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Identification of an Auxiliary Leader Peptide-Binding Protein Required for Azoline Formation in Ribosomal Natural Products

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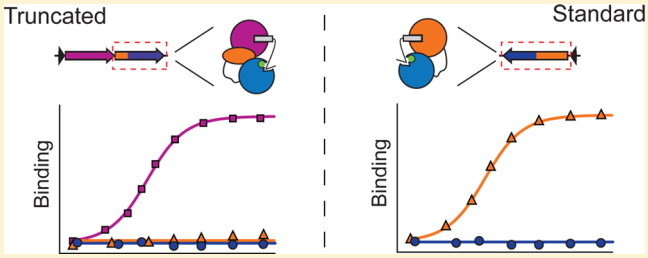
Supporting Information

ABSTRACT: Thiazole/oxazole-modified microcins (TOMMs) are a class of post-translationally modified peptide natural products bearing azole and azoline heterocycles. The first step in heterocycle formation is carried out by a two component cyclodehydratase comprised of an E1 ubiquitin-activating and a YcaO superfamily member. Recent studies have demonstrated that the YcaO domain is responsible for cyclodehydration, while the TOMM E1 homologue is responsible for peptide recognition during azoline formation. Although all characterized TOMM biosynthetic clusters contain this canonical TOMM E1 homologue (C domain), we also identified a second, highly divergent E1 superfamily member, annotated as an Ocin-ThiF-like protein (F protein), associated with more than 300 TOMM biosynthetic clusters. Here we describe the *in vitro* reconstitution of a novel TOMM cyclodehydratase from such a cluster and demonstrate that this auxiliary protein is required for cyclodehydration. Using a combination of biophysical techniques, we demonstrate that the F protein, rather than the C domain, is responsible for engaging the peptide substrate. The C domain instead appears to serve as a scaffolding protein, bringing the catalytic YcaO domain and the peptide binding Ocin-ThiF-like protein into proximity. Our findings provide an updated biosynthetic framework that provides a foundation for the characterization and reconstitution of approximately 25% of bioinformatically identifiable TOMM synthetases.



INTRODUCTION

In thiazole/oxazole-modified microcin (TOMM) natural products, thiazoline and (methyl)oxazoline heterocycles are synthesized by a two-component cyclodehydratase composed of a ubiquitin-activating enzyme E1 homologue (C domain) and a member of the YcaO superfamily (D domain).[1,2] In approximately 50% of cases, the genes encoding the C and D domains are fused and are expressed as a single polypeptide (fused cyclodehydratase).[1] In many TOMMs, the azolines are oxidized to azoles by a flavin mononucleotide (FMN)-dependent dehydrogenase (B domain).[1,2] Previous research has demonstrated that the TOMM D domain catalyzes the ATP-dependent cyclodehydration reaction, while the TOMM C domain recognizes the peptide substrate through specific motifs within the N-terminal leader peptide and regulates D domain activity.[3−5] However, the biophysical basis for leader peptide binding and activity potentiation by the C domain has yet to be elucidated.

Nearly all members of the *Bacillus cereus* group (including *B. anthracis*) harbor an uncharacterized TOMM biosynthetic cluster, previously designated as a "heterocycloanthracin" (HCA) biosynthetic cluster (Figure 1).[6,7] Recently, the first report regarding the isolation of a HCA natural product has emerged;[8] however, the structure of the compound was not described. Here we report the reconstitution of the fused TOMM cyclodehydratase from the *Bacillus* sp. Al Hakam HCA cluster and demonstrate that cyclodehydratase activity is dependent on the presence of an uncharacterized protein (annotated as an Ocin-ThiF-like domain based on weak similarity to E1 superfamily members) found in all HCA clusters.[6] We show that the C domain of the fused HCA cyclodehydratase is N-terminally truncated compared to characterized TOMM C domains and is unable to bind the precursor peptide. Furthermore, we establish that the previously uncharacterized Ocin-ThiF-like protein is responsible for leader peptide binding. A bioinformatic survey of all currently identifiable TOMM clusters revealed that approximately 25% of biosynthetic gene clusters contain a similarly truncated TOMM C domain and that, in nearly every case, an Ocin-ThiF-like protein is found in the gene cluster.

RESULTS AND DISCUSSION

The genes corresponding to the *Bacillus* sp. Al Hakam HCA dehydrogenase (Figure 1; HcaB) and fused cyclodehydratase (Figure 1; HcaD) and two potential precursor peptides (HcaA and HcaA2; Figures 1 and S1; Supporting Information) were expressed in *E. coli* with N-terminal His_6 or maltose-binding

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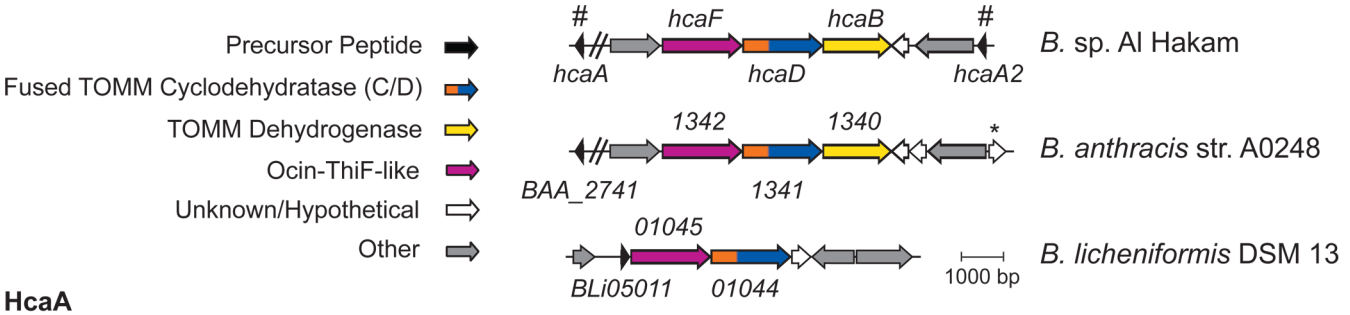

HcaA

MNQFQQELQSLNLNDYQTGNVVYWDPQQSQYPYYYIQDDARRCGGCGGCGGRCGGCGGRCGGCAGRCGGCVGCAGCFGCFNCWNWWII

HcaA2

MDLPACDAEANWVAFQISCANVSSLDSQARALYIVALMENSWSSETAGFGSFGSCGSSGRWARRPAHIYFTYFQ

Figure 1. HCA biosynthetic clusters. Open reading frame (ORF) diagrams for three representative HCA biosynthetic clusters are displayed along with putative precursor peptides from the *Bacillus* sp. Al Hakam cluster. #, ORF not annotated in GenBank. An unannotated homologue of *hcaA2* is present in *B. anthracis* within the ORF marked by an asterisk. Cyclizable residues are colored orange. The putative core peptide for HcaA is underlined.

protein (MBP) tags, respectively. While HcaD expressed well in *E. coli*, HcaB was heavily proteolyzed and purified without the requisite FMN cofactor (Figure S2). Attempts to obtain a full-length, holo HcaB from orthologous biosynthetic clusters were equally unsuccessful (Figure S2). When HcaA was treated with HcaD in the presence of ATP, no modification was observed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), even at an enzyme to substrate ratio of 1:2 (Figure 2). Given that a subset of HCA

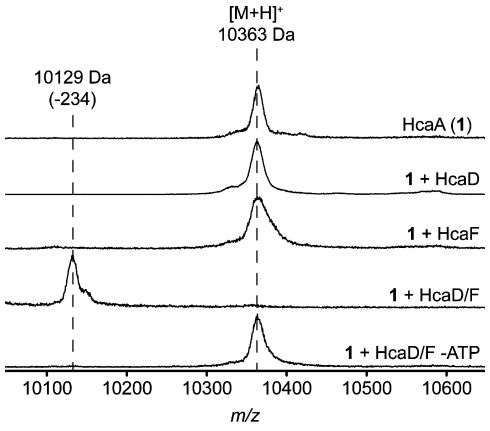

Figure 2. HcaF is required for cyclodehydratase activity. A MALDI-TOF mass spectral overlay for HcaA processing is displayed. The mass of the starting material and the fully cyclodehydrated species are displayed along with the mass shift relative to the unmodified peptide.

clusters lack a B domain,[6] and previous work with fused cyclodehydratases has demonstrated that azoline formation does not depend on the presence of a dehydrogenase even when it is present in the cluster,[9−11] we reasoned that HcaD activity could be dependent upon another, unidentified protein. Bioinformatic analysis identified a potential candidate, annotated as an Ocin-ThiF-like protein (hereafter referred to as a TOMM F protein), found adjacent to the cyclodehydratase in all HCA clusters (Figure 1; HcaF). When HcaF was expressed as a His_6-tagged fusion in *E. coli* and added to reactions containing HcaD, 13 azoline heterocycles (resulting in a −234 Da mass shift) were installed on HcaA (Figure 2). No modification was observed with HcaF alone, indicating that both HcaD and HcaF are required for cyclodehydration (Figure 2). Using iodoacetamide labeling, these modifications were localized to the 13 cysteines in the peptide (Figure S3). Heterocycle formation was ATP- and YcaO-dependent, as omission of ATP or mutagenesis of the ATP-binding site in the D (YcaO) domain of HcaD abolished activity (Figure 2 and Figure S4). Attempts to modify HcaA2 were unsuccessful, suggesting that this peptide is either not a substrate for the HCA cyclodehydratase or that an additional protein is required for modification (Figure S5). As HcaA2 is encoded within another gene (Figure 1),[7] we believe that the former hypothesis is more probable.

Consistent with other TOMM cyclodehydratases, HcaD and HcaF hydrolyzed ATP to ADP and phosphate (Figure S6 and S7).[9,12,13] As work with previous TOMM cyclodehydratases has demonstrated that ATP hydrolysis is tightly coupled to heterocycle formation,[12] we attempted to obtain kinetic parameters for HcaA modification using a previously described purine nucleoside phosphorylase (PNP)-dependent phosphate detection assay.[7,12,14] However, initial attempts to determine the rate of HcaA processing were stymied by the high nonspecific ATPase activity in HcaA control reactions. We reasoned that the high cysteine content of HcaA might account for the ATPase activity, and thus we generated truncations of HcaA lacking the C-terminal 36 (HcaA-C53*; * denotes stop codon) and 29 amino acids (HcaA-C60*; see Figure S1). Both truncated peptides displayed negligible ATPase activity and were fully processed when treated with HcaD and HcaF in the presence of ATP (Figure S3). The initial rates for each peptide demonstrated that both HcaD and HcaF were required for maximal ATP consumption and that both substrates were modified at similar rates (Figure S7). Moreover, control reactions revealed that removal of the N-terminal MBP-tag from the precursor peptide was not a prerequisite for processing (Figure S7). Consistent with previously characterized TOMM cyclodehydratases, ATP hydrolysis occurs even in the absence of substrate (Figure S6 and S7).[12] Unlike the previously characterized enzymes, the rate of background ATP hydrolysis is ∼25% the rate of the HcaA-saturated cyclodehydratase. Combined with the low apparent K_M for the HcaA derivatives and the slow rate of substrate processing, the determination of kinetic parameters was difficult. However, k_{obs} and apparent K_m can be approximated as 1.5 ± 0.1 min^{-1} and 5

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± 1 μM, respectively (Figure S7). These values are similar to the parameters obtained for the fused cyclodehydratase TruD (2.6 min^{-1}, 1 μM).[9]

Intrigued by the ability of the HCA cyclodehydratase to modify truncated analogs of HcaA, we sought to determine the order of cyclodehydration on the native substrate. However, a time course experiment performed with HcaA indicated that the enzyme processed the substrate with a high level of processivity, as partially cyclized species were not observed by MALDI-TOF-MS (Figure S8). When the reaction time course was repeated with HcaA-C60*, a similar result was obtained (Figure S8). As previously characterized TOMM cyclodehydratases have modified their substrates in a distributive fashion,[7,10,15] the processive modification of HcaA by HcaD is noteworthy.

Although studies performed on other TOMM synthetases have demonstrated that cyclodehydratase activity can be dependent on the presence of the TOMM B protein (dehydrogenase),[16−18] no other components have been shown to be required for azoline formation. As such, the absolute requirement for the TOMM F protein was unexpected. TOMM C domains have been shown to be responsible for binding the leader peptide.[3,5] Although the peptide-binding site has not been identified, recognition has been proposed to be accomplished by an N-terminal "peptide clamp" domain akin to that found in the microcin C7 maturation protein MccB.[3,19] This assignment is bolstered by a recent structure of the nisin dehydratase, NisB, bound to its leader peptide, which demonstrated that a homologous domain is responsible for precursor peptide recognition in the biosynthesis of type I lanthipeptides.[20] Comparing HcaD to other fused TOMM cyclodehydratases, we noticed that the enzyme was significantly shorter than previously characterized members and appeared to lack the putative "peptide clamp" (Figure S9). As such, we hypothesized that HcaF was involved in leader peptide recognition.

Using size-exclusion chromatography, we first established the oligomeric state of HcaD and HcaF. Individually, both proteins had retention volumes consistent with a monomer (Figure S10). When HcaD and HcaF were analyzed as a 1:1 mixture, a new peak was formed with a retention volume consistent with a 1:1 complex (Figure S10). When MBP-HcaA leader peptide (HcaA-LP; Figure S1) was added to samples containing HcaD, HcaF, or both proteins, a new peak was observed only in samples containing HcaF (Figure S10). In order to quantify the strength of this interaction, an MBP-free fluorescein-tagged version of HcaA-LP was generated, and binding to HcaD and HcaF was assessed by fluorescence polarization (FP). As before, HcaA-LP binding was only observed when HcaF was present (Figure 3), and the addition of HcaD did not affect binding. Importantly, the tight interaction between HcaF and HcaA obtained in this assay was consistent with the high level of processivity observed. Indeed, the large difference between the apparent K_M (5 μM) and K_d (37 nM) for HcaA indicates that k_{off} must be slower than k_{cat}, a requirement for processivity.

Based on the primary sequence of HcaF, it was unclear which region of the protein engaged the leader peptide. Although the protein displays weak similarity to E1 superfamily members (see Figure S11), attempts to locate a MccB-like clamp or a NisB-like peptide-binding domain in HcaF using BLAST were unsuccessful. This is not surprising given the high level divergence seen with these domains.[17,20] Further study of

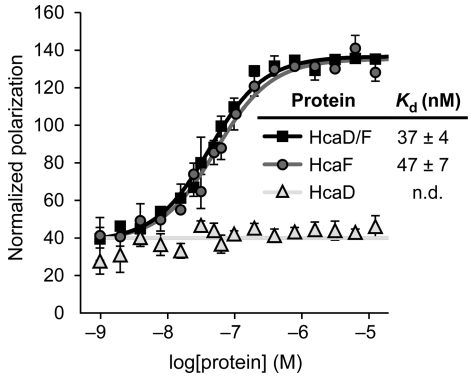

Figure 3. HcaF mediates leader peptide binding. Fluorescence polarization binding experiment with FITC-labeled HcaA-LP and HcaD, HcaF, or HcaD/F. Error bars represent standard deviation from the mean (n = 3). Curves were fit according to the methods. Error on K_d values is standard error of the mean determined from curve fitting; n.d., not detected.

HcaF will be required to identify the leader peptide-binding site.

Saturation transfer difference (STD) NMR spectroscopy can be used to detect binding between a protein and ligand;[21] in certain cases, this has been used for elucidating binding epitopes.[22] We performed ^{1}H STD-NMR to reveal the regions of HcaA-LP contacted by HcaF. Due to the solution instability of HcaA-LP under the conditions used for NMR (aggregation was seen after several hours, limiting the scope of available experiments) and the large number of overlapping, similar resonances (the peptide is replete with Asn, Gln, and Asp residues, for instance, which were difficult to differentiate in the absence of isotope labeling and multidimensional experiments), only partial assignment of the resonances was possible by ^{1}H and ^{1}H−^{1}H TOCSY (Table S3 and Figures S12 and S13). Nevertheless, resonances from a number of unique residues distributed throughout the peptide were assignable and used to assess contact with HcaF (Figure S14). These data implicated Met1 and Phe4 in binding and demonstrated that the assignable residues in the C-terminus of HcaA-LP did not closely contact HcaF. In addition to these unique residues, several Leu and Tyr residues appeared to be strongly binding; however, due to sequence redundancy, the exact positions could not be unambiguously assigned.

In order to resolve this ambiguity and to corroborate the STD-NMR data, alanine point substitutions were made to HcaA-LP, and recognition by HcaF was assessed using a competition-based FP assay (Table 1 and Figure S15). Consistent with the NMR data, mutation of either Met1 or Phe4 resulted in a 4- and 10-fold reduction in binding affinity, respectively. While all of the Leu mutations resulted in a 100-fold decrease in binding affinity, Tyr16 was the only Tyr residue (of six) that appeared to be critical for recognition. Combined with the STD-NMR data, these results suggest that HcaA-LP recognition occurs primarily through the N-terminal portion of the sequence. In order to test this further, a series of C-terminal truncations were made to the HcaA-LP and binding was assayed by competition FP (Table 1 and Figure S15). Truncations of HcaA-LP of up to 15 residues were tolerated without a decrease in binding affinity; however, removal of an additional five residues severely reduced binding. Combined, our data demonstrate that HcaF engages HcaA-LP through the N-terminal portion of the peptide and that hydrophobic

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Table 1. Competition FP Results for HcaA-LP Mutations[a]

HcaA-LP Mutant	IC_{50} (nM)	K_i (nM)
WT	310 ± 40	25 ± 3
M1A	1200 ± 400	180 ± 50
F4A	3500 ± 700	570 ± 110
L8A	~25,000	~4200
L11A	>50,000	>8500
L13A	>50,000	>8500
Y16A	>50,000	>8500
Y23A	320 ± 60	27 ± 5
Y31A	330 ± 50	28 ± 4
Y33A	380 ± 50	37 ± 5
Y34A	420 ± 40	44 ± 4
Y35A	290 ± 50	21 ± 4
V21*	~25,000	~4200
P26*	340 ± 30	30 ± 3
P32*	370 ± 50	35 ± 5
I36*	370 ± 80	35 ± 8

[a]Error on IC_{50} values is the standard error of the mean determined from curve fitting. Competitive K_i values were calculated from the experimentally-determined IC_{50}. Percent errors from the IC_{50} measurements were used to obtain the error for the calculated K_i values. An asterisk denotes a stop codon.

residues throughout this region are important for recognition. Notably, previous studies with the TOMM biosynthetic enzymes involved in streptolysin S and microcin B17 biosynthesis have demonstrated that leader peptide is primarily engaged through a FxxxB (B = V, I, or L) motif.[5,23] This suggests that a conserved strategy for peptide recognition has been propagated through at least part of the TOMM family.

Previous work with TOMM and lanthipeptide biosynthetic enzymes has demonstrated that select members have the ability to process substrates lacking leader peptides, albeit with reduced efficiency.[11,24−27] The decrease in processing efficiency can often be rescued in part by the addition of the leader peptide *in trans*. These studies have demonstrated that, in addition to providing substrate recognition, leader peptides activate their cognate biosynthetic machineries, presumably by biasing the enzymes to an active conformation. In order to determine if the HCA cyclodehydratase displays similar leader peptide-independent activity, HcaA derivatives lacking the leader peptide (HcaA-Core and HcaA-Core-C53*; Figure S1) were cloned and expressed as MBP-fusions. Following TEV protease cleavage to remove the MBP-tag, peptides were treated with HcaD and HcaF. Although both substrates were processed by the HCA cyclodehydratase, the efficiency of processing was severely reduced relative to the full-length variants (Figures 4 and S16). Addition of HPLC-purified HcaA-LP to reactions increased the efficiency of processing, suggesting that leader peptide binding activates the HCA cyclodehydratase. Consistent with the important role that the leader peptide plays in substrate binding, modification of the core peptides occurred in a distributive, not processive, fashion regardless of the addition of the leader peptide *in trans* (Figures 4 and S16).

As with reactions performed with full-length HcaA, controls lacking either HcaD or HcaF displayed no processing (Figures 4 and S16). This result suggests that HcaF potentiates HcaD activity in addition to binding HcaA-LP. Although our current data do not reveal the underlying cause of this potentiation, two possibilities include the presentation of the core peptide to the

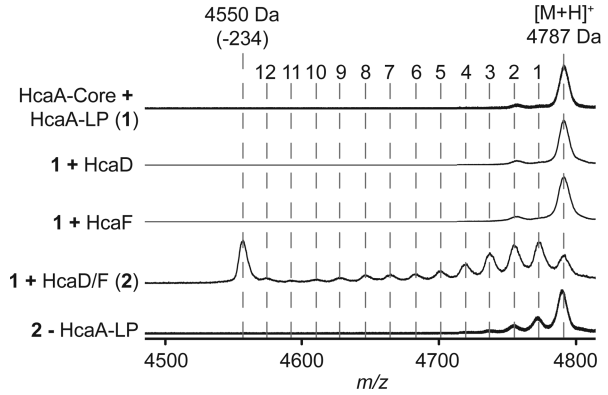

Figure 4. HcaA-LP activates the HCA cyclodehydratase. MALDI-TOF mass spectral overlay for HcaA-Core modification by HcaD/F is displayed. The mass of the starting material and the fully cyclodehydrated species are displayed along with the mass shift from the unmodified peptide. Dashed vertical guides are provided to indicate the expected masses for all partially cyclodehydrated species.

HcaD active site and the allosteric regulation of HcaD. Additional experiments will be required to unravel this phenomenon.

With the major roles for HcaF and the D (YcaO) domain of HcaD determined, we next attempted to determine the role of the C domain of HcaD. In order to accomplish this, we expressed each domain of HcaD (C domain: HcaD-E1; D domain: HcaD-YcaO) individually as an N-terminal His_6-tag fusion (Figure S2). Although the individual domains were inactive alone or with HcaF, a reaction containing all three components displayed cyclodehydratase activity (Figure S17). Based on this *in trans* activity, we reasoned that the interactions between the three components were still intact when HcaD was split into HcaD-E1 and HcaD-YcaO. Repeating the analytical size-exclusion chromatography experiment with the individual domains of HcaD clearly demonstrated that the C domain of HcaD was responsible for binding HcaF and the D domain of HcaD. This result is consistent with the naturally truncated C domain of HcaD serving as a scaffolding protein between the catalytic D domain and the leader peptide-binding, cyclodehydratase-activating TOMM F protein (Figures 5A and S17).

At the time of writing, there are over 300 bioinformatically identifiable TOMM F proteins in UniProt[28] distributed almost exclusively in HCA and thiopeptide biosynthetic clusters (Figures 5B, S18, and S19). Consistent with the function described above, 97% of TOMM F proteins are found in clusters containing N-terminally truncated fused TOMM cyclodehydratases (Figure S9). Although the sequence of the TOMM F protein is highly variable (Figure S11), the strong correlation between the presence of an F protein and the absence of the MccB-like peptide clamp on the C domain (Figure S9) suggests leader peptide binding will be a ubiquitous feature of this protein family.

■ CONCLUSIONS

We have reconstituted the activity of a novel TOMM cyclodehydratase found in nearly all members of the *B. cereus* group and have demonstrated that cyclodehydration is dependent on an uncharacterized biosynthetic protein, which we call the TOMM F protein. Through diverse biophysical assays, we have demonstrated that the TOMM F protein, rather than the C domain of the cyclodehydratase, is responsible for

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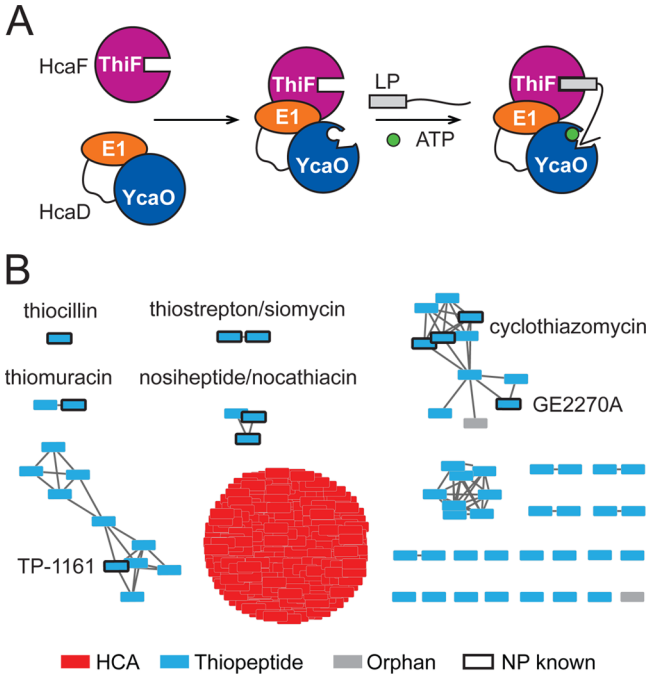

Figure 5. Model for TOMM F protein-dependent cyclodehydratases and distribution of TOMM F proteins. (A) A model for cyclodehydration in clusters containing an N-terminally truncated fused cyclodehydratase and a TOMM F protein is displayed. (B) A sequence similarity network of all bioinformatically identifiable TOMM F proteins is displayed. Each node represents a unique TOMM F protein. Lines connecting nodes share a BLAST Expect value (*E*-value) < 10^{-50}. Nodes are colored based on the class of natural product the cluster is predicted to produce. Nodes are bounded by a black box if the TOMM F protein is found in a cluster for which a natural product (NP) has been structurally characterized.

leader peptide recognition and cyclodehydratase activation. Our data are consistent with the C domain functioning as a scaffold to bring the catalytic D domain of the TOMM cyclodehydratase into contact with the substrate-binding TOMM F protein. Based on our bioinformatic analysis, we predict that the TOMM F protein will be required for the *in vitro* and *in vivo* biosynthesis of natural products from over 300 TOMM clusters (~25% of known TOMM clusters). Indeed, previous work with the nosiheptide (a thiopeptide) biosynthetic cluster demonstrated that the TOMM F protein was required for biosynthesis *in vivo*.[29] Although a biosynthetic role could not be provided at that time, we propose that the protein is required for precursor peptide recognition and cyclodehydratase activation. Additional studies will be necessary to confirm this hypothesis and to identify the leader peptide-binding domain in the TOMM F protein.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods and supporting figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04682.

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Notes

The authors declare no competing financial interest.

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