A Compound Inhibits Biofilm Formation of *Staphylococcus aureus* from *Streptomyces*

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Biofilm is one virulence factor of bacteria. It contributes not only to bacterial adherence to many kinds of infection-establishing surfaces, but also to bacterial resistance against antimicrobial agents and antiseptic agents. Inhibitors of bacterial biofilm formation should be useful in the prevention of infections. We found that a culture of *Streptomyces* sp. strain MC11024 showed inhibitory activity on biofilm formation by *Staphylococcus aureus* and isolated streptorubin B as an inhibitor of this formation in *S. aureus*. The biofilm formation of methicillin resistant *S. aureus* (MRSA) N315 was reduced to less than 30% at 1 µg/mL of streptorubin B, and at this concentration cell growth was not affected. Our study suggests that streptorubin B has the potential to be a leading compound of anti-infectious agents of *S. aureus*.

**Key words** biofilm; inhibitor; *Staphylococcus aureus*; *Streptomyces*; prodigiosin

Pathogenic bacteria produce many kinds of virulence factors and cause human infectious diseases. Among them, adherence factors are important for establishing infections.1,2) Such factors also confer adhering and staying on every surface including medical devices. One of such factors, biofilm, is a relatively flexible and sticky structure.3) Once bacteria form biofilm on the surface of medical devices set in the human body, it is difficult to remove the biofilm. It is also known that biofilm prevents the penetration of antibacterial drugs and helps bacteria escape from host immune systems.4) Some infections correlate with biofilm formation at the clinical site.5)

Biofilm formation by bacteria starts with the production of adhering factors. Bacterial cells attach to many kinds of surfaces via electrical charge and specific interactions. After that, bacteria develop biofilm by producing high viscosity extracellular matrix including polysaccharides, proteins, DNA and so on. After the biofilm has been developed, part of the bacteria in the biofilm releases some proteases, detaching them from the biofilm, and the small block of biofilm containing bacteria moves to other places.6) While bacteria attach to surfaces and mature biofilm, they also release many virulence factors.7)

*Staphylococcus aureus* is one of the major causes of biofilm associated infectious diseases.8) *S. aureus* produces adhesion factors and colonizes onto medical devices such as plastic catheters. Methicillin resistant *S. aureus* (MRSA) is frequently isolated at many clinical sites and it makes drug therapy much more difficult. Inhibitors of the biofilm formation of *S. aureus* are good candidates for anti-infectious agents, as such agents should work for not only preventing attachment to surfaces but also reducing resistance to antimicrobial agents and antiseptic agents.

Natural products from many origins have been good sources of leading compounds for bioactive drugs in clinical use. Actinomycetes have been one attractive natural resource for producing effective bioactive compounds including antibiotics. Antibiotics are thought to be utilized by producing organisms for their survival in the environment. It is also speculated that actinomycetes may produce other bioactive compounds such as biofilm inhibitors for the same purpose. So we have been trying to find inhibitors of biofilm formation by *S. aureus* in actinomycetes products.

**MATERIALS AND METHODS**

**Bacteria and Cultivation** MRSA N315 was cultured in brain heart infusion (BHI) broth (Nissui-seiyaku Inc., Japan) and incubated at 37°C under aerobic conditions. For maintenance of the strain MC11024, the strain was grown in starch-casein medium (1.0% starch, 0.03% casein, 0.2% NaCl, 0.2% K$_2$HPO$_4$, 0.005% MgSO$_4$, 0.002% CaCO$_3$, 0.001% FeSO$_4$, pH 7.2) at 30°C. A phylogenetic study of the strain MC11024 was carried out by 16S ribosomal DNA (rDNA) sequence analysis using the primers 27F (5'-AGAGTTTGATCCTGTCAGA G-3'), 514F (5'-GTGCGCCAGAGCCGCACGTTA-3'), 1114F (5'-GCAAGAGCGCACC-3'), 530R (5'-CCGGCGCTG TCTCGCAACGTA-3'), 936R (5'-GTCGCCGGCCGTCAT-3'), and 1492R (5'-TACGCGCTATTGGTGACGACT-3').

**Biofilm Formation Test** The effect of compounds on biofilm formation was evaluated using a modified semi-quantitative plate assay.9) Overnight cultures of *S. aureus* in the BHI broth were diluted to 1:100 by fresh BHI broth supplemented with 1.0% glucose. Individual wells of flat-bottomed 96-well polystyrene plates (Orange Scientific, Belgium) were filled with 100 µL of culture and 2.5 µL of sample solution, and incubated at 37°C. After 24 h of growth, the plates were washed twice with distilled water to remove unattached bacteria, and stained with a 0.1% crystal violet solution for 5 min. After the addition of 30% acetic acid to a well, the absorbance at 570 nm was measured to evaluate crystal violet amount. The testing was repeated at least three times.

**Measurement of Growth** Overnight cultures of *S. aureus* were diluted 1:100 with BHI broth supplemented with 1.0% glucose, and grown for 24 h in L-type tube TN-5L (ADVANTEC, Japan) at 37°C with shaking at 20 rpm, and OD$_{600}$ was measured every 10 min by using compact rocking

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incubator TVS062CA (ADVANTEC). Measurement of growth was repeated at least three times.

Drug Susceptibility Test The minimum inhibitory concentrations (MICs) of various antimicrobial agents were determined in Mueller–Hinton broth (Difco, Sparks, U.S.A.) by the two-fold dilution method according to the Clinical and Laboratory Standards Institute recommendations. Cells in the test medium (10^5 cells/mL) were incubated at 37°C for 20 h, and the growth was judged by unaided eye. The testing was repeated at least three times.

General Experimental Procedures NMR spectra were measured for analyzing structure using a JNM-ECA- 600 NMR spectrometer (JEOL, Japan) with tetramethylsilane as an internal standard in CDCl_3. HPLC analysis was carried out using a HPLC pump GL-7410 and a PDA detector GL-7452 (GL Science, Japan). For HPLC fractionations, a HPLC pump L-6200 (Hitachi, Japan) and a UV detector L-4200 (Hitachi, Japan) were used. Electrospray ionization-mass spectrometry (ESI-MS) spectra were measured for calculating molecular weight with an API-3200 triple quadrupole mass spectrometer (Applied Biosystems, Japan) and a MICROMASS Q-ToF PREMIER (Waters, Japan). UV-Vis spectra were measured with a V-630 spectrophotometer (JASCO, Japan). Optical densities were measured with a microplate reader SH-1000 Lab (Corona, Hitachinaka, Japan).

Fermentation of the Strain MC11024 A loop of spores of the strain were inoculated into starch-casein medium (10 mL) in a test tube, and incubated for 3 d at 30°C on a reciprocal shaker (120 rpm). The seed-culture was transferred into 250 mL starch-casein medium in a 500 mL Sakaguchi flask (40 flasks, total 12 L) and incubated for 7 d at 30°C on a reciprocal shaker (120 rpm).

Isolation of Compound The culture broth (12 L) was filtered and mycelia were extracted with methanol (MeOH) (2 L). After the removal of the solvent to dryness (988 mg) in vacuo, the residue was suspended in H_2O (2 L) and extracted with ethyl acetate (EtOAc) (2 L). The extract was applied on a silica gel column (Silica gel 60, i.d. 3.5×150 mm) and eluted with a stepwise gradient of hexane–EtOAc. Active fraction was further purified by HPLC (Cosmosil 5C_18-AR-II, i.d. 10×250 mm, detection 292 nm, flow rate 2.5 mL/min, a stepwise gradient of H_2O–CH_3CN) to obtain one active compound.

Physicochemical Properties and Spectroscopic Data of the Active Compound The active compound was dark-red powder and soluble in MeOH, EtOAc and chloroform (CHCl_3), but insoluble in water. High-resolution (HR) ESI-time-of-flight (TOF)-MS m/z: 392.2691 (Calcld for C_{26}H_{36}N_4O: 392.2702, [M+H]^+). MS m/z: 392.3 [M+H]^+. UV-Vis λ_{max} (MeOH) nm: 218, 297, 534. 1H-NMR (CDCl_3) δ: 12.7 (2H), 12.0 (1H), 7.24 (1H), 7.15 (1H), 6.93 (1H), 6.53 (IH), 6.36 (IH), 6.13 (IH), 4.04 (IH), 3.11 (2H), 2.54 (IH), 1.92–1.11 (1IH), 0.98–0.91 (2H), 0.82–0.77 (IH), –1.56 (IH). 13C-NMR (CDCl_3) δ: 122.1, 117.2, 111.7, 127.5, 120.7, 165.7, 58.7, 93.3, 147.8, 112.6, 154.2, 117.4, 150.9, 125.4, 29.9, 31.5, 29.1, 27.7, 25.4, 31.1, 37.4, 38.7, 30.4, 22.9, 14.1.

RESULTS

Taxonomy of the Active Compound Producing Strain MC11024 Almost complete 16S rDNA gene (1459 bp: Accession number AB973712) was sequenced and used for the phylogenetic study. Comparison of the sequence with known sequences of microbes in the GeneBank database was carried out by a BLAST search. The result showed that the 16S rDNA sequence of the strain exhibited 100.0% sequence similarities with ten actinomycetes including five Streptomyces strains such as Streptomyces sp. O29 and S. fradiae NBRC 12214 and NBRC 12215. Therefore, the strain MC11024 isolated from the soil obtained from Suita, Osaka, Japan was classified as a Streptomyces sp.

Screening, Isolation and Identification of Active Compound

The library of culture extracts of actinomycetes was used as a source for finding biofilm formation inhibitors of MRSA N315. All samples were solubilized in MeOH. We confirmed that the amount of MeOH used in biofilm formation and drug susceptibility tests did not affect them. Several extracts reduced the biofilm formation. One of them, the extract from the culture broth of Streptomyces sp. strain MC11024 reduced the biofilm formation of MRSA N315 by 49% in the presence of equal or less than the 1/8 concentration of the MIC.

The fermentation of strain MC11024 was performed in starch-casein medium with aeration. The mycelial MeOH extract was purified by column chromatographies, resulting in one active compound (10.0 mg). According to a search of the Dictionary of Natural Products Database based on its physicochemical properties and spectroscopic data, suggesting the compound is a relative compound of prodigiosin which is a family of tripyrrole red pigments. Since the 13C-NMR data and 1H-NMR data of the compound coincided with those in the literature, it was identified to be streptorubin B (Fig. 1). Streptorubin B has been reported as the metabolite of actinomycete strain B4358 and Streptomyces abikoensis.

Characterization of Streptorubin B The medium contained more than 32 µg/mL of streptorubin B showed very dark red color, and it was difficult to measure MIC. We could recognize the growth of S. aureus in the medium containing 16 µg/mL of streptorubin B. This means that the MIC of streptorubin B was more than or equal to 32 µg/mL, and the anti-S. aureus effect was not observed at 16 µg/mL of streptorubin B. The inhibitory effect of biofilm formation of MRSA N315 by streptorubin B was then measured. When 1 µg/mL, less than or equal to the 1/32 concentration of MIC, of streptorubin B was then measured. The inhibition was also evaluated at each concentration of streptorubin B. Biofilm formation was decreased less than 50% at even 0.17 µg/mL of streptorubin B. IC_{50} value (the concentration that inhibit 50% of biofilm formation) of streptorubin B was determined to be

![Structure of Streptorubin B](Fig. 1)
The Effect of Streptorubin B on the Growth of MRSA N315

There is a possibility that streptorubin B might have some effect on the cell growth of MRSA N315, and then secondarily inhibit the biofilm formation. We therefore tested the effect of streptorubin B on the cell growth of MRSA N315 at each concentration of streptorubin B (Fig. 3). The growth rate of MRSA N315 was obviously affected at 4 µg/mL, less than or equal to the 1/8 concentration of MIC, of streptorubin B. Streptorubin B showed a slight effect on growth at 2 µg/mL, but showed no effect at 1 µg/mL. Streptorubin B showed strong inhibition to biofilm formation of MRSA N315 at this concentration (Fig. 2). Although some or slight inhibitory effects on bacterial growth were observed in the presence of 2–4 µg/mL of streptorubin B, full growth was observed after 24 h.

DISCUSSION

Drug resistance is one of the most serious problems at clinical sites, and usage of antimicrobial drugs for treatment of infectious diseases caused by drug-resistant bacteria is limited. Under such circumstances, we have been focusing on the search for an inhibitor of biofilm formation. Biofilm is one bacterial virulence factor, and it is difficult to remove the bacterial biofilm once formed. We selected products of actinomycetes as the source for searching for such an inhibitor because actinomycetes are famous for producing a variety of biologically active substances including antibiotics.12)

We used the library of actinomycete culture extracts to find compounds that inhibit the biofilm formation of S. aureus. Several extracts showed an inhibitory effect on the biofilm formation of MRSA N315. We isolated one active compound from the product of Streptomyces sp. strain MC11024 and identified it as streptorubin B, a member of prodiginine. Recently prodiginines have again been attracting attention because they show a variety of bioactivities, antibiotic, anticancer, antimalarial, antiprotozoal, antifungal, immune-suppressive activities and so on.13–17)

The MIC of streptorubin B with MRSA N315 was more than or equal to 32 µg/mL, thus anti-MRSA activity is not strong. The biofilm formation of MRSA N315 was reduced to 27% at 1 µg/mL of streptorubin B. Biofilm formation was reduced to less than 50% even in the presence of 0.17 µg/mL, less than or equal to the 1/188 of the MIC. The cell growth of MRSA N315 in the shaking condition was affected by streptorubin B more than 2 µg/mL to some extent. However, bacterial full growth was observed at 24 h, where we usually judge the effect on biofilm formation, even in the presence of 2–4 µg/mL of streptorubin B. Though this culture condition of growth measurement is different from that of biofilm formation, it is speculated that inhibition of biofilm formation by streptorubin B at this concentration range is not due to some or slight effect on the growth. IC50 of streptorubin B against biofilm formation of MRSA N315 was calculated to be 0.22 µg/mL (0.56 µM). It has been reported that IC50 of other inhibitors of biofilm formation of S. aureus were more than 2.8 µM.18,19) Compared with such compounds, inhibitory effect of streptorubin B on biofilm formation by S. aureus is considerably strong. We only showed the inhibitory effect of biofilm formation on MRSA N315 in this paper. Further experiments should be needed. Prodigiosin has the ability of chelating metals and inducing oxidative DNA cleavage.20) The inhibitory effect of biofilm formation by streptorubin B may depend on these abilities. Although the target of streptorubin B is not clear yet, streptorubin B is an interesting compound for developing inhibitor of biofilm formation by S. aureus. Furthermore the cell growth of MRSA N315 was also affected in the presence of more than 2 µg/mL of streptorubin B, this compound might be useful.
The inhibitors of bacterial biofilm formation reduce the adherence of bacteria to the surface including medical devices and resistance against antibacterial drugs. Furthermore, they help human immune systems recognize bacteria. Thus, they and resistance against antibacterial drugs. Furthermore, they adherence of bacteria to the surface including medical devices by utilizing these kinds of anti-infectious compounds.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


