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Full Length Research Paper

Improvement of daptomycin yield by overexpression of the accessory genes of daptomycin gene cluster

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The effects of the accessory genes flanking the non-ribosomal peptide synthetase (NRPS) genes on daptomycin production were investigated by overexpression under the control of *ermE promoter via the integrative *Escherichia coli–Streptomyces* vector pIB139. The yield of daptomycin was promoted significantly when either of the upstream accessory genes of *dptE* and *dptF* or downstream accessory genes of *dptG*, *dptH*, *dptI* and *dptJ* was overexpressed individually or by a combined manner. The yield of daptomycin further increased to 869 ± 25 mg/L, approximately 42.6% higher than the parental strain of LC-54-16, when all of the six upstream and downstream accessory genes were overexpressed simultaneously. The results above suggested that the upstream and downstream accessory genes of NRPS had positive cooperativity in the biosynthesis of daptomycin. The transcriptional levels of both the upstream and the downstream accessory genes of NRPS in HP-EJ were approximately 2.5-fold as high as that in the parental strain of LC-54-16.**

Key words: Daptomycin production, accessory genes of NRPS, overexpression, *Streptomyces roseosporus*.

INTRODUCTION

Daptomycin is a member of the A21978C family of the cyclic anionic 13- amino acid lipopeptide, produced by a non-ribosomal peptide synthetase (NRPS) mechanism in *Streptomyces roseosporus* (Baltz et al., 2005). Daptomycin has a novel calcium-dependent mechanism, involving a binding action to bacteria cell membranes, which may account for its efficacy against strains resistant to other antibiotics (Zhou et al., 2009; Chamberlain et al., 2009). Cubicin® (daptomycin-for-injection) has been approved by FDA for the treatment of Gram-positive infection of skin structure since 2003 (Miao et al., 2005).

The market prospect of daptomycin is bright and promising and the demand of daptomycin has also been increasing. However, the yield of daptomycin needs to be further enhanced to reduce the cost of industrial production. Extensive studies on combinatorial biosynthesis of lipopeptide antibiotics derived from daptomycin have been conducted, since the daptomycin gene cluster (*dpt*) was characterized (Miao et al., 2006; Baltz et al., 2006). However, little information is available regarding the enhancement of daptomycin productivity by the genetic manipulation, though many exciting works have been done on production improving of antibiotics in other *Streptomyces*. For examples, the yield of antibiotics in *Streptomyces* can be improved significantly by increasing the availability of precursors (Stirrett et al., 2009). Malla et al. (2010) improved the productivity of doxorubicin greatly by overexpression of the regulatory genes of *dnrN*, *dnrI* and *metK1-sp* in *Streptomyces peucetius*; the yield of tautomycetin increased 2.4-fold by overexpression of the global antibiotic-simulating gene of *afsR2* (Park et al., 2009).

To improve the daptomycin production by manipulation of the whole large gene cluster (128 kb) is time consuming and uneconomical, not mention it may not be a wise choice, due to the complex pathway-specific regulatory mechanism of daptomycin biosynthesis. While, the accessory genes of NRPS play a very important role in the synthesis of lipopeptide antibiotics, which are involved in the supply of specific precursors, the regulation of the antibiotics biosynthesis, the secretion and the resistance of the self-produced antibiotics (Miao

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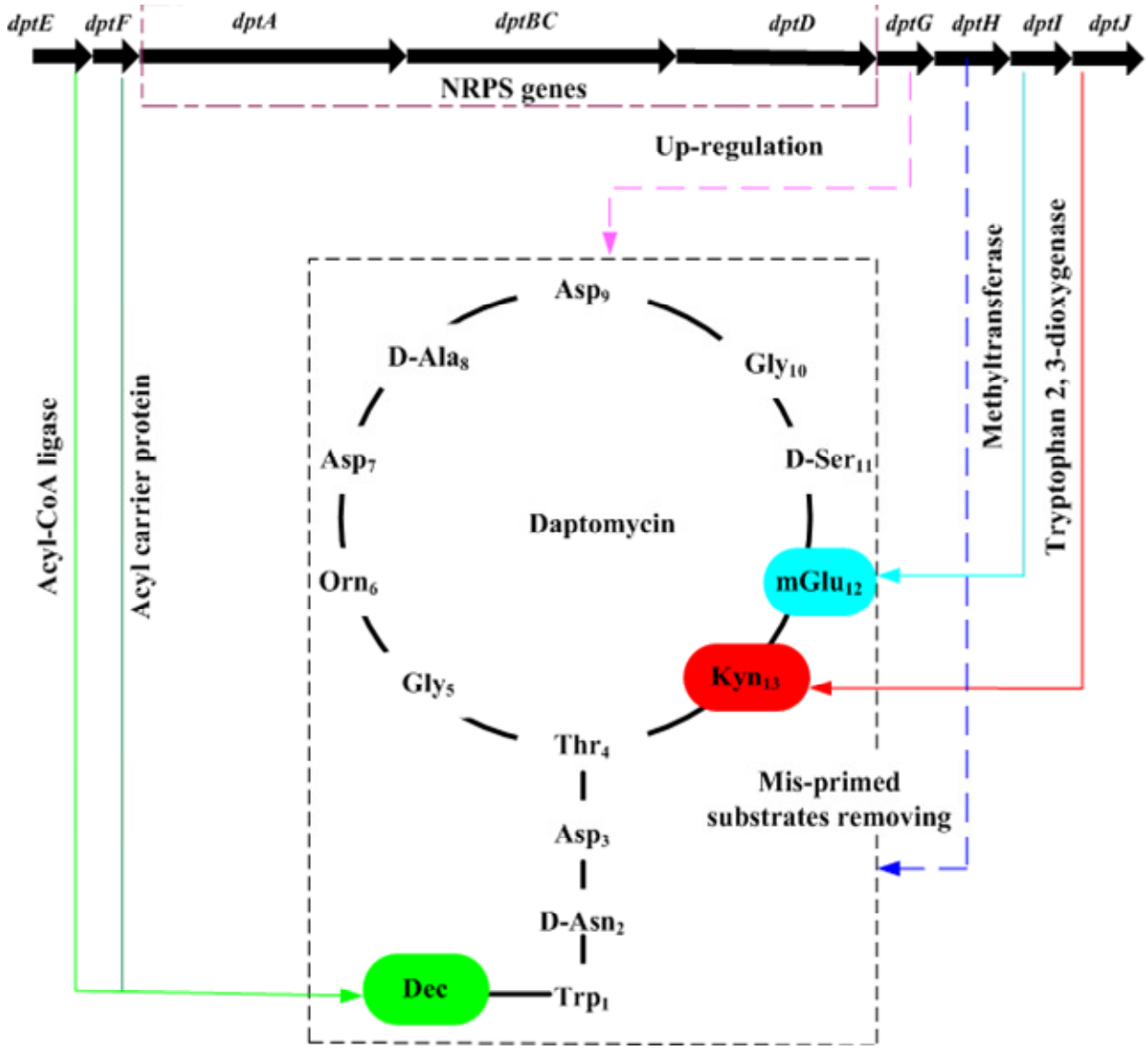

Figure 1. Functions of the accessory genes of NRPS on daptomycin biosynthesis.

et al., 2005; Nguyen et al., 2006). As shown in Figure 1, the genes of *dptE* and *dptF* located immediately upstream of NRPS are involved in the lipidation of daptomycin (Wittmann et al., 2008), while the genes of *dptG*, *dptH*, *dptI* and *dptJ* located immediately downstream of NRPS also play an important role in the regulation and in the supply of non-proteinogenic amino acids precursors.

The genes of *dptE* and *dptF* encode the acyl ACP synthetase and acyl carrier protein (ACP), respectively. N-decanoic acid is activated as a fatty acyl AMP and subsequently transferred on to DptF (Acyl carrier protein, ACP) by the acyl ACP synthetase of DptE, which initiates the biosynthesis of daptomycin. The *dptG* is proved to be a positive regulation gene, although its function mechanism is still not clear (Nguyen et al., 2006). The gene of *dptH* may involve in the removal of mis-primed substrates to improve the efficiency of NRPS (Miao et al., 2005), while the genes of *dptI* and *dptJ* encode key enzymes involved in the synthesis of nonproteinogenic amino acids precursors of 3-methyl-glutamic acid (3mGlu) and kynurenine (Kyn), respectively. However, few researches have been reported regarding the improvement of daptomycin production by manipulation of these ancillary genes, though they are crucial for the synthesis of daptomycin.

In this work, the NRPS upstream genes of *dptE* and *dptF* and the NRPS downstream genes of *dptG*, *dptH*, *dptI* and *dptJ* were cloned and overexpressed under the control of *ermE** promoter (Bibb et al., 1985) via the integrative *Escherichia coli–Streptomyces* vector pIB139 (Wilkinson et al., 2002) derivatives to improve the yield of

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

Strains/Plasmids	Relevant features	Source/ Reference
Strains		
S. roseosporus		
LC-54-16	Mutant, improved daptomaycin producer	NRRL11379
HP-E	LC-54-16 transformed with pIA1	This study
HP-F	LC-54-16 transformed with pIA2	This study
HP-EF	LC-54-16 transformed with pIA3	This study
HP-G	LC-54-16 transformed with pIA4	This study
HP-H	LC-54-16 transformed with pIA5	This study
HP-I	LC-54-16 transformed with pIA6	This study
HP-J	LC-54-16 transformed with pIA7	This study
HP-GH	LC-54-16 transformed with pIA8	This study
HP-GHI	LC-54-16 transformed with pIA9	This study
HP-GHIJ	LC-54-16 transformed with pIA10	This study
HP-EJ	LC-54-16 transformed with pIA11	This study
E. coli		This study
DH5α	Cloning host	Takara, Japan
ET-12567	pUZ8002, nonmethylating plasmid donor	Liu et al., 2006
Plasmid vectors		
pGEM®-T Easy	E. coli general cloning vector, Amp^r	Promega, USA
pIB139	pSET152 derivative	Wilkinson et al., 2002
pTA2		Toyobo, Japan
Recombinants		This study
pIA1	*dptE* in pIB139	This study
pIA2	*dptF* in pIB139	This study
pIA3	*dptEF* in pIB139	This study
pIA4	*dptG* in pIB139	This study
pIA5	*dptH* in pIB139	This study
pIA6	*dptI* in pIB139	This study
pIA7	*dptJ* in pIB139	This study
pIA8	*dptGH* in pIB139	This study
pIA9	*dptGHI* in pIB139	This study
pIA10	*dptGHIJ* in pIB139	This study
pIA11	*dptEFGHIJ* in pIB139	This study

daptomycin. Further, the transcriptional levels of over-expressed accessory genes were studied by reverse transcriptase-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Microorganism and plasmids

S. roseosporus LC-54-16 was a high-producing mutant of *S. roseosporus* NRRL11379, obtained by our laboratory via traditional mutagenesis and screen procedure (corresponding work is under review) and was used as a parent strain.

E. coli strain of DH5α was used as a cloning host, while *E. coli* ET12567 (pUZ8002) was used as the nonmethylating plasmid donor strain (Liu et al., 2006) for intergeneric conjugation with *S. roseosporus*. Integrative *E. coli-Streptomyces* vector pIB139 was used for gene expression in *S. roseosporus*. Both the *E. coli* ET12567 and vector pIB139 were presented by Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, China. pGEM®-T Easy vector (Promega, USA) was used as the cloning vectors in *E. coli*. Plasmids and strains used in this study are listed in Table 1.

Medium and culture conditions

Spores of *S. roseosporus* on slant were inoculated into 30 ml of R2YE medium in a 250-ml shake flask and incubated in an orbital shaker at 30°C and 220 rpm for 48 h. Cells growing exponentially were harvested for batch culture inoculation. The medium for daptomycin fermentation (g/L): glucose, 20; soluble starch, 20; casein, 5; yeast extract, 5; asparagines, 2; glutamine, 2; NH_4SO_4, 2; $K_2HPO_4{\cdot}3H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5 and $CaCO_3$, 0.2, dissolved in 1 L water, with pH adjusted to 7.5. Fermentations were carried out in a 7.5-L fermentor with 4 L medium (BIOFLO 110, New Brunswick Scientific Company, USA), and incubated at 30°C for 6 days, with

Table 2. Primers.

Primers	Sequence
dptE-F- NdeI	5'- TCGGTCATATGGGACTACGGACAGC-3'
dptE-R- XbaI	5'- CGACCTCTAGATCGACACGAACAGG-3'
dptF-F- NdeI	5'- ATGAACATATGCCCGCCCGAAGCG-3'
dptF-R- XbaI	5'- TCAGGTCTAGATGCGGTCGGCCAA-3'
dptEF-F- NdeI	5'- GGAGTGCATATGGTGAGTGAG -3'
dptEF-R- XbaI	5'- CATCTATCTAGAATCCCCTCAGG -3'
dptEF (lk)-F- KpnI-NdeI	5'- GTGGTACCCATATGGTGAGTGAG -3'
dptEF (lk)-R- HindIII	5'- CATCTCAAGCTTATCCCCTCAGG -3'
dptGJ (lk)-F- HindIII	5'- CGGGAAGCTTAGGAAACAT-3'
dptGJ (lk)-F- XbaI	5'- GTCAGTCTAGAGACGCTTGC-3'
dptG-F- NdeI	5'- CCATGCATATGGCCAACCCCTTCGAG-3'
dptG-R- XbaI	5'- TCAGGTCTAGACCGCGGTCGTCCGGT-3'
dptH-F- NdeI	5'- ATGAGCATATGGGCGACATCCAGGAT-3'
dptH-R- XbaI	5'- TCAGCTCTAGATCGTTCGGAAGAAAT-3'
dptI-F- NdeI	5'- ATGACCATATGCGTGCACGACTACCA-3'
dptI-R- XbaI	5'- TCCTGTCTAGACGCTGTCATGGTTTG-3'
dptJ-F- NdeI	5'- CGGGGCATATGCGCCACGTACCACGA-3'
dptJ-R- XbaI	5'- GCGCCTCTAGACCACGCCTCCGGGGG-3'
dptGH-F- NdeI	5'- GGGCATATG ATGGCCAACCCCTTCGAG-3'
dptGH-R- XbaI	5'- GCCTCTAGA TCAGCTCGTTCGGAAGAA-3'
dptGHI-F- NdeI	5'- GGGCATATG ATGGCCAACCCCTTCGAG-3'
dptGHI-R- XbaI	5'- GCCTCTAGA TCATGGTTTGCGTCCGTG-3'
dptGHIJ-F- NdeI	5'- TCCGTGCATATGAGGAAACAT-3'
dptGHIJ-R- XbaI	5'- CGTCAGTCTAGAGACGCTTGC-3'
rpsL-F	5'-GTATTCGACACACCCGACCG-3'
rpsL-R	5'-GAGGAGAGCTGTAGACCG-3'
pIB-F	5'-TTGCGCCCGATGCTAGTCG-3'
pIB-R	5'-GCACGACAGGTTTCCCGACTG-3'

the pH was automatically controlled at 7.0. N-decanoic acid was fed at 48 h after inoculation (0.2 g/L). All experiments were done in triplicate.

E. coli strains were cultured at 37°C in Luria–Bertani (LB) medium in both liquid broth and agar plates supplemented with the appropriate amount of antibiotics when necessary (ampicillin 100 µg/ml, chloromycetin 50 µg/ml, kanamycin 50 µg/ml and apramycin 50 µg/ml). Construction of recombinant plasmids and over-expression of accessory genes of NRPS

The PCR fragments were cloned into pGEM®-T Easy and sequenced. PCR products (NdeI- XbaI digested) were then ligated into NdeI-XbaI digested pIB139, generating recombinant plasmid of pIA1-pIA11. ET12567 (pUZ8002) facilitated the subsequent conjugation and integration into *S. roseosporus*-54-16 for overexpression. The exconjugants were verified by PCR (primers: pIB-F, pIB-R). The PCR fragment *dptEF* and *dptGHIJ* were interlinked by T-vector of pTA2 (Toyobo, Japan) via restriction sites of KpnI, HindIII and XbaI (primers: *dpt*EF (lk)-F- KpnI-NdeI, *dptEF* (lk)-R- HindIII; *dptGJ* (lk)-F- HindIII, *dptGJ* (lk)-F- XbaI). The primers utilized in this study are listed in the supplementary Table 2.

Analytical methods

The concentration of the daptomycin was measured by high performance liquid chromatography (HPLC) method (1100 Series, Agilent Company, USA) with a C-18 reversed-phase column (Lab Alliance, USA, 250×4.6 mm). The mobile phase consisted of 0.1% trifluoroacetic acid in water and in acetonitrile (55:45, v/v) and the flow rate was 1.0 ml/min. Dry cell weight (DCW) was determined by filtering the broth through a pre-weighed filter paper (Whatman GF/C) and dried at 80°C in a vacuum oven to constant weight.

Gene expression by RT-PCR

Total RNA was isolated from *S. roseosporus* cells by RNAprep pure Bacteria Kit (TianGen, China), in early-stationary phase, according to the protocol. The quantity and purity of RNA were determined by measuring the optical density at 260 nm and 280 nm and further determined by 1% formaldehyde agarose-gel electrophoresis. The gene specific primers of each gene are shown in supplementary Table 2. RT-PCR reactions were carried out in an iCycler (Bio-Rad, USA) using the QuantiTect SYBRgreen RT-PCR kit (Qiagen, Germany) according to the manual. The *rpsL* gene, housekeeping gene of *S. roseosporus*, was used as a constitutive reference (Rhee and Julian, 2006). RT-PCR was independently performed three times for each gene under identical condition. The relative abundance of each transcript with respect to the control sample was measured in triplicates and calculated according to the

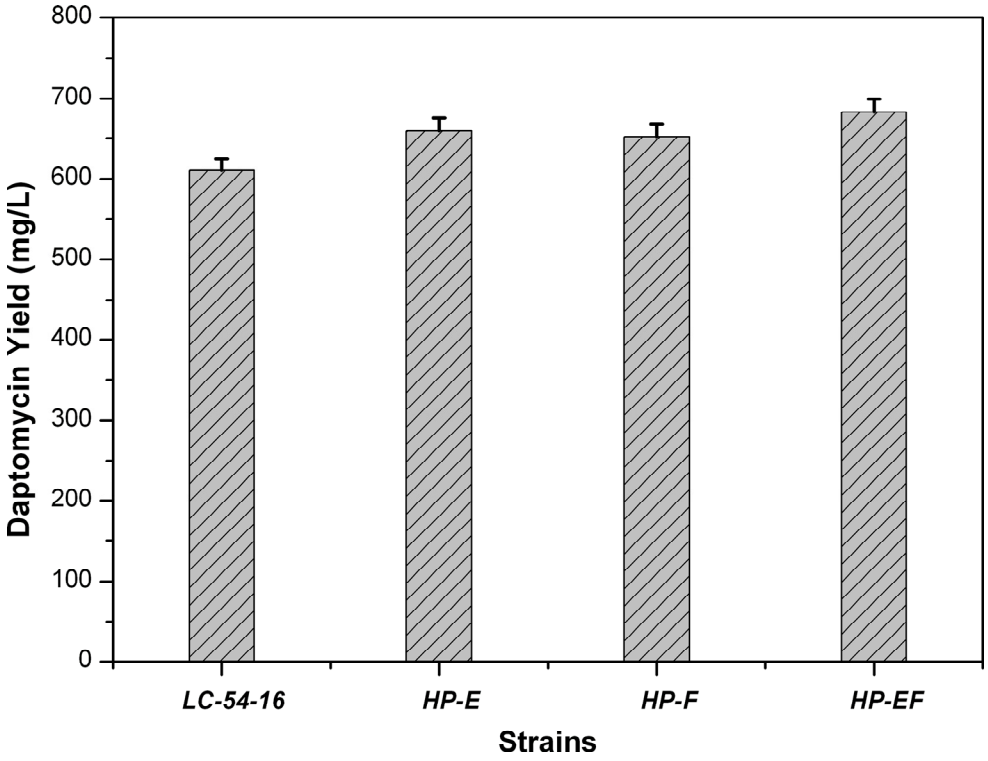

Figure 2. Effects of the NRPS upstream accessory genes on daptomycin production.

comparative Ct method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Effects of the NRPS upstream genes of *dptE* and *dptF* on daptomycin production

The effects of NRPS upstream accessory genes of *dptE* and *dptF* on daptomycin production were investigated by overexpressing these two genes under the control of constitutive strong promoter of *ermE** via pIB139. As shown in Figure 2, the production of daptomycin was enhanced notably, when either gene was overexpressed. The strain HP-E, which carried an additional copy of the *dptE*, showed about 8% higher in the yield of daptomycin than LC-54-16. While the daptomycin yield of the strain HP-F, with an additional copy of the *dptF*, enhanced approximately 6.9% compared with the parental strain of LC-54-16. Moreover, the daptomycin yield was further improved by overexpression of both the genes of *dptE* and *dptF*. This was reasonable since the genes of *dptE* and *dptF* played cooperatively an important role in the first step of daptomycin synthesis (Miao et al., 2005; Wittmann et al., 2008). The DptE protein could catalyze the formation of activated acyl CoA thioesters of C10 to C13 fatty acids which were transferred by the DptF protein to Trp_1 as an essential first step in the biosynthetic pathway of daptomycin. When decanoic acid was added during the fermentation process, the daptomycin (with C10 fatty acids tail) was the main ingredient of A21978C, which indicated that decanoic acid was the main substrate of DptE protein. The results above clarified that the activation of decanoic acid and condensation of decanoyl-CoA and Trp was crucial in daptomycin biosynthesis.

Effects of the NRPS downstream accessory genes on daptomycin production

The NRPS downstream accessory genes of *dptG*, *dptH*, *dptI* and *dptJ* were overexpressed individually or by a combined manner, in order to illuminate the effects of these genes on the daptomycin production. As shown in Figure 3, daptomycin production enhanced significantly by overexpression of the downstream accessory genes. The yield of daptomycin was enhanced 8.5, 4.9, 10.7 and 15.2%, respectively when the genes of *dptG*, *dptH*, *dptI* and *dptJ* were overexpressed separately. The daptomycin production was further enhanced by combined overexpression of the downstream accessory genes. The daptomycin yield of HP-GH (contained both additional copy of *dptG* and *dptH*) increased nearly 12% compared with the parental strain of LC-54-16. Further, the daptomycin yield of HP-GHI and HP-GHIJ reached 736 ± 20 mg/L and 818 ± 22 mg/L, respectively. The above results were consistent with the findings by Nguyen et al.

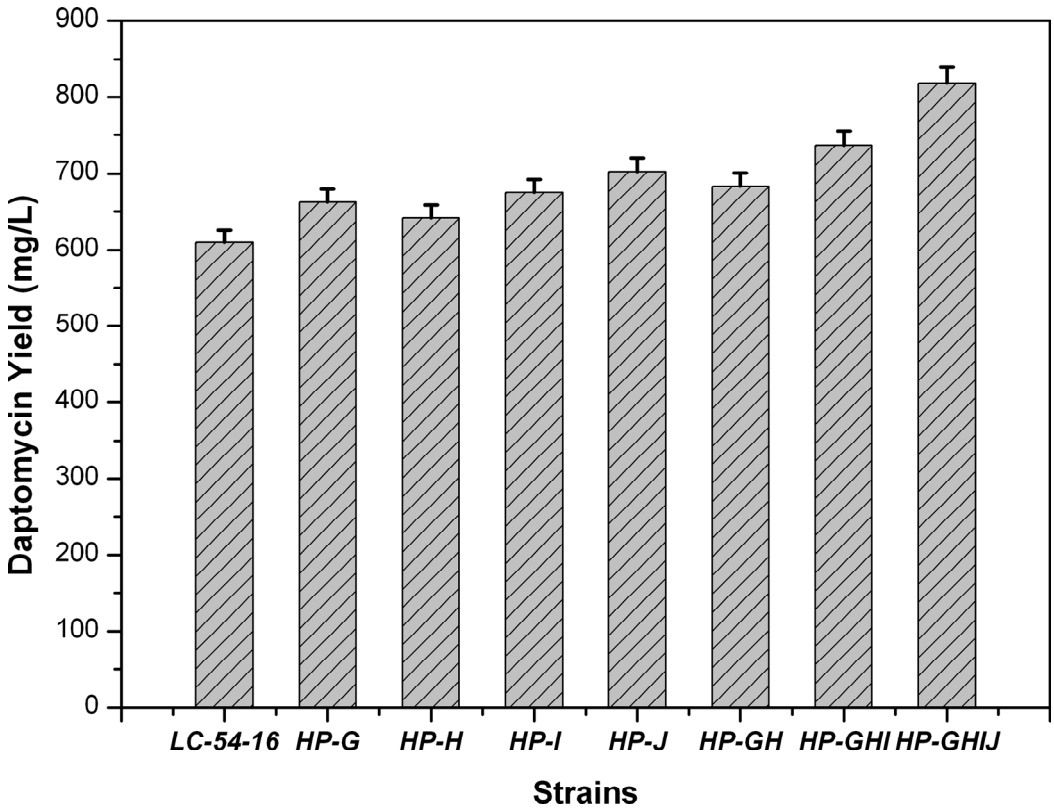

Figure 3. Effects of the NRPS downstream accessory genes on daptomycin production.

(2006), whose work team evaluated the effects of the downstream accessory genes by deletion and complementation. Comparative domain analyses suggested that *dptG* involved in the regulation of expression or export of antibiotics (Yeats et al., 2003). *dptH* is predicted to encode a type II thioesterase (Te II) , considering that the hydrolase-like protein encoded by the *dptH* gene bears the active site GXSXG motif of known thioesterases (Reimmann et al., 2004). Te II enzymes may be responsible for removal of mis-primed substrates from T- or ACP domains (Miao et al., 2005). Depletion of *dptH* reduced daptomycin production significantly (Nguyen et al., 2006). Thus, overexpression of *dptH* could enhance the efficiency of daptomycin production by clearing mis-incorporated substrates that block the pathway. The gene of *dptI* encodes the methyltransferase essential for 3mGlu formation (Miao et al., 2005; Nguyen et al., 2006; Mahlert et al., 2007). The significant enhancement of daptomycin yield in HP-I indicated that the 3mGlu was one of the important precursors of daptomycin biosynthesis. The *dptJ* encodes the tryptophan 2, 3-dioxygenase, an enzyme involved in the conversion of tryptophan (Trp) to kynurenine (Kyn) (Miao et al., 2005). The effect of *dptJ* on daptomycin production was the most significant, which may result from the deficiency of Kyn *in vivo* for daptomycin production. The results above were reasonable, since modified amino acids were not only important for biological activity, but also the bottlenecks for biosynthesis of antibiotics in many peptide antibiotics (Haug et al., 2004).

Enhancement of daptomycin production by overexpression both the upstream and downstream accessory genes

The studies above showed that all of the upstream and the downstream accessory genes of NRPS had notable influence in the promotion of daptomycin production. Thus, all of the upstream and the downstream accessory genes were overexpressed to further improve the daptomycin yield. As shown in Figure 4, the yield of daptomycin of HP-EJ was enhanced remarkably up to 869 ± 25 mg/L, which increased approximately 42.6% compared with the LC-54-16. While, there were no obvious differences in the cell growth. The results suggest that the upstream and downstream accessory genes of NRPS had positive cooperativity in the biosynthesis of daptomycin. The overexpression of the upstream and downstream accessory genes of NRPS was proved at the transcriptional level of by the RT-PCR (Figure 5). The transcriptional levels of both the upstream and the downstream accessory genes of NRPS in HP-EJ were approximately 2.5-fold as high as that in the

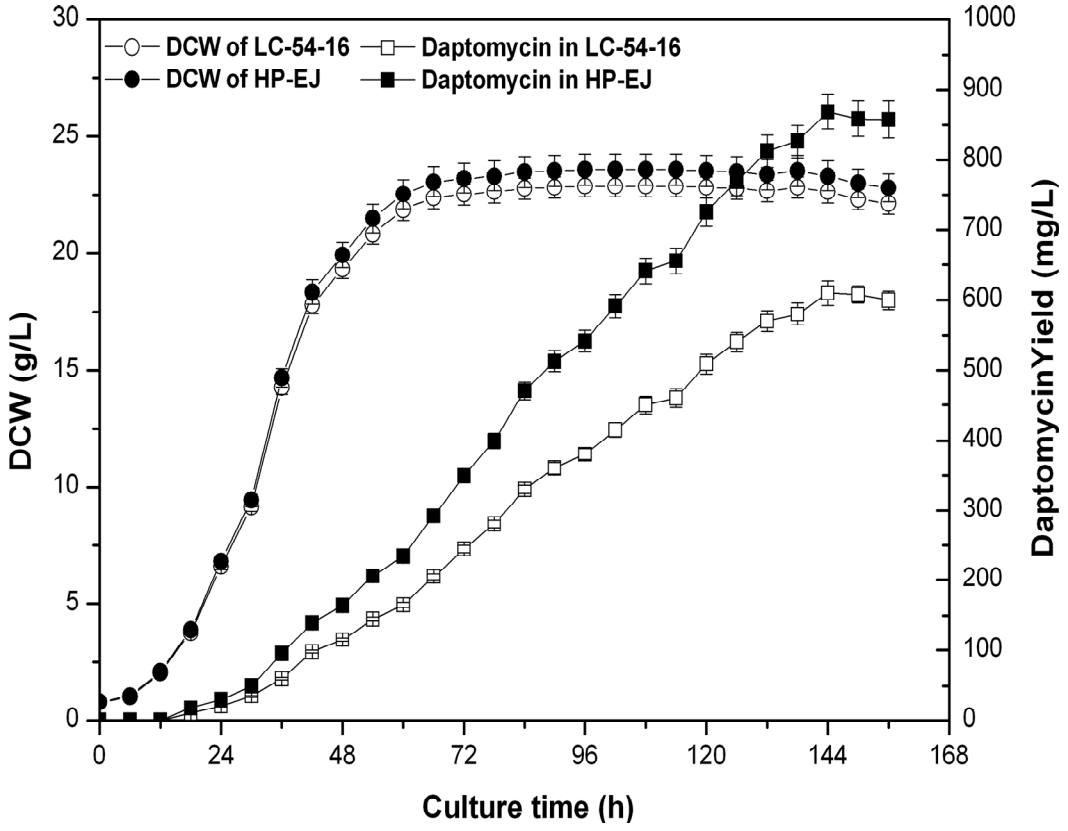

Figure 4. Enhancement of daptomycin production by overexpression both the upstream and downstream accessory genes.

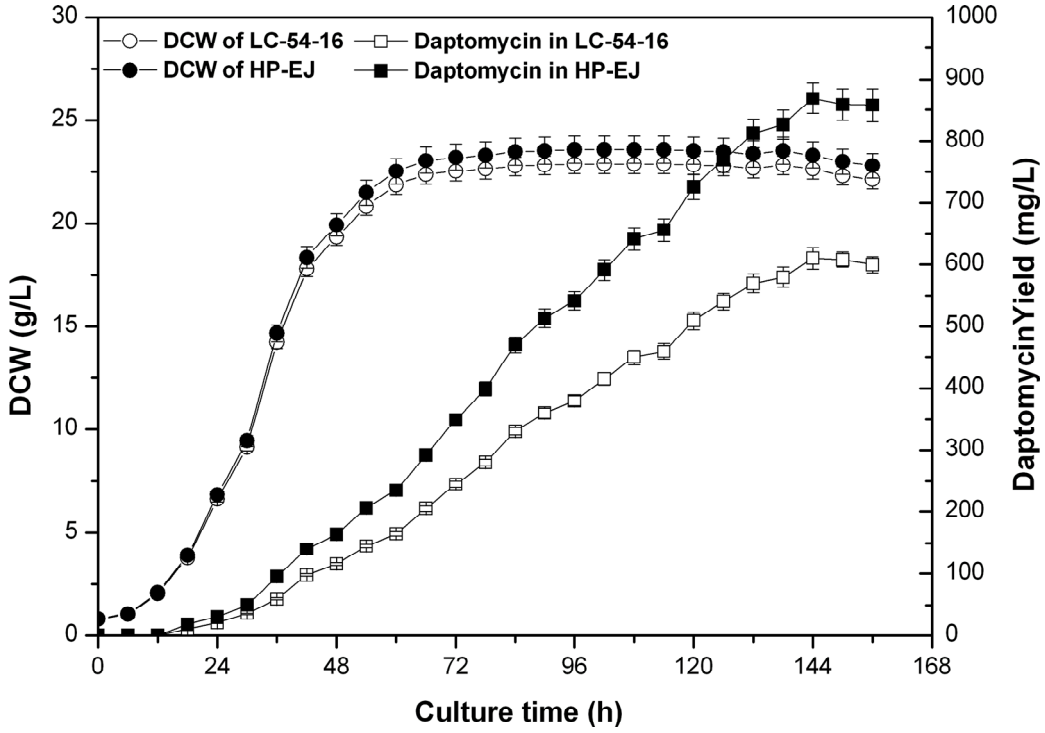

Figure 5. The comparison of gene expression by RT-PCR at early stationary phase.

parental strain of LC-54-16.

The results obtained in this work clarified that the pathway-specific regulatory genes and the genes involved in the supply of modified amino acids precursors located on both sides of NRPS genes were very important for daptomycin production. The whole *dpt* gene cluster comprises 64 ORFs, among which the three largest ORFs (42–44: *dptA*, *dptBC* and *dptD*) encodes the subunits of NRPS. Besides, all of the 61 accessory genes play an important role in daptomycin biosynthesis, since the daptomycin production would be inhibited at different levels, when either of genes was absent. Thus, to further elucidate the functions and effects of these accessory genes of NRPS on daptomycin production may offer an efficient way to further promote the yield of daptomycin.

Conclusion

In this work, the effects of the accessory genes flanking the NRPS genes on daptomycin production were investigated. The yield of daptomycin enhanced observably when each gene was overexpressed respectively and the daptomycin production was further improved, when either the upstream accessory genes or downstream accessory genes were overexpressed simultaneously. Finally, the daptomycin yield was increased to 869 ± 25 mg/L, when both the upstream and downstream accessory genes were overexpressed and the results of RT-PCR proved that the transcription level of the accessory genes was greatly promoted at the transcription level. It is suggested that this strategy for increasing the daptomycin production in *S. roseosporus* by manipulating the accessory genes flanking the NRPS genes may provide an alternative approach to enhancing the production of antibiotics in other *Streptomyces*.

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