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Antibacterial Potential of Termite-Associated *Streptomyces* spp

Ling-Feng Zhou,[‖] Jun Wu,[‖] Shuai Li, Qi Li, Li-Ping Jin, Cai-Ping Yin, and Ying-Lao Zhang*

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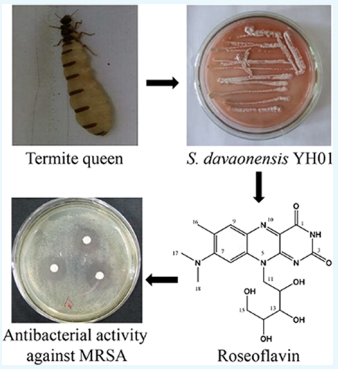
ABSTRACT: Twenty-one strains of termite-associated actinomycetes were tested for their activities against three bacteria. The results showed that nine strains showed bacteriostatic activities against at least one tested bacterium, and the actinomycete YH01, which was isolated from the body surface of the queen of *Odontotermes formosanus*, had potent antibacterial activity. The YH01 was further identified as *Streptomyces davaonensis*. Two metabolites roseoflavin (**1**) and 8-methylamino-8-demethyl-D-riboflavin (**2**) were isolated and purified from *S. davaonensis* YH01. Their structures were determined by NMR, MS, and the related literature. The metabolite **1** showed strong inhibition activities against *Bacillus subtilis* (MIC = 1.56 μg/mL) and *Staphylococcus aureus* (MIC = 3.125 μg/mL), which were comparable to referenced gentamycin sulfate, with MIC values of 1.56 and 1.56 μg/mL, respectively. Furthermore, the anti-MRSA potential of compound **1** was determined against nine kinds of MRSA strains, with inhibition zones in the ranges of 12.7−19.7 mm under a concentration of 15 μg/6 mm discs and 18.3−22.7 mm under a concentration of 30 μg/6 mm discs. However, metabolite **1** had no inhibitory effect on Gram-negative bacteria. These results suggested that roseoflavin produced by YH01 holds promise for use against Gram-positive bacteria, especially to MRSA.



INTRODUCTION

Antimicrobial resistance has long been an urgent problem for global health systems, and in recent decades, fewer and fewer new antimicrobials have been discovered.[1] Natural products are the source of most antimicrobials and are especially abundant in the metabolites of actinomyces.[2,3] However, most existing *Streptomyces* are found in soil, and most of the compounds derived from them are known compounds. New discoveries of natural antimicrobial products are needed to combat the emergence of multidrug-resistant pathogens.[4−7] The metabolites of some microorganisms occupying particular niches, which have been little studied, may be potential sources of new antimicrobial agents.

Insects are widely distributed in a variety of ecological niches, and their ability to live in unique habitats is often to promote symbiosis with their microbes.[8,9] There are 900,000 known species in the insect class and 2 to 30 million predicted species.[10] It is estimated that at least 15−20% of insects coexist with microorganisms.[11] The enormous insect species bred the large symbiotic microbial communities, which were sources of new antibiotic metabolites.[12−14] However, there are few studies on bioactive substances from insect symbionts.[15,16] In our ongoing identification of bioactive compounds from the insect-associated microbes,[17−19] 21 strains of termite-derived *Streptomyces* spp. were tested for their activities against three bacteria. We found that the methanol extract from the culture of the actinomycetes YH01, isolated from the queen of *Odontotermes formosanus*, exhibited potent antibacterial activities against several tested bacteria. Subsequent bioassay-guided separation of the crude extract afforded two main bioactive metabolites. The isolation, structural identification, and bioactivity of the two metabolites were reported in detail in this paper.

RESULTS

Antimicrobial Activities of Termite-Associated Actinomycetes. Twenty strains were isolated from the termite nest, and one strain was isolated from the body surface of the termite queen. The 21 strains of termite-associated actinomycetes were tested for their activities against three bacteria, and the results are shown in Table 1. Of the 21 actinomycetes, nine strains showed inhibition activities against at least one tested bacterium. The YH01 crude extract had potent antibacterial effect against *B. subtilis* (ATCC 6633) with a ZOI of 30 mm, which was better than referenced gentamicin sulfate with a ZOI value of 25 mm. The crude extract of YH01 also had a moderate antibacterial effect against *S. aureus* (ATCC 6538) with a ZOI of 15 mm, which was comparable to positive gentamicin sulfate with a ZOI value of 23 mm. However, it had no antibacterial activity against *E. coli* (ATCC 8739).

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Table 1. Inhibition Zone Diameters of Inhibiting Effects of Actinomycetes on Three Tested Bacteria (mm)

strain	*E. coli*	*S. aureus*	*B. subtilis*	strain	*E. coli*	*S. aureus*	*B. subtilis*
T45	NI[a]	NI	NI	T60	NI	NI	NI
T14	NI	NI	NI	T16	14.0 ± 0.1	12.0 ± 0.0	NI
T44	NI	14.5	14.5 ± 0.5	T55	17.5 ± 0.5	17.0 ± 1.0	NI
T21	NI	NI	NI	T9	13.0 ± 0.7	11.5 ± 0.7	14.3 ± 2.84
T17	18.3 ± 1.0	16.0 ± 0.5	NI	T1	17.1 ± 0.2	9.5 ± 0.0	12.0 ± 1.2
T25	NI	NI	NI	T38	NI	NI	NI
T10	NI	NI	NI	T54	19.8 ± 1.8	20.6 ± 0.9	NI
T61	11.3 ± 1.1	11.9 ± 0.1	11.6 ± 0.4	T57	NI	NI	NI
T19	NI	NI	NI	T58	NI	NI	NI
T40	NI	NI	NI	YH01	NI	15 ± 0.5	30 ± 1.2
T8	NI	NI	NI	PC[b]	23.0 ± 0.5	23.2 ± 0.2	25.5

[a]NI = not inhibited. [b]PC: gentamicin sulfate was used as a positive control.

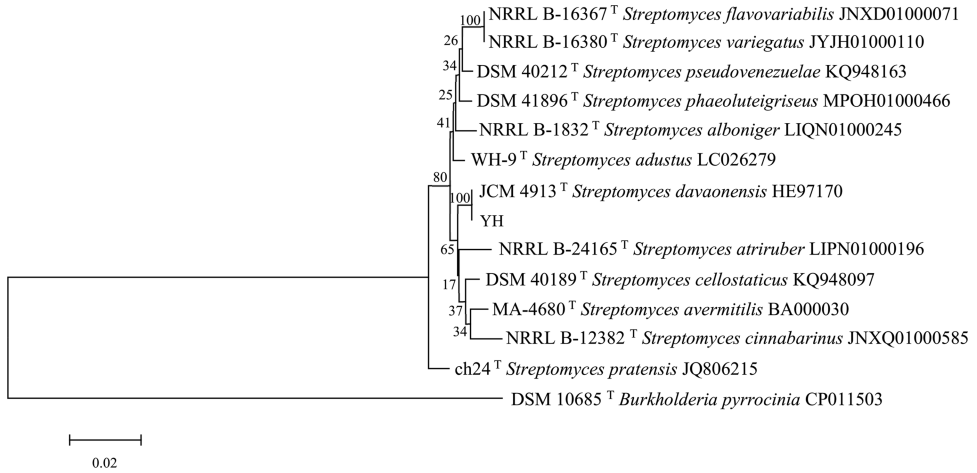

Figure 1. Phylogenetic tree obtained by neighbor joining analysis of 16S rRNA gene sequences showing the relationship between YH01 and related species belonging to genus *Streptomyces* obtained from the Ezbiocloud database. The numbers on branch nodes are bootstrap values (expressed as a percentage of 1000 replications).

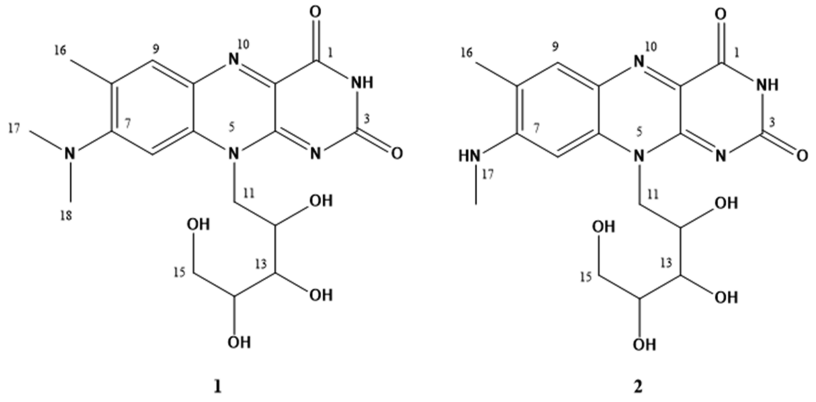

Figure 2. Chemical structures of secondary metabolites **1** and **2**.

Identification of the Actinomycetes. The identification of actinomycetes except YH01 has been completed, and relevant data have been published.[20] All of these actinomycetes belong to the genus *Streptomyces*.

The actinomycete YH01 grew well on Gause No. 1 solid medium. Aerial mycelium and substrate mycelium developed an opaque white color. The surface of the colony was dry and dense. A pink diffusible pigment was produced in the medium by YH01. Microscopic observation of YH01 showed long and straight spore hypha. At the same time, no transverse septum and no fracture were observed in the substrate mycelium. Based on morphological characteristics, the isolate YH01 was preliminarily identified as *Streptomyces* sp. It was further confirmed by 16S rRNA sequencing. The 1460 bp strain of the 16S rRNA gene was amplified from the YH01 strain, and its login number was MT242592. The phylogenetic tree shows that YH01 is closely related to *S. davaonensis* (JCM 4913T), with a sequence similarity of 99.8% (Figure 1). So, YH01 was identified as *S. davaonensis* based on morphological and molecular characteristics.

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Separation and Identification of the Antibacterial Compounds of YH01. Activity-monitored fractionation of the methanol extract of *S. davaonensis* YH01 yielded two active metabolites. The structures of the compounds (Figure 2) were determined by mass spectrometry, nuclear magnetic resonance spectroscopy, and the related literature.

Compound **1** (roseoflavin): red powder. ESI-MS: m/z $[M + H]^+$ 406 (calcd for $C_{18}H_{23}N_5O_6$ 405.1648), $[M + K]^+$ 445. ^1H NMR (DMSO-d_6) δ: 2.43 (3H, s, H-16), 3.01 (6H, s, H-17,18), 3.46 (1H, d, 5.3 Hz, H-12), 3.61 (3H, s, H-15), 4.25 (1H, s, H-14), 4.49 (1H, s, H-15), 4.53 (1H, s, H-15), 4.80 (2H, d, 5.4 Hz, H-11), 5.00 (1H, s, H-11), 5.18 (1H, s, H-13), 7.17 (1H, s, H-6), 7.75 (1H, s, H-9), 11.11 (1H, s, H-2). ^{13}C NMR (DMSO-d_6) δ: 20.5 (C-16), 43.5 (C-17), 47.4 (C-18), 63.8 (C-15), 69.3 (C-12), 73.1 (C-14), 73.8 (C-13), 102.3 (C-6), 129.8 (C-8), 131.7 (C-9), 132.0 (C-9a), 134.5 (C-5a), 134.5 (C-10a), 151.8 (C-7), 156.1 (C-4a), 159.2 (C-3), 161.0 (C-1). It indicated that compound **1** was roseoflavin.[21]

Compound **2** (8-methylamino-8-demethyl-D-riboflavin): yellow powder. ESI-MS: m/z $[M + H]^+$ 392 (calcd for $C_{17}H_{21}N_5O_6$ 391.1492), $[M + Na]^+$ 414, ^1H NMR (DMSO-d_6) δ: 2.25 (3H, s, H-16), 2.96 (3H, s, H-17), 3.46 (1H, d, 5.3 Hz, H-12), 3.61 (3H, s, H-15), 4.27 (1H, s, H-14), 4.49 (1H, s, H-15), 4.53 (1H, s, H-15), 4.95 (2H, d, 5.4 Hz, H-11), 5.00 (1H, s, H-11), 5.27 (1H, s, H-14), 6.75 (1H, s, H-6), 7.63 (1H, s, H-9), 10.91 (1H, s, H-2). It indicated that compound **2** was 8-methylamino-8-demethyl-D-riboflavin.[22]

Antibacterial Activity of Compound 1. The minimum inhibitory concentration (MIC) values of compound **1** against four bacteria are presented in Table 2. The results manifested that metabolite **1** could significantly inhibit the growth of *B. subtilis* and *S. aureus*, with MIC values of 1.56 and 3.125 μg/mL, respectively, which were comparable to the positive gentamicin sulfate with MIC values of 1.56 and 1.56 μg/mL, respectively. However, compound **1** could not inhibit the growth of Gram-negative *E. coli* and *S. typhimurium* under a maximum concentration of 25 μg/mL. Due to the small amount of compound **2**, its antibacterial activity was not investigated in this experiment.

Table 2. MICs (μg/mL) of Compound 1 against the Tested Bacteria

tested bacteria	1	gentamicin sulfate
B. subtilis (ATCC 6633)	1.56	1.56
S. aureus (ATCC 6538)	3.125	1.56
E. coli (ATCC 8739)	>25	12.5
S. typhimurium (CMCC(B) 50,115)	>25	3.125

We further tested the antibacterial activities of compound **1** against Gram-positive methicillin-resistant *S. aureus* (MRSA). Its inhibition zones against a variety of MRSA are presented in Table 3 and Figure 3. The results show that metabolite **1** presented significant antibacterial activities against all tested MRSA with inhibition zones in the ranges of 12.7−19.7 mm under a concentration of 15 μg/6 mm discs and 18.3−22.7 mm under a concentration of 30 μg/6 mm discs.

Table 3. Zones of Inhibition (mm) of Compound 1 against a Variety of MRSA

MRSA strain	30 μg/dics	15 μg/dics
MRSA01	18.3	15.3
MRSA02	21.0	19.0
MRSA03	18.7	17.3
MRSA04	20.3	12.7
MRSA05	22.7	19.7
MRSA06	20.7	18.3
MRSA07	21.7	18.7
MRSA08	21.0	19.3
MRSA09	18.3	16.0

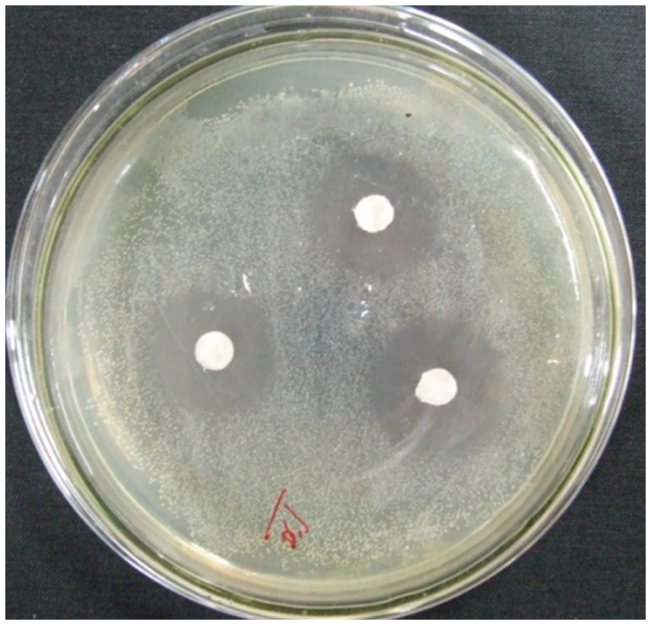

Figure 3. Antibacterial activity of compound **1** (30 μg/6 mm dics) against MRSA05.

DISCUSSION

Both compounds **1** and **2** were analogues of riboflavin (vitamin B_2), which was first obtained from milk in 1933.[23] The human body itself cannot synthesize vitamin B_2. The lack of this vitamin can easily cause diseases such as angular cheilitis, cheilitis, glossitis, eye conjunctivitis, and scrotal phlogistic.[24] Similar to the human body, insects cannot synthesize vitamin B compounds by themselves. However, some studies have found that the symbiotic bacteria could supply vitamins of the B group in significant amounts for their hosts.[25,26] So, as analogues of vitamin B_2, both compounds **1** and **2** may be related to the nutrient supply of the termite queen. This inspired us to think about the symbiotic relationship between the YH01 and the queen of *O. formosanus*. Is there an obligatory symbiosis between all termites and the species? Does roseoflavin only exist on the body surface of the queen to promote the nutritional supply? If there is a symbiotic relationship, how does this symbiosis pass on to the offspring termites? In order to understand the ecological function of YH01, these questions are worthy of further study.

From the perspective of the chemical structure, compound **1** is the analogue of compound **2**, and there is a slight difference at the position of 7, which are tertiary amine group and the secondary amine group, respectively. Furthermore, compound **2** is the precursor substance of **1** from the perspective of the biosynthesis pathway. During exploring the synthesis pathway of roseoflavin in *S. davawensis*, Jankowitsch et al. found a new dimethyl transferase ORF sda77220, which might catalyze the transformation of compound **2** to compound **1**.[27] This is

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consistent with our experimental result that the yield of compound **1** is much higher than that of compound **2**.

Many Gram-positive bacteria seem to be capable of acquiring riboflavin (vitamin B_2) from the environment, whereas most Gram-negative bacteria depend on the endogenous synthesis of this vitamin.[28] As a riboflavin analog, roseoflavin (**1**) can be taken up by the riboflavin importers of target cells. Thus, roseoflavin only acts against bacteria with riboflavin importers, which appear to be present mostly in Gram-positive bacteria.[29] This is consistent with our experimental result that compound **1** had an inhibitory effect on the tested Gram-positive bacteria but not on the tested Gram-negative bacteria.

The antibacterial mechanism of roseoflavin (**1**) was mainly due to potential riboswitch inhibition. For example, roseoflavin could directly bind to FMN riboswitch aptamers and downregulate the expression of the FMN riboswitch-*lacZ* reporter gene in *B. subtilis*.[30,31] In addition, the FMN riboswitch of *Listeria monocytogenes* was negatively affected by roseoflavin.[32] However, previous studies mainly focused on antibacterial activities against common pathogens by roseoflavin but less on drug-resistant bacteria. In the present study, roseoflavin showed excellent antibacterial activity against MRSA. This suggests that it has potential to resist clinically antibiotic-resistant bacteria, especially to MRSA.

CONCLUSIONS

Of the 21 strains of *Streptomyces* spp., nine actinomycetes showed antibacterial activities against at least one tested bacterium. One strain identified as *S. davaonensis* YH01 was isolated from the body surface of the queen of *O. formosanus* and had potent antibacterial activities. Two compounds roseoflavin (**1**) and 8-methylamino-8-demethyl-D-riboflavin (**2**) were obtained from the solid-state fermentation products of YH01. Metabolite **1** showed potent antibacterial activities against *B. subtilis*, *S. aureus*, and MRSA, which was comparable to referenced gentamycin sulfate. However, compound **1** had no inhibitory effect on Gram-negative bacteria.

MATERIALS AND METHODS

Isolation and Identification of Actinomycetes from the Nest and Queen of *O. formosanus*. The actinomycetes of the termite nest were isolated based on the methods detailed previously.[20] The strain YH01 was isolated from the queen of *O. formosanus* based on the method detailed elsewhere with some modifications.[33] Briefly, the queen of *O. formosanus* was captured in the suburb of Lanxi City, Zhejiang Province, China. The body surface of the queen was scratched by sterile cotton swabs one to two times and lightly coated with the Gause No. 1 solid medium. The media were supplemented with 0.1 g/L cycloheximide to suppress fungal growth. The plates were incubated at 28 °C for 3−4 days. A single colony was selected and purified to obtain pure actinomycete named YH01. Actinomycetes were identified by 16S rRNA sequencing and compared with the standard record.

Screening for Antibacterial Activities. The antimicrobial activities of actinomycetes from the nest of *O. formosanus* were evaluated using the Oxford cup method.[34] All tested bacteria were activated in LB liquid medium. Under aseptic conditions, an activated bacterial suspension was diluted with distilled water to 0.5−1 McIntosh turbidities. 200 μL of the above bacterial suspension was taken and evenly spread on the corresponding media. Fermented liquids of actinomycetes (100 μL of liquid filtered using a 0.22 μm filter membrane) were placed into the dish through the Oxford cup. The inhibition zone diameter was measured after incubation for 24 h at 37 °C. The experiment was repeated in triplicate for each sample.

The antimicrobial activities of actinomycete from the body surface of the queen of *O. formosanus* were evaluated by the disc diffusion method.[35] All tested bacteria were cultured overnight at 37 °C in Mueller Hinton broth (MHB), and then 200 μL of suspension of the tested microorganisms (10^8 cfu/mL) was spread on the beef extract peptone solid medium. Filter paper discs containing 5 μL of crude extract solution (6 mg/mL) were applied to the surface of solid plates. The plates were incubated at 37 °C for 18−24 h, and then the diameter of the inhibitory zone was measured. Gentamicin sulfate at the same concentration was selected as positive control. All tests were performed in triplicate.

Fermentation and Extraction of YH01. The fermentation and extraction of target strain YH01 were performed according to the methods detailed previously[36] with some modifications. The cultured actinomycete on the Gause No. 1 solid medium was mixed with distilled water to form a spore suspension with a concentration of 1.0×10^5 per mL. 5 mL of spore suspension was inoculated in a 1 L Erlenmeyer flask containing 300 mL of Gause No. 1 solid medium and fermented at 28 ± 1 °C for 15−20 days until the medium color turned bright red.

Isolation and Identification of the Active Compounds of YH01. A total of 15 L of fermentation product was divided into uniform small pieces with a medicine spoon. Methanol was used to extract the leachate for 24 h. This process was repeated many times until the extract has no obvious color. Methanol was then removed in vacuum to obtain 5.5 g of crude extract. The crude extract was eluted using an inverse silica gel column with a gradient of MeOH/H_2O (40:60−100:0, v/v) to obtain four fractions (Fr-1 to Fr-4). Fr-1 was further isolated on silica gel (MeOH/H_2O, 40:60−60:40) and purified on a Sephadex LH-20 (MeOH) to yield compound **1** (150 mg). Fr-2 was further separated by semiprepared HPLC (Agilent Prostar 218 Prep-LC03030623) using a C18 reversed-phase column (Cosmosil 10 mm I.D. × 250 mm) to give compound **2** (3 mg). HPLC parameters were as follows: the mobile phase MeOH/H_2O was 35%, the temperature of the column box was set at 30 °C, and the detection wavelength was 280 nm, with a flow rate of 2 mL/min. The peak time of compound **2** was 25.6 min.

The structures of the secondary metabolites were identified by spectral analysis. ^1H and ^{13}C nuclear magnetic resonance (NMR) were measured at 400 MHz using a Bruker AVANCE-400 (Bruker, Switzerland) spectrometer, and chemical shifts were reported as parts per million (δ) by referring to the solvent signals and tetramethylsilane (TMS) as internal standards. The electrospray ionization mass spectrometry (ESI-MS) spectra were acquired on a Mariner Mass 5304 instrument.

Detection of Antibacterial Activity of the YH01 Compound. The minimum inhibitory concentrations (MICs) of the purified compound against bacteria were determined using the microbroth dilution method in disposable 96-well microtiter dishes.[37] First, the isolated compound was prepared in a 200 μg/mL solution with liquid MHB (supplemented with 4% Tween 80 and 1% DMSO, v/v).

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A series of concentration gradients (ranging from 100 to 0.78 μg/mL) were then obtained by multiple 2-fold dilutions and inoculated into a single microplate well (100 μL/well) with a micropipette. Then, a standard amount (100 μL) of the tested microbes (10^5 cfu/mL) was added per well and incubated 24 h at 37 °C for *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), and *Salmonella typhimurium* (CMCC(B) 50,115). The culture medium and microorganisms without the compound were used as negative controls. The antibacterial activities were determined by comparing the experimental group with the negative control group. The MIC was defined as the minimum concentration of compounds at which the bacterial growth was inhibited, as indicated by the absence of turbidity. Three replicates were used for each test, with gentamicin sulfate as the reference compound.

MRSA was tested with the Kirby−Bauer disk diffusion technique using a cefoxitin (30 μg) disk, and the strains of *S. aureus* with zones of diameter ≤21 mm were regarded as methicillin-resistant. All MRSA strains were obtained from clinical patients in Zhejiang Jinhua Guangfu Hospital. The anti-MRSA assay was performed using the agar well diffusion susceptibility test as described by CLSI.[38]

AUTHOR INFORMATION

Corresponding Author

Ying-Lao Zhang − *College of Life Sciences, Anhui Agricultural University, Hefei 230036, People's Republic of China;* orcid.org/0000-0001-7599-9400; Phone: +86-551-657862169; Email: zhangyl@ahau.edu.cn

Authors

Ling-Feng Zhou − *College of Life Sciences, Anhui Agricultural University, Hefei 230036, People's Republic of China*

Jun Wu − *College of Life Sciences, Anhui Agricultural University, Hefei 230036, People's Republic of China*

Shuai Li − *College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua 321004, People's Republic of China*

Qi Li − *Zhejiang Jinhua Guangfu Hospital, Jinhua 321004, People's Republic of China*

Li-Ping Jin − *College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua 321004, People's Republic of China*

Cai-Ping Yin − *College of Life Sciences, Anhui Agricultural University, Hefei 230036, People's Republic of China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05580

Author Contributions

‖L.-F.Z. and J.W. have contributed equally to this work. Y.-L.Z. designed the research. Y.-L.Z. supervised the study. L.-F.Z. wrote the manuscript. J.W. consulted the literature and analyzed the results. S.L. performed the experiments and analyzed the data. All authors revised the manuscript and approved the final version for submission.

Notes

The authors declare no competing financial interest.

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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