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Methanobactin and the Link Between Copper and Bacterial Methane Oxidation

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SUMMARY

Methanobactins (mbs) are low molecular mass (< 1,200 Da) copper-binding peptides, or chalkophores, produced by many methane-oxidizing bacteria (methanotrophs). These molecules exhibit similarities to certain iron-binding siderophores, but are expressed and secreted in response to copper limitation. Structurally, mbs are characterized by a pair of heterocyclic rings with associated thioamide groups that form the copper coordination site. One of the rings is always an oxazolone and the second ring an oxazolone, imidazolone, or a pyrazinedione moiety. The mb molecule originates from a peptide precursor that undergoes a series of post-translational modifications including: (1) ring formation; (2) cleavage of a leader peptide sequence, and, in some cases, (3) addition of a sulfate group.

Functionally, mbs represent the extracellular component of a copper acquisition system. Consistent with this role in copper acquisition, mbs have a high affinity for copper ions. Following binding, mbs rapidly reduce Cu^{2+} to Cu^{1+}. In addition to copper, mbs will bind most transition metals and near transition metals and protect the host methanotroph as well as other bacteria from toxic metals. Several other physiological functions have been assigned to mbs, based primarily on their redox and metal binding properties. In this review, we examine the current state of knowledge of this novel type of metal-binding peptide. We also explore its potential applications, how mbs may alter the bioavailability of multiple metals, as well as the many roles mbs may play in the physiology of methanotrophs.

INTRODUCTION

Mbs were first identified in aerobic methane oxidizing bacteria (methanotrophs). This remarkable group of bacteria can grow using methane as their sole source of carbon and energy.

They are ubiquitous in environments where oxygen and methane are available, and play a major role in consuming much of the methane produced in the biosphere, thus mitigating its effects in global warming (1-4). Due to their growth on an inexpensive, readily available and, if generated via methanogenesis (5), a renewable carbon source, methanotrophs also have considerable potential for the production of bulk and fine chemicals and for bioremediation of pollutants in the environment (2, 6-8).

The first report of a bacterium growing on methane was by Söhngen, working in Beijerinck's laboratory in Delft in The Netherlands, who in 1906 reported the isolation of *Bacillus methanicus* from aquatic plants and pond water (9). It was not until 50 years later that this microbe was re-isolated and renamed *Pseudomonas methanica* (10, 11). A second methanotroph, *Methylococcus capsulatus* (Texas strain) was also isolated in 1966 (12). A landmark in methanotroph biology came in 1970 when Whittenbury and colleagues isolated and described, from a variety of terrestrial and freshwater environments, over 100 new aerobic methanotrophs growing on methane (13). They then devised a classification scheme, i.e. Type I vs. Type II, based on the ability of these methanotrophs to grow on methane, pathways of carbon assimilation, formation of resting stages (cysts and spores), morphology, the possession of complex intracytoplasmic membrane arrangements, and the mol% G + C content of their DNA. Subsequently, Bowman and colleagues isolated a similar number of methanotrophs from various environments, and classified them according to the scheme of Whittenbury and colleagues and to their 16S rRNA phylogeny (14, 15). It is remarkable that without any DNA sequencing at the time, the overall classification scheme of Whittenbury and colleagues still remains a robust and convenient way of grouping methanotrophs today.

Accordingly, there are currently 17 genera of methanotrophs within the family *Methylococcaceae.* In the class *Gammaproteobacteria*, known genera are *Methylobacter, Methylocaldum, Methylococcus, Methylogaea, Methyloglobulus, Methylohalobius, Methylomarinum, Methylomarinovum, Methylomicrobium, Methylomonas, Methyloparacoccus, Methyloprofundus, Methylosoma, Methylothermus, Methylosphaera, Methylosarcina* and *Methylovulum* (16-21). Within the class *Alphaproteobacteria*, the genera *Methylosinus* and *Methylocystis* are found in the family *Methylocystaceae*, and genera *Methylocella*, *Methyloferula* and *Methylocapsa* in the family *Beijerinkiaceae*. In the last 15 years, there have been increasing reports of facultative methanotrophs within the *Methylocella*, *Methylocapsa, and Methylocystis* genera that can use multi-compounds for growth in addition to methane (22-26). Also known today are filamentous methanotrophs from other genera, such as *Crenothrix* and *Clonothrix*, and non-proteobacterial (verrucomicrobial) methanotrophs of the genus *Methylacidiphilum* growing at high temperatures and low pH have also been discovered recently (27). Finally, it was shown that *Candidatus* Methylomirabilis oxyferans, a member of the NC10 phylum, generates dioxygen for the oxidation of methane despite being an obligate anaerobe (28, 29). Taken together, these data clearly illustrate the widespread nature of methanotrophic bacteria in most ecosystems of our planet.

Physiology and biochemistry of methanotrophs

Methanotrophs can use methane as an energy source, and also to provide carbon for all of their cellular constituents (6, 30, 31). The initial oxidation of methane to methanol is catalyzed by the enzyme methane monooxygenase (MMO). There are two structurally and biochemically distinct forms of MMO, a membrane-associated or a particulate MMO (pMMO), and a cytoplasmic or

soluble MMO (sMMO), which represent evolutionarily independent solutions to the same molecular problem of methane oxidation (32-37). The sMMO is a three-component binuclear iron active center monooxygenase that belongs to a large group of bacterial hydrocarbon oxygenases, known as soluble diiron monooxygenases (SDIMOs) (38), which are also homologous to the R2 subunit of class I ribonucleotide reductase. Two very similar sMMO systems, from *Methylococcus capsulatus* (Bath) (39-43) and *Methylosinus trichosporium* OB3b (44-47) have been studied in detail. sMMO is encoded by a six-gene operon *mmoXYBZDC* and has three components: (1) a 250-kDa hydroxylase with an $\alpha_2\beta_2\gamma_2$ structure in which the α-subunits (MmoX) contain the binuclear iron active center where substrate oxygenation occurs; (2) a 39-kDa NAD(P)H-dependent reductase (MmoC) with flavin adenine dinucleotide (FAD) and Fe_2S_2 prosthetic groups; and (3), a 16-kDa component (MmoB) known as protein B or coupling/gating protein that does not contain prosthetic groups or metal ions (39, 48). There are X-ray crystal structures for the hydroxylase component (49-52), NMR-derived structures for protein B (39, 53, 54) and an NMR-derived structure for the flavin domain of the reductase (55). The complex formed by the three components has been studied structurally via small angle X-ray scattering analysis and biophysically by electron paramagnetic resonance spectroscopy, ultracentrifugation and calorimetric analysis (56, 57). The catalytic cycle of sMMO has been extensively studied, and excellent progress has been made toward understanding the mechanism of oxygen and hydrocarbon activation at the binuclear iron center (45) (45, 58-62). Detailed reviews of the structure and catalytic mechanisms of sMMO are available (6, 37, 47, 63, 64).

The pMMO, in contrast, is a copper- and possibly iron-containing, membrane-associated enzyme that is associated with unusual intracytoplasmic membranes that take the form of vesicular disks in type I methanotrophs and paired peripheral layers in type II organisms (65-75).

153 Intracytoplasmic membranes are enriched in pMMO and can be physically separated from the
154 cytoplasmic membrane on the basis of sedimentation velocity in sucrose density gradients (76).
155 An understanding of the structure and mechanism of pMMO has emerged more slowly than that
156 of sMMO because of losses of activity when the enzyme is solubilized. pMMO consists of three
157 polypeptides of approximately 49, 27 and 22 kDa encoded by the genes *pmoCAB* (77). There are
158 often multiple copies of these *pmo* genes in methanotrophs (78, 79). Recent studies have shown
159 that native pMMO forms a complex with methanol dehydrogenase (MeDH), which may supply
160 electrons to the pMMO (80, 81), similar to what has been found for the hydroxylamine
161 oxidoreductase and ammonia monooxygenase redox couples (82-85).
162 Some methanotrophs such as *M. capsulatus* (Bath) and *M. trichosporium* OB3b can
163 produce either form of MMO. Most known methanotrophs possess only pMMO, e.g.
164 *Methylomonas methanica*, *Methylomicrobium album* BG8, *Methylocystis parvus* OBBP, and the
165 verrucomicrobial and NC10 methanotrophs. Only a few methanotrophs within the
166 *Beijerinkiaceae* family, e.g., *Methylocella silvestris* and *Methyloferula stellata*, have sMMO but
167 do not possess pMMO (21, 86).
168 The methanol produced by MMO is oxidized to formaldehyde by a calcium or rare earth
169 dependent pyrrolquinoline quinone (PQQ) containing MeDH (87-91). Formaldehyde is an
170 important branch-point in the metabolism of methanotrophic metabolism, and represents the
171 point at which one-carbon (C1) intermediates can either be oxidized to CO_2 to derive energy or
172 assimilated into biomass. Since formaldehyde is toxic, methanotrophs must protect themselves
173 against accumulation of this metabolic intermediate. Multiple pathways for metabolism of
174 formaldehyde are found in methanotrophs (2, 26, 92-96). For example, oxidative dissimilation of
175 formaldehyde can occur by its conjugation to tetrahydromethanopterin (H_4MPT) (97, 98), via

dye-linked membrane-associated (93) or via NAD^+-dependent formaldehyde dehydrogenases (95, 96, 99). Formate, resulting from the oxidation of formaldehyde by formaldehyde dehydrogenases, is further oxidized to carbon dioxide by an NAD^+ -dependent formate dehydrogenase which generates NADH, which is then available for oxidation of methane, biosynthetic reactions, and energy generation within the cell (100-102). Methanotrophs also possess two pathways for fixation of formaldehyde into biomass, the serine and ribulose monophosphate (RuMP) cycles active in Alphaproteobacterial and Gammaproteobacterial methanotrophs respectively. The pathways of carbon fixation in methanotrophs have been reviewed extensively (see e.g. (6)).

The "copper-switch" in methanotrophs

Early attempts to characterize methane oxidation were complicated by different reports on the cellular location of the MMO. MMOs were described either as soluble or as membrane-associated, depending on the strain and, for some strains, on the reporting laboratory. Several groups initially reported activity in the particulate or membrane fraction (103, 104), whereas other groups detected activity in the soluble fraction (105, 106). Subsequent studies showed the cellular location varied with cultivation conditions. Oxygen-limitation was reported to induce methane oxidation in the soluble fraction in *M. trichosporium* OB3b (36, 107). However, it was subsequently shown that oxygen was not the regulatory factor, and that the switch between the membrane-associated and soluble activity was related to biomass concentration (108). The defining moment in the discovery of this "switch" was when Dalton and colleagues attempted to grow *Methylocystis parvus* OBBP to high cell densities in chemostat culture. They observed that cultures of *M. parvus* OBBP, when supplied with methane, air and a nitrate mineral salts solution

199 at relatively low cell densities, simply stopped growing. However, when additional trace
200 elements solution was added, the cultures immediately started growing again. The "secret
201 ingredient" in the trace elements solution was narrowed down to copper ions (108). It was
202 subsequently realized that *M. parvus* OBBP contained only pMMO, hence the high requirements
203 for copper ions, and did not contain a sMMO which would have allowed it to grow to the same
204 high cell densities observed at the time with *M. capsulatus* Bath and *M. trichosporium* OB3b,
205 under copper limitation. The latter strains, when confronted with the same conditions, switched
206 to expression of the sMMO and carried on growing (35, 109). Interestingly, copper had earlier
207 been shown to enhance growth of *Methanomonas margaritae*, a methanotroph which does not
208 contain sMMO, but these original observations were never investigated further (110).
209 Dalton and colleagues followed up their observations in detail and established the
210 existence of this "copper switch" i.e., the regulation of expression of the two different forms of
211 MMO in methanotrophs in response to the copper-to-biomass ratio of cultures of methanotrophs
212 which possess both sMMO and pMMO. This allowed them to explain many of the earlier
213 observations on metal-dependent growth of methanotrophs. For instance, they showed that in *M.*
214 *capsulatus* Bath, expression of the sMMO was only observed at high cell densities when copper
215 ions in the medium were depleted, whereas additions of excess copper ions allowed this
216 methanotroph to express active pMMO. Subsequently, Murrell and colleagues showed at the
217 molecular level that under growth with low concentrations of copper ions, expression of sMMO
218 was initiated at a σ^{54} promoter upstream of the sMMO gene cluster (*mmoXYBZDC*). Conversely,
219 under high copper-growth conditions, expression of sMMO was repressed, and high levels of
220 expression of the genes encoding pMMO (*pmoCAB*) allowed both *M. trichosporium* OB3b and
221 *M. capsulatus* Bath to grow using pMMO (34, 111-113).

Further research established that copper affects methanotrophic physiology and gene expression much more broadly. For example, it was found that the intracytoplasmic membrane content in methanotrophs increased with increasing copper in the growth medium (66, 109, 114). This was not totally unexpected, however: considering that the pMMO is localized in the intracytoplasmic membranes, then greater expression and activity of pMMO would logically require more of these membranes. More surprisingly, however, it was recently discovered that pMMO and the PQQ-linked MeDH encoded by the *mxa* operon form a super-complex anchored in the intracytoplasmic membranes, and that electron transfer from the PQQ-linked MeDH to pMMO *in vivo* may drive the oxidation of methane (80, 81). In support of this latter finding, it was recently found that not only does expression of *pmo* genes increase with increasing copper, but that of genes in the *mxa* operon does so as well (91).

It has also been shown by proteomics that additional steps in the oxidation of methane to carbon dioxide are overexpressed with increasing availability of copper, as that of proteins involved in lipid, cell wall, and membrane synthesis (66, 115). Conversely, the ability of methanotrophs to direct carbon from methane to poly-3-hydroxybutyrate increases with decreasing copper availability (66, 116), suggesting that to some extent energy metabolism of methanotrophs is controlled by copper. Such a conclusion was actually already reached earlier by Dalton and colleagues, who showed that the biomass yield and carbon conversion efficiency in methanotrophs when grown on methane increased as copper increased, i.e., when methanotrophs switch from expressing sMMO to pMMO (117). Finally, it was found that the "surfaceome", or proteins on the outer surface of the outer membrane, are also controlled by copper availability in some methanotrophs. The expression of several multi-*c*-type cytochromes

and proteins believed to be involved in copper uptake also vary, and in most cases decrease, as copper availability increases (118-123).

Recently, a new family of copper storage proteins, the Csps, have been discovered in *M. trichosporium* OB3b (124). This bacterium possesses three Csps; Csp1 and Csp2 have predicted twin arginine translocase (Tat) targeting signal peptides and are therefore thought to be exported after folding, as well as the cytosolic Csp3. Csp1 forms a tetramer of four helix bundles that can bind up to 52 Cu^{1+} ions via Cys residues that point into the core of the bundle. Switchover to sMMO is accelerated in Δ*csp1*/*csp2* compared to wild type suggesting that these proteins play a role in storing copper for the pMMO and provide an internal copper source when copper becomes limiting. Under such conditions mb is produced that can readily remove all Cu^{1+} from Csp1 and therefore may play a role in helping to utilize Csp1-bound copper.

Evidence suggesting a copper-specific uptake system,

The first evidence for a copper-specific uptake system and for the production of an extracellular copper-binding ligand came during the phenotypic characterization of constitutive sMMO mutants ($sMMO^C$) of *M. trichosporium* OB3b (125, 126). Phelps *et al*. (126) isolated five $sMMO^C$ mutants by culturing *M. trichosporium* OB3b in the presence of dichloromethane, acting as a mutagen following its co-metabolic transformation by methane monooxygenase to formyl chloride. In addition to the $sMMO^C$ phenotype, $sMMO^C$ mutants were defective in copper acquisition (125, 127, 128). A drastic increase in soluble versus insoluble copper in the culture medium was also observed and fostered speculation on the production of an extracellular Cu^{2+}-complexing agent(s) analogous to Fe^{3+}-complexing siderophores (128). Subsequent studies

revealed the presence of a low molecular mass copper binding ligand, although the identity of this compound was not determined (125, 127).

Initial identification and isolation of methanobactin (a copper-binding compound or "chalkophore")

Somewhat paradoxically, the "copper-binding ligand" or "copper-binding compound" was first isolated from *M capsulatus* Bath during the purification of pMMO, and thus under conditions of high copper concentration (73). Separation of this copper-binding compound from the pMMO revealed a low molecular mass yellow fluorescence copper-containing molecule. Its color and fluorescence properties of this molecule were similar to those of the water-soluble pigment observed in cells cultured in low copper medium (73). Subsequent characterization of this water-soluble pigment revealed that it was identical to the copper-binding compound that co-purified with the pMMO (73, 128). The production of water-soluble pigments by methanotrophs had been noted during the initial isolation of many type strains over 40 years ago, but was associated with cells cultured in low iron medium (13). The copper-binding compound was eventually termed methanobactin (mb), based on the molecule's antimicrobial activity towards Gram-positive bacteria (129, 130). Once identified, this copper-binding compound was isolated or identified in a number of different methanotrophs, including *Methylomicrobium album* BG8 (131), *Methylocystis* strain SB2 (132), *Methylocystis rosea* (133), *Methylocystis hirsuta* (133), and *Methylocystis* strain M (133). In this review, we will focus on mbs whose primary structures have been determined from methanotrophs with sequenced genomes, i.e. *M. trichosporium* OB3b, *Methylocystis strain* SB2 and *Methylocystis rosea*.

289 **Methanobactins as chalkophores**

290 Functionally, mbs are similar to siderophores. Like siderophores, they are all low molecular
291 mass (<1,200 Da) compounds produced by bacteria under low copper conditions (125, 126, 128,
292 134-136). Following the nomenclature of siderophores, which is Greek: iron-bearing or iron-
293 carrying, mbs are chalkophores, which is Greek: copper-bearing or copper-carrying (137, 138).
294 Mbs are currently the only known representatives of this group. Consistent with their role as the
295 extracellular component of a copper acquisition system, mbs have some of the highest known
296 binding affinities for copper ions (2, 130, 136, 139-141) (Table 1).

297 Although chalkophores and siderophores share a number of properties, these two groups
298 of metal-binding compounds can be distinguished in a number of ways. With the exception of
299 the phytosiderophore domoic acid (142), siderophores are expressed under iron limitation,
300 whereas chalkophores are expressed under copper limitation. Many siderophores bind copper,
301 and chalkophores can bind iron (142-148), however, different metal binding constants
302 characterize the two groups and colorimetric assays have been developed to distinguish between
303 them based on this difference (136, 149, 150). Structurally, chalkophores differ from
304 siderophores by their typical heterocyclic rings and associated thioamide groups (Fig. 1 and 2).
305 Different ring systems have characteristic UV-visible absorbance, circular dichroism (CD) and
306 fluorescent spectral properties, which can be used for identification and to characterize the metal
307 binding properties of the molecule (131, 133, 134, 136, 137, 141, 148, 151-153). Excitation
308 energy transfer occurs between the two rings in mb (134, 154), as observed for the chromophores
309 of light harvesting complexes (155-157), resulting in the fluorescent properties of mbs. For
310 example, the emission intensity of mbs increases with selective hydrolysis of the one of the

311 rings, and emission intensity often increases following metal addition (132, 134-136, 140, 148,
312 154). Again, this property can be used in both identification and characterization of mbs.
313 However, not all of the properties just described are sufficient to identify mbs. For
314 instance, *Clostridium cellulolyticum* produces a copper binding secondary metabolite,
315 closthioamide, with a molecular mass similar to mbs (158-160), and like mbs, closthioamide has
316 thioamide groups, will reduce Cu^{2+} to Cu^{1+}, and has a high Cu^{1+} binding affinity ($>10^{15}$ M^{-1}).
317 Closthioamine will also test positive with the copper-chrome azural S (Cu-CAS) assay, a liquid
318 or plate assay used to screen for mb production (136, 149, 161). However, closthioamide can be
319 distinguished spectroscopically from mbs, e.g. closthioamide shows a single UV-visible
320 absorption maximum at 270 nm arising from its two characteristic phenolic groups separated by
321 six thioamide moieties. Furthermore, closthioamide is able to form a dinuclear Cu^{1+} complex.
322 Also in contrast to mbs, closthioamide synthesis is not regulated by copper, and unlike mbs, the
323 molecule is believed to be produced by a polyketide synthase (see below).
324 Similarly, under low copper conditions, *Paracoccus denitrificans* also produces a low
325 molecular mass 716.18 Da porphyin, coproporphyrin III, that appears to be involved in copper
326 acquisition (162). Unfortunately, the copper binding properties were not reported in the initial
327 publication and we are not aware of any follow-up studies. Coproporphyrin III has a typical
328 heme-UV-visible absorption spectra and shows different γ, α, β maxima depending on the metal
329 coordinated by the heme group, allowing it to be distinguished from mb based on this property.
330

331 **STRUCTURAL PROPERTIES OF METHANOBACTINS**

332 **Structural diversity and core features**

The core features of all structurally characterized mbs are shown in Figs. 1 and 2. As already mentioned above, mbs are modified peptides characterized by the presence of one oxazolone ring and a second oxazolone, imidazolone or pyrazinedione ring, which are separated by 2 – 4 amino acid residues (see below, Biosynthesis of Methanobactin). Each ring has an adjacent thioamide group. Structurally, mbs can be divided into two types (Figs. 3 and 4). One type (Group I) is represented by mb from *M. trichosporium* OB3b (mb-OB3b) (Figures 1A, 3A and 4). In addition to these core properties, mb-OB3b also contains Cys residues in the mature peptide, which are linked by an intramolecular disulfide bond (Fig. 1A and 3A). Cu^{1+}-mb-OB3b has a pyramid-like shape with the metal coordination site at the base (133, 137, 141). The disulfide bond is present in both apo- and Cu^{1+}-mb-OB3b. Mb-OB3b is structurally the most complex mb characterized so far, and is currently the only structurally characterized representative of this mb type. However, based on sequence similarity and alignments, the putative mbs from *Methylosinus* sp. strain LW3 (mb-LW3), *Methylosinus sp.* strain LW5 (mb-LW4), *Methylosinus* sp. strain PW1 (mb-PW1) and one of the two mbs from *Methylocystis parvus* OBBP (mb-OBBP(1)) would also fall within this group (Fig. 4). In this group the core peptide is predicted to contain 2 or more Cys in the mature peptide and/or to contain additional ring(s). If the Cys are not incorporated into a ring, the second Cys and either the third or fourth Cys are predicted to form an intermolecular disulfide bond.

The second group (group II) is represented by mbs whose primary structures have been determined in *Methylocystis rosea* and *Methylocystis* strain SB2. In this group, mbs lack the Cys residues in the mature peptide, are smaller and probably less rigid due to the absence of the disulfide bond found in mb-OB3b. Heterocyclic rings are separated by two or three amino acids. In contrast to members of group II mbs from methanotrophic organisms, putative mbs from non-

356 methanotrophs (mb-B-8, mb-14-3, mb-B510, mb-21721) contain four Cys in the apo-protein.
357 However, based on the location of Cys residues, we predict all 4 Cys are incorporated in the two
358 heterocyclic rings. Mbs from the structurally characterized members in this group contain a
359 sulfate group, which appears to aid in the formation of a tight bend in the molecule (Figs. 3B) by
360 making a hydrogen bond with the backbone amide of Ser2. The sulfate group also increases
361 affinity for copper ions (133). Overall, mbs from the *Methylocystis* strains display a hairpin-like
362 shape (Figs. 3B and 3C) (133). The conserved T/S adjacent to the putative C- terminal ring
363 suggest the other members of this group also contain a sulfate group (Fig. 4).
364 Truncated forms of mb have been identified for all structurally characterized mbs (133,
365 136, 141), which results from the loss of one or more C-terminal amino acid residues (Figs. 1
366 and 4. The different forms show very similar copper binding properties (133, 141), but distinct
367 reduction potentials (133, 141) and spectral properties (136). The mechanism leading to the loss
368 C-terminal amino acids remains an open question, but does not appear to involve N-protonation
369 as observed for microcin B17 (163), since the C-terminal oxazolone ring of mb remains intact. In
370 microcin B16, this reaction results in autoproteolysis of the ring and protein splicing. In
371 *Methylocystis* strains the sulfate group can also be lost, which does affect copper affinity (133).
372 Mbs thus have a number of unusual structural features. Modifications of two residues to
373 form oxazolone or imidazolone rings are rare, but have been observed in several classes of
374 ribosomally-synthesized and post-translationally modified peptide (RiPP) secondary metabolites
375 (163-166). Pyrazinedione rings are even more uncommon, and have only been detected in the
376 non-amino acid containing molecules selerominol (167) and flutimide (168) from fungi. The
377 presence of thioamide groups in natural products is also rare and has only been identified in
378 closthioamide from *Clostridium cellulolyticum* (160) and thioviridamide from *Streptomyces*

379 *olivovirdis* (169, 170). Although rare, *O*-sulfonation of Ser or Thr have been reported in
380 eukaryotic proteins (171), but to our knowledge mb is the only bacterial peptide that contains this
381 post-translational modification and is ribosomally produced, as described below.
382
383 **Copper coordination site**
384 In all mbs that have been structurally characterized, Cu^{1+} is coordinated in a similar
385 manner by a N_2S_2 ligand set with a distorted tetrahedral geometry (Fig. 3) (133, 137, 141). The
386 C-terminal S- and N- ligands in the *Methylocystis* mbs are switched compared to their position in
387 mb-OB3b (133), however, the resulting coordination geometry is very similar. The coordination
388 sites are stabilized by the two 5-membered chelate rings formed upon ligation and a number of
389 hydrogen bonding and π-interactions (Fig. 3), for example between the backbone amide of Cys3
390 and a coordinating sulfur in Cu^{1+}-mb-OB3b and a π-anion interaction between the sulfate and the
391 pyrazidione ring in *Methylocystis* mbs (133, 141).
392
393 **METAL BINDING PROPERTIES**
394 **Binding and reduction of the primary metal– copper**
395 Mb binds both Cu^{2+} and Cu^{1+} and the binding by mb appears to depend on pH (141, 172) and on
396 the Cu^{2+}/Cu^{1+} to mb ratio (134, 140, 172) (Table 1). As discussed below, mb is able to complex
397 both soluble and insoluble forms of Cu^{1+} and Cu^{2+}. Thermodynamic, spectral and kinetic studies
398 have been carried out on the addition of Cu^{2+} to mb-OB3b and *Methylocystis* strain SB2 (mb-
399 SB2) (134, 140). These studies (134, 140, 172) are complicated by the fact that mb rapidly
400 reduces of Cu^{2+} to Cu^{1+}. Since the oxidation state of copper responsible for the high bonding
401 constant for experiments in which Cu^{2+} has been added to apo-mbs is not known, we will

402 indicate that a mixture of these oxidation states is present using Cu^{2+}/Cu^{1+} from this point on. At
403 low Cu^{2+}/Cu^{1+} to mb-OB3b ratios, mb-OB3b initially binds Cu^{2+}/Cu^{1+} as an oligomer/tetramer
404 (134, 140). Pre-steady state kinetic data also suggest that the initial binding of metal is only on
405 one of the rings and its associated thioamide. In mb-OB3b, initial Cu^{2+}/Cu^{1+} coordination is to
406 the oxazolone A, followed by a short (8 – 10 ms) lag period and then coordination to oxazolone
407 B (140). At higher Cu^{2+}/Cu^{1+} to mb-OB3b ratios, mb-OB3b coordinates Cu^{2+}/Cu^{1+} as a dimer
408 followed by a monomer at Cu^{2+}/Cu^{1+} to mb-OB3b ratios above 0.5 Cu^{2+} per mb-OB3b. Mb-SB2
409 appears to follow a similar tetramer-dimer-monomer binding sequence depending on the copper
410 to mb ratio. For mb-SB2, the initial binding of Cu^{2+}/Cu^{1+} is to the imidazolone ring followed by
411 coordination to the oxazolone ring (154).
412 A number of different methods have been used to determine metal binding affinity
413 constants for mbs (Table 1). Affinities for Cu^{1+} can be determined from competition studies
414 using a well established approach (173-175) with chromophoric ligand such as bathocuproine
415 disulfonate. The measurement of the reduction potential of the Cu-mb then allows the Cu^{2+}
416 affinity to be calculated. The Cu^{1+} affinity is ~10^{21} M^{-1} for all mbs analyzed using this approach
417 (133, 141), and is one of the highest known for biological systems. Such a high affinity raises
418 the issue of how copper is released from mb within a methanotroph. The reduction of the
419 disulfide, in mb-OB3b (141), and removal of the sulfate, in *Methylocystis* mbs (133), do
420 decreases the Cu^{1+} affinity by ~2 order of magnitude, but the values are still high. Given that the
421 Cu^{2+} affinities are significantly weaker (Table 1) oxidation may be utilized for release of the
422 metal, which may be more likely for those Cu-mbs with lower reduction potentials, such as Cu-
423 mb-CSC1 (133).

Other copper chelating compounds, primarily for Cu^{2+}, have also been used to estimate affinities (91, 125, 128, 133, 141), as well as isothermal titration calorimetry (134, 140), and displacement isothermal titration calorimetry (134) (Table 1). Mbs have been shown to solubilize and bind insoluble forms of Cu^{1+} under anaerobic conditions (140), and to extract Cu from copper minerals (176), humic material (177), and glass (178). The ability of mbs to extract copper from copper-containing minerals as well as other organic molecules should be considered in modeling bioavailable sources of copper (179-183).

Binding of other metals

In addition to copper, mb-OB3b and mb-SB2 have been shown to bind a number of transition and near transition metals (135, 148, 154, 184)(Table 1). In general, the spectral properties following binding are unique for each metal, and can be used in initial analysis of metal binding and in metal competition studies (135, 154). The primary function of mbs appears to be copper acquisition and the capacity to bind other metals appears to be an inadvertent consequence of the Cu^{1+}-coordination site. The peptide backbone of mbs appears to influence the binding of other metals. For example, the order of metal binding preference by mb-OB3b is $Cu^{2+}/Cu^{1+} = Hg^{2+} = Au^{3+} > Zn^{2+} > Cd^{2+} > Co^{2+} > Fe^{3+} > Mn^{2+} > Ni^{2+}$, where as the metal binding preference for mb-SB2 is $Au^{3+} > Hg^{2+} > Cu^{2+}/Cu^{1+} > Zn^{2+} > Cd^{2+} = Co^{2+} = Fe^{3+} > Mn^{2+} > Ni^{2+}$ (135, 154).

Although strong binding of other metals such as mercury by mbs may seem counter-productive for methanotrophic growth, it may actually be of some benefit. Specifically, by strongly binding mercury, mbs may significantly alter its speciation and bioavailability, thereby reducing its toxicity. Indeed, growth studies showed that in the presence of mercury, added as mercuric chloride, methanotrophic growth was completely inhibited, but growth did occur when

447 mb was also added (184). Interestingly, such a protective effect was seen in a variety of
448 methanotrophs when exposed to mercury and mb-OB3b. These results suggest mb secreted by
449 any methanotroph could serve to protect the broader microbial community from mercury toxicity
450 (184).

451 Further indirect evidence of the importance of mb binding to metals other than copper
452 was afforded by the observation that in *M. trichosporium* OB3b sMMO is expressed and active
453 in the presence of both gold and copper. This finding was unexpected since, as discussed above,
454 copper strongly represses expression of sMMO. Also, the amount of copper associated with
455 biomass significantly decreased in the presence of gold, as compared to when gold was not
456 added (91). This suggests that mb-OB3b is limited in its ability to bind copper in the presence of
457 gold. Indeed, if mb was pre-loaded with copper and added exogenously to *M. trichosporium*
458 OB3b, sMMO expression was not observed, and copper levels associated with biomass increased
459 (91). These findings suggest that although methanotrophs synthesize mb for copper uptake, such
460 uptake may be compromised if other metals such as gold are also present due to their ability to
461 compete effectively for binding to mb. These observations suggest that in such situations,
462 sMMO may be expressed and active even in the presence of copper, which may alter the
463 methanotrophic community structure as well as activity.

464

465 **Metal reduction**

466 As mentioned above, another property exhibited by all mbs examined so far is their
467 ability to reduce Cu^{2+} to Cu^{1+} (133, 134, 140, 141, 185). In addition, mb-OB3b and mb-SB2
468 have also been shown to reduce Au^{3+} and Ag^{+} to Au^{0} and Ag^{0}, respectively (135, 148). In the
469 case of gold, no other oxidation states were observed. In contrast, no change in the oxidation

states of Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, and Ni^{2+} was observed following binding by mb-OB3b and mb-SB2 (135, 148). Surprisingly, in the absence of an external reductant, both mb-OB3b and mb-SB2 have been shown to reduce multiple Cu^{2+} and Au^{3+} ions, which raise the question of the electron donor for this reaction.

BIOSYNTHESIS OF METHANOBACTIN

Identification of the polypeptide precursor of methanobactin

Although a potential peptide sequence precursor of mb-OB3b was identified following hydrolysis of the oxazolone rings (132), the question whether this peptide was assembled by a non-ribosomal peptide synthetase or encoded by DNA and synthetized on the ribosome remained. Determination of the genome sequence of *M. trichosporium* OB3b helped resolve this question. BLAST searches using the predicted mb peptide precursor revealed a short ORF with a perfect match at a location within the *M. trichosporium* OB3b genome sequence where no protein product had been predicted (Fig. 4). The genome region of the putative mb precursor matching sequence in *M. trichosporium* OB3b had a number of distinctive and striking features (Fig. 5; Table 2). These included (a) a precursor peptide composed of leader and core peptide sequences followed by a stop codon, as expected for post-translationally modified peptide natural products (164); (b) a potential cleavage site between the leader and core peptide, suggestive of secretion; (c) genes upstream and downstream of the mb precursor gene encoding protein sequences compatible with possible roles in maturation of the mb precursor sequence, transport, and regulation of mb biosynthesis (Fig. 5, Table 2).

Elaboration on this initial search revealed a series of genomes containing gene clusters with characteristics matching that of the *M. trichosporium* OB3b mb gene cluster, e.g. in

493 *Methylocystis parvus* OBBP (139, 186, 187), *Methylosinus* sp. LW3 (186), as well as non-
494 methanotrophs *Azospirillum* sp. B510 (132, 139), *Azospirillum* sp. B506 (186), *Pseudomonas*
495 *extremaustralis* (139), *Pseudomonas extremaustralis* substrain *laumondii* TT01 (186), *Tistrella*
496 *mobilis* (139), *Gluconacetobacter* sp. SXCC (132, 186). *Gluconacetobacter oboediens* (186,
497 187) *Methylobacterium* sp. B34 (186) ,*Cupriavidus basilensis* B-8 (186), *Photorhabdus*
498 *luminescens* (186) and *Vibrio caribbenthicus* BAA-2122 (186). Notably, this list not only
499 includes methanotrophic strains known to produce mb for which a genome sequence was
500 available, but also in the genome of other strains, suggesting that such gene clusters may encode
501 peptide-derived products with more diverse functions than those associated with mbs.
502 Considering the mb precursor peptides identified so far, it is striking that the leader peptides are
503 much more strongly conserved than core peptide sequences (Fig. 4). It is also interesting to note
504 that sequence conservation does not follow taxonomic affiliation, and that identical precursor
505 sequences are found in gene clusters of strains from different genera (Fig. 4). This strongly
506 suggests that horizontal gene transfer has contributed to dissemination of mb gene clusters in the
507 environment, and indeed, mobile genetic elements have often been identified in the sequences
508 immediately adjacent to mb gene clusters (186).
509 The lack of sequence conservation in mb core peptides also implies that designing
510 sequence motifs to detect mb gene clusters is challenging, and that database hits using sequence
511 motifs based on known mb precursor sequences need to be substantiated with other evidence. In
512 our view, for a sequence hit to be considered indicative of a mb precursor, additional evidence
513 should include (a) the presence of the only conserved genes in the mb gene cluster (Fig. 5), i.e.
514 the genes for mb biosynthesis cassette proteins MbnB and MbnC, in the immediate vicinity of
515 the mb peptide precursor; and (b), the presence of 2 or more cysteines in the core mb peptide

516 sequence (Fig. 4; Table 2). Regarding the latter, it is striking that mb peptide precursors
517 identified so far contain either 2 or at least 4 cysteines, just as in the two types of mbs
518 characterized so far. Nevertheless, the spacing between cysteine residues in the mb peptide
519 precursor sequence appears to be quite variable, with 1 or 2 residues between the first and the
520 second cysteine, and if present, 2-4 residues between the second and the third cysteine, and 1-3
521 residues between the third and the fourth cysteine, respectively (Fig. 4). It is also striking to note
522 that "doublets" of two cysteine residues immediately adjacent to each other have only been
523 found so far in putative mb precursor peptides of presumably non-methanotrophic strains (Fig.
524 4).

525 The overall structure of mb gene clusters and related gene clusters is beyond the scope of
526 this review, but their analysis, using a combination of sequence-based, motif-based and gene
527 synteny-based searches, will undoubtedly yield many interesting findings in the future, as more
528 mb gene clusters are identified in the increasing number of microbial genomes that are being
529 sequenced. Summarizing the quite large diversity of mb gene clusters that have already been
530 detected (see e.g. Kenney and Rosenzweig (186)), two major types of mb gene clusters seem to
531 emerge with respect to the localisation of the *mbnA* gene for the mb precursor peptide (Fig. 5).
532 Whereas *mbnA* is always directly upstream and in the same orientation of *mbnB* and *mbnC*, it
533 may be found as a short orf, as exemplified for strain OB3b (Fig. 5), or actually represent the 3'-
534 end of regulatory gene *mbnI*, as in strains SB2 and SV97 (Fig. 5). The implications of the latter
535 localization of the *mbnA* gene, notably in terms of regulation of mb expression in strains with
536 this gene arrangement (also see Regulation of Gene Expression Section below), are completely
537 unknown at present. Currently, the cytochrome *c* peroxidase MbnH (Fig. 5; Table 2), as well as
538 the FAD-dependent oxidoreductase MbnF, present instead or sometimes in addition to MbnH in

539 methanotroph gene clusters (186, 187) are likely candidates to be involved in the oxidation steps
540 required for ring formation (see below, and Fig. 6). In addition, the aminotransferase MbnN
541 found in the mb-OB3b, but not the mb-SB2 gene cluster (Figure 5) may be involved in formation
542 of the *N*-terminal keto-isopropyl group from Ile1 in the peptide precursor, and the
543 sulfotransferase MbnS found in the mb-SB2 and mb-rosea, but not the mb-OB3b gene cluster
544 may catalyse sulfonation of the threonine (see Fig. 1 and 5). Two other gene products, TonB
545 receptor, multidrug and toxin extrusion (MATE) protein, Fig. 5; Table 2) have been suggested
546 (186, 187) to be involved in uptake and secretion of mature mbs, respectively. The reader is
547 referred to a recent review by Kenney and Rosenzweig (186) for a detailed description and
548 phylogenetic analysis of several mb gene clusters.
549
550 **Current hypotheses on the nature of the mb biosynthesis pathway**
551 Despite the identification of the genetic determinants of mb production, the precise mechanism
552 involved in maturation of mbs from peptide precursors still represents an open question. The
553 presence of heterocyclic rings would suggest a pathway similar to that of other post-ribosomal
554 peptide synthesis (PRPS) proteins (163-166, 188). For instance, in the PRPS protein microcin
555 B17, McbB (cyclodehydrase), McbC (FMN-dehydrogenase) and McbD convert –Gly-Cys- and
556 –Gly-Ser-dipeptide sequences into thiazole and oxazole rings, respectively (163). In this
557 reaction sequence, the cyclodehydrase catalyzes ring formation via the amine bond with the thiol
558 or alcohol, followed by oxidation by an FMN-dehydrogenase (163-166). In mb, ring formation
559 is initiated from an –X-Cys-dipeptide sequence, resulting in formation of either an oxazolone,
560 imidazolone or pyrazinedione ring with a neighboring thioamide group. If the catalytic sequence
561 in ring formation in mb followed the microcin B17 example, the thiol group on Cys from mb

562 would have to be replaced by a hydroxyl group, possibly with an amine intermediate. Then the
563 thioamide group would have to be introduced via an amide to thioamide replacement, as
564 proposed for two other natural products with thioamide groups (153), closthioamide from
565 *Clostridium cellulolyticum* (160) and thioviridamide from *Streptomyces olivoviridis* (169, 170).
566 As an alternative, the thioamide group of mbs was proposed to originate from the Cys thiol, as
567 hydrolysis of the oxazolone rings resulted in a -X-Gly-thioamide sequence (132). Based on the
568 precursor peptide sequences that have now been identified, the latter reaction mechanism now
569 appears highly likely.
570 The absence of a cyclodehydrase-like gene in mb gene clusters also suggest that ring
571 formation differs from that of other natural products in other aspects as well. The simplest and
572 shortest reaction sequence in oxazolone ring formation in mbs would involve an oxidation
573 followed by a rearrangement that changes the connectivity of the peptide backbone (Fig. 6). In
574 such a scheme, Cys thiols would likely require to be protected against oxidation (blue circle), as
575 protein-linked thioesters, or alternatively as disulfides (as shown in Fig. 6), possibly via
576 glutathionylation as observed with MetE in *Escherichia.coli* (189). This reaction may occur
577 during the initial peroxidase reaction, which is one of the proteins of the mb gene cluster (Table
578 2). The reaction sequence could operate during formation of both oxazolone rings of mb-OB3b,
579 from Leu1 and Cys2, and from Pro7 and Cys8 of the precursor peptide, respectively.
580 *Methylocystis* strain SB2 and *M. rosea* share the same mb gene clusters and identical precursor
581 peptides (Figs. 4 and 5), but the mature mb of *Methylocystis* strain SB2 (132) as characterized
582 contains an imidazolone ring, whereas the mb from *M. rosea* has a pyrazinedione ring that is also
583 present in the *Methylocystis* strain M mb (133). However, imidazolone and pyrazinedione rings
584 are actually isomeric and can be interconverted by a simple sequence of hydration,

585 rearrangement, and dehydration reactions (190). Nevertheless, the mechanism of formation of
586 this ring is likely to be more complex than that for oxazolone ring formation (Fig. 6). One
587 possible sequence would replace the transamination reaction, proposed for mb-OB3b, with an
588 oxidative deamination of the N-terminal amine, which would produce ammonia that could be
589 used for subsequent aminolysis of the oxazolone ring (Fig. 6). A condensation reaction would
590 then lead to cyclization and the formation of the imidazolone, or with an intervening
591 rearrangement step, to the pyrazinedione. It should be noted that the *Methylocystis* species lack
592 the aminotransferase found in the mb-OB3b gene cluster. Alternate reaction schemes would
593 involve two changes of connectivity in the peptide backbone, and not just one as proposed for
594 oxazolone ring formation (Fig. 6). Clearly, the formation of the different types of heterocyclic
595 ring systems in mbs now needs to be addressed experimentally. The production and
596 characterization of mutants will hopefully help in this respect, and such studies have now been
597 initiated (187).
598
599 **REGULATION OF GENE EXPRESSION**
600 The discovery of the mb biosynthesis gene cluster raised two main questions. First, is mb
601 biosynthesis regulated with respect to copper? Second, what is the role of mb in the "copper-
602 switch" controlling the expression of the two forms of MMO? The first question was easily
603 answered through the use of reverse-transcription-quantitative polymerase chain reaction (RT-
604 qPCR) using primers specific to some gene(s) of the *mbn* operon. Initial studies found that in *M.*
605 *trichosporium* OB3b, expression of *mbnA*, the gene encoding the polypeptide precursor of mb,
606 did indeed vary with respect to copper, with expression dropping over three orders of magnitude
607 when copper concentrations increased from zero (no amendment) to 1 μM copper, and

608 expression was largely invariant at higher copper concentrations (139). Such a decrease in
609 expression was reflected in the finding that mb in the spent medium was highest at copper
610 concentrations less than 1 μM, and dropped ~5-fold when the copper concentration was
611 increased to 5 μM (134, 136, 140). Given that the two forms of MMO are also regulated by
612 copper, it was possible that mb was directly involved in this copper-switch. Further genetic
613 analyses, however, found that it is not (139). RT-qPCR of *mmoX* and *pmoA* from both *M.*
614 *trichosporium* OB3b wild type and a *mbnA* mutant where a gentamycin cassette was inserted,
615 knocking out *mbnA*, showed that *mmoX* and *pmoA* expression followed similar patterns in both
616 strains as the copper-biomass ratio varied, but that the magnitude of such changes were lower in
617 the *mbnA*::Gm^R mutant as compared to the wild type strain (139). To answer the second
618 question above, another mutant was constructed in which the *mmo* operon was deleted. In this
619 mutant the "copper-switch" was inverted, i.e., *pmoA* expression was greatest in the absence of
620 copper, and dropped ~two orders of magnitude when copper concentrations increased (139).
621 Further, expression of *mbnA* was largely invariant in this mutant with respect to copper. Since
622 all deleted genes in the *mmo* operon with the exception of *mmoD* are known to encode
623 polypeptides of sMMO, and since MmoD is not required for sMMO activity (32, 51, 63), these
624 expression data suggest that MmoD is a key component of the copper-switch, MmoD serving to
625 regulate expression of mb, and mb then amplifying the magnitude of the response in the copper
626 switch.

627 On the basis of these findings, a model for the regulation of expression of the *mmo* and
628 *pmo* operons by mb and MmoD was proposed (139). During growth at low copper : biomass
629 ratios, MmoD protein represses expression of the *pmo* operon and also up-regulates expression
630 of the *mmo* operon, including that of *mmoR* and *mmoG* shown previously to play key roles in

regulating *mmo* expression (113). Further, MmoD is postulated to enhance expression of mb, which serves to amplify expression of the *mmo* operon. During growth under high copper, mb binds copper and can no longer enhance the expression of the *mmo* operon. Under these conditions, it is presumed that MmoD also binds copper and no longer represses expression of the *pmo* operon or induces expression of sMMO or mb.

There are, however, a number of issues with the model as proposed in Fig. 7. For such a model to be accurate, *mmoD* would be required to be constitutively expressed with respect to copper, in order for sMMO and mb expression to occur after copper is removed. This version of the model implies, however, that as copper : biomass ratio increases, expression of the entire *mmo* operon no longer occurs. Recent qRT-PCR analysis, however, show that *mmoD*, unlike *mmoX*, is constitutively expressed with respect to copper (Semrau, unpublished data). Nevertheless, expression of *mmoD,* as that of *mmoX*, increases with increasing addition of exogenous mb, suggesting that expression of *mmoD* is at least partly co-regulated with that of the entire *mmo* operon.

In addition, this model, although explaining the genetic basis for the copper-switch in methanotrophs, does not explicitly consider the role of two genes known to play roles in the regulation of the *mmo* operon, i.e., *mmoR* and *mmoG* upstream of *mmoX* (113, 191). Based on available data, it appears that *mmoR* and *mmoG* do not play a significant role in the copper-switch, i.e., they do not appear to have any control over expression of the *pmo* operon. It is possible, however, that regulation of the *mmo* operon is the result of a concerted interaction of mb and/or MmoD with these gene products.

This model also ignores the possibility that the presence of putative regulatory genes (e.g. *mbnI* and *mbnR*, Fig. 5) in mb gene clusters, which may play a role in regulating gene

654 expression, particularly the mb gene cluster and/or the *mmo* operon. Given this, a revised model
655 of the copper-switch in methanotrophs is now proposed in Fig 8. Here MmoD is again
656 postulated to be a key component of the copper switch, but MbnI is proposed to be responsible
657 for inducing expression of the mb gene cluster, as well as *mmoR* and *mmoG*. Collectively, mb,
658 MmoR and MmoG interact to induce expression from the σ^N promoter upstream of *mmoX*, while
659 MbnI binds to the σ^{70} promoter upstream of *mmoY*. In this updated and revised current model,
660 *mmoD* is constitutively expressed, but associates with copper when it is present, preventing
661 repression of the *pmo* operon or expression of the mb gene clusters. Further experiments will
662 show whether this new model is correct, but it is already clear that the exact details of the copper
663 switch mechanism are more complex than previously thought.
664
665 **PHYSIOLOGICAL FUNCTION(S)**
666 **Copper acquisition**
667 Although mb has a high affinity for copper, its importance in copper acquisition by
668 methanotrophs is still unclear. Non-native Cu^{1+}-mbs have been shown to be taken up by
669 methanotrophs and facilitate switchover to pMMO (133). Copper uptake and switchover is faster
670 if the native mb is used, but still takes more than 24 hours. However, the role of mb in meeting
671 the overall copper requirements of the cell is still in question. It has been found that *M.*
672 *trichosporium* OB3b possesses at least two mechanisms for copper uptake, the first one clearly
673 based on mb and involving active transport of copper-mb complexes and another, non-specific
674 passive transport pathway (192). It was also found that the amount of copper associated with
675 biomass was the same regardless of whether copper was added as $CuSO_4$ or as the Cu^{1+}-mb
676 complex at the same concentration. Subsequently, it was found for *M. trichosporium* OB3b that

677 when the gene for the precursor polypeptide of mb (*mbnA)* is knocked out, copper is still
678 associated with biomass, and increases with increasing amounts of copper in the growth medium
679 as does copper found associated with wild type cultures. The role of mb in copper uptake thus
680 remains unclear. It should be kept in mind that these studies were performed using well-defined
681 growth medium with limited diversity of copper speciation and little if any copper-containing
682 precipitates. It is tempting to speculate that mb may have a more significant role in copper
683 uptake *in situ*, where copper speciation and distribution will be much more complex, and will
684 include copper associated with a wide range of organic materials (e.g., humic and fulvic acids),
685 as well as found either sorbed onto or part of various mineral phrases. Indeed, it has been shown
686 that expression of *pmoA* is strongly dependent on the form of copper present, with copper
687 associated with metal oxides found to induce smaller changes in *pmoA* expression, and that
688 concomitant addition of mb increased the magnitude of the copper switch in *M. trichosporium*
689 OB3b (176, 193).
690
691 **Methanobactins: a role as signaling molecules?**
692 Given the small size of mbs, and their secretion into the environment when copper availability is
693 low, a role for mbs as signaling molecules may be envisaged. Indeed, addition of mb-OB3b
694 enhances expression of *mmoX* in in cultures of both wild type *M. trichosporium* OB3b and its
695 *mbnA*::Gm^R mutant (187) (139, 184), suggesting that mb may control gene expression by acting
696 as a signaling molecule. Further, given that all known forms of mb have significant structural
697 similarity, mbs may allow for cross-species communication. Recent studies show that this is
698 indeed the case. Mb-SB2 increased both *mmoX* expression and sMMO activity in *M.*
699 *trichosporium* OB3b in the presence of copper. However, it had no significant effect ($P > 0.05$)

on expression of either *pmoA* or *mbnA*, nor did it increase the amount of cell-associated copper (91). The addition of mb-SB2 preloaded with copper, however, reduced both *mmoX* and *mbnA* expression when *M. trichosporium* OB3b was grown in the absence of any added copper, and no sMMO activity was detected. These latter results were likely due to the increased amount of cell-associated copper, and may reflect the role of mb in copper uptake.

Membrane development

Several studies have demonstrated a direct correlation between intracytoplasmic membrane development and expression of pMMO in methanotrophs (33, 35, 66, 109, 114, 194). As stated above, *mbnA* plays a secondary role in the regulation of both the membrane bound and soluble MMO (see Regulation of Gene Expression). However, deletion of *mmoD* along with the rest of the sMMO operon, had little effect on intracytoplasmic membrane development in *M. trichosporium* OB3b (Fig. 9C and 9D). In contrast, a deletion mutation in the *mbnA* structural gene for mb, appears to effect intracytoplasmic membrane development well beyond its effects on pMMO expression (187) (Figs. 9E and 9F). Currently, the effect(s) of mb on intracytoplasmic membrane development is observational, and additional research is required to determine whether mb is directly involved in the development of these membranes in methanotrophs.

Detoxification of reactive oxygen species

Copper is one of the essential metals required by microorganisms for growth (179). Like iron, however, copper in oxygenated solutions can generate a variety of toxic reactive oxygen species via Fenton and Haber-Weiss reactions (195-197). The coordination of copper by mb is likely to

723 reduce this toxicity, as observed with the iron and copper-binding siderophore, schizokinen
724 (146). Nevertheless, both copper-bound and copper-free forms of mb-OB3b have been shown to
725 reduce dioxygen to superoxide in the presence of a reductant (198). In general, this would
726 represent a daunting problem for bacteria that accumulate high concentrations of Cu-mb.
727 Perhaps not unexpectedly, the superoxide dismutase (SOD) activity of Cu-mb is approximately
728 30,000 times higher than the oxidase activity associated with either mb-OB3b or Cu-mb-OB3b.
729 In addition, in the presence of a reductant, Cu-mb-OB3b also has a hydrogen peroxide reductase
730 (HPR) activity which is approximately 50 times the oxidase activity (198). The overall result of
731 these three activities is the reduction of O_2 to H_2O with concomitant loss of reductant. In
732 addition to their protective roles in the context of mb catalyzed oxidations, high SOD and HPR
733 activities of mb are also likely to provide protection against oxygen radicals that are potentially
734 formed during methane oxidation and respiration of dioxygen (196, 199, 200), and may thus
735 explain the stabilizing effects of mb-OB3b on pMMO activity in cell free extracts (66, 73, 198).
736 Similar activities have also been observed for mb-SB2 (Bandow et al., unpublished results), and
737 we predict this is a property of all mbs.
738
739 **MEDICAL, INDUSTRIAL AND ENVIRONMENTAL IMPLICATIONS**
740 **Metal mobilization or immobilization for remediation of polluted subsurface environments.**
741 Based on the published yields of mb in laboratory cultures (134, 136), methanotrophs are
742 predicted to export 3 – 50 copies of mb per cell per second depending on the copper
743 concentration during growth. Given this high export rate, and the fact that mb can bind most
744 transition and near transition metals, it is likely that mb is able to influence metal mobilization
745 and thus bioavailability of different metals in soil systems, and that this affects the structure and

746 functioning of microbial communities. Recent studies have shown that mb will bind both
747 inorganic and organic forms of mercury (154) and thereby protect the host bacterium as well as
748 other bacteria from mercury toxicity (184). However, additional studies are required to answer
749 which metals are affected in their mobility by mbs, and the effects of mbs on the soil microflora.
750
751 **Formation of gold nanoparticles**
752 The potential application of gold nanoparticles as anti-inflammatory agents, in the targeting of
753 cancer cells, detection of melamine, as biosensors, and in glucose oxidation has motivated the
754 development of various production methods for such materials (201-206). Most approaches
755 require either high (> 100°C) or low (-18°C) temperatures in the presence of reducing and
756 stabilizing agents. Alternatively, microbially-mediated production of gold nanoparticles, both
757 intracellularly and extracellularly, has also been extensively investigated (207). However, the
758 size distribution of gold nanoparticles produced by microbial processes is usually quite broad,
759 ranging in size from 10-6000 nm (208). Further, most reported processes yield particles with a
760 wide variety of shapes (spherical, octahedral, triangular, irregular, etc.), and rates of formation
761 vary over a large range, from minutes to hours (209). Crucially, both mb-OB3b and mb-SB2
762 have been shown to efficiently reduce Au^{3+} ions to metallic gold (typically >5 ions/mb), and in
763 so doing to form well-defined size distributions of spherical nanoparticles (135, 148). In a
764 further study with mb-SB2, gold spherical nanoparticles of well-defined sizes, 2.0 ± 0.7 nm,
765 were formed, with mb-SB2 suggested to act both as a reductant and as a stabilizing agent,
766 thereby demonstrating that use of mb-SB2 represents a viable approach to produce well-defined
767 gold nanoparticles in this size range (135).
768

Possible therapeutic treatment of human ailments

Copper has been implicated as a key factor in the development of a variety of human ailments, perhaps most notably Wilson's disease (210, 211), in which affected individuals have mutations that inactivate the Cu-transporting P-type ATPase ATP7B (212) impeding copper export to the bile, and causing copper accumulation in the liver. Based on *in vivo* and *in vitro* studies, copper accumulation specifically affects liver mitochondria, via copper-induced modification of membrane protein thiols leading to multiprotein cross-linking (213). In the final stages of Wilson's disease, oxidative damage, primarily in the liver, is observed. Other symptoms of Wilson's disease include serious neurological issues that can lead to additional psychiatric disorders (210, 214, 215).

There is as yet no cure for Wilson's disease. Currently, treatment consists of one or more of the following options: (1) the use of chelating agents to remove copper via enhanced urine excretion; (2) implementation of a low copper diet, and (3) zinc supplements added to the diet to stimulate production of the copper binding protein metallothionein (210, 214). Of these options, the use of chelating agents is the most commonly prescribed treatment, with two drugs, penicillamine and trientine, currently approved by the US Food and Drug Administration (FDA). Penicillamine, a breakdown product of penicillin, binds copper (216) and also induces the production of metallothionein. Although the clinical benefit of penicillamine is widely reported, it has serious side effects including bone marrow suppression, degeneration of elastic tissue, and proteinuria. In a large fraction of cases (20-50%), neurological deterioration is observed (217). In such cases, the polyamine trientine, which has fewer reported side effects compared to penicillamine (218), and a comparable if not higher affinity for copper (135), is substituted. In both penicillamine and trientine treatments, copper is excreted through the urine and not the bile.

Another chelating agent, tetrathiomolybdate, allows for copper to be excreted from liver primarily into the blood, but some evidence suggests that part of the copper may also be transferred to the bile (219), and that insoluble copper is found in both liver and kidneys at tetrathiomolybdate:copper ratios greater than two (219-221). It is unclear what the physiological impact of such an increased burden of copper in the blood, as well as the formation of insoluble copper.

Mbs may represent a superior alternative as a copper chelating agent to treat Wilson's disease, as indicated by preliminary studies. In the animal model for Wilson disease, the Long Evans Cinnamon (LEC) rat, intravenous application of mb-OB3b extracted copper from the liver into the bile as Cu-mb-OB3b, without measurable side-effects (213, 222). It was also found that mb-OB3b reversed both the buildup of copper in liver mitochondria and protein cross-linking, allowing the liver to function normally (213), and also stripped copper from metallothionein (222). Insoluble forms of copper were not observed. Lastly, and in contrast to other chelators used for the treatment of Wilson disease, mb-OB3b was shown to work in circumstances of advanced liver failure, further suggesting that mb may be a viable alternative in the treatment of Wilson's disease in the future. It is also tempting to speculate that the use of mbs may be of interest in other copper-associated pathologies as well, including certain forms of cancer (223-226). For example, it was recently reported that active oncogenesis of a wide variety of cancers is directly affected the presence of copper through the role of BRAF kinase (226), since disrupting kinase copper binding through site-directed mutagenesis or preventing copper uptake through the addition of tetrathiomolybdate inhibited tumorigenesis. In our view, these observations make it clear that mbs, first discovered as an elusive factor associated with the

814 exclusively bacterial process of methane utilization, may hold great promise as useful molecules
815 to humankind as well.
816
817 **CONCLUSIONS**
818 The combination of heterocyclic ring and an associated thioamide is a unique structural
819 feature of mbs among all natural products, and the primary cause of the capabilities observed for
820 these molecules. Although rare, oxazolone and imidazolone rings have been identified in several
821 ribosomally synthesized and post-translationally modified natural products, and this feature
822 appears to be responsible for the observed antimicrobial and metal binding properties of such
823 molecules. Pyrazinediones, in contrast, have so far only been observed in two non-ribosomally
824 synthesized natural products, seleriminol and flutimide, which show activity against several
825 cancer cell lines and antiviral activity, respectively. As for thioamide groups, they also confer
826 antimicrobial and metal binding properties in the two natural products identified so far that
827 contain this functional group. Crucially, combining both these structural features in small
828 peptide derivatives such as mbs appears to enhance properties including copper acquisition,
829 metal detoxification, detoxification of reactive oxygen species, electron transfer, nanoparticle
830 formation, and gene regulation. Production of high concentrations of mbs by a ubiquitous
831 microbial group also raises a number of ecological questions such as of its effects on metal
832 bioavailability and mobilization, as well as on the structure and functioning of microbial
833 communities in the environment. From an application viewpoint, mbs now have demonstrated
834 potential for the production of nanoparticles as well as in the treatment of copper-related
835 diseases, and we predict a variety of other commercial and medical applications have yet to be
836 identified.

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841 **Figure Legends**

842 Fig. 1. Chemical structures of full-length mbs from *M. trichosporium* OB3b (A) (141, 151),
843 *Methylocystis* strain sp.M (B)(133), *M. hirsuta* CSC1 (C)(133), *M. rosea* (D)(133) and
844 *Methylocystis* strain sp. SB2 (E)(132). Stared amino acids are observed in some but not
845 all samples.

846 Fig. 2. Core features of mbs. Abbreviations; AA, amino acid(s); AA – amine, amino acid minus
847 amine group. R-groups can be Arg, Ile, Met, or Pro.

848 Fig. 3. Structures of Cu^{1+}-mb-OB3b (A), and the Cu^{1+}-mbs from *Methylocystis* strain sp. M (B)
849 and *M. hirsuta* CSC1 (C), both with a Thr residue missing at the C-terminus compared to
850 the largest form isolated. The copper ions are represented as gray spheres, the oxazolone
851 (oxa) and pyrazinedione (pyra) rings are labeled as are the coordinating atoms.
852 Hydrogen-bonding interactions are shown in (B) and (C) as dashed orange lines.

853 Fig. 4. Mb precursor peptides. Sequences were detected in bacteria of known genome sequence,
854 from methanotrophs with structurally characterized mbs as well as from other
855 methanotrophs; and from selected non-methanotrophs, directly from the DNA sequence
856 using Fuzztran in Mobyle (http://mobyle.pasteur.fr), with the optimized mb sequence
857 motif (Prosite format) [ILMV]-[AIKST](1,3)-[IV]-[KNRT]-[IV]-X-[AKQ]-[KRT]-X-
858 [ILM]-X-[IV]-X-[GV]-R-X(2)-[AL]-X-C(1,2)-[GA](0,1)-[ST](0,2)-X(0,2)-C(1,2) as a
859 query. Only sequences also featuring downstream mb biosynthesis cassette genes *mbnB*
860 and *mbnC* (see text, and Table 2) are shown. Known and predicted leader sequences and
861 sequences not observed in the final product are shown in black, sequences detected in
862 bacteria of known genome sequence from methanotrophs with mbs whose primary
863 structures have been determined are shown in red, sequences detected in bacteria of

known genome sequence from methanotrophs are shown in blue, and sequences detected in bacteria of known genome sequence from non-methanotrophs are shown in green. Amino acids observed in some but not all samples are shown in tan. Grey background amino acids represent the amino acid pair shown or predicted to be post-translationally modified into an oxazolone, imidazolone or pyrazinedione group in structurally characterized mbs; underlined C represent Cys residues known or predicted to be present in the mature peptide. Abbreviations: mb from *M. trichosporium* OB3b (mb-OB3b), *Methylosinus* sp. strains LW3 (mb-LW3), LW4 (mb-LW4), PW1 (mb-PW1), *M. parvus* OBBP (mb-OBBP*), M. rosea* (mb-rosea), *Methylocystis* strains SB2 (mb-SB2), SC2 (mb-SC2), and LW5 (mb-LW5), *Cupriavidus basiliensis* B-8 (mb-B-8), *Pseudomonas extremaustralis* 14-3 (mb-14-3), *Azospirillum* sp. strain B510 (mb-B510), *Tistrella mobilis* KA081020-065 (mb-mobilis), *Comamonas composti* DSM 21721 (mb-21721) and *Gluconoacetobacter* sp. strain SXCC. Numbers in brackets after strain are used in species with more than one mb gene cluster. Stop codons are indicated by a star, and gaps in the sequence alignment by a hyphen.

Fig. 5. Mb gene clusters. Gene clusters of complete genomes of methanotrophs *M. trichosporium* OB3b (OB3b), *Methylocystis* sp. SB2 and *M. rosea* SV97 (SB2/SV97) that produce mb whose primary structure are known (Fig. 1). Shown are the gene for the mb peptide precursor mbnA (black) and associated genes for the mb biosynthesis cassette protein MbnB (red), mb biosynthesis cassette protein MbnC (orange), MATE efflux pump MbnM (dark brown), aminotransferase MbnN (yellow), di-heme cytochrome *c* peroxidase MbnH (dark green) and associated MbnP of unknown function (light green), FAD-dependent

887 oxidoreductase MbnF (violet), sulfotransferase MbnS (dark blue), TonB-dependent
888 receptor domain MbnT (light brown), FecI-like RNA polymerase sigma-70 domain MbnI
889 (light blue), and FecR-like membrane sensor MbnR (light purple). Other genes of unknown
890 function are shown in white, and genes flanking the clusters with predicted functions not
891 thought to be associated with mb maturation or transport are shown in grey.
892 Fig. 6. Proposed reaction schemes for biosynthesis of the oxazoline rings with associated
893 thioamide groups via a tandem two-step sequence of peroxidation and dehydration
894 reactions. Cysteine thiols are likely protected against oxidation, possibly as disulfides
895 involving one of the proteins of the mb gene cluster (blue circles). For imidazolone and
896 pyrazinedione ring formation, oxazolone rings are modified via a
897 transamination/deamination step followed by an aminolysis step to open the oxazolone
898 ring followed by ring formation and dehydration.
899 Fig. 7. Model for the regulation of gene expression in *mmo*, *pmo* and *mbn* gene clusters by
900 copper, mb and MmoD. (A) low copper : biomass ratio; (B) high copper : biomass ratio.
901 From Semrau et al. (187).
902 Fig. 8. Revised regulatory scheme for expression of *mmo*, *pmo* and *mbn* gene clusters as a
903 function of copper, mb and MbnI. (A) low copper : biomass ratio; (B) high copper :
904 biomass ratio.
905 Fig. 9. Transmission electron micrographs of wild type *M. trichosporium* OB3b (A and B), *M.*
906 *trichosporium* OB3b sMMO deletion mutant (C and D) and *M. trichosporium* OB3b mb
907 deletion mutant (E and F) cultured in nitrate mineral salts medium (A, C and D) and
908 NMS medium amended with 5 μM $CuSO_4$ (B, D, and F).

Cover Figure. Physiological functions and potential commercial and medical applications of methanobactin (center structure), a post translationally modified copper binding protein.

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Alan A. DiSpirito received his B.S. in biology from Providence College, M.S. and Ph.D. in microbiology from The Ohio State University with Olli H. Tuovinen and Patrick Dugan. His postdoctoral training was with Alan B. Hooper at The University of Minnesota and with Mary E. Lidstrom at The University of Washington-Seattle, Center for Great Lakes Studies and California Institute of Technology. He was an Assistant Professor at The University of Texas at Arlington before moving to the Department of Microbiology at Iowa State University. He is currently a Professor in the Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology at Iowa State University. His basic research interest is in the basic metabolism, environmental importance and commercial application of methanotrophic and chemoautotrophic bacteria. Current research projects include structural and functional characterization of methanobactins, mechanism of methanobactin biosynthesis and the influence of methanobactin on metal mobility in soil systems.

Jeremy D. Semrau, Arthur F. Thurnau Professor, has appointments in the College of Engineering and the College of Literature Science and Arts at the University of Michigan. His research primarily focuses on methanotrophs, with a particular emphasis on the biochemistry, genetics, and regulation of methane monooxygenase and methanobactin synthesis. As part of his research, Professor Semrau has also isolated and characterized novel facultative methanotrophs, developed applications of methanotrophy for use in bioremediation and biofuel production, as well as developed novel applications of methanobactin for the production of gold nanoparticles and the treatment of Wilson's disease. He holds several patents in these areas as well as has published many papers, abstracts, and book chapters on fundamental and applied aspects of

methanotrophy. Professor Semrau is currently on the Editorial Board of Applied and Environmental Microbiology.

Colin Murrell is Professor in Microbiology in the School of Environmental Sciences and Director of the Earth and Life Systems Alliance (ELSA) at the University of East Anglia, UK. He has wide ranging research interests centered round the bacterial metabolism of methane and other one carbon compounds such as methanol, methylamines, methanesulfonate, dimethyl sulfide, methyl halides and also isoprene in the terrestrial, aquatic and marine environment. Other areas of research include the microbiology of the rhizosphere, sea-surface microlayer, caves, alkaline soda lakes, saltmarshes, cold water corals and cultural heritage microbiology, regulation of gene expression by metals, microbial genomics, metagenomics, bioremediation, biocatalysis and industrial biotechnology. His research over the past 35 years has resulted in around 280 publications and six edited books. Murrell is member of the Editorial Boards of Environmental Microbiology, The ISME Journal and FEMS Microbiology Letters, and was the Chair of the 2015 Gordon Research Conference on Applied and Environmental Microbiology. Murrell is Vice President of The International Society for Microbial Ecology and was recently elected Member of the European Molecular Biology Organisation and Member of the European Academy of Microbiology.

Warren H. Gallagher was born in Providence, Rhode Island in 1952 and received his A.B. degree in chemistry and biology from Albion College, Albion, Michigan in 1974. He went on to receive his Ph.D. in biophysics from the University of Pittsburgh in 1980 under the direction of Prof. Max Lauffer. He then held a couple of postdoctoral positions with Prof. Victor Bloomfield

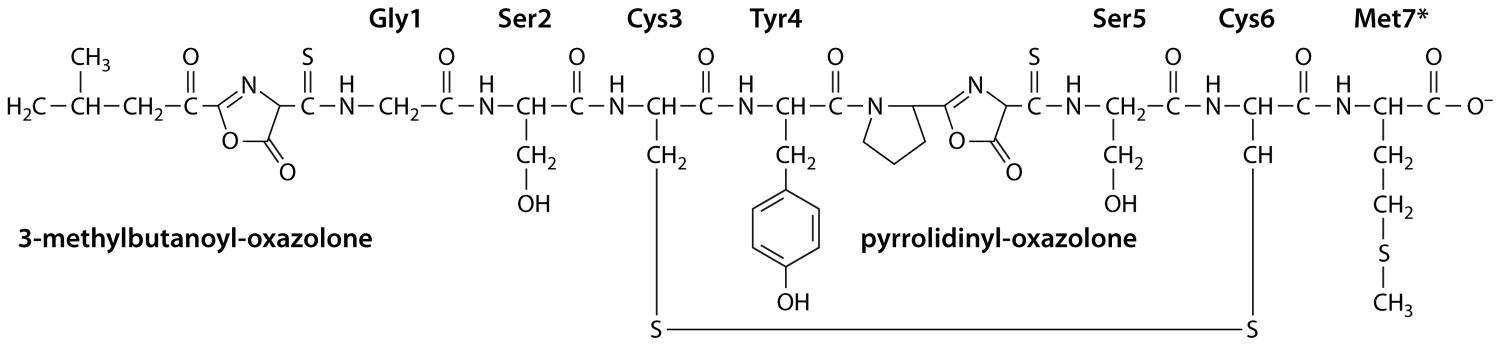
and Prof. Clare Woodward at the University of Minnesota, Twin Cities, before joining the chemistry faculty at the University of Wisconsin-Eau Claire, where his current title is Professor and Chair of Chemistry. He has a long term interest in using spectroscopic methods to determine the structures of peptide-derived molecules and over the past several years the methanobactins have presented him and his students with some exciting challenges that demanded detective-like solutions.

Chris Dennison received his PhD in Inorganic Chemistry (1994) from Newcastle University with Prof. A. G. Sykes FRS and Prof. W. McFarlane and then worked as a postdoctoral fellow with Prof. G. W. Canters at Leiden University. He was appointed as a Lecturer at University College Dublin in 1997, returning to Newcastle University in 1999 as a Lecturer in Inorganic Chemistry in the Department of Chemistry. In 2004 he became a Senior Lecturer in the Institute for Cell and Molecular Biosciences (Medical School) and Professor of Biological Chemistry in 2010. He studies the role of metals, and particularly copper, in biological systems. This has included the analysis of electron-transfer proteins and multi-copper redox enzymes. More recently work has focused on copper homeostasis in a wide range of organisms, including understanding how methane-oxidising bacteria acquire, handle and store copper.

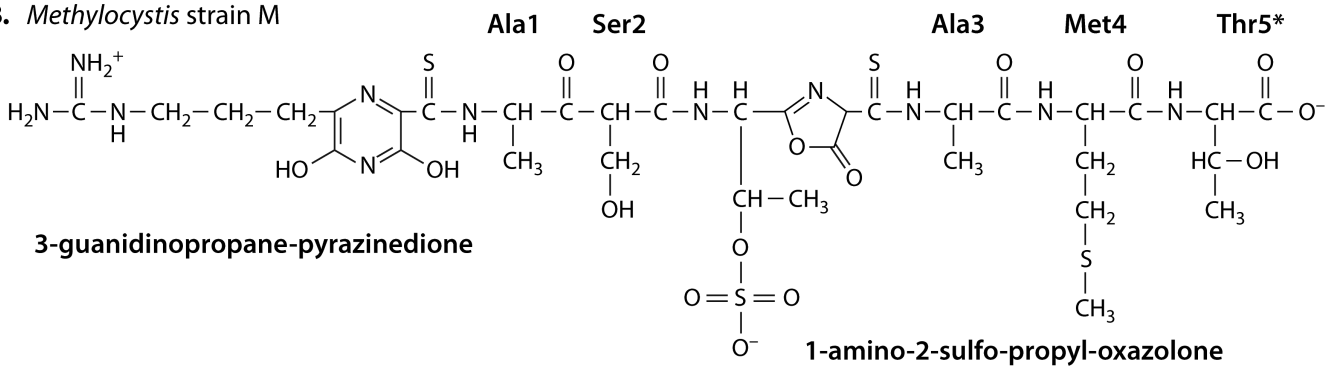
Stéphane Vuilleumier studied natural sciences at ETH Zurich, Switzerland, and obtained a PhD in organic chemistry at University of Basel, Switzerland. After a postdoc in protein engineering at University of Cambridge, UK, he returned to ETH Zurich, where he became a group leader at the Institute of Microbiology and obtained his habilitation. He is Professor of Environmental Biology and Microbiology in Strasbourg, France, since 2002, heads a CNRS research team, and

shares responsibility for the Chemistry and Biology Master at Université de Strasbourg. In 2005, his interest in bacterial metabolism of halogenated methanes has led him to become involved in international collaborative genome sequencing projects of methylotrophic and methanotrophic bacteria at JGI (USA) and Genoscope (France), and in their subsequent analysis using the MicroScope platform at Genoscope. Stéphane Vuilleumier is elected Chair of the 2016 Gordon Research Conference "Molecular Basis of Microbial One-Carbon Metabolism".

A. *Methylosinus trichosporium* OB3b



B. *Methylocystis* strain M



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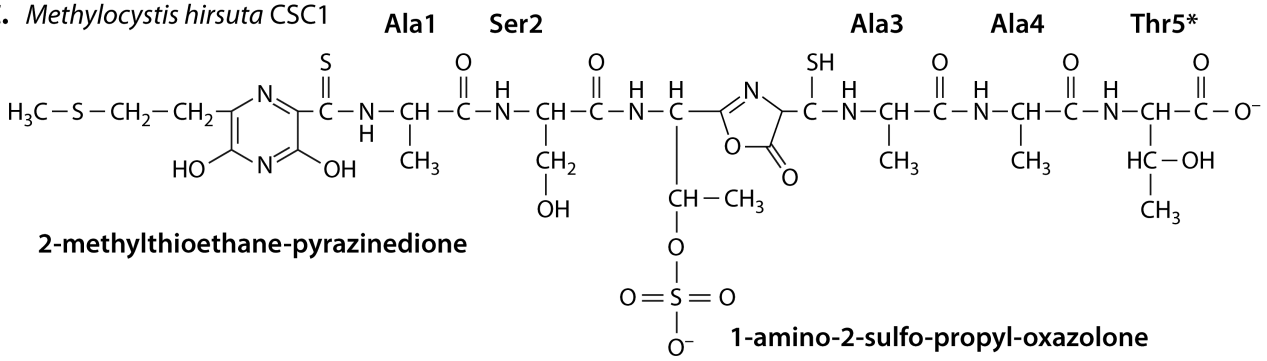

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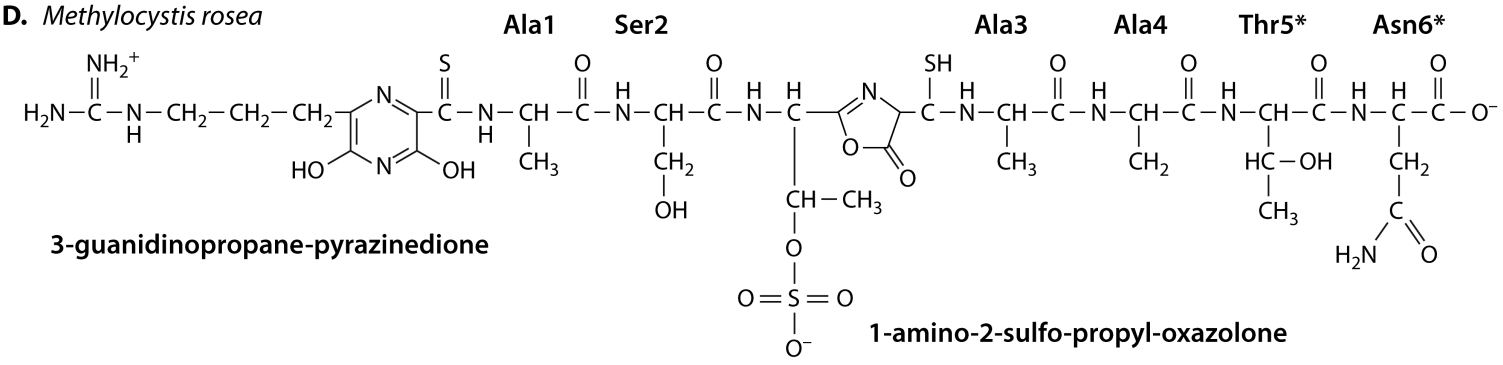

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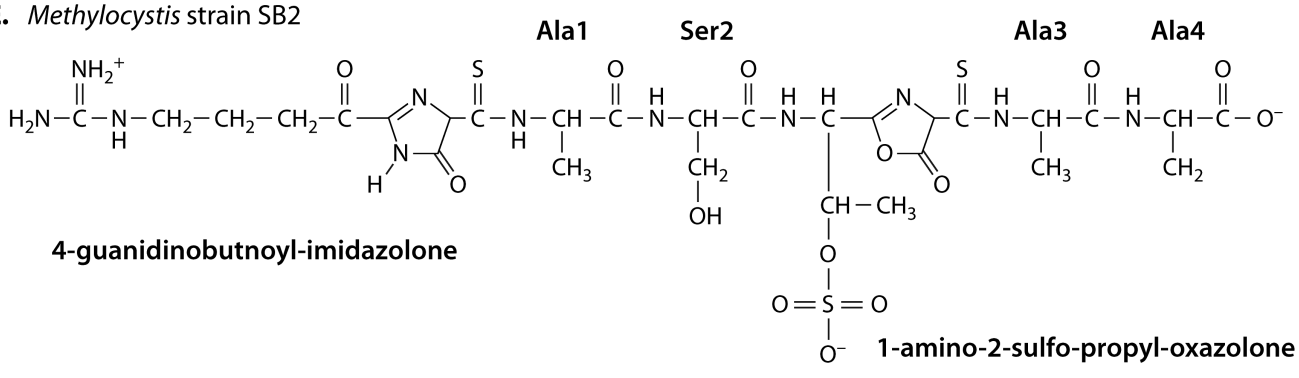
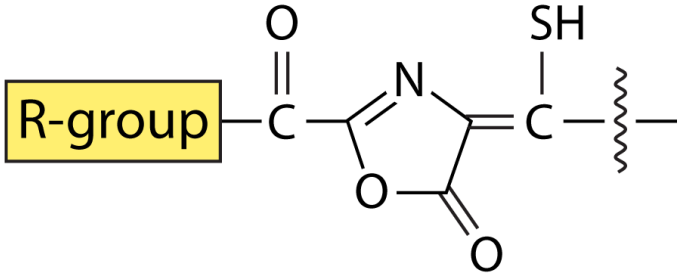
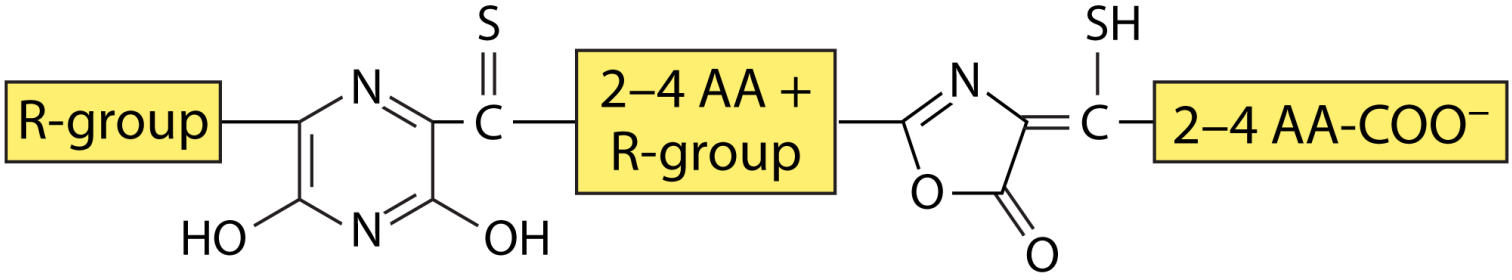
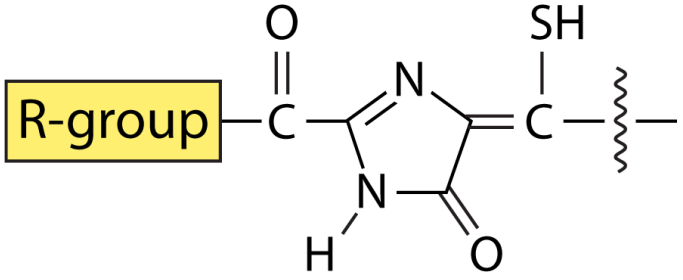




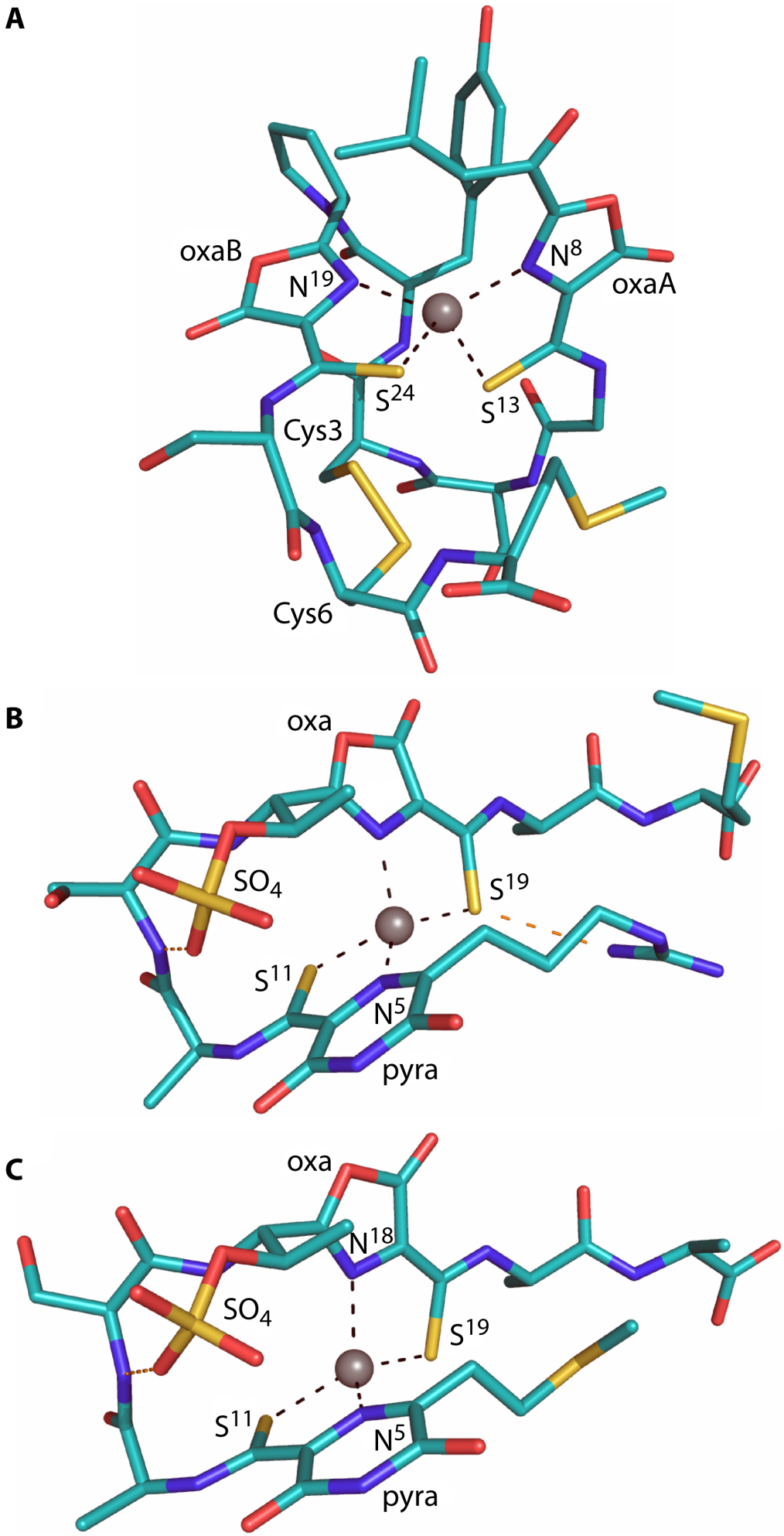
Oxazolone

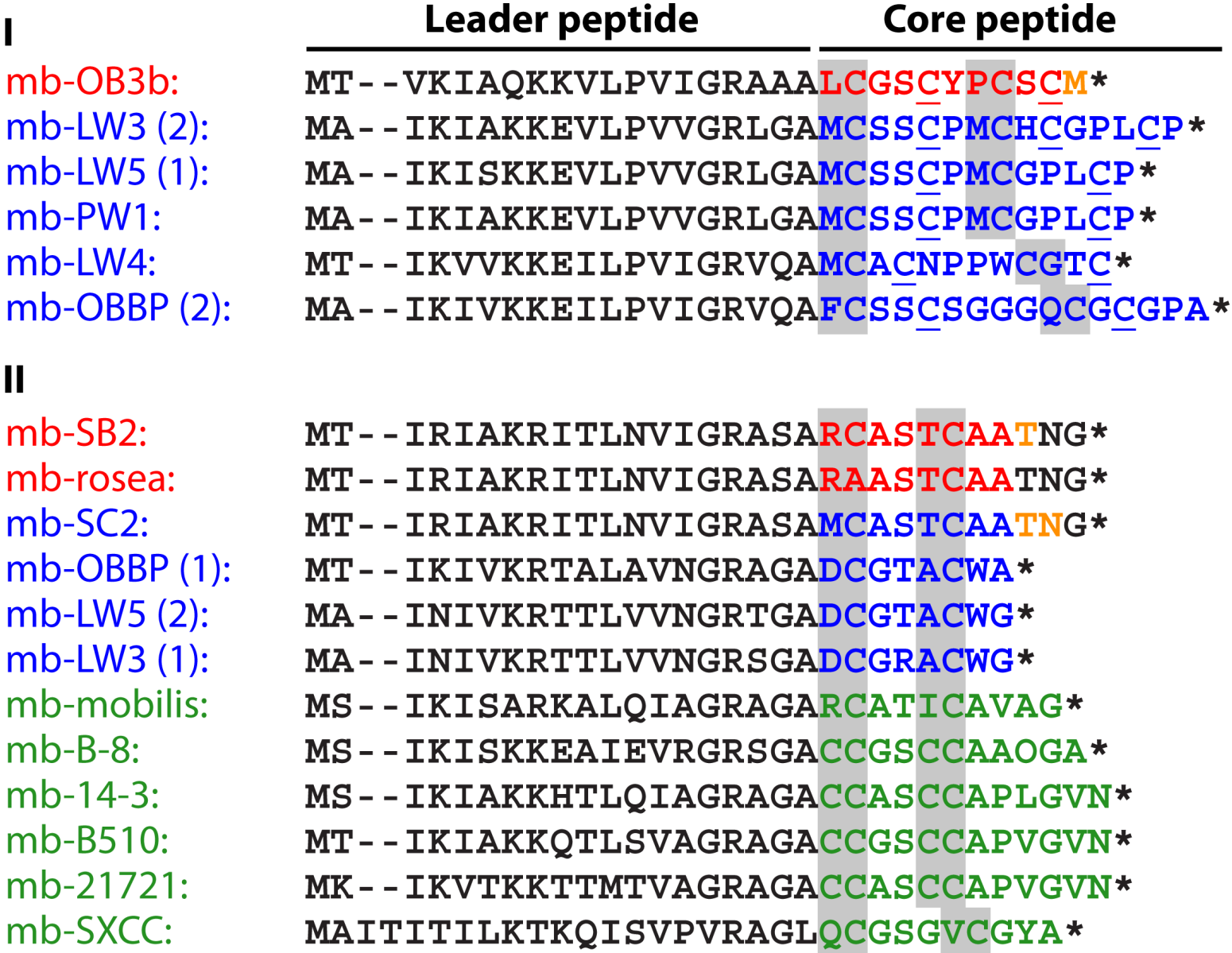


Pyrazinedione

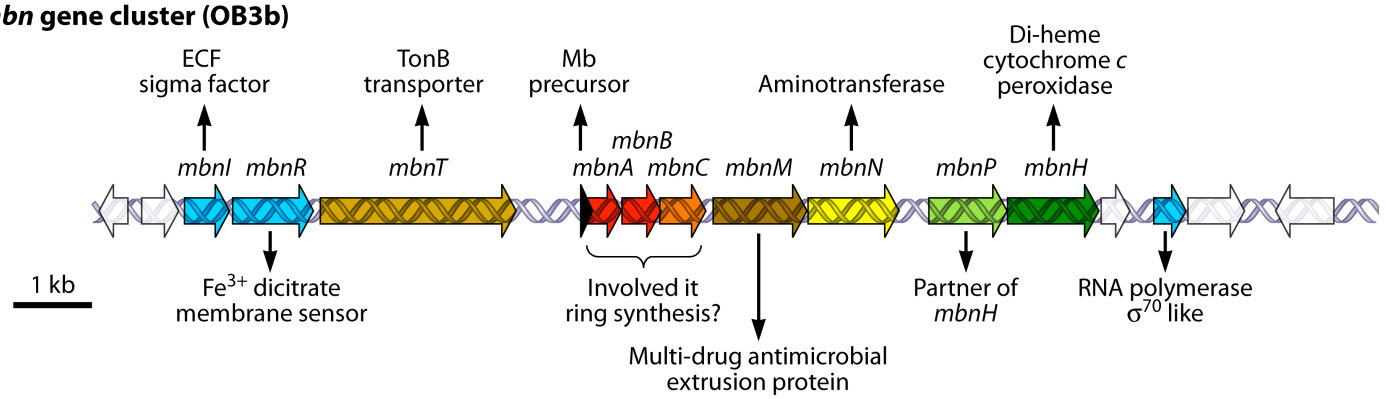


Imidazolone

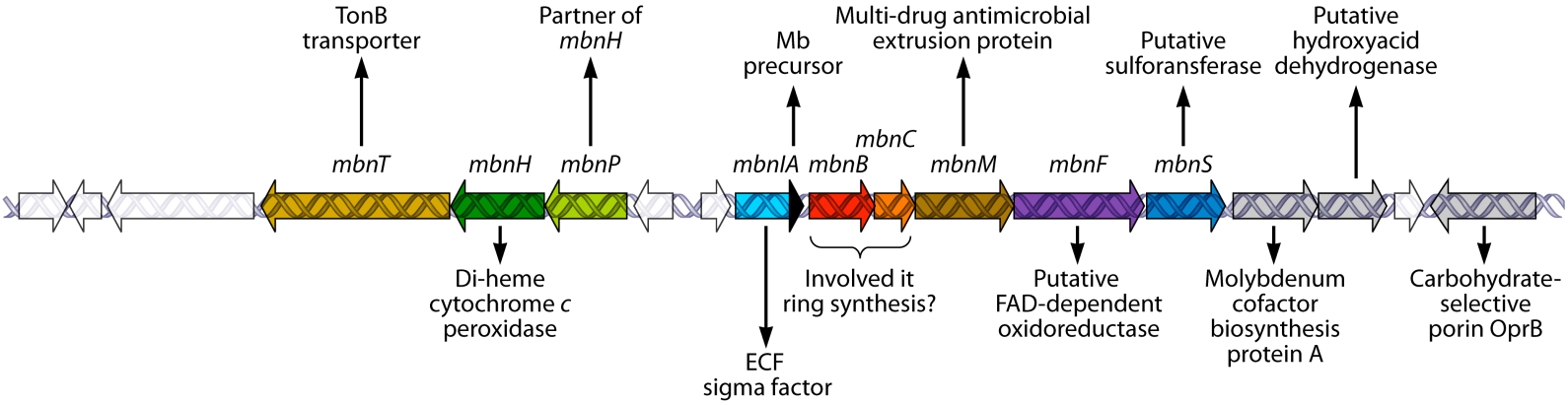

A
oxaB
N19
N8
oxaA
S24
S13
Cys3
Cys6
B
oxa
SO4
S19
S11
N5
pyra
C
oxa
N18
SO4
S19
S11
N5
pyra

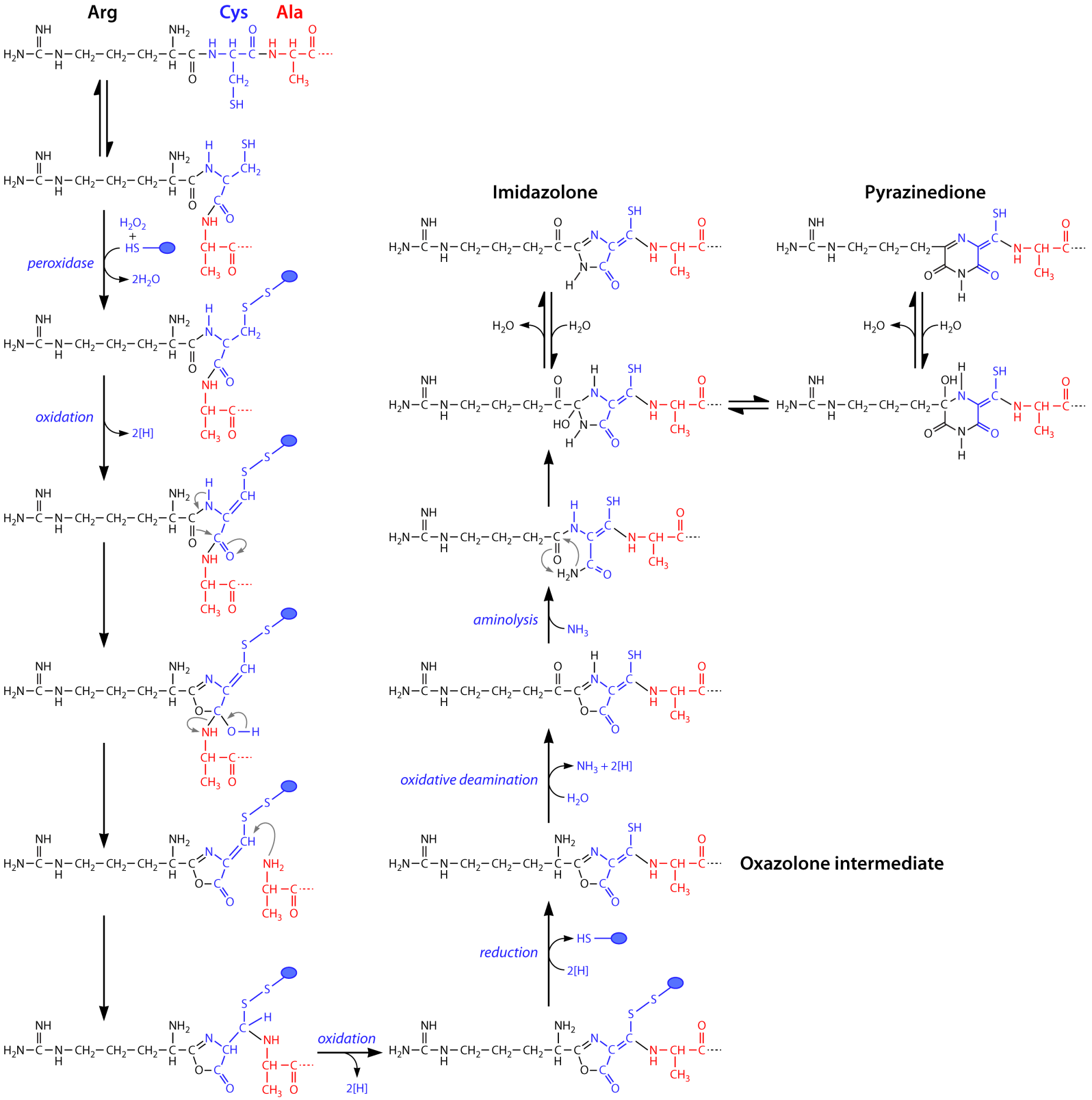

I
Leader peptide
Core peptide
mb-OB3b: MT--VKIAQKKVLPVIGRAAALCGSCYPCSCM*
mb-LW3 (2): MA--IKIAKKEVLPVVGRLGAMCSSCPMCHCGPLCP*
mb-LW5 (1): MA--IKISKKEVLPVVGRLGAMCSSCPMCGPLCP*
mb-PW1: MA--IKIAKKEVLPVVGRLGAMCSSCPMCGPLCP*
mb-LW4: MT--IKVVKKEILPVIGRVQAMCACNPPWCGTC*
mb-OBBP (2): MA--IKIVKKEILPVIGRVQAFCSSCSGGGQCGCGPA*
II
mb-SB2: MT--IRIAKRITLNVIGRASARCASTCAATNG*
mb-rosea: MT--IRIAKRITLNVIGRASARAASTCAATNG*
mb-SC2: MT--IRIAKRITLNVIGRASAMCASTCAATNG*
mb-OBBP (1): MT--IKIVKRTALAVNGRAGADCGTACWA*
mb-LW5 (2): MA--INIVKRTTLVVNGRTGADCGTACWG*
mb-LW3 (1): MA--INIVKRTTLVVNGRSGADCGRACWG*
mb-mobilis: MS--IKISARKALQIAGRAGARCATICAVAG*
mb-B-8: MS--IKISKKEAIEVRGRSGACCGSCCAAOGA*
mb-14-3: MS--IKIAKKHTLQIAGRAGACCASCCAPLGVN*
mb-B510: MT--IKIAKKQTLSVAGRAGACCGSCCAPVGVN*
mb-21721: MK--IKVTKKTTMTVAGRAGACCASCCAPVGVN*
mb-SXCC: MAITITILKTKQISVPVRAGLQCGSGVCGYA*

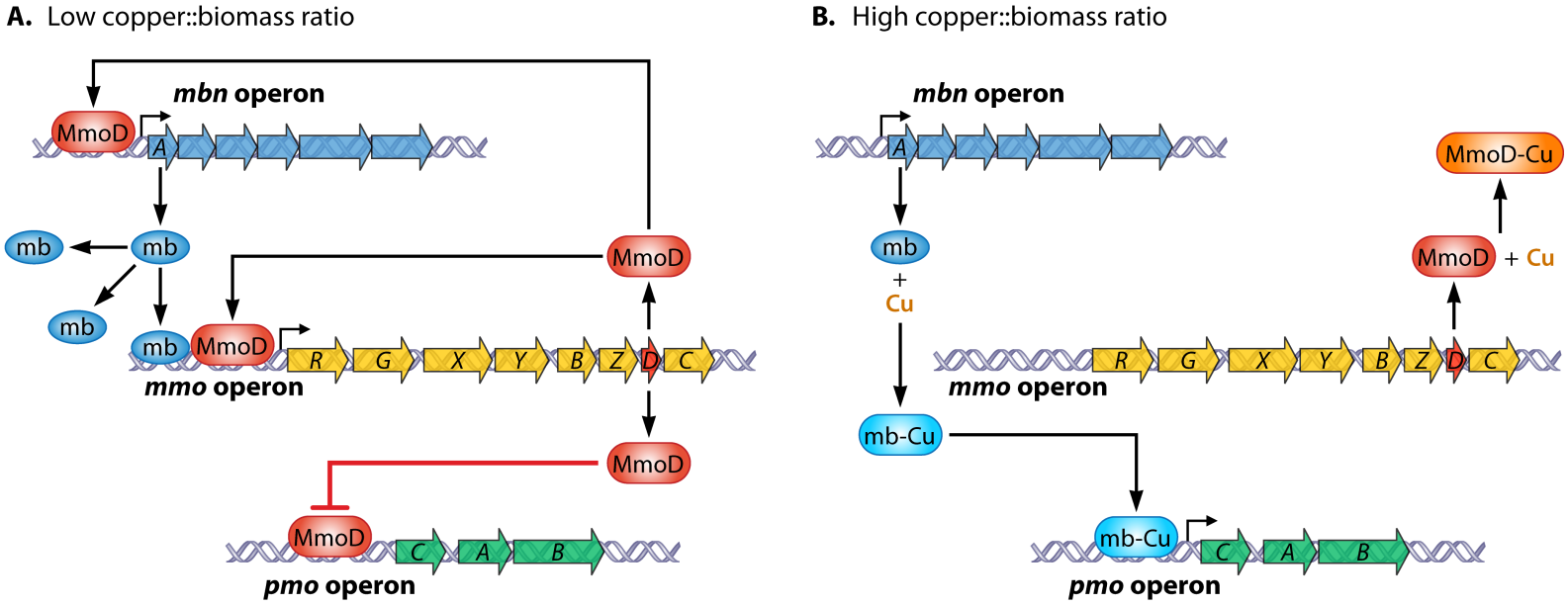
mbn gene cluster (OB3b)

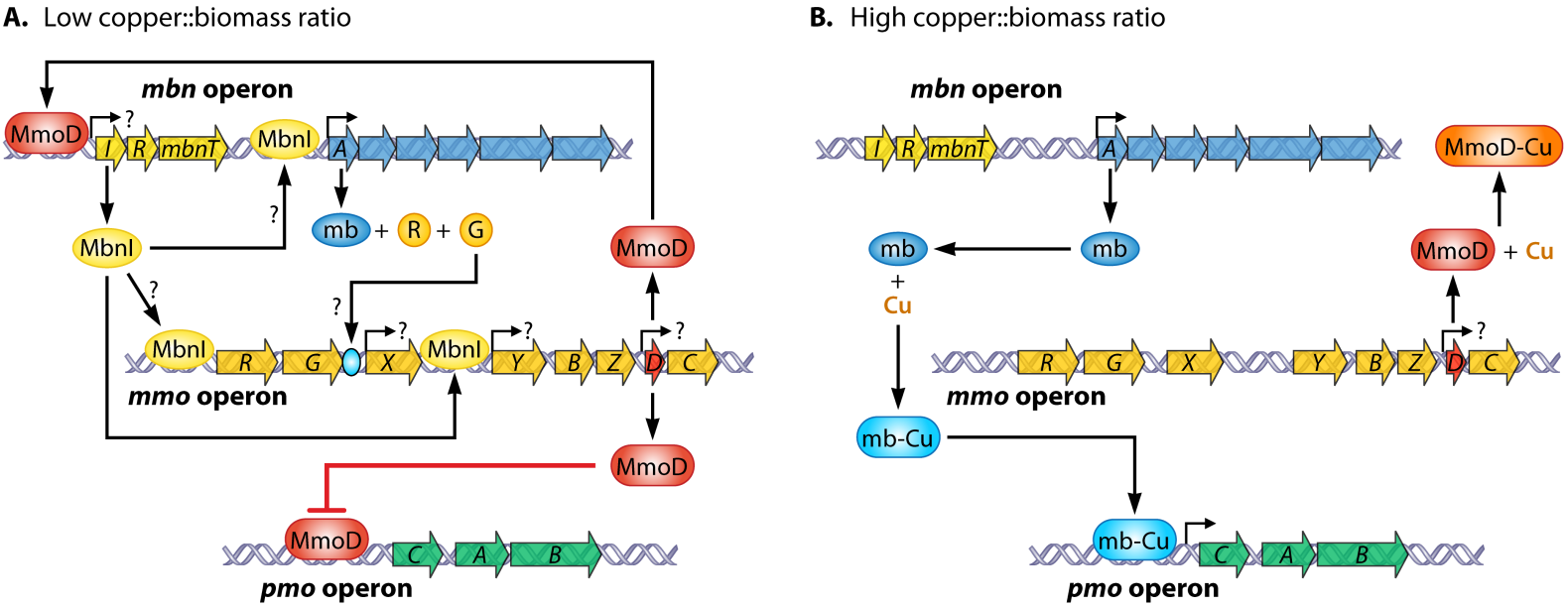


mbn gene cluster (SB2/SV97)




Arg
Cys
Ala
peroxidase
H_2O_2
+
HS
$2H_2O$
oxidation
2[H]
oxidation
2[H]
reduction
HS
2[H]
oxidative deamination
$NH_3 + 2[H]$
H_2O
aminolysis
NH_3
Imidazolone
Pyrazinedione
Oxazolone intermediate


A. Low copper::biomass ratio
mbn operon
MmoD
A
mb
mb
mb
mb
MmoD
MmoD
R
G
X
Y
B
Z
D
C
mmo operon
MmoD
MmoD
C
A
B
pmo operon
B. High copper::biomass ratio
mbn operon
A
MmoD-Cu
mb
+
Cu
MmoD
+ Cu
R
G
X
Y
B
Z
D
C
mmo operon
mb-Cu
mb-Cu
C
A
B
pmo operon


A. Low copper::biomass ratio
mbn operon
MmoD
I
R
mbnT
MbnI
A
mb + R + G
MbnI
MmoD
MbnI
R
G
X
MbnI
Y
B
Z
D
C
mmo operon
MmoD
MmoD
C
A
B
pmo operon
B. High copper::biomass ratio
mbn operon
I
R
mbnT
A
MmoD-Cu
mb
mb
MmoD + Cu
+
Cu
R
G
X
Y
B
Z
D
C
mmo operon
mb-Cu
mb-Cu
C
A
B
pmo operon

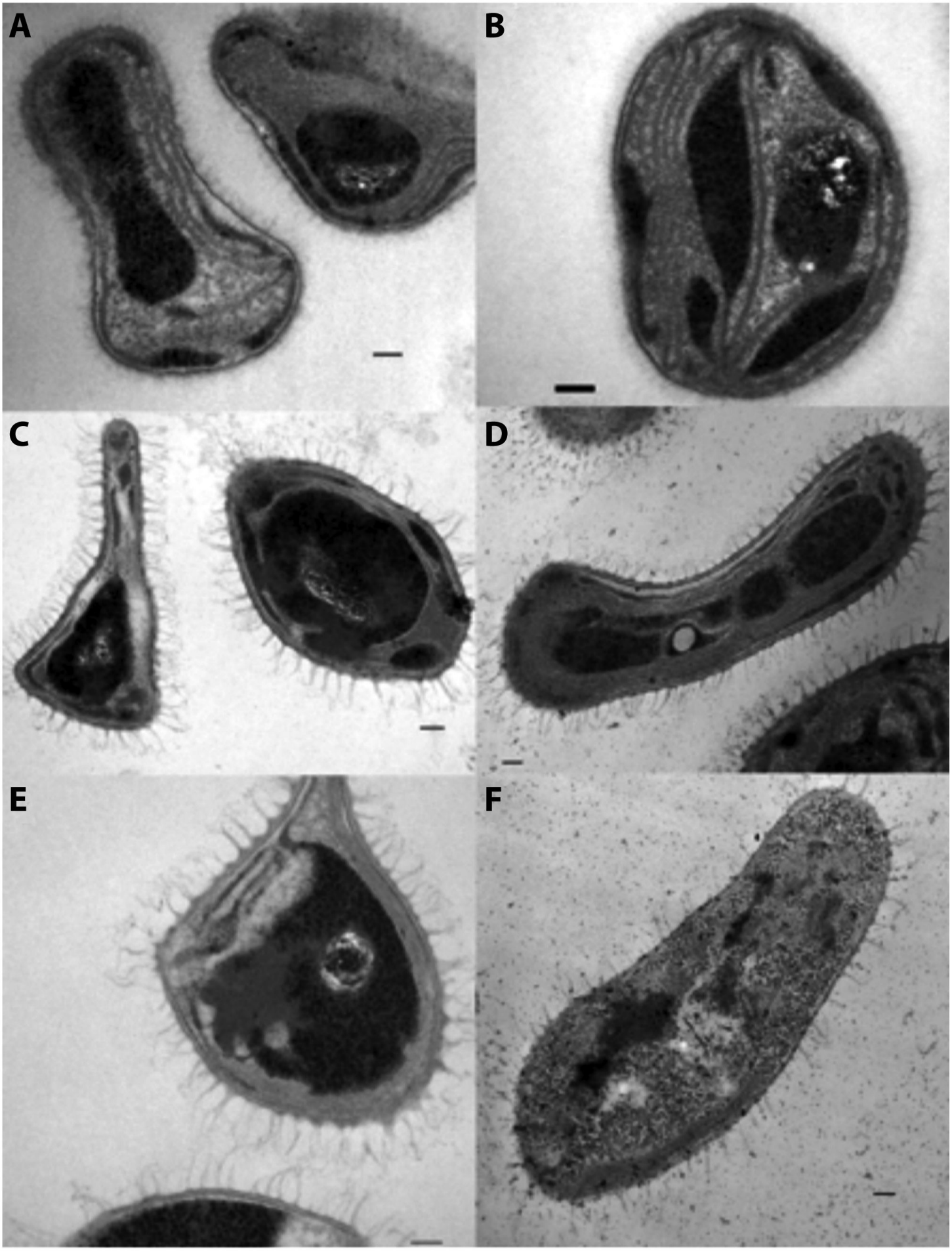

A
B
C
D
E
F

Table 1. Metal binding affinities, *K*, of mbs from *M. trichosporium* OB3b (mb-OB3b), *Methylocystis* strain M (mb-M), *Methylocystis hirsuta* CSC1 (mb-CSC1), *Methylocystis* rosea (mb-rosea), and *Methylocystis* strain SB2 (mb-SB2). Abbreviations, ITC, isothermal titration calorimetry; PT, potentiometric titration; DITC, displacement isothermal titration calorimetry; mb, methanobactin; mb_2, mb dimer; mb_4, mb tetramer; UTF, unable to fit; BCS, bathocuproine disulfonate; TRIEN, triethylenetetramine.

				K **(M^{-1})**						
mb	**Method**	**pH**	**Reference**	**Cu^{1+}**	**Cu^{+2}**	**Cu^{2+}/Cu^{1+}** [a]	**Au^{3+}**	**Ag^{1+}**	**Hg^{2+}**	**Pb^{2+}**
mb_4-OB3b	ITC	6.8	140, 148	-	-	3.2×10^{34}	1.0×10^{40}	-	UTF	-
mb_2-OB3b		6.8		-	-	2.6×10^{8}	1.0×10^{5}	2.6×10^{7}	9.9×10^{9}	-
mb-OB3b		6.8		-	-	6.5×10^{6}	1.8×10^{5}	4.7×10^{4}	9.0×10^{5}	6.8×10^{5}
mb_4-OB3b	PT	6.8	172	-	-	1.0×10^{58}	-	-	-	-
mb_2-OB3b		6.8		-	-	1.0×10^{52}	-	-	-	-
mb-OB3b		6.8		-	-	1.0×10^{25}	-	-	-	-
mb-OB3b	Competition titrations with BCS	7.5	141	$\sim 7 \times 10^{20}$		-				
mb-OB3b		7.5		-	$\sim 3 \times 10^{12}$ [b]		-	-	-	-
mb-M	Competition against mb-OB3b	7.5	133	$\sim 1 \times 10^{21}$			-	-	-	-
mb-M		7.5			$\sim 2 \times 10^{11}$ [b]	-	-	-	-	-
mb-CSC1	Competition titrations with BCS	7.5	133	$\sim 7 \times 10^{20}$			-	-	-	-
mb-CSC1		7.5			$\sim 5 \times 10^{14}$ [b]	-	-	-	-	-
mb-rosea [c]	Competition against mb-OB3b	7.5	133	$\sim 5 \times 10^{20}$	-		-	-	-	-

mb-rosea		7.5			$\sim 7 \times 10^{11}$ [b]	-	-	-	-	-
mb_4-SB2	DITC	6.9	154	-	-	7.6×10^{26}	-	-	-	-
mb_2.SB2	DITC	6.6		-	-	1.2×10^{9}	-	-	-	-
mb-SB2	DITC	6.9		-	-	1.0×10^{6}	-	-	-	-
mb-SB2	Competition titrations with TRIEN	6.8		-	-	1.9×10^{27}				
mb-SB2	ITC	6.8		-	-	-	-	1.2×10^{8}	-	-

				K **(M^{-1})**					
mb	**Method**	**pH**	**Reference**	**Cd^{2+}**	**Co^{2+}**	**Fe^{3+}**	**Mn^{2+}**	**Ni^{2+}**	**Zn^{2+}**
mb_4-OB3b	ITC	6.8	148	1.3×10^{6}	-	9.7×10^{5}	-	-	4.5×10^{6}
mb_2.OB3b		6.8		1.1×10^{7}	1.1×10^{6}	1.7×10^{5}	7.7×10^{5}	4.9×10^{5}	1.8×10^{4}

[a] Copper added as $Cu^{2+}$and therefore the oxidation state of the copper bound by mb is in question due to its ability to reduce Cu^{2+} to Cu^{1+}.

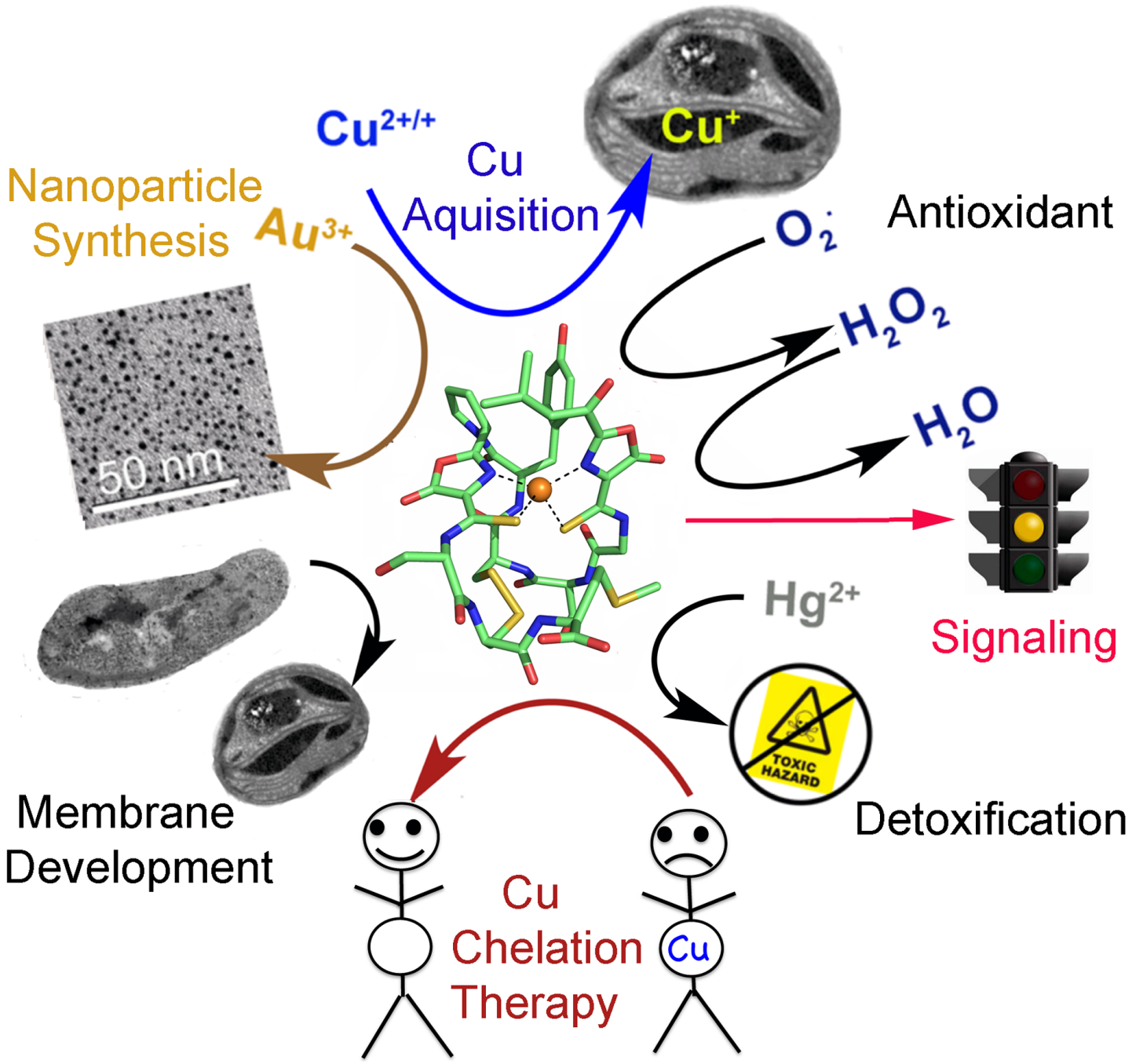
[b] Calculated using the reduction potential of the Cu^{2+}-mb/Cu^{1+}-mb couple.

[c] For the form of mb-rosea missing the C-terminal Asn and Thr residues.

Table 2 Genes in methanobactin gene cluster.

Gene	Proposed Annotation	Characteristics/Comments	Interproscan ID
mbnA	Mb peptide precursor	short ORF with N-terminal leader peptide	N/A
mbnB	mb biosynthesis cassette protein MbnB	TIM barrel enzyme (aldolase/isomerase type)	IPR026432, IPR0078801
mbnC	mb biosynthesis cassette protein MbnC	Less well defined than MbnB	IPR023973
mbnE	FAD-dependent oxidoreductase	often also found when *mbnH* and *mbnP* are missing	IPR003042
mbnH	di-heme cytochrome *c* peroxidase		IPR023929, IPR004852
mbnI	RNA polymerase siga-70 domain	Putative σ^{70} factor. In some cases (e.g. strains SB2, SC2, SV97), methanobactin precursor peptide is the C-terminal peptide of MbnI	
mbnM	MATE efflux pump	multi antimicrobial extrusion protein	IPR004839, IPR005814
mbnN	aminotransferase	rarely found (e.g. strain OB3b, LW4), pyridoxal-phosphate dependent	IPR023977
mbnP	partner of *mbnH*	conserved protein of unknown function	
mbnR		FecR-like, putative sigma factor activator	IPR000863

mbnS	sulfotransferase	rarely found (e. g. strain SB2, SC2, SV97); sulfonation of Thr?	IPR012373
mbmT	TonB-dependent receptor	plug domain, large membrane protein family	IPR000531


Cu2+/+
Cu
Aquisition
Cu+
Nanoparticle
Synthesis
Au3+
50 nm
O2
Antioxidant
H2O2
H2O
Signaling
Hg2+
TOXIC
HAZARD
Detoxification
Membrane
Development
Cu
Chelation
Therapy
Cu