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Streptomyces-derived actinomycin D inhibits biofilm formation by *Staphylococcus aureus* and its hemolytic activity

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ABSTRACT

Staphylococcus aureus is a versatile human pathogen that produces diverse virulence factors, and its biofilm cells are difficult to eradicate due to their inherent ability to tolerate antibiotics. The anti-biofilm activities of the spent media of 252 diverse endophytic microorganisms were investigated using three *S. aureus* strains. An attempt was made to identify anti-biofilm compounds in active spent media and to assess their anti-hemolytic activities and hydrophobicities in order to investigate action mechanisms. Unlike other antibiotics, actinomycin D (0.5 μg ml^{-1}) from *Streptomyces parvulus* significantly inhibited biofilm formation by all three *S. aureus* strains. Actinomycin D inhibited slime production in *S. aureus* and it inhibited hemolysis by *S. aureus* and caused *S. aureus* cells to become less hydrophobic, thus supporting its anti-biofilm effect. In addition, surface coatings containing actinomycin D prevented *S. aureus* biofilm formation on glass surfaces. Given these results, FDA-approved actinomycin D warrants further attention as a potential antivirulence agent against *S. aureus* infections.

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Actinomycin D; biofilm; hemolysis; hydrophobicity; *Staphylococcus aureus*

Introduction

The long-term use of antibiotics has generated multidrug resistant bacteria. *Staphylococcus aureus* is a versatile pathogen of humans and animals that is responsible for many outbreaks of nosocomial infections, and often exhibits antibiotic resistance (Lowy 1998). *S. aureus* preferentially attaches to various surfaces, producing a self-produced extracellular polymeric matrix (a biofilm) and cells within these biofilms are difficult to eradicate with antibiotics because of their inherent antibiotic tolerance, defense systems, and external stresses (Costerton et al. 1999). Furthermore, subinhibitory concentrations of several antibiotics often increase biofilm formation (Hoffman et al. 2005; Linares et al. 2006; Kaplan et al. 2012). Since conventional antimicrobial treatments are usually unsuccessful at eradicating biofilms, new antimicrobial agents are urgently required to control *S. aureus* biofilms.

Diverse mechanisms and environmental cues contribute to biofilm formation by *S. aureus*. For example, quorum sensing, c-di-GMP, protease, DNase, cis-2-decenoic acid, D-amino acids, phenol-soluble polypeptides, and pH change can all affect biofilm formation by *S. aureus* (O'Gara 2007; Boles & Horswill 2011; Arciola et al. 2012; Otto 2013). In addition, α-toxin produced by *S. aureus* causes hemolysis, and contributes to biofilm formation (Caiazza & O'Toole 2003) and cell surface hydrophobicity, which may also play a role (Pagedar et al. 2010).

In natural niches, bacteria coexist in multispecies communities and compete with each other for resources and space. As a result, many bacteria have developed the ability to adapt to environmental niches. For example, actinobacteria produce antibacterials (Mahajan & Balachandran 2012), antifungals (Gupte et al. 2002), and a number of useful anti-tumor drugs (Flick & Gifford 1984). Recently, some actinomycete species were reported to produce *S. aureus* biofilm inhibitors. Methanol extracts of coral-associated *Streptomyces akiyoshiensis* (Bakkiyaraj & Pandian 2010), extracellular proteases from *Streptomyces* sp. BFI 250 and *Kribbella* sp. BFI 1562 (Park, Lee, Kim et al. 2012), alnumycin D from *Streptomyces albus*, granaticin B from *Streptomyces violaceoruber* (Oja et al. 2015), and streptorubin B from *Streptomyces* sp. MC11204 (Suzuki et al. 2015) were shown to inhibit biofilm formation and also disrupt pre-established *S. aureus* biofilms. Also, ethyl

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acetate extracts of *Bacillus firmus* and *Vibrio parahemolyticus* have been shown to exhibit anti-biofilm activity against *S. aureus* (Gowrishankar et al. 2012).

In this study, attempts were made to identify new *S. aureus* biofilm inhibitors in a library of diverse endophytic microorganisms. Confocal microscopy, scanning electron microscopy, hemolysis analysis, and a hydrophobicity assay were used to determine the mechanisms responsible for biofilm inhibition. In addition, a biodegradable PLGA (poly(lactic-co-glycolic acid)) film (Zodrow et al. 2012) incorporating biofilm inhibitor actinomycin D was produced and its ability to prevent biofilm formation by *S. aureus* was investigated.

Materials and methods

Bacterial strains, growth measurements, and materials

Two methicillin-sensitive *S. aureus* strains (MSSA; ATCC 25923 and ATCC 6538), and one MRSA strain (ATCC 33591) were used in the study. Experiments were conducted at 37°C in LB medium for the MSSA strains and in LB medium containing 0.2% glucose for the MRSA strain. For cell growth measurements, optical densities were measured at 600 nm using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). Each experiment was performed using at least three independent cultures.

A culture library of diverse endophytic microorganisms was established at the Microbial Exploitation Lab of KRIBB (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea). Initially, 252 endophytic microorganisms were isolated from 25 plants in Korea. Source plants and information on sequenced-endophytic microorganisms are detailed in Table S1 (supplemental material). In order to isolate endophytic microorganisms, 25 different plant samples were pretreated with 5% NaClO for 5 min, 2.5% $Na_2S_2O_3$ for 10 min, 75% ethanol for 3 min, and 10% $NaHCO_3$ for 10 min and the plant samples were maintained at 28°C for seven days on three different agar plates, *viz.* R2A agar plates containing 5 g of proteose peptone (Difco), 0.5 g of dextrose, 0.5 g of soluble starch, 0.3 g of sodium pyruvate, 0.3 g of dipotassium phosphate, 0.05 g of magnesium sulfate, and 15 g of agar per liter (pH 7.0), TYAR agar plates containing 5 g of tryptone glucose, 3 g of yeast extract, 0.7 g of calcium chloride and 15 g of agar l^{-1} (pH 7.0), or humic acid-vitamin agar (pH 7.0). The strain of *Streptomyces* sp. that most inhibited biofilm formation by *S. aureus* was cultured for seven days in R2A liquid medium at 28°C and 140 rpm. Spent medium was passed through a 0.45 µm filter to completely remove all bacteria and kept at 4°C for further usage. The most active *Streptomyces* sp. strain # 56 was deposited as EB120060 at KRIBB. Standard actinomycin D extracted from *Streptomyces* sp. was purchased from Sigma-Aldrich (St Louis, MO, USA).

16S rRNA gene sequencing

Genomic DNA was prepared using the method previously described (Tamaoka & Komagata 1984). The 16S rRNA gene was amplified by PCR with the forward primer Eubac 27F and the reverse primer 1492R (DeLong 1992). Direct sequencing of PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3730XL, Applied Biosystems, Carlsbad, CA, USA). 16S rRNA strain sequences were compared with available sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine approximate phylogenetic affiliations. Sequence similarity values were computed using the EzTaxon server (http://www.eztaxon.org/; see Chun et al. 2007).

Crystal-violet biofilm assay

A static biofilm formation assay was performed in 96-well polystyrene plates (SPL Life Sciences, Pocheon, Korea), as previously reported (Pratt & Kolter 1998; Park, Lee, Cho et al. 2012). Briefly, cells were inoculated into LB medium (total volume 300 µl) at an initial turbidity of 0.05 at 600 nm and cultured with or without spent media for 24 h without shaking at 37°C. Biofilms were stained with crystal violet, dissolved in 95% ethanol, and absorbances were measured at OD_{570} to quantify total biofilm formation. Cell growth in 96-well plates was also measured at OD_{620}. Biofilm formation and static cell growth results are the averages of at least 12 replicate wells.

HPLC analysis of actinomycin D

High-performance liquid chromatography (HPLC) was used to measure the concentrations of actinomycin D in the spent medium of *Streptomyces parvulus*, as previously reported (Damen et al. 2009). Actinomycin D levels were measured by reverse-phase HPLC using a 4.6 × 250 mm Agilent HC-C18 column (Agilent Technology, Santa Clara, CA, USA) and 1 mM ammonium acetate-acetonitrile at a flow rate of 0.4 ml min^{-1} by gradient elution (Damen et al. 2009) using a photodiode array detector. Under these conditions, the retention time and the absorbance maximum of actinomycin D were 3.7 min and 450 nm. Spent bacterial culture medium was filtered through a 0.2 µm syringe filter before injection, and commercial actinomycin D (Sigma-Aldrich) was used as a standard for HPLC and UV/visible spectral studies.

Confocal laser microscopy and COMSTAT analysis

S. aureus biofilm formation in the presence of biofilm inhibitors was also evaluated by confocal laser microscopy (Nikon Eclipse Ti, Tokyo, Japan) and compared to *S. aureus* biofilms grown in medium alone. MSSA 6538 cells were stained with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Molecular Probes, Inc., Eugene, OR, USA) (Weston & Parish 1990) and samples were visualized using a 20× objective and an Ar laser (excitation wavelength 488 nm, emission wavelength 500 to 550 nm). All confocal images of the same strains were captured under the same conditions. Color confocal images were constructed using NIS-Elements C version 3.2 (Nikon Eclipse). In each experiment, at least four random positions of two independent cultures were observed, and 20 planar images were analyzed per position.

To quantify biofilm formation, color confocal images were converted to gray scale using ImageJ (http://imagej.nih.gov/ij/), and COMSTAT (Heydorn et al. 2000) was used to quantify biomass (μm^3 μm^{-2} substratum area), mean thickness (μm), substratum coverage (%), and maximum thickness (μm). Thresholding was fixed for all image stacks, and at least four positions and 20 planar images per position were analyzed.

Scanning electron microscope (SEM)-based biofilm assay

Scanning electron microscopy was used to observe biofilm cells, as previously described (Lee et al. 2011). Briefly, *S. aureus* strain MSSA 6538 cells were inoculated onto a nylon filter (0.5×0.5 mm square) at an initial OD_{600} of 0.05. Cells on nylon filters were incubated in the presence of *S. parvulus* (0.2 or 1%) or actinomycin D (0.02 or 0.1 μg ml^{-1}) at 37°C for 24 h without shaking. After critical-point drying, specimens were examined under a SEM (S-4100; Hitachi, Tokyo, Japan) at 15 kV and 10,000×.

Slime assay using Congo red agar (CRA)

Colony morphologies and phenotypic changes were investigated using CRA, as previously described (Freeman et al. 1989). The CRA was composed of 37 g l^{-1} of brain–heart infusion broth (BD Biosciences, Franklin Lakes, NJ, USA), 36 g l^{-1} of sucrose (Sigma-Aldrich), 15 g l^{-1} of agar (BD Biosciences), and 0.8 g l^{-1} of Congo red (Sigma-Aldrich). *S. aureus* cells on CRA were incubated with and without actinomycin D for 24 h at 37°C before taking images.

Hemolysis assay

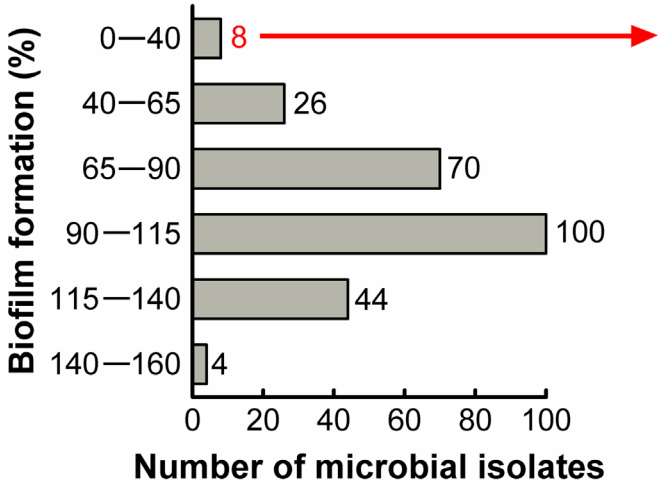
The efficacies of human red blood cell lysis were measured using whole cultures of *S. aureus* grown in the presence of the biofilm inhibitors, as described previously (Lee et al. 2013). Briefly, *S. aureus* cells were diluted at 1:100 in LB medium and cultured with or without test compounds for 16 h at 250 rpm. Cell cultures (including cells and culture supernatants) were then added to diluted human red blood cells (previously separated by centrifugation at 890×g for 2 min and washed three times with PBS buffer) in PBS buffer (330 μl red blood cells per 10 ml of PBS buffer). To determine hemolytic activities, mixtures of blood and *S. aureus* (200 μl of cell culture) were incubated at 37°C for 1 h at 250 rpm. Supernatants were collected by centrifugation at 16,600×g for 10 min and optical densities were measured at 543 nm.

Cell-surface hydrophobicity assay

Cell surface hydrophobicity was assayed as previously reported (Pérez et al. 1998; Häussler et al. 2003). Briefly, after incubation for 20 h in LB with shaking at 250 rpm, *S. aureus* cell cultures were harvested by centrifugation at 7,000×g for 5 min. Harvested cells were suspended in 2 ml of PBS and mixed with 400 μl of xylene (Sigma-Aldrich) at a ratio of 5:1 by vortexing for 120 s. Mixtures were allowed to stand for 30 min at room temperature to enable phase separation. Aqueous phase absorbances were measured at OD_{600}. The decrease in the OD of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(OD_o - OD)/OD_o] \times 100$, where OD_o and OD are the OD before and after extraction with xylene. Experiments were performed using two independent cultures per condition.

Preparation of actinomycin D load surface coatings

To fabricate biofilm inhibitor loaded films, biodegradable poly(D,L-lactide-coglycolide) (PLGA) was used, as previously reported (Zodrow et al. 2012). Briefly, actinomycin D (0, 0.2, or 1.0 μg ml^{-1}, final concentration) was mixed with 2% PLGA dissolved in chloroform, and 30 μl of this mixture were applied to slide-glass bottomed dishes to produce coatings of diameter 0.7–0.8 cm. The dishes were then air-dried for 1 h and UV sterilized for 4 h. For forming biofilms on glass surfaces, *S. aureus* cells were re-inoculated at a 1:100 dilution ratio into LB medium and incubated at 37°C for 24 h. After staining *S. aureus* cells with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Molecular Probes, Inc.) (Weston & Parish 1990), planktonic cells were discarded, and biofilm cells in PBS buffer were visualized by confocal laser microscopy.



Isolate no.	Growth (%)	Biofilm (%)
56	35 ± 4	8 ± 6
128	39 ± 7	18 ± 5
156	23 ± 3	10 ± 2
180	94 ± 3	38 ± 1
203	87 ± 1	27 ± 5
242	19 ± 2	13 ± 1
247	90 ± 4	34 ± 6
251	18 ± 3	9 ± 1

Figure 1. Anti-biofilm screening and the inhibitory effect of endophytic microbial spent culture medium on *S. aureus* biofilm formation. Histogram of *S. aureus* biofilm formation in the presence of the spent media of the 252 endophytic microorganisms. Biofilm screening for *S. aureus* ATCC 6538 was performed at a concentration of 1% (v/v) spent medium in 96-well plates over 24 h. As a negative control, bacterial culture medium (R2A) 1% (v/v) was used. The numbers on the tops of bars indicate numbers of spent media. The eight most active spent media are shown in the table with *S. aureus* growth and biofilm formation data.

Results

Spent culture medium of Streptomyces parvulus inhibited biofilm formation by S. aureus

To identify anti-biofilm compounds, the spent media of 252 endophytic bacterial species were tested against *S. aureus* MSSA 6538 in 96-well plates at 1% (v/v) concentration to minimize growth inhibition. Screening demonstrated the ability to control *S. aureus* biofilm formation varied (Figure 1). Detailed information on *S. aureus* growth and biofilm formation in the presence of the spent media of the 252 endophytic bacterial species is provided in Table S1. The library of endophytic microorganisms is diverse as there are 17 different genera among the 71 sequenced bacteria, including 31 *Bacillus* spp., 10 *Sphingomonas* spp., six *Enterobacter* spp., four *Streptomyces* spp., and 12 other genera. Spent media that inhibited biofilm formation by ≥ 60% were scored as primary hits and eight spent media were further confirmed with three independent cultures (Figure 1). Notably, four hits (#56, 156, 242, and #251) inhibited *S. aureus* biofilm formation by > 85%. #56 was the most active and reduced bacterial growth less than the other three strains and became the focus of further study (Figure 1).

16S rRNA gene sequence of strain #56 (1,426 nucleotides) was determined and a BLAST search showed that it was *Streptomyces parvulus* (GenBank accession number: KJ200636.1) with a sequence similarity of 100%. This strain was deposited as EB120060 at KRIBB. *S. parvulus* was originally isolated from the plant *Codonopsis lanceolata* in South Korea.

Further biofilm experiments showed that the spent medium of *S. parvulus* inhibited biofilm formation by all three strains of *S. aureus* in a dose-dependent manner (Figure 2A, B, and C). Specifically, at 1% concentration, it decreased MSSA 6538 and MRSA 33591 biofilm formation by ≥ 90% and by MSSA 25923 by ≥ 80%. However, the use of spent medium in the range 0.1–0.2% increased biofilm formation by the MRSA strain.

Identification and anti-biofilm activity of actinomycin D

The colonies and spent medium of *S. parvulus* were yellow, presumably due to the presence of actinomycin D (Figure 3A and B) in accordance with a previous study (Waksman & Woodruff 1940). Compared to the authentic actinomycin D in HPLC (Figure 3C), the mean level of actinomycin D in the spent culture medium of *S. parvulus* was 12 ± 1 μg ml^{-1}.

Corroborating the results with the spent medium of *S. parvulus* (Figure 2A, B, and C), actinomycin D extracted from *Streptomyces* sp. (Sigma-Aldrich) effectively and inhibited biofilm formation by all three *S. aureus* strains in a dose-dependent manner (Figure 3D, E, and F). Specifically, it decreased biofilm formation by MSSA 6538, MSSA 25923, and MRSA 33591 at 0.1 μg ml^{-1} by ≥ 80%, ≥ 70%, and ≥ 80%, respectively. MSSA 6538 was more sensitive to actinomycin D than the other two strains by one order of magnitude. Unlike the biofilm increase in MRSA in low concentrations of spent medium of *S. parvulus* (0.1 and 0.2% in Figure 2C), actinomycin D dose-dependently decreased biofilm formation over all

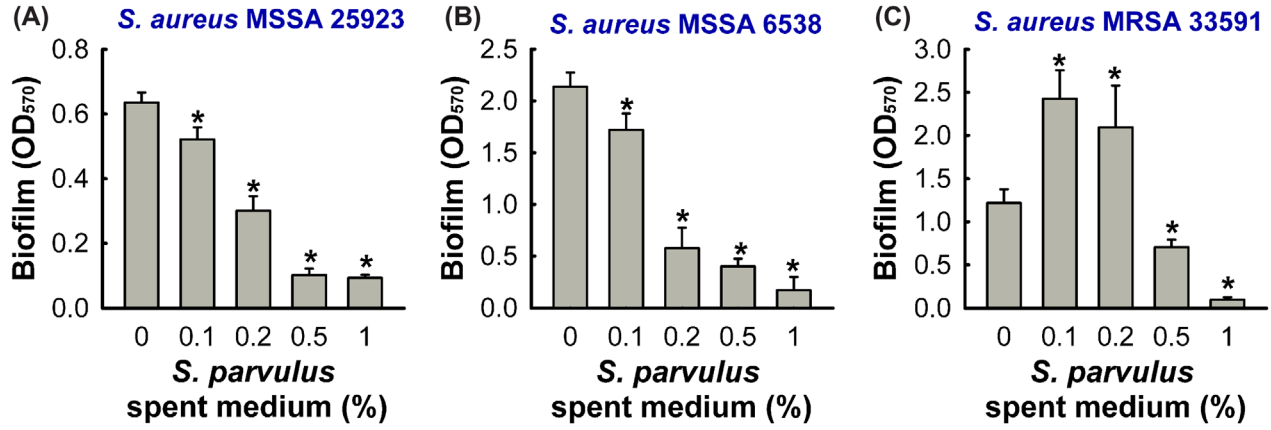


Figure 2. The effects of *S. parvulus* spent medium on biofilm formation by three *S. aureus* strains. Two methicillin-sensitive *S. aureus* strains (MSSA, ATCC 25923 and ATCC 6538) and a methicillin-resistant *S. aureus* strain (MRSA, ATCC 33591) were used (A, B, and C). Four independent experiments were conducted (total 12 wells). Error bars indicate SDs. *$p<0.05$ *vs* the control group.

concentrations (Figure 3F), which indicates the biofilm increase was not due to the presence of actinomycin D and probably due to some unknown components in the spent medium of *S. parvulus*. These results indicate that actinomycin D is probably the component produced by *S. parvulus* responsible for inhibiting *S. aureus* biofilm formation.

Confocal laser microscopy and SEM were also used to analyze changes in biofilm formation. In line with the 96-well biofilm quantitative data (Figures 2 and 3), fluorescent images indicated that both the spent culture medium of *S. parvulus* and actinomycin D dose-dependently inhibited *S. aureus* MSSA 6538 biofilm formation (Figure 4A). SEM analysis revealed fewer biofilm cells attached to nylon filters in the presence of either *S. parvulus* culture spent medium or actinomycin D, but no morphologic abnormality was observed in the presence of actinomycin D (Figure 4B). Similarly, the anti-biofilm activities of the spent medium of *S. parvulus* and actinomycin D against MSSA 25923, and MRSA 33591 were also observed (Figures S1 and S2).

Biofilm inhibition was further confirmed by COMSTAT analysis. The spent culture medium of *S. parvulus* or actinomycin D reduced all four measured parameters (biomass, mean thickness, substratum coverage, and maximum thickness) of MSSA 6538 biofilm (Table 1). Specifically, biomass (volume/area) and mean thickness were reduced more than 98% by the spent medium of *S. parvulus* (1%, v/v) or actinomycin D (0.1 μg ml^{-1}), and substratum coverage was also reduced 20-fold by *S. parvulus* or actinomycin D. MIC values of the spent culture medium of *S. parvulus* and actinomycin D were 10% (v/v) and 0.5–1.0 μg ml^{-1} against *S. aureus* strains, which is consistent with previous reports for use of this compound as an antimicrobial (Kirk 1960). Hence, biofilm inhibition by actinomycin D occurred at concentrations 10-fold lower than the MIC. *S. aureus* cell growth curves were also measured in the presence of spent culture medium of *S. parvulus* or actinomycin D (Figure S3). As was expected, 0.1 × MIC (0.1 μg ml^{-1}) of actinomycin D slightly delayed cell growth as generation times for an untreated control and the addition of actinomycin D (0.1 μg ml^{-1}) were 26 ± 1 and 34 ± 1 min, respectively. The cell growth and microscopic results indicate that inhibition of *S. aureus* biofilm formation by actinomycin D is due to anti-biofilm activity rather than antimicrobial activity.

Inhibition of slime production by actinomycin D

Slime is defined as the extracellular polymeric substance (a main component of biofilm) that is mainly formed by PIA in staphylococci strains (Hall-Stoodley et al. 2004). Hence, Congo red plates were used to detect slime production by *S. aureus* strains. In accordance with biofilm inhibition in the 96-well plate and microscopic observation, slime production by all three *S. aureus* strains was markedly reduced by actinomycin D (Figure 5).

Anti-hemolytic activity of actinomycin D in S. aureus

S. aureus produces α-toxin that causes hemolysis (Song et al. 1996) and contributes to biofilm formation (Caiazza & O'Toole 2003); therefore the effect of actinomycin D on blood hemolysis by *S. aureus* was investigated. Interestingly, spent culture medium of *S. parvulus* and actinomycin D dose-dependently inhibited hemolysis by *S. aureus* (Figure 6), while three negative controls, such as LB medium, spent medium of *S. parvulus* (1%, v/v), or actinomycin D (0.1 μg ml^{-1}) in the absence of *S. aureus* cells, did not cause hemolysis. Notably, 0.1 × or 0.01 × MIC

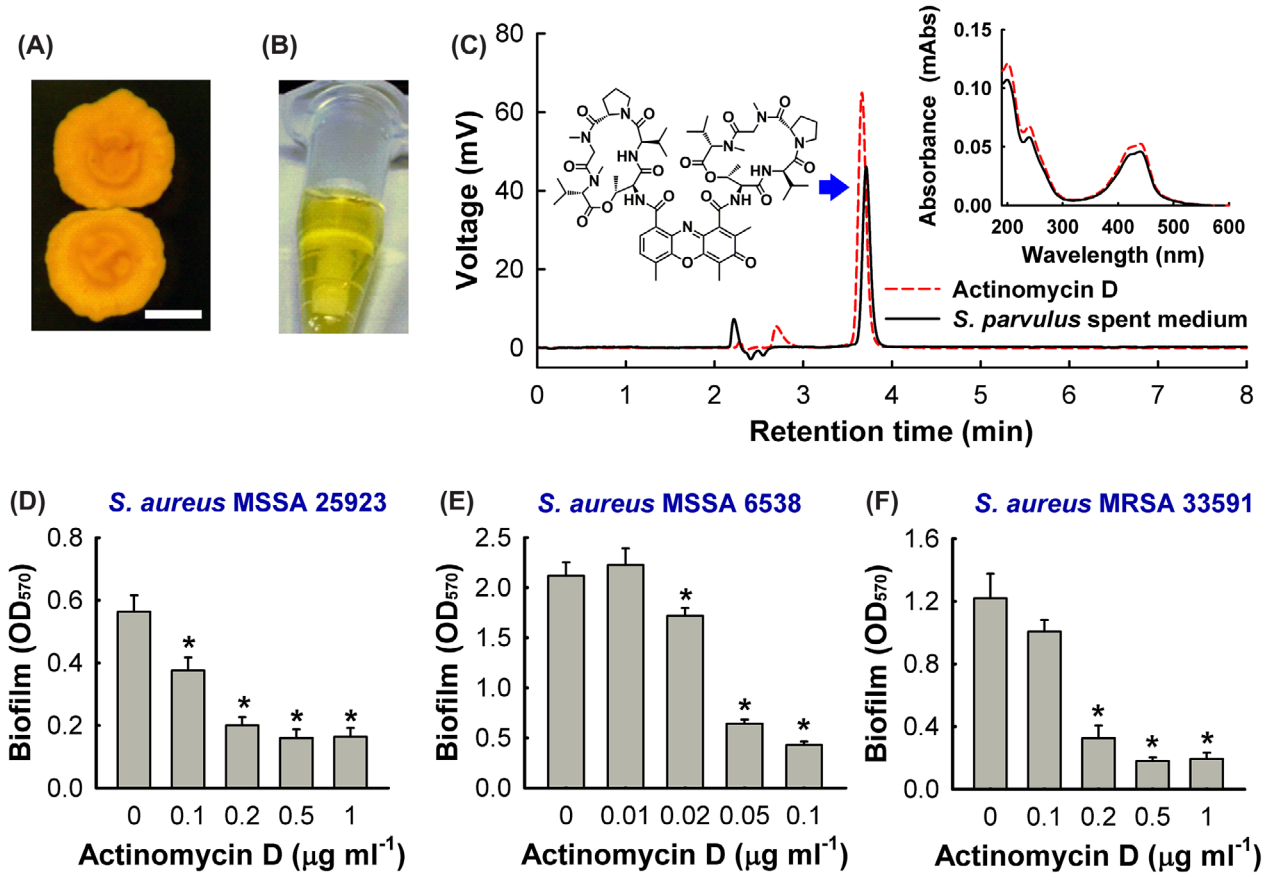


Figure 3. Actinomycin D from *S. parvulus*: its identification and anti-biofilm activity. (A) Colony morphology of *S. parvulus*. (B) Aqueous phase of the spent medium of *S. parvulus*. (C) HPLC analysis of the spent medium of *S. parvulus*. Chemical structure and UV/visible spectra of the spent medium of *S. parvulus* (1%) and of actinomycin D (20 μg ml^{-1}) are shown as insets. (D–F) The inhibitory effects of actinomycin D on biofilm formation by the three *S. aureus* strains examined. Biofilm formation (OD_{570}) by *S. aureus* was quantified in the presence of actinomycin D after culture in 96-well plates for 24 h without shaking. Four independent experiments were conducted (total 12 wells). Error bars indicate SDs. *$p<0.05$ *vs* the control group.

of spent culture medium of *S. parvulus* (0.1%, v/v) or actinomycin D (0.01 μg ml^{-1}) reduced hemolytic activity by ≥ 85%, suggesting inhibition of *S. aureus* biofilm formation by actinomycin D is partially associated with its inhibition of hemolytic activity. Furthermore, actinomycin D clearly reduces the hemolysis virulence factor of *S. aureus*.

Actinomycin D decreased cell-surface hydrophobicity

Since surface hydrophobicity plays a role in *S. aureus* biofilm formation, as it facilitates adherence to hydrophobic surfaces (Pagedar et al. 2010), a cell-surface hydrophobicity assay was performed to examine the mechanism underlying the inhibitory effect of actinomycin D on *S. aureus* biofilm formation. It was found the addition of spent culture medium of *S. parvulus* or actinomycin D caused cells to become less hydrophobic (Figure 7), which explains at least in part the inhibitory effect of actinomycin D on biofilm reduction.

Biofilm inhibition on solid surfaces by an actinomycin D loaded coating

PLGA polymer containing actinomycin D (0.2× MIC, 0.2 μg ml^{-1}) markedly reduced biofilm formation on a glass surface of all three *S. aureus* strains (Figure 8), but the numbers of planktonic cells were not affected by the PLGA/actinomycin D coating (data not shown). COMSTAT analysis confirmed that actinomycin D (0.2× MIC, 0.2 μg ml^{-1}) reduced biofilm biomass, mean thickness, substratum coverage, and maximum thickness of the three *S. aureus* biofilms (Table 2). Also, actinomycin D was more active in inhibiting biofilm formation by the MSSA 25923 strain than the MSSA 6538 strain. Specifically, biomass and mean thickness were reduced > 90% by actinomycin D (1.0 μg ml^{-1}) in MSSA 25923 and biomass and mean thickness were reduced 60% in MSSA 6538. Also, it is notable that PLGA coating alone produced less biofilms (biomass and mean thickness) than no coating in all three *S. aureus* strains (Tables 1 and 2).

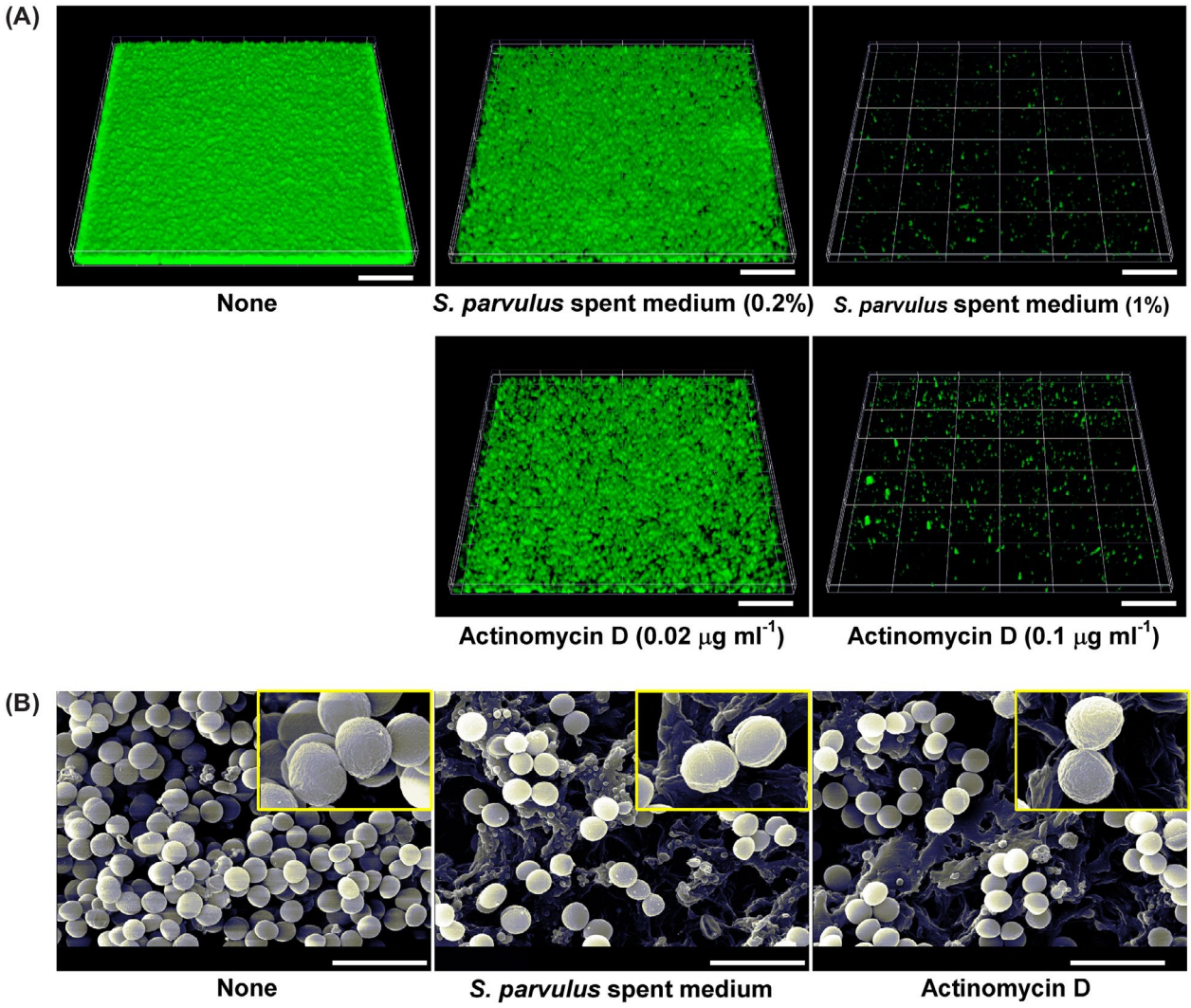


Figure 4. Microscopic observations of the effects of actinomycin D on biofilms. Biofilm formation by *S. aureus* strain ATCC 6538 was analyzed after 24 h in 96-well plates by (A) confocal laser microscopy and (B) SEM. SEM was used to examine biofilm cells grown on pieces of 0.45 μm nylon filters in the presence of *S. parvulus* (1%) or actinomycin D (0.1 μg ml^{-1}). At least two independent experiments were conducted. The scale bars represent 100 μm (A) and 3 μm (B), respectively.

Table 1. COMSTAT analysis of *S. aureus* 6538 biofilms at different concentrations of inhibitory agents.

	Concentration	Volume/area (μm^3 μm^{-2})	Mean thickness (μm)	Substratum coverage (%)	Maximum thickness (μm)
S. parvulus spent medium (%)	0	21 ± 2	20 ± 2	99 ± 1	39 ± 2
	0.2	5 ± 1	5 ± 1	53 ± 2	24 ± 1
	1.0	0.3 ± 0.1	0.3 ± 0.2	4 ± 1	19 ± 1
Actinomycin D (μg ml^{-1})	0	22 ± 2	20 ± 2	99 ± 1	39 ± 2
	0.02	3 ± 2	3.5 ± 0.4	45 ± 9	26 ± 2
	0.1	0.36 ± 0.03	0.39 ± 0.03	5 ± 1	19 ± 1

Discussion

Chronic *S. aureus* infections are frequently associated with biofilms that are difficult to eradicate with conventional antibiotics, and thus an ever-increasing amount of research is aimed at identifying new anti-biofilm agents and at understanding how bacteria control biofilm formation. This study demonstrates for the first time that the FDA-approved antitumor agent actinomycin D exhibits anti-biofilm and anti-hemolytic activity against *S. aureus*.

Actinomycin D (also called Dactinomycin) was the first antibiotic shown to have anti-cancer activity (Hollstei 1974). It was initially isolated from *Streptomyces* sp. in 1940 (Waksman & Woodruff 1940), approved by the FDA in 1964, and has been used to treat tumors and as

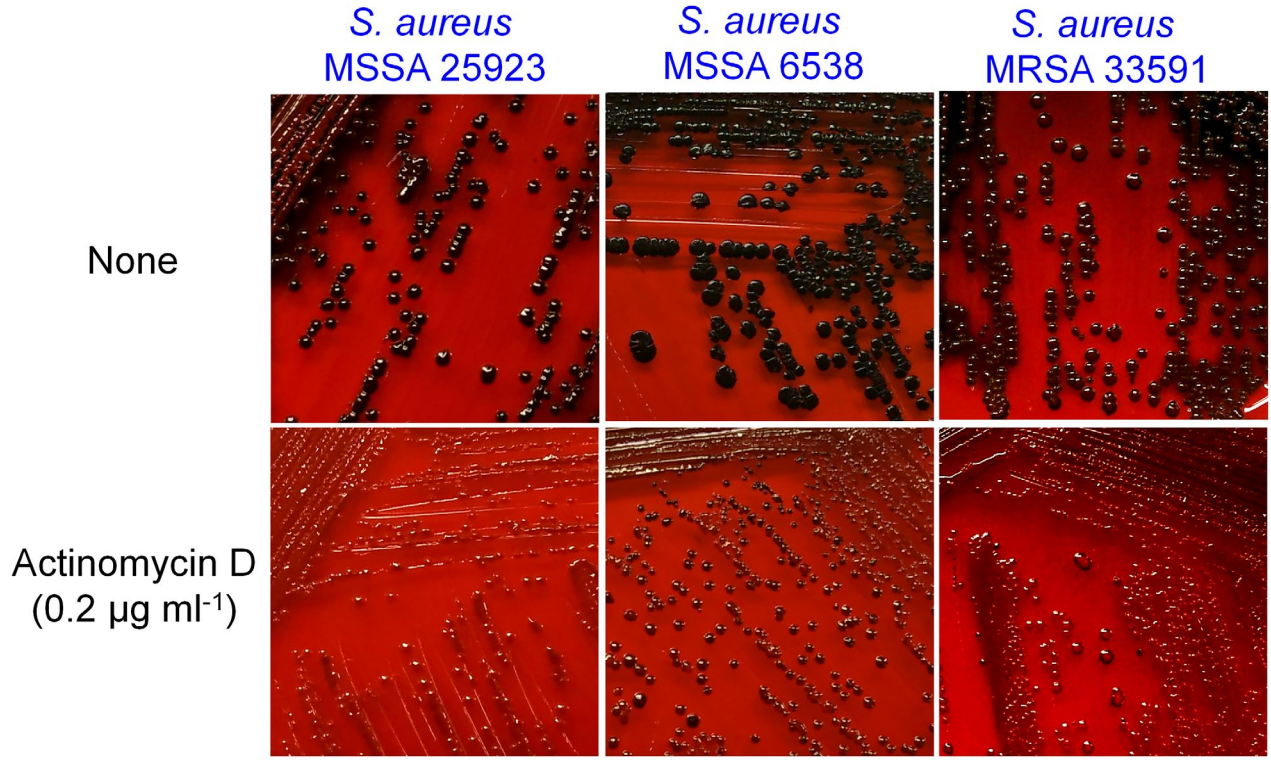


Figure 5. Inhibition of slime production by actinomycin D**.** Slime production was analyzed using Congo red agar plates. *S. aureus* was cultured with and without actinomycin D (0.2 μg ml^{-1}) on Congo red agar plates for 24 h at 37°C. Three independent experiments were conducted and one set of representative results is shown.

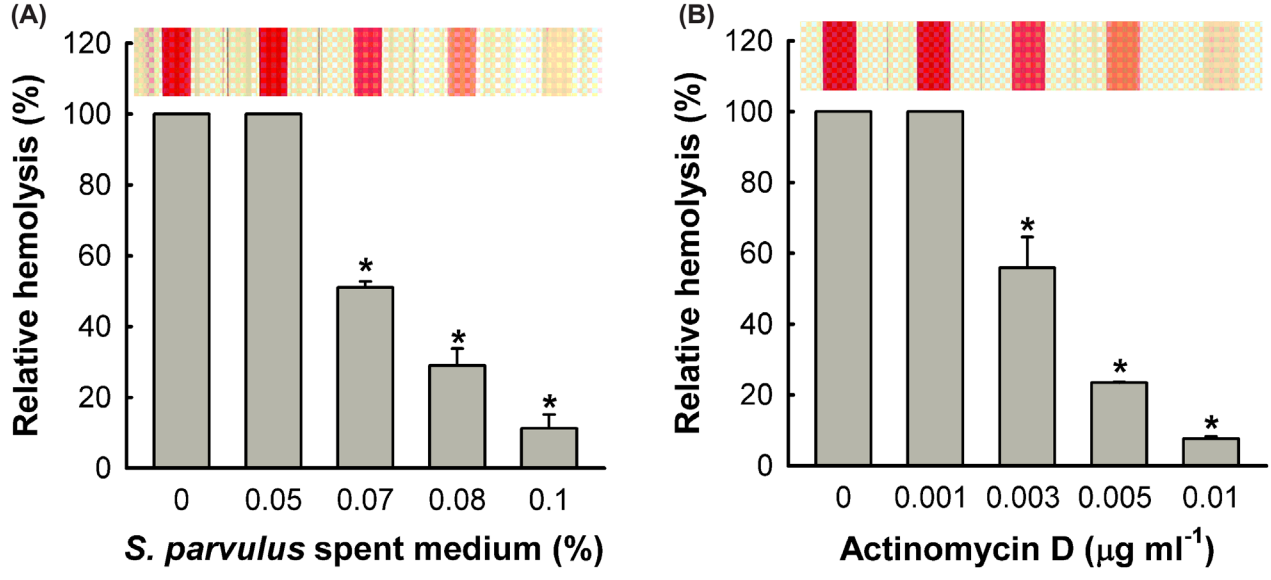


Figure 6. Anti-hemolytic activities of (A) the spent medium of *S. parvulus* and (B) actinomycin D. The hemolysis of human blood by *S. aureus* ATCC 6538 was quantified in the presence of *S. parvulus* spent medium or actinomycin D after incubation for 16 h. Images of the spectrophotometer cuvettes are shown. *$p<0.05$ *vs* the control group.

an antibiotic for many years. Actinomycin D inhibits the initiation of RNA synthesis in *S. aureus* and has powerful bacteriostatic effects on many Gram-positive bacteria (Kirk 1960). Although the development of resistance to actinomycin D by *Bacillus subtilis* is well known (Sterlini & Mandelst 1969), little is known about resistance to it by *S. aureus*. Interestingly, actinomycin D, unlike other antibiotics, was found to completely suppress the ability of

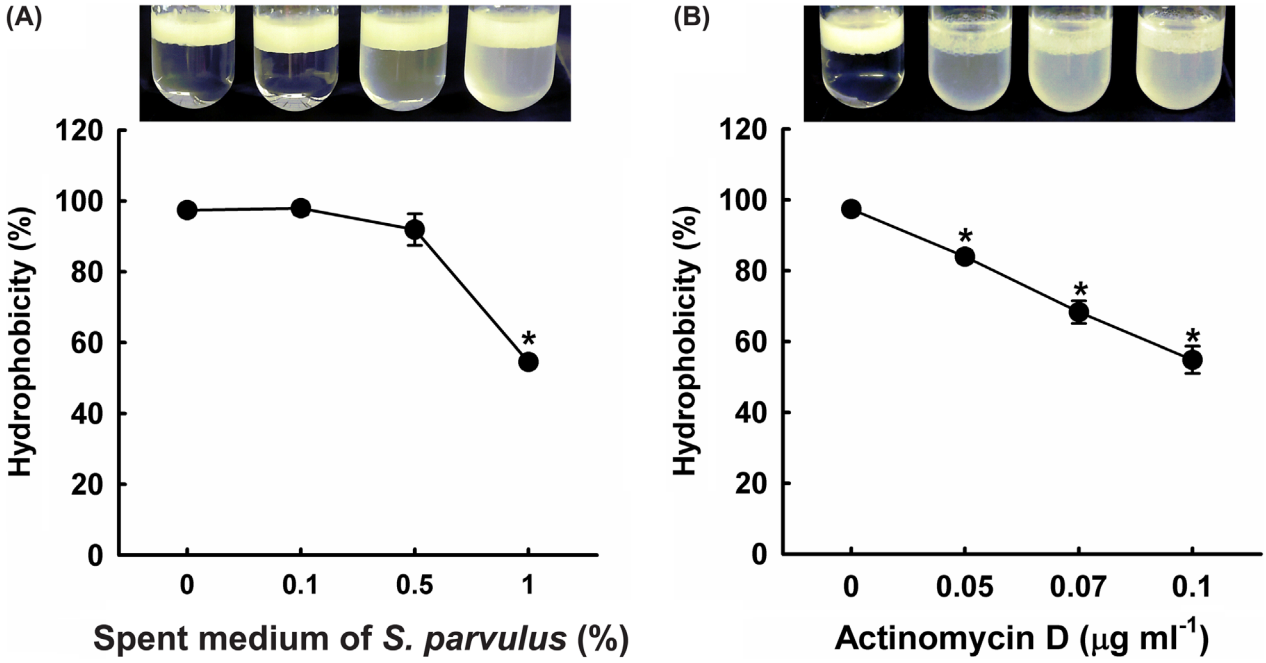


Figure 7. Effects of (A) the spent medium of *S. parvulus* and (B) actinomycin D on the hydrophobicities of cell surfaces. *S. aureus* ATCC 6538 cells were grown for 20 h, and hydrophobicity assays were performed by hexadecane extraction. Aqueous phase absorbance was measured at OD_{600} and high turbidity indicated less hydrophobicity. Tested tubes are shown. *$p<0.05$ *vs* the control group.

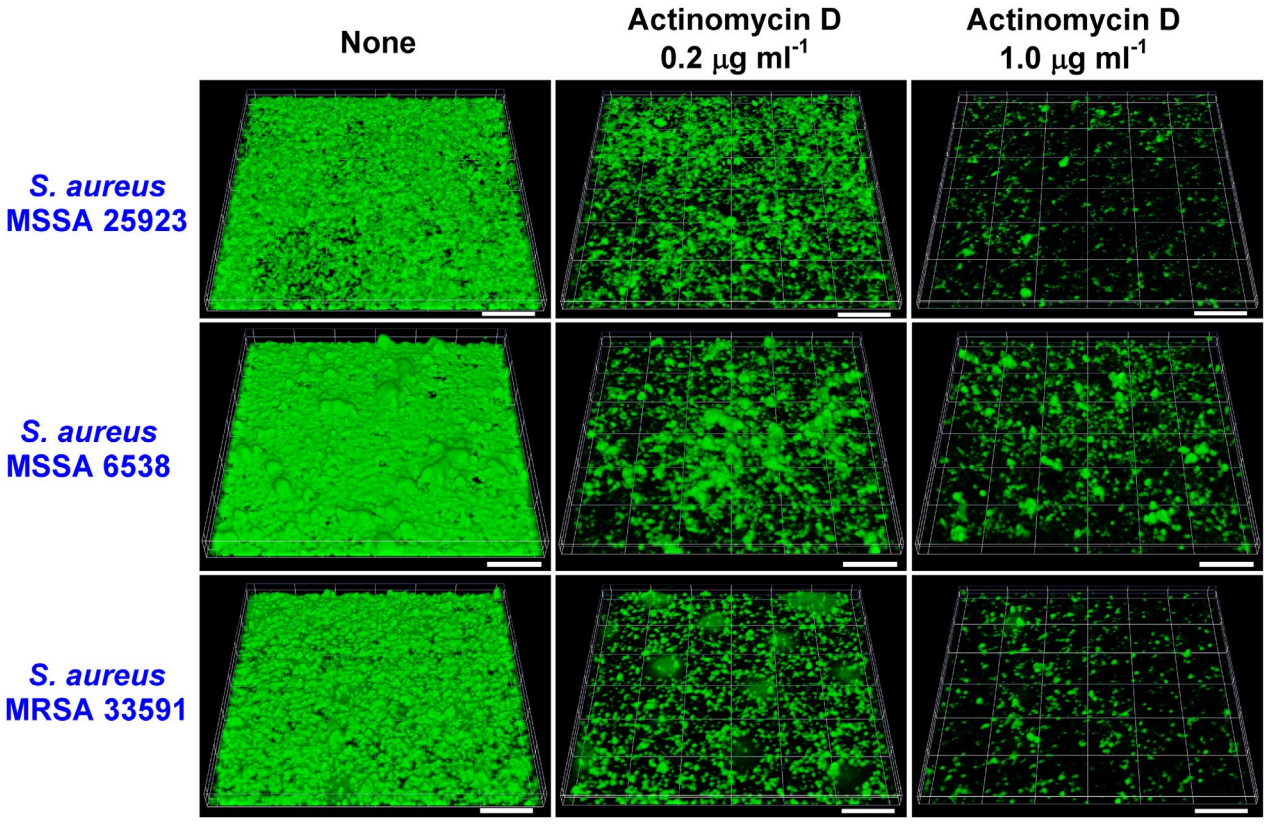


Figure 8. Inhibition of biofilm formation by a PLGA coating containing actinomycin D. Biofilm formation by *S. aureus* MSSA 25923, MSSA 6538, and MRSA 33591 was investigated by confocal laser microscopy. Actinomycin D (0.2 or 1.0 μg ml^{-1} (final concentration)) was mixed into PLGA and biofilms were grown for 24 h without shaking on glass-bottomed dishes. Scale bars represent 50 μm.

Table 2. COMSTAT analysis of *S. aureus* biofilms on PLGA containing different concentrations of actinomycin D.

Strain	Actinomycin D (μg ml^{-1})	Volume/area (μm^3 μm^{-2})	Mean thickness (μm)	Substratum coverage (%)	Maximum thickness (μm)
MSSA25923	0	4.2 ± 0.4	4.3 ± 0.4	88 ± 1	13 ± 1
	0.2	1.4 ± 0.4	1.6 ± 0.5	59 ± 8	10 ± 1
	1.0	0.3 ± 0.1	0.3 ± 0.1	22 ± 6	9 ± 1
MSSA 6538	0	6.0 ± 1.0	5.0 ± 1.0	96 ± 3	21 ± 3
	0.2	2.2 ± 0.4	1.8 ± 0.4	65 ± 8	15 ± 1
	1.0	1.5 ± 0.3	1.4 ± 0.2	41 ± 11	14 ± 1
MRSA	0	4.4 ± 0.6	3.7 ± 0.5	95 ± 8	16 ± 1
33591	0.2	1.9 ± 0.5	1.6 ± 0.4	62 ± 15	14 ± 1
	1.0	0.6 ± 0.1	0.5 ± 0.1	27 ± 5	11 ± 1

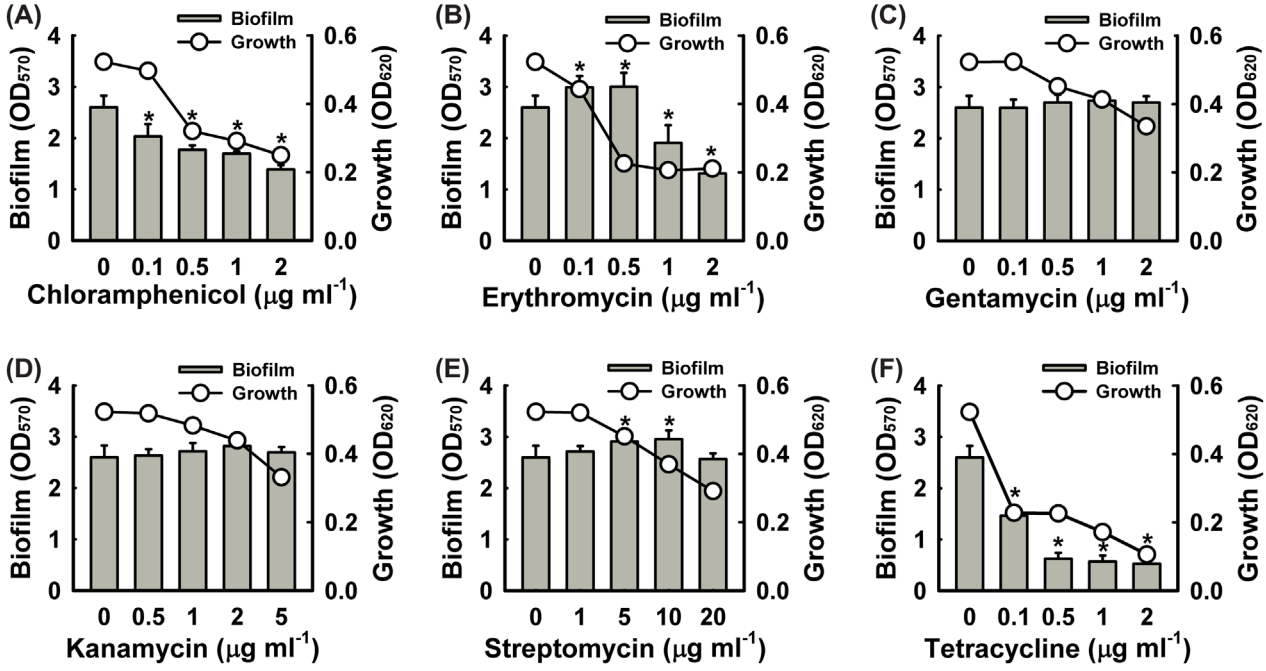


Figure 9. Effect of six antibiotics on biofilm formation by *S. aureus*. Biofilm formation (OD_{570}) and cell growth (OD_{620}) of *S. aureus* ATCC 6538 were quantified in the presence of antibiotics after culture in 96-well plates for 24 h without shaking. Two independent experiments were conducted (total 12 wells). Error bars indicate SDs. *$p<0.05$ *vs* the control group.

S. aureus to recover from thermal injury (Iandolo & Ordal 1966). Thus, it could be speculated that its anti-biofilm effect is associated with low tolerance of actinomycin D by *S. aureus*.

Since subinhibitory concentrations of several antibiotics often increase biofilm formation (Hoffman et al. 2005; Linares et al. 2006; Kaplan et al. 2012), the effects on *S. aureus* biofilm formation of six other antibiotics, chloramphenicol, erythromycin, gentamycin, kanamycin, and streptomycin, were investigated at subinhibitory concentrations. Five antibiotics were less effective at inhibiting *S. aureus* biofilm formation than actinomycin D and inhibited cell growth (Figure 9). For tetracycline, this result concurs with a previously reported result that tetracycline exhibits anti-biofilm and antibacterial activity (Monzón et al. 2001). Since actinomycin D derivatives also have antibiotic (Brockmann 1960) and antitumor activities (Olano et al. 2009), it would be interesting to investigate their effects on *S. aureus* biofilms.

The mechanism of *S. aureus* biofilm formation is a complex process that involves many environmental factors (Boles & Horswill 2011). In particular, protease treatment and the activation of *agr* quorum-sensing in *S. aureus* inhibited biofilm formation and dispersed established biofilms (Boles & Horswill 2008; Park et al. 2012; Lister & Horswill 2014). However, in the present study, actinomycin D up to 0.2 μg ml^{-1} did not disperse a pre-existing biofilm of *S. aureus* (Figure S4), which suggests actinomycin D is not associated with biofilm dispersal systems such as *agr* quorum sensing or the actions of proteases. Further investigation will be required to understand the genetic and molecular mechanism of actinomycin D using metaproteomics and metatranscriptomics approaches.

Bacterial surface hydrophobicity significantly affects biofilm formation, and generally bacteria with hydrophobic properties prefer hydrophobic material surfaces (Dunne 2002). It has been shown *S. aureus* favors hydrophobic surfaces (Pagedar et al. 2010) and that oligomer-based biofilm inhibitors decrease the hydrophobicity of *S. aureus* cells (Lee et al. 2015). Therefore, it appears that a decrease in cell-surface hydrophobicity prevents attachment of S. *aureus* cells to the surfaces of plastic wells (Figure 3), nylon membranes (Figure 4B), and glass (Figure 8). The above-mentioned findings suggest that increasing the surface hydrophilicities of medical devises may offer a means of inhibiting biofilm formation by *S. aureus*, which is the most frequent cause of infections associated with indwelling medical devices (Otto 2008). Recently, PLGA coatings incorporated with biofilm inhibitors (cinnamaldehyde, carvacrol, and thermoresponsive oligo(N-vinylcaprolactam)) have been shown to exhibit antibacterial and anti-biofilm activities against *S. aureus* (Nostro et al. 2012; Zodrow et al. 2012; Lee et al. 2015). The present study demonstrated for the first time the anti-biofilm efficacy of PLGA/actinomycin D coatings on glass against *S. aureus* (Figure 8), and suggests studies on actinomycin D coatings on medical and engineering materials *in vitro* and *in vivo* might be worthwhile.

The inhibition of biofilm formation and toxin production could provide an alternative means of reducing bacterial virulence of antibiotic resistant *S. aureus* strains. The results indicate actinomycin D exhibits anti-biofilm and anti-hemolytic activities. Actinomycin D warrants further attention as a potential antivirulence strategy against persistent *S. aureus* infection.

Disclosure statement

No potential conflict of interest was reported by the authors.

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