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Comparative metabolomics reveals streptophenazines with anti-methicillin-resistant *Staphylococcus aureus* activity derived from *Streptomyces albovinaceus* strain WA10-1-8 isolated from *Periplaneta americana*

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Abstract

Background Streptophenazines, a class of phenazine compounds with a variety of alkyl side chains and activity against methicillin-resistant *Staphylococcus aureus* (MRSA), are mainly derived from soil or marine microbial secondary metabolites. However, the discovered phenazine compounds still do not meet the needs of the development of anti-MRSA lead compounds. Here, we examined secondary metabolites of *Streptomyces albovinaceus* WA10-1-8 isolated from *Periplaneta americana*, for streptophenazines with anti-MRSA activity.

Results In this study, a guidance method combining high-performance liquid chromatography-ultraviolet (HPLC-UV) with molecular networking analysis was used to isolate and identify a series of streptophenazines (A–T) from *S. albovinaceus* WA10-1-8. Among them, a new streptophenazine containing a dihydroxyalkyl chain structure named streptophenazine T was isolated and identified for the first time. The results of bioactivity assays showed that streptophenazine T had anti-MRSA activity with a minimum inhibitory concentration (MIC) of 150.23 μM, while the MICs of streptophenazine A, B, G, and F were 37.74-146.12 μM.

Conclusions This study was the first to report multiple streptophenazine compounds with anti-MRSA activity expressed by *Streptomyces* isolated from insect niches. These results provided a valuable reference for future exploration of new streptophenazine compounds with activity against drug-resistant bacteria.

Keywords *Streptomycetes*, Streptophenazine, Metabolomics, MRSA, *Periplaneta americana*

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the greatest threats among globally pandemic pathogens on account of its high morbidity and mortality rates [1, 2]. MRSA, in addition to being resistant to methicillin-beta-lactam classes, also exhibits significant resistance to ceftaroline, oxacillin, and cephalosporin class antibiotics [3, 4]. Although the commonly used antibiotics, such as vancomycin, daptomycin, linezolid, etc., still exhibit strong inhibitory effects against MRSA, the irregular and persistent use may lead MRSA to develop a tolerance to these drugs [5–7]. Therefore, it is necessary to discover new anti-MRSA drugs or lead compounds. Natural products have been a significant source of various approved antibiotics, and remains one of the most popular sources for seeking new and effective antimicrobial lead compounds [8]. Among them, *Streptomyces* is an important source of many active natural products [9]. Streptophenazines are a special subgroup of phenazine derivatives with variable alkyl side chains, which has been firstly discovered in the marine *Streptomycete* sp. HB202 [10]. It has been reported that streptophenazine B exhibits significant anti-MRSA activity [11]. However, the discovered phenazine compounds from soil or marine *Streptomycete* still do not meet the needs of the development in anti-MRSA lead compounds.

Insects are one of the most widespread organisms on Earth. The digestive systems and glands of these insects harbor a variety of symbiotic microorganisms, and their diverse living environments result in significant differences in the types and quantities of these microorganisms [12]. Several studies have reported the significant potential and application value of insect symbiotic microorganisms in the production of new bioactive natural products [13–15].

P. americanas are among the most widely distributed insects in nature and have long been considered pests that pose a threat to human health [16]. On account of the unique living environments, there are abundant and large number of microorganisms colonizing in the digestive tract of *P. americana*s [17], suggesting that it may be a huge reservoir of microbial natural products. In previous studies, we have been isolated 159 strains of actinomycetes from the intestinal tract of 13 *P. americanas* and it has been found a series of lead compounds with antimicrobial activities [18–20].

In this study, we have isolated and identified a series of streptophenazine compounds (A–T) from the symbiotic *S. albovinaceus* strain WA10-1-8 of *P. americana* by using a guidance method combining high-performance liquid chromatography-ultraviolet (HPLC-UV) with molecular networking analysis. A new streptophenazine compound with anti-MRSA activity (MIC = 150.23 μM) named streptophenazine T was found for the first time. The results of this study revealed that the secondary metabolites of symbiotic *Streptomyces* of *P. americana* might be an important source of new streptophenazine compounds, providing new insights into the development of anti-MRSA or other drug-resistant bacteria lead compounds from symbiotic microorganisms of *P. americana* and other insects.

Materials and methods

Test strain, chemicals and media

The strain of bacteria were tested: Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 which were obtained from Guangdong culture collection center. The genomic DNA isolation kit was purchased from Takara (Beijing, China). Microbiological media were purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd (GuangZhou, China). High-performance liquid chromatography (HPLC) grade methanol was purchased from Honeywell. Solvents used for extraction and column chromatography were of analytical grade which were obtained from Guangdong Guanghua Sci-Tech Co., Ltd (GuangZhou, China).

Primary antimicrobial activity assay

Primary screening for antimicrobial activity was performed by growing cultures on Luria-Bertan (LB) agar plates swabbed with the MRSA suspension ($1–5 \times 10^4$ CFU/mL). 100 μL of the ethyl acetate extract obtained from strain WA10-1-8 was added to an oxford cup on the LB agar plates and the incubated at 28 ℃ for 12–14 h. 100 μL of the vancomycin was used as positive control with the concentration of 128 μg/mL and methanol was used as a negative control. The experiment was repeated in triplicate for each test strain to record the average diameter of the inhibition zone [21].

Isolation and identification of *S. Albovinaceus* WA10-1-8

The *P. americana* samples collected from the wild were rinsed with running water, soaked in 75% ethanol for 2 min, and then disinfected with 3.5% sodium hypochlorite for 2 min. Afterward, they were washed three times with sterile water and dried with sterile absorbent paper. The abdomen of the *P. americana* was dissected using a scalpel, and its intestines were removed. The intestines were placed in a homogenizer with sterile water for grinding, and then diluted to the desired concentrations (10 mg/L, 0.005 mg/L, 0.0025 mg/L). Next, samples at different dilution concentrations were taken and subjected to separation using the plate dilution method and streak plate method. Each dilution concentration was repeated three times and incubated in a 28 ℃ incubator for 3–7 d until the colonies grow. Colonies with distinct characteristics were selected and further purified on NA

plates (nutrient agar medium). The purified colonies were stored on slant ISP-2 medium for preservation.

S. albovinaceus WA10-1-8 was inoculated on Gause's synthetic agar no.1 and cultured in constant-temperature incubator at 28 ℃ for 4–5 d, during which time the culture had entered the logarithmic growth phase. *Streptomyces* strain WA10-1-8 was preliminarily identified based on colony morphological characteristics on Gause's synthetic agar no.1 and gram staining characteristics under microscope. Whole genomic DNA of strain WA10-1-8 was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma) according to manufacturer's instructions. After that, used universal bacterial primer set 27 F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3′) to amplify 16 S rRNA gene [22]. PCR was performed in a total volume 50 µL reaction containing of 1 µL DNA template, 1 µL upstream primer (Invitrogen), 1 µL downstream primer (Invitrogen), 25 µL PCR Premix (Invitrogen) and 22 µL sterile ddH_2O. Thermocycler conditions began with an initial denaturation step at 94 °C for 4 min followed by 35 cycles each consisting of 30 s at 94 °C, 30 s at 55 °C for annealing, and 30 s at 72 °C for extension followed by a post-cycle extension at 72 °C for 5 min. The resulting PCR products were verified by electrophoresis on a 1.5% agarose gel and was sequenced by BGI Genomics Institute (GuangDong, China). The sequenced 16 S rRNA sequence was uploaded to the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), and used BLAST software to compare all sequences available in GenBank [23]. Sequences were aligned with Clustal software and MEGA 11 was used to carry out a phylogenetic tree of the alignment using the maximum likelihood method [24].

Detection of anti-MRSA activity and HPLC analysis of metabolites from *S. Albovinaceus* WA10-1-8

Metabolite containing maximum content of streptophenazines and optimal anti-MRSA activity are obtained by changing fermentation temperature. We evaluated the effect of culture temperature from 24 ℃ – 38 ℃ on the change of streptophenazines in metabolites.

At the end of the fermentation cycle the mycelium and supernatant was separated by centrifugation at 8,000 rpm for 15 min. Collection of supernatant and extraction of three times with equal volume of ethyl acetate to obtain fermented crude extract. Finally, The anti-MRSA activity (MRSA ATCC 43300) was assessed using the crude extract, and crude extracts were determined by high-performance liquid chromatography (HPLC). The samples were analyzed triplicate by analytical HPLC after filtering use 0.22 µm syringe filter. The injection volume was set at 10 µL and the chromatographic method was constituted by a gradient of mixtures of solvents A (0.1% acetic acid in H_2O) and B (methanol) of: 0–5 min (10% B); 5–40 min (10–100% B); 40–50 min(100% B); 50–51 min (100 – 10% B); 51–60 min (10% B).

Fermentation, extraction, isolation of streptophenazine compounds

S. albovinaceus WA10-1-8 a single colony inoculated in 100 mL ISP1 liquid medium and cultivated as seed medium in 28 °C, 180 rpm thermostatic shaker for 2 d. RA medium was used as the fermentation medium: maltose extract (10 g/L), glucose (10 g/L), maltose (10 g/L), corn syrup (5 g/L), soluble starch (20 g/L), calcium carbonate (2 g/L) and trace elements (100 µL/L). 5% of seed medium was transferred into 500 mL Erlenmeyer flask containing 300 mL of RA medium as production flasks and incubated at 34 °C and 180 rpm for 15 d. The total 7 g crude extract was obtained from 72 L of fermentation, which were extracted with an equal volume of ethyl acetate.

The chromatography separation of the fermentation crude extract was performed using a silica gel column (50-cm height×4.5-cm diameter) with 200 g of 200–300 mesh silica gel powder as the stationary phase. The separation was carried out using petroleum ether-ethyl acetate systems (9:1, 8:2, 7:3, 6:4 v/v) and dichloromethane-methanol systems (9:1, 8:2, 7:3, 6:4 v/v) for gradient elution, with each solvent system eluted for 5 column volumes (1500 mL). All fractions from the silica gel column were pooled based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis. The characteristic chromatographic absorption peaks of streptophenazine were detected in pooled fractions 2, 3, and 7.

Fraction 2 was separated using a Sephadex LH-20 column (70-cm height×3-cm diameter) with approximately 1000 mL of 100% methanol, and collected the eluted fractions. Fraction Fr-2-2 contained the target compound, and was further separated using Sephadex LH-20 column with approximately 600 mL of 100% methanol elution, yielding fractions Fr-2-2-1 and Fr-2-2-2. Fraction Fr-2-2-1 was purified by semi-preparative HPLC to obtain compound **1** (15.3 mg) and compound **2** (11 mg).

Fraction 3 was separated using a Sephadex LH-20 column (70-cm height×3-cm diameter) with approximately 1000 mL of 100% methanol for isocratic elution, yielding fractions Fr-3-1 ~ Fr-3-3. Fr-3-2 and Fr-3-3 contained the target compound. Fr-3-2 was purified by semi-preparative HPLC to obtain compound **3** (7.5 mg). Fr-3-3 was further separated using a Sephadex LH-20 column (70-cm height×3-cm diameter) with approximately 350 mL of 100% methanol for isocratic elution, yielding fractions Fr-3-3-1 and Fr-3-3-2. Fr-3-3-2 was purified by semi-preparative HPLC to obtain compound **4** (7.0 mg).

Fraction 7 was separated using a Sephadex LH-20 column (70-cm height×3-cm diameter) with approximately 1200 mL of 100% methanol for isocratic elution. Fraction Fr-7-2 was further separated using a Sephadex LH-20 column (40-cm height×2.5-cm diameter) with approximately 200 mL of 100% methanol for isocratic elution, yielding fractions Fr-7-2-1 and Fr-7-2-2. Fraction Fr-7-2-1 was purified by semi-preparative HPLC to obtain compound **5** (5.5 mg), and Fr-7-5 was purified by semi-preparative HPLC to obtain compound **6** (4.0 mg).

The semi-preparative HPLC conditions for the purification of compounds **1**–**4** were as follows: a methanol-water system (60%:40%) as the mobile phase, with a sample injection volume of 100 µL, a flow rate of 2.5 mL/min, a column temperature of 25 °C, detection at dual wavelengths of 250 nm and 360 nm, and a YMC-Pack ODS-AQ 250 × 10.0 mm I.D., S-5 µm, 12 nm column type. The semi-preparative HPLC conditions for the purification of compounds **5**–**6** were as follows: a methanol-water system (30%:70%) as the mobile phase, with a sample injection volume of 100 µL, a flow rate of 2.5 mL/min, a column temperature of 25 °C, detection at dual wavelengths of 250 nm and 360 nm, and a YMC-Pack ODS-AQ 250 × 10.0 mm I.D., S-5 µm, 12 nm column type.

Streptophenazine T (**6**): yellow solid, soluble in methanol, DMSO and water; $[\alpha]^{20}_{D}$ −23.35 (*c* 0.01, MeOH); UV(MeOH) λ_{max} (logε) 205(4.56), 251(4.76), 348(3.91), 365(4.08) nm; IR (KBr) ν_{max} 3304, 2943, 2831, 1448, 1409, 1107, 1022 cm^{-1}; ECD (10 mg/L, MeOH) λ_{max} (Δε) 251 (−6.3) nm. ^{1}H NMR (CH_3OH-d_4, 500 MHz) and ^{13}C NMR (CH_3OH-d_4, 500 MHz) see Table 1; HRESIMS *m/z* 427.18582 $[M+H]^+$ (calcd for $C_{23}H_{27}N_2O_6$: 427.17944).

Table 1 1D and 2D NMR data in MeOH-d_4 for streptophenazine T (6)

Position	δ_C, type	δ_H, J [Hz]	HMBC	COSY
1	131.9, C			
2	136.4, CH	8.87 d (7.01)	4, 10a, 1-*C*OOH	3
3	132.8, CH	8.14 m	2, 4, 4a	2, 4
4	135.04, CH	8.64 d (8.6)	2, 10a	3
4a	142.2, C			
5a	141.8, C			
6	142.0, C			
7	129.0, CH	8.14 m	1′, 5a, 6a, 9	8
8	130.1, CH	8.14 m	6, 7, 9a	7, 9
9	127.2, CH	8.3 d (7.7)	6, 7, 9a, 10a	8
9a	140.1, C			
10a	139.8, C			
1′	69.2, CH	6.24 d (7.3)	5a, 6, 7, 2′, 3′, 2′-*C*OOCH$_3$	2′
2′	53.3, CH	3.29 m	1′, 3′, 4′	2′, 3′
3′	29.1, CH_2	1.75 m	4′	2′, 4′
4′	21.8, CH_2	1.32 m	3′, 5′	3′, 4′
5′	42.9, CH_2	1.32 m	3′, 4′, 6′	4′
6′	69.7, C			
7′	27.7, CH_3	1.09 s	4′, 5′, 6′, 8′	
8′	27.5, CH_3	1.09 s	4′, 5′, 6′, 7′	
1-*C*OOH	167.2, C			
2′-*C*OOCH$_3$	175.0, C			
2′-COO*CH*$_3$	50.9, CH_3	3.64 s	2′-*C*OOCH$_3$	

GNPS Molecular networking analysis and MS/MS spectra annotation

Using the Global Natural Product Social Molecular Networking (GNPS) platform (https://gnps.ucsd.edu) to construct a molecular network [25]. The MS^2 data of crude extracts and solvents (blank) were converted into mzXML file format using the MSConvertGUI tool and uploaded to the GNPS platform. The precursor ion mass tolerance was set to 0.02 Da, the fragment ion tolerance was set to 0.02 Da, and the cosine score was 0.7, with a maximum molecular family size of 100 for the construction of the molecular network (MN). The molecular network was visualized as a node and edge network using Cytoscape (3.9.1) [26]. Node sizes and edge thicknesses were automatically adjusted according to precursor ion abundance and the relationships between precursor ions, with redundant nodes manually removed. Additionally, various databases and tools were used for the manual annotation of spectra of interest, including Pubchem, Sci-finder, and Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID, https://cfmid.wishartlab.com/) [27].

Spectroscopic analysis

The detection of HRESIMS and HRESIMS/MS was performed using a Thermo Scientific Ultimate 3000 system connected to a Q Exactive Orbitrap detector, equipped with a Hypersil Gold C18 column (100 × 2.1 mm, 1.9 µm). The crude extract was dissolved and diluted to 1 mg/mL in chromatographic methanol. The solvent system consisted of 0.1% acetic acid solution (A) and methanol (B). The gradient program was: 0–5 min (10% B), 5–40 min (10–100% B), 40–50 min (100% B). All 1D and 2D NMR spectra were obtained using a Bruker AVANCE III 500 MHz NMR spectrometer (Bruker Company, Germany). Analytical HPLC was performed on a Waters 2695–2996 high-performance liquid chromatography system, using a reverse-phase YMC-Pack ODS-AQ column (250 × 4.6 mm, 5 µm, YMC Company, Japan). Semi-preparative HPLC was performed on a Waters high-performance liquid chromatography system, using a YMC-Pack ODS-AQ column (250 × 10.0 mm, 5 µm, YMC Company, Japan).

Minimum inhibitory concentration (MIC) testing

The minimum inhibitory concentration (MIC) of streptophenazine compounds against MRSA ATCC43300 was measured using the broth dilution method [28]. In

this experiment, MRSA was cultured in LB broth at 37 °C until the cell density reached approximately $1–5 \times 10^6$ CFU/ml. Streptophenazine compounds were tested in the concentration range of 0–128 μg/ml. The anti-MRSA activity was observed after 12 h of incubation at 37 °C. The lowest concentration of the compound that displayed antibacterial activity was recorded as the MIC value. The experiment was repeated three times, with vancomycin used as a positive control.

Scanning electron microscopy analysis

The tested bacterial suspension (1.5×10^5 CFU/mL) were treated with 1×MIC of the compounds for 12 h at 37 °C. The supernatant was removed by centrifuged and then the tested strain were placed on a cover glass and fixed overnight with 2.5% glutaraldehyde at 4 ℃. Fixed samples were washed with 1×PBS three times for 20 min each and dehydrated in increasing concentrations of ethanol (20, 40, 60, 80, and 100%). The coverslips were finally dried then analyzed by Quanta FEG 200 FESEM at an accelerating voltage of 2–19 kV under standard operating conditions.

Result

Identification and antimicrobial activity of *S. Albovinaceus* WA10-1-8

According to the morphological characteristics and comparison results of 16 S rRNA sequence showed in (Fig. 1), strain WA10-1-8 was identified as a *Streptomyces* species. The strain had quite good growth and abundant spores (powder) on the Gause's synthetic agar no. 1 plates, showed light brown aerial mycelia with gray diffusible pigments (Fig. 1A). The Gram staining result showed strain WA10-1-8 was the Gram-positive bacterium, and the elongated and curved hyphae were visible under an optical microscope (Fig. 1B). Elongated and branched hyphae attached to round or oval spores are observed under SEM (Fig. 1C). The 16 S rRNA sequence and the taxonomic position in the phylogenetic tree indicated that strain WA10-1-8 belongs to the *Streptomyces* genus, with 99.8% identity to *Streptomyces albovinaceus* CSSP418 (NR 115373) (GenBank Accession Number: KY206803) (Fig. 1E). Therefore, strain WA10-1-8 belongs to the *albovinaceus* species, and its deposit number was GDMCC NO. 4.422. We conducted an antibacterial activity screening of the metabolites from *S. albovinaceus* WA10-1-8 and found that it exhibited good activity against MRSA ATCC 43300. Consequently, MRSA was selected as the indicator bacterium for bioactivity-guided isolation of compounds (Fig. 1D).

HPLC-UV and molecular networking analysis of *S. Albovinaceus* WA10-1-8

The metabolites of *S. albovinaceus* WA10-1-8 exhibit certain anti-MRSA properties, although the bacteriostatic effect is relatively weak. Therefore, we attempted to stimulate the metabolic potential of *S. albovinaceus* WA10-1-8 and increase the expression of antimicrobial substances through modifying the cultivation conditions. In exploratory experiments on changing the cultivation temperature, HPLC analysis revealed significant changes in the metabolites of *S. albovinaceus* WA10-1-8 at a cultivation temperature of 34 °C. Mainly exhibited a significant increase in the chromatographic peak abundance at 40 min (Fig. 2A). Additionally, the metabolites obtained under the 34 °C cultivation condition showed significantly enhanced anti-MRSA activity, compared to other cultivation temperature conditions (Fig. 2B). To assess whether *S. albovinaceus* WA10-1-8 produced previously unreported antibacterial compounds under the 34 °C cultivation condition. We analyzed the metabolites obtained at 34 °C and 28 °C using ESI-HRMS^2 combined with the Global Natural Products Social Molecular Networking (GNPS) molecular networking method. A comparison of the positive ESI ion chromatograms of the metabolites obtained at 34 °C and 28 °C showed significant ion peak differences at 35–38 min, corresponding to molecular ions of *m/z* 425.2068 and 439.2223 (Fig. 2C). In the molecular network, these ions indicated towers a molecular cluster containing 17 nodes (Fig. 2D). Among them, node **1** (*m/z* 425.2065 $[M+H]^+$) had the highest abundance with a relatively higher content under the 34 °C condition compared to the 28 °C condition. Other compounds represented by the nodes were either not produced or were in very low abundance under the 28 °C condition. Based on the molecular weight and previously reported data in the literature, we speculated that node **1** is streptophenazine A, a phenazine compound with variable alkyl side chains. Node **2** had the second-highest abundance and featured a molecular ion of *m/z* 439.2223, corresponding to the molecular formula $C_{25}H_{31}N_2O_5^+$ (calcd. *m/z* 439.2227), speculated to be streptophenazine G. In addition, based on the protonated molecular ions and fragmentation patterns in the MS^2 spectra, we predicted five other nodes (**4**–**8**) within the molecular cluster that potentially represent new compounds, as indicated by the red circles in Fig. 2D. All potential new compounds' MS^2 spectra and annotations are provided in Figs. S1-S5 and Table S1.

Isolation and identification of multiple streptophenazines from *S. Albovinaceus* WA10-1-8

Based on the metabolomics analysis results, the *S. albovinaceus* WA10-1-8 produced several highly abundant streptophenazines when the cultivation temperature

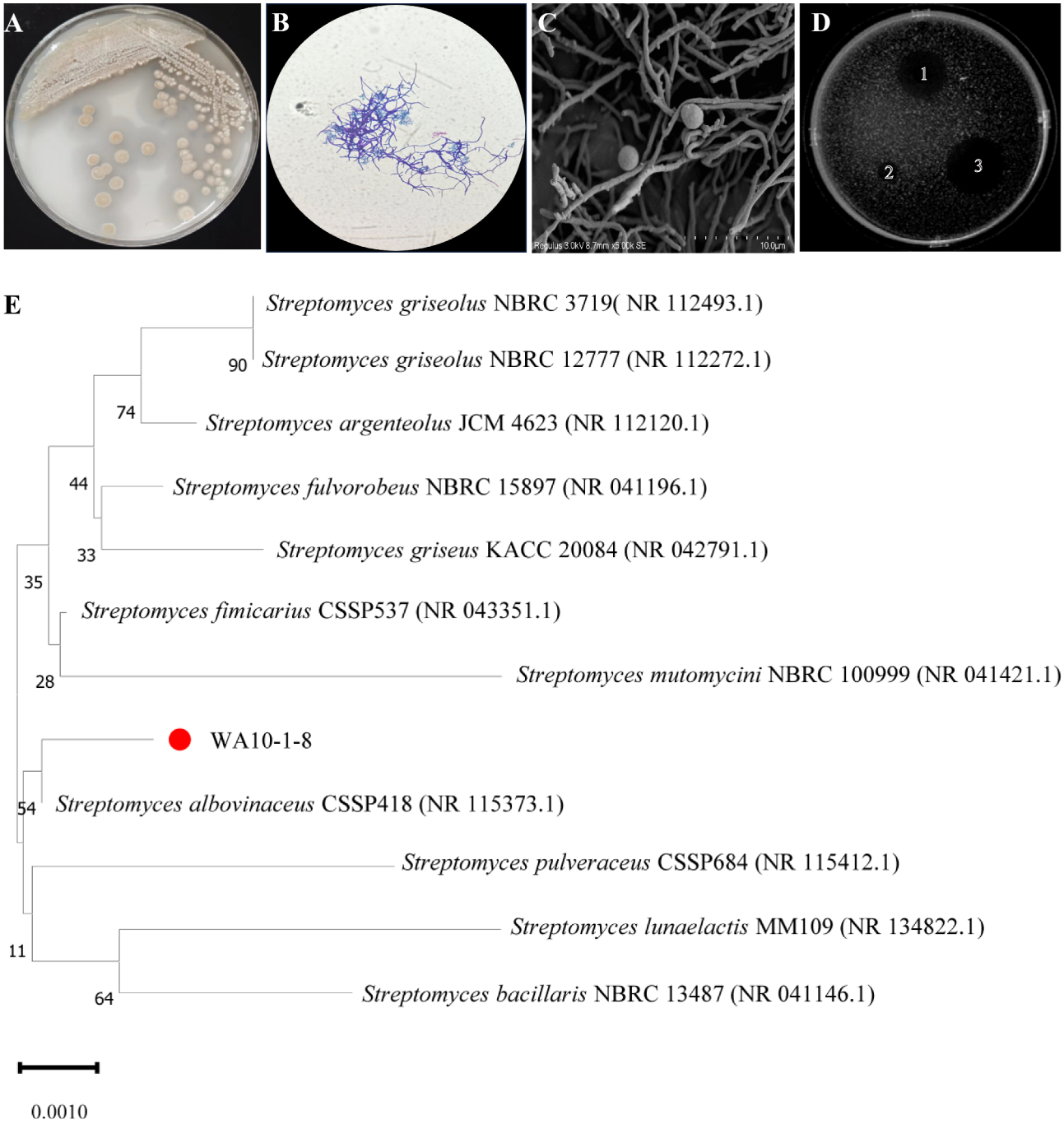

Fig. 1 The morphological characteristics, phylogenetic tree and anti-MRSA activity of *S. albovinaceus* WA10-1-8. (**A**) Culture on Gause's synthetic agar no. 1. (**B**) Observation of the culture under optical microscopy following Gram staining (100×). (**C**) Observation of the culture by scanning electron microscopy (5,000×). (**D**) The anti-MRSA activity of *S. albovinaceus* WA10-1-8, 1–3 refers to sample, blank control and positive control. The concentrations of sample was 5 mg/mL and positive control was 100 μg/mL and the experiment was performed in triplicate. (**E**) Maximum-likelihood phylogenetic tree based on 16 S rRNA gene sequences showing the positions of *S. albovinaceus* WA10-1-8

was 34 °C. These streptophenazines likely include both compounds with strong anti-MRSA activity and new streptophenazines. Therefore, we utilized the optimal cultivation conditions to scale up the culture of *S. albovinaceus* WA10-1-8. A total of 7.0 g of crude extract was subjected to silica gel column chromatography, yielding seven distinct fractions. The inhibitory activity of these seven fractions against MRSA ATCC43300 was evaluated using the Oxford cup method, with fractions 2, 3, and 7 showing significant activity, exhibiting inhibition zone diameters of 21.83 ± 0.61 mm, 17.40 ± 0.17 mm, and 14.37 ± 0.70 mm, respectively (Fig. 3). Compounds **1** and

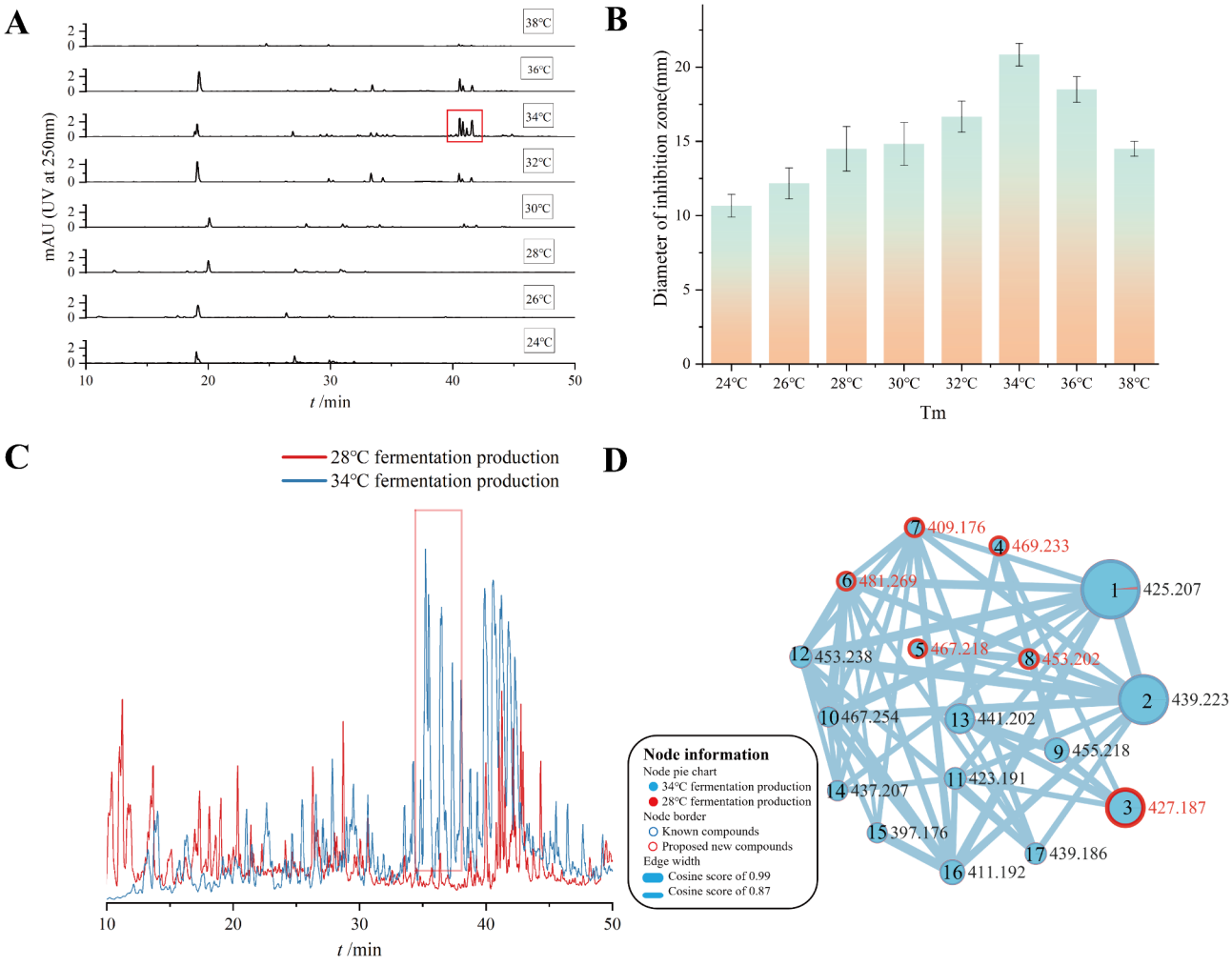

Fig. 2 Analysis of secondary metabolites of *S. albovinaceus* WA10-1-8 under different culture temperature conditions. (**A**) Variations in the metabolites of *S. albovinaceus* WA10-1-8 under different cultivation temperature conditions. Chromatographic peaks of streptophenazines highlighted in red boxes. (**B**) The size of the antibacterial zone of metabolites against MRSA under different cultivation temperature conditions. The experiment was repeated three times. (**C**) positive ESI ion chromatography of metabolites under 28 °C and 34 °C cultivation conditions. (**D**) Visualizing the features of culture extracts by GNPS molecular networking

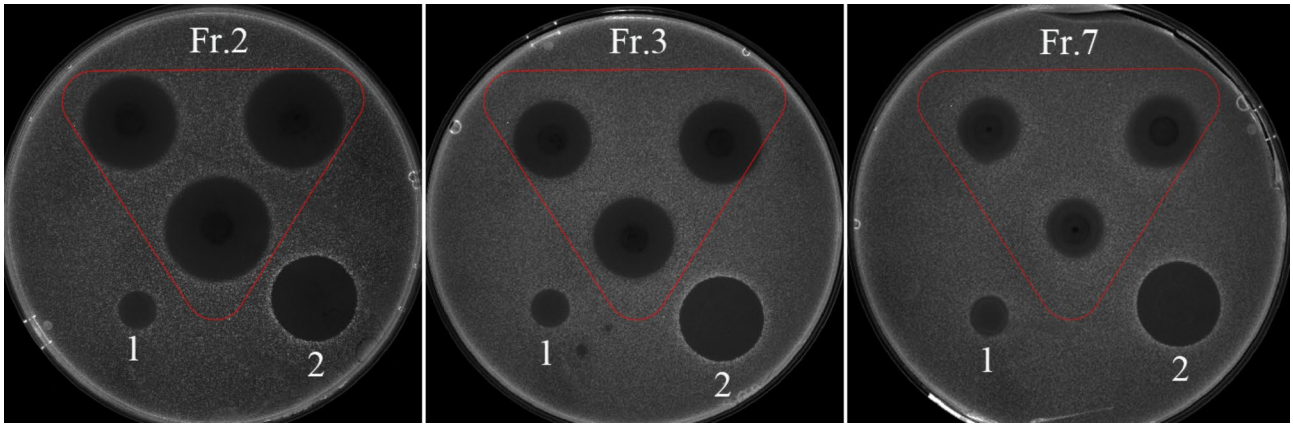

Fig. 3 The anti-MRSA activity of different fractions. 1: Blank control: methanol solution; 2: Positive drug: Vancomycin, 128 μg/mL; Fr.2, Fr.3 and Fr.7: Methanol solution with diferent fractions, 5 mg/mL

2 were isolated from fraction 2, compounds **3** and **4** from fraction 3, and compounds **5** and **6** from fraction 7.

Spectral analysis

The structures of the compounds were identified using 1D NMR, 2D NMR and HRESMS. Comparison by previously reported data identified Compound **1** as streptophenazine A, with *m/z* 425.20682 $[M+H]^+$, and a molecular formula of $C_{24}H_{28}N_2O_5$. Compound **2** as streptophenazine B, with *m/z* 425.20694 $[M+H]^+$, and a molecular formula of $C_{24}H_{28}N_2O_5$. Compound **3** as streptophenazine G, with *m/z* 439.22275 $[M+H]^+$, and a molecular formula of $C_{25}H_{31}N_2O_5$, Compound **4** as streptophenazine F, with *m/z* 439.22263 $[M+H]^+$, and a molecular formula of $C_{25}H_{31}N_2O_5$. Compound **5** as 1-carbomethoxyphenazine, with *m/z* 239.08105 $[M+H]^+$, and a molecular formula of $C_{14}H_{10}N_2O_2$. The structures of compounds **1**–**5** are shown in the Fig. 4. The ^1H NMR and ^{13}C NMR data of compounds **1**–**5** are presented in Tables S2-S3. The NMR spectra and HRESMS spectra are shown in Figs. S9-S23.

Compound **6** is a yellow solid. HRESMS results indicate its molecular weight is *m/z* 427.18582 $[M+H]^+$, which corresponds to node **3** in the molecular cluster, and it has the same MS^2 spectrum. The ^1H NMR spectrum of compound **6** shows six aromatic hydrogen proton signals: δ_H 8.87 (d, *J* = 7.0 Hz, 1H), δ_H 8.14 (m, *J* = 8.19–8.09 Hz, 3H), δ_H 8.64 (d, *J* = 8.6 Hz, 1H), and δ_H 8.32 (dd, *J* = 8.8, 1.3 Hz, 1H). These signals correspond to carbon signals δ_C 136.4, 132.8, 129.0, 130.1, 135.2, and 127.2 as determined by HSQC spectra. Additionally, there are six quaternary carbon signals at δ_C 131.9, 142.2, 141.8, 142.0, 140.1, and 139.8 (Table 1).Therefore, it is inferred that compound **6** is also a 1,6-disubstituted phenazine. HSQC and HMBC experiments indicate an unesterified carboxyl group at C-1, with a signal at δ_C 167.2 (1-COOH), and an ester carbonyl signal at δ_C 175.0, along with one methoxy proton signals at δ_H 3.64 (s, 3H) resonating with it. Moreover, ^1H-^1H COSY and HMBC experiments reveal an oxygen-linked methylene proton signal at δ_H 6.24 (d, *J* = 7.3 Hz, 1H) adjacent to a methyl proton signal at δ_H 3.27 (m, *J* = 3.31-3.24 Hz, 1H), with corresponding carbon signals at δ_C 69.2 (C-1') and δ_C 53.3 (C-2'). Additionally, HMBC spectra reveal another oxygen-linked carbon (δ_C 69.7) related to two methyl groups (δ_C 27.5, δ_C 27.7) (Table 1). ^1H-^1H COSY spectra show no correlation signals for the two methyl protons, indicating they are at the end of an alkyl chain connected to the oxygen-linked carbon. Furthermore, 2D NMR spectra reveal three additional aliphatic methylene carbon signals correlated with δ_H 1.75 (m, *J* = 1.80–1.71 Hz, 2H) and δ_H 1.33 (m, *J* = 1.39–1.28 Hz, 4 H) (Fig. 5A). A $^3J_{1'-2'}$ coupling constant of 7.2 Hz, a negative optical rotation value, and a negative Cotton effect at 251 nm indicate that compound **6** has a 1′S, 2′R configuration (Fig. 5B). Therefore, compound **6** is a new member of the streptophenazine family, corresponding to the molecular formula $C_{23}H_{26}N_2O_6$. We have named it streptophenazine T (Fig. 4). In summary, by optimizing the culture conditions of the strain and incorporating metabolomics guidance, we isolated a series of streptophenazine compounds from the metabolic products of *S. albovinaceus* WA10-1-8, including a

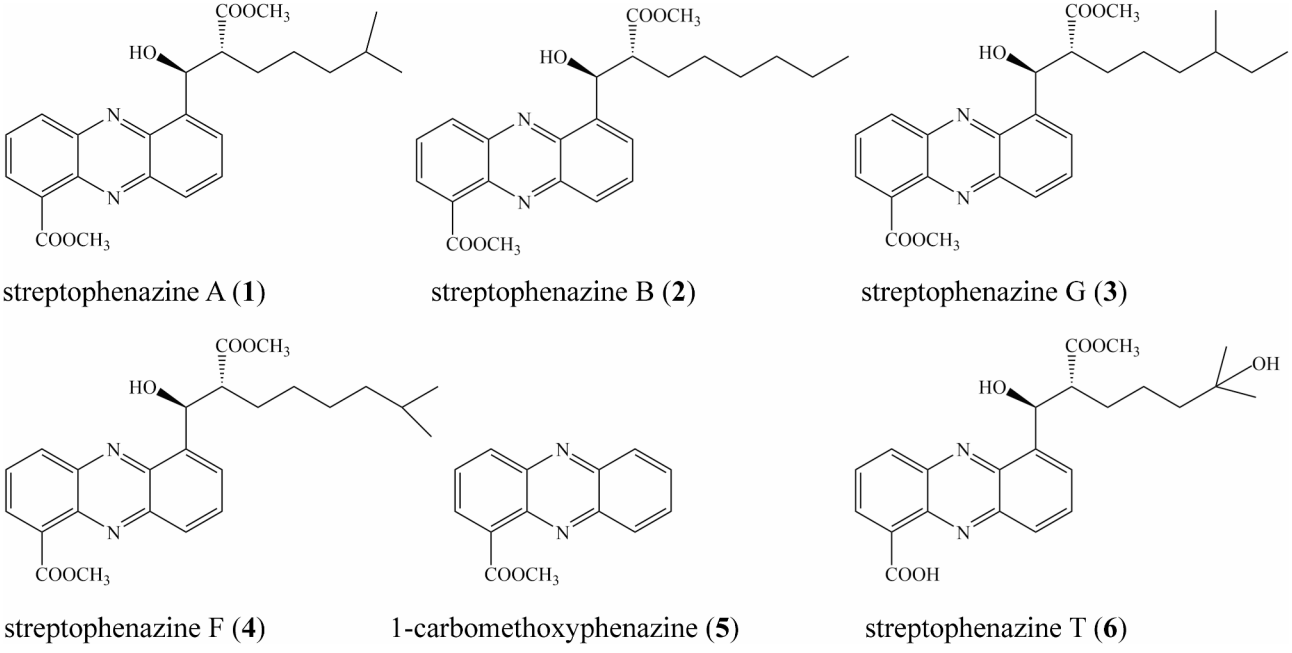

Fig. 4 Isolation and chemical structures of compounds **1**–**6**

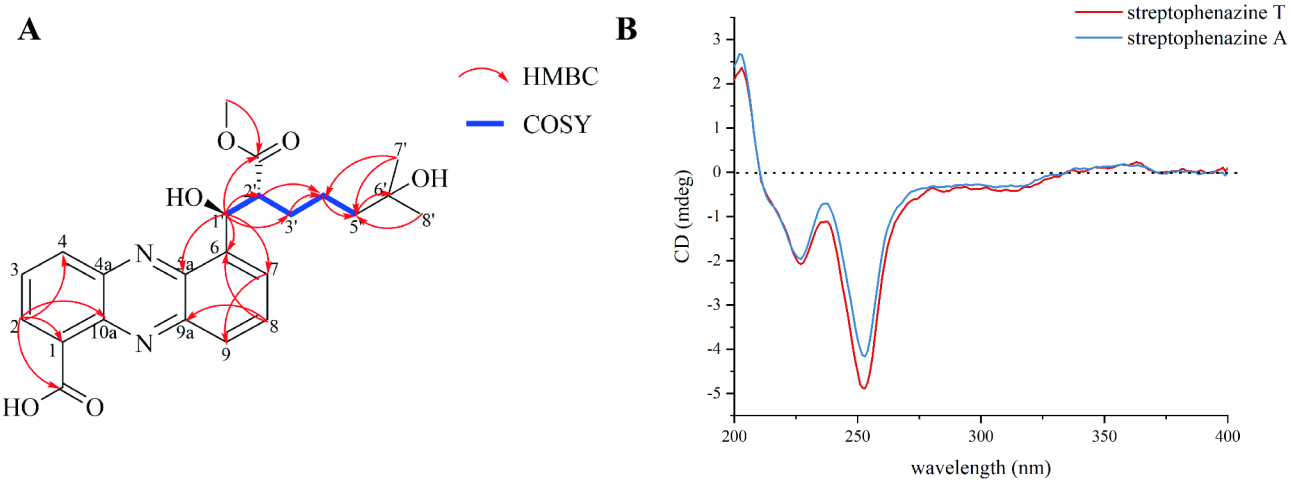

Fig. 5 The determination of the chemical structure and absolute configuration of streptophenazine T (**A**) Key HMBC and COSY correlations of streptophenazine T. (**B**) Comparison of the CD spectra of streptophenazine T and streptophenazine A

Table 2 The anti-MRSA activity of compounds 1–6 ($n=3$)

Compounds	MIC (μM)
Streptophenazine A (1)	75.47
Streptophenazine B (2)	37.74
Streptophenazine G (3)	146.12
Streptophenazine F (4)	73.06
1-carbomethoxyphenazine (5)	-
Streptophenazine T (6)	150.23
Vancomycin	0.69

-, no antibacterial activity

new compound, streptophenazine T. The NMR spectra and HRESMS spectra of compound **6** are shown in Fig. S21-S26.

Anti-MRSA activity of the compounds 1–6

The minimum inhibitory concentrations (MIC) of compounds **1**–**6** against MRSA ATCC43300 were measured using the broth dilution method. As shown in Fig. S27 and Table 2, the MIC values were 75.47 μM for compound **1**, 37.74 μM for compound **2**, 146.12 μM for compound **3**, 73.06 μM for compound **4**, and 150.23 μM for compound **6**. Compound **5** showed no inhibitory activity against MRSA. Scanning electron microscopy (SEM) was used to observe the effects of compound **2** and compound **6** on bacterial morphology. The untreated MRSA cells exhibited smooth surfaces (Fig. 6A). However, after being treated with compound **2** and compound **6**, the cell membrane of MRSA were suffer from various degrees damage (Fig. 6B-C).

Disscussion

Over the past few decades, with the misuse of antibiotics, MRSA has become one of the most widespread and dangerous pathogens in hospitals and communities worldwide [29]. Moreover, the frequent and indiscriminate use of antibiotics, coupled with the encoding of some antibiotic resistance genes, has led to MRSA exhibiting resistance to multiple antimicrobial agents. Therefore, the demand for discovering new, safe, and effective anti-MRSA bioactive compounds is increasing [30]. The genus *Streptomyces*, the largest family within actinobacteria, has long been regarded as a treasure trove for producing various antibacterial substances [31, 32]. However, as antibacterial substances from traditional sources of *Streptomyces* are continuously being explored, attention has turned to insect symbiotic *Streptomyces*. *Streptomyces* from special habitats often evolve their genomes to adapt to their environments, resulting in different secondary metabolite synthesis strategies compared to those from common habitats. As one of the most widely distributed species in nature with the most complex living environments, insects are undoubtedly a new source of *Streptomyces* for producing new antibacterial compounds [33].

In this study, we isolated a strain WA10-1-8 with strong anti-MRSA ATCC43300 activity from the intestinal tract of *P. americana*. Based on 16 S rRNA sequencing and comparison, the closest related species was found to be *S. albovinaceus*, with a similarity of 99.8%. This is the first reported an anti-MRSA active strain of *S. albovinaceus* isolated from the *P. americana*. During the optimization of the cultivation conditions for *S. albovinaceus* WA10-1-8, we discovered that a moderate increase in cultivation temperature (34 °C) significantly alters the metabolic state and anti-MRSA activity of the strain. GNPS molecular networking and untargeted metabolomics analysis revealed that both the content and chemical diversity of streptophenazine compounds produced by *S. albovinaceus* WA10-1-8 increased significantly under the cultivation condition of 34 °C. Additionally, several potential new compounds were identified. Temperature regulation

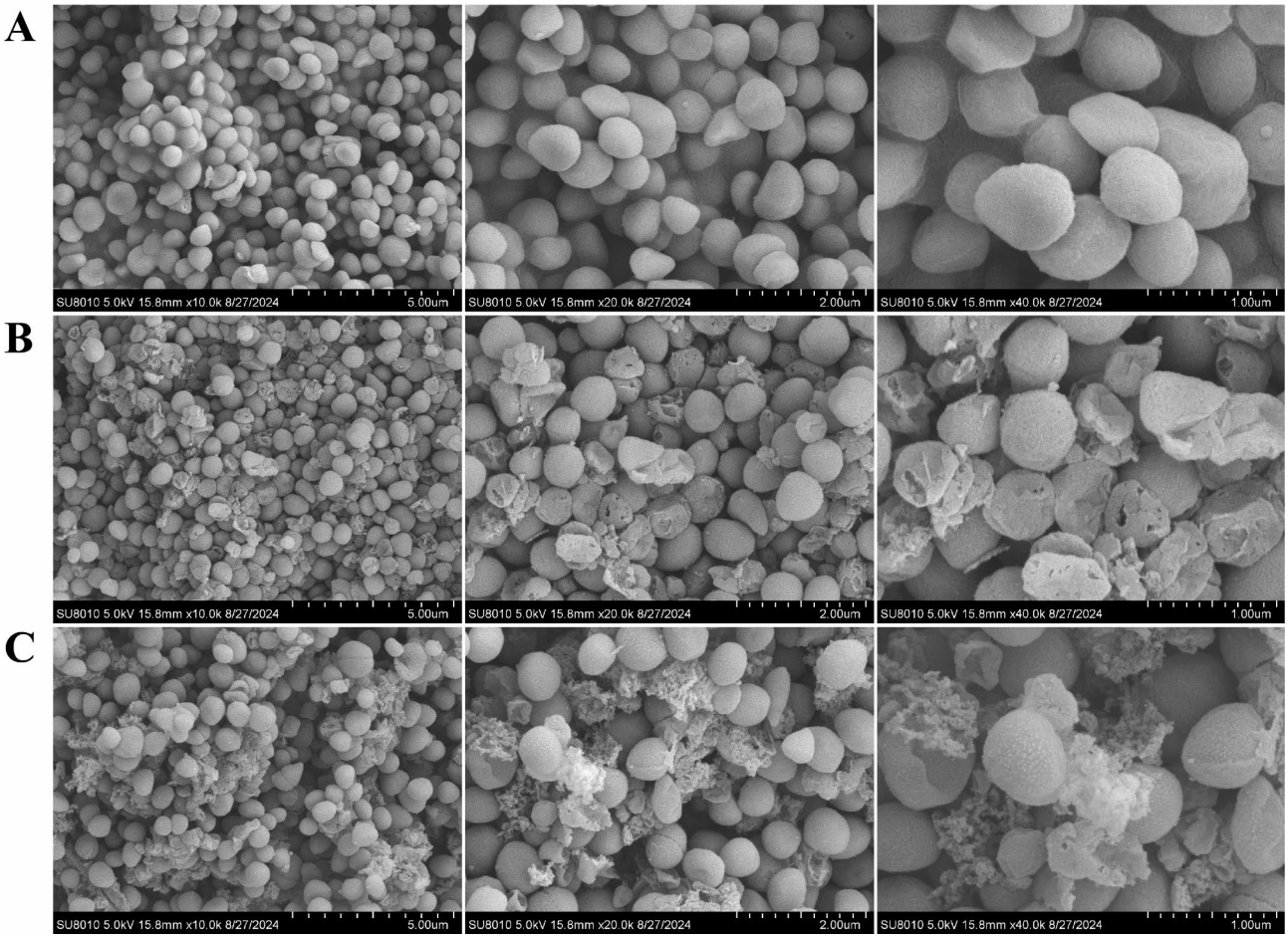

Fig. 6 Scanning electron microscopy (SEM) images of MRSA. (**A**) Untreated control MRSA cell. (**B**) Streptophenazine B (**2**) treated MRSA. (**C**) Streptophenazine T (**6**) treated MRSA

is crucial in microbial biosynthesis, as it promotes the expression of biosynthetic gene clusters by influencing the activity of relevant enzymes and gene transcription, thereby affecting the production of metabolites [34, 35]. An interesting example is the regulation of phenazine production in *Pseudomonas aeruginosa* by temperature [36, 37]. These temperature-dependent gene expression regulations enable the strain to flexibly adjust its metabolite production under different environmental conditions to meet survival and competitive needs. Similarly, for phenazine compounds, the expression of streptophenazines in *S. albovinaceus* WA10-1-8 may involve similar temperature regulation mechanisms, although the specific regulatory processes require further investigation.

According to previous reports, the production of streptophenazine compounds often exhibits rich chemical structural diversity, thereby possessing various pharmacological activities, including anticancer and antibacterial properties [38, 39]. This structural diversity-oriented biosynthesis is considered an evolutionary advantage, as compounds with different structures can target various biological sites and act synergistically as antibiotics [40]. The biosynthesis of such structurally diverse compounds by symbiotic bacteria may play a crucial role in the natural defense of insects [41]. For example, beewolf solitary digger wasps (*Philanthus* spp.) have evolved a unique mutualistic symbiosis with a *Streptomyces* strain known as *Candidatus Streptomyces* philanthi, utilizing nine different antibiotics produced by this strain to protect their larvae from bacterial or fungal infections [42].

Based on metabolomics-guided analysis, five structurally distinct streptophenazines were selectively isolated from the fermentation product of *S. albovinaceus* WA10-1-8, including four known streptophenazines: streptophenazine A (**1**), streptophenazine B (**2**), streptophenazine G (**3**), streptophenazine F (**4**), and one new compound, streptophenazine T (**6**), along with a simple phenazine compound, 1-carbomethoxyphenazine (**5**). The MIC determination results indicated that all five streptophenazine compounds exhibited strong inhibitory activity against MRSA, with MIC values in the range of 37.74-150.23 μM. In addition, Maya I. Mitova et al.,

Nantiya Bunbamrung et al. demonstrated that streptophenazine A and streptophenazine B effectively inhibited Gram-positive bacteria, including *Bacillus subtilis* and *Bacillus cereus*, and inhibited various cancer cell lines, including MCF-7, KB and NCI-H187 [10, 38]. Streptophenazine G, in addition to exhibiting antibacterial activity against *Bacillus subtilis* and *Staphylococcus epidermidis*, also inhibits the enzyme phosphodiesterase (PDE 4B), indicating dual potential for both antibacterial and anti-inflammatory effects [43]. These findings provide valuable insights for future structural optimization and drug development, emphasizing the need for further investigation of these compounds.

Conclusions

Through metabolomics-guided analysis, we successfully isolated several known streptophenazine compounds with anti-MRSA activity, as well as a new streptophenazine, from the *P. americana* symbiotic *Streptomyces* for the first time. This indicates that streptophenazine compounds may serve as promising lead compounds against MRSA. However, the in vivo anti-MRSA effects, toxicity, and underlying mechanisms of streptophenazines require further investigation. Moreover, this study demonstrates the immense potential of insects symbiotic *Streptomyces* in the discovery of new antibiotics, anticancer agents, and other therapeutic streptophenazine compounds.

Abbreviations

GNPS	Global Natural Products Social Molecular Networking
MRSA	Methicillin-resistant Staphylococcus aureus
MIC	Minimum inhibitory concentration
HPLC	High-performance liquid chromatography
NMR	Nuclear Magnetic Resonance
HR-ESI-MS	High-resolution electrospray ionization mass spectroscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03789-9.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

XBJ and JWdesigned the study. LCZ, JLT, DH, JXH, RYZ and JYL performed the experiments, analysed the data and prepared figures. LCZ and JW edited and modified the manuscript.

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Data availability

16 S rRNA sequencing reads have been deposited in the National Center for Biotechnology Information (NCBI) under accession number: KY206803. The strain WA10-1-8 has been deposited in the Guangdong Microbial Culture Collection Center (GDMCC) with the preservation number: GDMCC No. 4.422. Mass spectral files are accessible from the MassIVE repository accession ID：MSV000096434 (http://massive.ucsd.edu/ProteoSAFe/status.jsptask=932674dd310946118dde3ccc8d6cf5fd), reviewers can access the Mass spectral files using the username: MSV000096434_reviewer and the password: 2ZbgbE6ZV567×4li. The GNPS feature based molecular networking job is available at http://gnps.ucsd.edu/ProteoSAFe/status.jsptask=33899ea203244457bd4504f13de6af8e.

Declarations

Ethics approval and consent to participate

Clinical trial number: not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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