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MbnC is not required for the formation of the N-terminal oxazolone in the methanobactin from *Methylosinus trichosporium* OB3b.

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Running title: Role of MbnC in Methanobactin Biosynthesis

ABSTRACT Methanobactins (MBs) are ribosomally synthesized and post-translationally modified peptides (RiPPs) produced by methanotrophs for copper uptake. The post-translational modification that define MBs is the formation of two heterocyclic groups with associated thioamines from X-Cys dipeptide sequences. Both heterocyclic groups in the MB from *Methylosinus trichosporium* OB3b (MB-OB3b) are oxazolone groups. The precursor gene for MB-OB3b, *mbnA*, which is part of a gene cluster that contains both annotated and unannotated genes. One of those unannotated genes, *mbnC*, is found in all MB operons, and in conjunction with *mbnB*, is reported to be involved in the formation of both heterocyclic groups in all MBs. To determine the function of *mbnC*, a deletion mutation was constructed in *M. trichosporium* OB3b, and the MB produced from the Δ*mbn*C mutant was purified and structurally characterized by UV-visible absorption spectroscopy, mass spectrometry and solution NMR spectroscopy. MB-OB3b from Δ*mbn*C was missing the C-terminal Met and also found to contain a Pro and a Cys in place of the pyrrolidiny-oxazolone-thioamide group. These results demonstrate MbnC is required for the formation of the C-terminal pyrrolidinyl-oxazolone-thioamide group from the Pro-Cys dipeptide, but not for the formation of the N-terminal 3-methylbutanol-oxazolone-thioamide group from the N-terminal dipeptide Leu-Cys.

IMPORTANCE A number of environmental and medical applications have been proposed for MBs, including bioremediation of toxic metals, nanoparticle formation, as well as for the treatment of copper- and iron-related diseases. However, before MBs can be modified and optimized for any specific application, the biosynthetic pathway for MB production must be defined. The discovery that *mbnC* is involved in the formation of the C-terminal oxazolone

group with associated thioamide but not for the formation of the N-terminal oxazolone group with associated thioamide in *M. trichosporium* OB3b suggests the enzymes responsible for post-translational modification(s) of the two oxazolone groups are not identical.

KEYWORDS methanobactin • chalkophore• methanotroph • aerobic methane oxidation • ribosomally synthesized and posttranslational modified peptide

Methanobactins (MBs) are low molecular mass (<1,300 Da), post-translationally modified copper binding peptides excreted by some methanotrophs as the extracellular component of a copper acquisition system (1-7). Structurally MBs are characterized by the presence of a C-terminal oxazolone group with a C2-associated thioamide and by the presence of an N-terminal oxazolone, imidazolone or pyrazinedione group with an associated thioamide. Some MBs also contain a sulfate group in-place of the hydroxyl group on a Tyr adjacent to the C-terminal oxazolone group. The best characterized MB is from *Methylosinus trichosporium* OB3b and the post-translational modifications for this MB involves: (1) deamination of the N-terminal Leu, (2) conversion of the N-terminal Leu-Cys dipeptide to 1-(N-(mercapto-(5-oxo-2- (3-methylbutanoyl)oxazol-(Z)-4-ylidene)methyl), (3) conversion of the C-terminal Pro-Cys dipeptide into pyrrolidin-2-yl-(mercapto-(5-oxo-oxazol-(Z)-4- ylidene)methyl); and (4) cleavage of the leader sequence (2, 4, 5, 8-11).

The gene encoding the MB precursor peptide, *mbnA*, (5, 10) is found in a gene cluster that contains both genes of known function such as *mbnB* (5, 11), *mbnN* (9), *mbn*T (12) as well as unannotated genes such as *mbnC* (5, 10, 11, 13, 14). MbnB is a member of TIM barrel family as well as the DUF692 family of diiron enzymes (11, 14). In heterologous expression studies in *Escherichia coli*, MbnBC was shown to catalyze a dioxygen-dependent four electron oxidation of Pro-Cys in MbnA (11, 14, 15). The role(s) of MbnB and MbnC could not be separately determined as attempts to separately purify these gene products in *E. coli* failed (11). From these data, it has been argued that MbnBC must act in concert and by doing so create both heterocyclic groups in MBs (11). Such conclusions, however, appear to be premature for several reasons. First, the reported spectra (11) only shows the presence of the C-terminal oxazolone

group, not the N-terminal oxazolone group as the 394 nm absorption maximum is missing. Second, the absorption maximum at 302 nm, diagnostic for the presence of N-terminal oxazolone group was absent (5, 8, 16). Third, no structural data was provided to support the presence of both oxazolone groups. To examine if MbnBC act in concert and are involved in the formation of both oxazolone groups in *M. trichosporium* OB3b, a deletion mutation for MnbC was constructed *(*Δ*mbn*C). The results show MbnC is required for the formation of the C-terminal oxazolone group, but not for the formation of the N-terminal oxazolone group.

RESULTS

Generation of Δ*mbn*C

The Δ*mbnAN* strain previously constructed whereby *mbnABCMN* were deleted using a sucrose counter selected technique (9), was back complemented with *mbnABMN* through selective amplification and ligation of *mbnAB* with *mbnMN*, deleting *mbnC*, and inserting this ligation product into pTJS140, creating pWG104 (Table 1). Successful removal of *mbnC* from this product was confirmed via sequencing (data not shown). The native σ^{70}-dependent promoter upstream of *mbnA* was also incorporated into pWG104, and expression of *mbnABMN* but not *mbnC* (from pWG104), as well as *mbnPH* (from the chromosome) was confirmed via RT-PCR (Figs. S1 and S2).

UV-visible absorption and mass spectrometry of metal-free MB from *M. trichosporium* OB3b *ΔmbnC*

Comparison of the UV-visible absorption spectra of MB from *M. trichosporium* OB3b *ΔmbnC* to wildtype MB-OB3b suggested the of presence of the N-terminal oxazolone group, but the absence of C-terminal oxazolone (Figs. 1 and S3). The molecular mass of native,

full length MB-OB3b is 1154 Da, and MB-OB3b lacking the C-terminal Met is 1023 Da. It should be noted that both forms of MB-OB3b are present in most MB-OB3b preparations (2, 5, 17). The molecular mass of ΔMbnC was 1,018Da as determined by electrospray ionization (ESI) MS/MS (Fig. 2), which was within 1Da of the predicted molecular mass of MB-OB3b in which only one oxazolone group was formed. Taken together, the UV-visible absorption spectra and molecular mass data suggest Δ*mbn*C lacked the C-terminal Met as well as the N-terminal oxazolone group with a 1-(*N*-[mercapto-(5-oxo-2-(3-methylbutanoyl)oxazol-(*Z*)-4-ylidene)methyl]-GSCYPCSC predicted structure (Fig. 3B). In contrast to wild-type MB-OB3b, the C-terminal Met was never observed in MbnC.

Chemical Structure of metal free Δ*mbn*C as determined by NMR spectroscopy. Metal-free MB has multiple conformations, making structural studies of MBs via solution NMR or crystallography difficult (Fig. S4). In prior structural studies of MB, the addition of Cu^{2+} (which is bound and reduced to Cu^{1+} by native MB-OB3b) stabilizes MB-OB3b into one conformation, allowing for crystal formation and NMR characterization (Fig. S4) (2-5, 8, 18). Our initial efforts to investigate the structure of the MB intermediate produced by the Δ*mbn*C strain via NMR were unsuccessful. In contrast to native MB, the MB intermediate from the Δ*mbn*C strain bound, but did not reduce Cu^{2+} to Cu^{+}, resulting in peak broadening from paramagnetic Cu^{2+}. This necessitated a different strategy. Substituting other metals with similar binding behavior for copper, Au^{3+}, Zn^{2+}, Co^{2+} and Ni^{2+} also failed to produce well-behaved complexes. Therefore, it was necessary to examine metal-free Δ*mbn*C.

115 At standard temperature and pressure, Δ*mbn*C undergoes exchange between multiple
116 conformations on an intermediate time scale, leading to excessive line broadening (Fig. S5). In
117 order to slow down the rate of exchange and reduce line broadening, we sampled various
118 temperature and hydrostatic pressure conditions. We found that 2D ^{1}H-^{15}N NMR spectra of
119 Δ*mbn*C recorded at high pressure (3000 bar) and low temperature (265 K) (18,19) show
120 significantly reduced line broadening and gave excellent spectra in the absence of copper (Fig.
121 4).

122 A series of NMR experiments were conducted on Δ*mbn*C, including homonuclear
123 correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating-frame nuclear
124 Overhauser effect spectroscopy (ROESY), ^{1}H-^{15}N and ^{1}H-^{13}C heteronuclear single-quantum
125 correlation spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy
126 (HMBC). These experiments enabled assigning all non-hydroxyl ^{1}H, non-conjugated ^{13}C, and all
127 ^{15}N resonances (Table 2 and Figs.4 and S6). The assigned chemical shifts show that the MB from
128 Δ*mbn*C contains 8 amino acids - 3Cys, 2Ser, 1Gly, 1Tyr and 1Pro and 1 oxazolone group (Fig. 4).
129 The 1D ^{15}N experiment showed a peak at 109ppm that was absent from the [^{1}H, ^{15}N]-HSQC
130 spectra and was assigned to proline. However, the glycine nitrogen peak was especially broad,
131 and could only be assigned with the ^{1}H-^{15}N-HSQC. Finally, while the 1D ^{15}N experiment had
132 several resonances around 180ppm - likely due to hydrolysis and deprotonation - only one of
133 them had a correlation with ^{1}H in the ^{1}H-^{15}N-HSQC indicating a single oxazolone group. The
134 NMR results are consistent with the UV-visible absorption spectra, and the ESI-MS results and
135 with the structure shown in Fig. 3B.

DISCUSSION

Due to the variability in the core sequences of structurally characterized MBs, it is difficult to use *mbnA* to screen the potential ability of microbes to produce MB. Instead, *mbnB* and *mbn*C sequences are commonly used as they are found in all known *mbn* gene clusters (5, 13). All known MBs contain two heterocyclic rings, with the N-terminal ring found to be either an oxazolone, pyrazinedione or imidazolone ring, while the C-terminal ring always found to be an oxazolone. Given these data, it could be presumed that MbnBC is involved in the formation of the C-terminal oxazolone group along with an associated thioamide, while the N-terminal oxazolone groups is formed via a different process such as the involvement of an aminotransferase as concluded earlier (5, 9, 10, 13).

Other researchers have attempted to elucidate the role of MbnB and MbnC in methanobactin maturation (11). These individuals were unable to separately heterologously express soluble protein from either MbnB or MbnC , but were able to co-heterologously expressed MbnBC as a heterodimeric complex. In studies where the MbnA precursor polypeptide was incubated with this MbnBC complex, the authors conclude that MbnBC was involved in the formation of both oxazolone groups and the associated thioamides of MB-OB3b. It should be noted, however, that in this study, no structural evidence (i.e., solution NMR data) was provided to definitively show the presence of either ring, rather such conclusions were largely based on mass spectral analyses of MbnA after incubation with the MbnBC complex. Further, the authors assumed that since their construct did not contain the N-terminal aminotransferase, MbnN, the extended conjugation resulting from this reaction would result in both oxazolone groups having identical absorption maxima. The idea that the extended

158 conjugation of the N-terminal oxazolone could be responsible for the bathochromic shift was
159 first proposed as a possible reason for the 50nm shift in the absorption maxima by Krentz *et al.*
160 (5). Kenny *et al*. used this theory to bolster their claim that both oxazolone groups were
161 present in the product from their heterologous system, with both oxazolone groups showing
162 the identical absorption spectra (11). The evidence to support this claim came from their
163 *ΔmbnN* strain in *M. trichosporium* OB3b. MbnN is responsible for the deamination of the N-
164 terminal Leu in *M. trichosporium* OB3b extending the conjugation one additional double bond.
165 In this study the authors claim they can stabilize the MB produced by the *ΔmbnN* strain by the
166 addition of copper before purification. UV-visible absorption spectra of copper containing-
167 ΔMbnN suggest the possible presence of two-oxazolone groups but additional evidence no
168 additional evidence was provided supporting this claim.
169 This observation was surprising as the MB produced by *ΔmbnN* strain in our laboratory
170 showed similar UV-visible absorption spectra throughout the growth cycle suggesting the
171 absence of the N-terminal oxazolone group (Fig. S7). In addition, the UV-visible absorption
172 spectra, LC-MS/MS, FT-ICR-MS, amino acid analysis, number of thiol groups, copper binding
173 properties, and pattern of acid hydrolysis demonstrate the absence of the N-terminal
174 oxazolone group in ΔMbnN (9).
175 Additional evidence that the bathochromic shift in MBs with two oxazolone groups is
176 unlikely to solely arise from the addition of one double bond following deamination of the N-
177 terminal amine comes from examination of the group I MB from *Methylocystis parvus* OBBP.
178 Acid hydrolysis of the MB from *M. parvus* OBBP shows a similar hydrolysis pattern to that
179 observed with the MB from *M. trichosporium* OB3b, demonstrating the presence of two

oxazolone groups, with absorption maxima at 340 and 390nm (Fig. S8). However, both MB operons from *M. parvus* OBBP lack *mbnN* and without deamination of the N-terminal Phe, the conjugation around the N-terminal oxazolone group would not be extended. It is possible that another aminotransferase in the *M. parvus* OBBP genome may catalyze deamination of the N-terminal Phe. However, this appears unlikely as deamination of the N-terminal amino acid has never been observed in structurally characterized MBs from operons lacking *mbn*N (3, 5). The results suggest deamination of the N-terminal amino acid is not solely responsible for the 40 – 50nm absorption maxima difference between oxazolone groups in MBs. The absence of either the N-terminal or C-terminal oxazolone group in a small (0.5-2%) fraction of most MB-OB3b preparations (Fig. S3) also questions the suggestion that the absorption maxima difference between the N-terminal and C-terminal oxazolone groups is due solely to extending the conjugation of an additional double bond introduced following the deamination reaction.

The results presented here confirms MbnC is required for the formation of the C-terminal oxazolone group (Fig. 5). However, the results presented here also demonstrates MbnC is not required for the formation of the N-terminal oxazolone group in *M. trichosporium* OB3b suggesting the formation of the two hetercyclic groups with associated thioamides from XC dipeptides do not utilize the same enzyme(s). Future studies will determine if MbnB is involved in the formation of the N-terminal oxazolone, pyranzinedione or imidazolone groups. Resolution of the pathway and enzymes responsible for the post-translational modifications required for the synthesis of MB in methanotrophic bacteria will aid in the production of MBs derivatives with pharmacological properties specific for different metal-related diseases (19-24) as well as for environmental applications(10, 25).

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions.

Plasmid construction was accomplished using *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) as described previously (9). Plasmids used and constructed during this study are shown in Table 1. The donor strain for conjugation of plasmids into *Methylosinus trichosporium* OB3b was *E. coli* S17-1 (26). *E. coli* strains were cultivated at 37°C in Luria broth medium (Dot scientific, Burton, MI). Methanotrophic strains (i.e., *M. trichosporium* OB3b wildtype, *M. trichosporium* OB3b Δ*mbnAN*, *M. trichosporium* OB3b Δ*mbnC*, *Methylocystis* sp. strain SB2, and *Methylocystis parvus* OBBP) were cultivated at 30°C on nitrate mineral salts (NMS) medium (27), either in 250 ml flasks with side-arms at 200 rpm or in a 15-liter New Brunswick Bioflow 310 fermenter (Eppendorf, Hauppauge, NY,USA) using methane as the sole carbon and energy source. Where necessary, filter-sterilized solutions of copper (as $CuCl_2$) and spectinomycin were added to culture media aseptically. A working concentration of 20 $\mu g \bullet ml^{-1}$ spectinomycin was used for maintaining pWG104 in the *M. trichosporium* OB3b Δ*mbn*AN deletion mutant (i.e., *M. trichosporium* OB3b Δ*mbnC*). Chemicals were purchased from Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) with American Chemical Society reagent grade or better.

For ^{15}N NMR, $K^{14}NO_3$ in NMS media was replaced with $K^{15}NO_3$ (Cambridge Isotope Laboratories, Cambridge, MA, USA).

General DNA Methods, transformation and conjugation.

223 DNA purification and plasmid extraction were performed using QIAquick and QIAprep kits from
224 Qiagen following the manufacturer's instruction. DNA cloning, preparation of chemically
225 competent cells, and plasmid transformation with *E. coli* were performed according to (28) .
226 Enzymes used for restriction digestion and ligation were purchased from New England Biolabs
227 (Ipswich, MA). PCR of DNA for cloning purposes was accomplished using iProof-High Fidelity
228 polymerase (Bio-Rad, Hercules, CA, USA). PCR for general purposes was accomplished using
229 GoTaq DNA polymerase (Promega, Fitchburg, WI, USA). PCR programs were set according to
230 manufacturers' suggestion. Plasmid pWG104 was conjugated into *M. trichosporium* OB3b
231 Δ*mbnAN* with *E. coli* S17.1 as the donor strain as described by Martin and Murrell (29).
232
233 **Construction of *M. trichosporium* OB3b Δ*mbnC* strain**
234 Previously a mutant of *M. trichosporium* was constructed where *mbnABCMN* was deleted using
235 a counter-selection technique (9). To characterize the function of *mbnC*, a Δ*mbnC* mutant was
236 constructed by introducing pWG104 expression vector into the Δ*mbnAN* mutant. pWG104 was
237 constructed by cloning two separate DNA fragments, one being a 1.9-kb DNA fragment of
238 *mbnAB* (created via use of primers mbnANf and mbn66) and the other being a 2.5 DNA
239 fragment of *mbnMN* (create via use of primers mbn70 and mbnANr), leaving out *mbnC*. These
240 two fragments were amplified with BamHI restriction sites as indicated in Fig S2. These were
241 then ligated together and cloned into the broad host range vector pTJS140 at the KpnI site.
242
243 **Extraction of RNA and reverse transcription-PCR (RT-PCR)**

244 To check the expression of genes restored to the *M. trichosporium* OB3b ⊿*mbnC* mutant (e.g.
245 *mbnA*, *B, M* and *N*), genes associated with MB remaining in the chromosome (*mbnPH*), as well
246 as the absence of *mbnC*, RNA from the ⊿*mbnC* mutant was collected, purified, and reverse
247 transcribed to cDNA to perform RT-PCR. Total RNA was isolated as described earlier (9). Briefly,
248 the ⊿*mbnC* mutant was grown to the exponential phase, and RNA extracted using a phenol-
249 chloroform method modified from Griffiths *et al*. (30). Collected RNA was purified and removal
250 of DNA confirmed by the absence of 16S rRNA PCR product from PCR reactions. The same
251 amount of RNA (500ng) was used for reverse transcription by SuperScript III reverse
252 transcriptase (Invitrogen, Carlsbad, CA) for all reactions. RT-PCR analyses were performed to
253 confirm the expression of *mbnABMNPH* as well as the absence of *mbnC* using primers listed in
254 Table 1.
255
256 **Isolation of MB *from M. trichosporium* OB3b, *Methylocystis* strain SB2, *Methylocystis***
257 ***parvaus* OBBP and Δ*mbn*C.** MBs from all three methanotrophs were purified as previously
258 described (31).
259 **UV-visible absorption spectra.** UV-visible absorption spectra of MbnC¯, HPLC fractions
260 from MB preparations from *M. trichosporium* OB3b and *Methylocystis strain* SB2 and from the
261 MB from *M. parvus* OBBP were determined as previously describes (32, 33). Acid hydrolysis of
262 the oxazolone groups in the MB from *M. parvus* OBBP was carried out in 85µM acetic acid as
263 previously described (32)
264 **Structural Characterization of ΔMbnC**. UV-visible spectroscopy was recorded on a Cary
265 50 (Agilent, Santa Clara, CA, USA). Electrospray ionization (ESI)MS/MS was performed on an

Agilent LC using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Waltham, MA, USA) with an HCD fragmentation cell and an Agilent 1260 Infinity Capillary Pump with an Agilent Zorbax SB-C18, 0.5mm x 150mm, 5 micron, using 0.1% formic acid/water and 0.1% formic acid/acetonitrile as buffers A and B, respectively. NMR experiments were performed on a Bruker Advance 700 (Bruker Allentown PA, USA) with a Bruker 5 mm TCI 700 H/C/N cryoprobe or on a Bruker Advance 800 with a Bruker 5 mm TCI 800 H/C/N cryoprobe. NMR solutions were made using 15-40mg uniformly ^{15}N-MB-OB3b in a 90:10 H_20:D_2O mixture at pH 6.5. Unless otherwise specified, all experiments were run at 265 K and 3 kbar. Samples were placed in 3 kbar-rated sapphire NMR tubes (Daedalus Innovations LLC, Beverdam, VA, USA) and high pressure was generated by an Xtreme 60 (Daedalus Innovations). Analysis was performed in Mnova (Mestrelab Research, Escondido, CA, USA).

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409 **Figure Legends**

410 Fig. 1. UV-visible absorption spectra of MB-OB3b (blue) and ΔMbnC (red). Abbreviations; OxaA,
411 oxazolone A or the N-terminal oxazolone group; OxaB, oxazolone B or the C-terminal
412 oxazolone group.

413 Fig. 2. LC-ESI-MS of methanobactin from Δ*mbnC*.

414

415 Fig 3 (A) Structure of wild-type MB-OB3b, with the labile terminal methionine in gray. (B)
416 Proposed structure of ΔMbnC based on UV-visible absorption spectra, LC-MS and NMR
417 analysis, the differences between MB-OB3b-Met and ΔMbnC are highlighted in red. C.
418 Amino acid sequence of (a) wild-type MB-OB3b minus the C-terminal Met and (b) ΔMbnC.

419

420 Fig. 4. 800 MHz (^{1}H,^{15}N)-HSQC spectrum of uniformly ^{15}N-labeled ΔMbnC in 90% 9 mM
421 phosphate buffer, pH 6.5, and 10% D_2O at 265K and 3000 bar. The horizontal and vertical
422 1D spectra are ^{1}H and ^{15}N spectra, respectively.

423

424 Fig. 5. A. MB-OB3b gene cluster. Genes with known involvement in MB-OB3b synthesis and
425 transport are shown in blue. B. Proposed genes involved in the biosynthesis of the
426 oxazolones rings with associated thioamides from MbnA. Additional, yet to be identified
427 genes may also be involved in the formation of oxazolone groups.

428

429 Table 1. Strains, plasmids, and primers used in this study.
430

Strains/Plasmids	Description	Restriction site	Reference/Source
Escherichia coli			
TOP10	F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*) *7697 galU galK rpsL* (StrR) *endA1 nupG*		Invitrogen
S17.1 *λpir*	*recA1 thi pro hsdR-* RP4-2Tc::Mu Km::Tn*7 λpir*		(26)
Methylosinus trichosporium			
OB3b	Wild-type strain		
Δ*mbnAN*	*mbnABCMN* deleted		(9)
Δ*mbnC*	Δ*mbnAN* carrying pWG104		this study
Plasmids			
pTJS140	Broad-host-range cloning vector; Mob Ap^r Sp^r Sm^r *lacZ*		(34)
pWG104	pTJS140 carrying *mbnABMN* with its native promoter		this study
Primers			
mbnANf	ATTTTTggtaccGACGTTCGGGTCTTCTTCGC	KpnI	(9)
mbnANr	ATTTTTggtaccCGCCTCTAGATCATTCCGAC	KpnI	(9)
mbn66	ATTTTTggatccCGAACAATGTGTGCCAGTAG	BamHI	this study
mbn70	ATTTTTggatccGTTCGGCTATTTCCTGACGC	BamHI	this study
qmbnA_FO	TGGAAACTCCCTTAGGAGGAA		(35)
qmbnA_RO	CTGCACGGATAGCACGAAC		(35)
qmbnB_F1	TGGTCCAGCAGATGATCAAAG		this study
qmbnB_R2	TTCCCGAGCTTCTCCAATTC		this study
dmbnC_F	GGGAGAACAACCTCGCTTT		this study

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dmbnC_R	CTTCCCAGCACGATCTGAC	this study
qmbnM_F	GCTAGGCTGGCTCCTTTATC	this study
qmbnM_R	GATGTTGACCACAAACCGAAAG	this study
qmbnN_F	CGATTCCATCCTTTCCGATGT	this study
qmbnN_R	CACTTTCGAAGACAAGGAGAGA	this study
qmbnP_F	AAAGGGAAGCACACACCCAT	this study
qmbnP_R	GTCGTGTTCTTGGCCGGATT	this study
qmbnH_F	ACTTACCGAAATACATCCCGC	this study
qmbnH_R	CGGAGAGGCGCTTATCGTAG	this study

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434

435 **Table 2.** ^{1}H, ^{13}C, and ^{15}N resonances for metal free ΔMbnC.

Residue	Atom	Chemical Shifts (ppm) ^{1}H	^{13}C	^{15}N	Residue	Atom	Chemical Shifts (ppm) ^{1}H	^{13}C	^{15}N
3-Methyl-	C^{1}		174.6		Tyr^{4}	H^{N}	7.44		
butanoyl	C^{2}		50.5			H^{α}	2.96		
	C^{3}		38.0			H^{β}	2.79		
	C^{4}		19.6			H^{β}	1.20		
	C^{5}		19.6			$H^{2,6}$	6.11		
	H^{2}	4.15				$H^{3,5}$	6.45		
	H^{3}	2.17			Pro^{5}	N^{1}			109.6
	H^{3}	2.72				C^{2}		67.3	
	H^{4}	1.88				C^{3}		21.1	
	H^{5}	1.80				C^{4}		39.5	
Oxazolone	N			180.1		C^{5}		55.2	
	H^{N}	7.61				H^{2}	3.67		
Gly^{1}	N			125.1		H^{3}	1.06		
	C					H^{3}	2.13		
	C^{α}		26.6			H^{4}	1.28		
	H^{N}	9.57				H^{4}	2.29		
	H^{α}	1.46				H^{5}	2.79		
Ser^{2}	N			114.3		H^{5}	2.96		
	C		181.6		Cys^{6}	N			127.9
	C^{α}		72			C		136.3	
	C^{β}					C^{α}		53.3	
	H^{N}	8.19				C^{β}		49.3	
	H^{α}	4.14				H^{N}	8.43		
	H^{β}	3.98				H^{α}	3.96		
	H^{β}	1.41				H^{β}	3.23		
Cys^{3}	N			118.1		H^{β}	1.38		
	C		173.0		Ser^{7}	N			117.5
	C^{α}		71.2			C			
	C^{β}		35.6			C^{α}		51.6	
	H^{N}	7.93				C^{β}		45.0	
	H^{α}	3.96				H^{N}	8.90		

	H^{β}	3.23				H^{α}	4.19		
	H^{β}	1.37				H^{β}	3.25		
Tyr^{4}	N			121.5		H^{β}	1.48		
	C				Cys^{8}	N			112.4
	C^{α}		48.9			C		172.6	
	C^{β}		35.6			C^{α}		42.3	
	C^{1}					C^{β}		21.1	
	$C^{2,6}$					H^{N}	8.47		
	$C^{3,5}$		135.4			H^{α}	3.69		
	C^{4}					H^{β}	3.55		
						H^{β}	0.97		

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Table 2. ^{1}H, ^{13}C, and ^{15}N resonances for metal free ΔMbnC.

Residue	Atom	Chemical Shifts (ppm) ^{1}H	^{13}C	^{15}N	Residue	Atom	Chemical Shifts (ppm) ^{1}H	^{13}C	^{15}N
3-Methyl-	C^{1}		174.6		Tyr^{4}	H^{N}	7.44		
butanoyl	C^{2}		50.5			H^{α}	2.96		
	C^{3}		38.0			H^{β}	2.79		
	C^{4}		19.6			H^{β}	1.20		
	C^{5}		19.6			$H^{2,6}$	6.11		
	H^{2}	4.15				$H^{3,5}$	6.45		
	H^{3}	2.17			Pro^{5}	N^{1}			109.6
	H^{3}	2.72				C^{2}		67.3	
	H^{4}	1.88				C^{3}		21.1	
	H^{5}	1.80				C^{4}		39.5	
Oxazolone	N			180.1		C^{5}		55.2	
	H^{N}	7.61				H^{2}	3.67		
Gly^{1}	N			125.1		H^{3}	1.06		
	C					H^{3}	2.13		
	C^{α}		26.6			H^{4}	1.28		
	H^{N}	9.57				H^{4}	2.29		
	H^{α}	1.46				H^{5}	2.79		
Ser^{2}	N			114.3		H^{5}	2.96		
	C		181.6		Cys^{6}	N			127.9
	C^{α}		72			C		136.3	
	C^{β}					C^{α}		53.3	
	H^{N}	8.19				C^{β}		49.3	
	H^{α}	4.14				H^{N}	8.43		
	H^{β}	3.98				H^{α}	3.96		
	H^{β}	1.41				H^{β}	3.23		
Cys^{3}	N			118.1		H^{β}	1.38		
	C		173.0		Ser^{7}	N			117.5
	C^{α}		71.2			C			
	C^{β}		35.6			C^{α}		51.6	
	H^{N}	7.93				C^{β}		45.0	
	H^{α}	3.96				H^{N}	8.90		
	H^{β}	3.23				H^{α}	4.19		
	H^{β}	1.37				H^{β}	3.25		

Tyr^4	N		121.5		H^{β}	1.48		
	C			Cys^8	N			112.4
	C^{α}	48.9			C		172.6	
	C^{β}	35.6			C^{α}		42.3	
	C^1				C^{β}		21.1	
	$C^{2,6}$				H^N	8.47		
	$C^{3,5}$	135.4			H^{α}	3.69		
	C^4				H^{β}	3.55		
					H^{β}	0.97