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Heterologous Expression of Daptomycin Biosynthetic Gene Cluster Via *Streptomyces* Artificial Chromosome Vector System S

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The heterologous expression of the *Streptomyces* natural product (NP) biosynthetic gene cluster (BGC) has become an attractive strategy for the activation, titer improvement, and refactoring of valuable and cryptic NP BGCs. Previously, a *Streptomyces* artificial chromosomal vector system, pSBAC, was applied successfully to the precise cloning of large-sized polyketide BGCs, including immunosuppressant tautomycetin and antibiotic pikromycin, which led to stable and comparable production in several heterologous hosts. To further validate the pSBAC system as a generally applicable heterologous expression system, the daptomycin BGC of *S. roseosporus* was cloned and expressed heterologously in a model *Streptomyces* cell factory. A 65-kb daptomycin BGC, which belongs to a non-ribosomal polypeptide synthetase (NRPS) family, was cloned precisely into the pSBAC which resulted in 28.9 mg/l of daptomycin and its derivatives in *S. coelicolor* M511(a daptomycin non-producing heterologous host). These results suggest that a pSBAC-driven heterologous expression strategy is an ideal approach for producing low and inconsistent *Streptomyces* NRPS-family NPs, such as daptomycin, which are produced low and inconsistent in native host.

Keywords: *Streptomyces* artificial chromosome, daptomycin, biosynthetic gene cluster, heterologous expression

Introduction

The screening and development of *Streptomyces* natural products (NPs) as antibiotics, antifungals, antivirals, immunosuppressants, and anti-cancer drugs have played essential roles in human medicine for the past several decades [1–5]. Recent post-genomic approaches have stimulated the development of microbial genome mining to identify previously unsuspected and low-titer NP biosynthetic gene clusters (BGCs) [6–9]. The heterologous expression of *Streptomyces* NP BGC has become an attractive method for the titer improvement and pathway engineering of a range of potentially valuable *Streptomyces* NPs. Because the typical size of *Streptomyces* NP BGC is usually larger than 20 kb (sometimes over 100 kb), a range of strategies, such as a transformation-associated recombination (TAR) system, integrase-mediated recombination (IR) system, and plasmid *Streptomyces* bacterial artificial chromosome (pSBAC) system, have been developed to isolate large-sized *Streptomyces* NP BGCs [10–13].

The pSBAC system is an efficient tool for *Streptomyces* heterologous expression with site-specific restriction site insertion, recombinant pSBAC *in vivo* rescue, and *E. coli-Streptomyces* intergeneric conjugation [13]. Previously, meridamycin (MER, ~95 kb), tautomycetin (TMC, ~80 kb), and pikromycin (PIK, ~60 kb) BGCs were isolated successfully as a single giant recombinant plasmid using the pSBAC system and those BGCs were expressed functionally in several *Streptomyces* cell factories, such as *S. lividans* and *S. coelicolor* [12–14]. These results showed that the pSBAC system can be an effective platform technology for the precise cloning and functional overexpression of large-sized BGC of any potentially valuable low-titer metabolite in *Streptomyces* and its physiologically related actinomycetes. Because all three BGCs previously cloned in the pSBAC system belong to the polyketide synthase (PKS) family, it could be argued whether a different structural family BGCs, such as non-ribosomal peptide synthase (NRPS), are

also feasible for pSBAC-driven heterologous expression.

Daptomycin is an FDA-approved highly-valuable antibiotic exhibiting strong bioactivity against Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant enterococci (VRE), and other antibiotic resistant strains [15–17]. Daptomycin belongs to A21978C family of lipopeptides containing thirteen amino acids produced by *S. roseosporus* [18]. The three major components, A21989$C_{1\text{-}3}$, have 11-, 12- or 13-carbon branched-chain fatty acids, respectively, attached to the terminal amino group of L-Trp [18]. After the fatty acid side chains of A21978C are removed by incubation with *Actinoplanes utahensis* or with the *A. utahensis* deacylase produced by recombinant *S. lividans*, daptomycin can be produced by reacylation of the cyclic peptide with n-decanoyl fatty acid [19]. Alternatively, for daptomycin production biosynthetically, decanoic acid can be supplied to cultures of *S. roseosporus* to allow the direct incorporation of the straight chain supplement [20].

Previously, the heterologous expression of daptomycin BGC was performed to improve the production and generate novel analogues. A 128-kb fragment and a 157-kb fragment, including 65-kb daptomycin BGC, were isolated by the construction of a BAC library and IR system, respectively [21]. Because these clones contained huge extra sequences, which are not related to daptomycin production, it might impart some genetic burden to the heterologous host. Here, the exact daptomycin BGC was isolated precisely using the pSBAC system and the recombinant pSBAC was introduced into a model surrogate host, *S. coelicolor* M511 [22]. This was the first example of NRPS BGC to be cloned into the pSBAC-driven heterologous expression system. The daptomycin and its derivatives were produced successfully in the heterologous host, and their levels of production varied according to decanoic acid feeding into the culture media.

Material and Method

Bacterial Strains and Culture Media

Table 1 lists the bacterial strains and plasmids used in this study. *E. coli* strains were cultured in *Luria-Bertani* (LB) broth or agar supplemented with the appropriate antibiotics at 37°C. Spores and hyphal fragments of *S. roseosporus* ATCC31568 were grown on DA1 agar medium (0.4% glucose, 1% malt extract, 0.4% yeast extract, 0.2% $CaCO_3$, and 1.5% agar) for five days at 30°C [23]. The MS agar medium was used for the sporulation of *S. coelicolor*. For the production of daptomycin and its derivatives, *S. coelicolor* were grown in TSB media for two days and cultured for five days in R5 media at 30°C. Conjugation was carried out on a modified ISP4 medium.

Insertion of Recombinant pSBAC in Vicinity of Daptomycin Biosynthetic Gene Cluster

attP-int$_{\Phi BT1}$ was removed from pSBAC by *Avr*II digestion and

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/reference
Plasmid		
pATTP	*attP-int*$_{\Phi C31}$-containing pGEM-T easy vector	This work
pSBAC	*accIII(IV)*, *oriT*, *attP-int*, backbone of pCC1BAC	[10]
pSA	Modified pSBAC which deleted *attP-int*	[11]
pSAMD	Modified pSBAC with deleted *attP-int* and inserted *dptP* fragment and *Nhe*I site	This work
pDPT	pSAMD with 70kb DNA insert containing whole *dpt* gene cluster	This work
pDPT001	pDPT with *attP-int*$_{\Phi C31}$	This work
E. coli		
EPI300	F-*mrc*-A-D (*mrr-hsd*RMS-*mcr*BC) *trfA* host for cloning and amplification of various BAC vectors and constructs derived from it	Epicenter
S17-1	*E. coli* host for transferring various plasmids into *Streptomyces* via conjugation	
ET12567/pUZ8002	*E. coli* host for transferring various plasmids into *Streptomyces* via conjugation	
Streptomyces roseosporus		
ATCC31568	Original daptomycin-producing strain	[18]
Streptomyces coelicolor		
M511	*ΔactII-orf4*-deleted M145, Non daptomycin-producing strain	[20]
DPT101	M511 with pDPT001	This work

ligation (named pSA) to isolate the daptomycin biosynthetic gene cluster from the chromosome by NheI digestion and ligation. To integrate the pSA into the vicinity of *dptP* by homologous recombination, a 1,720-bp DNA fragment (HR fragment) was amplified by PCR using the genomic DNA of the *S. roseosporus* ATCC31568 wild-type strain as a template. The amplified PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel and purified using a DNA extraction kit. The amplified PCR products were then ligated into RBC T&A cloning vector (Real Biotech Co., Taiwan). The ligated vector was sequenced completely to confirm its integrity (Macrogen, Korea). The HR fragment, which was digested using EcoRI and HindIII, was cloned into pSA to generate pSAMD. The pSAMD was then introduced into *E. coli* S17-1 and conjugated directly with *S. roseosporus* ATCC31568 by homologous recombination. The desired mutant was selected on apramycin-included DA1 agar medium, and its genotypes were verified by PCR.

Isolation of the Entire Daptomycin Biosynthetic Gene Cluster into pSBAC

To isolate the entire daptomycin biosynthetic gene cluster, the pSAMD containing the *S. roseosporus* strain was cultured in TSB media for 1 day at 30°C, and the genomic DNA of the mutant was prepared using a Wizard genomic DNA purification kit (Promega, USA). The genomic DNA was digested using the restriction enzyme, NheI, purified, and concentrated by ethanol precipitation before self-ligation using T4 ligase (TaKaRa, Japan). After desalting, the ligation mixture was used for the electroporation of *E. coli* EPI300. Recombinant colonies were selected on an apramycin-containing LB medium. The plasmids were isolated using a TIANprep Mini Plasmid Kit (Tiangen, China), and screened by PCR using DptM, DptA, DptA-B-C, DptD, DptI, and DptRI check primers in daptomycin cluster to identify pDPT.

A 1,989-bp DNA fragment including the *attP-int*$_{\Phi C31}$ and *Avr*II was amplified by PCR using the pSET152 vector as a template. The amplified PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel, purified using a DNA extraction kit, and then ligated into a pGEM-T Easy vector (named pATTP). The pATTP vector was completely sequenced to ensure integrity (Macrogen, Korea). The *attP-int*$_{\Phi C31}$ was digested by *Avr*II and cloned into pDPT to generate pDPT001.

Production and Analysis of Daptomycin and Its Derivatives

Spores of *S. coelicolor* M511 and its mutants prepared from MS medium were inoculated into 250 ml baffled flasks containing 50 ml TSB and shaken at 230 rpm for two days. A 1 ml volume of seed culture was inoculated into the flasks containing 50 ml of R5 medium and then fermented at 30°C on a rotary shaker at 220 rpm for five days. Decanoic acid and methyl oleate were used as the feeding medium. After 48 h of fermentation, the feeding medium (final concentration, 1 mM) was added to the fermentation media once after 48 h or two, four, and six times (every 12, 18, 36 h) after 48 h. The culture broth was mixed with an equal volume of methanol to disrupt the cells and harvested by centrifugation. The supernatant was concentrated using a vacuum concentrator and dissolved in methanol. The final extracts were analyzed by HPLC (high pressure liquid chromatography) on a reverse phase column (Agilent ZORBAX SB-C18, USA) at a flow rate of 1 ml/min with UV detection at 222 nm. Solutions A (H_2O containing 0.01% TFA) and B (acetonitrile containing 0.01% TFA) were used to isolate daptomycin and the compounds of the A21978C family.

Mass spectrometric (MS) data were obtained by LC-MS analysis on Triple TOF 5600+ (AB Sciex, USA) system using electrospray ionization in positive ion mode, with a scan range of 50 ~ 2000 *m/z*. The LC method was run at 40°C on a Phenomenex Kinetex 1.7u C18 (2.1 mm × 150 mm, 1.7 um). The initial composition of 95% water, 10% acetonitrile and 0.01% formic acid were maintained for 3 min, followed by a linear gradient to 95% acetonitrile and 0.01% formic acid over 21 min, and this composition was held 25 min before re-equilibration; the flow rate was 0.4 ml/min. The electrospray capillary voltage was 5.5 kV and capillary temperature was maintained at 500°C.

Antibacterial Assays against *Staphylococcus aureus*

The antimicrobial activities of the daptomycin and its derivatives were assessed using the paper disc diffusion method. The bioassay was performed using *Staphylococcus aureus* as the indicator organism. A 1 ml sample of overnight-cultured *St. aureus* was mixed with 10 ml of a sterile solution of 1% agarose in H_2O. A 5 ml volume of the agarose/growth media mixture was added to a prewarmed NA medium and solidified for 30 min. The discs (6 mm diameter) were saturated with 10 µl of the extracts (dissolved in methanol) and placed onto an NA medium overlaid with *St. aureus*. After incubation for 20 h at 37°C, the diameter of the inhibition zone surrounding the discs, resulting from the diffusion of bacterial compounds, was then measured.

Results and Discussion

Isolation of the Daptomycin Biosynthetic Gene Cluster Using the pSBAC System

Unique restriction enzyme site in both border regions of NP BGC is necessary to isolate the BGC using pSBAC system. While the MER BGC was isolated using the unique restriction enzyme *Mfe*I sites present in the border region of MER BGC, artificial XbaI or HindIII sites were inserted precisely near the borders of the TMC BGC and PIK BGC to capture the BGCs. No unique restriction enzyme sites were available at the border regions of TMC BGC and PIK BGC for pSBAC cloning. Fortunately, the genome sequence analysis of *S. roseosporus* ATCC31568 revealed two NheI restriction enzyme sites at the both border regions of the daptomycin BGC (Fig. 1). To integrate a pSBAC vector into the vicinity of the *dptP*, a 1,720-bp gene fragment was

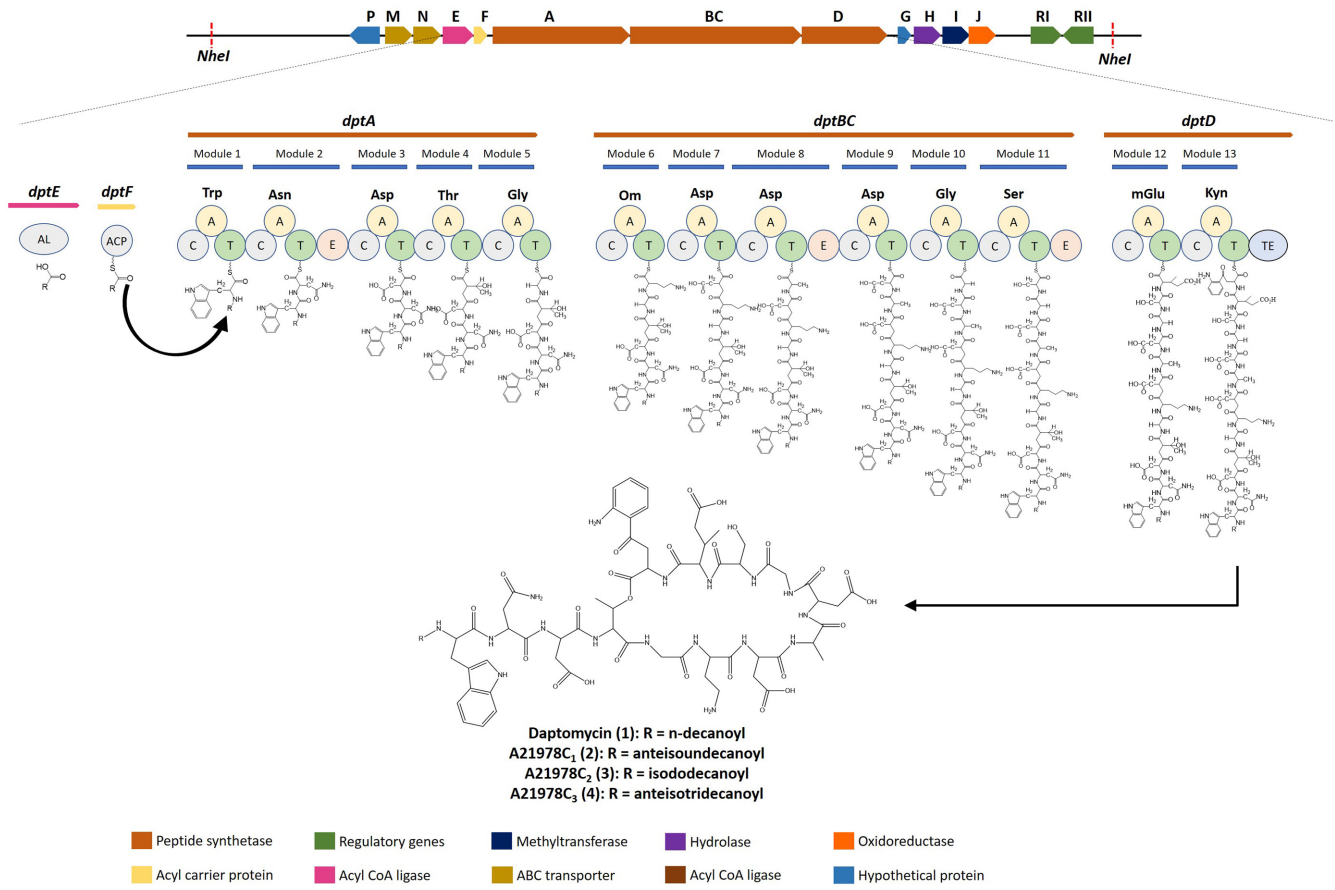

Fig. 1. Daptomycin biosynthetic pathway and its biosynthetic gene cluster.

cloned into a pSA using In-Fusion cloning kit. The resulting construct was called pSAMD, which was then introduced directly into the daptomycin-producing *S. roseosporus*. The integration of pSAMD was confirmed by PCR analysis (data not shown). The NheI-digested total chromosomal DNA fractions were then self-ligated and transformed directly into *E. coli* EPI300 (Fig. 2). The transformants were selected using apramycin antibiotics and confirmed by PCR (Fig. S1). As a final step of pSBAC cloning, *attP-int*$_{\Phi C31}$ was introduced into the daptomycin BGC-captured vector for the stability of daptomycin BGC in a heterologous host. The isolated TMC BGC or PIK BGC were expressed stably in *Stretpomyces* cell factories via integration using ΦBT1 integrase. On the other hand, the integration efficiency of the ΦBT1 *attP-int* system was quite low. Therefore, to increase the integration efficiency, the ΦC31 *attP-int* system, which is an advantage for high integration efficiency and a broad host range, was utilized for the integration of daptomycin BGC into *S. coelicolor*. The *Avr*II-digested *attP-int*$_{\Phi C31}$ from pATTP was ligated with pDPT, and the entire daptomycin BGC-containing modified pSBAC, called pDPT001, was constructed successfully for heterologous expression.

Using the pSBAC system, the precise cloning of the target cluster can be achieved through site-specific chromosomal integration of the vector and unique restriction sites as well as in vivo plasmid rescue. Previously, the daptomycin biosynthetic gene cluster was cloned by the construction of the BAC library, but approximately the 60 kb region of the BAC clone was not associated with the daptomycin biosynthetic gene cluster. Here, the daptomycin biosynthetic gene cluster (~65 kb) was isolated precisely using pSBAC and endogenous restriction enzyme, NheI, sites. Therefore, isolation of the daptomycin biosynthetic gene cluster can be another example of the isolation of natural product biosynthetic gene cluster belonging to not only type I polyketide, but also non-ribosomal polypeptide using the pSBAC system.

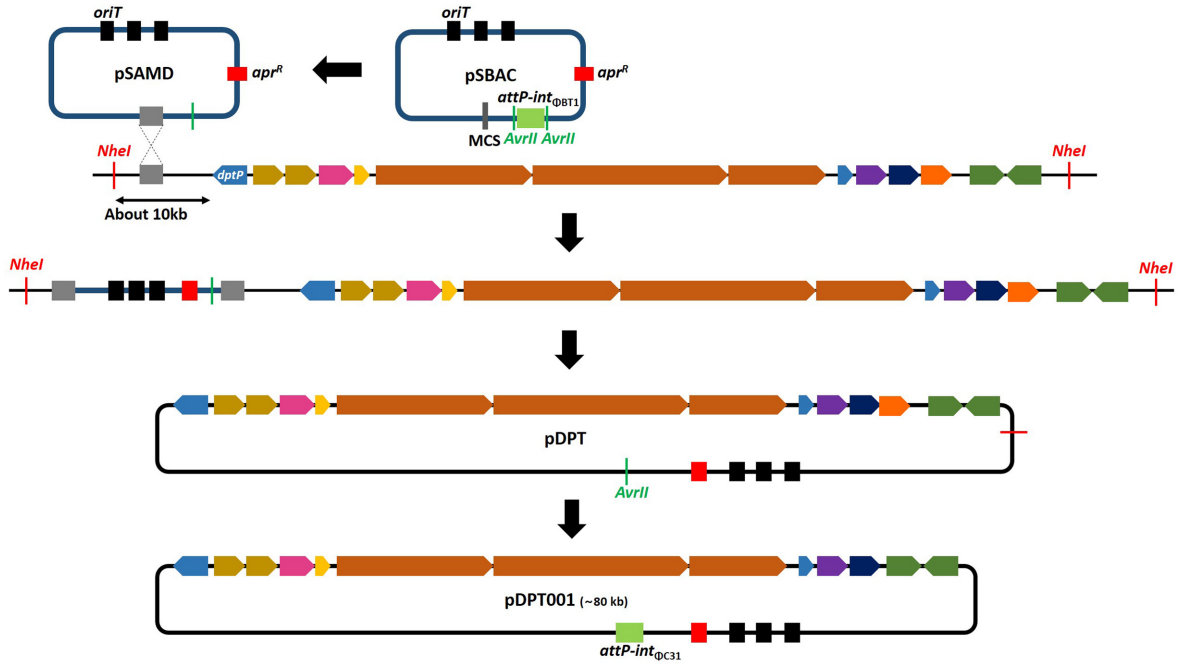

Fig. 2. Schematic description of pDPT001 construction.

Modified pSBAC called pSAMD was constructed by removing *attP-int*$_{\Phi BT1}$ and inserting 1,720-bp sequences in the vicinity of *dptP*. pSAMD was then introduced into the chromosomal DNA via homologous recombination. The daptomycin biosynthetic gene cluster was isolated by NheI digestion of the modified chromosomal DNA and its self-ligation, called pDPT. A DNA fragment containing *attP-int*$_{\Phi C31}$ was inserted into the *Avr*II recognition site of pDPT to generate pDPT001.

Heterologous Expression of Daptomycin Biosynthetic Gene Cluster in *Streptomyces coelicolor*

To confirm the heterologous and functional expression of the daptomycin BGC, the newly formed pDPT001 was introduced into *S. coelicolor* M511 through conjugation (named *S. coelicolor* DPT101). *S. coelicolor* M511 is one of the most widely used *Streptomyces* surrogate strains for heterologous expression, which is variant of *S. coelicolor* M145 with deletion of *actII-orf4* for repression the actinorhodin cluster that might be a competitive pathway for heterologous BGC. The constructed strain was cultured along with the parental strain, *S. coelicolor* M511. HPLC analysis revealed highly noticeable peaks showing identical retention times to the authentic daptomycin only in the *S. coelicolor* DPT101 (Fig. 3A). LC-MS analyses with the collected fraction of the peak between 26 min and 28 min showed that the *m/z* of **1** was 810.8630, and its UV max was approximately 222 nm, which is characteristic of the daptomycin. This suggests that daptomycin was produced heterologously in *S. coelicolor* M511 (Fig. S2). Compounds 2, 3, and 4 were also identified as daptomycin derivatives, A21798C$_{1\text{-}3}$ (Fig. S2). A21978C$_{1\text{-}3}$ containing a cyclic anionic 13-amino acid lipopeptide can be distinguished by the 11 to 13-carbon branched-chain fatty acyl moiety attached to the N-terminal L-Trp. This fatty acid comprises *n*-decanoic acid for daptomycin, *anteiso*-undecanoic acid, *iso*-dodecanoic acid, and *anteiso*-tridecanoic acid for A21798C$_{1\text{-}3,}$ respectively. In contrast, the production of daptomycin and its derivatives were inconsistent in *S. roseosporus* (data not shown); the production yields of daptomycin were 3.24 mg/l and the total yields of daptomycin and its derivatives were 24.22 mg/l in the heterologous host (Fig. 3B). This shows that the pSBAC-driven heterologous expression of an entire daptomycin BGC resulted in consistent production of daptomycin in a surrogate host *S. coelicolor*. As expected, the antimicrobial activity against *Staphylococcus aureus* was detected only in the extracts of DPT101 (Fig. 3C).

Previously, the *attP-int*$_{\Phi BT1}$ system was used for the stable expression of TMC BGC and PIK BGC in heterologous hosts, despite its integration efficiency being very low. In this study, the phage ΦC31 *attP-int* system, which has an advantage for high integration efficiency, was utilized for the integration of daptomycin BGC into *S. coelicolor*. After conjugation, many colonies (> 30 colonies) were shown in the ΦC31 system while only a few colonies were observed in the ΦBT1 system (data not shown). Integration into the

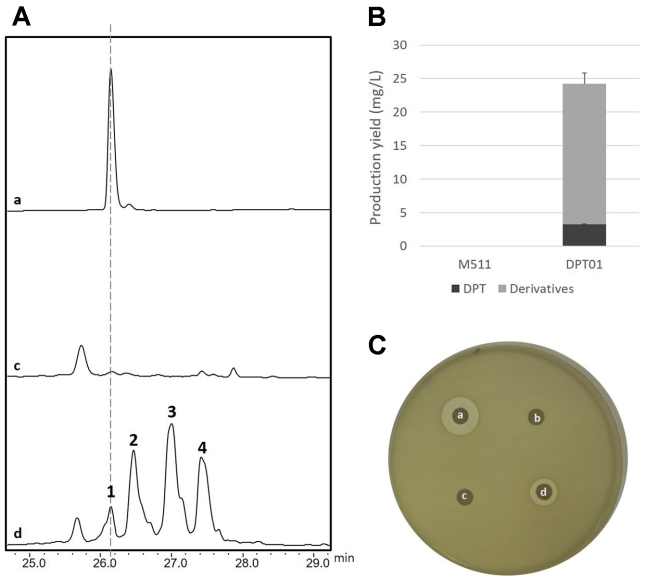

Fig. 3. (**A**) HPLC analysis of DPT101. (**B**) Production yield of daptomycin and its derivatives. (**C**) Antimicrobial assay against *Staphylococcus aureus*. a, authentic daptomycin 5 mg; b, culture media with decanoic acid; c, *S. coelicolor* M511 wild type; d, *S. coelicolor* DPT101.

ΦC31 *attB* site in *S. coelicolor* did not affect the production of daptomycin and its derivatives. These results suggest that the pSBAC system combined with the ΦC31 system can be used for the development of various NRPS-driven NP BGCs.

Heterologous Production of Daptomycin Via Decanoic Acid Feeding

The biosynthesis of daptomycin is initiated by the condensation of decanoic acid (a 10-carbon branched chain fatty acid) and L-Trp [24]. Therefore, the addition of decanoic acid to the fermentation medium is essential for daptomycin production [20]. However, decanoic acid feeding should be optimized because high concentration of decanoic acid could affect the surrogate host cell growth. To improve daptomycin production in a *S. coelicolor* DPT101 strain, 1 mM decanoic acid was added to the shake flask culture once after 48 h or two, four and six times after 48 h [25, 26]. Compared to repeated feeding of decanoic acid, two, four and six mM of feeding medium is added among the cultures tested. Although precursor was fed repeatedly or once in higher concentration, it resulted in similar level of daptomycin and its derivatives production (Fig. 4). These results suggested that adding higher amount of decanoic acid has no effect on improvement of

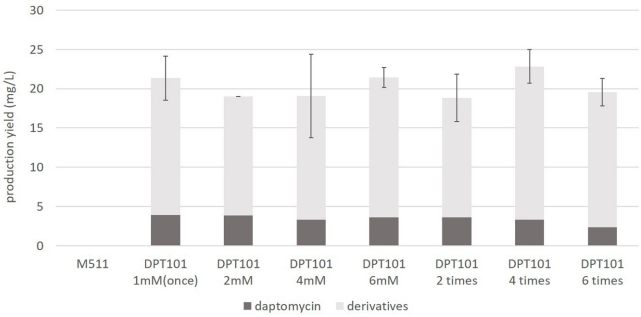

Fig. 4. Comparison of the production of daptomycin and its derivatives by optimization of the number of times decanoic acid was fed in DPT101.
Quantitative production yield of daptomycin and its derivatives.

daptomycin production yield for *S. coelicolor* DPT101. Increasing daptomycin production yield in a heterologous strain still needs further improvement with several strategies such as BGC refactoring, regulatory network optimization, and heterologous expression in more diverse *Streptomyces* cell factories. In summary, the 65-kb daptomycin BGC was cloned successfully in a single pSBAC vector system and expressed functionally in a heterologous host, suggesting that a pSBAC-driven heterologous expression strategy is an efficient approach for the production of *Streptomyces* NRPS-family NP BGC.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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