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MbtH-Like Proteins as Integral Components of Bacterial Nonribosomal Peptide Synthetases[†]

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ABSTRACT: The biosynthesis of many natural products of clinical interest involves large, multidomain enzymes called nonribosomal peptide synthetases (NRPSs). In bacteria, many of the gene clusters coding for NRPSs also code for a member of the MbtH-like protein superfamily, which are small proteins of unknown function. Using MbtH-like proteins from three separate NRPS systems, we show that these proteins copurify with the NRPSs and influence amino acid activation. As a consequence, MbtH-like proteins are integral components of NRPSs.

Nonribosomal peptide synthetases (NRPSs) are involved in the assembly of natural products of clinical interest such as the antibacterial drugs vancomycin, daptomycin, and capreomycin. A basic understanding how NRPSs catalyze the assembly of such molecules from simple precursors has been established (*1*). During assembly, each precursor is activated, covalently tethered to the NRPS, and then directionally condensed into the growing molecule by a set of catalytic domains grouped together as modules. Each module is typically composed of an adenylation (A) domain that recognizes and activates each precursor and tethers them to a peptidyl carrier protein (PCP) domain as a thioester. Condensation (C) domains subsequently catalyze directional bond formation between two PCP-linked precursors. Additional domains can add functionality to the precursors or govern their release from the NRPS. The repeating domain/modular structure of NRPSs provides an assembly line-like logic to the biosynthesis of the associated natural products.

In bacteria, many gene clusters coding for the NRPS involved in the production of natural products also code for a small (~70 amino acid) protein containing three conserved tryptophan residues. These proteins have been named the MbtH-like protein superfamily based on their similarity to MbtH from the mycobactin biosynthesis gene cluster (*2*). The production of some NRPS-dependent natural products requires an MbtH-like protein (*3*, *4*), but how these proteins influence production is unknown. Coproduction of an MbtH-like protein with an NRPS component enhances protein production levels (*5*). A direct role in catalysis has been questioned by a report that the enterobactin (ENT) NRPS is functional *in vitro* in the absence of the associated MbtH-like protein (*6*). Structural work on the MbtH-like protein from the pyroverdine system (*3*) and MbtH itself (*7*) did not reveal any motifs suggestive of a catalytic site. Instead, a role in protein−protein interactions was proposed.

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We are investigating the biosynthesis of the antituberculosis drugs capreomycin (CMN) and viomycin (VIO) to better understand NRPS enzymology and develop new derivatives of these drugs using combinatorial biosynthesis. These structurally related nonribosomal peptides are assembled by NRPSs that generate a cyclic pentapeptide core that can be appended by a sixth amino acid (Figure S1, Supporting Information) (*8*, *9*). In addition to the NRPS components, the associated gene clusters code for MbtH-like proteins. This provided us with two related NRPS systems to address questions concerning peptide assembly and the role the MbtH-like proteins play in this process.

We proposed that β-lysine (β-Lys) addition to the cyclic pentapeptide core of these antibiotics is catalyzed by NRPS components CmnM (C domain) and CmnO (A-PCP didomain) for CMN production and the corresponding homologues, VioM and VioO, for VIO production (*8*, *9*). We heterologously overexpressed both CmnO and VioO separately in *Escherichia coli* with N-terminal hexa-histidine (H6-CmnO, H6-VioO) affinity tags and purified them to near homogeneity (Figure 1A). We tested each protein for amino acid activation using standard ATP/PP_i exchange assays but failed to detect activity with β-Lys or any of the other amino acids found in CMN and VIO. CmnO was also inactive when produced with a C-terminal H6 tag (CmnO-H6) or an N-terminal maltose-binding protein tag (MBP-CmnO). H6-CmnO was also inactive when heterologously produced in *Streptomyces lividans*.

We next investigated whether the presence of the MbtH-like proteins CmnN or VioN influenced the activity of H6-CmnO or H6-VioO, respectively. CmnN and VioN were targeted because their associated genes are immediately upstream of *cmnO* and *vioO* in their respective gene clusters (*8*, *9*). The addition of purified CmnN to the CmnO reactions and VioN to the VioO reactions resulted in β-Lys activation (Figure 1B). The addition of purified CmnN also enabled CmnO-H6, MBP-CmnO, and H6-CmnO from *S. lividans* to activate β-Lys. Varying the concentration of MbtH-like protein relative to the amount of A-PCP didomain determined that optimal β-Lys activation was achieved when the MbtH-like protein was in excess (Figure S2, Supporting Information). This suggests that MbtH-like proteins are not needed in catalytic amounts, but rather they are needed in stoichiometric amounts to form a complex with the A-PCP didomain proteins. CmnN and VioN were able to stimulate β-Lys activation by the noncognate A-PCP didomain (Figure S3, Supporting Information). Additionally, the MbtH-like protein from the ENT pathway also stimulated β-Lys activation, albeit at a much lower level. These data are consistent with prior genetic data suggesting functional overlap between similar MbtH-like proteins (*4*). These data show that *in vitro* β-Lys activation by the

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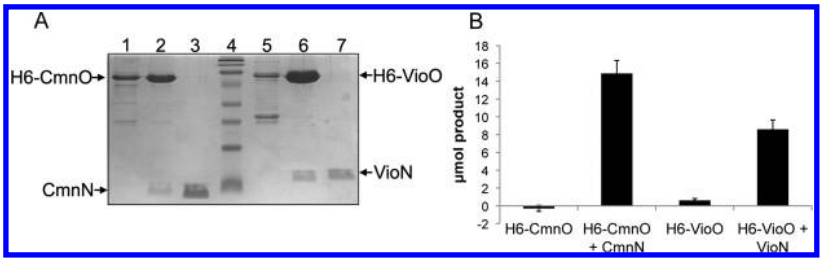

FIGURE 1: Purified proteins and assessment of β-Lys activation using ATP/PPi exchange assays. (A) 15% SDS-PAGE/Coomassie blue staining of 2 μg of H6-CmnO (lane 1), 3 μg of H6-CmnO coproduced with CmnN (lane 2), 2 μg of CmnN (lane 3), molecular mass markers (7.1, 20.6, 28.9, 34.8, 49.1, 80.0, 124.0, 209.0 kDa; lane 4), 2 μg of H6-VioO (lane 5), 3 μg of H6-VioO coproduced with VioN (lane 6), 2 μg of VioN (lane 7). CmnN and VioN in lanes 2 and 6, respectively, were confirmed by mass spectrometry. (B) Comparison of β-Lys activation by 100 nM H6-CmnO alone or with 1.6 μM CmnN and 100 nM H6-VioO alone or with 1.6 μM VioN. Assays were run for 15 min (H6-CmnO-containing) or 1 h (H6-VioO-containing) with 1 mM β-Lys.

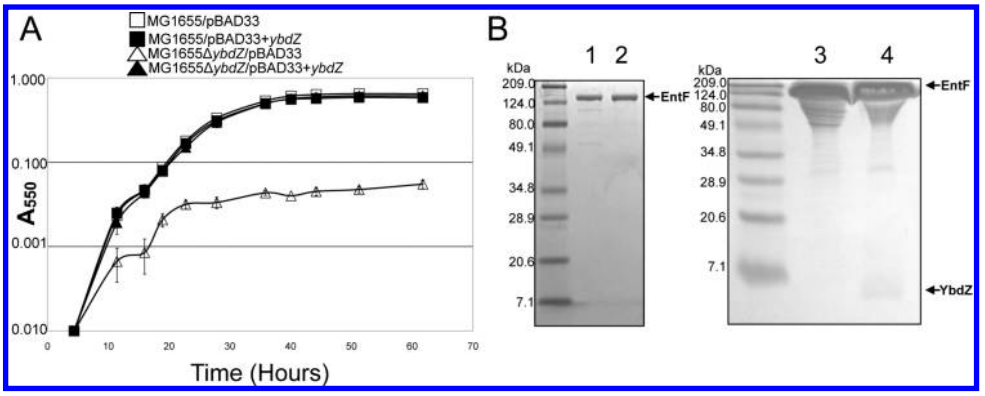

FIGURE 2: Analysis of ENT production *in vivo* and Purified EntF. (A) Growth curve comparing wild-type MG1655 to MG1655Δ*ybdZ* with an empty vector (pBAD33) or vector expressing *ybdZ* (pBAD33+*ybdZ*). (B) Analysis of purified EntF-H6 overproduced in the presence or absence of YbdZ: 1 μg EntF (lane 1), 1 μg EntF+YbdZ (lane 2), 17 μg EntF (lane 3), 17 μg EntF+YbdZ (lane 4); (left) 10−20% acrylamide gradient gel and (right) 15% acrylamide gel.

A domains of both CmnO and VioO requires an MbtH-like protein.

We reasoned if the MbtH-like proteins were forming a complex with the associated NRPS, the proteins would copurify when coexpressed in the same strain. To test this hypothesis, we overproduced H6-CmnO and untagged CmnN in the same *E. coli* strain and purified the H6-CmnO using Ni-affinity, anion-exchange, and size-exclusion chromatography. Analysis of the purified H6-CmnO by SDS-PAGE/Coomassie Blue staining determined that CmnN copurified with H6-CmnO (Figure 1A). Quantifying the amount of H6-CmnO and CmnN that copurified determined that the proteins were in ratio of 1.0 mol of H6-CmnO per 0.42 mol CmnN (Figure S4, Supporting Information). Similar results were found with H6-VioO and VioN. It is reasonable to propose that these proteins are in a 1:1 ratio but during purification a portion of the MbtH-like protein is lost. The H6-CmnO and H6-VioO proteins coproduced with CmnN and VioN, respectively, were both functional for β-Lys activation without the addition of independently purified MbtH-like protein; thus, the purified complexes were catalytically active. The copurified complexes were more active than the reconstituted complexes (Figure S5, Supporting Information), suggesting that coexpression of the NRPS components with an MbtH-like protein is important for a fully functional complex. Changing one of the conserved Trp residues in CmnN (W47A or W47F) yielded protein that neither copurified with H6-CmnO nor enabled H6-CmnO to be functional (Figure S6, Supporting Information). This confirmed that the observed H6-CmnO activities were due directly to the presence of wild-type CmnN.

We investigated whether the other A domains of the CMN NRPS required CmnN for optimal catalytic activity. The two A-PCP didomains of CmnA (CmnA-A1PCP1 and CmnA-A2PCP2), CmnF, and CmnG were overproduced in *E. coli*, with and without CmnN coexpression, and purified. Both H6-CmnA-A1PCP1 and H6-CmnA-A2PCP2 were catalytically active only when coexpressed with CmnN or when purified CmnN was added to the reaction mixtures (Figure S7, Supporting Information). The two CmnA A-PCP didomains copurified with CmnN in a ratio similar to that observed for H6-CmnO with CmnN and H6-VioO with VioN. To our surprise, neither H6-CmnF nor H6-CmnG copurified with CmnN. Both proteins were catalytically active in the absence of CmnN and coexpression with CmnN imparted no significant difference in the kinetic parameters (Table S1, Supporting Information). Addition of purified CmnN failed to alter H6-CmnF or H6-CmnG activity. These data suggest that in the same NRPS system there are A domains that are either dependent or independent of the MbtH-like proteins. At this time, it is not clear why there is such a distinction between A domains.

We were interested in whether our findings with the CMN and VIO NRPSs were translatable to other NRPS systems. We targeted the ENT siderophore biosynthetic pathway from *E. coli* because its biosynthetic gene cluster codes for an MbtH-like protein, YbdZ (*10*). Although prior studies suggest that YbdZ is not involved in NRPS catalysis (*6*), our findings with the CMN and VIO NRPSs made us reinvestigate this system.

We constructed an in-frame deletion of *ybdZ* from the chromosome of *E. coli* strain MG1655. The resulting strain was deficient in growth under iron-limiting conditions (Figure 2A), consistent with

Table 1: Kinetic Parameters of Substrate Activation

enzyme	amino or aryl acid	K_m[a]	V_{max}[a]
EntE	2,3-Dhb	2.9 ± 0.4	3168.2 ± 151.2
EntE+YbdZ	2,3-Dhb	1.9 ± 0.5	1164.2 ± 75.8
EntF[b]	L-Ser	7162.1 ± 413.5, 4589.5 ± 313.0	678.3 ± 13.1, 1342.2 ± 27.3
EntF+YbdZ[b]	L-Ser	655.9 ± 53.0, 240.1 ± 46.7	442.2 ± 11.7, 1066.5 ± 53.5

[a] K_m is in micromolar, V_{max} is in picomoles products/min/micrograms of protein. [b] The kinetic parameters for two different preparations are presented.

the inability of the strain to produce ENT. Introduction of a plasmid expressing *ybdZ* restored growth under iron-limiting conditions. These data, when combined with our observations with the CMN and VIO NRPSs, suggested that precursor activation by the ENT NRPS was compromised by the loss of YbdZ.

We overexpressed and purified EntE, an A domain specific for 2,3-dihydroxybenzoate (2,3-Dhb), and EntF, a multidomain NRPS with an A domain specific for L-Ser, in the presence or absence of YbdZ. Importantly, we inactivated the chromosomal copy of *ybdZ* in BL21(DE3); thus, the only source of YbdZ was from our *ybdZ*-expression plasmid. H6-EntE and YbdZ did not copurify and the kinetic parameters for 2,3-Dhb were similar whether or not H6-EntE was coproduced with YbdZ (Table 1). The addition of purified YbdZ to H6-EntE reactions also did not influence the observed activity. In contrast, EntF-H6 copurified with YbdZ (Figure 2B) and the level of EntF-H6 produced was higher in the presence of YbdZ. We determined that there were 0.6 mol of YbdZ for each mol of H6-EntF, similar to the ratio of MbtH-like protein to NRPS observed for the CMN and VIO NRPSs. A comparison of the kinetic parameters for L-Ser activation determined that EntF coexpressed with YbdZ had an average 15-fold higher affinity for L-Ser based on two independent purifications, but the V_{max} values were similar (Table 1). These data confirm that YbdZ plays a role in the catalytic function of EntF and likely explain the growth phenotype of the Δ*ybdZ* strain. In minimal medium, the intracellular concentration of L-Ser in *E. coli* varies from 68 to 150 μM (*11*). In the absence of YbdZ, the K_m of EntF for L-Ser is changed from near the *in vivo* concentration of L-Ser to ~15-fold higher. Thus, *E. coli* Δ*ybdZ* may not be able to produce enough ENT for efficient growth due to the lower affinity of EntF for L-Ser.

At first glance, our data appear inconsistent with previously published work on EntF; however, we believe our data are consistent with the prior work considering the new information we present. Prior analyses of overexpressed, full-length EntF were performed using an expression vector (pMS22) that overexpressed not only EntF but probably also YbdZ. This is consistent with the authors' finding that EntF yields increased when the *ybdZ* coding region was included on this expression vector (*12*), as we also observed that EntF levels were increased when YbdZ was present. At the time, the authors concluded that YbdZ was not produced from pMS22 because [^{35}S]-methionine-labeling studies detected EntF but not YbdZ; however, mature YbdZ does not contain any methionines. *E. coli* removes the N-terminal methionine of proteins that have alanine as the second residue as YbdZ does, therefore YbdZ cannot be detected using [^{35}S]-methionine labeling (*13*). Additionally, EntF produced from pMS22, and therefore with YbdZ, had a 23-fold lower K_m for L-Ser compared to the C-A didomain of EntF overproduced from a pET vector lacking the *ybdZ* gene (*14*). This is consistent with our observed 15-fold change in L-Ser affinity in the presence of YbdZ compared to its absence.

We have shown that MbtH-like proteins influence amino acid activation by NRPSs involved in the production of CMN, VIO, and ENT. These data have broad implications on NRPS enzymology. First, because A domains of the studied NRPSs were not equally dependent on MbtH-like proteins, understanding the reason for these differences will be essential for fully understanding NRPS enzymology. Second, much of the work on NRPS components has been performed without coexpression of the cognate MbtH-like protein. It will be interesting to know whether MbtH-like proteins influence the kinetics of these NRPSs as we observed with EntF. Third, it is not clear what impact correct or incorrect NRPS/MbtH-like protein pairing will have on combinatorial biosynthesis. Fourth, the coexpression of cognate NRPS/MbtH-like proteins may enable the biochemical characterization of NRPS components that have previously proven recalcitrant to analysis as we found with CmnO, VioO, CmnA-A1PCP1, and CmnA-A2PCP2. Finally, the knowledge that MbtH-like proteins are necessary for proper NRPS activity makes them excellent drug targets for antibacterial compounds that disrupt siderophore production by pathogens (*3*,*7*).

SUPPORTING INFORMATION AVAILABLE

Details of experimental procedures, Table S1, and Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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