Non-ribosomal peptide synthetase module fusions to produce derivatives of daptomycin in *Streptomyces roseosporus*

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Genetic engineering has been applied to reprogramme non-ribosomal peptide synthetases (NRPSs) to produce novel antibiotics, but little is known about what determines the efficiency of production. We explored module exchanges at nucleotide sequences encoding interpeptide linkers in *dptD*, a gene encoding a di-modular NRPS subunit that incorporates 3-methylglutamic acid (3mGlu₁₂) and kynurenine (Kyn₁₃) into daptomycin. Mutations causing amino acid substitutions, deletions or insertions in the inter-module linker had no negative effects on lipopeptide yields. Hybrid DptD subunits were generated by fusing the 3mGlu₁₂ module to terminal modules from calcium-dependent antibiotic (CDA) or A54145 NRPSs, and recombinants produced daptomycin analogues with Trp₁₃ or Ile₁₃ at high efficiencies. A recombinant expressing DptD with a hybrid Kyn₁₃ module containing a di-domain from a D-Asn module caused the production of a new daptomycin analogue containing Asn₁₃.

INTRODUCTION

Streptomyces roseosporus NRRL 15998 produces the A21978C complex of acidic lipopeptides of the daptomycin family, which consist of at least six factors with different fatty acids attached to the amino group of L-Trp₁ of the peptide (Baltz *et al.*, 2005; Debono *et al.*, 1987). (The subscripts refer to positions of amino acids in the peptide, starting with L-Trp₁, as discussed below and in Fig. 1b.) The three major fermentation components of the A21978C complex have 11-, 12- and 13-carbon branched-chain fatty acids, whereas the minor factor daptomycin, which can be enriched by feeding decanoic acid during fermentation (Baltz *et al.*, 2005; Huber *et al.*, 1988), has a 10-carbon straight chain (Fig. 1a). Daptomycin has been approved as Cubicin[®] (daptomycin-for-injection) for treatment of skin and skin structure infections caused by Gram-positive pathogens (Arbeit *et al.*, 2004), and for bacteraemia and endocarditis caused by *Staphylococcus aureus*, including strains resistant to methicillin (MRSA) (Fowler *et al.*, 2006).

Abbreviations: A, adenylation; C, condensation; CDA, calcium-dependent antibiotic; E, epimerization; ESI, electrospray ionization; D-hAsn, D-hydroxyasparagine; D-hPhg, D-hydroxyphenylglycine; HR-MS, high resolution mass spectrometry; Kyn, kynurenine; LC-MS, liquid chromatography-mass spectrometry; 3mGlu, 3-methylglutamic acid; mOAsp, methoxyaspartic acid; NRPS, non-ribosomal peptide synthetase; PCP, peptidyl carrier protein; Sar, sarcosine; T, thiolation; Te, thioesterase.

A supplementary table of primers is available with the online version of this paper.

The daptomycin biosynthetic gene cluster was cloned and sequenced, and the non-ribosomal peptide synthetase (NRPS) and other genes identified (Miao *et al.*, 2005; Fig. 1b). The cyclic, branched, 13-membered lipopeptide backbone of daptomycin is produced by three giant NRPS multi-enzymes, DptA, DptBC and DptD, encoded by the *dptA*, *dptBC* and *dptD* genes, respectively. NRPSs are composed of conserved repeating units (modules) each with an average size of 120 kDa (Sieber & Marahiel, 2005). Each module catalyses the incorporation of one specific amino acid in a sequence that is collinear with the modular organization in the DNA coding sequences. A typical module has three catalytic domains, responsible for peptide bond formation (condensation domain, C; Stachelhaus *et al.*, 1998), recognition and activation of substrate amino acids (adenylation domain, A; Stachelhaus *et al.*, 1999), and translocation (thiolation domain, T; or peptidyl carrier protein, PCP; Stachelhaus *et al.*, 1996). Modules may also contain accessory domains, such as epimerization (E) domains that catalyse the conversion of L-amino acids to D-isomers (Stachelhaus & Walsh, 2000). The terminal modules of NRPSs often have thioesterase (Te) domains involved in cleavage of the nascent peptide, releasing linear or cyclic peptides, or cyclic depsipeptides (Kohli & Walsh, 2003). Such modules are arranged as C-A-T-Te, and individual enzyme domains are connected by linker peptides.

Because of their collinear gene/protein module organization, NRPSs present promising targets for combinatorial

Fig. 1. Structures of lipopeptide antibiotics and organization of NRPS genes. (a) Chemical structures of daptomycin, A54145 (factor B1) and CDA. (b) Organization of the NRPS gene clusters of daptomycin, A54145 and CDA. The *dptA*, *dptBC* and *dptD* genes encode multi-modular NRPS enzymes, DptA, DptBC and DptD, that sequentially incorporate L-Trp₁-D-Asn₂-L-Asp₃-L-Thr₄-Gly₅, L-Orn₆-L-Asp₇-D-Ala₈-L-Asp₉-Gly₁₀-D-Ser₁₁ and L-3mGlu₁₂-L-Kyn₁₃, respectively. The *lptA*, *lptB*, *lptC* and *lptD* genes encode LptA, LptB, LptC and DptD that incorporate L-Trp₁-D-Glu₂-L-Asn₃-L-Thr₄-Sar₅, L-Ala₆-L-Asp₇, D-Lys₈-L-mOAsp₉-Gly₁₀-D-Asn₁₁ and L-3mGlu₁₂-L-Ile₁₃, respectively. The *cdaPS1*, *cdaPS2* and *cdaPS3* genes encode CdaPS1, CdaPS2 and CdaPS3 that incorporate L-Ser₁-L-Thr₂-D-Trp₃-L-Asp₄-L-Asp₅-D-hPhg₆, L-Asp₇-Gly₈-D-hAsn₉ and L-3mGlu₁₀-L-Trp₁₁, respectively. (See Baltz, 2008; Baltz *et al.*, 2005.)

biosynthesis (Baltz, 2008; Baltz *et al.*, 2005; Fischbach & Walsh, 2006; Sieber & Marahiel, 2005). Module exchanges in NRPS model systems have proven to be useful to generate peptides of a targeted sequence (Doekel & Marahiel, 2000; Mootz *et al.*, 2000), and several novel derivatives of surfactin have been produced by the targeted replacement of modules (Mootz *et al.*, 2002; Stachelhaus *et al.*, 1995; Yakimov *et al.*, 2000). In other *in vivo* studies, the specificity-conferring codes in the active sites of A domains (Stachelhaus *et al.*, 1999; Challis *et al.*, 2000) have been exploited to redirect surfactin biosynthesis by site-specific mutagenesis (Eppelmann *et al.*, 2002). Biochemical investigations on NRPSs have helped define rules of substrate specificity and promiscuity, and to define inter-domain linkers and inter-peptide docking domains, thereby providing tools to guide the engineering of NRPS biosynthetic genes to produce novel peptides *in vivo* (Fischbach & Walsh, 2006; Sieber & Marahiel, 2005).

The enzymic domains of NRPSs are connected by peptide linkers that have no apparent sequence conservation. The

linkers have been used *in vitro* (Doekel & Marahiel, 2000; Mootz *et al.*, 2000) and *in vivo* (Stachelhaus *et al.*, 1995; Nguyen *et al.*, 2006b) to engineer modular NRPSs by module and domain exchanges. Production levels of novel lipopeptides related to daptomycin, engineered by single module exchanges, ranged from 17 to 50 % of controls (Nguyen *et al.*, 2006b).

The functions of NRPS inter-domain linkers have not been extensively explored, although there has been speculation about a role in initiation of protein-protein interactions (Shen *et al.*, 2002). The 18 aa linker connecting the T (or PCP) and downstream C domains in a T-C di-domain of the tyrocidine synthetase TycC apparently exhibits considerable conformational flexibility, and the final seven amino acids lack defined interactions with either domain and are mobile in the PCP-C crystal structure (Samel *et al.*, 2007). The flexibility in this linker reflects the requirement for the T (PCP) to interact with the A domain and the preceding and following C domains to function in peptide assembly.

Daptomycin production can be restored in a *dptD* deletion mutant of *S. roseosporus* ($\Delta dptD$) with *dptD* expressed under the transcriptional control of the *ermEp** promoter by *trans*-complementation at an ectopic site in the chromosome (Coëffet-Le Gal *et al.*, 2006; Miao *et al.*, 2006b). This $\Delta dptD$ mutation can also be complemented by the genes *lptD* and *cdaPS3*, encoding the terminal subunits from the distantly related lipopeptide biosynthetic pathways of A54145 and calcium-dependent antibiotic (CDA), respectively, to produce novel hybrid lipopeptides (Baltz, 2008; Coëffet-Le Gal *et al.*, 2006; Miao *et al.*, 2006b; Nguyen *et al.*, 2006b). The analogues contained Trp or Ile/Val in place of Kyn₁₃ when subunits CdaPS3 (Hojati *et al.*, 2002) or LptD (Miao *et al.*, 2006a) were exchanged for the native DptD (Fig. 1a). This work indicated that the Te domains from the CdaPS3 and LptD subunits were able to cyclize heterologous peptides differing substantially from the native CDA and A54145 peptides, consistent with *in vitro* chemoenzymic studies with excised T-Te di-domains that generated lipopeptides related to daptomycin (Grünewald *et al.*, 2004; Kopp *et al.*, 2006). In the *in vivo* studies, the recombinants produced daptomycin derivatives containing Trp₁₃ or Ile₁₃/Val₁₃ in yields of about 50 % and 25 % of control, respectively (Coëffet-Le Gal *et al.*, 2006; Miao *et al.*, 2006b). The reduction in yields might be due to suboptimal interpeptide docking, substrate recognition or ring closure.

This heterologous whole-subunit *trans*-complementation system for combinatorial biosynthesis of daptomycin analogues provides a means to test parameters important for high productivity of engineered NRPSs. In particular, it can be used to explore the efficiency of producing hybrid molecules by fusing modules or exchanging domains from di-modular DptD, LptD and CdaPS3 subunits to engineer positions 12 and 13. This also has a practical advantage in that positions 12 and 13 are important determinants for antibacterial activity (Coëffet-Le Gal *et al.*, 2006; Miao *et al.*, 2006b; Nguyen *et al.*, 2006a).

In the present study, we generated module fusions at T-C linkers, and showed that the linker region can be modified by amino acid substitutions, and by deletion or insertion of four amino acids, without disrupting lipopeptide production. We also generated a functional domain exchange by fusing a heterologous C-A di-domain to the T-Te di-domain of DptD at the A-T linker, and the recombinant produced a novel derivative of daptomycin with Asn₁₃.

METHODS

Strains, growth conditions and conjugal transfer of plasmids.

Strains and plasmids are listed in Table 1. *S. roseosporus* was grown on trypticase soy (TS) agar or in TS broth at 30 °C. AS-1 agar and fermentation medium F10A were described previously (Miao *et al.*, 2006b). Plasmids were transferred from *Escherichia coli* ML22 into *S. roseosporus* UA378 ($\Delta dptD$) by conjugation (Miao *et al.*, 2006b). Exconjugants were selected for hygromycin resistance at 50 µg ml⁻¹ on AS-1 agar.

Antibiotic production, purification and assay. *S. roseosporus* strains containing recombinant plasmids were fermented in triplicate for 5 days as described previously (Miao *et al.*, 2006b) and supernatants of fermentation broths were tested for antibacterial activity. Samples (100 µl) were added to wells (5 mm diameter) embedded in AS-1 agar plates supplemented with 10 mM CaCl₂ and *Staphylococcus aureus* 42 (10⁵ c.f.u. ml⁻¹). After incubation at 37 °C overnight, the plates were inspected for zones of inhibition, and MICs were determined (Miao *et al.*, 2006b). Lipopeptides were quantified from broth cultures by HPLC and LC-MS using daptomycin as the standard, and were purified as described by Nguyen *et al.* (2006a).

Plasmid constructions. Expression plasmids for engineered *dptD* genes were constructed based on pRB04, a pHM11a-derived plasmid in which the *dptD* gene is expressed from the strong, constitutive *ermEp** promoter (Miao *et al.*, 2006b). The coding sequence of *dptD* was cloned by inserting a *BsrGI/BamHI* (blunted) fragment (primers CB312 and CB313). Primer CB313 (see Supplementary Table S1, available with the online version of this paper) contained artificial *AvrII* and *PmeI* sites that were positioned in the A-site of the T-C linker of *dptD* as shown in Fig. 2(b), giving rise to plasmid pCB301. This cloning step was repeated using primer pairs CB312/CB314 and CB312/CB315, respectively, to shift the *AvrII* site downstream to the B and C sites of the T-C linker (plasmids pCB302 and pCB303, respectively). Plasmid pCB302 was used to ligate in-frame an *AvrII/HpaI* fragment comprising the terminal modules (CATTe) for *dptD2* (Kyn), *lptD2* (Ile) and *cdaPS3-2* (Trp) (primer pairs CB323/CB325, CB327/CB329 and CB330/CB331) generating plasmids pCB304, pCB305 and pCB306, respectively. Plasmids pCB301 and pCB303 were used for cloning a *dptD2* fragment with varying 5' terminal ends to accommodate A and C sites of the T-C linker, generating plasmids pCB307 and pCB308 (using primer pairs CB322/CB325 for the A site and CB324/CB325 for the C-site, respectively). Plasmids pCB309 and pCB310 were constructed as above, but by switching the inserts to generate a deletion or insertion of 12 nt in the linker coding sequences (Table 1). C-A di-domains for *dptD2* and *lptC4* were amplified using primer pairs CB323/CB354 and CB355/CB356, respectively, and cloned into pCB302. CAT modules and C-A di-domains were generated using λ Red-mediated recombination (Datsenko & Wanner, 2000; Nguyen *et al.*, 2006b; Zhang *et al.*, 1998) in *E. coli* DH10B(pKD78) using the following primers: CB346/CB347 for *dptD2*(CAT), CB346/CB357 for *dptD2*(CA), CB352/CB353 for *lptC4*(CAT) and CB352/CB358 for *lptC4*(CA). The Te domain from *dptD* was amplified using the primers CB332 and CB333 and subcloned along with *spec* (amplified with CB357/CB358) using *PmeI* and *HindIII* to form the Te/*spec* cassette. This cassette was excised and cloned into *HpaI/HindIII* sites in conjunction with the CAT modules of Kyn and Asn, respectively, to give pCB401 (Kyn) and pCB407 (Asn). The T-Te di-domain from *dptD* was amplified using the primers CB359 and CB333 and subcloned along with *spec* (amplified with CB357/CB358) using *PmeI* and *HindIII* to form the TTe/*spec* cassette. This cassette was excised and cloned into *HpaI/HindIII* sites in conjunction with the C-A di-domains of Kyn and Asn, respectively, to give pCB409 (Kyn) and pCB410 (Asn). Cloning junctions were sequenced for confirmation.

RESULTS

Module fusions and domain exchanges in NRPS subunit DptD

Previous successful whole-subunit *trans*-complementation studies, using the di-modular DptD, and distantly related LptC and CdaPS3 (Baltz, 2008; Coëffet-Le Gal *et al.*, 2006;

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
<i>S. roseosporus</i>		
UA343	A21978C producer	NRRL 15998
UA117	<i>rpsL7</i> (Sm ^r)	Miao <i>et al.</i> (2006b)
UA378	$\Delta dptD::ermE$ <i>rpsL7</i>	Miao <i>et al.</i> (2006b)
SD301	UA378::pCB301	This study
SD302	UA378::pCB302	This study
SD303	UA378::pCB303	This study
SD304	UA378::pCB304	This study
SD305	UA378::pCB305	This study
SD306	UA378::pCB306	This study
SD307	UA378::pCB307	This study
SD308	UA378::pCB308	This study
SD309	UA378::pCB309	This study
SD310	UA378::pCB310	This study
SD401	UA378::pCB401	This study
SD407	UA378::pCB407	This study
SD409	UA378::pCB409	This study
SD410	UA378::pCB410	This study
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>endA1</i> <i>araD139</i> $\Delta(ara, leu)7697$ <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> (Sm ^R) <i>nupG</i>	Invitrogen
ML22	DH10B::pUZ8002	Coëffet-Le Gal <i>et al.</i> (2006)
Plasmids		
BW25113	<i>lacI</i> ^q <i>rrnB</i> _{T14} $\Delta lacZ_{WJ16}$ <i>hsdR514</i> $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	Datsenko & Wanner (2000)
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:: <i>dptD</i>	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)
pUZ8002	RK2 derivative defective in <i>oriT</i>	Coëffet-Le Gal <i>et al.</i> (2006)
pCB301	pHM11a:: <i>dptD1</i> <i>AvrII</i> 81172 (A site) $\Delta dptD2$	This study
pCB302	pHM11a:: <i>dptD1</i> <i>AvrII</i> 81178 (B site) $\Delta dptD2$	This study
pCB303	pHM11a:: <i>dptD1</i> <i>AvrII</i> 81184 (C site) $\Delta dptD2$	This study
pCB304	pCB302 ($\Delta dptD2$ B site):: <i>dptD2</i> (CATTe)	This study
pCB305	pCB302 ($\Delta dptD2$ B site):: <i>lptD2</i> (CATTe)	This study
pCB306	pCB302 ($\Delta dptD2$ B site):: <i>cdaPS3-2</i> (CATTe)	This study
pCB307	pCB301 ($\Delta dptD2$ A site):: <i>dptD2</i> (CATTe)	This study
pCB308	pCB303 ($\Delta dptD2$ C site):: <i>dptD2</i> (CATTe)	This study
pCB309	pCB301 ($\Delta dptD2$ C site):: <i>dptD2</i> (CATTe) $\Delta 81172-81184$	This study
pCB310	pCB303 ($\Delta dptD2$ A site):: <i>dptD2</i> (CATTe) +81172–81184	This study
pCB401	pCB302:: <i>dptD2</i> (CAT):: <i>dptD2</i> (Te)	This study
pCB407	pCB302:: <i>lptC4</i> (CAT):: <i>dptD2</i> (Te)	This study
pCB409	pCB302:: <i>dptD2</i> (CA):: <i>dptD2</i> (TTe)	This study
pCB410	pCB302:: <i>lptC4</i> (CA):: <i>dptD2</i> (TTe)	This study

†BAC, bacterial artificial chromosome.

Miao *et al.*, 2006b), suggested a relatively simple test system to explore the requirements for functional module and domain exchanges. The di-modular *dptD*, *lptD* and *cdaPS3* genes can be split into individual modules, and ligated in different combinations to produce hybrid di-modules; these can be tested for efficiency of *trans*-complementation of a *S. roseosporus* $\Delta dptD$ mutant. In this way, the interpeptide docking sequences can be kept constant by using the 3mGlu₁₂ module from *dptD*, and fusing it in

different ways to the Kyn₁₃, Ile₁₃ and Trp₁₁ modules from the *dptD*, *lptD* and *cdaPS3* genes, respectively. The efficiencies of lipopeptide production can be compared to the results of whole-subunit exchanges carried out previously. In describing the current experiments, we represent inter-domain linker regions as hyphens (e.g. C-A-T-C-A-T-Te for DptD and related di-modules), and use :: to represent fusion sites (e.g. C-A-T::C-A-T-Te or CAT::CATTe for fusion at the T-C linker).

cete lipopeptide linkers in Fig. 2(a) may span 20–22 aa, providing additional sites for module fusions.

Heterologous module fusions in DptD

Module fusions were generated to link the DptD module for 3mGlu₁₂ to the terminal modules for Trp₁₁ and Ile₁₃ from CDA and A54145 NRPSs, respectively (Fig. 1). The substrate specificities of these modules redirect the synthesis of daptomycin to incorporate Trp and Ile/Val, respectively, in place of Kyn₁₃. Both modules were introduced with their C-terminal Te domains in the CAT::CATTe fusions using the splice site B in the T-C linker (Fig. 2b and Fig. 3). These hybrid subunits restored lipopeptide biosynthesis in strain UA378 deleted for *dptD* at yields similar to those produced by recombinants reconstructed to produce daptomycin (Table 2). LC-MS analysis of fermentation broths detected the mass ions indicative of hybrid compounds with Trp₁₃ (1630, 1644 and 1658) and Ile₁₃ (1543, 1557, 1571 and 1585), respectively (Table 3). The mass ions (1529, 1543, 1557 and 1571) indicative of compounds containing Val₁₃ were also detected in lower abundance, consistent with the fact that LptD can also incorporate Val at lower efficiency than Ile (Baltz *et al.*, 2005; Miao *et al.*, 2006b). Similar results were obtained when the splice site C was used to generate hybrid DptD subunits (not shown).

Domain exchanges in DptD and the role of native TTe linkage

Although exchanges of the complete CATTe module of the daptomycin NRPS with similar CATTe modules from the

CDA and A54145 pathways were successful, the availability of modules containing Te domains is limited. It would be useful to be able to generate hybrid CATTe modules by excising CA or CAT from CAT or CATE modules, and ligating at A-T or T-Te linkers (Fig. 3). To test this, we chose the CATE module for D-Asn₁₁ from *lptC* (Miao *et al.*, 2006a; Fig. 1) to generate C-A-T::Te and C-A::T-Te fusions (Table 3, Fig. 3). As controls, similar homologous fusions were made to reconstruct the Kyn₁₃ module (strains SD401 and SD409; Table 1). These plasmids containing the hybrid *dptD* genes were introduced into *S. roseosporus* UA378 and fermentations of recombinant strains were carried out. The strains expressing the reconstituted Kyn₁₃ CAT::Te and CA::TTe fusions produced the predicted lipopeptides. The recombinant containing the Asn₁₃ CAT::Te fusion produced no detectable lipopeptides, but the strain with the CA::TTe fusion produced lipopeptides with ESI mass ions of 1558, 1572 and 1586 and UV maximal absorptions at 220 and 281 nm, as expected for the three novel compounds containing A21978C₁₋₃(Asn₁₃), at yields of about 36–60 mg l⁻¹. As a representative example, the structure of A21978C₁(Asn₁₃) was supported by its HR-MS data at *m/z* 1558.7042 [M+H]⁺. Furthermore, analysis of LC-MS/MS data revealed that the Asn residue was located in the depsipeptide ring, as expected (Fig. 4).

Biological activities of hybrid peptides

While most of the daptomycin derivatives were very active against *Staph. aureus* 42, they were slightly less potent than daptomycin. The MIC of daptomycin against *Staph. aureus* 42 is 0.5–1 µg ml⁻¹, whereas the MICs of the daptomycin

Fig. 3. Inter-domain linkers in NRPSs. Domain organization of DptD, LptD and CdaPS3 (CAT-CATTe). Alignments of the A-T linker, the T-C linker and the T-Te linker regions. Proposed linkers are boxed.

Table 3. Production of lipopeptides by recombinants containing reconstructed or hybrid *dptD* genes

The module and domain fusions of daptomycin NRPS using domains from *dptD* (Kyn), *lptD* (Ile), *cdaPS3* (Trp) and *lptC* (Asn) were constructed as described in Methods and in Table 1. The fusion sites are marked by ::. Fermentation broths of recombinants carrying plasmids encoding hybrid subunits were analysed by LC-MS. The heterologous domains from the A54145 and CDA pathways are italicized.

Strain	Fusion scheme	Predicted mass ions	Mass ions detected
SD304	CAT::CA _{Kyn} Te	1634, 1648, 1662	+
SD305	CAT::CA _{Ile} Te	1543, 1557, 1571, 1585	+
SD306	CAT::CA _{Trp} Te	1630, 1644, 1658	+
SD401	CAT::CA _{Kyn} T::Te	1634, 1648, 1662	+
SD407	CAT::CA _{Asn} T::Te	1558, 1572, 1586	–
SD409	CAT::CA _{Kyn} ::TTe	1634, 1648, 1662	+
SD410	CAT::CA _{Asn} ::TTe	1558, 1572, 1586	+

derivatives were 1 µg ml^{−1} for the Trp₁₃ derivative and 2–4 µg ml^{−1} for the Ile₁₃ derivative. This is consistent with results obtained previously with heterologous whole-subunit exchanges (Nguyen *et al.*, 2006a; Miao *et al.*,

2006b). All three Asn₁₃ derivatives had MICs of 128 µg ml^{−1}, further emphasizing the critical importance of position 13 for antibacterial activity (Nguyen *et al.*, 2006b; Miao *et al.*, 2006b).

Fig. 4. LC-MS/MS analysis of A21978C₁-(Asn₁₃) produced by strain SD410. (a) Chemical structure of A21978C₁(Asn₁₃) and six characteristic product ions b₁–b₃ and y₁₀–y₁₂. (b) MS/MS spectrum of A21978C₁-(Asn₁₃) and assignment of key product ions.

DISCUSSION

It is now feasible to reprogramme the amino acid specificity of NRPS multi-enzymes, but the factors determining high-efficiency *in vivo* productivity from hybrid enzymes are poorly understood. This needs to be addressed to link the application of combinatorial biosynthesis to industrial-scale fermentation production. In the present work, we addressed the efficiency of producing hybrid lipopeptides related to daptomycin by fusing complete modules at T-C linkers in the DptD subunit. We found that neither the sequence nor the length (within ± 4 aa) of a segment of the inter-module T-C linker is important for catalytic efficiency of DptD. This linker may simply act as a tether which ensures that domains are aligned and in close proximity.

The flexible T-C linker nucleotide sequences containing inserted restriction endonuclease sites were exploited to construct hybrid genes to redirect lipopeptide biosynthesis to produce hybrid compounds containing Trp₁₃ or Ile₁₃/Val₁₃ at levels comparable to those produced by recombinants producing the native daptomycin factors. This compares favourably with yields obtained using whole-subunit exchanges to produce the same hybrid molecules at about 50 % and 25 % of controls, respectively (Miao *et al.*, 2006b; Coëffet-Le Gal *et al.*, 2006). The high productivity of recombinant *S. roseosporus* strains containing heterologous module exchanges may be due in part to the use of homologous inter-peptide docking sites and promiscuous Te domains, but also must be due in part to the inherent flexibility of the inter-module linker. Thus two advantages of using module exchange, as opposed to subunit exchange, are that it conserves the natural inter-peptide docking sites required for efficient communication between DptBC and DptD, and it provides a mechanism to expand the number of substitutions at position 13, which is important for antibacterial activity (Nguyen *et al.*, 2006a; Miao *et al.*, 2006b).

To further explore the engineering of the daptomycin biosynthetic pathway at the terminal module, we made fusions at T-C linkers, keeping the 3mGlu₁₂ module intact, coupled with fusions at A-T or T-Te linkers to exchange the CA or CAT at position 13. Of these, the CAT::CA::TTe double fusion, using the C-A di-domain from the D-Asn₁₁ (CATTe) module of A54145 was successful, whereas the CAT::CAT::Te double fusion did not yield product. This is consistent with previous work (Zhou *et al.*, 2006) that demonstrated a functional interaction between T and Te in T-Te di-domains. T domains from typical CAT modules might not be expected to carry out this function efficiently, if at all. Thus maintaining the integrity of the T-Te di-domain may be an important consideration in engineering terminal modules containing Te domains.

The fermentation yield of the recombinant containing the CAT::CA::TTe double fusion in *dptD* to insert Asn₁₃ was about 30–50 % of the yields obtained with strains contain-

ing single module fusions. This promising approach provides a route to insert other amino acids at the important position 13. Suboptimal fermentation yields of recombinants may be addressed by focused mutagenesis, as has been demonstrated in the engineering of the mixed polyketide-peptide andrimid (Fischbach *et al.*, 2007).

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