SUPPORTING INFORMATION

MbtH-like proteins are integral components of bacterial non-ribosomal peptide synthetases

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Supplementary Results

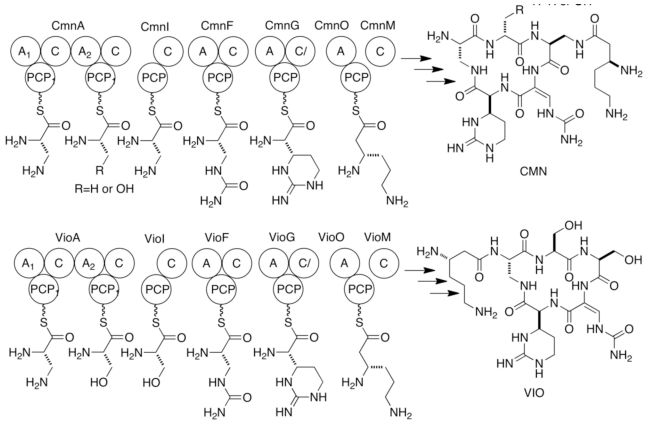

Figure S1. Schematics of the CMN and VIO NRPSs and chemical structure of CMN and VIO. Schematic of the CMN (top) and VIO (bottom) NRPSs highlighting the domain architecture and the amino acid activated by each module. The protein name is shown above the associated protein domains. The chemical structures of the final natural products are shown at the right. For simplicity, CMN is represented by structures of CMN IA (R=OH) and CMN IB (R=H). The CMN derivatives lacking β-Lys (CMN IIA and IIB) are not shown. The NRPS abbreviations are: A, adenylation domain; PCP, peptidyl carrier protein domain; C, condensation domain; C/ truncated condensation domain. For CmnA and VioA, the A and PCP domains are denoted as A1, PCP1, A2, PCP2 to clarify which domains are discussed in the text.

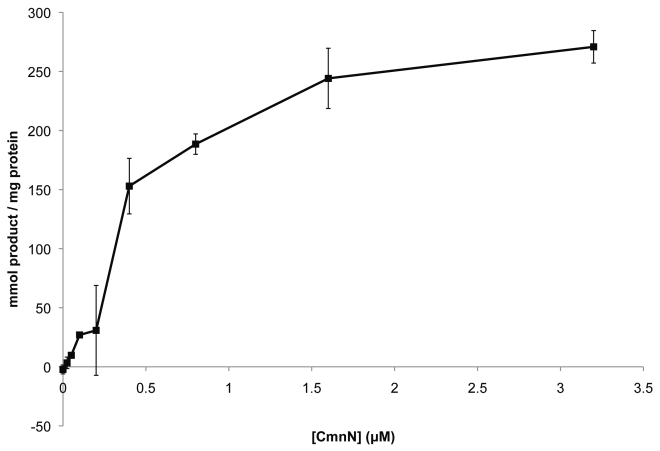

Figure S2. Increase in β-Lys activation by H6-CmnO as a function of increasing concentrations of CmnN. The concentration of H6-CmnO was held constant at 100 nM and the CmnN concentration was varied from 0 to 3.2 μM. The ATP/PP_i exchange assays were run for 15 min with β-Lys at 1 mM. Assays were performed in triplicate, and the standard deviations are shown. CmnN, in the absence of H6-CmnO, did not stimulate ATP/PP_i exchange under any conditions tested.

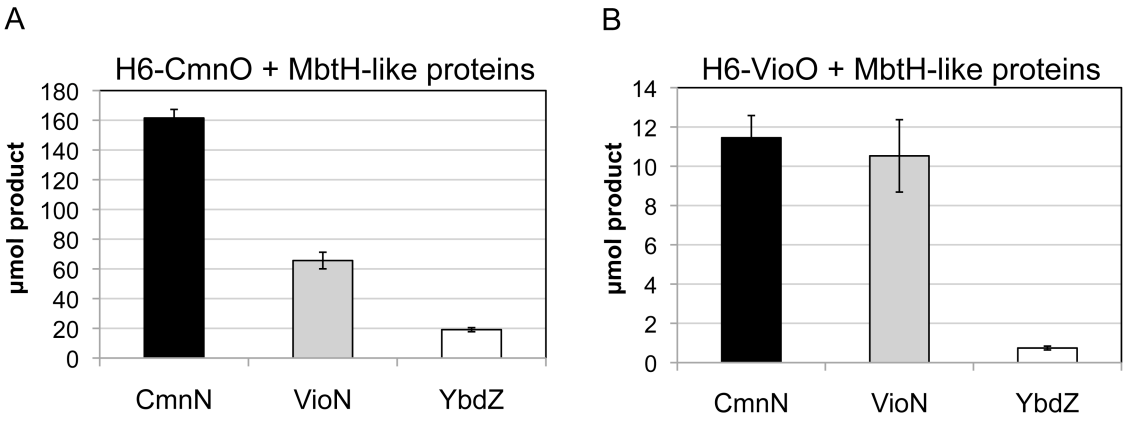

Figure S3. β-Lys activation stimulated by the presence of CmnN, VioN, or YbdZ. Quantification of the amount of product detected when 100 nM H6-CmnO (A) or H6-VioO (B) were incubated with 1.6 μM CmnN, VioN, or YbdZ. H6-CmnO containing assays were run for 15 min while H6-VioO assays were run for 1 hr. All assays contained 1 mM β-Lys. Assays were performed in triplicate, and the standard deviations are shown. The MbtH-like proteins, by themselves, did not stimulate ATP/PP_i exchange under any conditions tested. In the absence of an MbtH-like protein, no activity by H6-CmnO or H6-VioO was detected. Amino acid sequence identity/similarity between MbtH-like proteins: CmnN versus VioN, 75% identity/83% similarity; CmnN versus YbdZ, 27% identity/46% similarity; VioN versus YbdZ, 32% identity/50% identity.

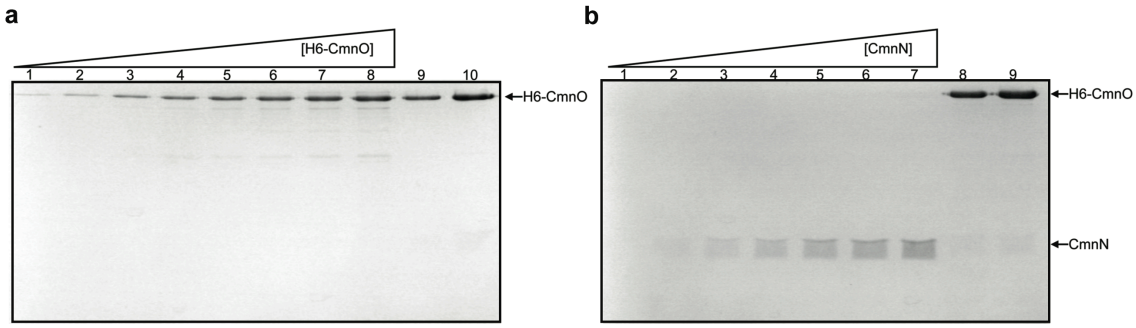

Figure S4. Quantifying H6-CmnO and CmnN when co-purified. (a) Increasing concentrations of H6-CmnO were loaded in lanes 1-8 (2.4, 4.7, 9.4, 14.1, 18.8, 23.5, 28.3, 35.3 nmol). 2.6 and 5.3 μl of co-purified H6-CmnO and CmnN were loaded in lanes 9 and 10, respectively. Using ImageJ64 version 1.43, the intensity of the protein bands in lanes 1-8 were used to generate a standard curve of nmol versus band intensity. This was used to estimate the number of nmol of H6-CmnO in lanes 9 and 10. (b) Increasing concentrations of CmnN were loaded in lanes 1-7 (17.6, 34.4, 52.1, 68.9, 86.5, 103.3). 5.3 and 10.6 μl of co-purified H6-CmnO were loaded in lanes 8 and 9. Using ImageJ64 version 1.43, the intensity of the protein bands in lanes 1-7 were used to generate a standard curve of nmol versus band intensity. This was used to estimate the number of nmol CmnN in lanes 8 and 9. The same type of analysis was performed for H6-VioO + VioN, H6-CmnA-A1PCP1 + CmnN, H6-CmnA-A2PCP2, and EntF-H6 + YbdZ.

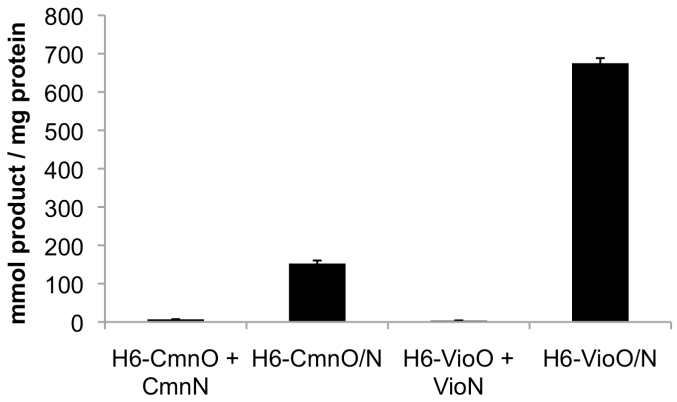

Figure S5. Comparison of β-Lys activation by 0.64 μg H6-CmnO with 2.0 μM CmnN (H6-CmnO+CmnN), 0.64 μg of total protein of H6-CmnO co-produced with CmnN (H6-CmnO/N), 0.66 μg H6-VioO with 2.0 μM VioN (H6-VioO+VioN), 0.66 μg of total protein of H6-VioO co-produced with VioN (H6-VioO/N). The mg protein refers to the amount of H6-CmnO or H6-VioO. Assays were run for either 15 min (H6-CmnO-containing) or 1 hr (H6-VioO-containing) with 1 mM β-Lys.

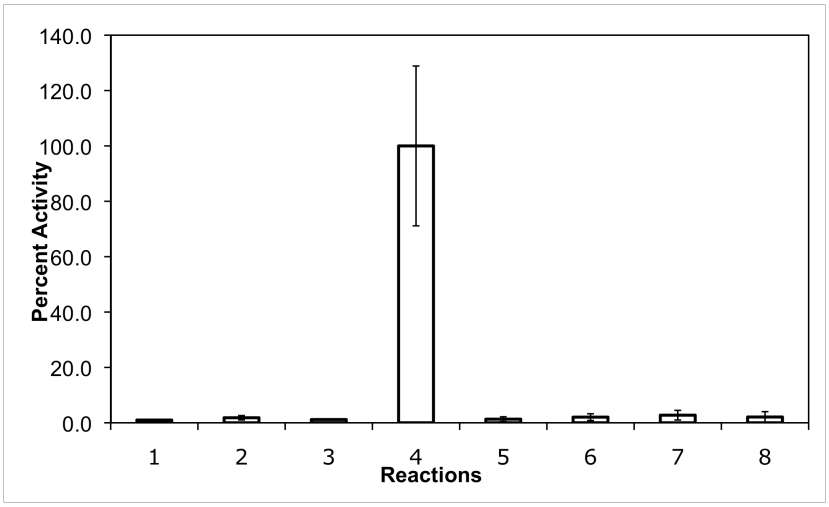

Figure S6. Comparison of β-Lys activation of H6-CmnO co-produced with wild-type CmnN, CmnN W47A, or CmnN W47F. Reactions are: 1, H6-CmnO no β-Lys; 2, H6-CmnO with 1 mM β-Lys; 3, H6-CmnO co-produced with CmnN no β-Lys; 4, H6-CmnO co-produced with CmnN with 1 mM β-Lys; 5, H6-CmnO co-produced with CmnN W47A no β-Lys; 6, H6-CmnO co-produced with CmnN W47A with 1mM β-Lys; 7, H6-CmnO co-produced with CmnN W47F no β-Lys; 8, H6-CmnO co-produced with CmnN W47F with 1 mM β-Lys. All assays were run for 30 min. The average activity of the H6-CmnO co-produced with wild-type CmnN was set at 100% and samples were normalized to this value. All assays were performed in triplicate, and the standard deviations are shown.

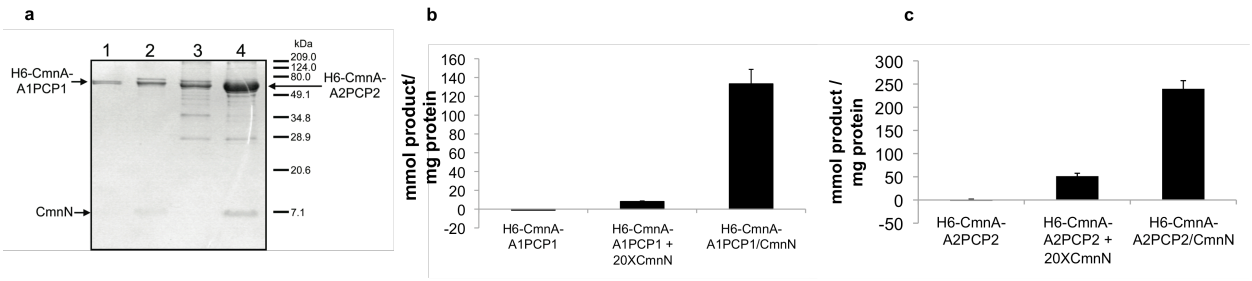

Figure S7. Purification and analysis of H6-CmnA-A1PCP1 and H6-CmnA-A2PCP2 in the presence or absence of CmnN. (a) 15% SDS-PAGE/Coomassie blue stained gel of: lane 1, H6-CmnA-A1PCP1 without CmnN co-production; lane 2, H6-CmnA-A1PCP1 with CmnN co-production; lane 3, H6-CmnA-A2PCP2 without CmnN co-production; lane 4, H6-CmnA-A2PCP2 with CmnN co-production. (b) 0.06 μg of H6-CmnA-A1PCP1 was used in all three assays, the reactions were run for 1 hr with 1 mM L-2,3-diaminopropionate. The 20XCmnN represents a reaction where 20X excess of CmnN was added relative to the amount of H6-CmnA-A1PCP1. The H6-CmnA-A1PCP1/CmnN is the co-produced and purified proteins. (c) 2 μg of H6-CmnA-A2PCP2 was used in all three assays. The reactions were run for 1 hr with 1 mM L-Ser. The 20XCmnN represents a reaction where 20X excess of CmnN was added relative to the amount of H6-CmnA-A2PCP2. The H6-CmnA-A2PCP2/CmnN is the co-produced and purified proteins. All assays were performed in triplicate, and the standard deviations are shown.

Table S1. Kinetic Parameters of Amino Acid Activation by CmnF and CmnG[a]

Enzyme	Amino Acid	K_m (μM)[a]	V_{max} (pmol/min/μg protein)
H6-CmnF	β-ureidoalanine	60.4 ± 11.0	38.5 ± 2.8
H6-CmnF + CmnN[b]	β-ureidoalanine	52.1 ± 7.5	46.5 ± 2.6
H6-CmnG	L-capreomycidine	4.3 ± 0.6	84.2 ± 3.5
H6-CmnG + CmnN[b]	L-capreomycidine	3.3 ± 0.3	87.9 ± 1.9

[a] Assays were run in triplicate, the calculated values and standard errors are shown.

[b] Indicates both proteins were co-produced together in *E. coli* prior to purification.

Table S2. List of Strains and Plasmids

Strains	Relevant characteristics	Source or reference
BL21(DE3)	F^-, *ompT*, *gal*, *dcm*, *lon*, *hsdSB*(rB^- mB^-), λ(DE3 [*lacI*, *lac*UV5-T7 gene 1, ind1, *sam*7, *nin*5])	Novagen
Rosetta™ (BL21(DE3)/pRARE)	BL21(DE3) containing a plasmid expressing rare tRNAs.	Novagen
BL21(DE3)*ybdZ*::aac(3)IV	BL21(DE3) with the *ybdZ* gene interrupted with an apramycin resistance gene.	this study
MG1655	F^-, λ^-, *ilvG*, *rfb*-50, *rph*-1	ATCC 700926
MG1655Δ*ybdZ*	MG1655 with a *ybdZ* deletion	this study
Streptomyces lividans 1326	WT	A. Gehring
Plasmids		
pET22b	T7 overexpression vector	Novagen
pET28a	T7 overexpression vector	Novagen
pET28b	T7 overexpression vector	Novagen
pIADL16	Maltose binding protein fusion overexpression vector	(*1*)
pGM202	Thiostrepton-inducible overexpression vector for Streptomyces	G. Muth
pBAD33	Arabinose-inducible expression vector	(*2*)
pTYB12	Chitin binding domain fusion overexpression vector	New England Biosciences
pTYB2	Chitin binding domain fusion overexpression vector	New England Biosciences
pACYCDuet-1	T7 coexpression vector	Novagen
pACYCDuet-1-MCS	pACYCDuet-1 with multiple cloning site deleted	this study

pET22b-cmnO	pET22b expressing *cmnO*	this study
pET28a-cmnO	pET28a expressing *cmnO*	this study
pIADL16-cmnO	pIADL16 expressing *cmnO*	this study
pGM202-cmnO	pGM202 expressing *cmnO*	this study
pET28b-vioO	pET28b expressing *vioO*	this study
pET28b-cmnA-A_1PCP_1	pET28b expressing the excised A1PCP1 domains from *cmnA*	this study
pET28b-cmnA-A_2PCP_2	pET28b expressing the excised A2PCP2 domains from *cmnA*	this study
pET28b-cmnG	pET28b expressing *cmnG*	this study
pET28b-cmnF	pET28b expressing *cmnF*	(*3*)
pET28b-entE	pET28b expressing *entE*	this study
pET28b-entF	pET28b expressing *entF*	this study
pBAD33-ybdZ	pBAD33 expressing *ybdZ*	this study
pTYB12-cmnN	pTYB12 expressing *cmnN*	this study
pTYB12-vioN	pTYB12 expressing *vioN*	this study
pTYB2-ybdZ	pTYB12 expressing *ybdZ*	this study
pACYCDuet-1-cmnN	pACYCDuet-1 expressing *cmnN*	this study
pACYCDuet-1-vioN	pACYCDuet-1 expressing *vioN*	this study
pACYCDuet-1-ybdZ	pACYCDuet-1 expressing *ybdZ*	this study
pACYCDuet-1-cmnN-W47A	pACYCDuet-1 producing CmnN with a W47A mutation	this study
pACYCDuet-1-cmnN-W47F	pACYCDuet-1 producing CmnN with a W47F mutation	this study

Table S3. Primers used to generate overexpression vectors[a]

CmnO-NdeI	GGAGGCCATATGACCGCGCTGCACCGCCTCGAC
CmnO-HindIII	GGACAAGCTTGTGCGGCTGGACGGGCACCGCGC
CmnO-XhoI	GGAGGATTTCTCGAGCCTCGGGGTCCGGCT
CmnO-BamHI	GGCTGTGGATCCCCTCGGGGTCCGGCT
	CCGCGCGGCAGCCATATGACCGCGGACGCCGCGC
cmnG-5'NdeI PIPE	TCG
	AGTGCGGCCGCAAGCTTTCACCGCCGCTCACCCG
cmnG-3'HindIII PIPE	CCAACG
cmnF-NdeI	GGAGAGCATATGACCCAGGTCGACTTCACCCGG
cmnF-HindIII	CCCGTTCGTAAGCTTCGAGCGCGGCGTCCGCGG
CmnN-NdeI	GGGGGACATATGGACACGTACCTGGTGGTCGTC
CmnN-XhoI	GGCCCGCCTCGAGGTCGAGGCGGTGCAGCGCGG
	ATAAGGAGATATACCATGGACACGTACCTGGTGGT
cmnN-DuetNcoI-5'	CGTC
	TTATGCGGCCGCAAGCTTGGTCGAGGCGGTGCAG
cmnN-DuetHindIII-3'	CGCGGTCA
	CCGCGCGGCAGCCATATGTCCGAGGACGAGCACC
CmnA-APCP1-NdeI-5’	GCCGG
	GAGTGCGGCCGCAAGCTTTCAGGACAGCAGCGCC
CmnA-APCP1-Stop-3’	GCCATCGC
	CCGCGCGGCAGCCATATGACCAGGGTCGCGGACC
CmnA-APCP2-NdeI-5’	TGCCGCTG
	GAGTGCGGCCGCAAGCTTTCAGTCGGCGCGCGTG
CmnA-APCP2-Stop-3’	CCGTCCAC
CmnN-W47A-5'	GAGACCGTGGCGACCGACATGCGC
CmnN-W47A-3'	GCGCATGTCGGTCGCCACGGTCTC
CmnN-W47F-5'	GAGACCGTGTTTACCGACATGCGC
CmnN-W47F-3'	GCGCATGTCGGTAAACACGGTCTC
VioO-NdeI	ATAACATATGACCACCATGCCCACGACCGGC
VIOO-SHindIII	TAATAAGCTTCCACGAGTTCCCGGCGGCGGTA
	CAGAATGCTGGTCATATGAACGACACCCCTGCGGA
VioNNdeIpTYB12	CACC
VioNXhoIpTYB12	CAGTCACCCGGGCTCGAGTCATGCGCGGGCGCTG

	AGCGGACG
	ATAAGGAGATATACCATGGACGACACCCCTGCGGA
vioN-DuetNcoI-5'	CACC
	TTATGCGGCCGCAAGCTTTCATGCGCGGGCGCTGA
vioN-DuetHindIII-3'	GCGGACG
	AGAAGGAGATATACCATGAGCCAGCATTTACCTTTG
EntF-5’-NcoI28b	GTCGCC
	GAGTGCGGCCGCAAGCTTCCTGTTTAGCGTTGCGC
EntF-5’-NoStop28b	GAATAAT
	ATAAGGAGATATACCATGGCATTCAGTAATCCCTTC
YbdZ-DuetNcoI-5'	GAT
	TTATGCGGCCGCAAGCTTGCTGGTTTCCATTTACG
YbdZ-DuetHindIII-3'	ACCGAGT
	TAAGAAGGAGATATACATATGGCATTCAGTAATCCC
YbdZ-pTYB2-NdeI-PIPE	TTC
	CTTGGCAAAGCACCCGGGTTGTGCCTCCTGCAACT
YbdZ-pTYB2-SmaI-PIPE	G

[a] All primers are listed in 5' to 3' direction.

Supplementary Methods

Bacterial strains, plasmids, and media. Bacterial strains used in this study are listed in Table S2. *E. coli* strains were propagated in LB liquid medium or on LB agar plates with the appropriate antibiotic selection. *S. lividans* 1326 strains were propagated on International Streptomyces Project Medium 2 (ISP2) plates or in Yeast Extract Malt Extract (YEME) liquid medium with the appropriate antibiotic (*4*).

The BL21(DE3)*ybdZ*::*acc(3)IV* strain was constructed by first generating a *ybdZ*::*acc(3)IV* mutation in the chromosome of *E. coli* strain BW25113 and then moving the mutation into BL21(DE3) by transduction. Briefly, the primers RedirectYbdZup 5'-ACCTCTGGCAACCACTTTTCCATGACAGGAGTTGAATATGATTCCGGGGATCCGTCGACC-3' and RedirectYbdZDown 5'-GCGACCAAAGGTAAATGCTGGCTCATTGTGCCTCCTGCAATGTAGGCTGGAGCTGCTTC-3' were used to PCR amplify the *aac(3)IV* gene from pIJ773 (John Innes Centre). The amplicon was electroporated into a BW25113/pIJ790 strain that was made eletrocompetent after growing under conditions to induce for the λ RED recombination enzyme (REDIRECT© technology [John Innes Centre])(*5, 6*). Strains containing the *ybdZ*::*acc(3)IV* mutation were selected on LB apramycin (100 μg ml^{-1}) at non-permissible temperatures for pIJ790 replication. Confirmation of the *ybdZ*::*acc(3)IV* mutation was accomplished using PCR amplification. Movement of the *ybdZ*::*acc(3)IV* mutation into BL21(DE3) involved standard bacteriophage P1 transductions (*7*). Confirmation of *ybdZ*::*acc(3)IV* insertion in BL21(DE3) was confirmed by PCR amplification and sequencing of the resulting amplicon.

Construction of the pACYCDuet-1 vector derivative lacking the multiple cloning site (MCS), pACYCDuet-1-MCS, was performed using PIPE cloning (*8*) using the following primers: pACYC-DuetNoMCS1 5'AAGGGAGAGCGTCGAGATCCCGG 3' and pACYC-DuetNoMCS2, 5' ATCTCGACGCTCTCCCTTATAACCCCTTGGGGCCTCTAA 3'. The MCS deletion was confirmed by PCR amplification and restriction digest analysis.

Generation of overexpression vectors. Plasmids used in this study are listed in Supplemental Table 2. All plasmids were constructed using PCR-based cloning except for pIADL16-cmnO, which was generated by subcloning the *Nde*I/*Hin*dIII *cmnO*-containing fragment from pET28a-cmnO into the corresponding sites of pIADL16. All *cmn* genes or gene fragments were PCR amplified from pCMN-P4C8RF-436 (*9*). The gene *vioO* was PCR amplified from pVIO-P4C3RH-436 (*3*). The genes *ybdZ, entE,* and *entF* were PCR amplified from chromosomal DNA of *E. coli* strain MG1655. All primers used for construction of overexpression vectors in this study are listed in Supplemental Table 3.

Purification of CmnO. The *cmnO* overexpression plasmids pET28a-cmnO, pET22b-cmnO, and pIADL16-cmnO were each transformed into BL21(DE3)/pRARE (Rosetta™, Novagen, Madison WI), selecting for chloramphenicol (15 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) resistance. We note that the activity of purified CmnO was similar when overproduced in BL21(DE3) and BL21(DE3)*ybdZ*::*acc(3)IV*. To overproduce H6-CmnO from each of these constructs, 6 X 1L LB (supplemented with chloramphenicol and kanamycin) cultures of each overexpression strain were grown at 25°C until an OD_{600} of 0.5 was reached. The cells were shifted to 4°C for 1 hr, isopropyl β-D-1-thiogalactopyranoside (IPTG; 100 μM) was added, and cells were shifted to 15°C and grown for 15 hr. Cells were harvested by centrifugation (10 min at 10,000 rpm, Beckman J2-21 centrifuge, KOMP Spin KA-9 rotor) and resuspended in buffer A (Tris-HCl pH8.0 [20 mM], NaCl [300 mM], glycerol [10% v/v]) containing imidazole (5 mM). The cells were broken by sonication (Fisher 550 Sonic Dismembrator, power = 5, 15 min sonication with 1

sec on, 1 sec off). Cell debris was removed by centrifugation (30 min at 15,000 rpm, Beckman J2-21 centrifuge, Beckman JA-25.50 rotor).

Clarified cell extract was incubated with 1 ml of Ni-NTA resin (Qiagen) for 2 hr at 4°C with gentle rocking. The resin was recovered and washed with buffer A containing imidazole (20 mM). H6-CmnO was eluted with a step gradient of buffer A plus varying concentrations of imidazole (40, 60, 80. 100, 250 mM). Fractions containing H6-CmnO, based on SDS-PAGE/Coomassie blue staining, were pooled and dialyzed at 4°C overnight against buffer B (Tris HCl, pH 8.0 [50 mM], NaCl [100 mM], glycerol [10% v/v]) using 3500 MWCO SnakeSkin® Pleated Dialysis Tubing (Thermo Scientific). The protein was concentrated using a Centriprep YM-3 (Amicon) and flash frozen in liquid nitrogen. The concentrations of H6-CmnO and CmnO-H6 were determined by using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) with bovine serum albumin (BSA) as a standard and by using the calculated molar extinction coefficient (56,170 M^{-1} cm^{-1}).

Cells overproducing maltose binding protein (MBP)-CmnO fusion protein were resuspended in buffer A, and clarified cell extract was generated as described for H6-CmnO. The clarified cell extract was incubated with 1.5 ml amylose resin (New England BioLabs) at 4°C for 2 hr with gentle rocking. MBP-CmnO was eluted with a step gradient of buffer A plus varying concentrations of maltose (1, 2, 4, 6, 8, 10 mM). Fractions containing MBP-CmnO, based on SDS-PAGE/Coomassie staining, were pooled, dialyzed (10000 MWCO SnakeSkin® Pleated Dialysis Tubing) against buffer B at 4°C overnight, and flash frozen in liquid nitrogen. The concentration of MBP-CmnO was determined by using the BCA Protein Assay kit using BSA as a standard.

The *cmnO* overexpression plasmid pGM202-cmnO was transformed into protoplasts of *S. lividans* 1326 using standard polyethylene glycol-assisted transformation methods (*10*) , and the transformants were selectied on kanamycin (200 μg ml^{-1})-containing medium. The transformants were streaked for isolation on kanamycin-containing ISP2 plates, and a single colony was used to inoculate 25 ml YEME medium supplemented with kanamycin (200 μg ml^{-1}). After 5-7 days of growth, shaking in baffled flasks at 30°C (200 rpm), cultures were harvested by centrifugation, washed with a 10.3% sucrose solution, resuspended in 2 ml 10.3% sucrose solution, and frozen at -20°C. Fifty-microliters of this frozen mycelial stock was used to inoculate 30 ml of kanamycin-containing YEME medium in a baffled flask and subsequently shaken (200 rpm) at 30°C for 9 days. Four-milliliters of this starter culture was used to inoculate 2 X 400 ml kanamycin-containing YEME in baffled flasks. The cultures were shaken (200 rpm) at 30°C for 4 days. Thiostrepton was added to a final concentration of 12.5 μg ml^{-1} to induce expression of H6-CmnO. After 24 h, the cultures were harvested by centrifugation and washed in 10.3 % sucrose. H6-CmnO was purified as described for described for Ni-NTA purification of *E. coli*-produced enzyme. The concentration of H6-CmnO was determined by using the BCA Protein Assay kit using BSA as a standard.

Purification of H6-VioO. The overexpression plasmid pET28b-vioO was transformed into BL21(DE3). The resulting strain was used to inoculate 6 X 1 L of LB medium supplemented with kanamycin (50 μg ml^{-1}) and the cultures were grown at 25°C for 24 hr. Purification of H6-VioO followed the purification scheme outlined above for H6-CmnO.

Co-production and purification of H6-CmnO with CmnN and H6-VioO with VioN. BL21(DE3)*ybdZ*::*acc(3)IV* was transformed with pET28b-CmnO and pACYCDuet-1-CmnN or pET28b-VioO and pACYCDuet-1-VioN. Transformants were selected on LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}). The transformants were used to inoculate 6 X 1L of LB supplemented with the appropriate antibiotics. The cultures were grown at 25°C until the OD_{600} reached 0.5 at which time the temperature was shifted to 15°C. After 1

hr, IPTG (100 μM) was added, and cells were grown for an additional 16h at 15°C. Clarified cell extract was generated following the same protocol as described for H6-CmnO.

The H6-CmnO and H6-VioO co-produced with CmnN and VioN, respectively, were purified using a three-step purification procedure. The first step was Ni-affinity chromatography as described above for H6-CmnO produced in the absence of CmnN. However, instead of flash freezing the proteins, they were dialyzed against buffer C (Tris HCl, pH 8.0 [50 mM], NaCl [25 mM], glycerol [10% v/v]) using 3500 MWCO SnakeSkin® Pleated Dialysis Tubing at 4°C overnight to prepare the proteins for the second step in the purification. The second step was strong anion-exchange chromatography using a 5 ml HiTrap Q Sepharose™ Fast Flow column (GE Healthcare Life Sciences). At a flow rate of 1 ml min^{-1} (using an AKTA™ prime FPLC; GE Healthcare Life Sciences), the proteins were bound to the column, the column was washed with 50 ml buffer C, and the proteins were eluted with a 100 ml gradient from 100% buffer C/0% buffer D (Tris-HCl, pH 8.0 [20 mM], NaCl [500 mM], glycerol [10% v/v]) to 0% buffer C/100% buffer D. Fractions containing H6-CmnO or H6-VioO were identified by SDS-PAGE/Coomassie blue staining and were pooled and dialyzed (3500 MWCO SnakeSkin® Pleated Dialysis Tubing) against buffer B at 4°C overnight. A final purification by size-exclusion chromatography was performed using a HiPrep 16/60 Sephacryl™ S-200 High Resolution column (GE Healthcare Life Sciences) at a flow rate of 0.4 ml min^{-1} (using a BioLogic LP system; BioRad) of buffer B. Fractions containing H6-CmnO or H6-VioO were identified by SDS-PAGE/Coomassie blue staining and pooled. The proteins were then concentrated (Centriprep YM-3) and flash frozen in liquid nitrogen. The concentrations of the purified proteins were determined by the Pierce BCA assay kit with BSA as a standard. The co-purification of CmnN or VioN was detectable by SDS-PAGE/Coomassie blue staining. Confirmation that CmnN co-purified with H6-CmnO was obtained by determining the mass of the protein using MALDI-TOF mass spectrometry (UW-Madison, Biotechnology Center). The observed *m*/*z* was 7118.6 ($[M+H]^+$), the theoretical *m*/*z* is 7126.0 ($[M+H]^+$). Additionally, the protein was only present when CmnN was co-expressed with H6-CmnO, it fails to co-purify with H6-CmnO when point mutations to a conserved Trp residue are generated, and had a similar migration in a 15% SDS-PAGE as purified CmnN (the purified CmnN contained three additional amino acids due to the CBD-tag cleavage reaction). Confirmation that VioN co-purified with H6-VioO was obtained using MALDI-TOF MS. The observed *m*/*z* was 6992.3 ($[M+H]^+$), the theoretical *m*/*z* is 6982.8 ($[M+H]^+$). Additionally, the protein that co-purified with H6-VioO had a similar migration in a 15% SDS-PAGE as purified VioN (the purified VioN contained three additional amino acids due to the CBD-tag cleavage reaction).

Co-production of CmnO and mutant CmnN. The gene *cmnN* was mutagenized using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using primers cmnNW47A5' and cmnNW47A3' for the W47A substitution or primers cmnNW47F5' and cmnNW47A3' for the W47F substitution. pACYCDuet-1-cmnN-W47A or pACYCDuet-1-cmnN-W47F was co-transformed into BL21(DE3)*ybdZ*::*aac(3)IV* with pET28b-cmnO, selecting for chloramphenicol (15 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) resistance. Overproduction and purification of H6-CmnO from both mutant CmnN co-production strains were performed as described for the co-production of H6-CmnO and CmnN. The concentrations of the purified proteins were determined by using the Pierce BCA Protein Assay kit with BSA as a standard. The co-purification of mutant CmnN proteins was analyzed by SDS-PAGE/Coomassie blue staining.

Purification of H6-CmnA-A1PCP1 and H6CmnA-A2PCP2 with and without CmnN. BL21(DE3)*ybdZ*::*acc(3)IV* was transformed with pET28b-CmnA-A1PCP1 and pACYCDuet-1-MCS, pET28b-CmnA-A1PCP1 and pACYCDuet-1-CmnN, pET28b-CmnA-A2PCP2 and

pACYCDuet-1-MCS, or pET28b-CmnA-A2PCP2 and pACYCDuet-1-CmnN. In all cases, transformants were selected on LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}). Transformants were used to inoculate 3 X 1L of LB containing the appropriate antibiotics. The overproduction and purification of H6-CmnA-A1PCP1 with our without CmnN coproduction and H6-CmnA-A2PCP2 with or without CmnN coproduction were performed as described for purification of H6-CmnO and H6-VioO with their respective MbtH-like protein homologs with one minor modification. The modification was the 5 ml HiTrap Q Sepharose™ Fast Flow column was washed with 25 ml of buffer B rather than 50 ml. The concentrations of the purified proteins were determined by using the Pierce BCA Protein Assay kit iwht BSA as a standard. Additionally, the calculated molar extinction coefficients of H6-CmnA-A1PCP1 (35,995 M^{-1} cm^{-1}) and H6-CmnA-A2PCP2 (30,035 M^{-1} cm^{-1}) were used to determine the molarity of the proteins purified in the absence of CmnN. The co-purification of mutant CmnN proteins was analyzed by SDS-PAGE/Coomassie blue staining. The identity of CmnN co-purifying with H6-CmnA-A1PCP1 and H6-CmnA-A2PCP2 was confirmed by MALDI-TOF MS. The theoretical *m*/*z* of CmnN is 7126.0 ($[M+H]^+$). The observed *m*/*z* of the protein that co-purified with CmnA-A1PCP1 was 7125.3 ($[M+H]^+$). The observed *m*/*z* of the protein that co-purified with CmnN-A2PCP2 was 7121.9 ($[M+H]^+$). Additionally, this protein was only present in the CmnA-A1PCP1 and CmnN-A2PCP2 preparations when CmnN was co-expressed.

Purification of H6-CmnF and H6-CmnG with and without CmnN co-production. BL21(DE3)*ybdZ*::*acc(3)IV* was transformed with pET28b-CmnF and pACYC-Duet1-MCS, pET28b-CmnF and pACYC-Duet1-CmnN, pET28b-CmnG and pACYC-Duet1-MCS, or pET28b-CmnG and pACYC-Duet1-CmnN. In all cases, transformants were selected on LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}). Transformants were used to inoculate 3 X 1L of LB containing the appropriate antibiotics. The overproduction and purification of H6-CmnF and H6-CmnG with or without CmnN coproduction were performed as described for purification of H6-CmnO-with CmnN. The concentrations of the purified proteins were determined by using the Pierce BCA Protein Assay kit with BSA as a standard. The co-purification of mutant CmnN proteins was analyzed by SDS-PAGE/Coomassie blue staining.

Purification of CmnN, VioN, and YbdZ. BL21(DE3) was transformed with pTYB12-vioN, pTYB12-cmnN, or pTYB2-ybdZ. Transformants were selected on LB medium containing ampicillin (100 μg ml^{-1}). To overproduce CBD-VioN for purification, 3 X 1L LB medium supplemented with ampicillin (100 μg ml^{-1}) were inoculated and grown at 25°C until OD_{600} of 0.5 was reached, and the cells were shifted to 15°C. After 1hr at this temperature, IPTG (100 μM) was added, and cells were grown for 18hr at 15°C. Cells were harvested by centrifugation and resuspended in buffer E (Tris-HCl pH 8.0 [20mM], NaCl [500mM]). Cells were broken by sonication, and cell debris was removed by centrifugation as described for H6-CmnO purification. The supernatant was passed over 1 ml chitin resin 1 ml (New England BioLabs) and washed with 20 ml of buffer F (Tris-HCl pH 8.0 [50 mM], NaCl [50 mM], glycerol [10 % v/v]). VioN was eluted from the column by CBD tag cleavage by flushing the column with 25 ml buffer F supplemented with dithiothreitol (DTT) (50 mM) and incubating at 4°C for 7 days. Cleaved protein was eluted with 15 ml buffer F. Fractions containing VioN, based on SDS-PAGE/Coomassie blue staining, were pooled and dialyzed (3500 MWCO SnakeSkin® Pleated Dialysis Tubing) against buffer F at 4°C overnight. Ammonium sulfate was used to preferentially precipitate VioN from any remaining CBD or CBD-VioN in 5% increments from 50% to 70% saturation. Pellets were resuspended in 4 ml buffer B, and fractions containing VioN, based on SDS-PAGE/Coomassie blue straining, were pooled and dialyzed (3,500 MWCO SnakeSkin® Pleated Dialysis Tubing) against buffer B overnight at 4°C and flash frozen in liquid nitrogen.

The protein concentration was determined using the calculated molar extinction coefficient (19,480 M^{-1} cm^{-1}).

Strain BL21(DE3)/pTYB12-cmnN was used to inoculate 3 X 1 L of LB containing ampicillin (100μg ml^{-1}). Overproduction of CBD-CmnN and cleavage from the CBD tag was performed as described above for VioN. Fractions containing CmnN, based on SDS-PAGE/Coomassie blue staining, were pooled and dialyzed against buffer G (Tris-HCl pH 8.0 [50mM], NaCl [25mM]) at 4°C overnight. CmnN was further purified by strong anion exchange chromatography using a 5 ml HiTrap Q Sepharose™ Fast Flow column (GE Healthcare Life Sciences) with a BioLogic LP System (BioRad, Hercules, CA) at 1 ml min^{-1} flow rate. CmnN was bound to the column, washed with 50 ml buffer G, and eluted with a 100 ml gradient from 100% buffer G/0% buffer H (Tris-HCl pH 8.0 [50mM], NaCl [500mM]) to 0% buffer G/100% buffer H. Fractions containing CmnN were identified based on SDS-PAGE/Coomassie blue staining. A final purification by size-exclusion chromatography was performed using a HiPrep 16/60 Sephacryl™ S-200 High Resolution column (GE Healthcare Life Sciences) at a flow rate of 0.4 ml min^{-1} (Biologic LP system) of buffer B. Fractions containing CmnN were pooled, concentrated (Centriprep YM-3), and flash frozen in liquid nitrogen. The protein concentration was also determined using the calculated molar extinction coefficient (19,480 M^{-1} cm^{-1}).

Strain BL21(DE3)/pTY2-ybdZ was used to inoculate 3 X 1 L of LB containing ampicillin (100 μg ml^{-1}). Overproduction of YbdZ-CBD and cleavage of the CBD tag was performed as described for CmnN and VioN, except DTT cleavage was carried out for 40 hr. After elution from chitin resin, fractions containing YbdZ based on SDS-PAGE/Coomassie blue staining were pooled and fractions were centrifuged through a Centriprep YM-3 at 1500 x *g* in a swinging bucket rotor in a Beckman-Coulter Allegra 6R centrifuge. The retentate volume was brought to 5 ml subjected to selective ammonium sulfate precipitation as described for VioN, followed by strong anion exchange chromatography as described for CmnN were performed. The purified protein was dialyzed against buffer B at 4°C overnight and flash frozen in liquid nitrogen. The protein concentration was determined using the calculated molar extinction coefficient (23,615 M^{-1} cm^{-1}).

Purification of EntF-H6 with and without YbdZ. BL21(DE3)*ybdZ*::*acc(3)IV* was transformed with pET28b-EntF and pACYCDuet-1-MCS or pET28b-EntF and pACYCDuet-1-YbdZ. Transformants were selected on LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}). EntF-H6 was purified following the same protocol as described for H6-CmnO with CmnN with a minor modification. Elution of EntF-H6 from the 5 ml HiTrap Q Sepharose™ Fast Flow column involved a 200 ml gradient from 100% buffer C/0% buffer D to 0% buffer C/100% buffer D. Fractions containing EntF-H6 were identified by SDS-PAGE/Coomassie blue staining, pooled, concentrated (Centriprep YM-3), and flash frozen in liquid nitrogen. The concentrations of the purified proteins were determined by Pierce BCA assay kit with BSA as a standard. The concentration of EntF-H6 purified without YbdZ was also determined using the calculated molar extinction coefficient (182,795 M^{-1} cm^{-1}). The co-purification of YbdZ was detectable by SDS-PAGE/Coomassie blue staining.

Purification of H6-EntE with and without YbdZ co-production. BL21(DE3)*ybdZ*::*acc(3)IV* was transformed with pET28b-EntE and pACYCDuet-1-MCS, pET28b-EntE and pACYCDuet-1-YbdZ. Transformants were selected on LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (15 μg ml^{-1}). H6-EntE overproduced without YbdZ was purified using Ni-NTA resin using the protocol described for H6-CmnO purification. H6-EntE overproduced with YbdZ was purified following the protocol described for purification of H6-CmnO co-produced with CmnN, but the did not include a size-exclusion chromatography step. The concentrations of the

purified proteins were determined using the Pierce BCA Protein Assay kit with BSA as a standard. The co-purification of YbdZ was assessed by SDS-PAGE/Coomassie blue staining.

Deletion of *ybdZ* from the chromosome of MG1655. A non-polar deletion was *ybdZ* was constructed using the temperature-sensitive plasmid pMAK705 (*11*). Briefly, 1 kilobase upstream of *ybdZ* was amplified using primers YbdZ/U/XbaI: 5' AATCTAGAATACTCGACGATAAAGCGCGT 3' and YbdZ/U/rev: 5' TAAATGCTGGCTCATATTCAACTCCTGTCATGG 3'. One kilobase downstream of *ybdZ* was amplified using primers YbdZ/D/for: 5' TGACAGGAGTTGAATATGAGCCAGCATTTACCT 3' and YbdZ/D/HindIII: 5' AAAAGCTTGATATTGAGTACCGGACCAA 3'. Using splicing by overlap extension, a PCR product was obtained that fused the 2 kb together. The resulting amplicon was cloned into pMAK705 using *Xba*I and *Hin*dIII, generating plasmid pMAK705-ybdZ. This plasmid was transformed into MG1655 and grown under a permissible temperature (28°C). The pMAK705-ybdZ plasmid was integrated into the *ent* region of the chromosome by growing the cells at a non-permissive temperature (37°C) while selecting for pMAK705-ybdZ retention. The integrated vector was subsequently resolved from the chromosome by growing the cells at permissive temperatures without selection for pMAK705-ybdZ. Cultures of this strain were dilution plated and colonies were screened for loss of pMAK705-ybdZ (sensitive to chloramphenicol [15 μg ml $^{-1}$]). Clones lacking *ybdZ* were identified by PCR amplification and sequencing of the resulting amplicon. The deletion resulted in the fusion of the *entF* gene to the start codon of *ybdZ*.

Construction of pBAD33-ybdZ. The 282 base pair region of *ybdZ* was amplified using the primers: YbdZ/NdeI: 5' ACAGGAGTTGCATATGGCATTCAGTAAT 3' and YbdZ/EcoRI: 5' AAGAATTCTGCCATCCAGATGCCGGGCT 3' and cloned into pET37b, generating pET37b-ybdZ. The *Xba*I/*Xho*I fragment of pET37b-ybdZ containing *ybdZ* and the ribosome-binding site of pET37b was subcloned into the *Xba*I/*Sal*I sites of pBAD33. The resulting plasmid, pBAD33-ybdZ, has *ybdZ* expression being controlled by an arabinose-inducible promoter.

Growth curves of *E. coli* MG1655 and MG1655Δ*ybdZ* with pBAD33 or pBAD33-ybdZ. Growth curves of strains were performed in Tris minimal medium (*12*) supplemented with L-arabinose (0.2% w/v), sodium succinate (0.2% w/v), chloramphenicol (15 μg ml^{-1}), and the iron chelator 2, 2'-dipyridyl (0.2 mM). Triplicate cultures were grown at 37°C and growth of the cells was monitored at OD_{550}.

ATP/PP_i exchange assays. ATP/PP_i exchange assays were performed as previously described (*13, 14*). Assays (100 μl) were performed with the following substrates and concentrations: Tris-HCl pH 7.5 (75 mM), $MgCl_2$ (10 mM), dithiothreitol (5mM), ATP pH 7.0 (3.5mM), $NaPP_i$ pH 7.0 (1mM), [^{32}P]PP_i (2.15 Ci mol^{-1}, PerkinElmer), amino or acyl acid (1 mM), and varying concentration of enzyme as noted in the associated Figures. All assays were performed at room temperature (~24°C) for varying time periods. The reactions were terminated by the addition of quench solution containing perchloric acid (3.5 % v/v), Na-PPi (100 mM), and activated charcoal (1.6% w/v). The charcoal was pelleted by centrifugation, and the pellets were washed twice with quench solution lacking activated charcoal prior to being counted in a scintillation counter. For assays in which MbtH-like protein orthologs were included, each ortholog was pre-incubated with the corresponding A-domain containing enzyme for 10-20 min at room temperature before the rest of the reaction mixture was added. For determination of the kinetic parameters, the amino or aryl acid concentrations were varied from 0.5- to 10-fold over the determined K_m. All assays were performed in the linear range of enzyme concentration and less than 10% substrate to product conversion. The kinetic parameters were determined by

fitting the data to the Michaelis-Menten equation using Kaleidagraph software (Synergy Software). The β-Lys was a gift from Prof. Helen Blackwell (UW-Madison), β-ureidoalanine was purchased from Acros, L-capreomycidine was enzymatically synthesized as previously described (*15*), and all other components were purchased from Sigma-Aldrich or Thermo-Fischer Scientific.

Determination of NRPS:MbtH-like protein stoichiometry. To determine the stoichiometry of H6-CmnO relative to the amount of co-purified CmnN a series of dilutions of known concentrations of independently purified H6-CmnO were run on a 15% SDS-PAGE adjacent to different dilutions of H6-CmnO co-purified with CmnN. The gel was stained with Coomassie blue and the intensities of the stained proteins in the dilution series were measured with ImageJ64 version 1.43 (http://rsb.info.nigh.gov/ij/) and plotted against the known μmol concentrations of H6-CmnO and CmnN. The plot was used used to calculate the μmol of H6-CmnO in the co-purified sample. This was repeated for dilutions of known concentrations of CmnN to determine the concentration of CmnN in the co-purified samples. From this, the molar ratio of the co-purified proteins was determined. Representative gels of this process are shown in Figure S4. The same process was repeated to determine the molar ratio of CmnN to co-purified H6-CmnA-A1PCP1 and EntF-H6 to co-purified YbdZ.

H6-VioO proved difficult to purify to homogeneity at any significant level when overproduced in the absence of VioN. This limitation resulted in only partially purified H6-VioO. The concentration of partially purified H6-VioO was estimated in the following manner. The amount of total protein in the sample was determined using BCA assays with BSA as a standard. Different dilutions of known protein concentration were visualized by SDS-PAGE/Coomassie blue staining. The fraction of protein associated with H6-VioO was determined by densitometry of the stained gel using ImageJ64 version 1.43; thus providing an with an estimate of H6-VioO concentration in mg ml^{-1}. We made the assumption that this value was representative of the true concentration of H6-VioO and converted this concentration to a molar concentration to estimate the number of moles of H6-VioO. Using this dilution series and a dilution series of VioN, we generated standard curves using ImageJ64 version 1.43 and used these to determine the ration of H6-VioO to VioN when co-purified.

H6-CmnA-A1PCP1 could be purified to near homogeneity when overproduced in the absence of CmnN, but the protein levels were very low and it proved difficult to accumulate high enough amounts to accurately determine the protein concentration using the calculated molar extinction coefficient of the protein. Instead, we used the BCA assay to determine the protein concentration and made the assumption that this value represented an accurate calculation of the true concentration of H6CmnA-A1PCP1. We subsequently used protein dilutions and ImageJ64 software to calculate the ration of the two co-purified proteins.

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