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# Characterization of the role of *copCD* in copper uptake and the 'copper-switch' in *Methylosinus trichosporium* OB3b

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**One sentence summary:** Using specific gene knockouts, the basis of the 'copper-switch' that controls the activity of microbes (methanotrophs) used for the valorization of methane is clarified.
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## ABSTRACT

Methanotrophs or methane-oxidizing bacteria exhibit a unique 'copper-switch' where expression of two forms of methane monooxygenase (MMO) is controlled by the availability of copper. In the absence of copper, a cytoplasmic or soluble methane monooxygenase (sMMO) is expressed. In the presence of copper, a membrane-bound or particulate methane monooxygenase (pMMO) is expressed. These two forms of MMO have very different properties, and elucidation of the basis of the copper-switch is of significant interest as methanotrophs are becoming increasingly popular for the valorization of methane. Recently, it was suggested via characterization of a mutant of *Methylosinus trichosporium* OB3b that expresses sMMO in the presence of copper (smmoC mutant) that the copper-switch may be based on *copCD*. These genes encode for a periplasmic copper-binding protein and an inner membrane protein, respectively, and are used by other bacteria for copper uptake. Specific knockouts of *copCD* in *M. trichosporium* OB3b wild type, however, show that these genes are not part of the copper-switch in methanotrophs, nor do they appear to be critical for copper uptake. Rather, it appears that the constitutive expression of sMMO in the smmoC mutant of *M. trichosporium* OB3b may be due to multiple lesions as smmoC was generated via random chemical mutagenesis.

**Keywords:** methanotrophy; methanobactin; copper; CopCD; methane monooxygenase

## INTRODUCTION

Given the current low price of methane, methanotrophs are becoming more and more attractive as a biological platform for the valorization of methane to a diverse range of products. These include biofuels, plastic percursors and osmoprotectants (Strong, Xie and Clarke 2015). Successful use of methanotrophy for any of these processes, however, requires detailed knowledge of methanotrophic metabolism. Interestingly, methanotrophic conversion of methane into multicarbon compounds can be controlled to a large extent simply by manipulating growth conditions. In particular, it has been long known that copper strongly affects methanotrophic activity, i.e. the 'copper-switch', first described in 1983 (Stanley *et al.* 1983). That is, in the absence of copper, some methanotrophs will express a cytoplasmic or soluble methane monooxygenase (sMMO). This form of MMO has a broad substrate range, is able to oxidize both aliphatic and aromatic hydrocarbons, and characterized as having a high turnover but relatively weak affinity for methane (Colby,

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Stirling and Dalton 1977; Burrows *et al.* 1984; Lee *et al.* 2006). In the presence of copper, a membrane-bound or particulate methane monooxygenase (pMMO) is expressed. pMMO has a narrow substrate range—only able to attack small aliphatic compounds—but has a higher affinity for methane (Burrows *et al.* 1984; Lee *et al.* 2006).

It is of great interest to understand the basis of the copper-switch as this could be key for greater control of methanotrophic activity, and thus for the valorization of methane. Currently, there are conflicting models for this switch. One model, proposed by us, is based on *mmoD*, a gene within the operon for the sMMO (*mmo* operon). In prior work, we showed that when *mmoD* was knocked out in the methanotrophic type strain, *Methylosinus trichosporium* OB3b, expression of pMMO was inverted as compared to *M. trichosporium* OB3b wild type, i.e. pMMO expression in the mutant was highest in the absence of copper and decreased with the addition of copper (Semrau *et al.* 2013). In this mutant, however, not only was *mmoD* deleted, so were genes encoding for polypeptides of sMMO. As such, independent assessment of sMMO expression was not possible. Subsequently, Yan *et al.* (2016) selectively removed *mmoD* from *Methylomicrobium buryatense* 5GB1C. In this mutant, no sMMO activity or expression was observed in the absence of copper, supporting our hypothesis that MmoD is a transcriptional activator for the sMMO operon.

Others disagree with this hypothesis. It has been reported that MmoD, when expressed and purified from *Escherichia coli*, does not bind either copper or DNA (Kenney, Sadek and Rosenzweig 2016). Instead, it has been suggested that *copD*, encoding for a copper importer, may be involved in copper uptake and/or the copper-switch in methanotrophs (Kenney, Sadek and Rosenzweig 2016). The rationale for this hypothesis was developed by comparing the genome of *M. trichosporium* OB3b wild type with that of a mutant generated via random chemical mutagenesis that constitutively expressed sMMO (smmoC) in the presence of copper. Interestingly, the smmoC mutant did not collect copper, suggesting that the phenotype of this mutant was due to specific lesions in genes involved in copper uptake. Several differences were observed, perhaps most notably *copD* in smmoC has a 22-bp deletion. *copD*, encoding for an inner membrane protein, has been found to be involved in copper uptake in other bacteria, and is commonly in an operon with *copC*, encoding for a periplasmic copper binding protein (Cha and Cooksey 1993; Koay *et al.* 2005; Wijekoon *et al* 2015). Thus, it was speculated that *copD* may play a critical role in the copper-switch and/or copper homeostasis in methanotrophs. Both conclusions are somewhat controversial, given that independent laboratories conclude that MmoD is involved in the copper-switch, and the role of methanobactin, a well-characterized copper binding agent shown to be important for copper uptake (Gu *et al.* 2016), is not explicitly considered in this model. Here we describe the phenotype of specific mutants to elucidate the role of *copD* in both copper uptake and the copper-switch in *M. trichosporium* OB3b.

## MATERIALS AND METHODS

### Growth conditions

*Methylosinus trichosporium* OB3b wild type and Δ*mbnAN* mutant (Gu *et al.* 2017) were grown on nitrate mineral salt (NMS) medium (Whittenbury, Phillips and Wilkinson 1970) at 30°C in 250 ml side-arm Erlenmeyer flasks with shaking at 200 rpm as described before (Gu *et al.* 2017). $CH_4$ was added at a methane-to-air ratio of 1:2 as the sole source of carbon. The newly constructed *M. trichosporium* OB3b *copCD*::$Gm^R$ and Δ*mbnAN* + *copCD*::$Gm^R$ mutants were maintained in NMS with 2.5 $\mu$g/ml gentamicin. Copper (as $CuCl_2$) was filter sterilized and added to NMS medium when necessary. The optical density at 600 nm ($OD_{600}$) was measured with a Genesys 20 Visible spectrophotometer (Spectronic Unicam, Waltham, MA, USA). *Escherichia coli* Top10 and S17 (Simon 1984) were grown in Luria-Bertani medium at 37°C.

### Knockout of *copCD*

Marker exchange mutagenesis was used to knock out *copCD* in *M. trichosporium* OB3b wild type and the Δ*mbnAN* mutant using the pK18*mobsacB* vector (Semrau *et al.* 2013). Two sets of primers (Table 1) were used to amplify two 1-kb DNA fragments flanking *copCD*. These two arms were ligated together at a *BamHI* site, and cloned into pK18*mobsacB* at *EcoRI* and *HindIII* sites. A gentamicin cassette from pS34-Gm was then inserted between the two arms at the *BamHI* site to create the plasmid pWG014.

**Table 1.** Primers used in this study.

| Primer | Target gene | Sequence[a] (5′–3′) | Reference |
|---|---|---|---|
| Arm A forward | *copCD* | ATTTTTaagcttCCGTTATCGCTATGTCGGGT[b] | This study |
| Arm A reverse | | ATTTTAggatccAATCTCTCATCGCTGAAAGC[b] | |
| Arm B forward | | ATTTTTggatccCAGCTTCATTCTTCGGCTCC[b] | This study |
| Arm B reverse | | ATTTTTgaattcCCGTTCCTCTCGCTGTTTTGC[b] | |
| *copCD*_f | *copCD* | GCCGTATCGCCCTTGTTATG | This study |
| *copCD*_r | | GGAGCCGAAGAATGAAGCTG | |
| pK18_bb_F | pK18*mobsacB* | CTCTGGTAAGGTTGGGAAGC | Gu *et al.* (2016) |
| pK18_bb_R | | GCAATATCACGGGTAGCCAA | |
| qcopC_FO | *copC* | GATCCTCGACTCGACTGGC | This study |
| qcopC_RO | | TTTCACGACATAGCTCCCGA | |
| qcopD_FO | *copD* | CCTATCTCACGAGCCATCCC | This study |
| qcopD_RO | | GAGCGGTCGATCAGGAAATG | |
| qpmoA_FO | *pmoA* | TTCTGGGGCTGGACCTAYTTC | Knapp *et al.* (2007) |
| qpmoA_RO | | CCGACAGCAGCAGGATGATG | |
| qmmoX_FO | *mmoX* | TCAACACCGATCTSAACAACG | Knapp *et al.* (2007) |
| qmmoX_RO | | TCCAGATTCCRCCCCAATCC | |

[a]Y, S and R are the IUPAC DNA codes for the C/T, C/G and A/G nucleobases, respectively.
[b]Lowercase letters indicate EcoRI, BamHI or HindIII restriction site sequences included in these primers.

pWG014 was then conjugated into *M. trichosporium* OB3b wild type and Δ*mbnAN* with help of *E. coli* S17 (Simon 1984) to create *copCD*::Gm$^R$ and Δ*mbnAN* + *copCD*::Gm$^R$ mutants. Conjugation colonies were transferred onto fresh NMS plates supplemented with nalidixic acid, to remove *E. coli* and gentamicin, to select for recombinants. The recombinants were screened for successful double homologous recombination by checking for kanamycin sensitivity and sucrose resistance, indicating loss of plasmid backbone. The genotypes were confirmed by PCR and sequencing.

### Nucleic acid extraction and reverse transcription

Genomic DNA and total RNA were extracted from different strains at the same growth point in the late exponential phase. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. RNA was isolated and purified using the method described before (Semrau *et al.* 2013; Gu *et al.* 2016). DNA-free total RNA was treated with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) for reverse transcription of mRNA to cDNA following the manufacturer's instructions.

### Quantitative PCR analyses

Differential expression of selected genes was tested by quantitative PCR (qPCR). The primers used for RT-qPCR are listed in Table 1. The qPCR reactions were performed as described before (Gu *et al.* 2016). Calibration curves (Fig. S1, Supporting Information) for each gene were used to calculate the gene transcripts per ng RNA and copy numbers per ng of DNA. Expression levels were determined via the absolute quantification method, where the ratio of transcript to copy number was determined from cDNA and DNA, respectively. For each condition, at least duplicate biological samples were analyzed.

### Metal measurements

Copper associated with the methanotrophic biomass was determined using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies, Santa Clara, CA). Cultures at late exponential phase were harvested for acid digestion as described previously (Kalidass *et al.* 2015). The copper contents of digested cell suspensions were subsequently analyzed using ICP-MS with 2% nitric acid as background. The ICP standards of copper were purchased as 1000 ppm stocks and serially diluted to create standard curves with measured correlation coefficients of $> 0.999$. Protein concentrations were converted from cell densities as $OD_{600}$ using a correlation obtained from Bradford assay (Bio-Rad, Hercules, CA) (Semrau *et al.* 2013). For each condition, at least duplicate biological samples were analyzed.

### Measurement of sMMO activity

sMMO activity was assayed using the method by Brusseau *et al.* (1990). A total of 1.6 mL of cells in late exponential phase were incubated with a few crystals of naphthalene for 1 h at 30°C with shaking in 2 mL screw-cap tubes. The suspensions were then pelleted by centrifugation. Supernatant (1.3 mL) was transferred to a new tube and 130 $\mu$L of 4.21 mM tetrazotized o-dianisidine was added. The absorbance of the mixture at 528 nm was immediately measured by an ultraviolet-visible spectrometer (Specord 250 plus, Analytik, Germany).

### Statistical analyses

Two-tailed Student's *t*-tests were performed in Excel for different strains grown under different conditions to determine if gene expression, sMMO activity and/or copper uptake were significantly different ($P < 0.05$). At least two biological replicates were measured for each condition and were included in these analyses.

## RESULTS

Using marker exchange mutagenesis, *copCD* from wild-type *Methylosinus trichosporium* OB3b and the Δ*mbnAN* mutant (Gu *et al.* 2017) was knocked out creating *copCD*::Gm$^R$ and Δ*mbnAN*+ *copCD*::Gm$^R$. The mutants are gentamicin-resistant, indicating successful insertion of the Gm cassette, and kanamycin-sensitive and sucrose-resistant, showing loss of the plasmid backbone. The genotypes of these mutants were verified by PCR (Fig. S2, Supporting Information) and Sanger sequencing (data not shown).

The phenotype of the *copCD*::Gm$^R$ and *mbnAN* + *copCD*::Gm$^R$ mutants was compared with that of wild-type *M. trichosporium* OB3b and the Δ*mbnAN* mutant. When grown in the presence of either 0 (no added) or 1 $\mu$M copper, all strains had similar amounts of copper associated with biomass (Fig. 1). Furthermore, gene expression in all three mutants, as well as in *M. trichosporium* OB3b wild type, showed clear evidence of the copper-switch (Fig. 2). Specifically, in the presence of 1 $\mu$M copper, expression of *mmoX* decreased by several orders of magnitude as compared to when no copper was added. Furthermore, *pmoA* expression increased by about an order of magnitude when copper was added. Interestingly, *mmoX* expression was slightly (but significantly) higher in the *copCD*::Gm$^R$ and Δ*mbnAN* + *copCD*::Gm$^R$ mutants as compared to wild-type *M. trichosporium* and the Δ*mbnAN* mutant in the presence of 1 $\mu$M copper. It should be noted, however, that in the presence of copper *mmoX* expression was low for any strain, i.e. <0.1 transcripts per gene copy number. Furthermore, no sMMO activity was observed for any strain in the presence of copper as determined via the naphthalene assay (Brusseau *et al.* 1990) (Fig. 3). This strongly suggests that CopCD alone is not the cause of constitutive sMMO activity in the smmoC mutant.

To determine if expression of *copCD* was affected by the availability of copper, expression of both *copC* and *copD* was monitored via RT-qPCR (Fig. 4). Expression of *copC* and *copD* did not change with increasing copper in wild-type *M. trichosporium* OB3b. *copC* and *copD* expression did increase slightly (~1.5-fold; $P < 0.05$) in Δ*mbnAN* with increasing copper, but expression was in any case very low (< 0.1 transcripts per gene copy number).

## DISCUSSION

Via genomic sequencing of a constitutive sMMO-expressing mutant created by Phelps, *et al* (1992), a 22-bp deletion in *copD* was observed by Kenney, Sadek and Rosenzweig (2016). Given that this mutant not only expresses sMMO in the presence of copper but also has reduced copper uptake as compared to *Methylosinus trichosporium* OB3b, it was speculated that CopD may be involved in both copper uptake and the copper-switch of *M. trichosporium* OB3b. To test these hypotheses, we constructed a specific knockout of *copCD* in both wild-type *M. trichosporium* OB3b and a previously constructed mutant where genes encoding for methanobactin synthesis were removed (Δ*mbnAN*). All strains exhibited similar responses to copper, i.e. all strains were still

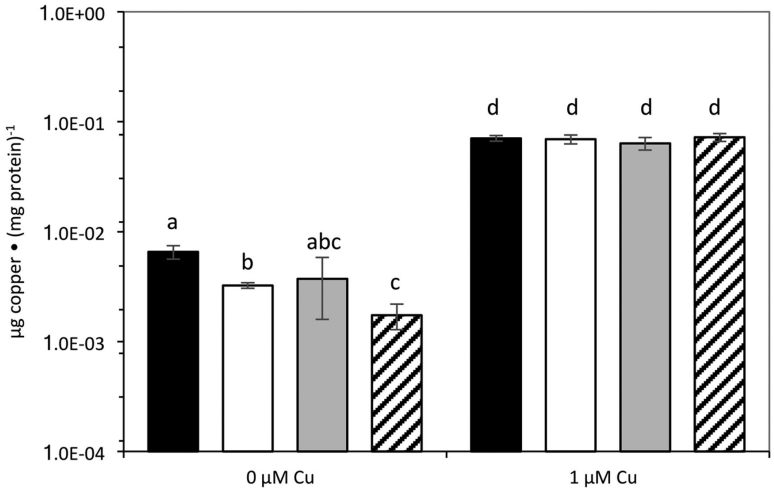


**Figure 1.** Copper associated with *M. trichosporium* OB3b wild type (black), Δ*mbnAN* (white), *copCD*::Gm$^R$ (grey) and Δ*mbnAN* + *copCD*::Gm$^R$ (striped) grown in the presence of either 0 or 1 $\mu$M copper. Columns indicated by different letters are significantly different ($P < 0.05$).

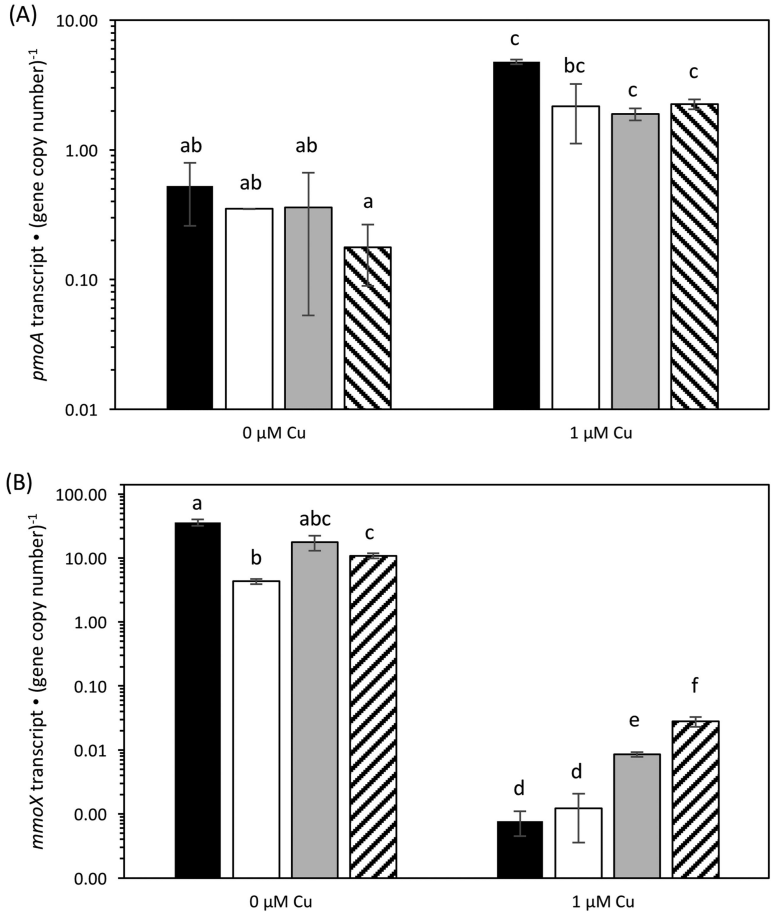


**Figure 2.** Expression of (**A**) *pmoA* and (**B**) *mmoX* in *M. trichosporium* OB3b wild type (black), Δ*mbnAN* (white), *copCD*::Gm$^R$ (gray) and Δ*mbnAN* + *copCD*::Gm$^R$ (striped) grown in the presence of either 0 or 1 $\mu$M copper. Columns indicated by different letters are significantly different ($P < 0.05$).

able to sequester copper and all strains exhibited the copper-switch. These data indicate that CopD likely does not play a role in the copper-switch. Furthermore, as discussed earlier, it is apparent that in addition to methanobactin, methanotrophs have a second mechanism for copper uptake (Balusubramanian, Kenney and Rosenzweig 2011; Gu *et al.* 2016). Given that the mutants defective in *copCD* still collected copper suggests that CopD and its partner CopC do not play a significant role in copper uptake, or that there is a third (unknown) mechanism by which *M. trichosporium* OB3b collects copper.

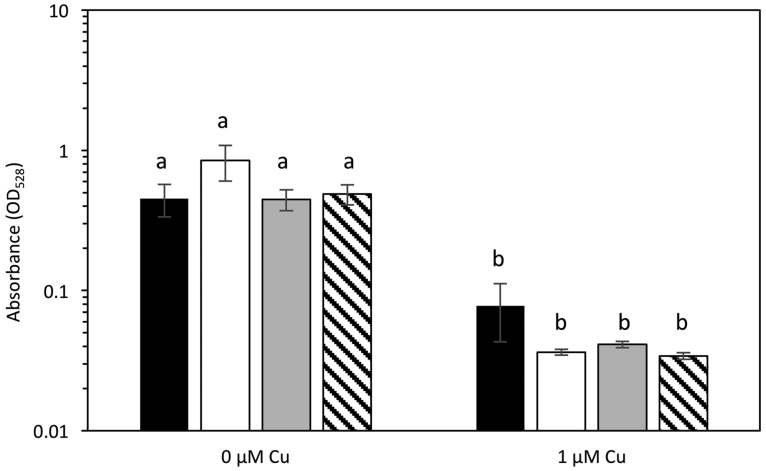


**Figure 3.** sMMO activities as determined via naphthalene oxidation. *Methylosinus trichosporium* OB3b wild type (black), Δ*mbnAN* (white), *copCD*::Gm$^R$ (gray) and Δ*mbnAN* + *copCD*::Gm$^R$ (striped) grown in the presence of either 0 or 1 $\mu$M copper. Columns indicated by different letters are significantly different ($P < 0.05$).

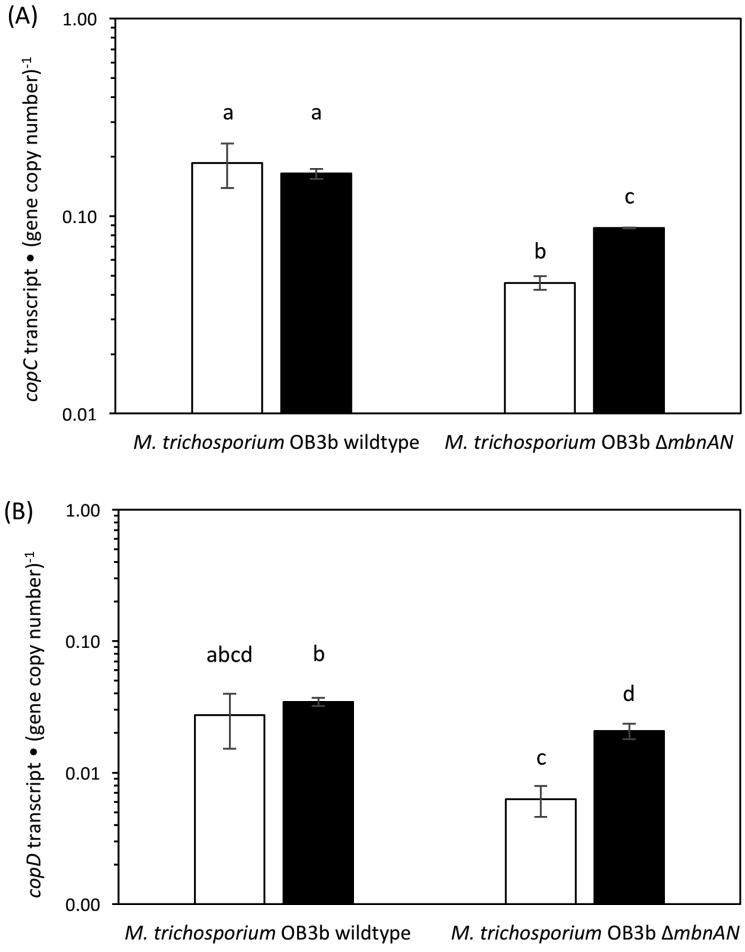


**Figure 4.** Expression of (**A**) *copC* and (**B**) *copD* in *M. trichosporium* OB3b wild type and Δ*mbnAN* grown in the presence of 0 (white) and 1 $\mu$M copper (black). Columns indicated by different letters are significantly different ($P < 0.05$).

Furthermore, we did not observe any changes in *copC* or *copD* expression with respect to copper in wild-type *M. trichosporium* OB3b, and only slight increases in the Δ*mbnAN* mutant. This is in contrast to the findings of Kenney, Sadek and Rosenzweig (2016) where mild upregulation in the presence of copper was reported. We cannot explain this difference, but the fact that at most mild increases have been observed in *copCD* gene expression in response to copper suggests that it may have a limited role in the copper-switch and/or copper uptake.

In conclusion, our selective knockout of *copCD* in *M. trichosporium* OB3b indicates that these genes, on their own, do not play a significant role in the copper-switch. Furthermore, if CopCD are involved in copper uptake, there is remarkable redundancy of copper uptake systems in *M. trichosporium* OB3b as copper uptake was observed when *copCD* was knocked out in either wild-type *M. trichosporium* OB3b or the Δ*mbnAN* mutant. Nonetheless, it is must be kept in mind that the smmoC mutant has no copper-switch. At this time, we cannot state why this is, but it should be noted that in addition to the 22-bp deletion in *copD*, several other lesions were noted in the genome of the smmoC mutant, including two kinases that may (or may not) be involved in copper uptake/sensing, as well as several hypothetical proteins. It may be that one of these lesions disrupted the copper-switch, or that some set of these mutations are responsible for the phenotype of the smmoC mutant.

## SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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***Conflict of interest.*** None declared.

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