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Chalkophores

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Abstract

Copper-binding metallophores, or chalkophores, play a role in microbial copper homeostasis that is analogous to that of siderophores in iron homeostasis. The best-studied chalkophores are members of the methanobactin (Mbn) family—ribosomally produced, posttranslationally modified natural products first identified as copper chelators responsible for copper uptake in methane-oxidizing bacteria. To date, Mbns have been characterized exclusively in those species, but there is genomic evidence for their production in a much wider range of bacteria. This review addresses the current state of knowledge regarding the function, biosynthesis, transport, and regulation of Mbns. While the roles of several proteins in these processes are supported by substantial genetic and biochemical evidence, key aspects of Mbn manufacture, handling, and regulation remain unclear. In addition, other natural products that have been proposed to mediate copper uptake as well as metallophores that have biologically relevant roles involving copper binding, but not copper uptake, are discussed.

Keywords

copper homeostasis; natural product; metallophore; chalkophore; methanobactin; siderophore; bioinorganic chemistry

INTRODUCTION

Metals play key roles in many biological processes. It has been estimated that up to half of all proteins contain a metal (1). While metals are purely structural elements in some proteins, the redox capabilities of transition metals are required for electron transport (2), and redox-active metals play a catalytic role in many families of enzymes (1). Metal deficiency is correspondingly a significant stressor that limits viability. However, the oxidative stress caused by surplus transition metals can also be fatal to a cell (3). All forms of life thus possess intricate metal homeostasis systems that govern both influx and efflux of metals, allowing the cell to maintain a carefully regulated pool of bioavailable metal in the correct oxidation state(s).

DISCLOSURE STATEMENT

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Efflux of transition metals has been extensively studied across all forms of life, but metal influx has received less attention. Iron acquisition in prokaryotes is the best-understood example. Despite the many biological processes that depend on iron, its low solubility under aerobic conditions and at neutral pH significantly limits its bioavailability (4). As a result, strategies have evolved to facilitate active iron uptake. The canonical microbial strategy is the use of siderophores, small iron-binding natural products that are secreted from cells and that bind extracellular iron with high affinity (5). The iron-bound siderophores are then taken back up into the cell, where the iron is liberated from the compound and incorporated into the cellular iron pool (6). These systems are particularly common in pathogenic bacteria, which compete with host cells for iron, and genomic cassettes related to siderophore biosynthesis and transport can be markers of virulence (7, 8). Similar strategies exist in fungi (9) and plants (10), but the focus of this review is bacterial metal acquisition.

Over the past 15 years, it has become increasingly apparent that this strategy is not limited to iron (11, 12). In species with a high need for other metals or in situations of metal deficiency, including host sequestration of metals from pathogenic bacteria, metallophores (biogenic ligands that facilitate metal ion uptake) can be deployed (13). Some metallophores also have secondary roles related to defense against metal toxicity. Although better known as siderophores, rhizoferrin and some desferrioxamines and pyoverdines have higher affinities for manganese than they do for iron (14–16). The broad-spectrum metallophore staphylopine binds both nickel and cobalt, and other compounds, including L-His_2, L-His/2-methyl-2,4-thiazolidinedicarboxylic acid, and yet-uncharacterized metallophores, are implicated in nickel uptake as well (17–19). Staphylopine can also bind zinc, as can yersiniabactin (in a biologically relevant role), and other putative zincophores such as coelibactin have been identified (17, 20, 21). Gold toxicity can be minimized by the chelation of the metal by delftibactin (22). Even molybdenum and vanadium have possible metallophores, in the form of azotobactin, aminochelin, and protochelin (23, 24). However, the best-studied family of noniron metallophores are copper-binding natural products known as chalkophores (*chalko-* is derived from the Greek word for copper), and chief among them are the methanobactins (Mbns) (25).

Mbns are peptidic natural products that chelate copper via paired nitrogen-containing heterocycles and thioamide/enethiol moieties (26) (Figure 1*a*). They have an exceedingly high affinity for copper and bind copper from soluble or mineral sources upon secretion (27). The copper-chelated Mbn (CuMbn) is then internalized as a source of copper for cells that have a high requirement for this metal (28). Significant inroads have been made into our understanding of Mbn transport, regulation, and biosynthesis, rendering Mbns the largest and best-understood group of chalkophores. Although Mbns are the major focus of this review, other natural products with proposed roles in copper uptake and metallophores with secondary roles involving copper are addressed as well.

METHANOBACTINS

Copper Homeostasis in Methanotrophs

Research into prokaryotic copper homeostasis has traditionally focused on copper efflux (29). While copper enzymes play important roles across all families of life, they are

comparatively few in number in prokaryotes (30), and Fenton chemistry mediated by unbound copper causes significant oxidative stress, rendering copper toxicity a significant risk (29). However, in species in which key metabolic enzymes require copper cofactors, copper deficiency is a major problem that can be addressed by the use of chalkophores. Methanotrophic bacteria, which oxidize methane as their sole carbon source, are a widespread group of bacteria with a high demand for copper (31), and Mbn was first identified in these bacteria.

In methanotrophic bacteria, two entirely unrelated families of metalloenzymes can carry out the aerobic oxidation of methane to methanol: a cytoplasmic iron enzyme, soluble methane monooxygenase (sMMO) (32), and a copper enzyme, particulate methane monooxygenase (pMMO) (33), which is found in intracytoplasmic membranes with unclear connectivity to periplasmic membranes (34) (Figure 2). Some methanotrophs possess both enzymes, and in these species, the two enzymes are reciprocally regulated by copper, with pMMO produced whenever sufficient copper is available (31, 35). pMMO is produced in massive quantities, composing nearly a fifth of the cellular protein mass (36). Since pMMO is an ~300-kDa trimeric enzyme with at least three copper sites (33), the resulting demand for copper far outstrips that of many other bacteria, and conditions that would provide more than enough copper for well-studied gram-negative *Pseudomonas* and *Escherichia* species constitute copper stress for methanotrophs. It is thus unsurprising that some of the best-understood copper acquisition (rather than copper efflux) systems have been identified in these bacteria.

The Identification of Methanobactins in Methanotrophs

Although the importance of copper to methanotrophy had been recognized a decade earlier, the first evidence for a dedicated copper uptake system in the form of Mbns came from a set of mutant strains constructed from the methanotroph *Methylosinus (Ms.) trichosporium* OB3b. A set of constitutively sMMO-producing strains was developed (37), and during the extensive screening of these strains, initial evidence was obtained for the existence of small extracellular copper-binding compounds (38). Within the next five years, two groups had attempted to characterize the compound, variously described as the CBL (copper-binding ligand) and the CBC (copper-binding compound), and though the compound was not fully purified or structurally characterized, it was correctly described as having a partly peptidic nature (39, 40).

Structural and Biophysical Characteristics of Methanobactin from *Methylosinus trichosporium* OB3b

Structural characterization of Mbn from *Ms. trichosporium* OB3b was not achieved until 2004, when that compound was successfully crystallized (26) and the term chalkophore was first coined. In this structure, a single copper ion is coordinated in a distorted tetrahedral geometry by two nitrogen-containing heterocycles and two neighboring thioamide or enethiol groups (26) (Figure 1*a*). Initially assigned as imidazolone rings, later NMR analysis showed that the heterocycles in the *Ms. trichosporium* OB3b Mbn are oxazolones (Oxa_A and Oxa_B) (41). The remainder of Mbn is peptidic, although replacement of the N-terminal primary amine with a carbonyl resulted in the initial misidentification of the neighboring leucine side chain as an isopropylester group (26, 41). The C-terminal methionine group is

sometimes absent in Mbn isolated from spent medium, and despite unaltered Cu(I)-binding capabilities, its reduction potential differs slightly (42). The etiology of this truncated Mbn is unclear.

The two oxazolone rings present in Mbn are responsible for two absorption features at 345 nm and 392 nm in the apo compound, and excitation at these wavelengths results in fluorescence emission in the 400–500 nm region (43). Local absorption maxima in the 330–350 nm range are common for oxazolone compounds, but extension of the heterocycle's conjugation system results in a bathochromic shift (44), as observed for Oxa_A in Mbn, which is adjacent to an additional ketone group in lieu of the N-terminal amine. Copper binding by the oxazolones both quenches fluorescence emission intensity and alters their absorption maxima (43). These rings are prone to acid-catalyzed hydrolysis when not stabilized by copper binding (43, 45).

Both Cu(II) and Cu(I) can bind Mbn, but binding is reductive because, at stoichiometric levels of copper and Mbn, only EPR-silent Cu(I)Mbn is present in the final compound (46), and even after binding Cu(II), only Cu(I)Mbn is present after the first 10 minutes (47). The mechanism of Cu(II) reduction to Cu(I) has not yet been elucidated. Under certain conditions, other copper:Mbn stoichiometries are observed, at least temporarily (48, 49), and Cu_2Mbn is sometimes observed when copper is added in excess.

Due to its high copper affinity, Mbn can extract copper from a wide range of otherwise biounavailable mineral sources, including borosilicate glass and humic acid species (50, 51). These observations are consistent with a major role for Mbn in liberating copper from organic-rich environments with a near-neutral pH, such as peatlands, rice paddies, lake sediments, and soils, which are some of the common habitats for methanotrophs (51, 52). Mbn can also bind metals other than copper. Some metal ions, including Ag(I), Au(III), Hg(II), Pb(II), and U(VI) appear to bind via a similar mechanism to Cu(I) and Cu(II), and for these metals, reductive binding is observed (53). Others, including Cd(II), Co(II), Fe(III), Ni(II), and Zn(II), appear to bind as bischelates and are not reduced (53). Measured binding constants for metals other than copper appear to be lower than that of Cu(I) by 10–15 orders of magnitude (53).

Early research probed not only interactions between Mbn and metals but also interactions between Mbn and the primary methanotroph copper-dependent enzyme pMMO. Mbn was initially reported to play a direct role in pMMO activity, though no evidence for a direct interaction has been found to date. There are conflicting data regarding whether or not CuMbn addition affects the activity of purified pMMO (47, 54). Apo Mbn addition has been reported to increase activity (54), but surplus copper or zinc binding can inactivate pMMO (55, 56), and it may be that apo Mbn is merely removing inhibitory metals.

Genome Mining Reveals a Wider World of Methanobactins

The mode of Mbn biosynthesis was unknown until 2010, but nonribosomal peptide synthesis (NRPS) was a common hypothesis (43, 57). This was ruled out by the sequencing of the *Ms. trichosporium* OB3b genome (58): An open reading frame encoding a 30-amino-acid peptide with a C-terminal sequence resembling the peptidic Mbn backbone was detected

(Figure 2). In addition, a homolog of that gene (now termed *mbnA*), along with homologs of the surrounding genes, was identified in the genome of the nonmethanotrophic diazotrophic endophyte *Azospirillum* sp. B510 (45). Genome mining identified 18 putative Mbn operons in 16 species, and in subsequent years that number has increased to 74 operons in 71 species, spanning both gram-negative and gram-positive bacteria (25, 59) (Figure 3*a,b*; Supplemental Figure 1). Construction of a knockout strain of *Ms. trichosporium* OB3b in which *mbnA* is eliminated eradicates Mbn production (60), consistent with a role in Mbn biosynthesis.

The presence of *mbnA*, which encodes a short (22–40 amino acid) peptide in all Mbn operons (59), is consistent not with NRPS but rather with the biosynthesis of ribosomally produced, posttranslationally modified natural products (RiPPs). Mbn-related gene names, terms, and abbreviations have been accordingly been assigned following the guidelines established by the RiPP research community (61). Also present in all Mbn operons are genes encoding two proteins termed MbnB and MbnC, which are proposed to play a role in Mbn biosynthesis (59) (Figure 3*a*). Some operons encode additional proteins with known or putative biosynthetic roles as well as regulatory and transport proteins; the current state of knowledge regarding all these proteins is described below. Phylogenetic analysis of the three core genes (*mbnABC*) along with analysis of operon content supports the division of Mbn operons into five or possibly six main groups (25, 59) (Figure 3). A less granular set of subdivisions has been proposed (62), although this division is not based on phylogeny or posttranslational modifications. Groups I, IIa, and IIb are found exclusively in methanotrophs. The remaining groups comprise nonmethanotrophic bacteria, and Mbn operons in nonmethanotrophs now significantly outnumber those found in methanotrophs (25). Additional genes encoding proteins related to further posttranslational modifications, transport, and regulation are also found in many operons (59) and are discussed at greater length below.

Notably, genes encoding proteins with roles in copper homeostasis are located near Mbn operons in most nonmethanotrophic Group III–IV Mbn operons (59) (Supplemental Figure 1). Many Group III and IV operons are near genes encoding noncanonical members of the CopC family of periplasmic copper-binding proteins (63, 64), which are often adjacent to genes encoding CopD inner-membrane copper importers (65). Other periplasmic copper-binding proteins are sometimes present, including homologs of the Sco1 proteins and of the DUF461/PCu$_A$C proteins, all implicated in assembly of the cytochrome *c* oxidase Cu$_A$ site (66, 67). Although no specific copper need has been identified in Mbn-producing nonmethanotrophs, a P-type ATPase involved in copper import has been identified in some *Pseudomonas* species, consistent with active uptake of copper in at least one Mbn-producing genus of nonmethanotrophs (68).

Finally, Mbn operons are frequently associated with mobile genetic elements connected to horizontal gene transfer (59) (Supplemental Figure 1). This is unsurprising, given the broad range of species in which Mbn operons are found as well as fact that, in many genera, only a few species appear to have Mbn operons. The wide distribution of these operons, their presence in primarily nonpathogenic bacteria, and their role in trace metal acquisition

suggest that they may serve as a fitness island rather than the virulence islands that are traditionally associated with siderophores (69).

Copper-Dependent Gene Regulation in Methanotrophs

Genome mining provided evidence for the conservation of genes associated with *mbnA*, but those assignments were based purely on bioinformatics. However, like siderophore biosynthesis, which is inhibited by excess iron (70), Mbn is produced primarily at low copper levels (40), suggesting that genes for Mbn biosynthesis should be repressed under high copper conditions. Furthermore, any regulatory patterns should be correlated with the well-attested copper-dependent reciprocal regulation of the two methane monooxygenases, referred to as the copper switch, in which sMMO is severely downregulated by copper and pMMO is highly produced under copper-abundant conditions (31) (Figure 2). Extensive time-dependent quantitative real-time PCR (qPCR) experiments in *Ms. trichosporium* OB3b verified these two predictions. First, copper-dependent downregulation of the Mbn operon is clearly observed: When 12.5 μM copper is added to copper-starved cells, the entire Mbn operon is downregulated, including the *mbnIRTABCMNPH* operon (59) as well as *mbnE*, a gene encoding a periplasmic binding protein that is separated from the bulk of the operon by mobile genetic elements (71). Second, the Mbn operon appears to be coregulated with the sMMO operon. These broad trends have been replicated in a whole transcriptome shotgun sequencing experiment, albeit with a simpler, time-independent copper starved/copper replete set of samples (72).

Interestingly, genes encoding regulatory proteins in both operons, including *mbnI* and *mbnR* in the *mbn* operon and *mmoR,* the recently identified *mmoF* (71, 73), and *mmoG* in the sMMO operon (Figure 2), exhibit similar patterns. All five genes are almost completely downregulated within 15 minutes of copper addition, while the transcription levels of the remaining genes in the two operons drop primarily over 5- and 24-hour time periods (71). These data suggest a model wherein MbnIR and MmoRFG activate Mbn and sMMO production, respectively, at low copper levels. Upon copper addition, the same copper-binding repressor inhibits transcription of both *mbnIR* and *mmoRFG*. As levels of the proteins encoded by those genes fall, transcription of the main operons falls as well, resulting in the characteristic copper-induced downregulation of sMMO and Mbn. This putative copper-binding repressor of *mbnIR* and *mmoRFG* has yet to be identified, although other genes encoding proteins with predicted roles in copper homeostasis were identified in this study (71).

Structural and Biochemical Characterization of Additional Methanobactins

Five Mbns other than the original compound from *Ms. trichosporium* OB3b have been at least partially characterized; all belong to Group I or Group IIa and are from *Methylocystis* (*Mc.*) or *Methylosinus* species (Figure 1*b–f*). The second characterized Mbn is from *Mc.* sp. SB2 and was investigated via NMR (45). It was reported to be smaller than the *Ms. trichosporium* OB3b Mbn, with a modified nine-amino-acid backbone (45) and different posttranslational modifications (Figure 1*b*). No intramolecular disulfide bond is present, and no N-terminal transamination occurs. Instead, an acid-insensitive heterocycle initially

assigned as an imidazolone ring is the first heterocycle, and an additional posttranslational modification is present in the form of a sulfonated threonine (45).

Three additional *Methylocystis* Mbns have been characterized, including compounds from *Mc. hirsuta* CSC1 (Figure 1*c*), *Mc.* sp. M (Figure 1*e*), and *Mc. rosea* SV97 (Figure 1*f*), which were studied using mass spectrometry and, for the first two compounds, X-ray crystallography (74). Like *Mc.* sp. SB2 CuMbn, these Mbns have two nitrogen-containing heterocycles and thioamides, no intramolecular disulfide bonds, and a sulfonated threonine (45, 74). Unlike *Mc.* sp. SB2 CuMbn (Figure 1*b*), the first nitrogen-containing heterocycle in these Mbns is not a five-membered imidazolone ring but a six-membered ring depicted as a pyrazinediol group (74) (Figure 1*c,e,f*). Given the exceedingly high sequence similarity between *Methylocystis* operons, the source of the discrepancy between these pyrazinediol groups and the imidazolone of *Mc.* sp. SB2 was unclear. However, a reexamination of both the NMR data for *Mc.* sp. SB2 and the X-ray crystallography data for the remaining *Methylocystis* species suggests a possible answer (75). The assignment of the first *Mc.* sp. SB2 heterocycle rests partly on the presence of a secondary amine in that heterocycle (45). If the six-membered ring present in *Methylocystis* Mbn crystal structures is not a pyrazinediol but a hydroxypyrazinone or pyrazinedione tautomer (Figure 1*g*), a six-membered ring would be visible via crystallography and a secondary amine would be observed by NMR and would account for the long-distance interactions observed in the [^{13}C-^{1}H]-heteronuclear multiple-bond correlation spectroscopy (HMBC) and [^{15}N-^{1}H]-heteronuclear single-quantum correlation spectroscopy (HSQC) experiments performed on *Mc.* sp. SB2 CuMbn (45, 75). In all four characterized *Methylocystis* species, analysis of the *mbnA* genes indicates that not one but several C-terminal residues are missing from the structurally characterized Mbns (25, 45, 74). As with *Ms. trichosporium* OB3b, it is unclear when and how these residues are lost.

A second *Methylosinus* CuMbn has recently been characterized from *Ms.* sp. LW4 (75) (Figure 1*d*). This Mbn resembles the compound produced by *Ms. trichosporium* OB3b, despite a dissimilar peptidic backbone. Posttranslational modifications include N-terminal transamination, formation of two oxazolone/thioamide moieties from two cysteines, and intramolecular disulfide formation, and like *Ms. trichosporium* OB3b Mbn (Figure 1*a*), Mbn from this *Methylosinus* species binds a single copper ion (reductively in the case of Cu(II)) in what is likely to be a similar geometry (75). No C-terminal amino acid loss is observed, possibly because the C-terminal amino acid is a cysteine that stabilized by disulfide formation.

Comparing the copper binding affinities of the growing number of characterized Mbns remains somewhat challenging. To date, no equivalent of the pM scale for iron affinity in siderophores has been established for chalkophores; pM values describe the amount of unbound iron present at equilibrium under a standard set of experimental conditions (76). The only Mbn for which a similarly systematic set of measurements has been undertaken is that from *Ms. trichosporium* OB3b, in which proton-independent stability constants were also calculated (49). Furthermore, chalkophores present similar challenges to siderophores: Copper–chalkophore binding strength can preclude direct measurement of metal binding via common techniques. Competition experiments against compounds such as the Cu(I) chelator

bathocuproine disulfonate or even other Mbns provide an alternative method that has been used for several Mbns (42, 74).

Under superstoichiometric copper-loading conditions, lower-affinity nonreductive binding of a second copper ion has been observed, for which potential binding sites include the C-terminal carboxylic group, the tyrosine phenol, the C-terminal methionine, and the two cysteines otherwise involved in a disulfide bond (49). Binding is pH sensitive, with higher affinities above neutral pH (42, 49). Despite these complexities, the copper binding properties for Mbns have been remarkably consistent: Cu(I) binding constants have converged at ~10^{21} M^{-1} (42, 74, 77). As additional structurally divergent Mbns are characterized, a wider range of binding affinities may emerge. Evidence has already been found that the presence or absence of certain posttranslational modifications such as threonine sulfonation in *Methylocystis* Mbns can tune affinity (74).

Current Models for Methanobactin Biosynthesis

Mbns belong to the RiPP class of natural products, but their biosynthetic machinery appears to be unique. Although heterocycle formation is common in RiPP natural products, no known RiPP biosynthetic enzymes are found in Mbn operons (61). Other features of RiPP biosynthetic machinery, including a peptide recognition element (78) often found in RiPP-related enzymes, are also absent. Instead, both new enzyme families and divergent members of well-characterized enzyme families are involved in Mbn biosynthesis.

MbnA: the precursor peptide—Like all RiPP systems, MbnA precursor peptides have multiple components, including a sequence involved in binding biosynthetic enzymes, the leader peptide, which is lost in the final natural product, and the core peptide, which forms the backbone of the final natural product (61). The positions within the MbnA sequence occupied by nitrogen-containing heterocycles in the final natural product are cysteines in the core peptide (45, 59). These cysteines targeted for modification are part of motifs; these motifs comprise the cysteine and two adjacent C-terminal residues, with the first C-terminal residue small (glycine, alanine, and sometimes serine) and the second residue frequently larger and slightly hydrophilic (most commonly serine and threonine) (59) (Figure 3*c*). While some RiPP systems favor specific flanking residues (79), many others do not (80, 81). In Mbns, cysteines that are not part of a modification motif remain unmodified and available to form disulfide bonds, as observed in the two structurally characterized *Methylosinus* Mbns (26, 75).

In a subset of operons found in certain *Methylocystis* species, *mbnA* genes are part of a longer gene encoding an extracytoplasmic function (ECF) sigma factor (59). Whether an alternate transcription start site results in the production of only the MbnA peptide or whether the full protein is produced is unclear. Precedent for the latter has been found in a subset of thiazole-oxazole modified microcins (TOMMs) produced from larger genes encoding a nitrile hydrolase-like sequence in lieu of a standard leader peptide (82). This extended leader peptide sequence is biochemically relevant in those TOMM families (83) but has not yet been investigated for Mbns.

MbnB and MbnC: the core biosynthesis machinery—Two genes encoding proteins known as MbnB and MbnC are present in all Mbn operons. MbnB is a member of the uncharacterized DUF692 family, while MbnC is a smaller protein of unknown function that belongs to no previously known family. Their conservation (Figure 3*a*) despite the lack of a known function led to suggestions that these two proteins play a role in Mbn biosynthesis (59). While originally annotated as two open reading frames in the *Ms. trichosporium* OB3b operon (45), *mbnB* is a single gene in other Mbn operons, and the pseudogene observed in the published *Ms. trichosporium* OB3b genome is not present in all lineages of that strain (71). Although RiPPs containing amino acid–derived heterocycles such as thiazol(in)es and (methyl)oxazol(in)es are common, particularly in the TOMM family (84, 85), no precedents for enzymatic oxazolone biosynthesis have been found. The sole characterized oxazolone-containing natural product family, jadomycins, receives these modifications as part of the nonenzymatic incorporation of amino acids (86). Thioamides are slightly less uncommon in natural products (87), with thioviridamide (88) and closthioamide (89) of particular interest.

However, none of the biosynthetic machinery found in any of these enzyme families is related to MbnB or MbnC. Azol(in)e formation in TOMM systems involves the recently characterized ATP-binding YcaO family (90, 91), but neither MbnB nor MbnC bears any similarity to those enzymes, and both lack nucleotide-binding motifs. Thioamide biosynthesis is less well understood, but a YcaO family enzyme has been proposed to be responsible for thioamide biosynthesis in thioviridamide as well (88). Additionally, unlike many enzymes involved in RiPP biosynthesis, MbnB and MbnC lack RiPP recognition elements (RREs), which are involved in precursor peptide binding (78).

Instead, recent work indicates that MbnB and MbnC form an entirely novel heterodimeric iron-containing enzyme (MbnBC) that carries out a four-electron oxidation on the MbnA substrate, forming an oxazolone and thioamide moiety from a cysteine in a single concerted rearrangement (92). The iron active site is located in the MbnB subunit, and some ambiguity remains regarding the composition and oxidation state of the active cofactor, with coupled di- and trinuclear iron centers as possibilities. Importantly, this mechanism of heterocycle formation appears to apply to MbnA/MbnBC pairs from all five operon groups, although Group V is sufficiently divergent so that its enzyme and substrate are not compatible with the other four groups. An alternative mechanism involving a catalytic thiol and several biosynthetically unusual steps has been suggested (62), but even apart from the lack of conserved cysteines in MbnB and MbnC, this model does not fit the biochemical data, which support an oxygen- and iron-dependent mechanism (92) (Figure 2*b*).

MbnS, MbnF, MbnD, and MbnX enact additional posttranslational modifications—Along with MbnA, MbnB, and MbnC, some Mbn operons encode other proteins with predicted roles in Mbn biosynthesis (Figure 3*a*). Like MbnB and MbnC, these enzymes also lack RREs (78). MbnS, a 3′-phosphoadenosyl-5′-phosphosulfate-dependent sulfotransferase, is likely responsible for threonine sulfonation in Group IIa Mbns (59, 93, 94) (Figure 1*b–f*). MbnF, a flavin-dependent oxidoreductase, is predicted to play a role in (hydroxy)pyrazin(edi)one biosynthesis in Group I and Group II Mbns (Figure 1*c–f*), catalyzing the transformation from oxazolone to (hydroxy)pyrazin(edi)one (59). The closest characterized flavin-dependent monooxygenase is involved in rebeccamycin biosynthesis

(95), but flavin enzymes catalyze a wide range of biosynthetic reactions (96). MbnD is a predicted dioxygenase that is believed to play a role in the biosynthesis of Group IIb Mbns, though the reaction that it catalyzes has not been identified and no Mbns from that subfamily have been structurally characterized (59). Group V Mbn operons also contain *mbnX,* a gene encoding a DUF692 enzyme that is only distantly related to MbnB (59). Both Group IIb and Group V MbnAs lack a second cysteine modification moiety (and in the case of Group V, another cysteine at all), meaning that they cannot bind copper via a canonical Mbn ligand set; one possibility might be modification of another amino acid to provide replacement metal-binding ligand(s). Finally, both Group I and Group IV operons contain genes encoding pyridoxal-5′-phosphate-dependent aminotransferases, MbnNs, believed to perform N-terminal transamination reactions (59).

A role in oxazolone biosynthesis has also been proposed for *Ms. trichosporium* OB3b MbnN owing to the absence of the first Mbn oxazolone ring in an Mbn intermediate secreted by an Δ*mbnN* strain (97). Given that no neighboring transamination reaction is necessary for the completion of the second oxazolone in any Mbn, why the first oxazolone would require it is unclear, particularly when aminotransferases are only present in a few Mbn operons (25). However, the observed mass is consistent with acid-catalyzed oxazolone hydrolysis (45), which in combination with the predicted lack of an N-terminal transamination reaction, provides a potential explanation for the missing oxazolone ring. Indeed, addition of copper during purification stabilizes the Mbn intermediate and prevents oxazolone degradation (92); the role of MbnN is thus predicted to be catalyzing a simple transamination reaction on this intermediate. A biosynthetic role has also been proposed for the diheme cytochrome *c* peroxidase MbnH and its partner protein MbnP (62); however, *mbnPH* genes are commonly associated with genes related to CuMbn uptake (25), and while the *Ms.* sp. LW4 Mbn operon lacks *mbnPH*, its Mbn contains oxazolone/thioamide moieties (75). On the basis of in vitro experiments with MbnBC, it does not appear that there is a role for either MbnN or MbnPH in oxazolone/thioamide biosynthesis (92).

Finally, all of the proposed biosynthetic enzymes are cytoplasmic. There are RiPP systems in which leader peptide cleavage or additional posttranslational modifications occur in the periplasm (98), but this is not likely to be the case in Mbn systems owing to the cytoplasmic nature of MbnN and MbnF, which require cytoplasmic leader peptide cleavage to access the N terminus of the core peptide. One gene conspicuously absent in all Mbn operons is a protease responsible for leader peptide cleavage. Whether MbnB or MbnC is capable of catalyzing MbnA leader peptide cleavage is unclear, although this proteolytic step has not been observed in vitro (92), or whether an unknown process is responsible.

Efflux and Influx of Methanobactins

Transport is vital for metallophores. Mature metallophores are secreted into the environment, where they can liberate otherwise biounavailable metal. The metal-bound compounds must then be taken back up into the cell, where the metal is released. These processes have been studied extensively in siderophores, and in particular, many active importers have been identified. Mbn trafficking appears to follow a similar model.

Methanobactin secretion via MbnM—The secretion of Mbn is thought to be mediated primarily by a group of inner membrane efflux pumps designated MbnMs (59). Genes encoding MbnMs are even present in the divergent gram-positive Group V operons (Figures 2 and 3*a*; Supplemental Figure 1). Group IV Mbn operons lack *mbnM* genes, and whether these operons are no longer functional or whether a different exporter is involved is unclear (25). MbnM belongs to the multidrug and toxic compound extrusion (MATE) family (99). MATE proteins function as proton/sodium antiporters (100), and previously identified members of this family are primarily involved in the efflux of cationic xenobiotic compounds (101). Confirmed export of a native natural product like Mbn by MbnM would significantly broaden the range of MATE-transported compounds.

Internalization of copper-chelated methanobactin via MbnT—Evidence for active transport of CuMbn predated both the classification of Mbn operons via genome mining (59) and the confirmation that the *Ms. trichosporium* OB3b operon is regulated by copper (71). CuMbn uptake is inhibited via proton pump uncouplers such as carbonyl cyanide-*m*-chlorophenylhydrazone, indicating an energy-dependent uptake process, while soluble copper is taken up by a passive process that is disrupted by porin inhibitors such as spermine (102). Furthermore, apo Mbn inhibits copper uptake via CuMbn (102), a phenomenon that is observed in siderophore uptake as well (103) and is consistent with the presence of a specific transporter.

Genome mining identified a candidate CuMbn importer, a TonB-dependent transporter (TBDT) found in Groups I–IV Mbn operons (59) and initially named MbnT (Figures 2 and 3*a*). TBDTs are responsible for the energy-dependent import of various metabolites, including siderophores (104) as well as heme, thiamine, cobalamin, colicins, sugars, and small soluble nickel and cobalt chelates (105). Additional bioinformatic analysis revealed that MbnTs fall into several classes. One group, the MbnT1s, are associated with Group I Mbns and are paired with ECF sigma factors (MbnIs) and anti–sigma factors (MbnRs) (59). These MbnT1s possess an ~100-amino-acid periplasmic N-terminal extension that mediates interactions between the outer membrane MbnT and the inner membrane MbnR, which then interacts with the cytoplasmic MbnI, allowing for cell surface-to-cytoplasm signaling upon the binding of Mbn (106) (Figure 2). Similar systems exist for enterobactin (107), pyoverdine (108), and other siderophores.

However, this signaling pathway is not present in other Mbn operon groups. The genes encoding MbnT2s, a second set of TBDTs found in Group II Mbn operons, do not display significant sequence similarity to *mbnT1s*, lack neighboring *mbnIR* genes, and lack the N-terminal extension necessary for *trans*-periplasmic signal transduction (28). A third group of TBDTs, the MbnT3s, are found in the nonmethanotrophic Groups III–IV. These importers resemble heme-importing TBDTs and are not closely related to MbnT2 or MbnT1s (28). No *mbnT* genes are found in Group V operons or elsewhere in the genomes of these species.

Siderophore piracy is common among organisms with a high need for iron (109), and chalkophore piracy can occur among methanotrophs. *Mc. hirsuta* CSC1 cells take up *Ms. trichosporium* OB3b CuMbn and vice versa, and *Ms. trichosporium* OB3b also take up CuMbn from *Mc.* sp. SB2. In both cases, the Mbn-mediated copper influx can trigger the

copper switch (74, 110). Mbn piracy is consistent with the presence of multiple *mbnT1* and *mbnT2* (and *mbnIRT*) genes in the genomes of many species lacking Mbn operons, including other methanotrophs as well as ammonia oxidizers (which produce an ammonia-oxidizing homolog of the copper enzyme pMMO) (28). Many methanotrophs in fact have multiple *mbnT* homologs, complicating functional studies of this transporter family. In *Ms. trichosporium* OB3b, two additional *mbnT1* homologs are part of non-operon copper-regulated *mbnIRTPH* cassettes, but their regulatory patterns differ from those of the in-operon *mbnT1* (28). These proteins possibly function in the uptake of non-native Mbns.

Disruption of the in-operon *mbnT1* gene of *Ms. trichosporium* OB3b significantly decreases uptake of copper provided as CuMbn (but not soluble copper), despite intact biosynthesis and secretion of the chalkophore (111). The continued uptake of soluble copper is consistent with earlier reports of a second passive method for soluble copper uptake in this species (102). In a similar mutant strain, isotopically labeled ^{65}CuMbn import by the wild-type but not Δ*mbnT1* strain alters the copper isotopic ratio of the wild-type cells (28). These results can be replicated in *Escherichia coli* cells heterologously expressing MbnT1 from *Ms. trichosporium* OB3b (28), providing strong evidence for the correct identification of this transporter.

Binding of CuMbn to MbnT transporters is observed in vitro. Surface plasmon resonance, a method not yet commonly used for siderophore/receptor studies, provides evidence for binding of *Ms. trichosporium* OB3b CuMbn to both its cognate MbnT1 (K_d value of 6 μM) and the in-operon MbnT2 from *Mc. rosea* SV97 (K_d value of 18 μM) (28). Although TBDTs can be quite specific, their promiscuity depends on what portion of the substrate is recognized. The peptidic backbones of Mbns can differ significantly, but the geometries of their copper binding sites are similar (26, 74). FpvA from *Pseudomonas aeruginosa* ATCC 15692 recognizes pyoverdine metal binding sites, permitting some binding to noncognate pyoverdines (112), and Mbn binding to MbnT could conceivably be similar. How these binding affinities correlate to internalization rates of noncognate Mbns has not yet been elucidated.

A new role for a periplasmic binding protein, MbnE—In many siderophore families, internalized compounds are transferred to chaperone proteins after import into the periplasm. Such a system had previously been proposed for Mbns (113), and Group IIa Mbn operons do in fact encode a gene for a periplasmic binding protein (PBP), MbnE (28) (Figures 2 and 3*a*). A similar gene adjacent to the Mbn operon in *Ms. trichosporium* OB3b is copper regulated, while other *Ms. trichosporium* OB3b PBPs belonging to the same protein family (solute binding protein family 5) are not (28, 71). Bioinformatic analyses indicate that MbnEs are related to the microcin C7 transporter YejA and the oligopeptide transporter OppA/AppA (28, 114, 115), both of which also bind substrates with peptidic backbones. Moreover, a 1.9-Å resolution crystal structure of the apo MbnE from *Mc. parvus* OBBP suggests both significant homology with AppA and the presence of a binding cavity sufficiently large to accommodate Mbn (28). Notably, MbnEs from three species (*Ms. trichosporium* OB3b, *Mc. hirsuta* CSC1, and *Mc. parvus* OBBP) bind cognate Mbns with K_d values of 6–12 μM but do not interact with noncognate Mbns (28). However, in the absence of an operon-adjacent ABC importer, the fate of CuMbns after MbnE binding has not yet

been ascertained. What happens to CuMbns after internalization in species that lack MbnE homologs is also not clear.

Copper release from methanobactin—The mechanism of copper release from CuMbn has not been explored experimentally. Some siderophores are degraded upon uptake (116), but degradation of a complex compound like Mbn is potentially wasteful. Recycling of Mbn after copper release is another possibility, similar to recycling of pyoverdine (117), carboxymycobactin (118), and ferrichrome (119). One way to release copper without chalkophore degradation could involve copper oxidation, analogous to iron reduction for release from some siderophores (120) and consistent with the lower binding affinity of Mbn for Cu(II) than for Cu(I) (42). Given the ability of Mbn to reduce Cu(II), additional proteins are possibly required to facilitate copper release.

Two candidates for such partner proteins are MbnH and MbnP, encoded by operons in Groups I–IV (Figure 3*a*). MbnH is MauG-like diheme cytochrome *c* peroxidase, and genes encoding MbnH homologs are almost always found adjacent to the gene encoding MbnP, a hypothetical protein with several highly conserved tryptophan residues (59). These proteins are reminiscent of MopE/CorA, copper-binding proteins secreted by some methanotrophs. MopE/CorA proteins bind copper using a posttranslationally modified tryptophan that is likely generated by a diheme cytochrome *c* peroxidase encoded by a neighboring gene (121, 122). However, there is no significant sequence similarity between MopE/CorA and MbnP, and though MbnP and MbnH are predicted to be periplasmic, there is no indication that they are secreted. Alternatively, MbnH might itself oxidize or even modify CuMbn, directly effecting copper release.

The location of copper release is also unclear. CuMbns interact with MbnEs (28), which may reflect ultimate internalization to the cytoplasm and release there. However, copper release mediated by MbnPH would be periplasmic. In *Ms. trichosporium* OB3b, many periplasmic proteins implicated in copper-related processes are present, including CopC, CopD, DUF461/PCu_AC, and Sco1 proteins (71) as well as the so-called copper sponge Csp1 (123) and the uncharacterized protein PmoD (71). Finally, pMMO itself houses a copper active site in the periplasmic portion of the PmoB subunit (55, 124), which is believed to be the final destination of most copper trafficked into methanotroph cells. For siderophores, demetalation can occur in the cytoplasm (e.g., pyochelin; 125) or the periplasm (e.g., pyoverdine; 117), and more work is needed to determine which is the better model for CuMbn copper release.

Secondary Roles for Methanobactin

Historically, metallophores were considered primarily in the context of their role in metal uptake and metal homeostasis. However, many metallophores, including methanobactin, appear to have a broad range of secondary roles, ranging from regulatory functions to protection against toxicity caused by metal or reactive oxygen species to biomedically relevant antibiotic or therapeutic functions (12).

Signaling factor—Self-regulation of production and uptake machinery, often via anti–sigma factor/sigma factor pairs that interact with the TBDT, is well documented for a range

of siderophores, including enterobactin and pyoverdine (126). As described above, *mbnIRT* cassettes may play a similar role in the import of Mbn in both Mbn producers and in species that are engaging in chalkophore piracy (28) (Figure 2). Other regulatory schemes are observed in some siderophore systems, such as the cytoplasmic uptake of pyochelin, which is required for activation of the AraC-family regulator PchR (127), but it is not known if similar systems are present in Mbn operon groups without *mbnIRT* cassettes.

A direct role has been proposed for Mbn in the methanotroph copper switch. Disruption of the *mbnA* gene dampens the effect of copper addition on both the sMMO and pMMO operons (60). Knockout of the sMMO operon, including a gene encoding a protein of unknown function termed MmoD, also perturbs the copper switch, as does knockout of *mmoD* alone (128). It was thus proposed that MmoD and Mbn work together to effect this regulatory switchover (60). However, neither Mbn nor sMMO operons are necessary for fully triggering the copper switch. Species lacking Mbn operons can still switch between sMMO and pMMO production (35, 73), indicating that Mbn is not primarily responsible for copper-dependent repression of the sMMO operon. Moreover, species lacking either the sMMO operon (and thus MmoD) or both the sMMO and Mbn operons still exhibit copper-responsive phenotypes, including decreased Mbn production and intracytoplasmic membrane formation (77, 129, 130).

MmoD involvement in the copper switch is particularly problematic. MmoD has no observed copper binding capacity and has an unusual set of conserved residues for a putative copper-binding protein. It also does not bind heparin, commonly used as a proxy for DNA binding (71). Despite reports to the contrary (62), *mmoD* is downregulated in response to copper in at least two species (71, 73), which would be unexpected for a copper-binding repressor. Finally, in the obligate sMMO-producing *Methylocella silvestris* BL2, which has MmoD but not the pMMO or Mbn operons, copper addition does not repress sMMO (131). Biochemical evidence for interactions between MmoD and the sMMO enzyme complex has been obtained, although the role of those interactions in vivo is uncertain; sMMO is active in vitro without MmoD (132, 133). These considerations suggest that Mbn and/or MmoD are not the primary regulatory elements governing the copper switch.

A second proposed model includes Mbn, MbnI, and MmoR, a transcription factor encoded by the sMMO operon (62). However, most methanotrophs lack Mbn operons, and it seems correspondingly unlikely that the variable genomic environments of multiple σ^{70}-regulated pMMO operons (134) have been retrofitted to support a σ^{ECF} regulatory binding site for MbnI or an Mbn-binding riboswitch. This is also true of sMMO operons, for which σ^{N} but not σ^{ECF} binding sites have been reported (135). There is no indication that MmoR might bind Mbn, particularly as similar regulators exist for distant sMMO homologs with no role in methane oxidation and no known copper switch (136, 137). Taken together, any direct role for Mbn in signaling beyond MbnIRT-mediated regulation of the *mbn* operon is speculative. In a more conservative model (Figure 2), most regulatory changes associated with Mbn production and CuMbn uptake result from the increase in the pool of bioavailable copper, with direct Mbn-mediated signaling limited to the MbnI regulon, likely consisting of just the *mbn* operon and possibly the non-operon *mbnIRTPH* clusters (28, 71).

Protection from metal toxicity—As mentioned above, Mbn binds some metals other than copper reductively (53). For example, Mbn binds Au(III), mediating the production of gold nanoparticles by reducing the metal to Au(0) (138). Although the measured affinity for copper is higher, optical spectroscopic data indicate that gold may be able to compete with copper to some extent, inhibiting copper uptake and the ensuing copper switch (139). The biological relevance of gold binding is not clear: Both gold toxicity (140) and mitigation of gold toxicity via biomineralization (141) are observed in other proteobacteria, such as *Cupriavidus metallidurans* CH34, but unlike methanotrophs, these species are known to tolerate extreme levels of heavy metals (142, 143).

Mercury binding to Mbns from *Ms. trichosporium* OB3b and *Mc.* sp. SB2 has also been investigated in some detail. Mbn can bind Hg(II) in the absence of and, to a lesser extent, the presence of copper, although the binding constant is 15 orders of magnitude lower than that of copper; because copper does not easily displace bound mercury, a role in protection against mercury toxicity has been suggested (144, 145). The affinity and specificity are somewhat compound specific, with *Mc.* sp. SB2 Mbn exhibiting less selectivity for copper (144, 145). A role for Mbn in methylmercury (CH_3Hg^+) degradation has also been suggested, although Mbn does not demethylate mercury on its own (146). It may be that the ability of Mbn to reduce methylmercury makes the substrate available to the demethylating enzyme (53, 147). While some methanotroph species have been found in environments replete in heavy metals (148), methanotrophs are present in a wide range of environments (52, 149), from both freshwater and marine sediments (150) to peat bogs (151), arctic tundra (152), and volcanic soil (153), and it is unclear if interactions between Mbn and mercury are relevant to the behavior of methanotrophs under common biological conditions.

Superoxide dismutase activity—The ability of Mbn not just to bind Cu(I) but to reductively bind Cu(II) (46), producing Cu(I)Mbn, hinted that CuMbn might be capable of more redox chemistry. CuMbn has been reported to exhibit oxidase, superoxide dismutase (SOD), and hydrogen peroxide reductase activities (54). Biological superoxide production and secretion by heterotrophic bacteria may be a source for oxidative stress in environments frequented by methanotrophs, such as lake sediments and terrestrial soils (154). Thus, SOD activity of secreted Mbns may be functionally relevant.

Antibiotic activities—Mbn from *Ms. trichosporium* OB3b has been reported to exhibit antibiotic activity against gram-positive bacteria (155). Any mechanism remains hypothetical, but the antibiotic activity of redox-active metal-binding natural products such as bleomycins is well documented (156), and uptake of surplus copper can be cytotoxic in its own right (29). Given that Mbn operons from Groups I–IV contain TBDTs predicted to enable CuMbn uptake along with additional genes with predicted roles in periplasmic copper homeostasis (59), a primary antibiotic role seems less likely in these groups.

By contrast, Mbns from Group V may well function as antibiotics or quorum-sensing compounds. All Group V species lack operon-associated and non-operon *mbnT* genes. While Group V compounds have not yet been characterized, the presence of only a single modifiable cysteine in their MbnA sequences (Figure 3*c*) suggests a notably different copper coordination environment, if they indeed bind copper (25). Several Group V Mbn operons

are found in *Streptomyces* species, which are prolific antibiotic producers. Some *Streptomyces* species play a symbiotic role, producing antibiotics that protect their hosts from other bacteria, and a similar phenomenon is observed in several Group V bacteria, including entomopathogenic *Photorhabdus* and *Xenorhabdus* species (157, 158). The *Vibrio* species that together form the third-largest group of Group V Mbn producers also possess a range of poorly characterized biosynthetic machinery for secondary metabolites (159). Further research is necessary to determine the role(s) of the Mbn-like compounds in these species.

Therapeutics—Mbns have also attracted interest as drug candidates. Similar to the treatment of hemochromatosis with the siderophore desferrioxamine (160, 161), Mbn is a potential therapeutic for Wilson disease, a human genetic disorder of copper overload (162). Current treatments have significant side effects, are incapable of mobilizing copper in both oxidation states, and/or chelate other physiologically relevant transition metals (163, 164). In a rat model of Wilson disease, Mbn can reverse acute liver failure (165, 166). Copper overload may also play a role in a range of neurological disorders (167), and if that role is verified, Mbns might be widely applicable as copper-chelating drugs. Finally, the modularity of Mbn biosynthesis and its peptidic nature could enable biological generation of diverse Mbn-like drug candidates, potentially with additional features such as the ability to utilize tissue-directed peptide targeting.

CHALKOPHORES BEYOND THE METHANOBACTIN FAMILY

Copper Uptake Mechanisms in Non-Methanobactin-Producing Methanotrophs

The production of Mbns by two species lacking Mbn operons (59), *Methylococcus capsulatus* (Bath) and *Methylomicrobium album* BG8 (168), has been reported. If these species do produce a chalkophore, it is not one directly related to the Mbn family. These compounds have significantly lower calculated copper affinities than other Mbns, bind iron, and contain non-negligible amounts of Cu(II) (168). As such, they are possibly metallophores that target some other metal but are capable of binding copper. The secreted protein MopE may instead substitute for high-affinity chalkophores in these species. In MopE, a tryptophan-to-kynurenine posttranslational modification likely carried out by a diheme cytochrome *c* peroxidase generates a high-affinity copper binding site (121, 169, 170).

Coproporphyrin III

Methanotrophs are not the only bacteria that rely on copper enzymes for key metabolic processes. Under anaerobic conditions or in the presence of nitrate, *Paracoccus denitrificans* uses copper enzymes for both respiration and denitrification (171, 172). Under copper-deficient conditions, less cell yield is obtained and a red compound is secreted (173). The addition of Cu-coproporphyrin III to the medium allows recovery of growth and limits secretion of the chromophore, identified as coproporphyrin III (Figure 4*a*), which turns red upon binding zinc in copper-free medium (173). This putative copper acquisition role is not universal, however. Coproporphyrin III is secreted at higher copper levels in a strain of

Rubrivivax gelatinosus that lacks the copper efflux pump CopA, which is inconsistent with a role in copper influx (174).

Yersiniabactin

As an iron-binding natural product produced by *Yersinia pestis*, the pathogen responsible for the bubonic plague, yersiniabactin (Ybt) (Figure 4*b*) was discovered nearly 20 years ago and was identified as a siderophore and a key component of the *Yersinia* high-pathogenicity island (175, 176). However, like a small number of other siderophores, including pyochelin and anguibactin, several of the iron-coordinating ligands are not the classical siderophore hydroxymate and phenolate-catecholate groups but rather are thiazoline and thiazolidine groups (175, 177). This divergent ligand set proved to be biochemically relevant when it was shown that Ybt produced by uropathogenic *E. coli* binds copper during infection (178). Under these conditions, Ybt binds Cu(II) competitively with Fe(III), protecting the cell from copper toxicity. Furthermore, this protective effect is not simply due to copper sequestration: Catecholate siderophores can bind Cu(II) and reduce it to the more cytotoxic Cu(I) form, and the higher copper affinity of Ybt eliminates that possibility (178). Copper release in cytokine-stimulated macrophages and copper overload in bacteria internalized by macrophages suggest that high external copper levels are a significant risk for pathogenic bacteria. Thus, a secondary protective role for less-specific metallophores is plausible (179).

Like CuMbn, copper-bound Ybt (CuYbt) exhibits SOD activity, potentially providing protection against phagocytic killing (180). Similar to staphylopine, Ybt's role as a broad-spectrum metallophore means that it is capable of interacting with more than just copper. Ybt has also been reported to play a role in Zn(II) acquisition in *Y. pestis*, although conflicting reports exist regarding its affinity for zinc, particularly in the presence of iron, and a range of metal-Ybt complexes are imported in a TonB-dependent manner (20, 181). Cu(II)Ybt does not competitively inhibit Fe(III)Ybt uptake, suggesting that if the compound acts as a true chalkophore to mediate copper acquisition, it does not do so under common growth conditions (181). However, under copper starvation conditions, uropathogenic *E. coli* does take up Cu(II)Ybt, followed by dissociation of the copper within the cell (and incorporation into the cellular copper pool) and finally recycling of this bifunctional siderophore/chalkophore (182). Notably, this process involves Cu(II)Ybt, unlike many of the other chalkophores that prefer Cu(I); this allows the chalkophore to coopt the siderophore reduction machinery, the YbtFQ enzyme complex, to liberate the metal after internalization, rather than requiring a second set of enzymes for copper uptake.

SF2768 and Other Diisonitrile Natural Products

A diisonitrile natural product that binds copper, SF2768 is produced via NRPS in the gram-positive bacterium *Streptomyces thioluteus* (183) (Figure 4*c*). Like siderophore and chalkophore operons, the SF2768 operon contains exporters [members of the major facilitator superfamily (MFS)] as well as importers (an ABC transporter annotated as an iron chelate importer). SF2768 binds Cu(I) and Cu(II), and like Mbn, it appears to be able to bind Cu(II) reductively, followed by reinternalization after copper binding (184). Similar operons were identified in other gram-positive species, and characterization of the resulting compounds in *Mycobacterium* species indicated that they form diisonitrile lipopeptides with

a possible role in zinc homeostasis (185). The broader structural and metal-binding diversity of these diisonitrile metallophores has yet to be explored, but it is clear that Mbns are not the only potential chalkophores found in gram-positive bacteria.

COPPER-BINDING SIDEROPHORES AND METALLOPHORES

Nicotianamine, Staphylopine, and Pseudopaline: Multi-Metal Metallophores

Plants also secrete metallophores, and as in bacteria, the best-known metallophores are iron-binding compounds known as phytosiderophores. However, some phytosiderophores are involved in the acquisition of other metals. Nicotianamine (Figure 5*a*) has been implicated in iron, copper, nickel, and zinc homeostasis (186). In some species, such as *Brassica carinata*, nicotianamine plays a role in copper acquisition during periods of copper starvation (187). Nicotianamine and related phytosiderophores are found primarily in eukaryotic and archaeal species, but nicotianamine synthase (NAS), a key *S*-adenosylmethionine (SAM)–dependent enzyme in nicotianamine biosynthesis, has prokaryotic homologs, including one in the genome of *Staphylococcus aureus* (17).

In *S. aureus*, the gene for the NAS homolog (*cntL*) is accompanied by an additional two biosynthetic genes: a gene encoding an epimerase (*cntK*) and a gene encoding an NAD(P)H-dependent dehydrogenase (*cntM*) (17). Also found in the operon are genes encoding an ABC family transporter thought to be involved in nickel import (*cntABCDEF*) and a MFS exporter (*cntE*) (17). Incubation of heterologously expressed biosynthetic components of the operon (CntK, CntL, and CntM) in the presence of L-histidine, SAM, pyruvate, and NADPH results in production of a natural product termed staphylopine, which exhibits significant structural similarity to nicotianamine (Figure 5*b*). Staphylopine binds divalent metal ions with similar K_d values and affinity order ($Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Fe^{2+}$) to nicotianamine (17). Strains with genetic disruptions of the key biosynthesis or importer genes exhibit reduced levels of Zn, Ni, Cu, and Co and decreased metal uptake when exposed to soluble extracellular metal, although results for copper appear to be affected by swift efflux (17). It is not yet clear what the primary role of this metallophore is in *S. aureus* or how a broad-spectrum metallophore is regulated in different environments. Staphylopine-like operons are found in other pathogenic species, including *P. aeruginosa*, in which the products of those genes are needed for growth within the host environment (188). Pseudopaline, the opine metallophore produced by these staphylopine-like operons, has recently been identified, and evidence that it plays a role in zinc and nickel import is available (189–191).

Pyochelin and Pyoverdine

The siderophores pyochelin (Pch) and pyoverdine (Pvd) are also produced by *P. aeruginosa*. Members of the partially peptidic Pvd family have a higher affinity for iron than Pch (192) (Figure 5*c,d*). Pch, like Ybt, contains thiazoline and thiazolidine groups, which facilitate binding of other divalent metal ions, including Cu(II) and Zn(II). While Pch binds Fe(III) in monochelate and bischelate modes, Cu(II)Pch and Zn(II)Pch complexes are exclusively bischelate. Pch is only weakly selective for Fe(III) over Cu(II) and Zn(II) (193). Similar to Ybt, Pch may not be a true chalkophore under standard physiological conditions. Binding of

Cu(II) to Pch and binding of Cu(II)Pch to the Pch importer are significant, but little uptake of Cu(II)Pch is observed in comparison with other divalent metal ions, including Ni(II) and Co(II) (193).

Pvds are also capable of binding a range of divalent metal ions, although the ligands have not been identified (Figure 5*d*). However, Cu(II)Pvd cannot inhibit uptake of Fe(III)Pvd and is taken up into the cell at a rate 37-fold lower than that of Fe(III)Pvd (194). As with Ybt, Pvd and Pch may sequester copper outside of the cell, playing a protective role (195). In support of this notion, not only does copper stress upregulate Pvd (and not Pch) biosynthesis (196), but also import of CuPvd upregulates Pvd biosynthetic machinery and not the Pvd importer, the TBDT FpvA (195). While both Pch and Pvd can bind copper and play some protective role against copper toxicity, Pvd appears to be more biologically relevant in this process. Operons for both siderophores are downregulated in conditions of copper starvation, suggesting that neither siderophore is primarily involved in copper uptake (197). Finally, *Pseudomonas* species are among the nonmethanotrophic species in which Mbn homologs have been detected, and the presence of both MbnT3 TBDTs and copper homeostasis machinery suggests that Mbns in these species are the primary chalkophores (59).

Pyridine-2,6-Dithiocarboxylic Acid

Pyridine-2,6-dithiocarboxylic acid (pdtc) (Figure 5*e*) is also secreted by *Pseudomonas* species. Like Pch and Pvd, it binds a range of metals in varying arrangements and stoichiometries, and like CuYbt, copper-bound pdtc (Cu-pdtc) is capable of redox cycling (198). Interestingly, like Mbn, copper binding is mediated by both nitrogen and sulfur ligands (198), and unsurprisingly, pdtc can also bind other soft and easily polarizable metals. Stabilization of harder transition metals like nickel and iron requires bischelate formation, and despite demonstrated iron binding (199), pdtc's role in iron uptake may rely partly on its ability to reduce iron (198, 200). Nevertheless, uptake of Fe(III)-pdtc and of Zn-pdtc (201, 202) has been the major biological focus, and whether pdtc is a true chalkophore is unclear. Cu-pdtc appears to have other unusual properties, including the ability to dechlorinate the carcinogen carbon tetrachloride (203).

Schizokinen and Marine Cyanobacterial Metallophores

Schizokinen is a citrate-containing hydroxamate siderophore originally identified in gram-positive *Bacillus* and cyanobacterial *Anabaena* species (204) (Figure 5*f*). The copper-binding abilities of siderophores produced by *Anabaena* species were noted as early as 1980 (205). While copper binding was clearly a secondary role for this siderophore, schizokinen's copper-binding abilities were proposed as an explanation for the resistance of cyanobacteria to copper sulfate treatments during algal blooms (206). There is no evidence that the TBDT that imports Fe(III)-schizokinen can import copper-bound schizokinen (207), but alternate copper uptake methods exist, including the TBDT IacT, which appears to be involved in both iron and copper uptake (208).

The marine environment contains other copper-complexing compounds, including classes of uncharacterized compounds with higher affinity (L1) and lower affinity (L2) (209). Other

specifically cyanobacterial metallophores appear to have roles related to copper, often for protection against copper toxicity (210). Under copper stress, *Synechococcus* species secrete an uncharacterized copper chelator with a binding affinity similar to that of L1 (211, 212). These compounds appear to be distinct from the schizokinen-like marine siderophores known as synechobactins (213), but whether they are primarily siderophores or their major role is in prevention of copper toxicity is unclear. The identification of marine metallophores with metal preferences other than iron remains an area of active research (214).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY POINTS

1. Metallophores other than siderophores play major roles in metal homeostasis. The best-studied nonsiderophores are the Mbn copper-binding chalkophores.
2. Mbns are RiPPs. The copper ligands comprise nitrogen-containing heterocycles and neighboring thioamide groups generated by core posttranslational modifications.
3. Mbn operons are found in a wide range of nonmethanotrophic bacteria, including gram-positive bacteria and human pathogens.
4. Increasing genetic and biochemical evidence has been found for the roles and mechanisms of the proteins involved in Mbn biosynthesis, regulation, and transport.
5. Other compounds with proposed chalkophore roles exist, indicating that chalkophore production is not restricted to methanotrophs or Mbns alone.
6. In some cases, metallophores that prefer other metals can bind copper with relatively high affinity, and copper binding that does not result in copper uptake may be a biologically relevant function of several siderophores.

FUTURE ISSUES

1. Characterization of new Mbns from additional species is necessary to reveal the full structural and biochemical diversity of the family.
2. Specific aspects of Mbn biochemistry remain to be elucidated, including mechanisms of leader peptide cleavage as well as mechanisms of copper release.
3. The roles of Mbns in nonmethanotrophs are unexplored. Such studies will provide new insight into the roles and mechanisms of copper influx in a wide range of bacteria.
4. Further work is required to uncover how Mbn production is regulated, and for methanotrophs in particular, regulatory processes of interest include any role in the copper switch.
5. The roles of additional compounds currently classified as siderophores in the homeostasis of metals other than iron should be explored. For copper in particular, siderophores with nitrogen-containing heterocycles merit investigation.
6. Chalkophores and copper-binding siderophores should be pursued as drug candidates for diseases in which copper overload or mismetalation occurs.

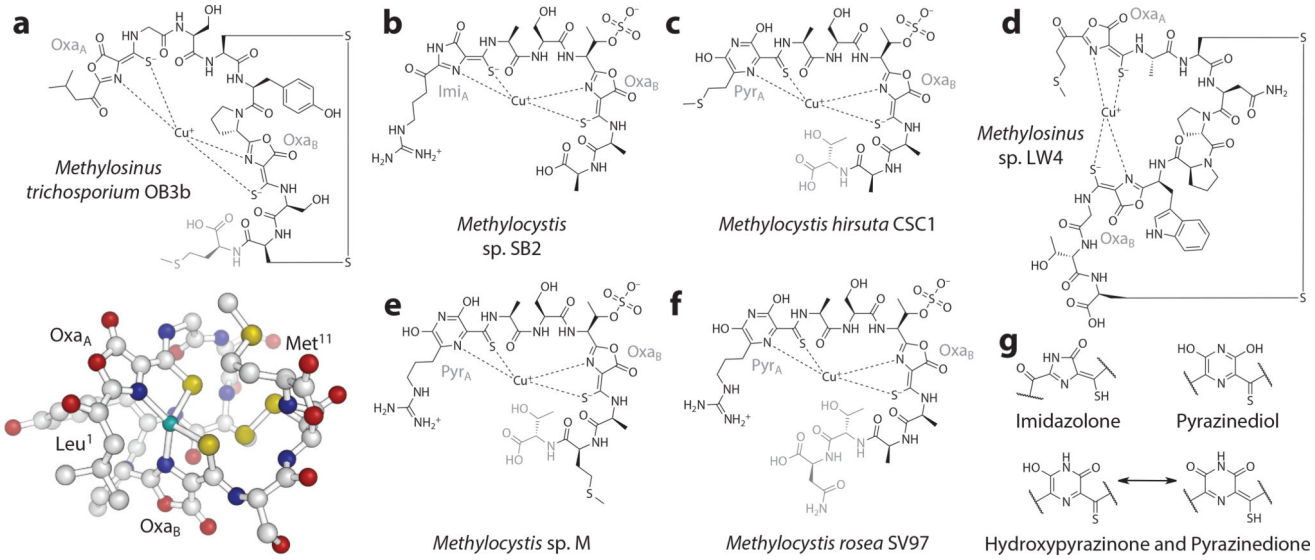

Figure 1.

Structures of methanobactins (Mbns) characterized to date. (*a*) The structure of copper-chelated Mbn (CuMbn) (*top*) from *Methylosinus* (*Ms.*) *trichosporium* OB3b. The C-terminal methionine, in gray, is sometimes absent from the final compound. The crystal structure (*bottom*) clearly shows the distorted tetrahedral geometry of the copper binding site. (*b*) The structure of CuMbn from *Methylocystis* (*Mc.*) sp. SB2. Based on the precursor peptide sequences for this and other *Methylocystis* Mbns, additional residues are present at the C terminus of these compounds during biosynthesis but have not yet been experimentally observed. (*c*) The structure of Mbn from *Mc. hirsuta* CSC1. Determined via X-ray crystallography, this and the remaining *Methylocystis* Mbns have six-membered rings (assigned as a pyrazinediol) as the first heterocycle. Residues in gray are sometimes absent. (*d*) The structure of *Ms.* sp. LW4 CuMbn, which, like *Ms. trichosoporium* OB3b CuMbn, contains two oxazolone rings and neighboring thioamide groups. (*e*) The structure of Mbn from *Mc.* sp. M. Residues in gray are sometimes absent. (*f*) The structure of CuMbn from *Mc. rosea* SV97. Residues in gray are sometimes absent. (*g*) Potential identities for the "N-terminal" heterocycle in Mbns from *Methylocystis* species. Figure adapted with permission from References 25 and 215.

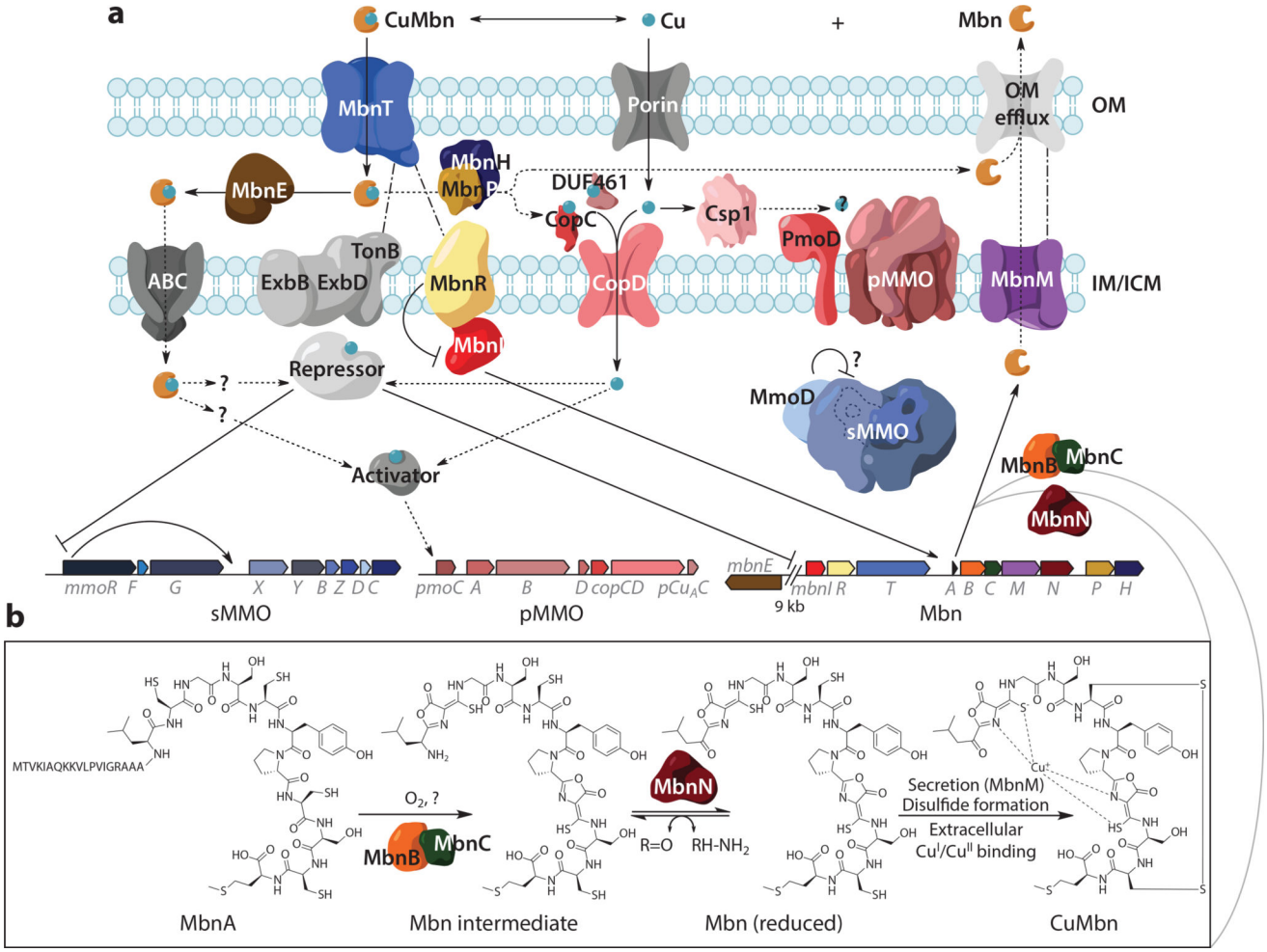

Figure 2.

Current model for processes related to (*a*) copper homeostasis and methanobactin (Mbn) transport, regulation, and (*b*) biosynthesis in *Methylosinus trichosporium* OB3b. Abbreviations: ABC, ATP-binding cassette transporter; CuMbn, copper-chelated Mbn; ICM, intracytoplasmic membrane; IM, inner membrane; OM, outer membrane; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase. Figure adapted with permission from References 25 and 215.

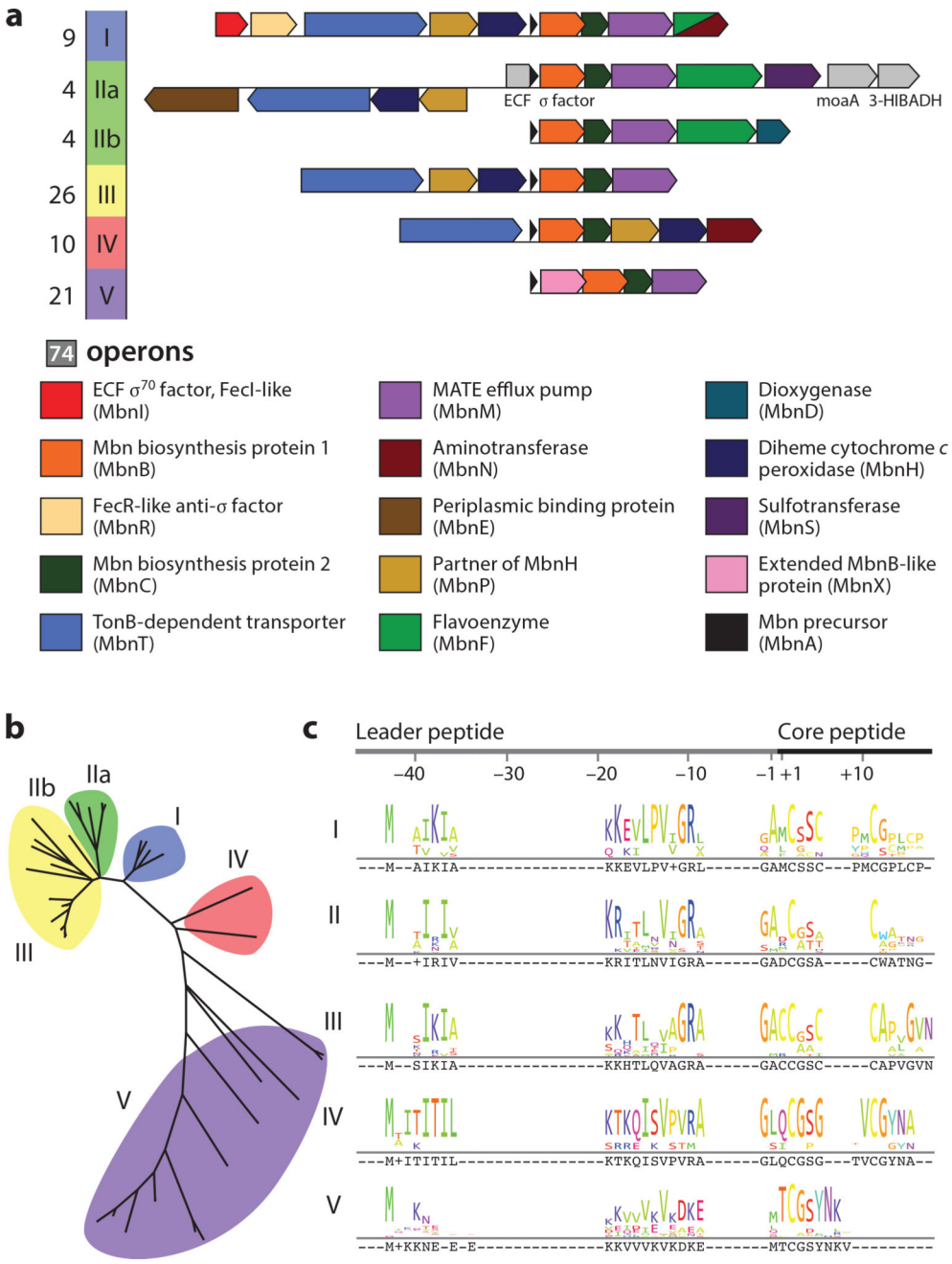

Figure 3.

Methanobactin (Mbn) operons. (*a*) Operon schematics for Mbn operon groups. (*b*) Phylogenetic tree of MbnB protein sequences, illustrating the divisions used to define the Mbn operon groups. (*c*) Sequence logos for MbnA sequences from different groups. Figure adapted with permission from References 25 and 215.

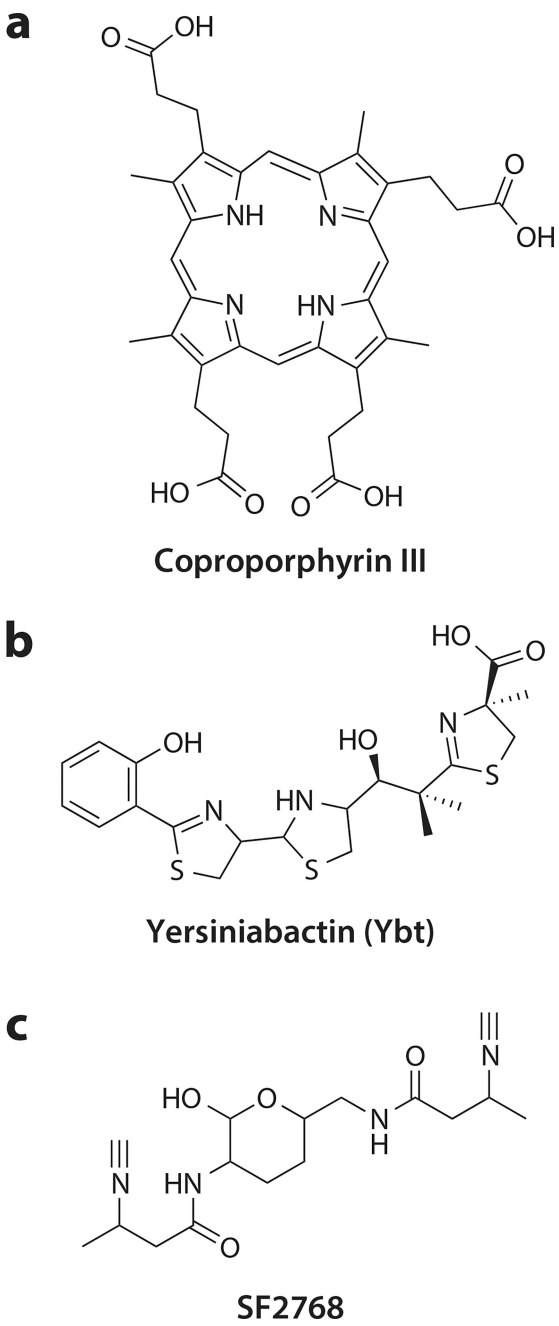

Figure 4.

Structures of putative chalkophores. (*a*) Coproporphyrin III is a porphyrin. Although not traditionally considered siderophores, porphyrin (heme) uptake is a source of iron for many species. (*b*) Yersiniabactin (Ybt) metal binding is partly mediated by heterocycles, which are not among the traditional catecholate, hydroxymate, and phenolate siderophore ligands. (*c*) SF2768 has unusual diisonitrile groups that are likely involved in copper binding.

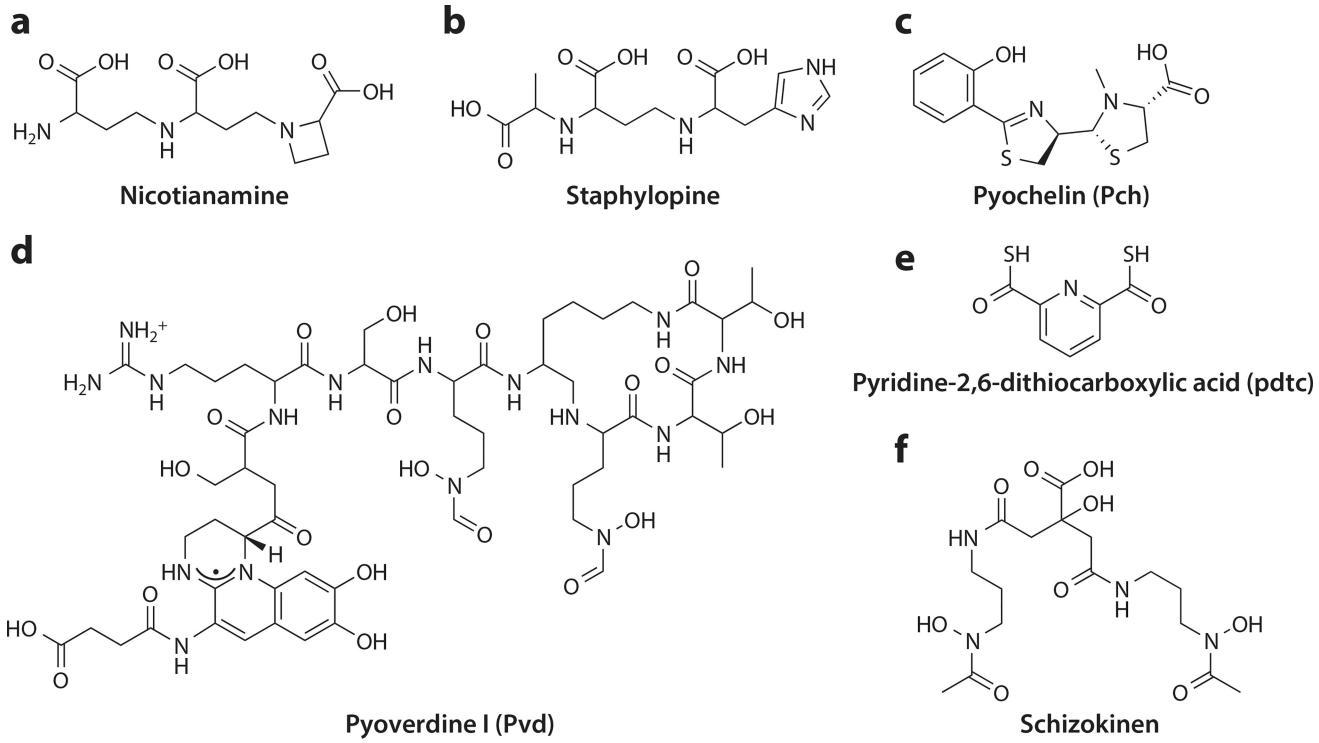

Figure 5.

Structures of select copper-binding siderophores. (*a*) Nicotianamine and (*b*) staphylopine share structural characteristics and biosynthesis genes, despite differing origins in eukaryotic and bacterial species. (*c*) Pyochelin (Pch) has a metal-chelating hydroxyphenol-thiazolyl-thiazolidinyl backbone similar to that of Yersiniabactin (Ybt). (*d*) Pyoverdine I (Pvd) is a member of the large family of primarily peptidic pyoverdine siderophores. (*e*) Pyridine-2,6-dithiocarboxylic acid (pdtc) is a small metal-binding secondary metabolite. (*f*) Schizokinen was originally identified in gram-positive *Bacillus* and cyanobacterial *Anabaena* species.