Chemistry & Biology 13, 269–276, March 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.chembiol.2005.12.012

Genetic Engineering in *Streptomyces roseosporus* to Produce Hybrid Lipopeptide Antibiotics

Vivian Miao,[1] Marie-Francois Coëffet-Le Gal,[1] Kien Nguyen,[1] Paul Brian,[1] Julia Penn,[2] Andrew Whiting,[2] Jonathan Steele,[2] David Kau,[2] Steve Martin,[2] Robert Ford,[2] Trevor Gibson,[2] Mario Bouchard,[1] Stephen K. Wrigley,[2] and Richard H. Baltz[1,*]

[1]Cubist Pharmaceuticals, Inc.
65 Hayden Avenue
Lexington, Massachusetts 02421
[2]Cubist Pharmaceuticals, Inc.
545 Ipswich Road
Slough, Berkshire, SL1 4EQ
United Kingdom

Summary

Daptomycin is a lipopeptide antibiotic produced by a nonribosomal peptide synthetase (NRPS) in *Streptomyces roseosporus*. The holoenzyme is composed of three subunits, encoded by the *dptA*, *dptBC*, and *dptD* genes, each responsible for incorporating particular amino acids into the peptide. We introduced expression plasmids carrying *dptD* or NRPS genes encoding subunits from two related lipopeptide biosynthetic pathways into a daptomycin nonproducing strain of *S. roseosporus* harboring a deletion of *dptD*. All constructs successfully complemented the deletion in *trans*, generating three peptide cores related to daptomycin. When these were coupled with incomplete methylation of 1 amino acid and natural variation in the lipid side chain, 18 lipopeptides were generated. Substantial amounts of nine of these compounds were readily obtained by fermentation, and all displayed antibacterial activity against gram-positive pathogens.

Introduction

Cubicin (daptomycin-for-injection) is a lipopeptide antibiotic approved in the United States for treatment of skin and skin structure infections caused by gram-positive pathogens [1], including methicillin-resistant or vancomycin-resistant *Staphylococcus aureus* [2–5]. The active principle in Cubicin is daptomycin, a semisynthetic antibiotic produced by fermentation of *S. roseosporus*.

Daptomycin is a member of the A21978C family of acidic lipodepsipeptides [6]. These have a common 13 amino acid core cyclized by an intramolecular ester bond to make a 10-membered ring with a 3 residue side chain; attachment of different fatty acids to the N-terminal residue in the chain results in the predominant C_1, C_2, and C_3 factors (Figure 1). To produce daptomycin, decanoic acid is provided in the medium during fermentation to drive biosynthesis toward the n-decanoyl product [7]. A21978C is produced exclusively by *S. roseosporus*, but similar compounds, the calcium-dependent antibiotic (CDA) [8, 9] and members of the A54145 family [6, 10], are synthesized by *S. coelicolor* and *S. fradiae*, respectively (Figure 1).

Daptomycin is a complex molecule, and there have been a number of recent NMR studies aimed at defining its biologically active conformation [11–13]. The challenging nature of its structure has resulted in only limited attempts at synthesis and evaluation of daptomycin derivatives. Modifications of the acyl tail or derivatization at the δ-amino group of ornithine (Orn) have been successful [14–16], but these peripheral alterations have not yet yielded a compound with improved antibacterial properties compared to daptomycin. Total synthesis of an analog differing from daptomycin by substitution of Glu for 3-methyl-glutamic acid (3mGlu) at position 12 has recently been achieved through the use of isolated CDA thioesterase (cyclase) [17]. This approach enabled replacement of the acidic amino acid residues with Asn or Gln, and the results highlighted the importance of Asp_7 and Asp_9 for antibacterial activity.

In *S. roseosporus*, daptomycin is synthesized by a nonribosomal peptide synthetase (NRPS) via a thiotemplate mechanism [18–20]. NRPSs are typically composed of several subunits, each divided into a set of modules. An individual module is minimally comprised of adenylation (A), condensation (C), and thiolation (T) domains, and, collectively, the modules are responsible for recognizing specific amino acids and assembling them in the correct sequence. Modules can activate nonproteinogenic amino acids such as the kynurenine (Kyn) and Orn found in daptomycin, and they may include additional domains that modify the amino acids; e.g., epimerase (E) domains convert *L*-amino acids into their *D*-isomers. The specificity of the modules within the subunits and the order of amino acids in the peptide are generally colinear, and the last module has a thioesterase (Te) domain that releases the mature peptide from the enzyme.

The daptomycin biosynthetic gene cluster from *S. roseosporus* includes three genes, *dptA*, *dptBC*, and *dptD*, which encode the multimodular NRPS subunits (DptA, DptBC, and DptD) for assembling the peptide core (Figure 1). Other genes nearby are likely to be involved in coupling branched chain fatty acids to the N-terminal Trp_1 (*dptE*, *dptF*), error correction (*dptH*), or incorporation of $3mGlu_{12}$ and Kyn_{13} (*dptI*, *dptJ*) [21].

The cloned *dpt* gene cluster provides an opportunity to study daptomycin biosynthesis and to explore methods to build novel lipopetides by pathway engineering. This is essential for compounds that are commercially generated through fermentation. The ordered nature of nonribosomal peptide assembly lends itself well to genetic manipulation by domain or module exchange [22, 23]. Another approach, successful for engineering polyketides [24, 25] but not reported for peptides in vivo, is the substitution of entire subunits to create hybrid pathways and compounds. Here, we describe experiments in which genes encoding NRPS subunits from two other lipopeptide pathways were expressed in *S. roseosporus*, leading to the production

*Correspondence: rbaltz@cubist.com

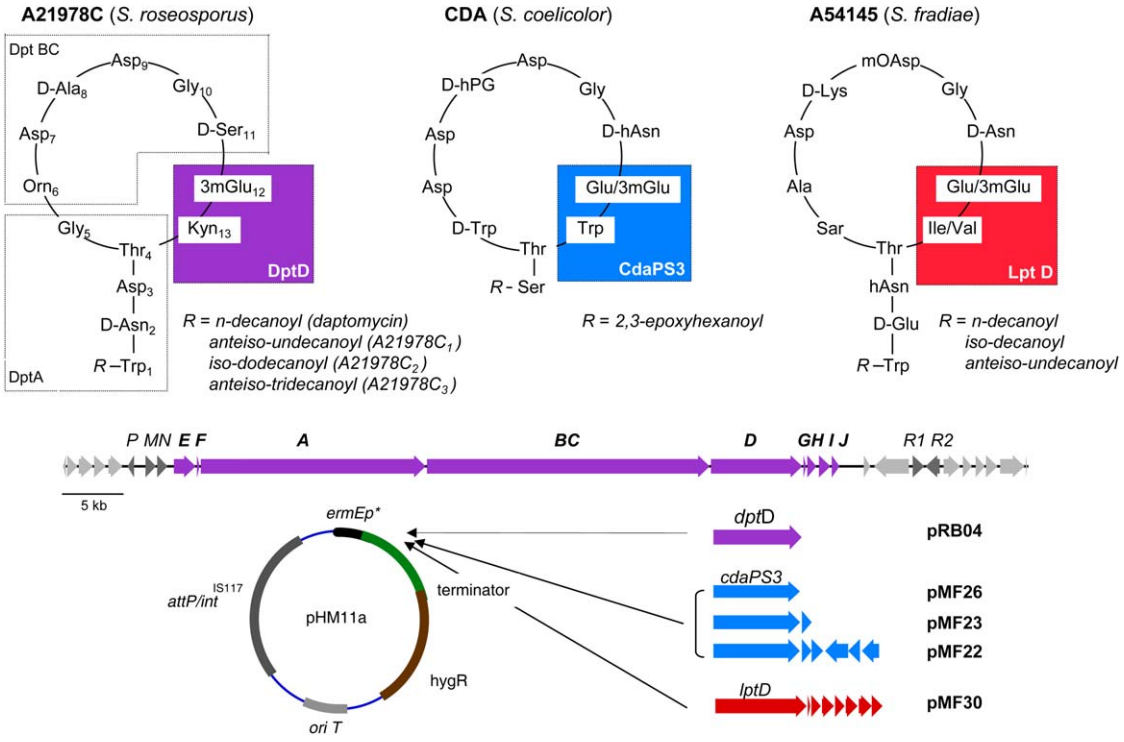


Figure 1. Lipopeptide Structures, Maps of the Daptomycin Biosynthetic Gene Cluster, and Expression Plasmids

A21978C, CDA, and A54145 are shown at the top. Amino acids incorporated by subunits DptA, DptBC, and DptD are delimited by dotted lines for daptomycin; those incorporated by CdaPS3 and LptD are indicated likewise for CDA and A54145. Abbreviations: hAsn, hydroxyasparagine; hPG, hydroxyphenylglycine; Sar, sarcosine; mOAsp, methoxy-Asp; others are as in the text. A21978C includes all acyl tail variants, although subscripts may be used for clarity; e.g., A21978C$_1$. The *dpt* genes are indicated by letter above the corresponding arrow on the scaled map at the center. Portions of the *cda* and *lpt* pathways (same scale as for *dpt*) used in complementation plasmids as well as pHM11a are shown at the bottom. Genes downstream of *cdaPS3* represented by block arrows (left to right): SCO3233 (Te), *hasP* (phosphotransferase), SCO3235, *asnO* (hydroxylase), and SCO3237. Genes downstream of *lptD*: *lptG*, *lptH* (Te), *lptJ*, *lptK* (O-MTase), *lptL* (Asn hydroxylase), *lptM*, and *lptN*.

of novel active analogs and further understanding of structure-activity relationships within the daptomycin molecule.

Results

Homologous Complementation: The *dptD* System

We first determined if complementation of a deletion in a multisubunit enzyme gene cluster could be achieved in *trans* by NRPS DNA introduced at an ectopic location. The *dptD* gene, encoding the last subunit of daptomycin NRPS, is comprised of the modules for incorporating 3mGlu and Kyn as well as the terminal Te domain (Figure 1). A Δ*dptD* deletion strain (UA378, Table 1) was generated in *S. roseosporus* UA117, a daptomycin producer, by replacement of *dptD* with a resistance marker. UA378 was confirmed to be blocked in A21978C production by HPLC analysis of shake flask fermentations (Figures 2A and 2B). The coding region of *dptD* was cloned under control of the strong constitutive *ermEp** promoter in pHM11a [26], an *E. coli*/streptomycete shuttle vector allowing for conjugative transfer and chromosomal integration at the *att*^{IS117} site. The resulting plasmid, pRB04, was introduced into the Δ*dptD* host, and exconjugants were characterized for lipopeptide production in shake flasks and compared with the control strain. While UA378 with pHM11a vector alone produced no A21978C, typical production was restored in transformants with pRB04 (Figure 2C). The yield of UA383 was very similar to that of UA374, a UA117+pHM11a control (202 ± 7 mg/l versus 210 ± 7 mg/l). These results showed that an NRPS subunit expressed ectopically under heterologous control could complement the remainder of the native enzyme complex in vivo. The very efficient functional restoration by pRB04 included not only the 3mGlu- and Kyn-incorporating activities of modules 12 and 13, but also the hydrolytic activity of

Table 1. Key *S. roseosporus* Strains and Plasmids

Strains	Genotype[a]	Lipopeptides Produced
UA117	*rpsL7* (Sm^r)	A21978C
UA374	UA117 (pHM11a)	A21978C
UA378	UA117 Δ*dptD*::*ermE*	(none)
UA383	UA378 (pRB04)	A21978C
UA416	UA378 (pMF26)	A21978C(mGlu/Glu$_{12}$Trp$_{13}$)
UA385	UA378 (pMF23)	A21978C(mGlu/Glu$_{12}$Trp$_{13}$)
UA384	UA378 (pMF22)	A21978C(mGlu/Glu$_{12}$Trp$_{13}$)
UA451	UA378 (pMF30)	A21978C(mGlu/Glu$_{12}$Ile/Val$_{13}$)
UA376	UA117 (pMF22)	A21978C, A21978C(mGlu/Glu$_{12}$Trp$_{13}$)
UA377	UA117 (pMF23)	A21978C, A21978C(mGlu/Glu$_{12}$Trp$_{13}$)

[a] Indicated by a host strain and plasmid (parenthetic), e.g., UA374 was constructed by integration of pHM11a into UA117.

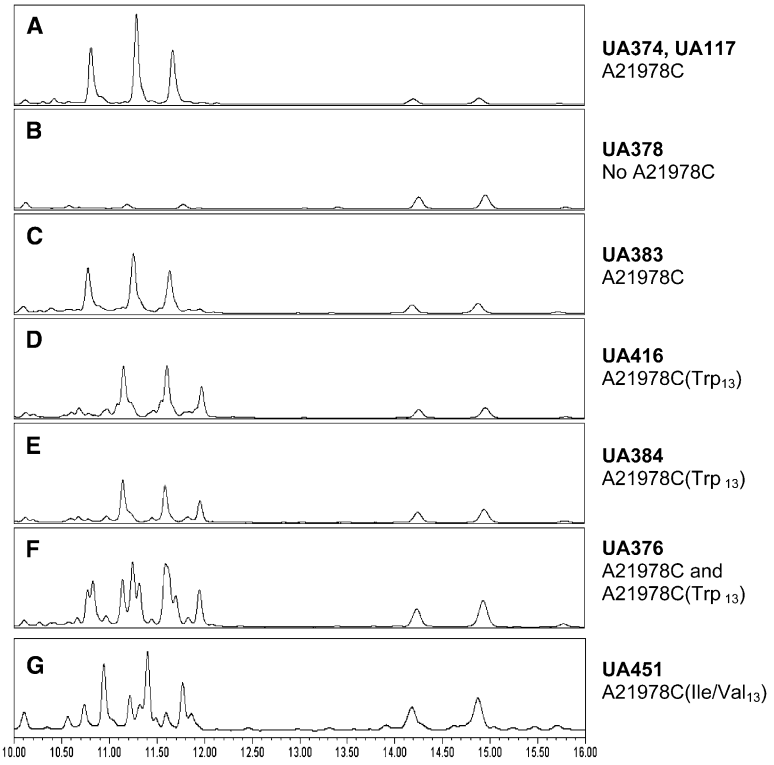


Figure 2. HPLC Analyses of Lipopeptide Production

(A–G) Absorbance at 224 nm was plotted with a (A–F) 0–0.6 AU or a (G) 0–0.1 AU vertical scale. Compounds and strains for which each panel is representative are listed; the strain associated with the chromatogram shown is in bold. (A) Native A21978C profile and production levels in *S. roseosporus* UA374 or UA117. Retention times (R_ts) of C_1, C_2, and C_3 factors = 10.8, 11.3, 11.7 min, respectively. (B) UA378 (Δ*dptD*). (C) UA383 (UA378 + *dptD*). (D) UA416 (UA378 + *cdaPS3*). Peak R_ts were 11.2, 11.6, and 12.0 min. (E) UA384 (UA378 + *cdaPS3*). (F) UA376 (UA117 + *cdaPS3)*. The complex profile includes those of A21978C, A21978C(Trp_{13}), and a third set of peaks that was slightly more polar than A21978C. LC-MS analysis of the new set indicated molecular ions (MH^+) at m/z 1651.7, 1665.7, and 1679.7 that are consistent with uncyclized A21978C lipopeptides or compounds produced by hydrolysis of the ester bond or one of the peptide bonds of A21978C, as has been reported for daptomycin [41, 42]. (G) UA451 (UA378 + *lptD*). The new, complex set of products includes A21978C(Ile/Val_{13}). Peaks at R_t = 14.2 and 14.9 min in all panels are unrelated peptidic compounds (M. Chu and J.-Q. Gu, personal communication) whose presence is not qualititatively affected by the current manipulations.

the Te domain that is needed to cyclize and release the mature peptide.

Heterologous Complementation: Novel Compounds

Introduction of subunits that bring substitutions into the peptide core would generate analogs of daptomycin to facilitate elucidation of structure-activity relationships, as well as provide a means to produce the desired derivatives by fermentation. The lipopeptides CDA and A54145 are also synthesized by NRPSs, and the last two residues in each compound are incorporated by a terminal dimodular subunit like DptD: where DptD incorporates 3mGlu-Kyn in daptomycin, CdaPS3 (encoded by *cdaPS3* in *S. coelicolor* [9]) incorporates Glu/3mGlu-Trp in CDA, and LptD (encoded by *lptD* in *S. fradiae* [27]) incorporates Glu/3mGlu-Ile and, at a lower frequency, Glu/3mGlu-Val in A54145 (Figure 1). For successful complementation, CdaPS3 and LptD must form functional multienzyme complexes with DptA and DptBC; the C domains at the start of the replacement subunits must accept a nonnative nascent peptide terminating in a heterologous amino acid, and the Te domains must cyclize hybrid substrates that differ substantially from their native peptides. The end of DptBC and the start of DptD, and the corresponding regions in the Cda and Lpt proteins, are similar in organization and sequence (Figure 3). The upstream subunits (donor) end at an E domain, while the downstream subunits (acceptor) begin with a C^{II} domain, a type generally following modules incorporating D or achiral amino acids [21]. At the sequence level, there are short extensions or docking sites that discriminate subunit-terminating

A

Pathway	donor subunit	acceptor subunit
dpt	. . . CA_{Ser} TE	$C^{II}A_{3mGlu}$T CA_{Kyn}T Te
cda	. . . CA_{nAsn}TE	$C^{II}A_{3mGlu/Glu}$T CA_{Trp}T Te
lpt	. . . CA_{Asn}TE	$C^{II}A_{3mGlu/Glu}$T $CA_{Ile/Val}$T Te

B

```
DptBC   TPSDVG-LAELSFAEIELLEDDWRTQG------MTQRAMEDILPLTPLQEGLLF   DptD
CdaPS2  TPSDVS-LTSLSEDDISRLESEWGSL-------MAKSGLEDILPLSPLQEGMLF CdaPS3
LptC    PLSDPPPLVELTDTELDQLEAEWKAG-------M-RGSLQDVLPLSPLQEGLLF   LptD
TycB3   TPSDLG-DEELSMEELENILE-WI--------MKKQENIAKIYPLTPLQEGMLF   TycC
TycA    TPSDFS-VKGLQMEEMDDIFELLANTLR--MSVFSKEQVQDMYALTPMQEGMLF   TycB
          **                                    * * *** **
```

Figure 3. Intersubunit Docking Sites

(A) Domain organization. C, C^{II}, A, T, E, and Te domains are described in the text.

(B) Terminal peptides on adjoining subunits. Hydrophilic residues are underlined, and acidic residues are shaded. Sequences include: Cda (GenBank AL035654, AL035707), Dpt (GenBank AY787762), Lpt (GenBank DQ118863), and tyrocidine (Tyc) proteins (GenBank AF004835). An asterisk indicates conserved amino acids; hyphens are inserted to facilitate alignment of the terminal conserved amino acid residues of the subunits, as indicated at the far left and right.

Table 2. Physicochemical Characteristics of A21978C and Recombinant Derivatives

Strain	Lipopeptide Products	λ_{max}	Molecular Weights of C_{1-3} Analogs C_1, C_2, C_3	Molecular Ions (MH^+) Observed by LC-MS
UA374	A21978C	224, 262, 365	1633.7, 1647.7, 1661.7	1634.7, 1648.7, 1662.7
UA383	A21978C	224, 262, 365	1633.7, 1647.7, 1661.7	1634.7, 1648.7, 1662.7
UA384, UA385, and UA416	A21978C(m$Glu_{12}Trp_{13}$)	220, 280, 288	1629.7, 1643.7, 1657.7	1616.0, 1630.4
	A21978C($Glu_{12}Trp_{13}$)	220, 280, 288	1615.7, 1629.7, 1643.7	1644.0, 1658.0
UA451	A21978C(m$Glu_{12}Ile_{13}$)	220, 280, 288	1556.7, 1570.8, 1584.8	1529.0, 1543.0,
	A21978C(m$Glu_{12}Val_{13}$)	220, 280, 288	1542.7, 1556.7, 1570.8	1557.0, 1571.0,
	A21978C($Glu_{12}Ile_{13}$)	220, 280, 288	1542.7, 1556.7, 1570.8	1585.0
	A21978C($Glu_{12}Val_{13}$)	220, 280, 288	1528.7, 1542.7, 1556.7	

domains from those that are internal. There is a very strong bias for hydrophilic, including acidic, residues among the ~25 C-terminal amino acids (starting at the "TPS" motif), and for hydrophilic residues among the ~15 N-terminal amino acids (ending at the "PLQEG" motif). The similarities in the features of the three subunits prompted the investigation of producing daptomycin analogs with substitutions of Kyn by another bulky aromatic residue, Trp, or by smaller aliphatic amino acids, Ile or Val.

The *cdaPS3* gene, present in plasmid pMF26, was introduced into UA378, and shake flask fermentations from the recombinant UA416 were analyzed. A family of compounds with UV and mass spectra consistent with analogs of A21978C in which Kyn has been replaced by Trp (A21978C[Trp_{13}]) was detected by HPLC-MS (Figure 2D; Table 2). UA416 produced 50% as much lipopeptide as UA374, containing pHM11a alone (70 mg/l versus 142 mg/l) in a 7 liter fermentation, and provided material for structure confirmation and biological evaluation. Comparison of the amide and aromatic regions of the ^1H NMR spectra of the purified A21978C(Trp_{13}) analogs with those of the A21978C lipopeptides clearly showed the absence of the Kyn signals and the appearance of a new set of signals, as expected for an additional Trp residue. Detailed analysis of the mass spectra of each of the purified Trp-containing compounds indicated that these were 5:1 mixtures of 3mGlu_{12}- and Glu_{12}-containing analogs, A21978C(Trp_{13}) and A21978C ($Glu_{12}Trp_{13}$), respectively, with the same acyl tails (exhibiting the same acyl-Trp losses upon fragmentation) that had cochromatographed during purification. The presence of A21978C(Trp_{13}) factors as major products was noteworthy, as the methyltransferase (MTase)-encoding gene proposed for methylation of Glu in CDA [9] was not introduced into *S. roseosporus*. This indicated that the MTase encoded by *dptI* and responsible for conversion of Glu to 3mGlu in A21978C [21] could accommodate the hybrid peptide. The presence of unmethylated compounds, however, also suggests that the *S. roseosporus* enzyme worked less efficiently with the hybrid, as Glu_{12} analogs of A21978C have not been observed in control fermentations.

In addition to pMF26, two other plasmids containing *cdaPS3* and flanking genes were tested in *S. roseosporus*. Plasmid pMF23 had an extra gene for a putative proof-reading Te (SCO3233), whereas pMF22 had five more genes, including *asnO* and *hasP*, which encode enzymes that are likely to catalyze the hydroxylation and phosphorylation on Asn in CDA [9] (Figure 1). Strains with these plasmids (Table 1) produced A21978C(Trp_{13}) analogs in shake flasks, but despite the inclusion of genes for potential Asn-modifying enzymes in pMF22, no phosphorylated or hydroxylated compounds were observed (Figure 2E).

Plasmids pMF22 and pMF23 were also tested in UA117, which has a complete *dpt* pathway. HPLC data from recombinants UA376 and UA377 both showed complex patterns within which profiles characteristic of the native A21978C factors and of the novel A21978C(Trp_{13}) factors were discernible, along with other lipopeptide components that require further characterization (Figure 2F). The total amount of lipopeptide detected was the same or slightly greater (106% for UA377; 121% for UA376) than that observed in UA374, and the ratio of native A21978C to A21978C(Trp_{13}) factors was 4:6 in both genotypes, suggesting that complementation to reconstitute an active enzyme occurred in a competitive manner.

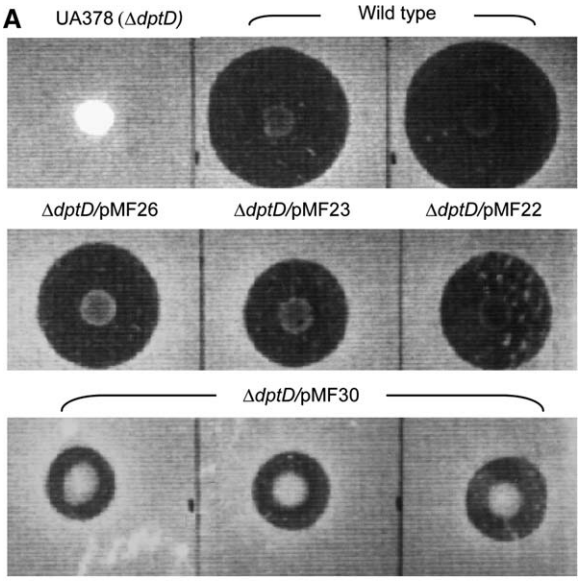
To further explore the production of daptomycin derivatives, the *lptD* gene, flanked downstream by seven genes, including two or three that may be involved in synthesis of hydroxyasparagine (hAsn) or methoxyaspartic acid (mOAsp), was cloned in pMF30 [27]. Recombinants carrying pMF30 produced a family of compounds with UV and mass spectra consistent with A21978C analogs having Ile or Val in place of Kyn_{13} and Glu_{12} or 3mGlu_{12} (Figure 2G; Table 2). Material for structural confirmation was obtained by fermentations in 7 liter vessels yielding 60 mg/l total lipopeptide. Although HPLC-MS analysis of the fermentation at harvest indicated the presence of both Glu- and 3mGlu-containing products, only the latter were present in quantities sufficient for isolation. Each of the expected A21978C (Ile_{13}) and A21978C(Val_{13}) products was purified and identified by NMR and mass spectroscopy. Inspection of the aromatic region of the ^1H NMR spectra indicated that the Kyn signals were absent, as was the Kyn β-CH_2 signal. Additionally, the high-field regions of the spectra showed the presence of two extra methyl groups for each Val- or Ile-containing product compared to its A21978C parent. As with the *cdaPS3*-bearing constructs, the *S. fradiae* gene for methylation of Glu in A54145 was not present in the complementing DNA. That both 3mGlu- and Glu-containing A21978C(Val_{13}) and A21978C(Ile_{13}) were detected confirms the flexibility in substrate recognition, albeit at a reduced efficiency, of the native *S. roseosporus* MTase.

Production of Hybrid Lipopeptides by Recombinants

In shake flasks, there was no adverse effect of homologous *trans*-complementation (pRB04) on production: yield was essentially the same as that of the vector carrying positive control, UA374. Production of Trp_{13}-substituted compounds ranged from 30%–50% yield compared to UA374, and the strains carrying pMF26 (*cdaPS3* only) yielded 50%. The pMF23- and pMF22-carrying strains produced 40% or 30% as much as control, suggesting a disadvantage conferred by the additional genes in these plasmids. Production by recombinants with pMF30, with *lptD* and seven other genes, may also have been decreased by the presence of the extra genes, but the 25% yield in these strains compared to control was adequate to easily detect the compounds and to generate enough material in 7 liter fermentations for structure elucidation and assessment of biological activity.

Antibacterial Activity of Recombinant Lipopeptides

Recombinants were initially assessed by indicator-seeded patch overlays of transformants. Strains containing pMF22, pMF23, and pMF26 exhibited a greater inhibition of *S. aureus* growth than strains with pMF30 (Figure 4A). As whole-cell assays reflect a combination of yield and efficacy, purified compounds were also tested against a panel of gram-positive pathogenic bacteria (Figure 4B). Compounds containing Trp_{13} instead of Kyn_{13} showed antibacterial activities similar to the A21978C controls, but compounds containing Ile_{13} or Val_{13} only showed comparable antibacterial activities when coupled with the longer fatty acid tails.



B

Compound	*S. aureus* 399*	*S. aureus* 42	*Enterococcus faecium* 14	*Enterococcus faecalis* 201
A21978C$_1$	1.6	1.6	6.3	3.1
A21978C$_1$ (Trp_{13})	1.6	0.8	6.3	12.5
A21978C$_1$ (Ile_{13})	3.1	3.1	12.5	12.5
A21978C$_1$ (Val_{13})	6.3	6.3	50.0	50.0
A21978C$_2$	0.4	0.8	1.6	0.8
A21978C$_2$ (Trp_{13})	0.8	0.8	6.3	6.3
A21978C$_2$ (Ile_{13})	1.6	1.6	6.3	6.3
A21978C$_2$ (Val_{13})	1.6	1.6	12.5	6.3
A21978C$_3$	0.8	0.8	1.6	0.4
A21978C$_3$ (Trp_{13})	0.8	0.8	3.1	3.1
A21978C$_3$ (Ile_{13})	1.6	1.6	3.1	3.1
A21978C$_3$ (Val_{13})	1.6	1.6	6.3	12.5

Figure 4. Biological Activity of Recombinant Strains and Purified Compounds

(A) Patch bioassay of representative strains.

(B) MIC (μg/ml) of purified compounds against a test panel.

Discussion

Daptomycin is the first lipopeptide antibiotic approved for the treatment of gram-positive pathogens. Its complex peptide core with unusual amino acids has presented limited opportunities for chemical modification to produce second-generation candidates for clinical development. To begin exploring the possibility of making modifications in the peptide by genetically engineering the NRPS genes, we cloned and characterized the daptomycin and A54145 biosynthetic gene clusters [21, 27], and in this study, we developed an NRPS *trans*-complementation system to generate novel compounds.

Similarities in the structures of the cyclic peptides CDA, A54145, and daptomycin, and in the organization of their respective NRPS genes, provided a system by which to test the engineering of daptomycin analogs by heterologous subunit exchanges. The in vivo reconstitution control showed a high complementation efficiency (~95%), as measured by A21978C production in the *S. roseosporus* Δ*dptD* deletion strain when *dptD* was expressed from an ectopic position under the control of *ermEp**. This promoter was chosen because it has been used effectively in expressing cloned secondary metabolism genes [26, 28]. Complementation with the terminal subunits from the other pathways led to the biosynthesis of active analogs of daptomycin containing Trp_{13}, Ile_{13}, or Val_{13} at production efficiencies ranging from 25% to 50% relative to a Kyn_{13} control. These levels were obtained without optimizing the expression of the heterologous subunits or the fermentation conditions; therefore, yield improvements should be possible by further engineering or fermentation development. Although identification and recovery of novel compounds is simpler with a Δ*dptD* host, heterologous *trans*-complementation was also observed when the *cdaPS3* gene was brought into a host with a complete *dpt* cluster. The roughly equivalent production of lipopeptides containing Kyn_{13} or Trp_{13} indicated that the heterologous CdaPS3 subunit competed well with the native subunit in formation of a functional hybrid NRPS multisubunit enzyme; in this case, the use of *ermEp** probably helped to enhance the expression of *cdaPS3*.

Contextual resemblances both physical (e.g., of domain types and order, including the Te) and biochemical (e.g., cyclization involving an analogously positioned Thr), as well as sequence conservation at the ends of the subunits, must all contribute to successful complementation. Restoration of the junction structure by the N-terminal interpeptide docking sites on the CdaPS3 and LptD proteins undoubtedly played a significant role. It is now established that elongation of type I

polyketides, peptides, and mixed polyketide/peptides that are assembled by PKS and NRPS mechanisms requires interpeptide docking sites to facilitate the appropriate communication between the subunits of the giant multisubunit enzymes [29–35]. Heterologous subunit exchanges have been successful at generating hybrid polyketide aglycones related to platenolide [24] and erythronolide [25], but the molecules were not glycosylated to produce active antibiotics. Our work extends this approach for the first time to peptides in vivo, and, in this case, the fully embellished products were active as antibiotics. Further work is needed to establish the relevance of the amino acid conservation and charge distribution observed in the docking sites of DptD, LptD, and CdaPS3, and their homologous and heterologous pairing subunits, and to optimize them for highly efficient expression of heterologous NRPS multienzymes.

The results here suggest that *trans*-complementation may be generalizable to other multisubunit NRPS systems if considerations are given to the design of subunit docking sites. Where there are no convenient natural subunits, it should be possible to follow the examples here and build subunits with different amino acid-incorporating specificities, within a framework optimized for intersubunit communication through selection of appropriate docking sites. Hahn and Stachelhaus [35] have shown that this is feasible with NRPSs from *Bacillus*.

The potential for accessory gene involvement in generating analogs was also exemplified here. Methylation of Glu in the Trp_{13}-, Ile_{13}-, or Val_{13}-containing analogs was accomplished by the native *S. roseosporus* MTase, but, interestingly, the methylation was incomplete, as is typical in the native production of CDA and A54145. Whether this was an inherent limitation of the *S. roseosporus* enzyme on the hybrid molecules, or whether other factors precluded complete methylation, remains to be determined.

Significance

This work demonstrates the utility of subunit *trans*-complementation as a method by which to engineer nonribosomally synthesized peptide antibiotics. In *S. roseosporus*, *trans*-complementation of the terminal dimodular daptomycin NRPS subunit with heterologous dimodular subunits was productive and robust. This was coupled with incomplete methylation of Glu and acylation with the three different natural fatty acid side chains to generate 18 new lipopeptide antibiotics. This approach may be expanded by coupling it with module exchanges to generate combinatorial libraries of lipopeptides that can also act as scaffolds for further chemical modifications directed at the development of second-generation derivatives of daptomycin with improved therapeutic properties.

Experimental Procedures

Media

Actinomycetes were routinely grown on trypticase soy agar (TSA) or in broth (TSB) in 125 ml flasks shaken at 200 rpm, 30ºC. Other common bacteriological media and procedures for handling organisms are as described [36]. Agar medium AS-1 and broth media A355 and F10A were described elsewhere [21, 37]. Broth medium A354 was comprised of 1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% casein, 4.6% MOPS, 0.1% antifoam A (pH 7).

Strains

UA117 is a spontaneous *S. roseosporus* NRRL 15998 derived mutant resistant to 50 μg/ml streptomycin (C. Wai, personal communication), and was the parent for all recombinants (Table 1). *S. coelicolor* M145 was used in constructing the BAC library. *Escherichia coli* DH10B carrying pUZ8002 was the conjugation host for all plasmids. Pathogens used in bioassays (below) were from the Cubist culture collection and are susceptible to daptomycin. Other strains were constructed as described below.

Strain Construction

To generate a Δ*dptD* deletion host in UA117, flanking regions 5′ (1.1 kb) and 3′ (1.5 kb) of *dptD* were amplified from pCV1, a BAC clone carrying a 128 kb insert of *S. roseosporus* DNA including the entire *dpt* gene cluster [21], and cloned into pNEB193 to build a targeting plasmid for gene replacement. The primer pairs used were 5′-GCGAAGCTTCTGGTGGCGCATCACCTGG-3′ and 5′-GCTCTAGATGGAAGTATGTCCTCCATCGC-3′ and 5′-CGGATCCCGCCGGCACCTGACCC-3′ and 5′-CCGAATTCCGCCTCCGAGTACATCGAGG-3′. A 1.4 kb fragment including the *ermE* gene, conferring resistance to 20 μg/ml lincomycin (ErmR), was amplified from *Saccharopolyspora erythraea* DNA with primers 5′-CGACTAGTTTGCGCCCGATGCTAGTC-3′ and 5′-GAACTAGTCAGAGGTTGATGTCGGCCC-3′, cut with SpeI, and ligated into an XbaI site between the *dpt* fragments. This *dpt-ermE-dpt* cassette was cloned into a derivative of pRHB538 [38], a *Streptomyces* replicative vector with *rep*^{ts}, *aac(3)IV* for resistance to 100 μg/ml apramycin (AmR), and the *S. roseosporus rpsL*$^{+}$ gene for dominant sensitivity to streptomycin (SmS), and it was conjugated into UA117. Selection was initially for the presence of the plasmid (AmR) and secondarily for plasmid curing after a temperature shift to 38ºC (SmR), and was followed by screening for gene replacement (ErmR, StrR, AmS). Four candidates were confirmed as Δ*dptD* mutants by Southern analysis [36]. UA378 was subsequently selected as the host for conjugative transfer of the expression plasmids (below) that could be selected for resistance to 100 μg/ml hygromycin.

Construction of Expression Plasmids

For pRB04, a 0.3 kb fragment at the start of *dptD* was amplified (primers were 5′-GGAATTCCATATGACGCAGCGCGCGATGG-3′ and 5′-GCGGATCCTTCTGCTCGGCGCCCTCG-3′) from pCV1, cut with NdeI and BamHI, and cloned into corresponding sites in pHM11a [26]. This adapted the translational start of *dptD* to that provided by pHM11a and introduced the unique natural BsrGI site within *dptD*. The plasmid was sequentially treated with BamHI, Klenow, and BsrGI, and the remainder of *dptD* was cloned in as a 7.2 kb BsrGI/ScaI fragment from pCV1.

For *cdaPS3* plasmids, DNA was obtained from a *S. coelicolor* M145 BAC library constructed by using methods described previously [21]. The 2000 clone library was screened by PCR with primers derived from *cda* (GenBank AL035654, AL035707). Clone pCV18, with a 51 kb insert, was determined by sequencing with vector primers P108 (5′-GCCTGGCCGTCGACATTTAGG-3′) and P113(5′-GGAGCTGACTGGGTTGAAGG-3′) and mapping to carry the complete sequence of all three *cdaPS* genes [9]. Complementing plasmids were constructed via an amplified adaptor fragment consisting of the 5′ end of the *cdaPS3* gene, delimited by P177 (5′-GGAATTCCATATGGCAAGTCCGGCTTG-3′) and P178 (5′-CGGGATCCGACCATGGTGTCGATACC-3′). This fragment, with a natural BsrGI site, was cloned as an NdeI/BamHI fragment into pHM11a to build pMF21. Fragments (13.2 kb BsrGI/BamHI, 7.6 kb BsrGI/EcoRI, and 6.9 kb BsrGI/DraIII) containing *cdaPS3* and flanking genes from pCV18 were cloned between the BsrGI and BamHI sites of pMF21 to build pMF22, pMF23, and pMF26, respectively (inserts, vector blunt ended with Klenow as needed).

For pMF30, a 13 kb BamHI fragment from *S. fradiae* cosmid pCV15 [27] was cloned into a derivative of pHM11a in which the multiple cloning site had been replaced (by using a mutagenic primer and PCR) by the sequence 5′-CCGTATCATATGCGCGGATCCGCTGTTCGCCGGCAC-3′. This includes the first 12 nucleotides of *lptD* (underlined), fused to the ATG start codon provided by the vector, as well

as a unique natural BamHI site from *lptD* suitable for cloning the fragment from pCV15.

All plasmids were introduced into *S. roseosporus* strains by conjugation with *E. coli* [38]. Some of the plasmids were introduced into UA378 and three other independently isolated Δ*dptD* mutants to verify reproducibility, and all gave equivalent fermentation results when complemented with pRB04, pMF22, and pMF23; therefore, UA378 was used for all subsequent experiments. Representative exconjugants were analyzed by Southern hybridization of EcoRI-digested chromosomal DNA with DIG-labled pHM11a. All gave banding patterns consistent with single plasmid insertions into a unique IS*117* insertion site. Recombinants containing plasmids inserted in the chromosome were grown in the presence of 100 μg/ml hygromycin on solid agar media, but without antibiotic in liquid culture.

Fermentation

Small-scale fermentations in 20 ml broth were conducted in 125 ml baffled flasks shaken at 200 rpm, 30ºC: starter cultures were grown in TSB for 48 hr, 1 ml starter culture was transferred to A355 and grown for 24 hr for seed, and 1 ml seed was transferred into F10A for production. Cultures were sampled 4–6 days later for HPLC analysis (below). At least two experiments were conducted on two or more strains per genotype, and representative results are presented in the Results. Large-scale fermentations were conducted in 7 liter fermenters stirred at 500 rpm, aerated at 1.0 vvm, and incubated at 30ºC. Seed stock was grown by inoculating mycelium from well-grown plate cultures into 250 ml A355 medium in 2 liter baffled flasks and shaken at 240 rpm at 30ºC for 24–26 hr; the entire seed culture was transferred to a fermenter containing 5 liters A354 medium and was harvested after 7 days.

Lipopeptide Purification and Characterization

Production cultures of UA416 (1 × 7 liters) and UA451 (2 × 7 liters) were centrifuged to remove biomass, and broths were loaded onto open glass columns packed with preconditioned Mitsubushi HP20 resin (60 × 300 mm) in H_2O. The loaded columns were washed with 1 liter H_2O, then eluted with 2.5 liters of methanol. The eluates were concentrated by rotary evaporation, lyophilized, and redissolved in water. Purification was achieved by preparative HPLC on a Waters Prep LC system and a Waters 40 × 200 mm Symmetry C8 6 μm radially compressed double cartridge with 40 × 10 mm guard with a linear gradient buffered with 0.4% trifluoroacetic acid (TFA). The gradient began with 35% (UA416) or 30% (UA451) aqueous acetonitrile (ACN), changed to 60% ACN over 18 min (UA416) or 20 min (UA451); the flow rate was 55 ml/min. From UA416, fractions containing lipopeptides were collected and lyophilized to yield 76 mg A21978C_1(Trp_{13}), 72 mg A21978C_2(Trp_{13}), and 35 mg A21978C_3(Trp_{13}). From UA451, the target components were collected as six semipure fractions and lyophilized. Each was further purified with a Symmetry C18 column (6 μm, 25 × 100 mm cartridge with 25 × 10 mm guard). Application of various isocratic mobile phase compositions (20 ml/min, buffered with 0.04% TFA) provided the compounds of interest to >95% purity: 34% aqueous ACN yielded 6 mg A21978C_1(Val_{13}) and 6 mg A21978C_1(Ile_{13}); 38% ACN yielded 4 mg A21978C_2(Val_{13}), 6 mg A21978C_2(Ile_{13}), and 4 mg A21978C_3(Val_{13}); and 36% ACN for 20 min, followed by a 10 min linear gradient to 43% can, afforded 11 mg A21978C_3(Ile_{13}). To monitor the presence of A21978C lipopeptides in shake flasks, culture samples were centrifuged to pellet the biomass, and 20 μl broth was analyzed by using a Waters HPLC system with a 4.6 × 150 mm Symmetry 3.5 μm column and a Phenomenex Security Guard C8 cartridge. The mobile phase, buffered with 0.01% TFA and flowing at 1.5 ml/min, was initially held at 10% aqueous ACN (2 min), linearly changed to 90% ACN over 18 min, held at 90% ACN (3 min), and followed by a wash in 10% ACN (8 min). Eluate absorbance was monitored at 224 nm. Yields were estimated by reference to a daptomycin standard (Cubist Pharmaceuticals, Inc., Manufacturing Dept.) run in triplicate, and areas under the curve corresponding to identified peaks were integrated and summed after manual baseline adjustment. A correction factor of 0.645 for yield of A21978C_3(Trp_{13}) with respect to daptomycin was made to allow for the relative absorbance at 224 nm of the new compound. Electrospray ionization liquid chromatography-mass spectrometry (ESI LC-MS) was performed as described previously (22). ^1H and ^{13}C NMR spectra were recorded at 308 K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz, respectively. TOCSY NMR spectra were recorded in CD_3OD by using standard methods.

Bioassays

Strains were patched on AS-1 agar for 5 days, then overlaid with soft nutrient agar containing $CaCl_2$ (5 mM final) and *S. aureus*, and incubated overnight at 37ºC. Minimum inhibitory concentrations (MICs) of purified compounds against test organisms (below) were determined by broth microdilution according to NCCLS guidelines, except that Mueller-Hinton broth was supplemented to 50 mg/l Ca^{2+} and assays were performed at 37ºC [39, 40].

Acknowledgments

We thank S. Sinnemann, K. She, M. Nichols, R. Brost, and S. Doekel for technical assistance, M. Chu and J.-Q. Gu for recommendations on lipopeptide quantification, M. Latif for NMR spectra, and N. Cotroneo for minimum inhibitory concentration tests. We are grateful to F. Tally, D. Keith, G. Shimer, and B. Eisensten for their encouragement.

Received: October 28, 2005
Revised: December 8, 2005
Accepted: December 19, 2005
Published online: March 24, 2006

References

1. Raja, A., LaBonte, J., Lebbos, J., and Kirkpatrick, P. (2003). Daptomycin. Nat. Rev. Drug Discov. *2*, 943–944.
2. Akins, R.L., and Rybak, M.J. (2001). Bactericidal activities of two daptomycin regimens against clinical strains of glycopeptide intermediate-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* isolates in an *in vitro* pharmacodynamic model with simulated endocardial vegetations. Antimicrob. Agents Chemother. *45*, 454–459.
3. King, A., and Phillips, I. (2001). The in vitro activity of daptomycin against 514 Gram-positive aerobic clinical isolates. J. Antimicrob. Chemother. *48*, 219–223.
4. Bozdogan, B., Esel, D., Whitener, C., Browne, F.A., and Appelbaum, P.C. (2003). Antibacterial susceptibility of a vancomycin-resistant *Staphylococcus aureus* strain isolated at the Hershey Medical Center. J. Antimicrob. Chemother. *52*, 864–868.
5. Cha, R., Brown, W.J., and Rybak, M.J. (2003). Bactericidal activities of daptomycin, quinupristin-dalfopristin, and linezolid against vancomycin-resistant *Staphylococcus aureus* in an *in vitro* pharmacodynamic model with simulated endocardial vegetations. Antimicrob. Agents Chemother. *47*, 3960–3963.
6. Baltz, R.H. (1997). Lipopeptide antibiotics produced by *Streptomyces roseosporus* and *Streptomyces fradiae*. In Biotechnology of Antibiotics Second Edition, W.R. Strohl, ed. (New York: Marcel Dekker, Inc.), pp. 415–435.
7. Huber, F.M., Pieper, R.L., and Tietz, A.J. (1988). The formation of daptomycin by supplying decanoic acid to *Streptomyces roseosporus* cultures producing the antibiotic complex A21978C. J. Biotechnol. *7*, 283–292.
8. Lakey, J.H., Lea, E.J., Rudd, B.A., Wright, H.M., and Hopwood, D.A. (1983). A new channel-forming antibiotic from *Streptomyces coelicolor A3(2)* which requires calcium for its activity. J. Gen. Microbiol. *129*, 3565–3573.
9. Hojati, Z., Milne, C., Harvey, B., Gordon, L., Borg, M., Flett, F., Wilkinson, B., Sidebottom, P.J., Rudd, B.A., Hayes, M.A., et al. (2002). Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem. Biol. *9*, 1175–1187.
10. Boeck, L.D., Papiska, H.R., Wetzel, R.W., Mynderse, J.S., Fukuda, D.S., Mertz, F.P., and Berry, D.M. (1990). A54145, a new lipopeptide antibiotic complex: discovery, taxonomy, fermentation and HPLC. J. Antibiot. (Tokyo) *43*, 587–593.

11. Jung, D., Rozek, A., Okon, M., and Hancock, R.E. (2004). Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem. Biol. *11*, 949–957.
12. Ball, L.J., Goult, C.M., Donarski, J.A., Micklefield, J., and Ramesh, V. (2004). NMR structure determination and calcium binding effects of lipopeptide antibiotic daptomycin. Org. Biomol. Chem. *2*, 1872–1878.
13. Rotondi, K.S., and Gierasch, L.M. (2005). A well-defined amphipathic conformation for the calcium-free cyclic lipopeptide antibiotic, daptomycin, in aqueous solution. Biopolymers *80*, 374–385.
14. Debono, M., Abbott, B.J., Molloy, R.M., Fukuda, D.S., Hunt, A.H., Daupert, V.M., Counter, F.T., Ott, J.L., Carrell, C.B., Howard, L.C., et al. (1988). Enzymatic and chemical modifications of lipopeptide antibiotic A21978C: the synthesis and evaluation of daptomycin (LY146032). J. Antibiot. (Tokyo) *41*, 1093–1105.
15. Hill, J., Siedlecki, J., Parr, I., Morytko, M., Yu, X., Zhang, Y., Silverman, J., Controneo, N., Laganas, V., Li, T., et al. (2003). Synthesis and biological activity of *N*-acylated ornithine analogues of daptomycin. Bioorg. Med. Chem. Lett. *13*, 4187–4191.
16. Siedlecki, J., Hill, J., Parr, I., Yu, X., Morytko, M., Zhang, Y., Silverman, J., Controneo, N., Laganas, V., Li, T., et al. (2003). Array synthesis of novel lipodepsipeptide. Bioorg. Med. Chem. Lett. *13*, 4245–4249.
17. Grunewald, J., Sieber, S.A., Mahlert, C., Linne, U., and Marahiel, M.A. (2004). Synthesis and derivatization of daptomycin: a chemoenzymatic route to acidic lipopeptide antibiotics. J. Am. Chem. Soc. *126*, 17025–17031.
18. Marahiel, M.A., Stachelhaus, T., and Mootz, H.D. (1997). Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. *97*, 2651–2674.
19. McHenney, M.A., Hosted, T.J., Dehoff, B.S., Rosteck, P.R., and Baltz, R.H. (1998). Molecular cloning and physical mapping of the daptomycin gene cluster from *Streptomyces roseosporus*. J. Bacteriol. *180*, 143–151.
20. Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat. Prod. Rep. *20*, 275–287.
21. Miao, V., Coëffet-Le Gal, M.-F., Brian, P., Brost, R., Penn, J., Whiting, A., Martin, S., Ford, R., Parr, R., Bouchard, M., et al. (2005). Cloning, sequence analysis and heterologous expression of the daptomycin biosynthetic gene cluster of *Streptomyces roseosporus*. Microbiol. *151*, 1507–1523.
22. Stachelhaus, T., Schneider, A., and Marahiel, M.A. (1995). Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science *269*, 69–72.
23. Cane, D.E., Walsh, C.T., and Khosla, C. (1998). Harnessing the biosynthetic code: combinations, permutations, and mutations. Science *282*, 63–68.
24. Kuhstoss, S., Huber, M., Turner, J.R., Paschal, J.W., and Rao, R.N. (1996). Production of a novel polyketide through the construction of a hybrid polyketide synthase. Gene *183*, 231–236.
25. Tang, L., Fu, H., and McDaniel, R. (2000). Formation of functional heterologous complexes using subunits from the picromycin, erythromycin and oleandomycin polyketide synthases. Chem. Biol. *7*, 77–84.
26. Motamedi, H., Shafiee, A., and Cai, S.J. (1995). Integrative vectors for heterologous gene expression in *Streptomyces spp*. Gene *160*, 25–31.
27. Miao, V., Brost, R., Chapple, J.P., She, K., Coëffet-Le Gal, M.-F., and Baltz, R.H. (2006). The lipopeptide antibiotic A54145 biosynthetic gene cluster from *Streptomyces fradiae*. J. Ind. Microbiol. Biotechnol. *33*, 129–140. 10.1007/s10295–005–0028–5.
28. Solenberg, P.J., Matsushima, P., Stack, D.R., Wilkie, S.C., Thompson, R.C., and Baltz, R.H. (1997). Production of hybrid glycopeptide antibiotics *in vitro* and in *Streptomyces toyocaensis*. Chem. Biol. *4*, 195–202.
29. Wu, N., Tsuji, S.Y., Cane, D.E., and Khosla, C. (2001). Assessing the balance between protein-protein interactions and enzyme-substrate interactions in the channeling of intermediates between polyketide synthase modules. J. Am. Chem. Soc. *123*, 6465–6474.
30. Tsuji, S.Y., Cane, D.E., and Khosla, C. (2001). Selective protein-protein interactions direct channeling of intermediates between polyketide synthase modules. Biochemistry *40*, 2326–2331.
31. Tsuji, S.Y., Wu, N., and Khosla, C. (2001). Intermodular communication in polyketide synthases: comparing the role of protein-protein interactions to those in other multidomain proteins. Biochemistry *40*, 2317–2325.
32. Wu, N., Cane, D.E., and Khosla, C. (2002). Quantitative analysis of the relative contributions of donor acyl carrier proteins, acceptor ketosynthases, and linker regions to intermodular transfer of intermediates in hybrid polyketide synthases. Biochemistry *41*, 5056–5066.
33. O'Connor, S.E., Walsh, C.T., and Liu, F. (2003). Biosynthesis of epothilone intermediates with alternate starter units: engineering polyketide-nonribosomal interfaces. Angew. Chem. Int. Ed. Engl. *42*, 3917–3921.
34. Liu, F., Garneau, S., and Walsh, C.T. (2004). Hybrid nonribosomal peptide-polyketide interfaces in epothilone biosynthesis; minimal requirements at N and C termini of EpoB for elongation. Chem. Biol. *11*, 1533–1542.
35. Hahn, M., and Stachelhaus, T. (2004). Selective interaction between nonribosomal peptide synthetases is facilitated by short communication-mediating domains. Proc. Natl. Acad. Sci. USA *101*, 15585–15590.
36. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D. (2000). Practical *Streptomyces* Genetics (Norwich, UK: John Innes Foundation).
37. Baltz, R.H. (1980). Genetic recombination by protoplast fusion in *Streptomyces*. Dev. Indust. Microbiol. *21*, 43–54.
38. Hosted, T.J., and Baltz, R.H. (1997). Use of *rpsL* for dominance selection and gene replacement in *Streptomyces roseosporus*. J. Bacteriol. *179*, 180–186.
39. Silverman, J.A., Oliver, N., Andrew, T., and Li, T. (2001). Resistance studies with daptomycin. Antimicrob. Agents Chemother. *45*, 1799–1802.
40. Wayne, P. (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Document M7–A6, Sixth Edition, NCCLS.
41. Kirsch, L.E., Molloy, R.M., Debono, M., Baker, P., and Farid, K.Z. (1989). Kinetics of the aspartyl transpeptidation of daptomycin, a novel lipopeptide antibiotic. Pharm. Res. *6*, 387–393.
42. Muangsiri, W., and Kirsch, L.E. (2001). The kinetics of the alkaline degradation of daptomycin. J. Pharm. Sci. *90*, 1066–1075.