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Improvement of daptomycin production via increased resistance to decanoic acid in *Streptomyces roseosporus*

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Daptomycin, a cyclic anionic lipopeptide compound produced by *Streptomyces roseosporus*, is used to treat skin infections caused by multi-drug resistant gram-positive pathogens. The biosynthesis of daptomycin is initiated by the condensation of decanoic acid (DA, a 10-carbon unit fatty acid) and the N-terminal L-tryptophan. So, the addition of DA to the fermentation medium is essential for increasing daptomycin production. However, increasing of DA concentration in the fermentation medium was not possible due to the high toxicity of DA. The previous studies reported that the cell growth of *S. roseosporus* was halted from 1 mM DA. In order to improve daptomycin production with increasing DA concentration in the medium, the DA-resistant *S. roseosporus* was developed via a sequential-adaptation method. The DA-resistant strain (DAR) showed complete resistance to 1 mM DA, and the daptomycin production was increased 1.4-fold (40.5 ± 0.7 mg/L) compared with the wild-type (28.5 ± 0.8 mg/L) at 1 mM DA. Additionally, the initial step of the daptomycin biosynthesis was enhanced by the overexpression of *dptE* and *dptF* in DAR. The *dptEF* overexpression DAR showed 3.9-fold (156.3 ± 8.2 mg/L) increase in the daptomycin production compared with DAR (40.1 ± 2.6 mg/L) at 1 mM DA.

[**Key words:** *Streptomyces roseosporus*; Daptomycin; Decanoic acid; Toxicity; Sequential adaptation]

Daptomycin, a cyclic anionic lipopeptide comprised of 13 amino acids, is produced by *Streptomyces roseosporus* NRRL 11379 and exhibits antibacterial activity against gram-positive (G(+)) pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), penicillin-resistant *Streptococcus pneumonia* (PRSP), and vancomycin-resistant *Enterococci* (VRE) (1–4). Daptomycin destabilizes phosphatidylglycerol-containing membrane bilayers in the presence of calcium ions, causing the disruption of G(+) cytoplasmic-membrane functioning (5). Consequently, potassium ions leak out from the cytoplasm due to the damaged cytoplasmic membrane, causing a loss of the membrane's functionality that finally leads to cell death (6). The FDA-approved Cubicin, which is a daptomycin injection, can be used to treat a G(+) infection of the skin structure (7).

Daptomycin and its analogs are synthesized by the following three non-ribosomal peptide synthetases (NRPSs): DptA, DptBC, and DptD (7–9). The biosynthesis of daptomycin is initiated by the actions of DptE and DptF that are encoded upstream of the NRPSs (9). DptE has the acyl-CoA ligase domain and it activates the fatty acid (FA) moiety using ATP; the activated FA is then transferred onto the DptF, and the process includes the acyl carrier protein (ACP) domain. The ACP-bound FA and L-tryptophan (L-Trp) are condensed by the N-terminal C-domain of the DptA initiation module. After initiation, the daptomycin backbone is elongated by the three NRPSs and then cyclized by the C-terminal thioesterase (TE) domain of DptD (10).

The rapid worldwide increase of antibiotic-resistant bacteria has increased the demand for daptomycin in the market; however, due to the low yield, it is difficult to increase the cost-effectiveness of industrial daptomycin production. Since the daptomycin biosynthesis pathway was revealed, many studies have been conducted to improve the efficiency of daptomycin production, including random mutagenesis, the overexpression of biosynthetic daptomycin genes, and the optimization of production conditions (11–14). Daptomycin comprises a 10-carbon branched-chain FA, which is derived from decanoic acid (DA) on the terminal amino group of L-Trp (15). Previous studies showed that supplementation of the fermentation medium with DA, a short-chain FA (SCFA), is essential for increasing daptomycin production (11,16,17).

Although there were many attempts to enhance the daptomycin production through various methods, the DA content in the medium has been strictly limited in all the methods, because of the high toxicity of DA in relation to *S. roseosporus* (16). Previous studies reported that the cell growth of *S. roseosporus* was halted from 1 mM DA and was severely inhibited at 2.5 mM DA (18). In order to overcome problems caused by DA, sodium-decanoate (SD), soluble form of DA, was used as a precursor of daptomycin, and it successfully led to increased daptomycin production (17). However, SD has not been preferred by industries due to its high price. So it is still needed to develop a method to solve the problems from the precursor of daptomycin. The previous study showed that

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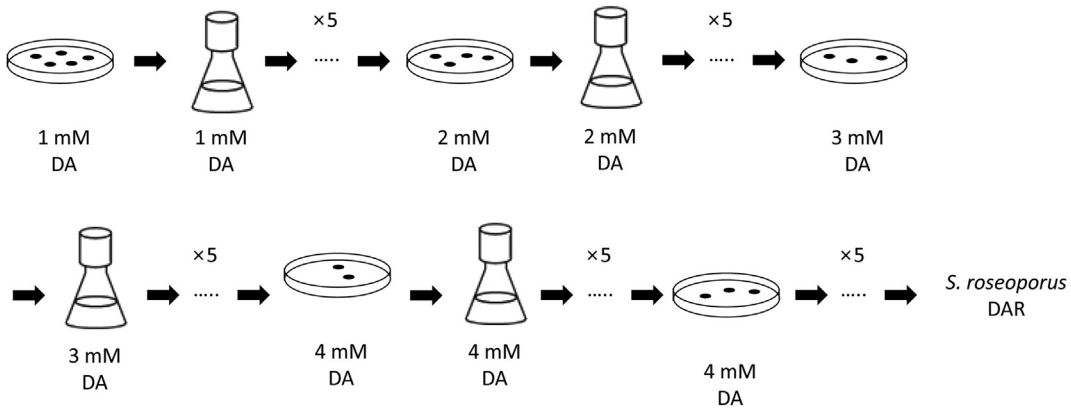

FIG. 1. Flow charts for the sequential adaptation method to develop DA tolerance in *S. roseosporus*.

overexpression of the initial condensation step in the daptomycin biosynthesis increased the production of daptomycin (18). These results led us to hypothesize that the daptomycin production can be increased by enhanced DA supply in *S. roseosporus*. To prove this, it was needed to find the solutions to overcome DA toxicity.

In yeast, DA increases the fluidity of the cell membrane, leading to conformational changes of the membrane proteins, and the increase of membrane fluidity is accompanied by an increase of reactive oxygen species (ROS) production (19,20). Moreover, it has been observed that DA upregulates genes involved in the coding of proteasome components and is also related to trehalose synthesis in *S. roseosporus* (18).

FAs have an ability to kill or inhibit the growth of bacteria (21). The antibacterial mechanism of FAs is not clearly understood, but most studies have proposed that the cell membrane is the major target of FAs (22). The antibacterial activity of a number of SCFAs is used to inhibit pathogens such as *Salmonella* spp. and *Vibrio* spp. (23–25). Interestingly, previous studies reported that FA supplementation increases the resistance of food-borne bacteria to antibacterial agents such as nisin and parabens (26). Moreover, the frequent exposure of *Salmonella typhimurium* to SCFAs such as acetate, propionate, and butyrate increased the virulence or tolerance to stress conditions via the increase of acid resistance (27–29). These results suggest that the adaptation of bacteria to SCFAs could increase the resistance to a toxic compound like DA.

In this study, we developed a DA-resistant strain from the wild-type *S. roseosporus* NRRL 11379 (NRRL 11379) using sequential adaptation to increase the DA feeding rate. Concurrently, we show that daptomycin production of the DA-resistant strain could be enhanced by genetic engineering method through the overexpression of *dptE* and *dptF*, the genes of the initial condensation step of daptomycin biosynthesis.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions All of the strains and plasmids used in this study are listed in Table S1. DH5α and ET12567/pUZ8002 are the *Escherichia coli* strains that were used for the subcloning and demethylation, respectively, of plasmid DNA according to the standard procedures (30,31). The *E. coli*–*Streptomyces* shuttle vector pSE34 was used for gene overexpression (32). Luria–Bertani agar and liquid media were used for growth of *E. coli* strains (31). ISP2 agar plates (33) were used for the sporulation and genetic manipulation of the NRRL 11379 strain and its mutant strains. Tryptic soy broth (TSB) (Difco, USA) was used for DA sequential adaptation or seed culture of the NRRL 11379 strain and its mutant strains. Each concentration of DA (1, 2, 3, and 4 mM) was added into the ISP2-agar medium with 0.1 % (v/v) TWEEN80 and the TSB-liquid medium with 0.1% (v/v) methyloleate. For the daptomycin fermentation, a main culture medium (1.65% (w/v) yeast extract, 0.043% (w/v) ammonium ferrous sulfate, 1.07% (w/v) glucose, 7.2% (w/v) maltodextrin, 0.72% (w/v) molasses, and 0.05% (v/v) antifoam SAG-471; pH was adjusted to 7.0 with NaOH) was used. The production of daptomycin was conducted by first inoculating a baffled 250 mL flask containing 50 mL of main culture medium with 50 mg (wet mass) of mycelium from the seed culture; the suspension was then cultivated for 144 h at 28°C, and 180 rpm. DA was added into the main cultures with methyloleate at 48 h after inoculation. Ampicillin (50 μg/mL), kanamycin (50 μg/mL), chloramphenicol (50 μg/mL), and thiostrepton (25 μg/mL) were also added to the media as required.

Sequential adaptation The NRRL 11379 strain was adapted through a subculture in the medium containing DA (Fig. 1). The spore suspension of the NRRL 11379 strain (20 μL) containing 1×10^8 spores/mL was inoculated into 50 mL of TSB medium and cultivated for 72 h at 28°C and 180 rpm. After 72 h of incubation, 200 μL of culture broth was transferred to ISP2 agar plate containing 1 mM DA, and then incubated for 7 days at 28°C. Each single colony that was formed on the agar plate was transferred into 50 mL of TSB medium containing 1 mM DA and incubated for 120 h at 28°C and 180 rpm. The culture broth was then subcultured five more times under the same conditions, and then 200 μL of culture broth was transferred to ISP2 agar plate containing 2 mM DA. A single colony that was formed on the agar plate was transferred and incubated in 50 mL of TSB medium containing 2 mM DA. After 5 times of subculture, 200 μL of culture broth was transferred to ISP2 agar plate containing 3 mM DA and incubated. A single colony that was formed on the agar plate was cultivated in 50 mL of TSB medium containing 3 mM DA. After subcultures, 200 μL of culture broth was transferred to ISP2 agar plate containing 4 mM DA. A single colony on the agar plate was subcultured in 50 mL of TSB medium containing 4 mM DA. After subcultures, 200 μL of culture broth was transferred to ISP2 agar plate containing 4 mM DA and incubated. A single colony that formed on the agar medium was subcultured five more times on ISP2 agar plate containing 4 mM DA to stabilize the strain. After the completed sequential-adaptation process, the strain showed a resistance to 4 mM DA and was therefore selected as the DA-resistant strain (DAR).

DNA manipulations The DNA extraction, manipulation, and transformation of the *E. coli* and *Streptomyces* were carried out according to the standard protocols (30,31). Restriction enzymes and molecular biology reagents were purchased from TAKARA and Sigma–Aldrich.

Gene overexpression The PCR primers that were used for the gene overexpression are listed in Table S2; for the overexpression of *dptE* and *dptF* (*dptEF*) in the NRRL 11379 strain, pSE34 was used (32). The primer pair OEF_F/OEF_R was designed to amplify the 2494-bp of *dptEF* (1794-bp of *dptE*; 270-bp of *dptF*) with its ribosomal binding site (RBS), and contains *Bam*HI and *Hin*dIII restriction sites. The PCR fragment of *dptEF* was digested with the *Bam*HI–*Hin*dIII, and was ligated into the *Bam*HI–*Hin*dIII sites of pSE34 to produce the overexpression vector pOEF; subsequently, the pOECF was introduced into the *E. coli* ET12567/pUZ8002 and then isolated for the purpose of demethylation (34). Protoplasts of the NRRL 11379 strain and DAR were prepared according to the standard methods (30), and were then transformed with the demethylated pOEF. Lastly, thiostrepton-resistant transformants were selected as the *dptEF*-overexpression strains 11,379/EF and DAR/EF. The vector-only control strains were also produced with the empty pSE34, and were designated 11,379/VC and DAR/VC.

Analysis of daptomycin production and cell growth To determine the occurrence of daptomycin production, the spore suspensions of the *S. roseosporus* strains were first inoculated into 50 mL of TSB medium before they were cultivated as seed cultures for 48 h at 28°C and 180 rpm. After 48 h of incubation, 50 mg of mycelium was harvested from the seed-culture broth and inoculated into 50 mL of the main culture medium, followed by cultivation for 144 h at 28°C and 180 rpm; the 1 mL culture broth was then collected and the same volume of MeOH was added. After 10 min of shaking, the solution was centrifuged for 5 min at 10,000 ×*g* and 20 μL of supernatant was injected into the HPLC system. A HPLC analysis for the identification of daptomycin production was performed using a modified version