Complementation of daptomycin *dptA* and *dptD* deletion mutations *in trans* and production of hybrid lipopeptide antibiotics

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Daptomycin is a lipopeptide antibiotic produced by *Streptomyces roseosporus* and recently commercialized as Cubicin® (daptomycin-for-injection) for treatment of skin and skin-structure infections caused by Gram-positive pathogens. Daptomycin is synthesized by a non-ribosomal peptide synthetase (NRPS) encoded by three overlapping genes, *dptA*, *dptBC* and *dptD*. The *dptE* and *dptF* genes, immediately upstream of *dptA*, are likely to be involved in the initiation of daptomycin biosynthesis by coupling decanoic acid to the N-terminal Trp. Analysis of RT-PCR data suggests that *dptE*, *dptF*, *dptA*, *dptBC*, *dptD* and possibly other *dpt* genes are transcribed as one large message; however, it has been demonstrated that sequential translation of these genes from a long transcript is not essential for robust daptomycin production. The *dptA* and the *dptD* genes were deleted from the *dpt* gene cluster, and expressed from ectopic positions in the chromosome under the control of the strong constitutive *ermEp** promoter to produce high levels of lipopeptides. This three-locus *trans*-complementation system was used to produce hybrid lipopeptide antibiotics by introducing the heterologous *lptD* and *cdaPS3* genes from *Streptomyces fradiae* and *Streptomyces coelicolor*, respectively, to complement the $\Delta dptD$ mutation.

INTRODUCTION

Streptomyces roseosporus produces A21978C, a mixture of acidic lipopeptide antibiotics, composed of a 13 amino acid peptide core coupled to one of several different chain-length fatty acids (Debono *et al.*, 1987). One member of the family, daptomycin, which is produced by feeding decanoic acid to the fermentation (Huber *et al.*, 1988), has been commercialized as Cubicin® for the treatment of skin and skin-structure infections caused by Gram-positive pathogens (Arbeit *et al.*, 2004; Eisenstein, 2004; Kirkpatrick *et al.*, 2003). The A21978C lipopeptides are synthesized by a large non-ribosomal peptide synthetase (NRPS) multienzyme composed of three subunits, DptA, DptBC and DptD, each containing two to six modules to direct the incorporation of the 13 amino acids (Fig. 1a). Each module has a complete set of domains responsible for amino acid condensation (C), adenylation (A) and thiolation (T) (Marahiel *et al.*, 1997; Schwarzer *et al.*, 2003), and three of the modules have epimerase (E) domains to convert L-amino acids to D-amino acids at positions 2, 8 and 11 (Miao *et al.*, 2005). The daptomycin NRPS subunits are encoded by three very large genes, *dptA* (17.5 kb), *dptBC* (22.0 kb) and *dptD* (7.1 kb) (Miao *et al.*, 2005) arranged in tandem (Fig. 1b). Two genes,

dptE (1.8 kb) and *dptF* (270 bp), directly upstream of *dptA*, show sequence similarities to acyl-CoA ligase and acyl carrier protein genes, respectively, and are likely to be involved in the acylation of the N-terminal amino acid, Trp, which initiates lipopeptide biosynthesis.

The manipulation of the cloned *S. roseosporus* daptomycin gene cluster provides a means to develop daptomycin analogues biosynthetically (Baltz *et al.*, 2006). The terminal NRPS gene, *dptD*, can be deleted and successfully complemented *in trans* by genes from related lipopeptide NRPS pathways that encode similar subunits, but with alternative modules to generate active new antibiotics (Miao *et al.*, 2006b). This prompted us to evaluate whether *dptA* could also be complemented *in trans*. Extending the *trans*-complementation platform to include *dptA* would further increase the opportunities to generate novel lipopeptide antibiotics by module exchanges (Baltz *et al.*, 2006). However, this approach is potentially more challenging as little is known about the transcriptional organization of the *dpt* pathway. The stop and start codons of the NRPS genes, *dptA*, *dptBC* and *dptD*, overlap and the intergenic regions between *dptE*, *dptF* and *dptA* are relatively small, less than 70 bp. It is possible that a promoter resides in the intergenic region upstream of *dptE*, or even further upstream, and that *dptE*, *dptF* and *dptA*, as well as *dptBC* and *dptD*, are

Abbreviation: NRPS, non-ribosomal peptide synthetase.

Fig. 1. Organization of *dpt* subunits and genes in *S. roseosporus* strains. (a) Structure of daptomycin. Amino acids incorporated by each subunit are as outlined. (b) Core *dpt* genes in native cluster (UA343 and UA117). (c) $\Delta dptD$ deletion (UA378) (d) In-frame deletion of *dptA* (UA474) and *dptA* with *ermEp** (angled arrow) introduced in front of *dptBC* (UA475).

transcribed on a single large mRNA. In *Streptomyces coelicolor*, it has been shown that there is a single promoter upstream of the three terminally overlapping NRPS genes encoding the calcium-dependent antibiotic CDA (Ryding *et al.*, 2002). If this is also the case for the daptomycin NRPS, it may be necessary to compensate for potential polar effects on downstream genes when engineering an upstream gene such as *dptA*.

To investigate the possibility of using additional engineered NRPS subunits (*dptA* and *dptD*) to generate novel analogues of daptomycin, and to acquire a better understanding of the transcriptional organization of the NRPS genes, we have explored the transcription of the *dpt* gene cluster using RT-PCR, and the reconstitution of the daptomycin biosynthetic pathway by expressing the three *dpt* NRPS genes from different chromosomal loci. We also evaluated the effects on product yield of placing a strong constitutive promoter, *ermEp** (Bibb *et al.*, 1994), either in front of *dptBC* at the native locus, or in front of *dptA* or *dptEFA* expressing from the *attB^{C31}* locus and demonstrated the utility of this system by producing hybrid lipopeptides by heterologous subunit exchanges with *lptD* (Miao *et al.*, 2006a, b) and *cdaPS3* (Hojati *et al.*, 2002).

METHODS

Strains and culture conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *Streptomyces* strains were routinely grown on trypticase soy agar or broth at 30 °C. AS-1 agar (Baltz, 1980), used for bioassays, and fermentation medium F10A and other procedures (Miao *et al.*, 2005, 2006b) were described previously. *Escherichia coli* DH10B (Invitrogen) was used for general cloning and ML22 was used as a donor strain for conjugative transfer of plasmids to *S. roseosporus*. *E. coli* BW25113 was used in all the recombination experiments (Datsenko & Wanner, 2000).

Staphylococcus aureus SA42 is susceptible to daptomycin and was used for microbiological assays. Antibiotics were added to the culture media at previously described concentrations (Kieser *et al.*, 2000; Sambrook *et al.*, 1989).

Construction of *S. roseosporus* $\Delta dptA$ $\Delta dptD$ deletion mutants.

S. roseosporus $\Delta dptA$ mutants were constructed by homologous recombination in UA378 ($\Delta dptD::ermE$), which was derived from UA117, an A21978C producer with a recessive mutation (*rpsL7*) conferring resistance to streptomycin (Sm^R) (Fig. 1c, Table 1). Mutants were constructed using a markerless *dptA* deletion cassette. Fragments of DNA upstream and downstream of *dptA* were subcloned or amplified by PCR and directionally cloned into *Hind*III–*Xba*I sites in pRHB538 (Hosted & Baltz, 1997) to leave an artificial *Xho*I site between them: the 3.2 kb upstream fragment (*dpt*:48381–51590, the coordinates refer to NCBI accession no. AY787762, the *dpt* gene cluster) included a portion of *dptN* and all of *dptE* and *dptF*; the 1.5 kb downstream fragment (*dpt*:69010–70520) consisted primarily of the 5' end of the *dptBC* gene. This construction deleted most of *dptA*, leaving a small open reading frame encoding 25 amino acids. Another version of this cassette was constructed by inserting at the *Xho*I site, upstream of *dptBC*, a fragment containing *ermEp** amplified by PCR from pHM11a (Motamedi *et al.*, 1995) using primers m97F (5'-CTCGAGGCGAGTGTCCGTTCGAGTGG-3') and m98R (5'-CTCGAGCATATGGGTCTCCTGTGGAC-3'). Each deletion plasmid was introduced into *S. roseosporus* UA378 by conjugation from *E. coli* (Bierman *et al.*, 1992; Hosted & Baltz, 1997). The pRHB538 vector carries an apramycin resistance (Am^R) marker and a dominant wild-type *rpsL* allele (Sm^S) that allows for the direct selection of gene replacements in Sm^R hosts carrying a mutant *rpsL* (Hosted & Baltz, 1997). Growth at 39 °C, a non-permissive temperature for plasmid replication (*ori^{ts}*), in the presence of *Am* selects for exconjugants with the plasmid integrated into the chromosome by a single crossover (Am^R). Subsequent selection on AS-1 agar plates containing *Sm* facilitates identification of recombinants that have eliminated vector sequences (Sm^S) after a second crossover exchanging the targeted gene with an in-frame deletion or a replacement cassette. Attempts to grow *S. roseosporus* at 39 °C during this experiment were not successful, but exconjugants with the desired in-frame deletions of

Table 1. Key parent strains and plasmids

Strains	Relevant characteristics	Reference
<i>S. roseosporus</i>		
UA343	A21978C producer	NRRL 15998
UA117	UA343 <i>rpsL7</i> (Sm ^R)	Miao <i>et al.</i> (2006b)
UA378	UA117 Δ dptD::ermE	Miao <i>et al.</i> (2006b)
UA474	UA378 Δ dptA Δ dptD::ermE	This study
UA475	UA378 Δ dptA <i>ermEp</i> *::dptBC Δ dptD::ermE	This study
MF6	UA474::pRB04	This study
MF40	UA475::pRB04	This study
MF54	MF6::pLT01	This study
MF77	MF6::pLT02	This study
MF85	MF6::pLT03	This study
MF171	MF40::pLT01	This study
MF110	MF40::pLT02	This study
MF101	MF40::pLT03	This study
MF159	UA474::pMF30::pLT01	This study
MF118	UA474::pMF30::pLT02	This study
MF125	UA474::pMF30::pLT03	This study
MF202	UA475::pMF30::pLT01	This study
MF133	UA475::pMF30::pLT02	This study
MF141	UA475::pMF30::pLT03	This study
MF63	UA474::pMF26::pLT01	This study
MF191	UA474::pMF26::pLT02	This study
MF193	UA474::pMF26::pLT03	This study
MF214	UA475::pMF26::pLT01	This study
<i>E. coli</i>		
DH10B	F [−] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ 139(<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ - <i>rpsL</i> (Sm ^R) <i>nupG</i>	Invitrogen
ML22	DH10B::pUZ8002	This study
BW25113	<i>lacI</i> ^f <i>rrnB</i> _{T14} Δ <i>lacZ</i> _{WJ16} <i>hsdR514</i> Δ <i>araBAD</i> _{AH33} Δ <i>rhaBAD</i> _{LD78}	Datsenko & Wanner (2000)
<i>S. aureus</i>		
SA42	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> Rosenbach	ATCC 29213
Plasmids		
pUZ8002	RK2 derivative with defective <i>oriT</i> (<i>aph</i>)	J. Wilson & D. H. Figurski, Dept of Microbiology, Columbia University, New York
pKD78	<i>repA101(ts)oriR101ParaB(gam bet exo)cat</i>	CGSC ID#80301
pKD119	<i>repA101(ts)oriR101ParaB(gam bet exo)tet</i>	CGSC ID#80304
pRHB538	<i>rpsL</i> <i>rep</i> ^{ts} Am ^R	Hosted & Baltz (1997)
pHM11a	Hm ^R <i>oriT</i> <i>ermEp</i> * <i>att/int</i> ^{IS117}	Motamedi <i>et al.</i> (1995)
pRB04	pHM11a::dptD	Miao <i>et al.</i> (2006b)
pMF26	pHM11a::cdaPS3	Miao <i>et al.</i> (2006b)
pMF30	pHM11a::lptD	Miao <i>et al.</i> (2006b)
pStreptoBAC V	BAC vector <i>oriT</i> <i>att/int</i> ^{φC31} Am ^R	Miao <i>et al.</i> (2005)
pLT01	pStreptoBAC::dptP dptM dptN dptE dptF dptA	This study
pLT02	pStreptoBAC::ermEp* dptE dptF dptA	This study
pLT03	pStreptoBAC::dptE dptF ermEp* dptA	This study

dptA were identified by unique PCR products representing the new junctions that result from successful double crossovers. Positive candidate clones were streaked, and individual colonies were reconfirmed by PCR. A strain with the in-frame deletion of *dptA* was designated UA474, and one with the deletion of *dptA* and insertion of *ermEp** upstream of *dptBC* was designated UA475 (Fig. 1d).

The *dptA* complementation plasmids were constructed by λ Red-mediated recombination (Datsenko & Wanner, 2000) from pCV1, a

BAC clone with a 128 kb insert that contains the entire *dpt* gene cluster and flanking genes (Miao *et al.*, 2005). The regions flanking the *dptE*, *dptF* and *dptA* group ('*dptEFA*') were replaced with marker gene cassettes: the region upstream of *dptEFA* (*dpt*:552–45576) was replaced by *aadA1* (which confers resistance to spectinomycin, NCBI accession no. AP002527), and the region downstream of *dptA* (*dpt*:69106–127392) was replaced by *aph*(2") (which confers resistance to gentamicin: Kao *et al.*, 2000). The resulting plasmid, pLT01 (Fig. 2a), includes a very small fragment (*dpt*:1–551) from the 5' end of

Fig. 2. Complementing plasmids. Schematic diagrams of the three pLT plasmids. For pLT01 (a), the white arrow represents the remaining three ORFs (truncated *dptP*, *dptM* and *dptN*). In pLT02 (b) and pLT03 (c), the DNA region upstream of *dptE* has been removed. A ribosome-binding site introduced upstream of the start of *dptE* or *dptA* is shown in bold.

the original insert in pCV1 and a 23.2 kb fragment (*dpt*:45861–69105) that extends from inside *dptP* to within the 5' end of *dptBC*, at 49 nt downstream of the start codon for *dptBC*. The DNA region (3560 bp) upstream of the *dptEFA* group has been kept in the pLT01 construct as there was no evidence of an obvious promoter sequence immediately upstream of *dptE*.

Two derivatives were constructed from pLT01: pLT02 by introducing a fragment containing *ermEp** upstream of *dptE*, after nucleotide 49420, and pLT03 by introducing the *ermEp** fragment upstream of *dptA*, after nucleotide 51567. In pLT02 and pLT03, the introduction of *ermEp** deleted the region upstream of *dptE*, including the *aadA1* marker and 3.5 kb of *S. roseosporus* DNA (*dpt*:45861–49420); this was achieved by λ Red-mediated exchange with *tetA* (which confers resistance to tetracycline, NCBI accession no. J01830) (Fig. 2b, c). The pStreptoBAC V vector backbone derived from pCV1 (Miao *et al.*, 2005) includes sequences for apramycin resistance [*Am^R*; *aac(3)IV*], conjugative transfer (*oriT*) and plasmid integration (*int/attP*) (Bierman *et al.*, 1992) in the bacterial chromosome (Table 1).

Construction of recombinant strains, bioassays and quantification of lipopeptides. All complementation plasmids were introduced by conjugation from *E. coli* and integrated into the *S. roseosporus* chromosome. The pLT plasmids integrate at *attB^{CS1}*, while pRB04, the *dptD* complementation plasmid (Miao *et al.*, 2006b), integrates at *attB^{IS117}* (Table 1). Integrations at these loci are neutral with respect to the production of A21978C (D. Alexander, personal communication). Plasmid pRB04 was introduced into the Δ *dptA* Δ *dptD* hosts and hygromycin-resistant (*Hm^R*) exconjugants (the phenotype conferred by pRB04), were then used as recipients for the pLT plasmids. Since the latter conferred *Am^R*, *Hm^RAm^R* exconjugants were patched on AS-1 agar, grown for 5 days at 30 °C and then screened for antimicrobial activity using a nutrient soft agar overlay containing CaCl_2 (5 mM final) and *Staph. aureus* SA42. At least ten recombinants which produced zones of inhibition after 18 h at 37 °C were then fermented in F10A broth in shake flasks at 30 °C. After 5 days, aliquots of each culture were clarified by centrifugation and evaluated for A21978C lipopeptides, by HPLC and LC-MS (Miao *et al.*, 2005, 2006b). Bioassays were also conducted by placing 50 μ l clarified broth into 5 mm diameter wells cut in plates of AS-1 agar containing 5 mM CaCl_2 and *Staph. aureus*, and inspecting for zones of inhibition after incubating the plates overnight at 37 °C. For each group of strains, three siblings were fermented and analysed, and one representative strain from each set was re-fermented in triplicate to obtain accurate yields. Comparisons of yields between the different strains were made from fermentations carried out concurrently.

Extraction of RNA and RT-PCR. *S. roseosporus* UA343, an A21978C-producing strain, was grown in duplicate in 25 ml TSB in 125 ml baffled flasks at 30 °C and 250 r.p.m. After 4 days, the

mycelium was harvested by rapid filtration through Miracloth (Calbiochem) and immediately frozen by immersion into liquid nitrogen. The frozen mycelium was ground under liquid nitrogen and total RNA was extracted using the RNeasy Midi kit (Qiagen). The sample was treated twice with DNase I from the DNA-free kit (Ambion). The integrity of total RNA was assessed by gel electrophoresis to visualize 16S and 23S rRNA and from this, to infer the quality of the mRNA. Test PCRs supplemented with DMSO (Expand Long Template kit, Roche) were performed with primers P219 and P220 (5'-GTATTTCGACACACCCGACCG-3' and 5'-GAGGAGAGCTGTAGACCG-3'; V. Miao, unpublished) to amplify a 516 bp region of the *rpsL* gene (Hosted & Baltz, 1997) from *S. roseosporus* DNA (positive control) and DNase I-treated RNA using a 55 °C annealing temperature. The treated RNA was considered suitable for RT-PCR if there was no amplified *rpsL* fragment present in the PCR reaction.

RT-PCR was performed using 20–23 nt primers (Table 2) and the Superscript one-step RT-PCR kit with Platinum *Taq* (Invitrogen), supplemented with RNAGuard RNase Inhibitor (Amersham Pharmacia) and DMSO on 500 ng RNA, in a total volume of 20 μ l. The RT-PCR programme for all reactions was: 50 °C for 30 min, 94 °C for 2 min, followed by one cycle of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, followed by 29 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally, 72 °C for 5 min. Two independent RNA preparations were used and RT-PCR was repeated in three experiments.

RESULTS

Analysis of transcription across the contiguous *dpt* genes

In the *dpt* cluster, the core genes are oriented in the same direction. The NRPS genes are overlapped and *dptD* is followed by a 73 bp region, including a 26 bp, nearly perfect inverted repeat with potential for secondary structure ($\Delta G = -26 \text{ kcal mol}^{-1}$; 109 kJ mol^{-1}) that may be involved in transcription termination. The predicted start codon of *dptA* is separated from the end of *dptF* by only 15 nt, while *dptE* and *dptF* are separated by 68 nt. Given the small intergenic regions between the genes, it is possible that a promoter resides in the 397 nt intergenic region upstream of *dptE*, or even further upstream, and leads to transcription of the core genes on a single transcript.

To determine if the core *dpt* genes, as well as *dptG* and *dptH* genes downstream of *dptD*, are transcribed from a large

Table 2. Primers for RT-PCR

Region*	Intergenic space (bp)	FPC†	RPC†	PCR product (bp)	FPC distance from start codon of downstream gene
<i>dptE</i> – <i>dptF</i>	68	51109	51519	410	175
<i>dptF</i> – <i>dptA</i>	15	51286	51861	575	283
<i>dptA</i> – <i>dptBC</i>	0	68395	69123	728	663
<i>dptBC</i> – <i>dptD</i>	0	90617	91317	700	454
<i>dptBC</i> – <i>dptD</i>	0	90617	91761	1144	454
<i>dptD</i> – <i>dptG</i>	73	97808	98499	691	476
<i>dptG</i> – <i>dptH</i>	39	98288	99192	904	261

*Defined by an adjacent gene pair.

†FPC and RPC: 5' coordinate of forward (FPC) or reverse (RPC) primers derived from GenBank accession no. AY787762.

polycistronic mRNA, we designed primers to amplify mRNA across the boundaries of the genes (Table 2). RNA from 4-day-old cultures of *S. roseosporus* UA343 grown in TSB was tested and RT-PCR products of the sizes predicted for amplification across the junctions of adjacent genes, *dptE*–*dptF*, *dptF*–*dptA*, *dptA*–*dptBC*, *dptBC*–*dptD*, *dptD*–*dptG*, *dptG*–*dptH* were obtained (Fig. 3). This suggested that the contiguous genes from *dptE* to *dptH* may be transcribed on a polycistronic transcript, or on overlapping large transcripts.

S. roseosporus Δ *dptA* Δ *dptD* host strains

It was shown previously that the last NRPS gene, *dptD*, can be deleted [strain UA378 (*dptD*::*ermE*), Table 1] and expressed from the *ermEp** promoter when plasmid pRB04 is inserted at the *attB*^{S117} site; the recombinant produced ~90 % of the control A21978C factors (Miao *et al.*, 2006b). In light of the possibility that many of the *dpt* genes may be transcribed as a polycistronic message, the deletions of the upstream gene, *dptA*, were designed to be in-frame. The Δ *dptA* mutants were constructed by homologous recombination in UA378 using PCR to screen Am^S colonies

for double-crossover mutants. The frequency of in-frame deletion was 1–2 % of Am^S colonies, demonstrating that homologous double-crossover recombination is efficient in *S. roseosporus* (Hosted & Baltz, 1997). The resulting Δ *dptA* Δ *dptD* strain, UA474, confirmed by PCR, should allow any promoters upstream of *dptA* to direct the transcription of *dptBC* and any other genes normally co-transcribed with *dptBC* (Fig. 1d). The same method was used to construct UA475, a deletion strain with an insertion of *ermEp** upstream of *dptBC* (Δ *dptA* *ermEp**::*dptBC* Δ *dptD*), in addition to any native promoters (Fig. 1d). UA474 and UA475 were both confirmed for the loss of A21978C production in fermentation broths by microbiological and HPLC analyses.

Expression of complementation plasmids in *S. roseosporus*

Six combinations of Δ *dptA* Δ *dptD* deletions doubly complemented with *dptEFA* and *dptD* outside of the *dpt* gene cluster were constructed. First, pRB04, which contains the *dptD* gene fused to the *ermEp** promoter, was integrated at the *attB*^{S117} site in the chromosome of strains UA474 and UA475, resulting in strains MF6 and MF40, respectively (Table 1). The *dptEFA*-containing plasmids pLT01, pLT02 or pLT03 were then introduced at the *attB*^{C31} site in MF6 and MF40 to generate six genotypes that represent the daptomycin NRPS genes with different combinations of promoters expressed from three different sites in the chromosome (Table 1). All complementations appeared successful when assayed microbiologically (Fig. 4), except MF171. We studied at least three recombinants for each group of complementations, and found that all members of the same group had the same fermentation and production characteristics. Therefore, we present detailed data for one representative strain from each group.

Effects of the promoters on A21978C production

To assess the optimum conditions for ectopic *trans*-complementation of *dpt* genes, the three NRPS genes were

Fig. 3. Transcriptional analysis across *dpt* gene junctions by RT-PCR. Total RNA was extracted from strain UA343 after 4 days growth at 30 °C and used as template to amplify products ranging in size from 400 bp to 1100 bp using primers spanning two adjacent genes. The 1 kb Plus DNA marker (Invitrogen) is on the last lane on the right.

Fig. 4. Antibacterial activity of the recombinants in a well bioassay. Zones of inhibition caused by fermentation broths from strains carrying various pLT plasmids are shown in the middle and bottom rows as labelled. Controls are on the top row.

expressed in different locations of the chromosome from their native promoter(s) (*dptEFA* and *dptBC*), or from the strong constitutive promoter, *ermEp** (*dptEFA*, *dptA*, *dptBC* and *dptD*) in all combinations. The presence of A21978C

Fig. 5. A21978C lipopeptide production by recombinants. After centrifugation, culture broths were analysed on a Waters HPLC system. The mobile phase, buffered with 0.01 % trifluoroacetic acid and flowing at 1.5 ml min⁻¹, was initially held at 10 % aqueous acetonitrile for 2 min, followed by a linear gradient over 18 min to 90 % acetonitrile at ambient temperature. The chromatogram shows a typical HPLC profile after fermentation of recombinants with a retention time similar to the one obtained with native A21978C₁₋₃ (11.2, 11.6 and 12.0 min respectively, Miao *et al.*, 2006b). The triplet peaks represent A21978C₁₋₃ factors.

lipopeptides in the fermentation broths of the *trans*-complemented strains was confirmed by mass spectrometry and HPLC. Despite the engineered modifications, the distribution of the native factors was similar (Fig. 5) to that described previously (elution between 11 and 12 min) (Debono *et al.*, 1987). *S. roseosporus* MF54, which has native *dpt* promoter sequences for *dptEFA* and *dptBC*, produced about 200 mg l⁻¹ A21978C factors, or 58 % of control (Table 3). *S. roseosporus* MF77 and MF85, which have *ermEp** upstream of *dptEFA* or *dptA*, respectively (Fig. 2b, c), produced 273 and 267 mg l⁻¹ A21978C factors, or 80 % and 79 % of control. Thus, the presence of *ermEp** in the *dptA* complementation plasmids clearly caused improved production relative to *dptA* expressed from a native promoter(s) (Table 3).

S. roseosporus MF171, which has *dptA* expressed from its native promoter(s) and *dptBC* expressed from *ermEp**, produced only 32 mg l⁻¹ of A21978C factors, or 9 % of control (Table 3). However, when *dptA* and *dptBC* were expressed individually from *ermEp** (MF110), the yields improved to 171 mg l⁻¹, or 50 % of control. Thus, of the six combinations tested, the best expression was obtained when the two ectopically expressed genes, *dptA* and *dptD*, were expressed from *ermEp**, and the *dptBC* gene, located in the original *dpt* locus, was expressed from its native promoter(s). There was no observable consequence of the positioning of *ermEp** in front of *dptEFA* or *dptA* genes on the levels of production of A21978C factors.

Heterologous *trans*-complementation to produce hybrid lipopeptides

In a previous study (Miao *et al.*, 2006b), the heterologous complementation of *dptD* by the *lptD* or the *cdaPS3* genes, driven by *ermEp** and inserted into the *attB*^{IS117} site, using plasmids pMF30 and pMF26, produced yields of hybrid molecules of 25 % and 50 %, respectively.

Having established robust production of A21978C factors in strains containing *dptA* and *dptD* inserted in ectopic positions of the chromosome under control of the *ermEp** promoter, we investigated the heterologous expression of *lptD* and *cdaPS3* in a strain that was deleted for *dptD*, but that has *dptA* expressing ectopically from *ermEp**, and *dptBC* expressing from its native promoter. MF125, containing the cloned *lptD* gene from the A54145 gene cluster (Miao *et al.*, 2006a) inserted into the *attB*^{IS117} site, produced 135 mg l⁻¹ or 40 % of the control, of the hybrid lipopeptides containing a mixture of Ile and Val at position 13, as previously described in a *dptD* deletion mutant (Miao *et al.*, 2006b) (Table 3). The HPLC profile was similar to the one obtained for the native and engineered A21978C factors, with retention times ranging between 11 and 12 min. MF193, containing the cloned *cdaPS3* inserted in the *attB*^{IS117} site, produced 235 mg l⁻¹ or 69 % of the control, of the hybrid lipopeptide containing Trp in position 13 (Table 3). Those yields are superior to the yields observed previously in the *dptD* single complementation (Miao *et al.*,

Table 3. Comparisons of the production yields of daptomycin analogues*

*The promoters used to drive expression (e.g. *dptA*-p) were either the natural promoter(s) from the *dpt* gene cluster (*Nat*) or the *ermEp** promoter (*Erm*). For expression of *dptA*, the *ermEp** promoter was inserted in front of *dptA* (*Erm-A*) or in front of *dptE* (*Erm-EFA*).

Strain	<i>dptA</i> -p	<i>dptBC</i> -p	<i>dptD</i> -p	<i>lptD</i> -p	<i>cdaPS3</i> -p	Lipopeptide (mg l ⁻¹)	Percentage of control
MF54	<i>Nat</i>	<i>Nat</i>	<i>Erm</i>	–	–	196 ± 42	58
MF77	<i>Erm-EFA</i>	<i>Nat</i>	<i>Erm</i>	–	–	273 ± 24	80
MF85	<i>Erm-A</i>	<i>Nat</i>	<i>Erm</i>	–	–	267 ± 35	79
MF171	<i>Nat</i>	<i>Erm</i>	<i>Erm</i>	–	–	32 ± 3	9
MF110	<i>Erm-EFA</i>	<i>Erm</i>	<i>Erm</i>	–	–	171 ± 11	50
MF101	<i>Erm-A</i>	<i>Erm</i>	<i>Erm</i>	–	–	169 ± 6	50
MF159	<i>Nat</i>	<i>Nat</i>	–	<i>Erm</i>	–	94 ± 21	28
MF118	<i>Erm-EFA</i>	<i>Nat</i>	–	<i>Erm</i>	–	120 ± 12	35
MF125	<i>Erm-A</i>	<i>Nat</i>	–	<i>Erm</i>	–	135 ± 6	40
MF202	<i>Nat</i>	<i>Erm</i>	–	<i>Erm</i>	–	38 ± 4	11
MF133	<i>Erm-EFA</i>	<i>Erm</i>	–	<i>Erm</i>	–	51 ± 9	15
MF141	<i>Erm-A</i>	<i>Erm</i>	–	<i>Erm</i>	–	64 ± 12	19
MF63	<i>Nat</i>	<i>Nat</i>	–	–	<i>Erm</i>	168 ± 11	49
MF191	<i>Erm-EFA</i>	<i>Nat</i>	–	–	<i>Erm</i>	121 ± 37	36
MF193	<i>Erm-A</i>	<i>Nat</i>	–	–	<i>Erm</i>	235 ± 29	69
MF214	<i>Nat</i>	<i>Erm</i>	–	–	<i>Erm</i>	33 ± 6	10
UA117	<i>Nat</i>	<i>Nat</i>	<i>Nat</i>	–	–	340 ± 4	100

2006b), demonstrating a positive effect of the *ermEp** driving the expression of *dptA*.

S. roseosporus MF202, which has *dptA* expressed from its native promoter(s), *dptBC* and *lptD* expressed from *ermEp**, produced only 38 mg l⁻¹ of hybrid lipopeptides, or 11 % of control (Table 3). When *dptA* and *dptBC* were expressed individually from *ermEp** (MF141), the yields improved slightly to 64 mg l⁻¹, or 19 % of control. Similarly, MF214, where *dptA* expressed from its native promoter(s), *dptBC* and *cdaPS3* expressed from *ermEp**, produced 33 mg l⁻¹ of hybrid lipopeptide, or 10 % of control (Table 3). No exconjugants were obtained when *dptBC* and *dptEFA* or *dptA* were driven by *ermEp**. Thus, as previously shown for *dptA* and *dptD* double homologous complementation, the best expression was obtained when *dptA*, and *lptD* or *cdaPS3* were expressed from *ermEp** and the *dptBC* gene was expressed from its native promoter at its native locus (Table 3).

DISCUSSION

It has been shown previously that a $\Delta dptD$ deletion mutation in *S. roseosporus* can be complemented *in trans* with a *dptD* gene under the control of *ermEp** (Miao *et al.*, 2006b). The $\Delta dptD$ gene was also complemented with the heterologous genes *lptD* from the A54145 pathway (Miao *et al.*, 2006a) and *cdaPS3* from the *S. coelicolor* pathway (Hojati *et al.*, 2002), both under control of *ermEp**. In this study, we have further explored this NRPS expression system and reconstituted the daptomycin pathway by *in trans* complementation of two NRPS subunits from ectopic loci in the *S. roseosporus* chromosome. We demonstrated that it is possible to have robust production of A21978C when genes

encoding DptBC, DptE and DptF proteins are expressed from the native locus, while those for subunits DptA and DptD are expressed from other chromosomal sites.

The organization of the daptomycin gene cluster was a consideration during design of the double *trans*-complementation, since deletion of *dptA* might disrupt transcription of the *dptBC* gene downstream. Large polycistronic transcripts have been found in some secondary metabolic pathways: a transcript greater than 16 kb, including six cephamycin C biosynthetic genes, is driven from the *pcbAB* promoter in *Nocardia lactamdurans* (Enguita *et al.*, 1998) and a transcript of 35 kb, including seven erythromycin biosynthetic genes, is generated from the *eryAI* promoter in *Saccharopolyspora erythraea* (Reeves *et al.*, 1999). The successful RT-PCR amplification across adjacent *dpt* genes from UA343 (*dptE*–*dptF*, *dptF*–*dptA*, *dptA*–*dptBC*, *dptBC*–*dptD*, *dptD*–*dptG*, *dptG*–*dptH*) allows the possibility of a single transcriptional unit. The forward primers were situated well upstream of the initiation codon of the downstream gene to exclude the possibility of encompassing untranslated leader sequences. In transcripts from other secondary metabolite biosynthetic pathways, there may be little or no leader (Reeves *et al.*, 1999), although transcription starting 133–180 nt upstream of the translation start has been reported, and in one case 69–70 nt were inside the 3' end of the gene upstream (Enguita *et al.*, 1998). The forward primer for the *dptA*–*dptBC* junction is over 600 nt upstream of the presumed initiation codon of *dptBC*, making capture of a product representing a leader sequence highly unlikely. Purine-rich sequences 5–7 nt upstream of the presumed translational starts of the terminally overlapping NRPS genes that may facilitate ribosome binding

leave open the possibility of translational coupling of a large polycistronic transcript for the entire 48.8 kb region. This type of organization and possible translational coupling has been described in the tylosin biosynthetic gene cluster (Cundliffe *et al.*, 2001).

Our model of a promoter upstream of *dptE* regulating transcription of a large polycistronic message, including the NRPS genes and other genes downstream, guided construction of the deletion host/complementation plasmid combinations. In the host UA474 and its derivative, MF6, the deletion of *dptA* leaves a small open reading frame to maintain the natural terminal overlap between *dptA* and *dptBC*. Restoration of A21978C production in MF6 after complementation, with the *dptEFA* genes outside the daptomycin gene cluster, showed that *dptBC* did not have a unique promoter within the deleted region of *dptA*, and further suggests that *dptBC* is regulated by a promoter upstream, either within *dptF*, *dptE* or further upstream of *dptE*: in an intact gene cluster, this promoter would also determine transcription of *dptA* in addition to *dptE*, *dptF* and *dptBC*. The functional expression of *dptA* from pLT01, which lacks an added promoter but contains *dptE*, *dptF* and three genes upstream (truncated *dptP*, *dptM* and *dptN*), is consistent with the presence of a native promoter upstream of *dptA*. While the possibilities of additional or secondary promoters are not excluded, the current observations showed that expression of the genes remaining at the native locus, *dptE*, *dptF* and *dptBC*, can be complemented by ectopic expression of *dptEFA* and *dptD* to restore antibiotic production. Similarly, in *Bacillus subtilis* (Guenzi *et al.*, 1998), the physical dissociation of the thioesterase from the last amino acid of the surfactin synthetase did not affect the level of expression of the lipopeptides.

We also investigated the impact of different promoters on lipopeptide production. The lipopeptide yield with *dptA* expressed *in trans* from a native promoter, *dptBC* from its native locus from a native promoter, and *dptD* *in trans* from *ermEp** was about 58 % of control (Table 3). In previous work (Miao *et al.*, 2006b), complementation of a single Δ *dptD* mutation by *dptD* *in trans* from *ermEp** yielded about 90 % of control. However, when both *dptA* and *dptD* were expressed ectopically from *ermEp**, and *dptBC* from a native promoter at the native locus, the yield was improved to 80 % of control; equivalent results were obtained by positioning *ermEp** in front of *dptEFA* or *dptA* (Table 3).

Given the excellent yields associated with *ermEp** regulation of *dptA* and *dptD*, it was surprising that when the *dptBC* gene at the native locus is regulated by *ermEp**, and *dptA* was expressed from a native promoter, the lipopeptide production was only 9 % of control. It is possible that the overproduction of the DptBC subunit from the native locus adversely affected the stoichiometry of the three subunits, or the expression of other genes.

The results in this study demonstrate that the *dpt* NRPS genes need not be expressed from the native *dpt* locus to

obtain high yields of A21978C factors. The *dptA* and *dptD* genes can be conveniently expressed from the *ermEp** promoter from ectopic positions in the chromosome. Using this configuration, we showed that *dptD* could be replaced by the heterologous *lptD* and *cdaPS3* to produce daptomycin analogues with substitutions at position 13 at 40 % and 69 % of control yields, respectively. This compares favourably with the constructs containing *dptA* (and all other *dpt* genes except *dptD*) present in the native *dpt* locus, where recombinants with *lptD* and *cdaPS3* produced 25 % and 50 % of control (Miao *et al.*, 2006b). The apparent improvement in yields may be due to the presence of *ermEp** driving the expression of *dptA*. This cloning system is now well suited to explore module exchanges in *dptA*, coupled with subunit exchanges for *dptD* to generate additional derivatives of daptomycin for evaluation. The demonstration of robust ectopic expression of two NRPS subunits in daptomycin biosynthesis, using *ermEp** to drive transcription, also suggests that ectopic expression driven by strong constitutive promoters might also work in other multi-modular, multi-subunit NRPS biosynthetic pathways to engineer peptide or mixed peptide/polyketide derivatives not readily amenable to chemical synthesis or scale up.

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