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Rational Design of a Bimodular Model System for the Investigation of Heterocyclization in Nonribosomal Peptide Biosynthesis

Thomas Duerfahrt,[1] Katrin Eppelmann,[1,3] Rolf Müller,[2] and Mohamed A. Marahiel[1,*]
Philipps-Universität Marburg
Fachbereich Chemie/Biochemie
Hans-Meerwein-Strasse
35043 Marburg
[2]Gesellschaft für Biotechnologische Forschung mbH (GBF)
Mascheroderweg 1
38124 Braunschweig
Germany

Summary

Cyclization (Cy) domains in NRPS catalyze the heterocyclization of cysteine and serine/threonine to thiazoline and oxazoline rings. A model system consisting of the first two modules of bacitracin synthetase A fused to the thioesterase (Te) domain of tyrocidine synthetase was constructed (BacA1-2-Te) and shown to be active in production of the heterocyclic IleCys$_{thiazoline}$. Based on this model system, the feasibility of Cy domain module fusions was investigated by replacing the BacA2 Cy-A-PCP-module with modules of MbtB and MtaD from the biosynthesis systems of mycobactin and myxothiazol, revealing the formation of novel heterocyclic dipeptides. To dissect the reaction sequence of the Cy domain in peptide bond formation and heterocyclization, several residues of the BacA1-2-Te Cy domain were analyzed by mutagenesis. Two mutants exhibited formation of the noncyclic dipeptide, providing clear evidence for the independence of condensation and cyclization.

Introduction

Numerous microorganisms utilize a nonribosomal process for the biosynthesis of small peptides that are mainly products of secondary metabolic pathways. Synthesis of these structurally diverse and pharmacologically interesting compounds is often mediated by multimodular nonribosomal peptide synthetases (NRPSs) as well as hybrid NRPS/polyketides synthase (PKS) systems [1]. Each module within these multifunctional NRPSs represents a functional unit of catalytic domains that is responsible for recognition, activation, and incorporation of one amino acid into the growing peptide product. An adenylation (A) domain specifically activates the substrate amino acid in an ATP-dependent reaction to generate the corresponding aminoacyl adenylate [2, 3]. Subsequently, the aminoacyl moiety is covalently bound to the 4′-phosphopantetheine cofactor (Ppant) of the adjoining peptidyl carrier protein (PCP) domain [4, 5]. Peptide bond formation between two adjacent aminoacyl/peptidyl-S-Ppant intermediates is catalyzed by a condensation (C) domain [6, 7]. These three core domains constitute the minimal elongation module C-A-PCP of NRPSs [8]. The release of the final peptide product is usually catalyzed by a thioesterase-like (Te) domain C terminally appended to the very last module [9, 10]. The number and organization of the iterated modules within the enzymatic NRPSs template determine the primary structure, size, and complexity of the product synthesized. The diversity of nonribosomal peptides can be further enlarged by optional domains that catalyze, e.g., epimerization [11], *N*-methylation [12], or *O*-methylation [13, 14] and are integrated within the corresponding module.

While integrated optional domains only lead to modification of the corresponding substrate amino acids, variations of the peptide backbone can be obtained by the replacement of C domains with heterocyclization (Cy) domains in modules processing either cysteine or serine/threonine residues [15]. The remarkable Cy domains catalyze amide bond formation, cyclization of the side chain nucleophile with the newly formed amides, and final dehydration and are therefore responsible for the formation of five-membered heterocyclic rings of the thiazoline and oxazoline family [16]. The sequential order of Cy domain-catalyzed reactions has not yet been determined but is thought to involve separate condensation and heterocyclization steps [17]. Once the heterocycle is formed, it can be further modified by the action of additional modifying domains [16]. Reductases like the NADPH-dependent PchG of pyochelin synthetase reduce the thiazoline heterocycle to the tetrahydro thiazolidine ring [18]. In contrast, FMN-containing oxidase (Ox) domains [19], which are present, for instance, within the hybrid PKS/NRPS of epothilone [20, 21], bleomycin [22], and myxothiazol [22] biosynthesis templates, oxidize the dehydro heterocycle by a two-electron transfer to give rise to the heteroaromatic thiazole or oxazole ring, respectively.

Cy domains and their accessory modifying domains lead to the formation of structurally diverse heterocycles with remarkable biochemical properties such as chelation of metal ions and interaction with protein, DNA, and RNA targets. Consequently, heterocyclic ring-containing peptides are of enormous pharmacological importance, as documented, i.e., by the antibiotic bacitracin A (**1**) [23, 24] as well as the antitumor agents bleomycin [25] and epothilone A (**2**) [26] (see Figure 1). Therefore, Cy domains represent an attractive tool for the engineering of hybrid NRPSs and the rational design of heterocyclic ring-containing compounds with potentially novel biological activities.

In order to establish the promising biocombinatorial potential of Cy domain-containing modules, we report here on the construction and exploitation of a bimodular model system for the investigation of heterocyclization in NRPS assembly lines. The model system constructed consists of the first two modules of the bacitracin biosynthesis template (BacA1-2, A-PCP-Cy-A-PCP; see

*Correspondence: marahiel@chemie.uni-marburg.de
[3] Current address: DSM Biotech GmbH, Karl-Heinz-Beckurts-Straße 13, 52428 Jülich, Germany.

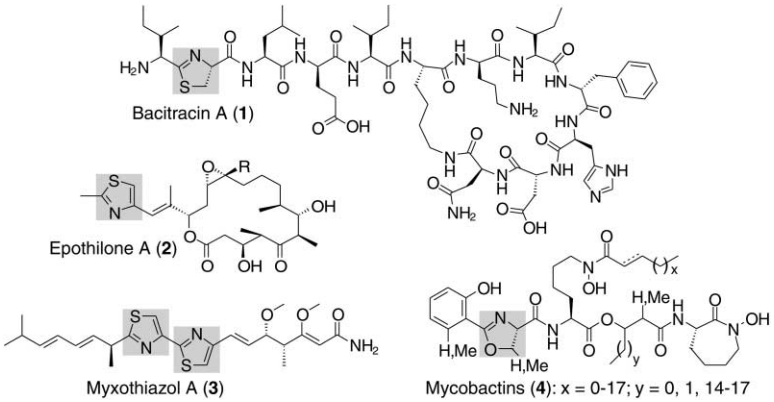

Figure 1. Five-Membered Heterocyclic Ring-Containing Secondary Metabolites from Different Microorganisms

Shown are bacitracin A (**1**) (*Bacillus licheniformis* ATCC 10716), epothilone A (**2**) (*Sorangium cellulosum* So ce90), myxothiazol (**3**) (*Stigmatella aurantiaca* DW4/3-1), and mycobactin A (**4**) (*Mycobacterium tuberculosis*). Heterocyclic rings are shaded in gray.

Figure 2), which are responsible for the formation of the N-terminal, thiazoline-containing dipeptide moiety $IleCys_{thiazoline}$ of the peptide antibiotic bacitracin A (**1**). The Te domain of the tyrocidine synthetase C was C terminally appended to the hybrid synthetase to ensure a catalytic product release.

The functionality of the artificial model NRPS BacA1-2-Te was demonstrated by the production of the predicted dipeptide $IleCys_{thiazoline}$. The sequential order between condensation and heterocyclization was examined by means of mutational studies. In addition, the genetic exchange of module BacA2 (Cy-A-PCP) with other Cy domain-containing modules derived from the myxothiazol (**3**) [14] and mycobactin (**4**) [27] NRPS biosynthesis clusters was used to demonstrate the practicability of module fusions with these special kinds of heterocyclic ring-forming modules. All rationally designed hybrid NRPS systems were capable of producing the respective oxazoline, oxazole, and thiazole-containing dipeptides.

Results and Discussion

In order to investigate heterocyclization reactions in nonribosomal peptide synthesis, we constructed an artificial bimodular NRPS model system that is based on the well-characterized biosynthesis cluster of bacitracin A (**1**). This branched cyclic thiazoline-containing dodecapeptide is produced in *Bacillus licheniformis* ATCC 10716 on a protein template consisting of the three NRPSs BacABC (see Figure 2A). Within the second module of BacA, the optional Cy domain, catalyzing formation of the N-terminal thiazoline ring, replaces the usual C domain.

The Bimodular Model System BacA1-2-Te

The bimodular model system that we constructed consists of the first two modules of the bacitracin biosynthetic system (BacA1-2, A-PCP/Cy-A-PCP; Figure 2A). The two modules specifically activate isoleucine and cysteine, respectively, and are capable of forming the dipeptide $IleCys_{thiazoline}$. In order to obtain multiple catalytic turnovers, we fused the Te domain of tyrocidine synthetase C (TycC) to the C terminus of the bimodular system. Te domains of different NRPSs catalyze the release of the respective peptide products. They have been shown to possess a broad substrate selectivity and to be functional when artificially fused in hybrid NRPSs [10]. Following previously described strategies, the TycC-Te domain was genetically appended to BacA1-2 so that the fusion site between both enzymes was located 39 amino acids downstream of the invariant serine residue (→ Ppant binding site) of the second PCP [8, 28]. Thus, the resulting model system BacA1-2-Te obtained had the domain order $[A(Ile)\text{-}PCP\text{-}Cy\text{-}A(Cys)\text{-}PCP]_{BacA1\text{-}2}\text{-}[Te]_{TycC6}$ (Figure 2D). For further biochemical characterization, the bimodular NRPS was heterologously produced in *E. coli* and purified as described in Experimental Procedures.

A prerequisite for the formation of the expected dipeptide $IleCys_{thiazoline}$ by the model system BacA1-2-Te is the integrity and activity of its catalytic domains, especially those involved in substrate recognition and activation. To investigate the activity of the A domains, the bimodular model enzyme was first subjected to amino acid-dependent ATP-PP_i-exchange reactions, whereby cognate and noncognate amino acids were tested [29]. As shown in Figure 3A, BacA1-2-Te revealed highest exchange activities for the expected substrates isoleucine (100%) and cysteine (84%) and only low side selectivity for the noncognate amino acids leucine (10%), valine (8%), and serine (4%).

After formation of the aminoacyl adenylate by the A domain, activated amino acids are covalently tethered to the Ppant moiety of the adjacent PCP domain. In order to probe the posttranslational modification of the PCPs with cofactor Ppant as well as the communication between A and PCP domains, covalent loading assays using radiolabeled amino acids were conducted [29]. As expected, both substrate amino acids, Ile and Cys, could be efficiently loaded onto the Ppant-carrier arm of the PCP domains in the presence of ATP (data not shown).

After the activity of the individual domains had been confirmed, we next investigated the capability of the bimodular model NRPS to catalyze the formation of the expected dipeptide product. To this end, BacA1-2-Te was incubated with ATP, isoleucine, and cysteine. At certain time points, samples were taken and immediately quenched and analyzed by HPLC/MS. As shown in Figure 3B, incubation of BacA1-2-Te with its substrates led to the time-dependent formation of the expected thiazoline-containing product isoleucinyl-thia-

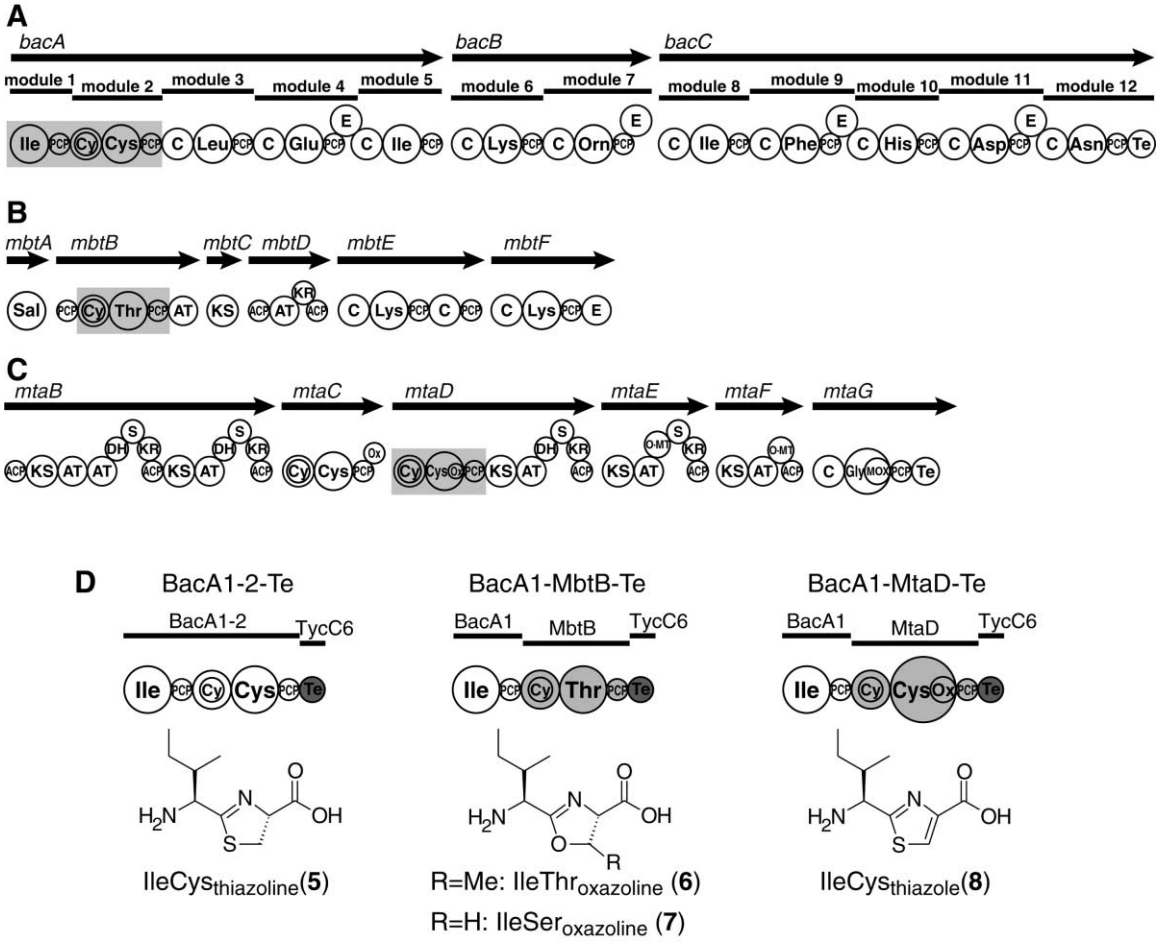

Figure 2. Biosynthesis Systems from which the Used Domains Originate and the Constructed Hybrid Peptide Synthetases

(A) The three enzymes involved in biosynthesis of bacitracin A are encoded by the genes *bacABC*. The Cy domain is located between the first and second module.

(B) Mycobactin synthesis is catalyzed by a PKS/NRPS hybrid system. The NRPS module of MbtB contains a threonine-processing Cy domain.

(C) Myxothiazol is also produced by a PKS/NRPS biosynthesis system. Two Cy domains are located in MtaC and MtaD. Domains and modules utilized in this study are shaded in gray.

(D) Hybrid bimodular NRPSs investigated in this study and their expected catalytic products. The expected product of this model synthetase BacA1-2-Te is the thiazoline-containing dipeptide $IleCys_{thiazoline}$ (5). The hybrid synthetases BacA1-MbtB-Te and BacA1-MtaD-Te were assumed to produce the oxazoline-containing products $IleThr_{oxazoline}$ (6) and $IleSer_{oxazoline}$ (7) (C) as well as the thiazole-product $IleCys_{thiazole}$ (8). Adenylation domains are identified by the three-letter code of the substrate. C, condensation domain; Cy, heterocyclization domain; E, epimerization domain; PCP, peptidyl carrier protein; Te, thioesterase domain; MOX, monooxygenase domain; O-MT, O-methyl transferase; Ox, oxidation domain; ACP, acyl carrier protein; AT acyl transferase; DH, β-hydroxy-acyl-ACP dehydratase; KR, β-ketoacyl-ACP reductase; KS β-ketoacyl-ACP synthase; S, spacer region.

zolinyl-cysteine ($IleCys_{thiazoline}$) (5). The identity of this product was confirmed by coupled ESI-MS analysis (at 14.3 min: $[M+H]^+ = 217$ *m/z*, calculated mass 217 *m/z*; $[M+Na] = 239$ *m/z*). In addition, a byproduct was detected at 17.2 min (retention time), which could be assigned to the thiazoline-containing tripeptide isoleucinyl-thiazolinyl-cysteinyl-cysteine (9) ($IleCys_{thiazoline}Cys$, calculated mass $[M+H]^+ = 320$ *m/z*; $[M+Na] = 342$ *m/z*). This byproduct is presumably formed by a noncatalyzed transthioesterification of the enzyme-bound $IleCys_{thiazoline}$-S-Ppant intermediate onto the thiol moiety of free cysteine molecules. Similar byproducts have already been reported for in vitro experiments with the yersiniabactin NRPS [30]. In a control reaction, we verified that no product was formed in the absence of one or the other substrate amino acid or ATP. The requirement of the Te domain for the product release is supported by analysis of the BacA1-2-enzyme, which has no Te fused. This construct shows absolutely no product formation (data not shown).

The presented data clearly show that fusion of the Cy domain-containing bimodule BacA1-2 with the TycC-Te domain led to the successful construction of a bimodular model NRPS, which can be exploited for the in vitro investigation of heterocyclization reactions in nonribosomal peptide biosynthesis. Both the activity of BacA1-2-Te and its robust turnover of the expected $IleCys_{thiazoline}$ product illustrate the practicability and potential of rationally designed hybrid NRPSs with Cy domain-containing modules. Furthermore, the result obtained for BacA1-

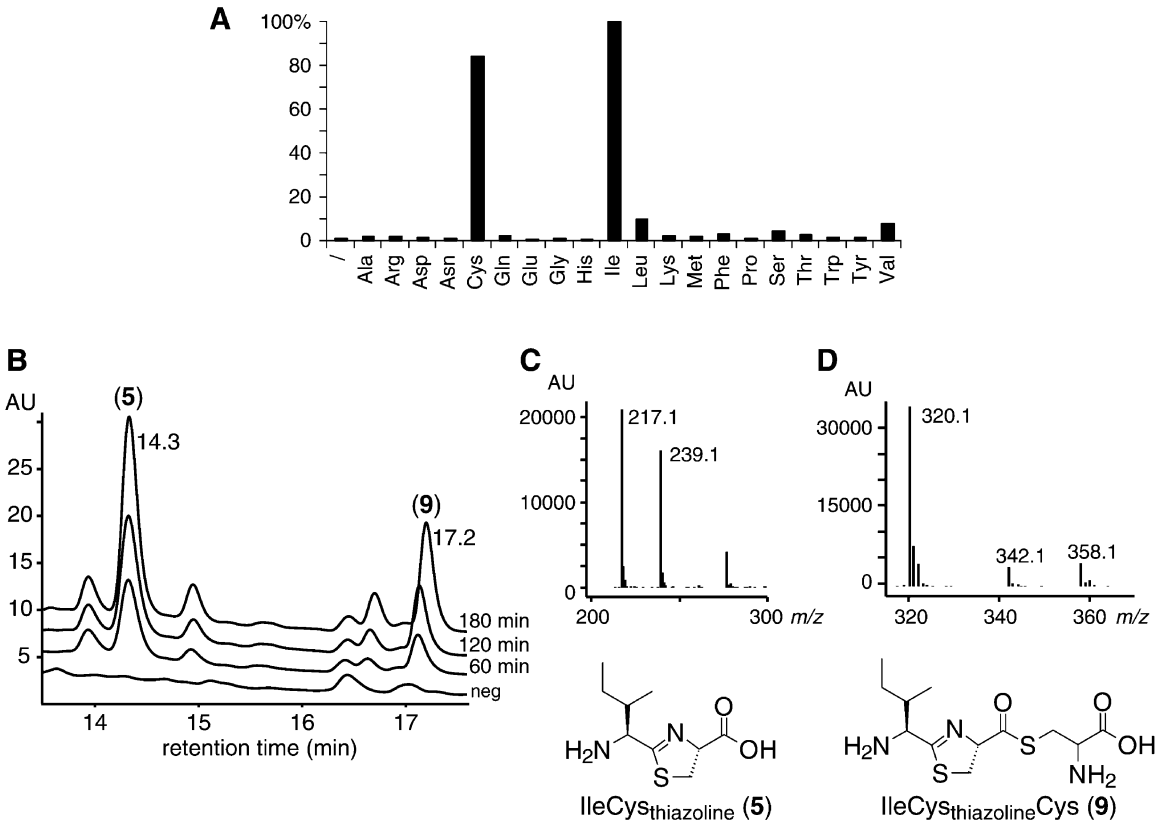

Figure 3. Biochemical Investigation of the Model System BacA1-2-Te

(A) The ATP-PP_i-exchange reaction of the model system BacA1-2-Te showed specific activation of isoleucine and cysteine.

(B) HPLC/MS analysis of product formation catalyzed by BacA1-2-Te showed time-dependent formation of two different products.

(C and D) The peaks at 14.3 min and 17.2 min could be assigned to the expected product $IleCys_{thiazoline}$ (**5**) ($[M+H]^+$ = 217 *m/z*; $[M+Na]^+$ = 239 *m/z*) and the noncatalytically formed product $IleCys_{thiazoline}Cys$ (**9**) ($[M+H]^+$ = 320 *m/z*; $[M+Na]^+$ = 342 *m/z*; $[M+K]^+$ = 358 *m/z*), respectively.

2-Te demonstrates once again the versatility of NRPS Te domains, since the TycC Te domain used in this work apparently also catalyzes the release of unusual products such as the thiazoline-containing dipeptide $IleCys_{thiazoline}$.

Hybrid Bimodular Constructs

The engineered bimodular model system BacA1-2-Te provides the basis for an exploitation of the biocombinatorial potential of Cy domain-containing modules. Up to now, analogous attempts for the construction of hybrid NRPSs were restricted to the fusion of modules consisting only of the basic core domains C, A, and PCP [8, 28, 31]. In order to demonstrate the practicality of comparable approaches for Cy domain-containing modules, we next set out to genetically substitute the original BacA2 Cy-A-PCP module of the model system with analogous modules derived from the mycobactin and myxothiazol NRPS cluster. The second module (MbtB) of the mycobactin NRPS of *Mycobacterium tuberculosis* has the domain arrangement Cy-A(Thr/Ser)-PCP (Figure 2B) and catalyzes the incorporation of the oxazoline heterocyclic ring (Figure 1) [27]. The fourth module (MtaD) of the myxothiazol biosynthetic cluster of *Stigmatella aurantiaca* DW4/3-1, in contrast, has the domain organization Cy-A(Cys)Ox-PCP (Figure 2C). Here, the oxidation (Ox) domain embedded between the core motifs A8 and A9 of the Cys-activating A domain catalyzes a two-electron transfer. Consequently, the corresponding module effects the incorporation of a heterocyclic thiazole ring (Figure 1).

For practical realization of the module swaps, the following two issues had to be kept in mind. First, it has been shown that C domains are selective for their respective acceptor amino acid, distinguishing both the chirality and the size of the side chain of the incoming monomeric nucleophile [32]. Taking into consideration the significant structural and functional homology between C and Cy domains, an analogous acceptor site selectivity of the Cy domains could not be excluded. Consequently, we decided to maintain the natural Cy-A junction and to use whole Cy-A-PCP modules. Second, based on strategies previously described for the construction of hybrid NRPSs, the optimal fusion site between PCP and C (or Cy) domains is located within short, nonconserved linker regions between the dedicated domains. Following this rule, the BacA1 module and the respective Cy domain-containing module were fused 38 amino acids downstream of the strictly conserved serine residue (→ Ppant binding site) of the PCP_{BacA1} domain and 21 or 23 amino acids upstream of the core motif Cy1 of the Cy domains of MtaD and MbtB, respectively

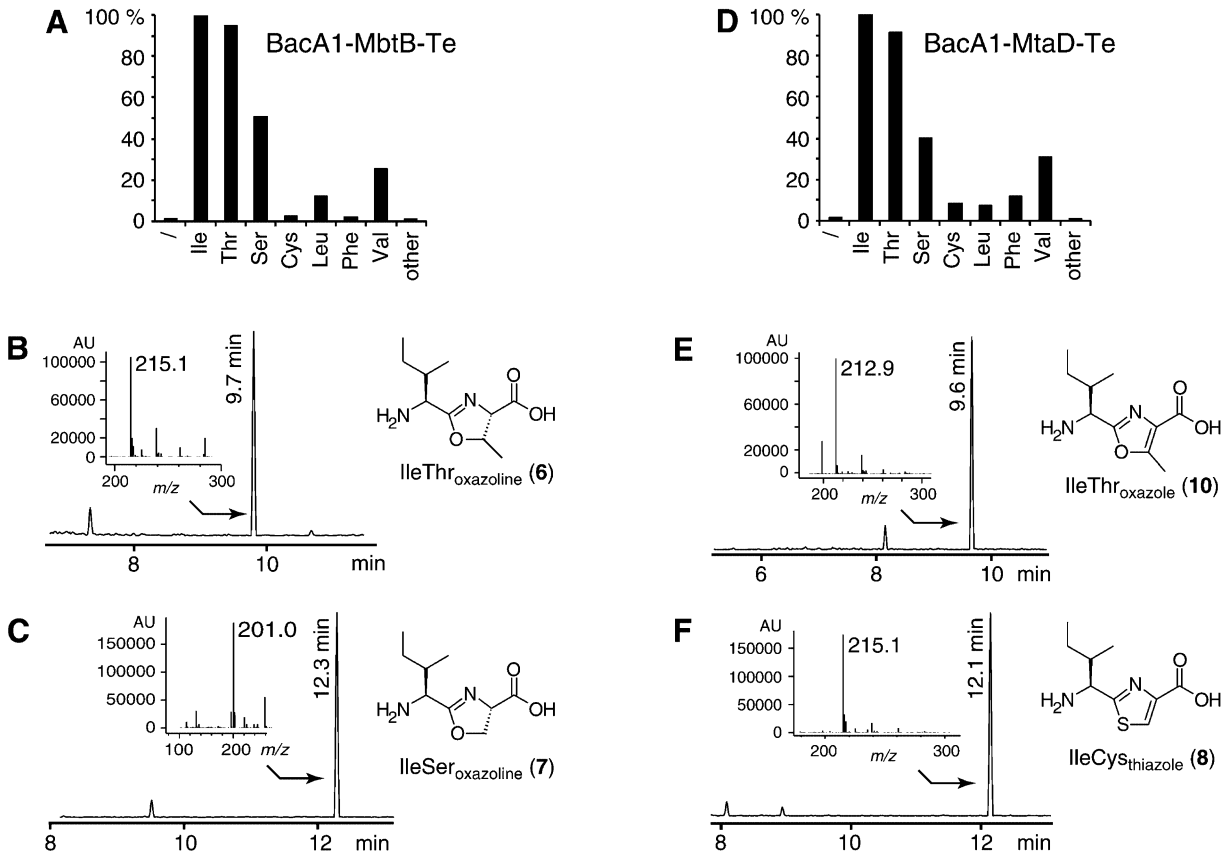

Figure 4. Biochemical Investigation of the Hybrid Synthetases BacA1-MbtB-Te and BacA1-MtaD-Te

(A) ATP-PP$_i$-exchange reaction showed expected activation of isoleucine, threonine, and serine by BacA1-MbtB-Te.

(B) Incubation of BacA1-MbtB-Te with the substrates isoleucine and threonine led to the formation of IleThr$_{oxazoline}$ (**6**) (9.7 min, $[M+H]^+ = 215$ *m/z*).

(C) Formation of IleSer$_{oxazoline}$ (7) (12.3 min, $[M+H]^+ = 201$ *m/z*) could be detected after incubation with isoleucine and serine.

(D) BacA1-Mta-Te recognized isoleucine, but only low activity could be observed for the second cognate amino acid cysteine. Instead, unexpected activation of threonine and serine was found.

(E) The hybrid synthetase BacA1-MtaD-Te was able to synthesize IleThr$_{oxazole}$ (**10**) (9.6 min, $[M+H]^+ = 213$ *m/z*) when incubated with isoleucine and threonine.

(F) IleCys$_{thiazole}$(**8**) (12.1 min, $[M+H]^+ = 215$ *m/z*) was found when the enzyme was provided with isoleucine and cysteine.

[15]. Likewise, and as already described for the construction of the model system BacA1-2-Te, the Te domain of TycC was fused 38 amino acids downstream of the conserved serine residue of the PCP domain of MtaD and MbtB.

Successive engineering led to the bimodular hybrid systems BacA1-MbtB-Te (domain arrangement [A(Ile)-PCP]$_{BacA1}$-[Cy-A(Thr)-PCP]$_{MbtB}$-[Te]$_{TycC6}$), and BacA1-MtaD-Te ([A(Ile)-PCP]$_{BacA1}$-[Cy-A(Cys)Ox-PCP]$_{MtaD}$-[Te]$_{TycC6}$) (Figure 2D). After heterologous production and purification, both hybrid enzymes were investigated for their catalytic activities and product formation according to the procedures described for the model system BacA1-2-Te.

The hybrid construct BacA1-MbtB-Te, comprising the initiation module of BacA1, the Cy-A-PCP module of MbtB, and the TycC-Te domain, was first tested using amino acid-dependent ATP-PP$_i$-exchange reactions. This assay revealed the expected activation of the substrates isoleucine (100%) and threonine (95%) as well as side selectivity for the noncognate amino acids serine (51%), valine (25%), leucine (13%), and cysteine (3%) (Figure 4A). Both amino acids isoleucine and threonine were also shown to be covalently incorporated into the hybrid enzyme, using the radioactive thioester formation assay (data not shown).

A striking feature of the initial tests of the hybrid NRPS BacA1-MbtB-Te was the high tolerance for the noncognate serine. This side selectivity, however, apparently represents a peculiarity of threonine-activating A domains (as present, i.e., in module MbtB) and has already been described for other systems, e.g., vibriobactin from *Vibrio cholerae* [33]. Likewise, concomitant activation of serine and threonine by MbtB in the mycobactin system leads to the formation of oxazoline and β-methyl oxazoline-containing derivatives, respectively. Correspondingly, the observed serine selectivity of hybrid synthetases BacA1-MbtB-Te is apparently not evoked by the artificial fusions, but rather an inherent feature of the threonine-activating A domain of MbtB.

Product formation activity of the hybrid synthetases BacA1-MbtB-Te was investigated, similarly to BacA1-2-Te, by coupled HPLC/MS analysis. As expected from the results of the ATP-PP$_i$-exchange reactions, the hybrid NRPS was able to synthesize both dipeptides, iso-

leucinyl-oxazolinyl-threonine ($IleThr_{oxazoline}$) (**6**) (at 9.7 min, $[M+H]^+ = 215$ *m/z,* calculated mass 215 *m/z*) and isoleucinyl-oxazolinyl-serine ($IleSer_{oxazoline}$) (**7**) (12.3 min, $[M+H]^+ = 201$ *m/z,* calculated mass 201 *m/z*), once provided with the corresponding substrate amino acids (Figures 4B and 4C). However, productivity of the hybrid synthetases BacA1-MbtB-Te was significantly impaired in both cases. Compared to the model system, we observed an approximately 80-fold decrease of product formation.

The second hybrid synthetase BacA1-MtaD-Te, comprising the initiation module of BacA1, the Cy-A(Ox)-PCP module of MtaD, and the TycC-Te domain, was also first analyzed in amino acid-dependent ATP-PP_i-exchange reactions. Considering both the primary structure of the natural product myxothiazol [Figure 1 (**3**)] [34] and the predictions made by the selectivity-conferring code (data not shown) [35], module MtaD was believed to recognize and activate Cys. Surprisingly, however, the assay revealed highest exchange activities for isoleucine (100%), threonine (91%), and serine (40%), whereas the proposed substrate amino acid cysteine was activated only inefficiently by BacA1-MtaD-Te (8%). Furthermore, the hybrid enzyme revealed tolerance for the activation of the noncognate amino acids valine (31%), phenylalanine (12%), and leucine (7%) (Figure 4D). The results of the ATP-PP_i-exchange reaction could be confirmed by the thioester formation assay, which revealed the covalent incorporation of isoleucine and threonine into the hybrid enzyme. Notwithstanding the observed low activation by BacA1-MtaD-Te, we found that small amounts of cysteine could also be covalently loaded onto the enzyme (data not shown).

A special feature of the hybrid synthetase BacA1-MtaD-Te is the presence of an Ox domain inserted into the A domain and catalyzing oxidation of the heterocyclic rings to give rise to the corresponding thiazole and oxazole derivatives. Thus, given the demonstrated activation of isoleucine (by module BacA1) as well as threonine and serine (by module MtaD), the hybrid NRPS should be capable of synthesizing the β-methyl oxazole and oxazole-containing dipeptides isoleucinyl-oxazolyl-threonine ($IleThr_{oxazole}$) (**10**) and isoleucinyl-oxazolyl-serine ($IleSer_{oxazole}$). Surprisingly, however, only production of the β-methyl oxazole-containing dipeptide $IleThr_{oxazole}$ (9.6 min, $[M+H]^+ = 213$ *m/z*; calculated mass 213 *m/z*) could be demonstrated (Figure 4E), while no formation of the $IleSer_{oxazole}$ product was observed. In an additional experiment, product formation by BacA1-MtaD-Te was investigated in the presence of substrates ATP, isoleucine, and cysteine, although the A domain of module MtaD exhibited only low side selectivity for cysteine in the ATP-PP_i-exchange reaction. HPLC/MS analysis revealed formation of isoleucinyl-thiazolyl-cystein ($IleCys_{thiazole}$) (**8**) (12.1 min, $[M+H]^+ = 215$ *m/z,* calculated mass 215 *m/z*) (Figure 4F), indicating that even low adenylation activity may be sufficient to bring about biosynthesis of the thiazole-containing dipeptide product.

Biochemical in vitro analysis of hybrid synthetase BacA1-MtaD-Te revealed several intriguing results that all can be traced back to the selectivity of individual domains of module MtaD (Cy-A(Ox)-PCP). The A domain of MtaD was believed to activate cysteine given (**1**) the predictions made by the selectivity-conferring code of NRPS A domains and (**2**) the primary structure of the peptide antibiotic myxothiazol. Surprisingly, amino acid-dependent ATP-PP_i-exchange reactions revealed highest exchange activity with noncognate amino acids threonine and serine. From both substrates, only threonine but not serine was incorporated into the dipeptide product synthesized, indicating that the MtaD-Cy domain apparently rejects serine. Even more striking was the observation that the hybrid enzyme BacA1-MtaD-Te did form the β-methyl oxazole-containing dipeptide $IleThr_{oxazole}$ (**10**), although the native myxothiazol biosynthesis system only synthesizes the thiazole-containing product. Feeding experiments with the producer strain *Stigmatella aurantiaca* DW 4/3-1 verified that exclusively cysteine is incorporated into the product [34], and no oxazole-containing variants are formed in vivo. Apparently, some editing barrier provided by the wild-type myxothiazol synthetase complex, which is missing in the hybrid enzyme BacA1-MtaD-Te, prevents the processing of threonine and formation of an oxazole-containing derivative. Recent studies support the idea of an effective editing function, since the deletion of the MtaD-Ox domain in the native myxothiazol system did not lead to formation of a thiazoline product but to a nonproducing *S. aurantiaca* strain [36].

The Ox domain of MtaD, which is integrated into the A domain, represents a very interesting member of the group of optional NRPS domains. The activity of the Ox domain in the BacA1-MtaD-Te-hybrid enzyme confirms previous studies that have shown that flavo-enzymes can be heterologously expressed in *E. coli* and purified in their FMN-containing form [22]. Recently, the Ox domain of epothilone synthetase B was purified as an active, stand-alone FMN-containing holo-enzyme [19]. In the same work, it was shown that the $FMNH_2$-Ox domain can be regenerated by molecular oxygen. Presumably, this oxygen-dependent regeneration guarantees the activity of the MtaD-Ox domain in the synthetase BacA1-MtaD-Te.

As demonstrated by the production of β-methyl oxazole and thiazole-containing dipeptides $IleThr_{oxazole}$ and $IleCys_{thiazole}$, the Ox domain of MtaD exhibits, like TycC-Te domain, broad substrate selectivity, making it a versatile tool for rational design of hybrid NRPSs. This biocombinatorial potential of optional Ox domains may be exploited in the future for the production of unprecedented thiazole- and oxazole-containing hybrid peptide antibiotics.

Cy Domain Mutants

Cy domains are optional domains of NRPS assembly lines that replace conventional C domains in modules that incorporate cysteine or serine/threonine residues by heterocyclization rather than simple amide bond ormation. The formation of the thiazoline and oxazoline rings requires three reaction steps: the condensation of the substrates, the formation of the five-membered heterocyclic ring, and the final dehydration. As shown in Figure 5B, two different routes are feasible to achieve this goal [16]. The peptide bond could be formed first, followed by the cyclization of the side chains and the

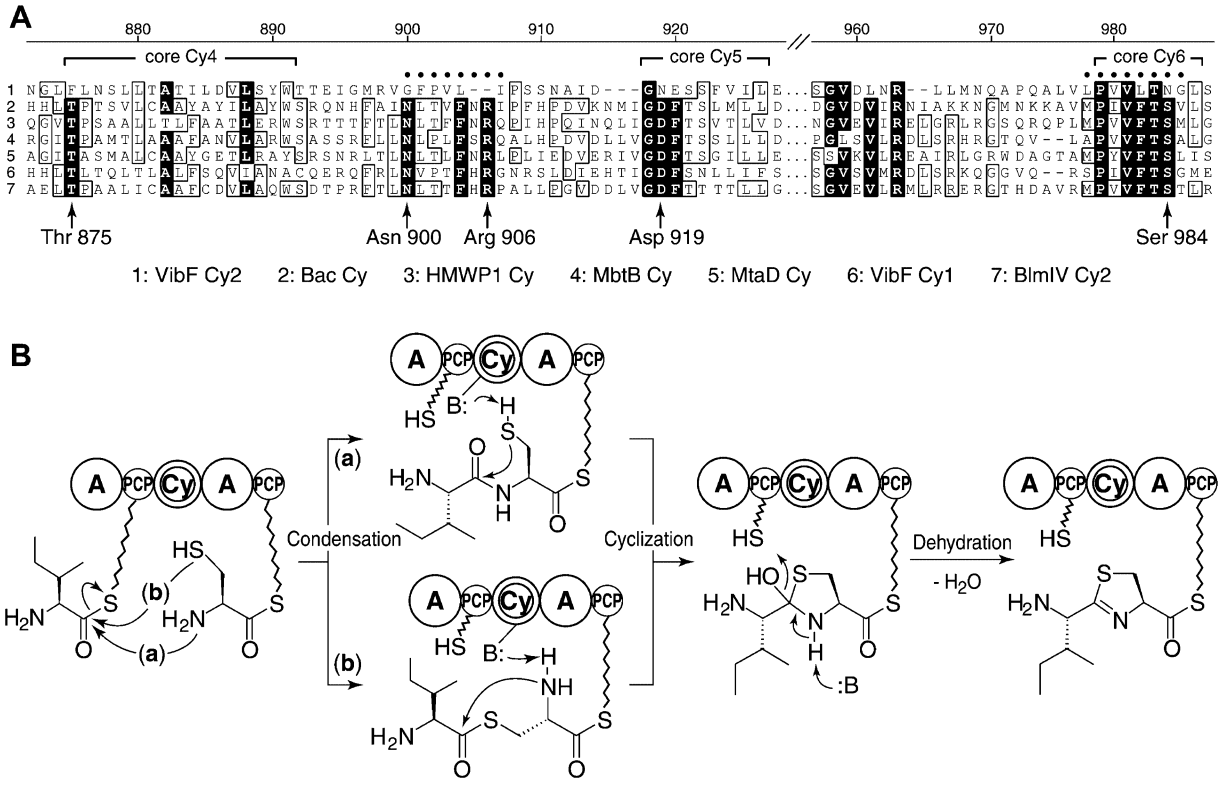

Figure 5. Partial Sequence Alignment of Cy Domains and Possible Reaction Mechanism

(A) Included are VibF Cy1 and Cy2 from the *Vibrio cholerae* vibriobactin synthetase, the Cy domains of BacA2 (bacitracin synthetase, *B. licheniformis*), HMWP1 (yersiniabactin synthetase, *Y. pestis*), MbtB (mycobactin synthetase, *M. tuberculosis*), MtaD (myxothiazol synthetase, *S. aurantiaca*), and the second Cy domain of BlmIV (Bleomycin synthetase *S. verticillus*). Residues conserved in at least four domains are boxed. Residues conserved in at least six domains are marked black. Residues that were mutated in the Cy domain of the model system BacA1-2-Te are indicated with arrows. The amino acids of the putative solvent channel are bulleted.

(B) Model for the reaction sequence of heterocyclization exemplified by the first two modules of bacitracin synthetase A. In the first step, the enzyme-bound amino acids are combined by a nucleophilic attack of either the cysteine amino group (a) or the heteroatom of the side chain (b) onto the carbonyl C of isoleucine. Next, a proton is abstracted by means of base catalysis, thereby enabling the attack of either the cysteine side chain or the amino group onto the amide bond carbonyl. This leads to the formation of a hydroxylated thiazolidine intermediate, which is subsequently dehydrated to give the final thiazoline-containing product.

final dehydration. Alternatively, a nucleophilic attack of the side-chain hydroxyl or thiol group could lead first to the formation of an oxoester or thioester, which is then further processed to yield the final five-membered heterocyclic ring product. In order to determine the chronological order of condensation and cyclization in the course of heterocyclization, we used mutational analysis of the Cy domain of our model system BacA1-2-Te.

Cy domains share striking structural and functional homology with C domains, which catalyze basic peptide bond formation only. Consequently, our goal was to identify those residues that distinguish Cy domains from C domains and are probably essential for cyclization of the side chain (see Figure 5) but are not involved in peptide bond formation. In order to determine possible candidates for the mutational analysis, we considered two important observations concerning NRPS Cy domains. First, the NRPS VibF involved in the biosynthesis of vibriobactin in *Vibrio cholerae* was shown to possess the unusual domain arrangement Cy-Cy-A-C-PCP-C. Interestingly, although the second Cy domain shares highest homology with other known Cy domains, it was shown to catalyze only peptide bond formation between 2,3-dihydroxybenzoate and threonine but was not able to perform cyclization of the side-chain nucleophile to form the corresponding heterocyclic ring [17, 33]. Secondly, previous studies revealed that the highly conserved motif DxxxxD located between the core motifs Cy2 and Cy3 is the Cy domain equivalent for the catalytic core of C domains (HHxxxDG) [7, 15, 17], indicating that this N-terminal portion of the Cy domains may be involved in the peptide bond-forming reaction only (Figure 5). Thus, in order to identify residues involved in cyclization but not peptide bond formation, we focused on the C-terminal portion of the Cy domains and carried out sequence comparisons between VibF-Cy2 and different Cy domains.

Figure 5A shows the sequence alignment of a stretch of approximately 120 aa between the core motifs Cy4 and Cy6 [15] (positions 872–987; numbering in accordance with the amino acid sequence of BacA). Within this region, we found nine invariant residues conserved between VibF-Cy2 and other Cy domains as well as nine residues that are strictly conserved among Cy domains but not present in VibF-Cy2. Among these latter resi-

dues, positions 904, 932, and 982 revealed only conservative substitutions between hydrophobic amino acids, and thus only the remaining six residues (T875, N900, R906, D919, F920, and S984) were selected and mutated to alanine. Mutation of position F920 failed for unknown reasons, but the remaining five mutants, BacA1-2-Te **T875A**, BacA1-2-Te **N900A**, BacA1-2-Te **D919A**, BacA1-2-Te **R906A**, and BacA1-2-Te **S984A**, were constructed successfully.

All five mutants were heterologously expressed in *E. coli*, purified to near homogeneity, and biochemically characterized using amino acid-dependent ATP-PP_i-exchange reactions as well as the covalent loading of radioactively labeled amino acids. These studies revealed that the mutant enzymes were not impaired in the activity and intramodular communication of the A and PCP domains compared with model system BacA1-2-Te. In order to investigate the activity of the mutated Cy domains, product formation assays were carried out as described above. On the supposition that the constructed Cy domain mutants may be affected in cyclization but not condensation activity, formation of the linear dipeptide isoleucinyl-cysteine (IleCys) (**11**) could be expected.

As demonstrated by HPLC/MS analysis of the reaction mixtures, the five mutant NRPSs can be categorized into three groups as follows.

Mutants BacA1-2-Te **T875A** and BacA1-2-Te **R906A** form the first group. Both encoded proteins failed to produce thiazoline-containing $IleCys_{thiazoline}$ (**5**) or linear IleCys (**11**), indicating that the mutations lead to an inactive Cy domain incapable of catalyzing condensation and/or cyclization. In this connection, it has to be stressed again that all constructs tested were not affected in the activation and covalent loading of their substrate amino acids.

Mutant BacA1-2-Te **D919A** revealed the same activity as the basic model BacA1-2-Te, indicating that this residue, although conserved between Cy domains, is not involved in the catalytic mechanism of the Cy domain. HPLC/MS analysis revealed the formation of both the thiazoline-containing dipeptide $IleCys_{thiazoline}$ (**5**) (at 14.1 min, $[M+H]^+ = 217$ *m/z*, calculated mass 217 *m/z*) and the byproduct $IleCys_{thiazoline}Cys$ (**9**) (at 17.1 min, $[M+H]^+ = 320$ *m/z*, calculated $[M+H]^+ = 320$ *m/z*). In addition, no differences in productivity could be observed compared to the model system.

Finally, mutants BacA1-2-Te **N900A** and BacA1-2-Te **S984A** were affected in cyclization but not condensation activity. In both cases, HPLC/MS analysis confirmed the production of expected linear dipeptide IleCys (**11**) at 12.8 min with the $[M+H]^+$ ion peak of 235 *m/z* (calculated mass $[M+H]^+ = 235$ *m/z*). In addition, formation of dimerized product $(IleCys)_2$ (**12**) could be observed (at 21.2 min, $[M+H]^+ = 467$ *m/z*, calculated mass $[M+H]^+ = 467$ *m/z*) (Figure 6). Here, dimerization is caused by the formation of a disulfide bond between the free thiol residues of the incorporated cysteine moieties. Consequently, since only the noncyclized product contains a free thiol group, detection of $(IleCys)_2$ dimer is only consistent with preceding formation of the linear dipeptide.

As mentioned above, C and Cy domains share significant structural and functional homology. Therefore, the crystal structure of the free-standing C domain VibH was supposed to be representative of the whole superfamily of condensation, epimerization, and cyclization domains [6]. Consequently, the existing structure of VibH can be exploited to discuss the possible function of the conserved Cy domain residues under investigation.

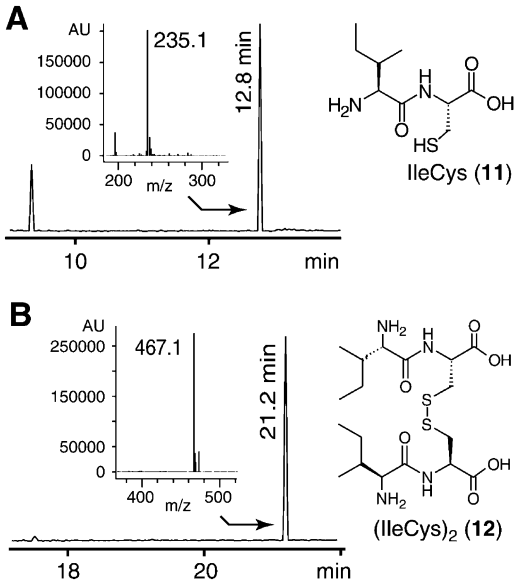

Figure 6. HPLC/MS Analysis of Product Formation Catalyzed by the Cy Domain Mutants

(A) The linear dipeptide IleCys (**11**) (12.8 min, $[M+H]^+ = 235$ *m/z*) was expected in cases where cyclization but not condensation was affected.

(B) Formation of a disulphide bond between two IleCys monomers led to the appearance of the dimerization product $(IleCys)_2$ (21.2 min, $[M+H]^+ = 467$ *m/z*) (**12**).

Sequence comparison between C and Cy domains revealed 13 conserved amino acid residues, of which the residue R906 is the only one that was investigated in this study. The 906-corresponding residue in the C domain was shown to be uninvolved in catalysis [7]. In contrast, the mutation R906A in BacA1-2-Te **R906A** led to an inactive enzyme, indicating an important role in Cy domain function. Presumably, the mutation of R906A led to structural alterations that affect the catalytic activity. From the crystal structure of VibH, it was concluded that R906 forms hydrogen bonds with other residues located within the solvent channel. Thus, mutation of R906 prevents formation of these hydrogen bonds that may cause structural changes, which will ultimately lead to an inactive Cy domain. In VibF-Cy2, which was also mutated and proved the opposite, other residues may take over that function.

Like R906, residues N900 and S984 are also proposed to be involved in the formation of the solvent channel. Both mutants, BacA1-2-Te **N900A** and BacA1-2-Te **S984A**, were affected in cyclization but not condensation activity. This observation confirmed studies on VibF that revealed a clear separation of condensation and cyclization catalyzed by the tandem domains Cy2 and Cy1, respectively [17, 33]. Secondary structure predic-

Table 1. Primers Used in This Study

Primer Name	Nucleotide Sequence
5′-bacA1-A	5′-TATACCATGGTTGCTAAACATTCATTAG-3′
3′-bacA2-T	5′-TTAAGATCTGTTTTTCAAATAGTGGTCC-3′
5′-tycTe	5′-ATAAGATCTCATAAGCGCTTTGAGAGCAG-3′
3′-tycTe	5′-TATGGATCCTTTCAGGATGAACAGTTCTTG-3′
3′-bacA1-T	5′-TATGGATCCTTCGATAAAAGCGCTCAATTC-3′
5′-mbtB-Cy	5′-ATAAGATCTACCGGCGTCGCGCCCAC-3′
3′-mbtB-T	5′-AATAGATCTGGTGAGCAACTGGGCCAACG-3′
5′-mtaD-Cy	5′-ATTAGATCTCGCTCCGCGAATCTTCAG-3′
3′-mtaD-T	5′-ATAAGATCTGTGGATGAACTCCGCCAG-3′
CyT875A	5′-CGGCATCATCATCTC**GCA**CCGACTTCCGTCCTTTG-3′
CyT875Arev	5′-CAAAGGACGGAAGTCGG**TGC**GAGATGATGATGCCG-3′
CyN900A	5′-CGGCAAAATCATTTTGCTATC**GCG**TTAACCGTATTTAACAGAATCCCG-3′
CyN900Arev	5′-CGGGATTCTGTTAAATACGGTTAA**CGC**GATAGCAAAATGATTTTGCCG-3′
CyR906A	5′-GCTATCAATTTAACCGTATTTAAC**GCG**ATCCCGTTTCATCCGGATGTCAAG-3′
CyR906Arev	5′-CTTGACATCCGGATGAAACGGGAT**CGC**GTTAAATACGGTTAAATTGATAGC-3′
CyD919A	5′-GGATGTCAAGAACATGATCGGA**GCC**TTTACCTCTTTAATGCTGCTGG-3′
CyD919Arev	5′-CCAGCAGCATTAAAGAGGTAAA**GGC**TCCGATCATGTTCTTGACATCC-3′
CyS984A	5′-GCCGATCGTATTCACA**GCT**GTGCTGATTGAAAATCCC-3′
CyS984Arev	5′-GGGATTTTCACTCAGCAC**AGC**TGTGAATACGATCGGC-3′

Restriction sites are underlined, and mutated codons are in bold.

tions and comparison with the structure of VibH [6] indicated that N900 is localized in the β strand 9, whereas S984 is part of β strand 11. Both residues are placed in the region of the solvent channel, which was described to pinpoint the C domain's active site. Apparently, cyclization in Cy domains is catalyzed in the same solvent channel, but by different residues, as the condensation reaction.

Significance

The rational design of NRPS templates is an important approach for the synthesis of novel peptide antibiotics. The structural diversity of these peptides is especially enhanced by structural particularities, which are normally affected by optional domains within the multimodular enzyme templates. One of the most remarkable optional domains is the Cy domain, responsible for the incorporation of five-membered rings of the thiazoline and oxazoline type by heterocyclization of cysteine and serine/threonine side chains. We report here the construction and exploitation of a bimodular model system for the investigation of heterocyclization in NRPS assembly lines. The functionality of the artificial model NRPS engineered was demonstrated by the production of the expected heterocyclic ring-containing dipeptide. Heterocyclization requires successive peptide bond formation, cyclization of the side chain, and dehydration. We examined the sequential order of heterocyclization by means of mutational analysis of the Cy domain and confirmed the independence of both condensation and cyclization reaction. In addition, the genetic exchange of various Cy domain-containing modules derived from different biosynthetic systems demonstrated the practicability of module fusions with this special kind of heterocyclic ring-forming module. Likewise, the engineered hybrid NRPSs proved the biocombinatorial potential of optional Ox domains. Thus, our study paves the way for the rational engineering of hybrid NRPSs, which may be exploited for the production of unprecedented heterocyclic ring-containing peptide antibiotics.

Experimental Procedures

Bacterial Strains and Culture Media

Escherichia coli strains Top10F′ (Invitrogen) and XL1-blue (Stratagene) were used for the construction and maintenance of the expression plasmids, whereas gene expressions were carried out in derivatives of *E. coli* BL21(λDE3) (Stratagene). All cells were grown in LB medium [37] supplemented, if appropriate, with 25 μg/ml kanamycin and 100 μg/ml ampicillin (final concentrations).

Construction of Expression Plasmids

All gene fragments required for the construction of the expression plasmids were amplified by using the Expand long-range kit (Roche). Modified oligonucleotides for mutagenesis and introduction of restriction sites for subsequent cloning were purchased from MWG Biotech and Qiagen Operon. Primers used in this study are listed in Table 1.

The plasmid p[A-PCP-Cy-A-PCP]$_{bacA1\text{-}2}$-[Te]$_{tycC6}$ for the overproduction of the model system BacA1-2-Te is a derivative of pQE60 vector (Qiagen). The 4956 bp gene fragment comprising *bacA1-2* was amplified from chromosomal DNA of *Bacillus licheniformis* ATCC 10716 using the primers 5′-bacA1-A and 3′-bacA2-T (see Table 1). After digestion with the endonucleases NcoI and BglII, the fragment was ligated into pQE60, previously cut in the same manner to give p[A-PCP-Cy-A-PCP]$_{bacA1\text{-}2}$. Subsequently, the 756 bp gene fragment encoding the Te domain of tyrocidine synthetase C (TycC) was amplified from chromosomal DNA of *Bacillus brevis* ATCC 8185 by using the oligonucleotides 5′-tycTe and 3′-tycTe digested with BglII and BamHI and ligated into the BglII-linearized plasmid p[A-PCP-Cy-A-PCP]$_{bacA1\text{-}2}$ to give p[A-PCP-Cy-A-PCP]$_{bacA1\text{-}2}$-[Te]$_{tycC6}$.

Plasmids for the overproduction of the hybrid bimodular NRPSs are based on the pQE60-derivative p[A-PCP]$_{bacA1}$. This plasmid was constructed by amplification of the *bacA1*gene from chromosomal DNA of *Bacillus licheniformis* ATCC 10716 using the primers 5′-bacA1-A and 3′-bacA1-T. After digestion with the endonucleases NcoI and BamHI, the 1829 bp DNA fragment was ligated into pQE60, which was cut in the same manner. Subsequently, the 3165 bp gene fragment encoding [Cy-A-PCP]$_{mbtB}$ was amplified from the plasmid pET28-mbtB using the oligonucleotides 5′-mbtB-Cy and 3′-mbtB-T. At the same time, the 4002 bp gene fragment comprising *mtaD* was amplified from the cosmid E25 [14] utilizing the oligonucleotides 5′-mtaD-Cy and 3′-mtaD-T. Both fragments were digested with BglII and ligated into the BglII/BamHI site of the plasmid p[A-PCP]$_{bacA1}$,

yielding the plasmids p[A-PCP]$_{bacA1}$-[Cy-A-PCP]$_{mbtB}$ and p[A-PCP]$_{bacA1}$-[Cy-A(Ox)-PCP]$_{mtaD}$, respectively. The integration of the thioesterase-encoding gene fragment of TycC into these plasmids was realized as described for p[A-PCP-Cy-A-PCP]$_{bacA1-2}$, giving rise to the final expression plasmids p[A-PCP] $_{bacA1}$-[Cy-A(Ox)-PCP]$_{mtaD}$-[Te]$_{tycC6}$ and p[A-PCP]$_{bacA1}$-[Cy-A(Ox)-PCP]$_{mtaD}$-[Te]$_{tycC6}$, respectively. The identity of all constructs was verified by restriction analysis.

The expression plasmids for Cy domain mutants are based on the plasmid p[A-PCP-Cy-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$. The mutations were integrated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the instructions of the manufacturer. p[A-PCP-Cy(**T875A**)-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$ was obtained using the mutagenesis primers CyT875A and CyT875Arev. For production of p[A-PCP-Cy(**N900A**)-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$, the primers CyN900A and CyN900Arev were used. The plasmids p[A-PCP-Cy(**R906A**)-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$ and p[A-PCP-Cy(**D919A**)-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$ were constructed with the primer pairs CyR906A/CyR906Arev and CyR919A/CyR919Arev, respectively. The fifth mutant plasmid p[A-PCP-Cy(**S984A**)-A-PCP]$_{bacA1-2}$-[Te]$_{tycC6}$ was obtained using the primers CyS984A and CyS984Arev.

Gene Expression in *Escherichia coli* and Purification of Recombinant NRPSs

Expression of the different recombinant NRPSs genes was carried out in *Escherichia coli* strain BL21(λDE3) [pREP4-*gsp*]. The helper plasmid pREP-*gsp* contains the gene for the 4′-phosphopantetheine transferase Gsp, whose coexpression ensures the in vivo modification of the dedicated PCP domains and is essential for the production of fully active, recombinant NRPSs [29]. Cells were grown at 30°C until the optical density at 620 nm (A_{620}) reached 0.6–0.7. NRPS gene expression was induced by the addition of 0.25 mM IPTG, and cells were allowed to grow for an additional 2.5 hr at 25°C. After harvesting and resuspension, cells were lysed by two passages through a French press (Polytec). His_6-tagged proteins could be purified to apparent homogeneity by single-step Ni^{2+}-affinity chromatography. Purity and concentration of the purified proteins were determined by SDS-PAGE and use of calculated extinction coefficients for their absorbance at 280 nm, respectively [38].

Pooled fractions were dialyzed against assay buffer (50 mM HEPES, 300 mM NaCl, 1 mM EDTA, pH 8.0) using HiTrap desalting columns (Amersham Biosciences). The hybrid enzymes [A(Ile)-PCP]$_{BacA1}$-[Cy-A(Thr)-PCP]$_{MbtB}$-[Te]$_{TycC6}$ BacA1-MbtB-Te and [A(Ile)-PCP]$_{BacA1}$-[Cy-A(Cys)Ox-PCP]$_{MtaD}$-[Te]$_{TycC6}$ (BacA1-MtaD-Te) were additionally concentrated using Amicon Ultra 100,000 MWCO (Millipore). Concentrations of the purified enzymes were determined using the calculated extinction coefficients for absorbance at 280 nm.

Enzyme Activity and Product Formation Assays

The amino acid-dependent ATP-pyrophosphate (ATP-PP_i)-exchange reaction was performed in assay buffer as described previously [29] to determine substrat selectivity and catalytic activity of recombinant NRPS A domains. Characterization of communication between adenylation (A) and peptidyl carrier protein (PCP) domain as well as the covalent incorporation of radiolabeled amino acids (ICN Biomedicals) within the recombinant NRPSs were performed as described previously [29].

For the investigation of product formation, 500 nM enzyme, 1 mM $MgCl_2$, 2 mM ATP, and 1 mM of each substrate amino acid were incubated in assay buffer in a total volume of 1 ml at 37°C for different periods of time and stopped by addition of 500 μl 1-butanol/chloroforme (4:1; vol/vol). After evaporation, the resulting pellet was resuspended in 100 μl 10% methanol and analyzed by HPLC/MS on a Hewlett Packard 1100 Series instrument using a C_{18}-reverse-phase column (C250/3 Nucleosil 120-3C_{18}-column, Macherey & Nagel, Düren, Germany) with H_2O/0.1% trifluoro acetic acid (TFA) (vol/vol) (buffer A) and acetonitrile/0.1% TFA (vol/vol) (buffer B). Samples could be separated by applying the following gradient at a flow rate of 0.3 ml min^{-1}: loading at 10% buffer B, linear gradient up to 60% in 30 min, linear gradient to 90% buffer B in 5 min, and holding 90% buffer B for 5 min. Products were monitored at 214 nm as well as in positive-ion mode over the *m/z* range 100–500.

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