Combinatorial biosynthesis of novel antibiotics related to daptomycin

Kien T. Nguyen, Daniel Ritz[†], Jian-Qiao Gu, Dylan Alexander, Min Chu, Vivian Miao[‡], Paul Brian, and Richard H. Baltz[§]

Department of Drug Discovery and Evaluation, Cubist Pharmaceuticals, 65 Hayden Avenue, Lexington, MA 02421

Communicated by Arnold L. Demain, Drew University, Madison, NJ, September 28, 2006 (received for review June 15, 2006)

Daptomycin, a cyclic lipopeptide produced by *Streptomyces roseosporus*, is the active ingredient of Cubicin (daptomycin-for-injection), a first-in-class antibiotic approved for treatment of skin and skin-structure infections caused by Gram-positive pathogens and bacteremia and endocarditis caused by *Staphylococcus aureus*, including methicillin-resistant strains. Genetic engineering of the nonribosomal peptide synthetase (NRPS) in the daptomycin biosynthetic pathway was exploited for the biosynthesis of novel active antibiotics. λ -Red-mediated recombination was used to exchange single or multiple modules in the DptBC subunit of the NRPS to modify the daptomycin cyclic peptide core. We combined module exchanges, NRPS subunit exchanges, inactivation of the tailoring enzyme glutamic acid 3-methyltransferase, and natural variations of the lipid tail to generate a library of novel lipopeptides, some of which were as active as daptomycin against Gram-positive bacteria. One compound was more potent against an *Escherichia coli* imp mutant that has increased outer membrane permeability. This study established a robust combinatorial biosynthesis platform to produce novel peptide antibiotics in sufficient quantities for antimicrobial screening and drug development.

cubicin | genetic engineering | nonribosomal peptide | *Streptomyces*

Daptomycin (Fig. 14) is a member of the A21978C family of cyclic anionic 13-amino acid lipopeptides produced by a nonribosomal peptide synthetase (NRPS) mechanism in *Streptomyces roseosporus* (1–3). During fermentation, three major compounds (A21978C_{1–3}) that have branched 11-, 12-, or 13-carbon chain fatty acids coupled to the N-terminal Trp residue normally are produced, but daptomycin, which has a straight 10-carbon chain fatty acid, is the predominant product if *n*-decanoic acid is provided during fermentation (3, 4). Daptomycin has potent *in vitro* bactericidal activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), vancomycin-resistant enterococci (VRE), and vancomycin-resistant *S. aureus* (VRSA) (3, 5–7). Cubicin (daptomycin-for-injection) was approved by the Food and Drug Administration for the treatment of skin and skin-structure infections caused by Gram-positive pathogens (5, 8) and bacteremia and endocarditis caused by *S. aureus*, including MRSA (9). Besides linezolid, daptomycin is the only novel antibiotic structural class to be approved in the last four decades.

Although daptomycin is efficacious for the indications discussed above, its analogs can be optimized for additional clinical uses, e.g., for the treatment of community-acquired pneumonia. A number of semisynthetic derivatives of daptomycin that contained modified lipid tails or side chains coupled through the δ -amino group of ornithine (Orn₆) were synthesized, but they represented limited structural diversity, and none had improved pharmacological properties (3, 10). Attempts to synthesize daptomycin derivatives with changes in the amino acid core by chemical (I. Parr, personal communication) and chemoenzymatic (11) methods have been hampered by the lack of commercially available 3-methyl-glutamic acid (for 3mGlu₁₂). The lack of suitable chemical approaches for the total synthesis of daptomycin analogs makes combinatorial biosynthesis an important alternative to generate and scale up derivatives of daptomycin for clinical evaluation.

The cloning and characterization of the biosynthetic gene clusters of daptomycin (12) and related lipopeptides, A54145 (13) and calcium-dependent antibiotic (CDA) (14) (Fig. 1), provided the starting materials for making derivatives of daptomycin. The daptomycin (*dpt*) biosynthetic gene cluster contains *dptA*, *dptBC*, and *dptD*, coding for the three NRPS subunits needed for incorporation of the 13 aa (Fig. 1B). As in other NRPSs, the daptomycin NRPS subunits are organized into modules (Figs. 1B and 2) with catalytic domains. Adenylation (A) domains selectively activate specific amino acids, and condensation (C) domains couple them to the peptide chain. Thiolation (T) domains located after A domains are pantetheinylated to provide an attachment point for the growing peptide chain as well as for the activated amino acids before condensation. Modules that incorporate D-amino acids usually contain epimerase (E) domains after T domains. The multimodular organization of NRPSs typically follows the colinearity rule in which the substrate binding specificity of the series of modules reflects the order of amino acid residues in the final products. Such organization can facilitate the construction of modified NRPSs in which defined functional modules (e.g., CAT or CATE) or domains (e.g., A or AT) are excised and replaced.

After some initial successes in producing novel compounds by manipulation of NRPSs using domain or module exchanges (15–18), most recent studies have focused on *in vitro* characterization of specific domains, substrate specificity, and intermodule communication by using purified proteins including truncated NRPSs (18). Several amino acid residues in the interpeptide docking sites were identified as important for maintaining or preventing functional interactions of NRPS subunits (19, 20). In an *in vitro* approach, rationally designed cyclic peptides and antibiotics were synthesized by using thioesterase-catalyzed chemoenzymatic biosynthesis (21, 22).

We recently described bioengineered daptomycin analogs in which Kyn₁₃ was substituted with Ile, Val, or Trp by using subunit exchange of the DptD subunit with the heterologous LptD and CdaPS3 subunits from the A54145 and CDA NRPSs, respectively (23–25). In the present study, we expanded the modification of the daptomycin amino acid core at residues nonconserved among structurally related lipopeptides by replacing single or multiple modules in the DptBC subunit with modules from daptomycin and A54145 NRPSs, and we combined them with DptD subunit exchanges, disruption of Glu₁₂ methylation (26), and natural lipid side-chain variations to generate a combinatorial library of novel lipopeptides. Characterization of purified compounds yielded valu-

Author contributions: K.T.N., D.R., J.-Q.G., M.C., V.M., P.B., and R.H.B. designed research; K.T.N., D.R., J.-Q.G., and D.A. performed research; D.A. contributed new reagents/analytic tools; K.T.N., D.R., J.-Q.G., M.C., P.B., and R.H.B. analyzed data; and K.T.N., D.R., V.M., P.B., and R.H.B. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: NRPS, nonribosomal peptide synthetase; CDA, calcium-dependent antibiotic; A, adenylation; C, condensation; T, thiolation; E, epimerase; MIC, minimal inhibitory concentration.

[†]Present address: Actelion Pharmaceuticals Ltd., 4123 Allschwil, Switzerland.

[‡]Present address: University of British Columbia, Vancouver, BC, Canada V6T 1Z3.

[§]To whom correspondence should be addressed. E-mail: r.baltz@cubist.com.

© 2006 by The National Academy of Sciences of the USA

Fig. 1. Lipopeptide antibiotics and daptomycin gene cluster. (A) Structurally related lipopeptides daptomycin, A54145 and CDA. (B) Subunit and module organizations of the daptomycin gene cluster.

able information on structural features critical for antibiotic activity and identified candidates for further modification and clinical development.

Results

Experimental System for Combinatorial Biosynthesis. To carry out combinatorial biosynthesis, we used different strains of *S. roseosporus* deleted for different sets of daptomycin genes (Table 3, which is published as supporting information on the PNAS web site, and Fig. 3) as hosts to express engineered *dptBC* genes, and we used heterologous *lptD* and *cdaPS3* in place of *dptD*, with and without the *dptI* gene essential for the Glu₁₂ methylation. We used λ-Red-mediated recombination (27) to exchange new domains or modules into the *dptBC* gene at specific intradomain linker sites (outlined in Figs. 2 and 4).

Control experiments indicated that strain UA431 ($\Delta dptEFABCDGHJ$ mutant) was fully complemented when pDA300 containing *dptEFABCDGHJ* was inserted at the ϕ C31 *attB* site. Likewise, KN100 ($\Delta dptBCD$ mutant) was fully complemented by coexpress-

ing plasmids pKN24 (*dptBC*) and pRB04 (*dptD*), which integrate into the chromosome at ϕ C31 *attB* and IS117 *attB* sites, respectively. In KN125 (Δ *dptBCDGHJ* mutant), coexpression of pKN24 and pRB04 led to production of a series of compounds containing Glu₁₂ instead of 3mGlu₁₂ (26). Replacement of *dptD* by *cdaPS3* and *lptD* resulted in the production of novel compounds in which Kyn₁₃ was replaced by Trp and Ile or Val (because of the substrate flexibility of LptD), respectively (23, 24). To introduce and recombine new changes at positions 8 or 11 with those at positions 12 and 13, plasmids with module exchanges in *dptBC* were introduced into a series of KN100- and KN125-derived strains carrying pRB04 (*dptD*), pMF23 (*cdaPS3*), or pMF30 (*lptD*) (Table 3 and Fig. 3).

Exchange of Homologous Modules Within DptBC. New domains/modules can be introduced into DptBC between splicing sites at the ends of CAT or CATE modules: two sites are within the T–C linker (TC1 and TC2) and one is within each of the T–E or E–C linkers (TE1 and EC1; Fig. 4), giving four possible configurations for CAT or CATE exchanges. Modules 8 and 11, comprising the CA₁₀TE

Fig. 2. λ -Red-mediated recombination for construction of hybrid *dptBC*. The coding region of domains (CAT, in this example) of module D-Ala₈ was replaced by an antibiotic resistance cassette flanked by rare restriction sites. The cassette was then excised, and the linearized plasmid was used in ligation, at the compatible restriction sites, with the gap repair cloned fragments coding for other domains.

Fig. 3. Daptomycin gene content in deletion mutants and expression plasmids. (A) NRP5 deletion mutants as expression hosts. (B) Expression plasmids for hybrid NRP5 pathways. Relative locations of domain and module exchanges on *dptBC* are marked by filled squares. Promoters that may drive expression of *dpt* subunit genes are indicated as bent arrows.

and CA_{Ser}TE domains, are highly homologous (12), making them good candidates for reciprocal module exchanges. Because leaving the native E domains in place may preserve homologous intermodule and interpeptide interactions, exchanges of CAT were made in pDA300, which carries the entire *dpt* NRPS gene cluster (Fig. 3B). λ -Red-mediated recombination (27) was used to introduce a *tetA* gene to delete the DNA sequence coding for CA_{Ala}T domains of the D-Ala₈ module between TC2 and TE1 (Figs. 2 and 4). The *tetA* gene then was replaced by a DNA sequence coding for CA_{Ser}T from the D-Ser₁₁ module (Fig. 2), resulting in pDR2153. A reciprocal construct (pDR2158) in which CA_{Ser}T of D-Ser₁₁ was replaced by CA_{Ala}T was made in a similar manner. To monitor the effects of inserting restriction sites in the linker region, CA_{Ala}T and CA_{Ser}T also were reintroduced at positions 8 and 11, respectively.

S. roseosporus UA431 strains expressing either one of these control plasmids produced native lipopeptides at control levels (200–250 mg/liter). When pDR2153 containing CA_{Ser}T exchanged for CA_{Ala}T was introduced into UA431, lipopeptides with mass ions of 1,636, 1,650, and 1,664 were produced, consistent with the incorporation of D-Ser at position 8. Similarly, when pDR2158 containing CA_{Ala}T exchanged for CA_{Ser}T was introduced into UA431, three mass ions of 1,604, 1,618, and 1,632 were detected, consistent with the incorporation of D-Ala at position 11. The levels of production of the lipopeptides were 35 mg/liter and 100 mg/liter, respectively, and the purified compounds were as potent as daptomycin against *S. aureus* (Table 1).

Exchange of Single Heterologous Modules. To introduce the D-Asn₁₁ module from the A54145 pathway (13) at positions 8 and 11, we made module exchanges in pKN24 expressing only *dptBC* (Fig. 3B). Fusions at the splicing sites TC1 and TE1 or EC1 generated four CA_{Asn}T or CA_{Asn}TE exchanges at positions 8 and 11 (see Fig. 4). The engineered plasmids were introduced into KN156, lacking only *dptBC*, and recombinants containing CAT inserted at TC1–TE1 or CATE at TC1–EC1 produced mass ions of 1,677, 1,691, and 1,705, consistent with the incorporation of D-Asn at position 8, and 1,660, 1,674, and 1,688, consistent with the incorporation of D-Asn at position 11. The yields of compounds containing D-Asn₈ or D-Asn₁₁ were 19 and 33 mg/liter in CAT exchanges and 6 and 19 mg/liter in CATE exchanges, respectively. Similar results were obtained when D-Asn₁₁ was introduced by using the splicing site TC2 in combination with TE1 or EC1 sites (Fig. 4). These results indicate that functional module exchanges can be made at different sites in the linkers, that linker regions can be modified to accommodate new restriction sites, and that the native E domains of the D-Ala₈ and D-Ser₁₁ modules can catalyze the epimerization of L-Ala, L-Ser, and L-Asn. CAT exchanges that left the native E domain in place seemed to be superior to CATE exchanges, perhaps because the native E domain may provide better protein–protein interactions between adjacent modules in *dptBC* and between the hybrid *dptBC* and native *dptD* proteins. The purified compound containing D-Asn₁₁ had antibiotic activity comparable with that of daptomycin, but that containing D-Asn₈ was less active (Table 1).

Fig. 4. Amino acid sequence alignment of linkers of modules from the biosynthetic pathways of *S. roseosporus* daptomycin (Sr), *S. fradiae* A54145 (Sf), and *Streptomyces coelicolor* CDA (Sc). These modules incorporate the amino acid residues specified in single-letter codes on the left. Fusion sites are indicated. The * marks the stop of LptB followed by the first amino acid residue of LptC.

Discussion

Combinatorial engineering of polyketide biosynthetic pathways has been extensively explored (29), but only a small number of successful *in vivo* exchanges of single modules have been reported for NRPS-derived compounds (15–18). In this study, we used combinatorial biosynthesis to generate daptomycin analogs. We demonstrated exchange of single and multiple modules from heterologous pathways, and we combined them with subunit exchanges, blockage of Glu methylation, and natural variation in side-chain lipidation to generate a library of hybrid lipopeptide pathways. Thirty novel NRPS biosynthetic pathways were constructed, and many produced novel active antibiotics in quantities sufficient for unambiguous analytical verification, purification, and antimicrobial characterization. These results are significant because many factors are required for successful engineering of NRPS pathways, including functional protein–protein interactions within and between enzyme subunits, effective peptide coupling, and cyclization of hybrid compounds (16–18). Interestingly, in one experiment, the six-module subunit DptBC was successfully replaced by a new arrangement comprising a truncated two-module subunit (DptB*) that was required to interact with a four-module subunit (LptC) to function. There are no apparent dedicated interpeptide docking sequences between LptB and LptC normally (13), so the interaction between DptB* and LptC may function by a different mechanism, perhaps translational frameshifting, to bypass the stop codon. Biochemical studies (30) suggested the native A54145 NRPS is composed of two large and one small subunit, instead of four subunits, consistent with read-through of the stop codon between *lptB* and *lptC*.

The building of novel compounds around the scaffold of daptomycin has helped to initiate a deeper understanding of structure–activity relationships in the cyclic peptide core. Although none of the hybrid compounds produced by combinatorial biosynthesis is superior to daptomycin, several have equivalent potencies. It is clear from this and other work (23, 25) that the amino acids present at positions 12 and 13 play key roles in determining antibacterial potency, whereas substitutions at position 11 seem to have little effect (Table 1). Substitutions at position 8 were well tolerated in the case of Ser and Lys (in conjunction with Asn at position 11), but substitution of Asn reduced the potency. The disubstitution of Lys₈ and Asn₁₁ was particularly interesting in that it generated a compound with an MIC of 1 μ g/ml. This result indicated that although the replacement of the small and hydrophobic D-Ala by the larger, basic D-Lys and D-Ser by D-Asn increases the basic charge from +1 to +2 and net charge from –3 to –2, it does not affect the antibiotic potency significantly in daptomycin-sensitive strains. However, the compound was less active than daptomycin against strains with reduced susceptibility to daptomycin, indicating that the overall charge on the molecule may be an important factor to consider when designing molecules with increased activity against daptomycin-resistant pathogens.

The compounds described in this study represent only a fraction of the possibilities offered by combinatorial biosynthesis. The significant structural diversity already generated can be further expanded by medicinal chemistry toward an even larger set of molecules to explore as potential candidates for clinical development: e.g., more extensive substitutions of the fatty acid tail, which previous studies have shown can have a pronounced effect on potency and toxicity of daptomycin related lipopeptides (2, 3, 10), and modifications of the amino and hydroxyl groups of the introduced residues such as D-Lys₈ and D-Ser₈, respectively. Introduction of modules specific for modified amino acids together with their tailoring enzymes (e.g., hydroxylase and methyltransferase for Asp) would provide additional options to generate diversity.

In conclusion, this study has demonstrated that combinatorial biosynthesis is an effective approach to generate novel lipopeptides related to daptomycin, an important antibiotic recently approved for clinical use that cannot be modified effectively by medicinal

chemistry alone. The combinatorial biosynthesis methodology described here generated many new derivatives with modifications in the core peptide that have provided valuable information on features of the molecule required for good antibacterial activity. The demonstration of functional module exchanges *in vivo* at different interdomain linker sites should be applicable to other secondary metabolites produced by NRPS or mixed NRPS/polyketide synthetase pathways.

Materials and Methods

Media, Growth Conditions, and General Methods. Bacterial strains and plasmids are given in Table 3. *S. roseosporus* strains were grown at 30°C on AS-1 agar or liquid TSB medium (12). Lipopeptides produced in fermentations were identified by electrospray-ionization liquid chromatography-MS, quantified by HPLC using daptomycin as the standard, and purified as described (23). Plasmids were transformed into *E. coli* by electroporation and transferred to *S. roseosporus* by conjugation (23). MICs were determined by using microdilution techniques in calcium-supplemented Mueller Hinton broth (MHB) (12). *E. coli* strains were grown in LB medium (31) at 37°C except when a temperature-sensitive λ -Red plasmid (pKD78 or pKD119) was present. For the latter, cells were grown at 30°C and λ -Red expression was induced for 2 h by L-arabinose (final 0.2% wt/vol) (27). *S. aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *S. pneumoniae* strains were grown at 37°C in MHB. Antibiotics were added into media at the following final concentrations: 100 μ g/ml ampicillin (Ap); 50 μ g/ml apramycin (Am); 25 μ g/ml chloramphenicol (Cm); 50 μ g/ml gentamicin (Gen); 50 μ g/ml hygromycin (Hyg); 50 μ g/ml kanamycin (Kan); 50 μ g/ml nalidixic acid (Nal); 50 μ g/ml spectinomycin (Spc); 15 μ g/ml tetracycline (Tet); and 50 μ g/ml thiostrepton (Ts). The following antibiotic resistance cassettes were PCR-amplified and used in plasmid deletions by λ -Red-mediated recombination: *aad9*(Spc^r) [National Center for Biotechnology Information (NCBI) accession no. AP002527], *aph*(2'')-Ib(Gen^r) (NCBI accession no. AF207840) available under the control of the phage T5 promoter integrated into the *E. coli* chromosome (CM141; C. Monahan, personal communication), *bla*(Ap^r) (NCBI accession no. J01749), and *tetA*(Tet^r) (NCBI accession no. J01749) available from a multiple-component cassette (K.T.N., unpublished data).

Construction of Deletion Cassettes. λ -Red-mediated recombination (27) was used to introduce PCR-generated cassettes [Long Template Expand PCR kit (Roche, Indianapolis, IN) or Advantage GC-2 polymerase mix (Clontech, Mountain View, CA)] and delete targeted sequences of the cloned *dpt* gene cluster. The cassettes may contain a resistance gene [*aad9*, *aph*(2'')-Ib, *bla*, or *tetA*] alone or in combination with the strong promoter *ermEp** (32). Primers (Table 5, which is published as supporting information on the PNAS web site) used for amplification of the deletion cassette were composed of a terminal 45- to 50-nt gene-specific sequence for targeted recombination and a proximal 20-nt universal sequence (single-underlined in Table 5) for portable use of these primers with multiple-resistance gene templates. Some of these cassettes are flanked by engineered restriction sites AvrII or PmeI (double-underlined in Table 5). The PCR products were purified, concentrated to 100 ng/ μ l, and used to electroporate into arabinose-induced *E. coli* BW25113 carrying a λ -Red system-expressing plasmid (pKD78 or pKD119) and the targeted Am^r plasmid (pCV1, pDA300, pDR2153, pDR2158, or pKN24). Plasmids recovered from transformants grown on AS-1 agar containing Am and Ap, Gen, Spc, or Tet were purified and checked by restriction analysis and sequencing of the fusion sites.

Construction of pDA300 and pKN24 by λ -Red-Mediated Recombination. pDA300 and pKN24 were constructed by truncation of pCV1, a BAC clone that contains the daptomycin biosynthetic gene cluster (NCBI accession no. AY787762) (12). For construction of pDA300,

the region upstream of the *dptMN* locus (Figs. 1 and 3) was replaced by *aad9* amplified by the primers Sp6 del3 and Sp6 del4 (Table 5). The region downstream of *dptI* (Figs. 1 and 3) was replaced by *bla* using the primers GTC del1 and GTC del2. For construction of pKN24, the region upstream of *dptBC* (nucleotides 191 to 69,057, NCBI accession no. AY787762) was replaced by an *aad9-ermEp** cassette PCR-amplified by using primers Sp6Del-1-2 and *dptBC-ermEp* from a template (D.A., unpublished data) containing the *aad9* gene, followed by the *ermEp** promoter and an optimized ribosome binding site (32). The region downstream of *dptBC* (nucleotides 91,140 to 127,346) then was replaced by the *bla* gene with primers DptD-3'::amp and primer GTC del2.

Deletion of Domains and Modules by λ -Red-Mediated Recombination.

Deletions at modules D-Ala₈ or D-Ser₁₁ were done by using splicing sites in the T-C linker (TC1 or TC2) and in the T-E linker (TE1) or the E-C linker (EC1) (Figs. 2 and 4). For TC2-TE1 deletions, the primers Ala8_AvrII-left and Ala8_PmeI-right were used to amplify the deletion *tetA* cassette. For TC1-TE1 deletions at modules 8 and 11, the primer 8.B-AvrII and 8.CAT-PmeI were used to amplify the *aph*(2") gene. As the homologous regions of the primers are almost identical for the D-Ala₈ and D-Ser₁₁ linker regions, individual disruptions of both modules were obtained by using the same linear PCR cassette. Because there is a single base mismatch (C6339G) in the T-C linker preceding modules 8 and 11, recombination introduced an Ala to Gly mutation in the T-C linker preceding the D-Ser₁₁ module. For TC1-EC1 deletions at module 8 or 11, the *aph*(2") gene was amplified by primer 8.B-AvrII and 8-CATE2-PmeI or 11-CATE2-PmeI. By using a similar protocol, the DNA sequence coding for D-Ala₈-Asp₉-Gly₁₀-D-Ser₁₁ on pKN24 was replaced by *aph*(2") amplified by using primers 8.B-AvrII and 11.SuE-PmeI; the latter anneals to a sequence downstream of the *dptBC* stop codon.

Gap-Repair Cloning by λ -Red-Mediated Recombination. The 3.1-kb DNA fragments coding for the CAT domains of D-Ala₈ and D-Ser₈ were cloned from pCV1 by the "gap-repair" method using λ -Red-mediated recombination (Fig. 2). Primers Ala-8-gap L and Ala-8-gap R were used to amplify the origin of replication and *bla* region of pBR322. The PCR product has 45 bp at each end that are homologous to the 5' and 3' ends of the CAT domains of both

D-Ala₈ and D-Ser₈ modules (which are 100% identical in nucleotide sequence), flanked by unique NheI (5' end) and HpaI (3' end) sites. After transformation of this PCR product into arabinose-induced *E. coli* BW25113 carrying pKD78 and pCV1, recombination between the 45-bp terminal sequences of the linear pBR322 PCR fragment and the homologous regions in pCV1 fills in the gap between the ends of pBR322 with fragments from the *dpt* gene cluster to generate a circular pBR322 derivative that confers Ap^r to *E. coli*. The insert ends were checked by sequencing. To clone modules from the A54145 biosynthetic pathway, pBR322 PCR fragments were transformed into *E. coli* BW25113 carrying pKD119 and pCB01. For cloning the D-Asn₁₁ module, PCR product was generated by using primers Lpt-N11-B-P13 and Lpt-N11-CAT-P14 (for the TC1-TE1 fragment) or Lpt-N11-CATE2-P14 (for the TC1-EC1 fragment). For cloning D-Lys₈-OmAsp₉-Gly₁₀-D-Asn₁₁, PCR primers Lpt-K8-B-P13 and Lpt-N11-SUE-P14 were used. The inserts were excised by NheI and HpaI digestion and gel-purified.

Construction of Hybrid *dptBC* Plasmids. The antibiotic cassette that had been inserted to replace modules in pKN24 was excised by digestion with AvrII and PmeI, and the linearized plasmid was ligated to compatible NheI and HpaI restriction sites of the above-described fragments (Fig. 2). The ligation mix was redigested with AvrII to eliminate self-ligated plasmids, dialyzed, and transformed into *E. coli* DH10B. The final hybrid plasmids were recovered from colonies grown on LB plates containing Am and checked by restriction digestion and sequencing of the fusion sites.

Deletion of *dptD* and the *dptGHII* Locus in pDA300 Derivatives (pDR2153 and pDR2158). *dptD* and *dptGHII* in pDR2153 and pDR2158 were replaced by the *aph*(2") cassette using λ -Red-mediated recombination. The cassette was amplified by using primers dptD-del1 and dptD-del3.

We dedicate this paper to the memory of Dr. Francis P. (Frank) Tally. We thank C. Monahan (Cubist Pharmaceuticals) for providing *E. coli* CM141, S. Doekel (Cubist Pharmaceuticals) for providing pCB01, X. He and V. Rajgarhia for fermentation support, D. Kau and S. Wrigley for purification and liquid chromatography-MS analyses of some compounds, N. Cotroneo for MIC determinations, and J. Silverman and J. Alder for comments on the manuscript. This work was supported in part by National Institutes of Health Small Business Innovation Research Grant 2 R44 GM068173-08.

- Debono M, Barnhart M, Carrell CB, Hoffmann JA, Occolowitz JL, Abbott BJ, Fukuda DS, Hamill RL, Biemann K, Herlihy WC (1987) *J Antibiot* 40:761-777.
- Debono M, Abbott BJ, Molloy RM, Fukuda DS, Hunt AH, Daupert VM, Counter FT, Ott JL, Carrell CB, Howard LC, et al. (1988) *J Antibiot* 41:1093-1105.
- Baltz RH, Miao V, Wrigley SK (2005) *Nat Prod Rep* 22:717-741.
- Huber FM, Pieper RL, Tietz AJ (1988) *J Biotechnol* 7:283-292.
- Arbeit RD, Maki D, Tally FP, Campanaro E, Eisenstein BI (2004) *Clin Infect Dis* 38:1673-1681.
- Eisenstein BI (2004) *Expert Opin Investig Drugs* 13:1159-1169.
- Raja A, LaBonte J, Lebbos J, Kirkpatrick P (2003) *Nat Rev Drug Discov* 2:943-944.
- Schriever CA, Fernandez C, Rodvold KA, Danziger LH (2005) *Am J Health Syst Pharm* 62:1145-1158.
- Fowler VG, Boucher HW, Corey GR, Abrutyn E, Karchmer AW, Rupp ME, Levine DP, Chambers HF, Tally FP, Vigliani GA, et al. (2006) *N Engl J Med* 355:653-665.
- Hill J, Siedlecki J, Parr I, Morytko M, Yu X, Zhang Y, Silverman J, Controneo N, Laganas V, Li T, et al. (2003) *Bioorg Med Chem Lett* 13:4187-4191.
- Grünewald J, Sieber SA, Mählert C, Linne U, Marahiel MA (2004) *J Am Chem Soc* 126:17025-17031.
- 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) *Microbiology* 151:1507-1523.
- Miao V, Brost R, Chapple J, She K, Coëffet-Le Gal M-F, Baltz RH (2006) *J Ind Microbiol Biotechnol* 33:129-140.
- Hojati Z, Milne C, Harvey B, Gordon L, Borg M, Flett F, Wilkinson B, Sidebottom PJ, Rudd BA, Hayes MA, et al. (2002) *Chem Biol* 9:1175-1187.
- Stachelhaus T, Schneider A, Marahiel MA (1995) *Science* 269:69-72.
- Schwarzer D, Finking R, Marahiel MA (2003) *Nat Prod Rep* 20:275-287.
- Finking R, Marahiel MA (2004) *Annu Rev Microbiol* 58:453-488.
- Sieber SA, Marahiel MA (2005) *Chem Rev* 105:715-738.
- Hahn M, Stachelhaus T (2004) *Proc Natl Acad Sci USA* 101:15585-15590.
- Hahn M, Stachelhaus T (2006) *Proc Natl Acad Sci USA* 103:275-280.
- Kohli RM, Walsh CT (2003) *Chem Commun (Camb)* 3:297-307.
- Grünewald J, Marahiel MA (2006) *Microbiol Mol Biol Rev* 70:121-146.
- Miao V, Coëffet-Le Gal M-F, Nguyen K, Brian P, Penn J, Whiting A, Steele J, Kau D, Martin S, Gibson T, et al. (2006) *Chem Biol* 13:269-276.
- Coëffet-Le Gal M-F, Thurston L, Rich P, Miao V, Baltz RH (2006) *Microbiology* 152:2993-3001.
- Baltz RH, Brian P, Miao V, Wrigley SK (2006) *J Ind Microbiol Biotechnol* 33:66-74.
- Nguyen KT, Kau D, Gu JQ, Brian P, Wrigley S, Baltz RH, Miao V (2006) *Mol Microbiol* 61:1294-1307.
- Datsenko KA, Wanner BL (2000) *Proc Natl Acad Sci USA* 97:6640-6645.
- Eggert US, Ruiz N, Falcone BV, Branstrom AA, Goldman RC, Silhavy TJ, Kahne D (2001) *Science* 294:361-364.
- Weissman KJ, Leadlay PF (2005) *Nat Rev Microbiol* 3:925-936.
- Wessels P, von Döhren H, Kleinkauf H (1996) *Eur J Biochem* 242:665-673.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Plainville, NY).
- Bibb MJ, Janssen GR, Ward JM (1985) *Gene* 38:215-226.