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Breeding of High Daptomycin-Producing Strain by Streptomycin Resistance Superposition

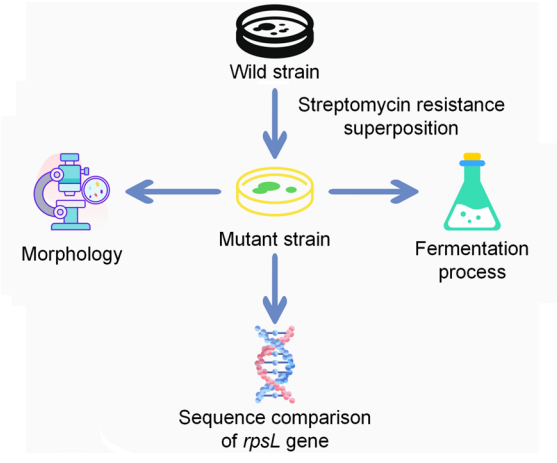
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Abstract

Daptomycin is a cyclolipopeptide antibiotic produced by *Streptomyces roseosporus*. It is widely used to treat drug-resistant bacterial infections; however, daptomycin yield in wild strains is very low. To improve the daptomycin production by the strain BNCC 342432, a modified method of ribosome engineering with superposition of streptomycin resistance was adopted in this study. The highest-yield mutant strain SR-2620 was obtained by increasing streptomycin resistance of BNCC 342432, and achieved daptomycin production of 38.5 mg/l in shake-flask fermentation, 1.79-fold higher than the parent strain and its heredity stability was stable. The morphological characteristics of the two strains were significantly different, and the 440th base G of the *rpsL* gene in the mutant strain was deleted, which resulted in a frameshift mutation. Our results demonstrate that gradually increasing strain resistance to streptomycin was an effective breeding method to improve daptomycin yield in *S. roseosporus*.



K e y w o r d s: daptomycin, streptomycin resistance, strain breeding, fermentation, *Streptomyces roseosporus*

Introduction

Daptomycin is a cyclic lipopeptide antibiotic produced by *Streptomyces roseosporus*, which is formed from a cyclic peptide chain connected to a decane side chain by tryptophan at the N-terminal. The molecular formula is $C_{72}H_{101}N_{17}O_{26}$, and its molecular weight is 1,620.67 (Osorio et al. 2021). Daptomycin is soluble in water, small molecular organic solvents, alkaline, and acidic solutions with pH > 3.5. It is slightly soluble in acetone and chloroform. When the pH of a solution is less than 2 or greater than 9, daptomycin is easily denatured and irreversible (Liao et al. 2012; Tótoli et al. 2015). Daptomycin has broad-spectrum resistance to Gram-positive bacteria and can kill most clinically isolated Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and penicillin-resistant *Streptococcus pneumoniae* (Jung et al. 2004; Matsuo et al. 2021). Its unique antibacterial properties make it difficult to produce cross-resistance with other antibiotics (Ng et al. 2014; Zuttion et al. 2020; Liu et al. 2021b), suggesting that it has promise in the clinical treatment of drug-resistant bacterial infections.

To improve the daptomycin productivity, a great deal of effort is committed enhancing the production of daptomycin through strain improvement, which has primarily focused on physical and chemical mutagenesis and genome recombination. Wang et al. (2020) used ultraviolet, microwave, LiCl, and compound mutagenesis to increase daptomycin yield by 20.79% compared with the original strain. Gao et al. (2016) used

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plasma and UV compound mutagenesis combined with sodium glutamate resistance screening to obtain a high-yield strain and achieved daptomycin production of 3.9 g/l in a 5 l fermentor. Using UV and nitrosoguanidine, Yu et al. (2014) screened eight strains with high daptomycin production to find an initial population for genome shuffling. A high yield strain was screened after the fourth round of fusion and achieved daptomycin production of 38.5 mg/l in a 7.5 l fermentor, which was 3.7-fold higher than the original strain (Yu et al. 2014). Zhang (2021) selected a daptomycin-producing strain by combining UV mutagenesis with NTG mutagenesis and genome rearrangement, and its yield was 1.59-fold higher than that of the original strain.

Compared with the traditional physical and chemical mutagenesis technology, ribosome engineering technology is simple and more effective. It modifies the ribosomal structure of microorganisms via resistance mutation technology so that the regulatory genes related to secondary metabolism are activated directly or indirectly, improving the synthesis ability of secondary metabolites (Ochi et al. 2004; Ochi 2007). This method can be used to increase the yield of secondary metabolites in almost all actinomycetes. More than ten new structural molecules have been discovered from the more than 100 actinomycetes, and the production efficiency of nearly 30 secondary metabolites have been enhanced (Xie et al. 2022). Liu et al. (2021a) produced a new carrimycin high-producing strain RFP40-6-8 using ribosomal engineering technology, with a yield approximately 6-fold higher than the original strain. Lu et al. (2018) used ribosomal engineering technology, and ultraviolet mutagenesis technology to modify *Streptomyces milbesii*. They finally obtained a strain with, 72.5% higher yield of milbemycin than the original one (Lu et al. 2018). However, so far, there seem to be few reports of increasing daptomycin production by ribosome engineering.

In this study, a genetically stable mutant strain SR-2620 of *S. roseosporus* was screened by improved ribosomal engineering. Subsequently, the mutant was compared with the original strain in morphology, genetics, and fermentation process. The results demonstrate that gradually increasing strain resistance to streptomycin is an effective breeding method to improve daptomycin yield in *S. roseosporus*.

Experimental

Materials and Methods

Microorganism and medium. *S. roseosporus* strain of BNCC 342432 was used as a parent strain and stored in the form of spores in 15% glycerol (v/v) at –80°C in our lab. DT solid medium was as follows: glucose 4 g/l, malt powder 10 g/l, yeast powder 4 g/l, calcium carbonate 2 g/l, agar 20 g/l, pH 7.0. TSB liquid medium: tryptone soybean broth 30 g/l. Seed medium: glucose 5 g/l, maltodextrin 15 g/l, peptone 10 g/l, yeast powder 10 g/l, pH 7.0. Fermentation medium: glucose 10 g/l, maltodextrin 80 g/l, molasses 5 g/l, soybean cake powder 30 g/l, gluten powder 5 g/l, calcium carbonate 2 g/l, nickel chloride hexahydrate 0.2 g/l, sodium molybdate dihydrate 0.1 g/l, ammonium ferrous sulfate hexahydrate 0.66 g/l, oxalic acid 4 g/l, pH 7.5.

Preparation of single spore suspension. The spore suspension of *S. roseosporus* strain of BNCC 342432 preserved in a glycerol tube was removed from the refrigerator at –80°C. The spore suspension of the original strain was spread on DT solid medium and cultivated for 8–10 days at 30°C until spores matured. After the spores matured, they were washed into the 10 ml EP tube with sterile water and filtered with four layers of filter paper after shaking for 10 min. The final concentration of spore suspension was approximately 10^7–10^8 CFU/ml.

Determination of minimum inhibitory concentration. The streptomycin solution (0.5 mg/ml) filtered by the 0.22 μm filter membrane was added to the DT solid medium to form resistant plates with different concentration gradients (0, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 μg/ml). The 200 μl of spores' suspension were spread on DT plates containing different concentrations of streptomycin and incubated at 30°C for 8–10 days to determine its MIC.

Acquisition of high-yield daptomycin strain with streptomycin resistance. The 200 μl of spores' suspension of *S. roseosporus* BNCC 342432 was spread on DT plates containing 1.4 μg/ml streptomycin and incubated at 30°C for 8–10 days. The single colony was picked up into a 24-well plate and continued to grow. Subsequently, the single colony growing well in a 24-well plate was inoculated into a 100 ml conical flask containing 20 ml seed medium and incubated at 30°C for 2 days with 250 r/min. Then the 2 ml seed liquid was absorbed by a liquid pipette and transferred to the 100 ml conical flask containing 18 ml fermentation medium at 30°C for six days with 250 r/min. The daptomycin production was finally detected by HPLC (Shimadzu Corporation, Japan).

The top five mutants with the highest daptomycin production in shake-flask fermentation were chosen as starting strains for the second round of ribosome engineering using 1.6 μg/ml streptomycin. In the third round, 1.8 μg/ml streptomycin was employed, and other conditions were the same as in the first round. The antibiotic concentration was increased by 0.2 μg/ml in each round until the strains no longer produced a sizeable positive mutation.

Determination of daptomycin production. 10 ml of fermentation broth was placed in a 15 ml centrifuge tube and centrifuged at 12,000 r/min for 10 minutes.

1 ml of supernatant was placed in a new 15 ml centrifuge tube, and 4 ml of anhydrous ethanol was added. An ultrasonic cleaner was used to shake for 10 minutes and centrifuged again at 12,000 r/min for 10 minutes. 1 ml of supernatant was absorbed with a disposable aseptic syringe (1 ml) and filtered into the HPLC sampling bottle through a 0.22 μm organic filter membrane to be tested. HPLC operating conditions were as follows: the analytical column was InertSustain C18 (GL Sciences, Japan) (4.6 mm × 250 mm, 5.0 μm); the flow rate was 1 ml/min, and the wavelength was 220 nm; the column temperature was 40°C; the injection volume was 10 μl. The mobile phase consisted of 0.1 % trifluoroacetic acid in distilled water and acetonitrile (55:45, v/v); isometric elution was used to collect the signal for 20 minutes.

Genetic stability verification of the mutant. After the ribosome engineering, several high-yield strains were continuously transferred for five generations from the streptomycin-resistant plates. HPLC determined the daptomycin yield of each generation, and the best strain was selected.

Morphology comparison between the original and mutant strain. The spore suspensions of BNCC 342432 and the spore suspensions of mutant strains were spread on DT plates separately, while their colony morphology, spore color, production, and growth rate were all observed.

Analysis of mutations in *rpsL* gene. BNCC 342432 and the mutant strain were inoculated into two 100 ml conical flasks with 20 ml TSB medium and incubated at 30°C for two days with 250 r/min. The supernatant was discarded by centrifugation at 12,000 r/min for 2 min. The bacteria were poured into the mortar and ground with liquid nitrogen, the DNA of the two strains was extracted by a bacterial DNA extraction kit, and the resulting DNA was stored at –20°C for further experiments. The bacterial DNA extraction kit was purchased from the Shanghai Sangon Company (China).

PCR reaction system: 1 μl of DNA template, 12.5 μl of San Taq PCR Mix enzyme, 1 μl of upstream primer, and 25 μl of downstream primer. PCR amplification system: Step one, pre-denaturation at 95°C for 5 minutes. Step two was denaturation at 95°C for 30 seconds, annealing at 40°C for 30 seconds, extension at 72°C for 30 seconds, for 30 cycles. Step three was extension at 72°C for 10 minutes. The prmier of *rpsL:* F: 5'-ATTCGGCACACAGAAAC-3'; R: 5'-AGAGGAGAACCGTAGAC-3'. The 3 μl PCR product was verified by 1% agarose gel electrophoresis. The purified PCR products were directly sequenced by the Shanghai Sangon Company, and the data were analyzed by DNAMAN 8.0 (LynnonBiosoft, USA).

Performance comparison between the wild and mutant strain by natural fermentation in shaking flasks. During the shake-flask fermentation, samples of culture filtrates of parent strain BNCC 342432 and mutant strain were harvested at several set times (24, 48, 72, 96, 120, 144, and 168 h). The dry cell weight (DCW), total sugar content, ammonia nitrogen concentration, and daptomycin yield were then determined and repeated three times at each time point.

The mycelium dry weight was determined as follows: the filter paper was dried at 85°C in the oven to a constant weight, weighed, and set aside. The 10 ml of fermentation broth was centrifuged for 10 min at 12,000 r/min, washed twice with deionized water, filtered on filter paper, dried in the oven at 85°C to a constant weight, and weighed.

The residual sugar was determined by anthrone colorimetry, while the ammonia nitrogen was determined using the reference method (Xie et al. 2005).

Results

Determination of minimum inhibitory concentration of streptomycin. The spore suspension of BNCC 342432 was spread on streptomycin-resistant plates with different concentrations and cultured at 30°C for 8 days. Fig. 1 demonstrates that the original strain grew

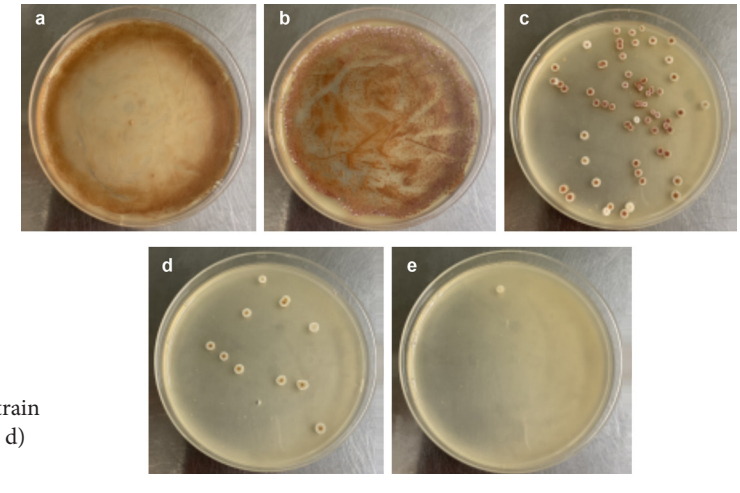


Fig. 1. The growth of *Streptomyces roseosporus* original strain on DT plates with a) 0 μg/ml, b) 0.8 μg/ml, c) 1.0 μg/ml, d) 1.2 μg/ml, e) 1.4 μg/ml streptomycin, respectively.

well in the plate with a streptomycin concentration of 0.8 μg/ml, and the colony number gradually decreased as the streptomycin concentration increased (Fig. 1). When the streptomycin concentration was 1.4 μg/ml, there was almost no strain on the plate. Therefore, a streptomycin concentration of 1.4 μg/ml was selected as the MIC of the original strain.

Screening of streptomycin-resistant mutants. A total of 518 strains were screened in streptomycin-resistant plates. Shake flask fermentation of these strains was performed, and the daptomycin production of the strains was determined by HPLC. The preliminary screening results are shown in Fig. 2. Using the daptomycin yield 10.3 mg/l of the original strain BNCC 342432 as an index, a total of 167 positive mutants were obtained under the stimulation of streptomycin resistance, and the positive mutation rate reached 32.2%. The 50 strains with the highest yield were re-screened according to the primary screening method, and six high-yield mutants were obtained. A daptomycin producing strain, SR-2620,

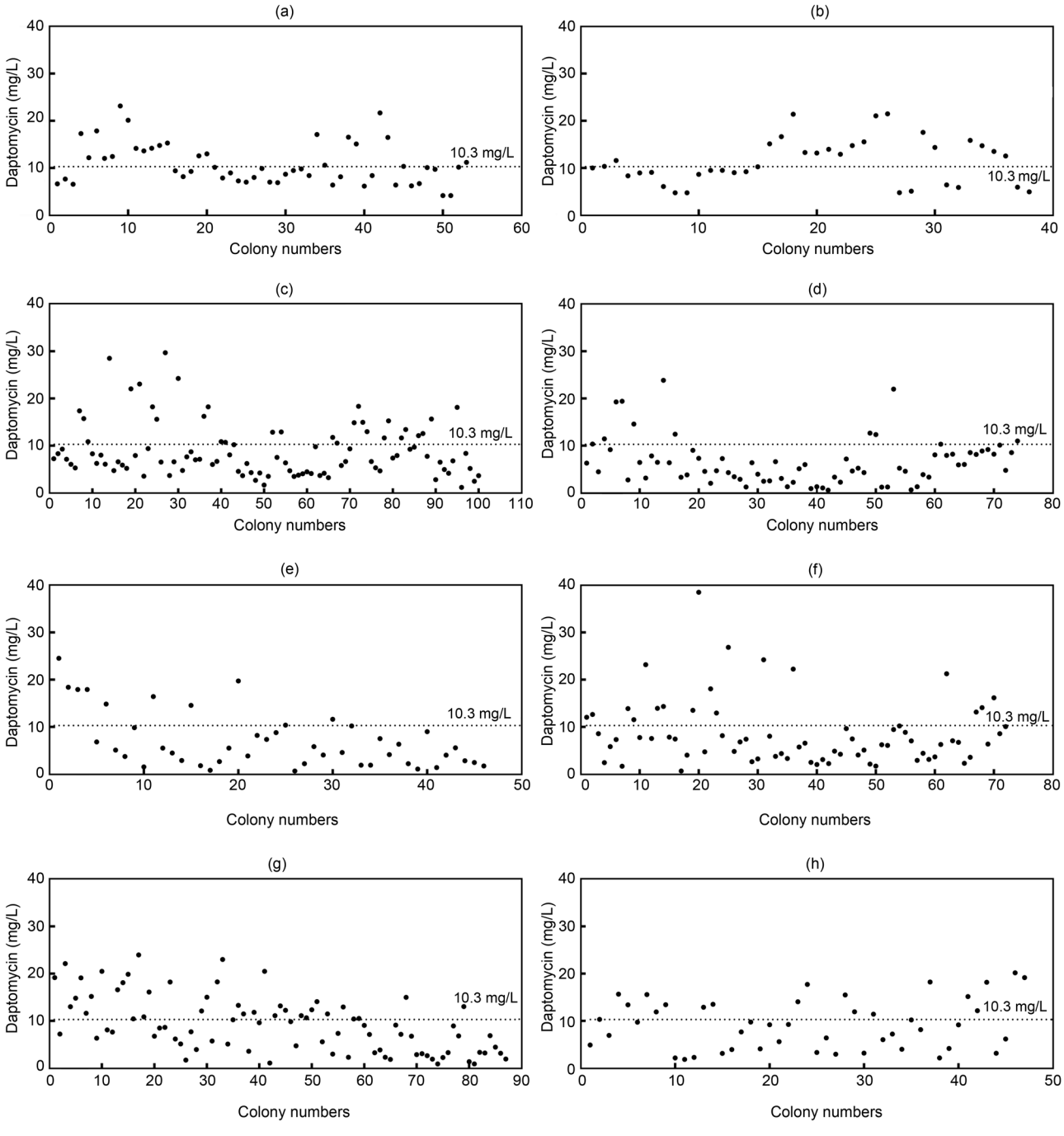


Fig. 2. Daptomycin production by *Streptomyces roseosporus* mutants screened by DT plates with streptomycin.at a concentration of a) 6 μg/ml, b) 1.8 μg/ml, c) 2.0 μg/ml, d) 2.2 μg/ml, e) 2.4 μg/ml, f) 2.6 μg/ml, g) 2.8 μg/ml, and h) 3.0 μg/ml. The dashed line represents daptomycin production of the parent strain.

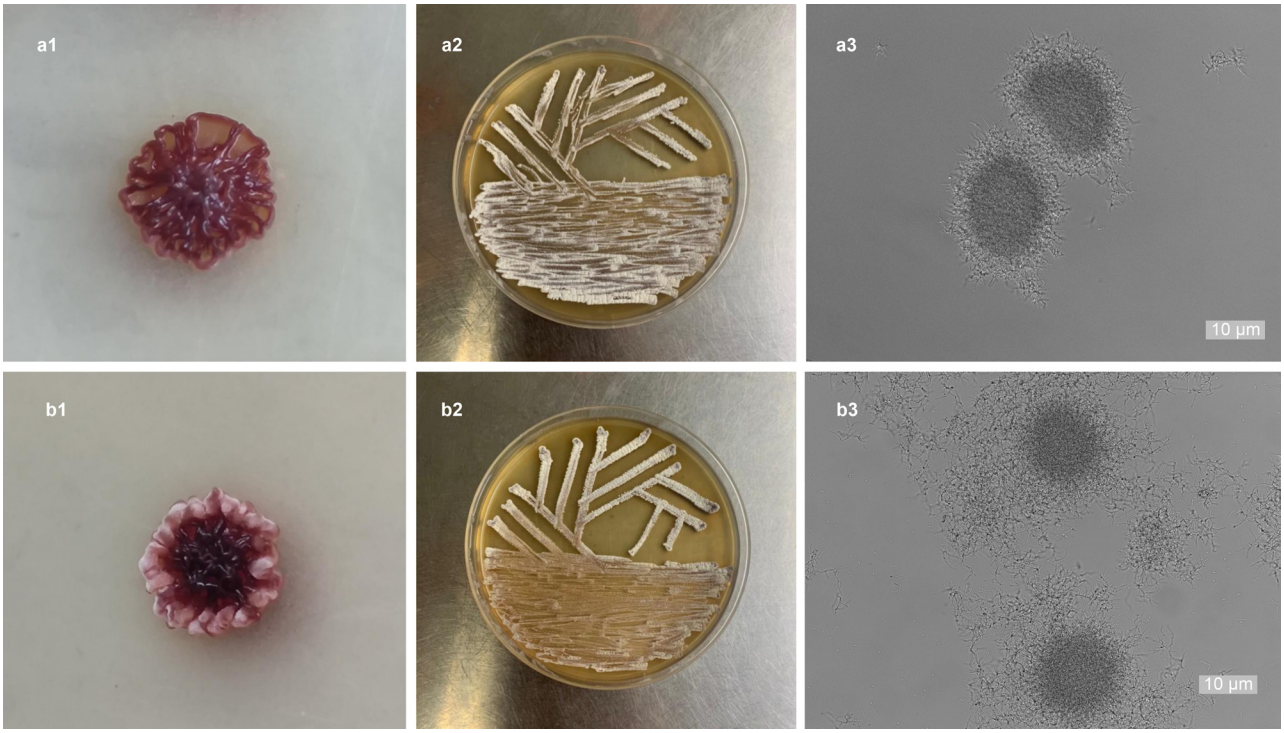


Fig. 3. a1) and b1) Colony and individual morphology of *Streptomyces roseosporus* strains BNCC 342432 and SR-2620; a2) and b2) spore growth capacity of BNCC 342432 and SR-2620; a3) and b3) mycelial pellet morphology of BNCC 342432 and SR-2620.

was screened in DT plates containing 2.6 µg/ml streptomycin, and its yield reached 38.5 µg/ml (Fig. 2).

Genetic stability verification of the mutant. The genetic stability verification of six high-yield mutants was performed. As shown in Table I, the daptomycin production in SR-2009, SR-2017, and SR-2026 were 20%, 36.8%, and 14.9% lower than that of the first generation, respectively. The high-yield mutants SR-2023, SR-2401, and SR-2620 were relatively stable (Table I). Among them, SR-2620 had the most substantial ability to produce daptomycin, so the target mutant strain was determined to be SR-2620.

Morphology comparison between the original and mutant strain. After streptomycin-resistant selection, the bacterial morphology significantly changed. As shown in Fig. 3, the mutant SR-2620 was fuller than BNCC 342432, and the periphery of the strain was white and translucent (Fig. 3a1 and 3b1). The sporulation ability of the mutant SR-2620 was slightly weaker than BNCC 342432 (Fig. 3a2 and 3b2). Some studies have demonstrated that under the stimulation of antibiotics, the yield of secondary metabolites of the strain will increase while the sporulation ability will decrease, which is called the "cost of resistance" (Andersson and Levin 1999; Nishimura et al. 2007). After the two strains were cultured in a seed medium for two days, the morphology of the mycelial pellet was observed under the differential interference microscope. Fig. 3 shows

Table I
Stability experiment on high-yield daptomycin strains.

Strain	First generation of daptomycin production (mg/l)	Second generation of daptomycin production (mg/l)	Third generation of daptomycin production (mg/l)	Fourth generation of daptomycin production (mg/l)	Fifth generation of daptomycin production (mg/l)
BNCC 342432	10.5 ± 0.2	9.9 ± 0.2	10.3 ± 0.2	9.7 ± 0.2	10.4 ± 0.2
SR-2009	28.5 ± 0.2	30.0 ± 0.2	24.3 ± 0.2	25.0 ± 0.2	22.8 ± 0.2
SR-2017	23.1 ± 0.2	21.6 ± 0.2	22.8 ± 0.2	19.8 ± 0.2	14.6 ± 0.2
SR-2023	29.7 ± 0.2	28.8 ± 0.2	30.6 ± 0.2	29.2 ± 0.2	27.2 ± 0.2
SR-2026	24.2 ± 0.2	22.9 ± 0.2	21.8 ± 0.2	20.1 ± 0.2	20.6 ± 0.2
SR-2401	24.5 ± 0.2	25.5 ± 0.2	23.4 ± 0.2	22.5 ± 0.2	24.2 ± 0.2
SR-2620	38.5 ± 0.2	36.9 ± 0.2	41.3 ± 0.2	36.0 ± 0.2	38.5 ± 0.2

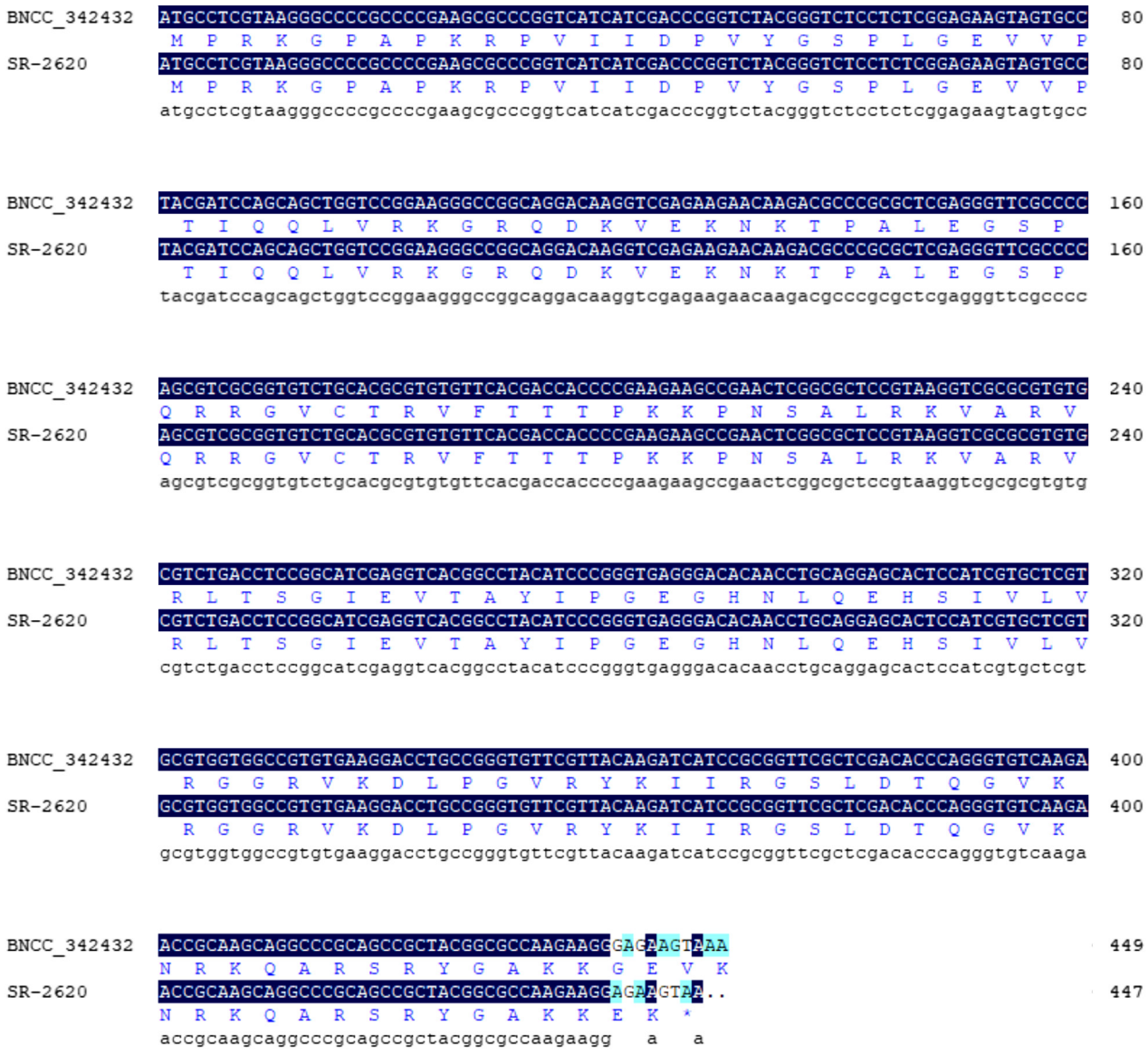


Fig. 4. Sequence comparison of the *rpsL* gene between *Streptomyces roseosporus* BNCC 342432 and SR-2620 strains.

that the mycelial pellet of BNCC 342432 is oval, the mycelium is more concentrated; the morphology of SR-2620 are regular, and the mycelium are loose (Fig. 3a3 and 3b3).

Analysis of mutations in the *rpsL* gene. Some reports have shown that increasing the yield of secondary metabolites and streptomycin resistance in *Streptomyces* is related to mutations in the *rpsL* (ribosomal protein S12) gene (Cai et al. 2012). Thus, the *rpsL* gene of the original strain BNCC 342432 and the mutant strain SR-2620 were amplified by PCR. The length of the fragment was approximately 500 bp and the purified PCR products were directly sequenced by Shanghai Sangon Company. The date was analyzed by DNAMAN 8.0 (Fig. 4). The results demonstrated that the 440th base G of SR-2620 was deleted, resulting in a frameshift mutation, which led to the early termination of translation. Considering that the mutant strain grows well and the yield of daptomycin is high, it is considered that this gene mutation is beneficial. However, the specific mechanism of the increase of daptomycin production caused by a base deletion in mutant strains is not clear.

Performance comparison between the wild and mutant strain by natural fermentation in shaking flasks. The performance of daptomycin fermentation of the BNCC 342432 and the mutant SR-2620 in flasks is shown in Fig. 5. The trend of total sugar consumption was similar during the fermentation process, and the total sugar consumption rate of SR-2620 was higher than BNCC 342432. It indicates that the growth and metabolism levels of the high-yield strain were higher than that of the original strain. The DCW of SR-2620 was higher than BNCC 342432 and reached 14.7 g/l in 100 h, which was approximately 2.9 g/l higher than that of the BNCC 342432 (about 11.8 g/l). Compared with daptomycin yield, the daptomycin synthesis rate

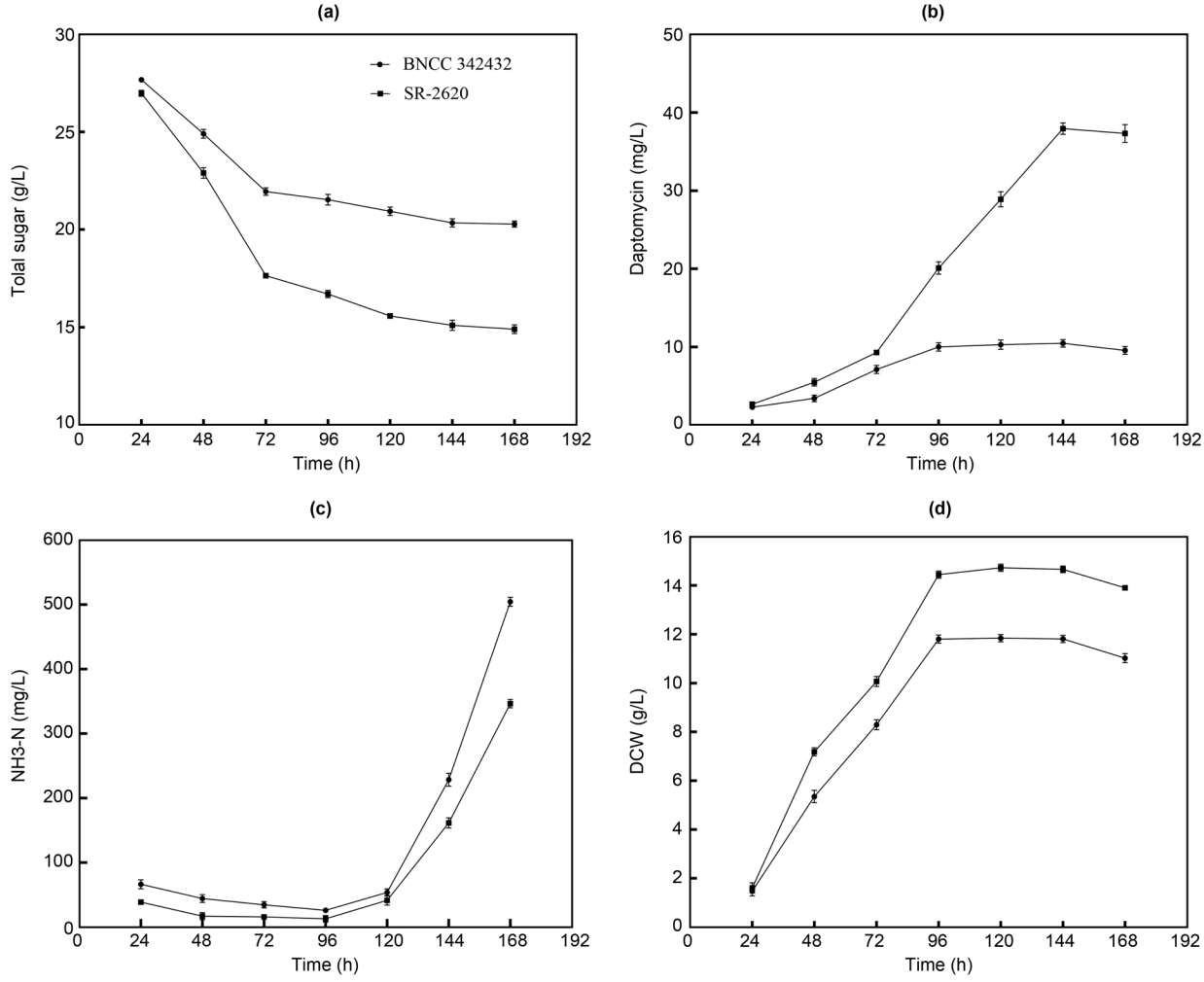


Fig. 5. Comparison of shake flask fermentation between *Streptomyces roseosporus* BNCC 342432 and SR-2620 strains.

of SR-2620 always exceeded that of the original strain and reached the highest yield of 38.4 mg/l in 140 h, which was approximately 3.7-fold higher than BNCC 342432 (Fig. 5). The ammonia nitrogen concentration first decreased and then increased since the early cell growth consumed ammonia nitrogen to synthesize amino acids. In later fermentation, when secondary metabolites were produced, the cell autolysis and ammonia nitrogen accumulation increased the concentration of ammonia nitrogen. Some studies have demonstrated that high ammonia nitrogen concentrations are not conducive to daptomycin synthesis, and will stop synthesis of daptomycin (Wang 2007).

Discussion

Ribosomal engineering technology modifies essential components of gene expression, RNA polymerase, and ribosome, to cause mutations in ribosomal-related genes. It then regulates the secondary metabolic pathway of microorganisms, resulting in the overexpression of secondary metabolites. Through transcriptome and metabonomic analysis, Lopatniuk et al. (2019) confirmed that the technology of practical introduction mutations into *rpsL* and *rsmG* could be widely used to improve biosynthetic gene clusters (BGC) expression levels. It indicates that ribosomal engineering technologies by using streptomycin as an antibiotic can affect the production of secondary metabolites of actinomycetes. In 2004, Ochi et al. first proposed the concept of "ribosomal engineering", which is still widely used for actinobacterial strain improvement today. However, current studies only underwent a round of antibiotic stimulation to improve the yield of secondary metabolites of actinomycetes. Wu et al. (2016) screened a ε-polylysine-producing strain with a high yield of 3.3 g/l by stimulating *Streptomyces albicans* with the streptomycin. Liu et al. (2019a) obtained the strain with a high avilamycin yield after a round of stimulation by spreading the *Streptomyces viridoehrongenes* gs77 on the resistant plate streptomycin at a concentration equal to 1 MIC.

The intensity of a round of antibiotic stimulation is relatively low, which can cause heredity instability of the high yield performance of the strain. In this study, a modified method of ribosomal engineering with gradually increasing the stimulation intensity of streptomycin was used to screen for the high-production strain. In this process, the secondary metabolites of the strain were improved. Finally, a streptomycin-resistant mutant SR-2620 with significantly increased daptomycin yield was obtained in a DT plate containing 2.8 μg/ml of streptomycin, while its yield in the shake flask reached 38.5 mg/l. It was 3.7-fold higher than BNCC 342432. In our subsequent studies, we have taken the mutant strain SR-2620 as the starting strain and continued to improve the yield of daptomycin by using the breeding method in this study after changing a new antibiotic. The yield of the new mutant strain has reached 73 mg/l by shaking flask fermentation. Our experimental results prove that this method is feasible and effective. Some studies have demonstrated that the superposition of several antibiotics will further increase the yield of secondary metabolites of the strain (Tamehiro et al. 2003; Wang et al. 2008). In the future study, we will use other new antibiotics to apply continuous resistance stimulation to the strain to improve the yield of daptomycin further.

In this study, a mutant strain SR-2620 was screened by modified ribosome engineering and achieved daptomycin production of 38.5 mg/l in shake-flask fermentation. However, the shaking flask fermentation makes it impossible to control the nitrogen source, carbon source, dissolved oxygen, and pH during the fermentation process. Zhou and Zhang (2018) used a 100 l fermentor feeding experiment to reach the daptomycin titer of 2,276 mg/l. Liu et al. (2019b) optimized the fermentation process using the response surface method and increased daptomycin production by 132%. Therefore, we will carry out a fermentation tank experiment and optimize fermentation conditions to further improve the yield of daptomycin in future research.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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