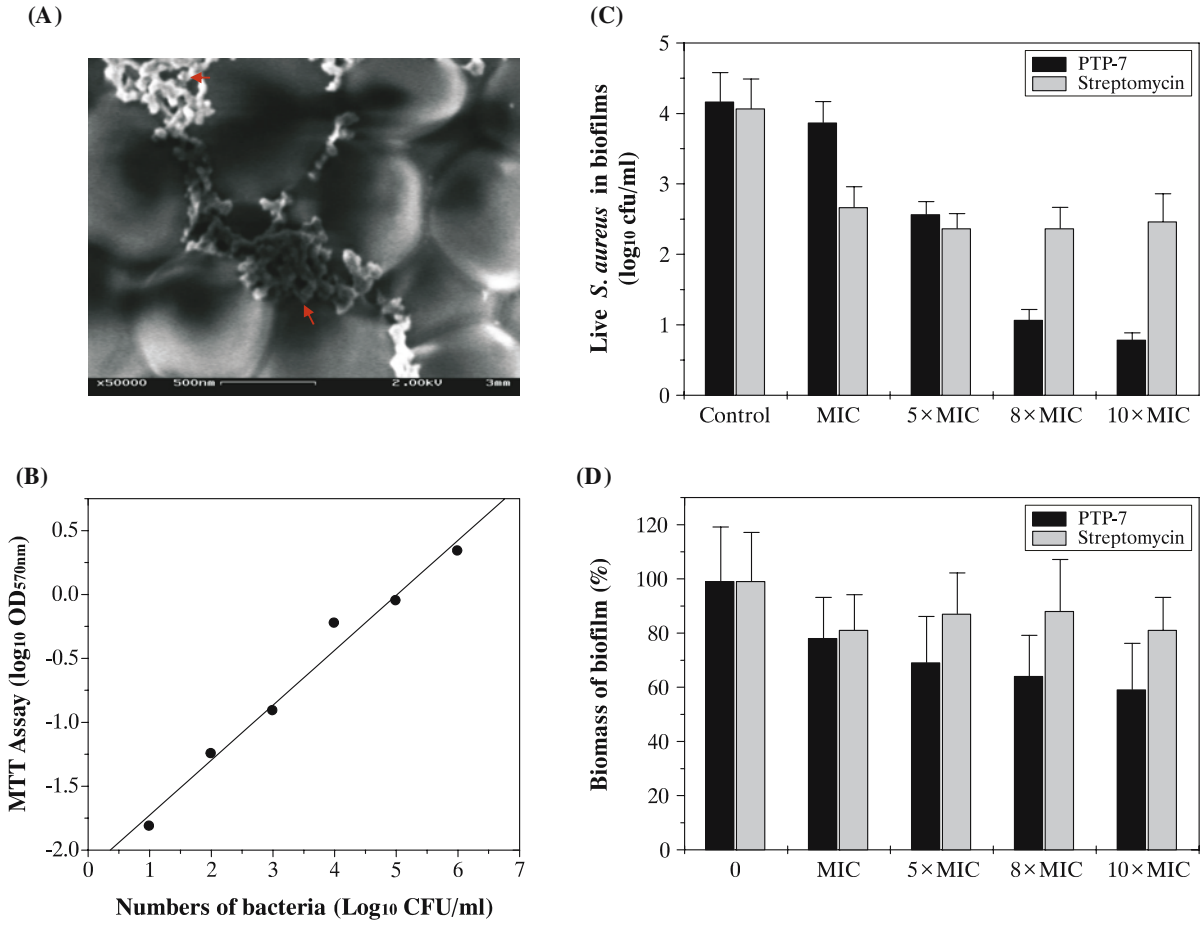

Fig. 3. (A) The SEM images of one-day old *S. aureus* (ATCC 29213) biofilms. Extracellular polymeric matrix was indicated by arrows. (B) A representative correlation between CFU counting and bacterial viability assay using MTT method. (C) The viability of *S. aureus* in biofilms treated by PTP-7 or streptomycin for 2 h as measured using MTT assay. (D) The biomass of biofilms treated by PTP-7 or streptomycin for 2 h as measured using crystal violet staining. Data represents the mean and SD of three independent tests.

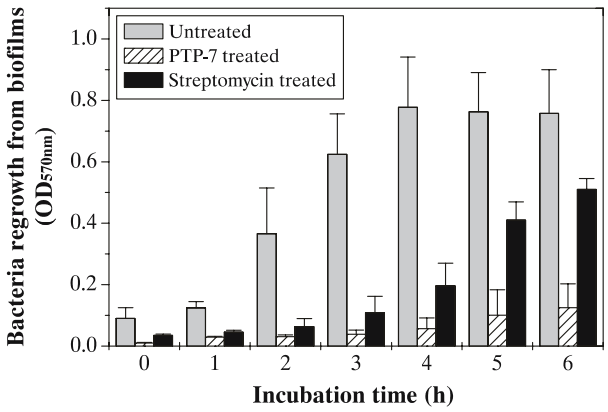

Fig. 4. *S. aureus* (ATCC 29213) biofilms grew on 96-well plates were treated with PTP-7 or streptomycin at a concentration of 8×MIC for 2 h. After washed, biofilms in wells of 96-well plates were fed with fresh TSBG medium and incubated at 37°C for up to 6 h. Bacteria and biofilm re-growth from treated biofilms was monitored by recording absorbance changes at 570 nm. Data represents the mean and SD of three independent tests.

formed *S. aureus* in biofilms (Fig. 3C). We did not see any further improved anti-biofilm effects as the concentrations of streptomycin were increased from 5×MIC to 10×MIC and above. The number of live *S. aureus* in biofilms was decreased by less than two-log (~97% killing) at 10×MIC concentration of streptomycin. It was also interesting to find that slight biomass loss was observed in PTP-7 but not streptomycin treated *S. aureus* biofilms (Fig. 3D). It seemed that streptomycin killed *S. aureus* in biofilms *in situ* while peptide PTP-7 at high concentrations demonstrated weak biofilm dispersion ability in addition to its anti-biofilm activity. Data collected from biofilm re-growth experiments supported the results from MTT assay (Fig. 4). Significantly delayed bacteria/biofilm re-growth was observed in PTP-7 treated biofilms after the peptide was withdrawn.

It is known that extracellular polymeric substance (EPS) secreted by bacteria is the main structural constituent of the extracellular matrix in biofilms. The presence of EPS has been considered as a diffusion barrier to antimicrobial agents, especially positively charged antibiotics, by affecting their diffusion in biofilms and uptake by bacteria (Costerton *et al.*, 2007). PTP-7 (FLGALFKALSKLL) is a positively charged

Table 2. Activity of PTP-7 tested on planktonic *S. aureus* (29213) cells under various conditions

	Control	Alginate (μg/ml)			NaCl (mM)			$MgCl_2$ (mM)			pH	
		50	100	500	50	100	500	2	4	10	7.5	5.5
MIC (μM)	4	4	4	4	4	4	4	4	4	4	4	2

peptide carrying two net positive charges. The measured zeta potential of PTP-7 in saline solution (pH=7.2) was +8.5 mV, suggesting that PTP-7 is a moderate positive molecule in solutions with physiological pH. However, alginate, the negatively charged EPS isolated from *P. aeruginosa* biofilms, did not show the expected inhibitory effects on the antibacterial activity of PTP-7 (Table 2). In addition to alginate, several negatively charged synthetic polymers including heparin and poly(acrylic acid) were also tried, and none of them demonstrated significant effects on the antibacterial activity of PTP-7 (data not shown). These results imply that peptide PTP-7 might have very weak or no interactions with the EPS of biofilms. To further reveal the anti-biofilm mechanism of PTP-7, *S. aureus* in biofilms were stained using the LIVE/DEAD staining kit and visualized under confocol microscopy. Propidium iodide (red fluorescence color) is a cell membrane impermeable nucleic acid probe thus only staining dead bacteria with damaged cell membranes. On the contrary, another nucleic acid probe in the LIVE/DEAD staining kit, SYTO 9 (green fluorescence color), is permeable to healthy cell membranes and thus bacteria with both damaged (dead) and intact (live) cell membranes are stained green. Images included in Fig. 5 showed the distributions of live and dead *S. aureus* in treated biofilms. Dead bacteria (red in color) were observed throughout the entire architecture of biofilms treated by PTP-7. This result is consistent with data obtained from the peptide-EPS interaction study (Table 1) and suggests the free diffusion of PTP-7 peptide in *S. aureus* biofilms. It has been observed that the diffusion coefficient of some small molecules through the biofilm extracellular matrix is roughly equivalent to that of water (Dunne *et al*., 1993; Jefferson *et al*., 2005).

It is also known that cells of the same microbial species can live in extremely different physiological environments in a biofilm because both the pH and the concentration of metal ions differ significantly in different regions of a biofilm (Costerton *et al*., 1999; Vroom *et al*., 1999; Welin *et al*., 2003). For examples, the pHs decrease quite remarkably over the short distances from the surface to the center of a biofilm, and the average pH value inside a *S. aureus* biofilm is around 6.5 (Costerton *et al*., 1999; Vroom *et al*., 1999). Despite very good diffusion coefficients, a significant number of antibiotics have been found to be inactive in the environments inside biofilms (Dunne *et al.*, 1993; Pascual, 2002; Walters III *et al.*, 2003; Jefferson *et al.*, 2005). Stable antibacterial activity of PTP-7 at acidic pHs (Table 2) and in solutions with varied compositions (Table 1) and metal ion concentrations (Table 2) ensures its activity to bacteria in biofilms.

It was interesting that the majority of *S. aureus* in streptomycin treated biofilms were still alive (green in color) (Fig. 5). Obviously, despite its high activity on antibiotic resistant *S. aureus* (Table 1), streptomycin only affected some of *S. aureus* in biofilms. This result is consistent with previous findings that most small molecule antibiotics only kill metabolically active bacteria but do not affect persisters in biofilms (Mack, 2003, 2004; Costerton *et al*., 2007). Since PTP-7 killed bacteria through the unique membrane-acting mechanism by causing cell lysis (Fig. 1), PTP-7 could maintain strong antibacterial activity to genetical and physiological variants of *S. aureus* present in biofilms. Therefore, the unique sequence/structure and high stability together with the resistant bacteria killing ability of PTP-7 confers its anti-biofilm ability.

Biofilm-associated bacteria can exhibit up to 1,000 times higher resistance to antibiotics than planktonic bacteria (Mack, 2003, 2004; Costerton *et al*., 2007). This often leads to the failure of conventional antibiotic therapy in bacterial infections (Chuard *et al*., 1991). *Staphylococcus aureus* is one of the most common etiological agents of hospital- and community-acquired infections (Madhuri Shireen *et al*., 2009). This study sheds light on applying antibacterial peptides in the treatment of biofilm-related various infections in which conventional antibiotic therapies have failed. In addition to biofilm killing ability, another unbeatable advantage of antibacterial peptide over conventional small molecule antibiotics is that bacterial resistance to peptides can hardly be developed (Zasloff, 2002).

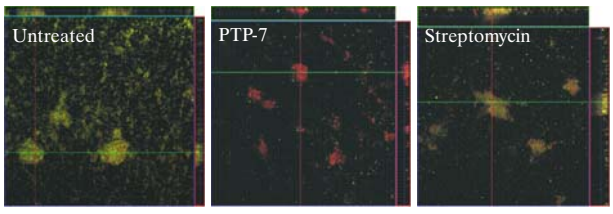

Fig. 5. *S. aureus* (ATCC 29213) biofilms grew on LabTek 8-well cover- glass chambers were treated with PTP-7 (middle) or streptomycin (left) at a concentration of 8×MIC for 2 h. *S. aureus* in biofilms were then stained using the LIVE/DEAD staining kit and visualized under cofocal microscopy. Merged images were produced using Zeiss LSM Image Browser software.

Acknowledgements

Thanks for the help from Jinwoo Park in acquiring biofilm SEM images and Christian Traba in preparing MTT-CFU standard curve. This work was supported by NIH grant GM081874 and AI072748.

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