

# Methanobactin and MmoD work in concert to act as the ‘copper-switch’ in methanotrophs

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## Summary

**Biological oxidation of methane to methanol by aerobic bacteria is catalysed by two different enzymes, the cytoplasmic or soluble methane monooxygenase (sMMO) and the membrane-bound or particulate methane monooxygenase (pMMO). Expression of MMOs is controlled by a ‘copper-switch’, i.e. sMMO is only expressed at very low copper : biomass ratios, while pMMO expression increases as this ratio increases. Methanotrophs synthesize a chalkophore, methanobactin, for the binding and import of copper. Previous work suggested that methanobactin was formed from a polypeptide precursor. Here we report that deletion of the gene suspected to encode for this precursor, *mbnA*, in *Methylosinus***

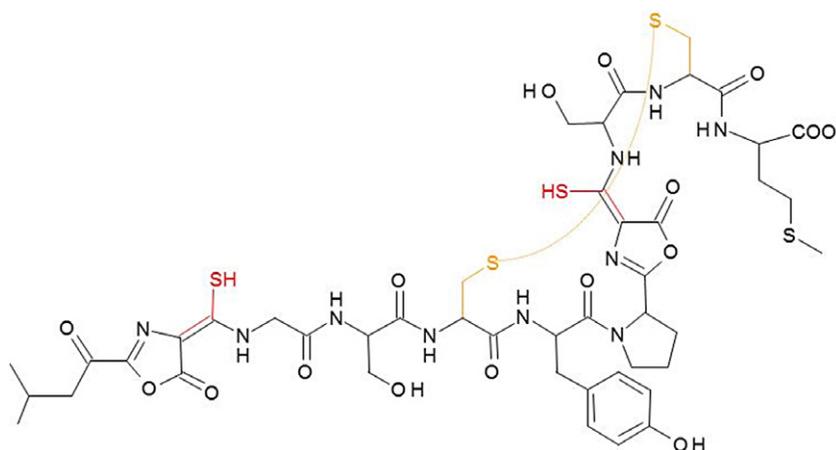
***trichosporium* OB3b, abolishes methanobactin production. Further, gene expression assays indicate that methanobactin, together with another polypeptide of previously unknown function, MmoD, play key roles in regulating expression of MMOs. Based on these data, we propose a general model explaining how expression of the MMO operons is regulated by copper, methanobactin and MmoD. The basis of the ‘copper-switch’ is MmoD, and methanobactin amplifies the magnitude of the switch. Bioinformatic analysis of bacterial genomes indicates that the production of methanobactin-like compounds is not confined to methanotrophs, suggesting that its use as a metal-binding agent and/or role in gene regulation may be widespread in nature.**

## Introduction

It is estimated that globally, ~ 566 million metric tons of methane are emitted from both natural and anthropogenic sources (Anderson *et al.*, 2010). Much less methane is emitted to the atmosphere than is generated via methanogenic activity, as it is estimated that up to 100 million metric tons of methane per year are oxidized in the environment by methane-oxidizing bacteria, or methanotrophs (Smith *et al.*, 2000). Methanotrophs are widespread, and are found, for example, in freshwater and marine sediments, soils, wetlands and rice paddies (Semrau *et al.*, 2010). Most aerobic methanotrophs group phylogenetically within the  $\gamma$ - and  $\alpha$ -*Proteobacteria* (Semrau *et al.*, 2010), although aerobic methanotrophs from *Verrucomicrobia* have been identified (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). In addition, a unique organism, *Methylomirabilis oxyfera*, of the NC10 phylum couples methane oxidation to nitrite reduction, but actually utilizes oxygen for methane oxidation via the production of dioxygen from the reduction of nitrite to nitric oxide, which is then converted via a nitric oxide dismutase to generate dioxygen, along with dinitrogen (Ettwig *et al.*, 2010).

All aerobic methanotrophs use methane monooxygenase (MMO) to convert methane to methanol in the first step of methane oxidation to CO<sub>2</sub>. Two forms of MMO have been characterized, the cytoplasmic or soluble form of methane monooxygenase (sMMO) and the

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**Fig. 1.** Primary structure of methanobactin from *M. trichosporium* OB3b (Krentz *et al.*, 2010).

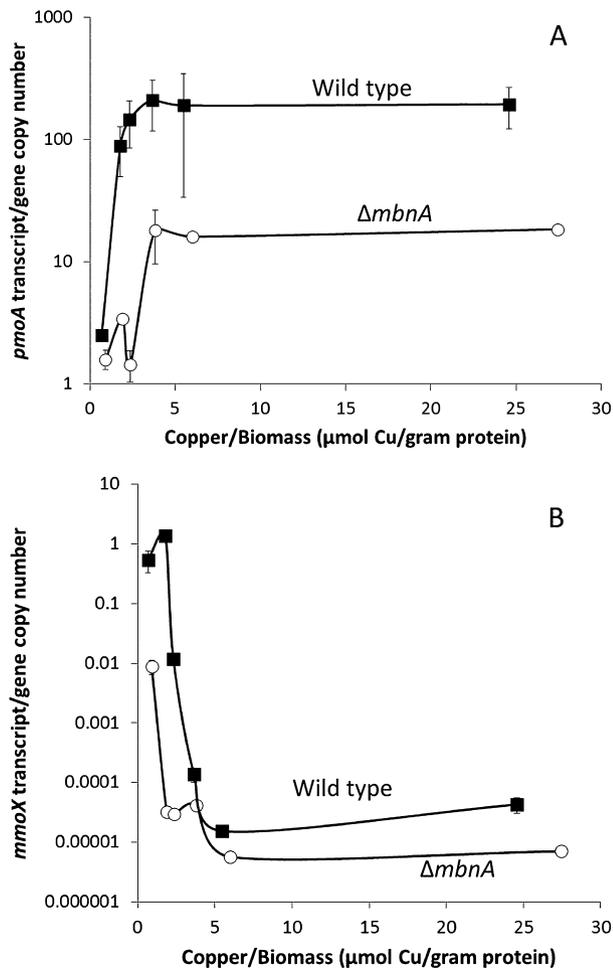
membrane-bound or particulate methane monooxygenase (pMMO; Stanley *et al.*, 1983). The sMMO, an iron-containing enzyme, is only expressed by a small number of methanotrophs and typically then only under conditions of copper deficiency (Dedysh *et al.*, 2000; 2004; Dunfield *et al.*, 2003; Semrau *et al.*, 2010; Vorobev *et al.*, 2011). Most extant methanotrophs, including those that have the ability to synthesize sMMO, also express pMMO, a copper containing enzyme, and such expression and activity increases with increasing copper availability (Lontoh and Semrau, 1998; Choi *et al.*, 2003), i.e. there is a 'copper-switch' that regulates the expression of the two forms of MMO. These two distinct MMOs have different kinetics for methane oxidation, with sMMO having a much higher turnover, but lower affinity for methane than pMMO (Lee *et al.*, 2006). As a result, the type of MMO expressed can have a significant impact on overall *in situ* methane consumption rates.

The copper requirement for  $\gamma$ - and  $\alpha$ -*Proteobacteria* methanotrophs expressing pMMO is quite high, approximately 10-fold higher than the copper requirement observed in other microorganisms (Nguyen *et al.*, 1994; 1998; Zahn and DiSpirito, 1996; Morton *et al.*, 2000a; 2000b; Choi *et al.*, 2003). As these methanotrophs have a high demand for copper, they have an effective mechanism to sequester greater than typical 'catalytic quantities' of copper from their environment (Semrau *et al.*, 2010). Indeed, aerobic methanotrophic bacteria of the  $\alpha$ - and  $\gamma$ -*Proteobacteria* have been found to synthesize and excrete a chalkophore (a siderophore-like molecule) known as methanobactin (Kim *et al.*, 2004; Choi *et al.*, 2010; Krentz *et al.*, 2010; El Ghazouani *et al.*, 2012). Previous studies have shown that methanobactin can increase the bioavailability of copper for methanotrophs, and as such, may play a role in the 'copper-switch' that controls expression of the two forms of MMO (Knapp

*et al.*, 2007). Other studies have also shown that that in the absence of copper, methanobactin will bind a variety of transition and near transition metals and may have a significant effect on the mobility of metals in soil and aquatic systems (Choi *et al.*, 2006a).

Methanobactins from five methanotrophs have been characterized. Each is a small (< 1200 Dalton) modified polypeptide with two five- or six-member rings (either imidazole, oxazolone or pyrazinedione rings) with associated enethiol groups that binds copper with affinities of greater than  $10^{21} \text{ M}^{-1}$  (Choi *et al.*, 2006b; Krentz *et al.*, 2010; Bandow *et al.*, 2012; El Ghazouani *et al.*, 2012). The first identified methanobactin, from *Methylophilum trichosporium* OB3b, is a modified polypeptide (Fig. 1) with two oxazolone rings that are responsible for the high-affinity binding of copper (Behling *et al.*, 2008). Acid treatment of methanobactin suggested that these oxazolone rings could be derived from proteinogenic amino acids, and thus methanobactin could be genetically encoded (Krentz *et al.*, 2010). Bioinformatic analysis by Krentz and colleagues (2010) of the sequenced genome of *M. trichosporium* OB3b (Stein *et al.*, 2010) revealed one open reading frame (orf) with a putative polypeptide precursor for methanobactin – **MTVKIAQKKVLPVIGRAAAALCGSCYPCSCM** (the polypeptide precursor region is underlined in bold).

The objectives of this study were to determine, using a combination of experimental and bioinformatic approaches, if this orf does indeed encode for the polypeptide precursor of methanobactin, what role methanobactin has in the copper-switch of methanotrophs, and how widespread the ability to produce methanobactin might be. The obtained results in this work lead us to propose a new model for the 'copper-switch' in methanotrophs and suggest that methanobactin production may not be limited to methanotrophs.



**Fig. 2.** Quantitative gene expression in *M. trichosporium* OB3b wild type and  $\Delta mbnA$  mutant. cDNA levels were normalized to the gene copy number as determined via qPCR on DNA.

A. *pmoA* expression in wild type (■) and  $\Delta mbnA$  mutant (○). B. *mmoX* expression in wild type (■) and  $\Delta mbnA$  mutant (○). Error bars indicate the standard deviation of triplicate samples. Symbol size is greater than the calculated standard deviation where error bars are not apparent.

## Results and discussion

### Construction of mutant strain defective in methanobactin production

Historically, genetic manipulation of methanotrophs has been difficult, due both to a limited set of useful methodologies for the introduction of foreign DNA into methanotrophs and to the small range of substrates these microorganisms can utilize for growth (i.e. methane and methanol). Despite these past difficulties, a mutant was constructed in this work in which the putative precursor gene of methanobactin (hereafter referred to as *mbnA* for *methanobactin* synthesis) was deleted by marker-exchange mutagenesis (Fig. S1). No methanobactin production was observed in the  $\Delta mbnA$  mutant of

*M. trichosporium* OB3b under any copper : biomass ratio, indicating that *mbnA* is essential for methanobactin synthesis, as determined via analysis of the spent medium and use of copper-chrome azural S (Cu-CAS) plates as specific assays for methanobactin production (Fig. S2). In contrast, as much as  $\sim 60 \mu\text{g}\cdot\text{ml}^{-1}$  was observed in the spent medium of wild-type *M. trichosporium* OB3b (Choi *et al.*, 2010; Semrau *et al.*, 2010), and Cu-CAS plates clearly showed uptake of copper by wild-type cultures (Fig. S2).

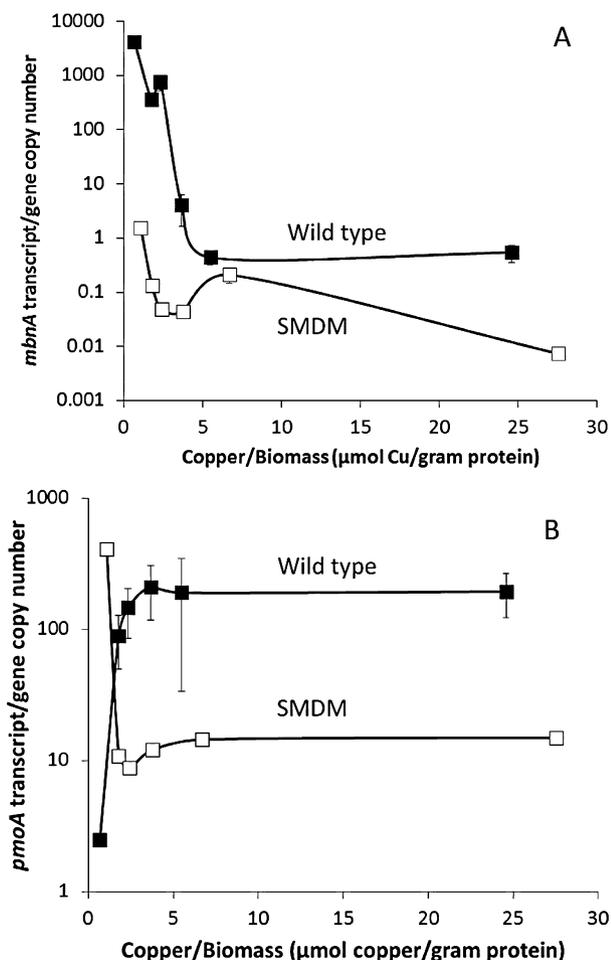
### Quantification of gene expression

Expression of *pmoA* and *mmoX* (genes encoding for key polypeptides of pMMO and sMMO respectively) in this mutant were then compared with wild-type *M. trichosporium* OB3b and quantified over a wide range of copper : biomass ratios using qPCR. As predicted, when the copper : biomass ratio increased, *mmoX* expression decreased by over four orders of magnitude in *M. trichosporium* OB3b wild type, whereas *pmoA* expression increased 78-fold (Fig. 2A and B; see Fig. S3 for qPCR calibration). It was also observed that although expression of *mmoX* and *pmoA* was reduced in the  $\Delta mbnA$  mutant as compared with *M. trichosporium* OB3b wild type regardless of the copper : biomass ratio, the expression pattern in the mutant was similar to that observed in wild type, i.e. as the copper : biomass ratio increased, *mmoX* expression decreased by three orders of magnitude while *pmoA* expression increased 10-fold.

Collectively, these data: (i) experimentally demonstrate that *mbnA* encodes for the peptide precursor of methanobactin, and (ii) surprisingly suggest that methanobactin is not essential for the copper switch regulating expression of genes encoding for polypeptides of pMMO and sMMO, although it amplifies the expression of MMO enzymes in *M. trichosporium* OB3b in response to copper ions.

### Analyses of an *mmo* deletion mutant

Analyses of a mutant of *M. trichosporium* OB3b in which the genes encoding for polypeptides of sMMO (*mmoXYBZDC*) had been deleted by marker-exchange mutagenesis (mutant SMDM; Borodina *et al.*, 2007), suggested that some element of the *mmo* operon is responsible for the copper switch. Quantitative PCR of cDNA and DNA of *mbnA* showed that its expression was orders of magnitude lower in the SMDM mutant than that found in wild-type *M. trichosporium* OB3b at low copper : biomass ratios. Unlike wild type, expression of *mbnA* remained low over the entire range of copper : biomass ratios investigated (Fig. 3A). Copper uptake by the SMDM mutant was evident from methanobactin assays using Cu-CAS plates



**Fig. 3.** Quantitative gene expression in *M. trichosporium* OB3b wild type and SMDM mutant. cDNA levels were normalized to the gene copy number as determined via qPCR on DNA.

A. *mbnA* expression in wild type (■) and SMDM mutant (□). B. *pmoA* expression in wild type (■) and SMDM mutant (□). Error bars indicate the standard deviation of triplicate samples. Symbol size is greater than the calculated standard deviation where error bars are not apparent.

(Fig. S2), indicating that methanobactin was synthesized. It should be noted that no other chalkophores have been identified in *M. trichosporium* OB3b, thus the positive results from the Cu-CAS plates can only be attributed to methanobactin.

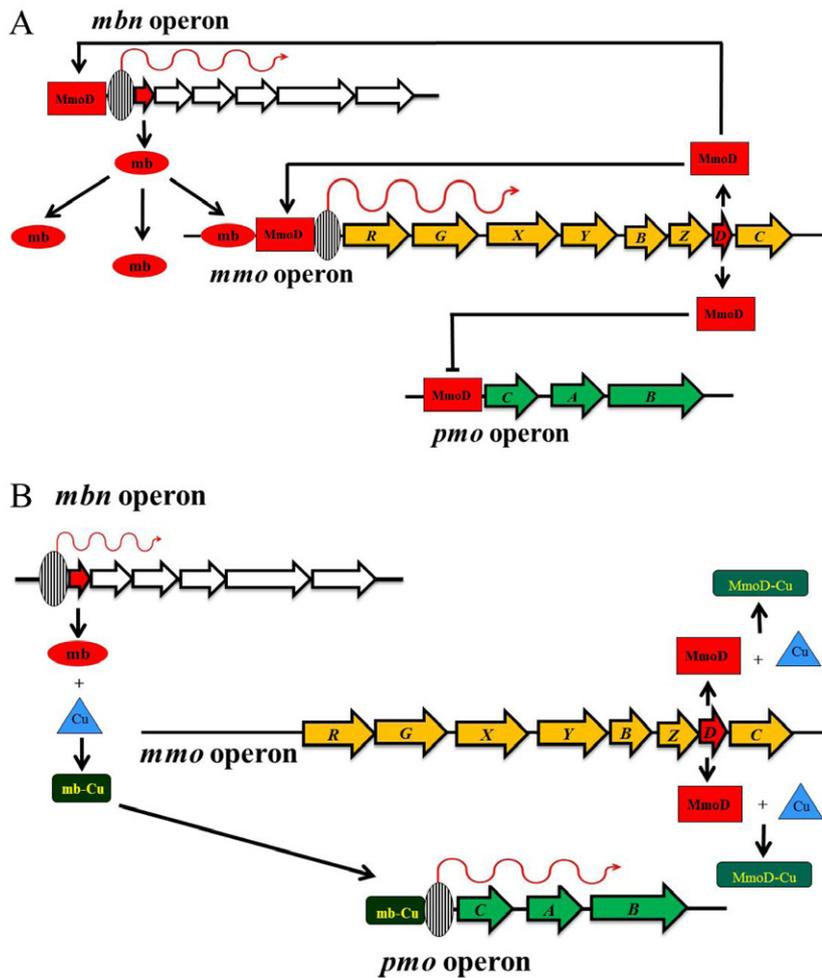
Moreover, expression of *pmoA* in the SMDM mutant was markedly different from that in the wild-type strain (Fig. 3B). In the SMDM mutant, *pmoA* expression was greatest in the absence of any added copper ( $1.1 \mu\text{mol copper-g protein}^{-1}$ ), and *pmoA* expression dropped  $\sim 40$ -fold when the copper biomass ratio was increased to  $1.8 \mu\text{mol-g protein}^{-1}$ . By comparison, in wild-type *M. trichosporium* OB3b, *pmoA* expression increased  $\sim 35$ -fold as the copper : biomass ratio changed from  $0.67$  to  $1.77 \mu\text{mol copper-g protein}^{-1}$ .

This suggests that the *mmo* operon itself strongly participates in the 'copper switch' of methane oxidation. Most of the genes of the operon already have clearly assigned functions. Genes *mmoX*, *mmoY* and *mmoZ*, encode for the polypeptides of the hydroxylase subunit of the sMMO (where methane oxidation and oxygen reduction occurs), while *mmoC* encodes for the reductase subunit (where the *in vivo* reductant binds) and *mmoB* encodes for a protein that shuttles electrons from the reductase subunit of sMMO to the hydroxylase subunit. It has been shown that MmoB serves to ensure sMMO acts as a monooxygenase and not a NADH oxidase (Green and Dalton, 1985; Lipscomb, 1994; Lee *et al.*, 2013). In contrast, *mmoD* encodes a protein of unknown function not essential for sMMO activity since it was not found in active purified preparations of sMMO (Fox *et al.*, 1989), although it has been speculated that it might be involved in copper sensing (Merx and Lippard, 2002). The primary structure of MmoD is predicted to bind DNA (Wang and Brown, 2006; Fig. S4), suggesting that it may be involved in the regulation of gene expression.

#### Proposed model for regulation of *mmo* and *pmo* operons

Given these findings, we propose a new general model (Fig. 4) for the regulation in *M. trichosporium* OB3b of expression of the *mmo* and *pmo* operons, as well as of the operon (hereafter termed the *mbn* operon) that includes gene *mbnA* encoding the methanobactin precursor peptide. During growth of *M. trichosporium* OB3b at low copper : biomass ratios (Fig. 4A), the MmoD protein represses expression of the *pmo* operon and also upregulates expression of the *mmo* and *mbn* operons. Methanobactin then serves to increase expression of the *mmo* operon, which results in more stringent repression of the *pmo* operon by MmoD. During growth at high copper : biomass ratios (Fig. 4B), methanobactin binds copper and can no longer enhance the expression of the *mmo* operon. This causes expression of the *mmo* operon to decrease, leading to reduced *mbnA* expression and thus reduced methanobactin production. Under these conditions, the MmoD protein binds copper and no longer represses expression of the *pmo* operon or induces expression of the *mmo* operon.

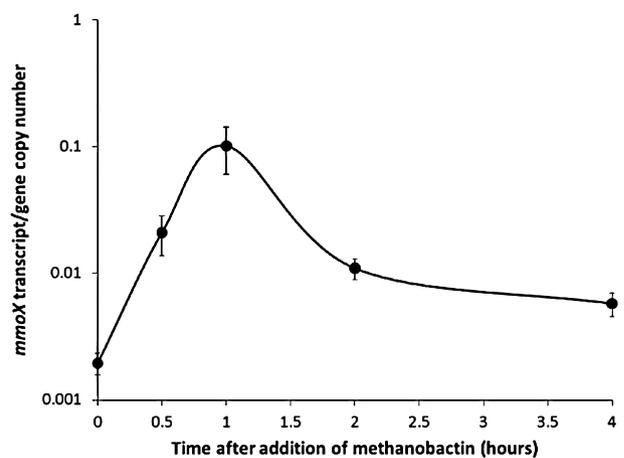
This model yields several testable hypotheses. For example, increased *mmoX* expression should be observed when methanobactin is supplied to the  $\Delta\text{mbnA}$  mutant cultured at a low copper : biomass ratio. Such experiments were performed, and indeed, *mmoX* expression increased 50-fold after the addition of  $20 \mu\text{g}\cdot\text{ml}^{-1}$  of methanobactin (Fig. 5). The model proposed here is also consistent with the phenotypes of the constitutive sMMO mutants, sMMO<sup>C</sup>, isolated by Phelps and colleagues (1992). Physi-



**Fig. 4.** Proposed regulatory scheme of *mno*, *pmo* and *mbn* operons by copper, methanobactin and MmoD: (A) low copper : biomass ratio; (B) high copper : biomass ratio.

ological characterization of two sMMO<sup>C</sup> mutants, PP319 and PP359, showed these mutants constitutively expressed both methanobactin, previously called copper-binding compound, and the sMMO, regardless of the copper concentration in the growth medium (Phelps *et al.*, 1992; DiSpirito *et al.*, 1998). Physiological characterization of the sMMO<sup>C</sup> mutants PP319 and PP359 suggested they were both defective in the acquisition system for copper-containing methanobactin (DiSpirito *et al.*, 1998). The model proposed here would predict a mutation in the copper-containing methanobactin uptake system would constitutively express both methanobactin and sMMO, as observed in both PP319 and PP359.

Finally, as shown in the general regulatory model Fig. 4, methanobactin and MmoD interact to increase expression of the *mno* operon. It is also possible that the methanobactin–copper complex may interact with other as yet unknown proteins, to enhance expression of the *pmo* operon.



**Fig. 5.** Quantitative expression of *mnoX* in a *M. trichosporium* OB3b  $\Delta mbnA$  mutant after addition of  $20 \mu\text{g}\cdot\text{ml}^{-1}$  methanobactin at  $t = 0$  h. cDNA quantities were normalized to the gene copy number as determined by qPCR on DNA. Error bars indicate the standard deviation of triplicate samples.

*Bioinformatic analyses*

Previously, analyses of published microbial genomes have shown that many bacteria – methanotrophs as well as heterotrophs – contain the methanobactin precursor gene, i.e. the methanotroph *Methylocystis* sp. SC2 and the heterotrophs *Azospirillum* sp. B510, *Tistrella mobilis*, *Gluconacetobacter* sp. SXCC-1, *Gluconacetobacter oboediens* and *Pseudomonas extremasutralis* (Krentz *et al.*, 2010; Haft *et al.*, 2013). More detailed analyses of the organization of the gene cluster shows that in these gene clusters, always immediately adjacent are two common genes, *mbnB* and *mbnC*, encoding for unknown functions, but possibly for peroxidase or dehydrogenase activity needed for conversion of the amino acids in the methanobactin precursor polypeptide to the observed oxazolone rings found in methanobactin (Fig. S5). Furthermore, in all gene clusters, genes belonging to families of proteins including MauG [a di-haem enzyme required for biosynthesis of tryptophan tryptophylquinone from tryptophan (Jensen *et al.*, 2010)] and TonB-dependent transporters are also located nearby to the putative *mbn* operon (Fig. S5), suggesting that these genes may be required for the biosynthesis of methanobactin and transport of methanobactin–copper complexes respectively. Transcriptional analyses using reverse transcription-polymerase chain reaction shows that *mbnA* is part of an operon that includes at least *mbnB* and *mbnC* (Fig. S6). Based on these findings, a hypothetical pathway for methanobactin maturation in *M. trichosporium* OB3b, requiring only three catalytic activities all potentially encoded by the methanobactin gene cluster, is proposed (Fig. S7). Further analysis of predicted methanobactin precursor polypeptide sequences shows that in all strains considered, the amino acid sequence is strongly conserved, with multiple cysteine residues spaced throughout that suggest that all may utilize cysteine residues for the formation of five- or six-member rings used for metal binding (Fig. S8). Finally, most intriguing is our finding of the localization of the methanobactin precursor peptide as a C-terminal extension of the RNA polymerase  $\sigma^{24}$  factor in the recently published genome of *Methylocystis* sp. SC2 (Dam *et al.*, 2012). To our knowledge, such a finding is unprecedented, and suggests unexpected complexities in the biosynthesis of methanobactin and its possible role in gene regulation, which warrant further detailed analysis.

In conclusion, we demonstrate that methanobactin, a recently discovered chalkophore produced by aerobic methanotrophs that play a vital role in the global carbon cycle, is genetically encoded and ribosomally synthesized. Given that methanobactin plays a critical role in methanotrophic activity, and has potential in therapeutic treatment of Wilson's disease (Summer *et al.*, 2011;

Zischka *et al.*, 2011), the discovery of its genetic basis provides opportunities to optimize methanobactin production and also to manipulate the activity of methanotrophs. The discovery of methanobactin peptide precursors and associated genes in non-methanotrophs also suggests that production of methanobactin may be more widespread in bacteria than previously thought. Finally, the elusive nature of the copper switch in methanotrophs involves MmoD, with methanobactin serving to amplify the magnitude of the bacterial response to copper. This likely represents a hitherto unknown but possibly general mechanism for metal-regulated gene expression in bacteria, and clearly warrants further detailed studies.

**Experimental procedures***Construction of  $\Delta mbnA$  and SMDM mutants*

Two DNA regions 3' and 5' of the orf encoding for the putative polypeptide precursor (*mbnA*) of *M. trichosporium* OB3b were amplified by PCR [arm A and arm B respectively (Fig. S1)]. Additional restriction site sequences were included into the primers to facilitate cloning of PCR products. Products A and B were digested with BamHI, separated by agarose gel electrophoresis and then purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The two arms were ligated and the ligation product used as template for PCR amplification to yield DNA fragment AB. Fragment AB was cloned into pK18mobsacB yielding construct pAK06. Plasmid p34S-Gm was cut with BamHI to produce a DNA fragment of 865 bp, which contained the gentamicin resistance (GmR) gene, which was cloned via the BamHI site between product A and B, to give the final targeting construct pAK066, which was used to transform *Escherichia coli* S17.1  $\lambda$ pir (Herrero *et al.*, 1990) by electroporation. Conjugation between the donor (*E. coli* S17.1 containing the pAK066 construct) and the recipient (*M. trichosporium* OB3b) strains was carried out according to previously described methods (Martin and Murrell, 1995). The resulting transconjugants were selected by plating onto NMS medium containing gentamicin (5  $\mu\text{g}\cdot\text{ml}^{-1}$ ). To ensure that no cells of *E. coli* remained as a background, colonies of the resultant *M. trichosporium* OB3b  $\Delta mbnA$  mutant were cultured in flasks containing nitrate mineral salt (NMS) medium supplemented with nalidixic acid (10  $\mu\text{g}\cdot\text{ml}^{-1}$ ). Identification of double homologous recombinants and the genotype of the cross-over mutant was confirmed by PCR and sequencing. The SMDM mutant was constructed and described previously (Borodina *et al.*, 2007).

*Growth conditions*

*Methylosinus trichosporium* OB3b and its  $\Delta mbnA$  and SMDM mutants were grown to late-exponential phase on NMS medium (Whittenbury *et al.*, 1970) at 30°C in 250 ml Erlenmeyer flasks shaken at 225 r.p.m. in methane-to-air ratio of 1 : 2. Varying concentrations of copper as  $\text{CuSO}_4$  (0.1  $\mu\text{M}$ , 0.2  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$  and 5  $\mu\text{M}$ ) were added to the medium. For SMDM and  $\Delta mbnA$  mutants, 5  $\mu\text{g}\cdot\text{ml}^{-1}$  and 2.5  $\mu\text{g}\cdot\text{ml}^{-1}$

gentamicin respectively, was added to the NMS medium. In one set of experiments, methanobactin was added to cultures of the  $\Delta mbnA$  mutant at a concentration of  $20 \mu\text{g}\cdot\text{ml}^{-1}$ . Cultures were grown to mid-exponential phase ( $\text{OD}_{600} \sim 0.7$ ) and then collected for subsequent assays of gene expression. Methanobactin was purified as previously described (Bandow *et al.*, 2011).

#### Protein estimation

Protein was measured using the Bradford assay (Bio-Rad Laboratories) after concentrating 5 ml of the culture to 1 ml and digesting the culture in 2 M NaOH (0.4 ml 5 M NaOH per 1.0 ml of culture) at  $98^\circ\text{C}$  for 15 min. A plot of protein concentration of cultures of *M. trichosporium* OB3b cells at densities ( $\text{OD}_{600}$ ) ranging between 0.2 and 0.9 yielded a linear regression coefficient of 0.9917. This correlation was used to calculate protein concentration for all cultures.

#### Extraction of DNA and RNA

Late-exponential-phase cells were harvested by centrifuging at  $5000 g$  for 10 min at  $4^\circ\text{C}$ . The cell pellets were suspended in 1 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 1% (w/v) hexadecyltrimethylammonium bromide (CTAB)], followed by bead beating and three freeze-thaw cycles. DNA was extracted using phenol-chloroform (Yoon *et al.*, 2011). RNA was extracted by resuspending the cell pellet in 0.75 ml of RNA extraction buffer (0.2 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer, pH 7.5; 5% CTAB in 2.4 M NaCl). This sample was subjected to bead-beating (three times, consisting of 30 s at 4800 r.p.m. with 1 min on ice between each cycle) in a 2 ml vial which consisted of 0.5 g of 0.1 mm zirconia-silica beads (Biospec Products),  $35 \mu\text{l}$  of SDS 20%,  $35 \mu\text{l}$  of laurylsarcosine 20% and  $750 \mu\text{l}$  of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The samples were then centrifuged at 14,000 r.p.m. for 5 min at  $4^\circ\text{C}$ . The aqueous phase was mixed with an equal volume of chloroform-isoamyl alcohol (24 : 1) and centrifuged at 14 000 r.p.m. for 5 min at  $4^\circ\text{C}$ . RNA was precipitated by adding  $\text{MgCl}_2$  (final concentration, 2.5 mM), 0.1 volume of 3 M sodium acetate and 0.7 volume of isopropanol and incubating overnight at  $-80^\circ\text{C}$ . RNA was recovered by centrifugation at 14 000 r.p.m. for 30 min at  $4^\circ\text{C}$ . The DNase treatment was carried out using the RNase free DNase set (Qiagen) in accordance with the manufacturer's instructions. The RNA samples were further purified using the RNeasy Plus kit (Qiagen) using gDNA eliminator columns and RNeasy Mini Spin columns according to manufacturer's instructions. To check for any DNA contamination, PCR was performed with extracted RNA as template. RNA was reverse transcribed to obtain cDNA by using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions.

#### Real-time quantitative PCR analyses

Real-time PCR (q-PCR) and real-time reverse transcription PCR (q-RT-PCR) were performed to quantify the expression of 16S rRNA, *pmoA*, *mmoX* and *mbnA* genes in *M. trichosporium* OB3b wild type, SMDM and  $\Delta mbnA$  mutants grown with

methane under varying copper concentrations. All cultures were grown in 100 ml of NMS medium. DNA and RNA subsequently extracted after the cultures reached an  $\text{OD}_{600}$  of  $\sim 0.7$ . To preserve samples, 5 ml of a stop solution (10% water-saturated phenol in 200 proof ethanol) to 50 ml of culture media. Previously developed primers were employed for *pmoA* and *mmoX* gene amplification (Knapp *et al.*, 2007). Specific primers for methanobactin gene (*mbnA*) were designed from sequences (NZ\_ADVE01000073.1) of the methanobactin gene cluster of *M. trichosporium* OB3b. The forward and reverse primers (5'-3') for *mbnA* orf amplification were TGGAACTCCCT TAGGAG GAA and CTGCAC GGATAGCAGAAC respectively, to produce an amplicon 107 bp in length. *pmoA*, *mmoX* and *mbnA* gene and transcript copy numbers were calculated from measured  $C_t$  values using calibration curves based on plasmid preparations with known, *pmoA* ( $10^7$ – $10^{12}$  per microlitre), *mmoX* ( $10^3$ – $10^{11}$  per microlitre) and *mbnA* ( $10^7$ – $10^{13}$  per microlitre) copy numbers (Fig. S3). Both DNA and mRNA were extracted from one culture at each copper-biomass ratio. qPCR (DNA as template) and qRT-PCR (cDNA as template) was then performed in triplicate for each targeted gene using RealMasterMix SYBR ROX solution (5 Prime, Gaithersburg, MD) according to the manufacturer's instructions. A three-step cycle, with an initial denaturation at  $94^\circ\text{C}$  for 2 min and 40 cycles of denaturation ( $94^\circ\text{C}$  for 15 s), annealing ( $52^\circ\text{C}$  for 20 s) and extension ( $68^\circ\text{C}$  for 30 s), was performed. The average of the transcript to gene copy ratio was then determined by dividing the measured gene expression by the measured gene copy number. The standard deviation of the ratio was then determined by propagating the error associated with both expression and gene copy number assuming that both measurements were independent using standard statistical techniques (Ku, 1966).

For measurement of *mbnA* from *M. trichosporium* OB3b after the addition of  $20 \mu\text{g}\cdot\text{ml}^{-1}$  of methanobactin, cultures were first grown in 3 l of NMS with no added copper. The culture was grown to a final  $\text{OD}_{600}$  of 1.5 to give a final copper to biomass of  $0.5 \mu\text{mol Cu mg}^{-1}$  protein. Five subsamples (50 ml each) of the initial 3 l culture were then added to 250 ml flasks for the measurement of *mmoX* expression before and after the addition of methanobactin ( $t = 0, 0.5, 1.0, 2.0$  and  $4.0$  h). At the designated time point, 5 ml of the stop mixture described above was then added to prevent further gene expression or degradation of mRNA. qPCR and qRT-PCR was then performed in triplicate using RealMasterMix SYBR ROX solution (5 Prime, Gaithersburg, MD) according to the manufacturer's instructions.

#### Detection of polycistronic transcripts from *mbn* operon

The following primer sets were used to detect polycistronic transcripts from the *mbn* operon: Primer 1F – GTGG GGCGATCAAGTAGGTA; Primer 1R – CGTGAAGTTGA AGCCGATCT; Primer 2R – CCATTGCTCGACCTTGCT; 3R – AGATCGGCGTCGATTCTAAC; Primer 4R – GCCAGAGC CATGAGCAAT. 1F was used in conjunction with all reverse primers to give PCR products of 230 bp (1F-1R), 572 bp (1F-2R), 1052 bp (1F-3R) and 1651 bp (1F-4R) respectively. PCR was performed using PCR supermix (Invitrogen) by heating to  $94^\circ\text{C}$  for 3 min, followed by 30 cycles of denaturation ( $94^\circ\text{C}$  for 1 min), annealing ( $48^\circ\text{C}$  for 1 min) and

extension (72°C for 1 min). A final extension step was carried out at 72°C for 10 min.

#### Characterization of methanobactin production

Production of methanobactin was determined using the Cu-CAS assay and by direct analysis of the spent media (Yoon *et al.*, 2010; Bandow *et al.*, 2011). If no methanobactin was detected in spent media, the spent media was passed through a dianion HP-20 column, and eluted as previously described by Bandow and colleagues (2012), to concentrate the sample at least 40-fold. The concentrated sample was then re-examined for the presence of methanobactin.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Construction of  $\Delta mbnA$  mutant via marker-exchange mutagenesis.

**Fig. S2.** Split NMS/50  $\mu$ M Cu-CAS plates for detection of chalkophore production over time by *M. trichosporium* OB3b wild type, SMDM mutant and  $\Delta mbnA$  mutant. Negative controls were constructed without any culturing of any micro-organism. Note decoloration of Cu-CAS agar by *M. trichosporium* OB3b wt and SMDM mutant, indicating production of methanobactin.

**Fig. S3.** qPCR standard curves for *mbnA*, *pmoA* and *mmoX*.

**Fig. S4.** BindN prediction of DNA-binding residues of MmoD. Binding residues predicted to be involved in DNA binding are labelled with '+' and labelled in red; non-binding residues labelled with '-' and labelled in green. Confidence of such prediction ranges from 0 (lowest) to 9 (highest).

**Fig. S5.** Methanobactin gene clusters in complete genomes. Gene clusters with unannotated methanobactin peptide precursors similar to that detected in *Methylosinus trichosporium* sp. OB3b (genes shown: MettrDRAFT\_3892 > MettrDRAFT\_3901; Accession No. NZ\_ADVE00000000; Krentz et al., 2010) were detected in *Azospirillum* sp. B510 (AZL\_007900 > AZL\_007960; Accession No. NC\_013854); *Methylocystis* sp. SC2 (BN69\_1382 – BN69\_1392; Accession No. NC\_018485); in *Methylocystis parvus* OBBP (Accession No. AJTV000000000); in *Tistrella mobilis* TMO\_3374 – TMO\_3368; Accession No. NC\_017956); in *Gluconacetobacter* sp. SXCC (SXCC\_01996 – SXCC\_01987 (Accession No. NZ\_AFCH000000000); an identical gene cluster was detected in *Gluconacetobacter oboediens*, (Accession No. CADT000000000). The position and orientation of the methanobactin precursor is shown by a red triangle. Black, Methanobactin biosynthesis cassette protein MbnB/DUF692 (TIGR04159, PF05114). Light grey, AZL\_007950 family protein, (TIGR04061), methanobactin biosynthesis cassette protein MbnC (TIGR4060, PF05114).

Violet, multi antimicrobial extrusion protein (MATE) (PF015544). Dark grey, putative aminotransferase (PF00155 or PF00202). Light green, AZL\_007920/MXAN\_0976 family protein (TIGR04052) often found near by MXAN\_0977 family. Dark green, di-haem enzyme, MXAN\_0977 family (TIGR04039, PF03150), a family of di-haem proteins related to the di-haem cytochrome *c* peroxidase and to MauG. Light blue, RNA polymerase sigma factor, sigma-70 family (TIGR02937, PF07638). Dark blue, TonB-dependent receptor plug domain, and TonB-dependent receptor (TIGR01783 or TIGR1785/TIGR1786, PF07660-PF07715-PF00593). White, other proteins with no significant identity to proteins encoded by the methanobactin gene cluster of *Methylosinus trichosporium* OB3b.

**Fig. S6.** RT-PCR of the *mbn* gene cluster. Polycistronic mRNA transcripts as noted by red lines were found that include *mbnA* and at least *mbnB* and *mbnC* (black and grey respectively).

**Fig. S7.** Proposed reaction scheme for oxazolone ring formation in the biosynthesis of methanobactin from its DNA-encoded peptide precursor. An identical tandem two-step sequence of peroxidation and dehydration reactions, flanking a central concerted cyclization reaction with a change in peptide backbone connectivity, is proposed for the formation of both oxazolone rings of methanobactin, from Leu1 and Cys2, and from Pro7 and Cys8 of the precursor peptide respectively. The cysteine thiol is shown protected against oxidation (-SR), possibly as a thioester formed by reaction with a carboxylate group of one of the gene products of the methanobactin gene cluster involved in oxazolone ring formation. Apart from the peroxidase activity of the MauG-like protein (dark green in Fig. S5), none of the proposed functions have yet been ascribed to a gene product encoded in the methanobactin gene cluster. A possible exception is the transaminase gene (Fig. S5), which may be involved in formation of the N-terminal keto-isopropyl group from Ile1 of the peptide precursor. Two other gene products (TonB-dependent receptor, MATE protein, Fig. S5) may be involved in secretion of mature methanobactin.

**Fig. S8.** Aligned methanobactin precursor peptide sequences. Potential methanobactin precursor peptide sequences were searched for directly on unannotated genome DNA sequences using the Fuzztran program from the Emboss bioinformatics suite implemented on the Mobyle portal (<http://mobyle.pasteur.fr>). For this, the sequence pattern [MI]-[ST]-[IV]-X-I-X(5)-[IL]-X-[VI]-X(2)-RA-[GS](0,1)-[AL]-X-C-[GA]-[ST]-X(0,2)-C(1,2), defined on the basis of previously detected methanobactin precursor peptide homologues (Krentz et al., 2010), was used. Sequence conservation is indicated by a colon, and sequence identity by a star. The position of the potential cleavage site during methanobactin maturation is shown by a hash. Cysteine residues involved in oxazolone rings and copper binding in mature methanobactin of *Methylosinus trichosporium* OB3b is highlighted in red, and those involved in a disulfide bridge are shown in orange. The cysteine residues in other putative methanobactin precursors are shown in blue.