Mutagenesis of a Copper P-Type ATPase Encoding Gene in *Methylococcus capsulatus* (Bath) Results in Copper-Resistance

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***Abstract*—Copper is an essential micronutrient for all living cells, however, it is extremely toxic at high concentrations and thus copper homeostasis is required to be tightly regulated. *copA* encodes for a copper-translocating P-type ATPase (CopA) and plays a vital role in copper homeostasis and is involved in copper transport across membranes of many organisms. Little is known about copper homeostasis in *Methylococcus capsulatus* although copper has a significant physiological role in this methanotroph. In this study we investigated the disruption of a CopA1 homologue (MCA2072; *copA1*) in *M. capsulatus* (Bath) by insertional inactivation mutagenesis. The resulting mutant, *M. capsulatus* Δ*copA1*, was copper resistant to elevated copper concentrations (100 μM) than the wild-type strain (80 μM). Furthermore, the intracellular copper measurements revealed that Δ*copA1* accumulated half the amount of copper when compared with the wild-type. No observed phenotypic difference between the mutant strain and wild-type related to growth at different silver concentrations. These observations suggest that *M. capsulatus* CopA1 has a key role in copper homeostasis.**

Index Terms*—CopA, copper homeostasis, P-type ATPases, *Methylococcus capsulatus

I. Introduction

Copper is a vital element required as cofactors for many enzymes that are essential for many for all living organisms. However, copper is extremely toxic at high concentrations [1]. Therefore, copper uptake and the intracellular copper quota must be precisely controlled. Many proteins are involved in coordination of copper homeostasis to be delivered to copper-containing proteins and sub-cellular compartments [2].

Copper homeostatic systems have been studied in both Gram-negative (*e.g*., *Escherichia coli*) and Gram-positive bacteria (*e.g*., *Enterococcus hirae*). In both examples, it has been shown that, *copA* encodes for a copper-translocating P-type ATPase (CopA) which is a main component in copper homeostasis and transports copper across membranes [2], [3]. P-type ATPases are a family of membrane proteins which are ubiquitous in all life forms, and they are acting as pumps for several ions. They do this function by utilizing the energy released from ATP hydrolysis to build an electrochemical potential gradient across the membranes [4]. The heavy metal transporters, P_{1B}-type ATPases, are a subgroup of P-type ATPases [5].

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Copper has a significant physiological role in *Methylococcus capsulatus* Bath which is a Gram-negative bacterium that utilizes methane, a potent greenhouse gas, as sole carbon and energy source [6], [7]. In this bacterium, the expression and activity of the key enzyme in methane metabolism; methane monooxygenase (MMO), is controlled by copper-to-biomass ratio [8]. There are two forms of MMO; one associated with intracytoplasmic membranes, the particulate methane monooxygenase (pMMO) and the cytoplasmic or soluble methane monooxygenase (sMMO). sMMO is expressed at low copper-to-biomass ratios and pMMO is expressed at high copper to-biomass ratios growth conditions [9]. Copper also increases the synthesis of an extensive network of intracytoplasmic membranes [10] and is the active center metal of pMMO [11]

Little is known about copper homeostasis in *M. capsulatus* although copper has a significant physiological role in this methanotroph. The genome sequencing of *M. capsulatus* led to the identification of four copper transport homologues [12]. Among them, three copper translocating P-type ATPase homologues; MCA0705, MCA0805 and MCA2072 were identified. The current study focuses on one of these genes, which is a copper translocating P-type ATPase homologue; MCA2072 (*copA1*). It is of interest to understand the copper transport system in this methanotroph due to the essential role of copper in regulating MMO expression. The aim of the current study was therefore to explore whether CopA is involved in the copper trafficking in *M. capsulatus*. To achieve this goal, a targeted mutagenesis approach was used to generate a mutant in *M. capsulatus copA1*. The mutant strain, Δ*copA1*, was subsequently characterized and compared to wild-type *M. capsulatus*.

II. Materials and Methods

Growth media and strains: Nitrate mineral salt (NMS) medium [13] was used to grow *M. capsulatus*. NMS agar plates were prepared with the addition of 2 % (w/v) Bacto (Difco) agar before autoclaving. *M. capsulatus* grown on NMS agar plates was incubated in a methane-rich atmosphere, in a gas-tight container, at 45 ^{o}C. During the 5-8 days incubation, methane was replenished about 3-4 times until colonies formed. *M. capsulatus* was grown in 250 ml Quickfit conical flasks which contained 50 ml NMS medium, sealed with suba-seals, gassed with 20 % v/v methane and incubated at 45 ^{o}C on a shaking incubator at 200 r.p.m. Growth was monitored by measuring the optical density (OD_{540} nm). Strains of *Escherichia coli* were grown on Luria-Bertani (LB) agar

plates [14]. The filter-sterilized antibiotics were added to media as required at the following final concentrations: kanamycin (25μg ml^{-1}) or gentamicin (5 μg ml^{-1}). All bacterial strains, plasmids and primers used in this study are shown in Table I.

TABLE I: BACTERIAL STRAINS, PLASMIDS AND PRIMERS USED IN THE STUDY.

Strain, plasmid or primer	Description	Source/ reference
Strains		
M. capsulatus (Bath)	Wild-type	University of Warwick Culture Collection
*M.capsulatus ΔcopA1*1	Δ MCA2072 (*copA*1); Gm^R	This study
E. coli S1 7.1 λ*pir*	*recA1 thi pro hsdR*⁻ RP4-2Tc::Mu-Km:: Tn7λ*pir*	[15]
Plasmids		
pK18mob	Km^R; RP4-mob, mobilizable cloning vector	[16]
pAK444	pCR2.1–TOPO containing 1067 bp *copA1* fragment	This study
pAK04	Km^R, pK18mob*sac*B with 1,513 bp *copA1* fragment *Eco*RI – *Hin*dIII insert	This study
pAK044	Gm^R, Km^R pK18mob*sac*B with 1,513 bp *copA1* fragment *Eco*RI –*Hin*dIII insert	This study
Primers		
COPA1F635-*Eco* RI	5' **GAATTC**CCCTCGAACGCA TGCAAATC 3'	This study
COPA1R2147-*Hin*dIII	5' **AAGCTT**AAACCGCGTTGA AGGAGGTG 3'	This study
US_COPA1_F13	5' TCGGTATGCTCAGGGTGT TG 3'	This study
DS_COPA1_246 4	5' GTGCCTTCTTCGAGCTTG AC 3'	This study
GENF37	5' GACATAAGCCTGTTCGGT TC 3'	This study
GENR851	5' GCGGCGTTGTGACAATTT AC 3'	This study

A. DNA Manipulation

Genomic DNA of *M. capsulatus* was extracted and stored at -20 °C. Plasmids preparations were extracted and purified from *E. coli* cultures using the QIAprep Miniprep Kit (Qiagen) according to the manufacturer's instructions.

B. Polymerase Chain Reaction (PCR)

PCR amplifications were carried out in 50 μl total volume of reaction mixtures using a Hybaid Touchdown Thermal Cycling System. *Taq* DNA polymerase and dNTPs were obtained from Fermentas. Primers used to amplify target DNA were synthesized by Invitrogen (Table 1). Amplification was performed using 30 cycles of 94 °C for 1 min, 55 °C annealing temperature for 1 min and extension at 72 °C for 1 min per 1kb of DNA amplified, followed by a final extension step at 72 °C for 10 min.

C. Cloning

An insertional inactivation mutagenesis technique was used to disrupt *copA1*, to determine the function of this gene. *copA1* DNA fragments were amplified using the primers COPA1F635-*Eco*R1 and COPA1R2147-*Hin*dIII. The purified DNA fragments were cloned into pCR2.1-TOPO to give the constructs pAK444. Then, *copA1* DNA fragments were cloned to plasmid vector, pK18mob*sacB* via *Eco*R1 and *Hin*dIII restriction sites respectively, to give the constructs pAK02. The gentamicin resistance cassette (Gm^R) was cloned via the *Pst*1 restriction site in the *copA3* DNA fragments to give the final constructs, pAK044, which were electroporated into *E. coli* strain S17.1 λpir [15]. This was then used as a donor strain for conjugating targeting constructs into *M. capsulatus*.

D. Conjugation

Conjugation of plasmid from *E. coli* into *M. capsulatus* was based on the method of Martin & Murrell [17].

1) Confirmation of the genotype of *ΔcopA1*

Screening of the transconjugants was carried out by plating the resulting strains onto NMS plates supplemented with gentamicin. Then, PCR amplifications were performed using primers specific for gentamicin cassette and for the flanking regions of the target *copA1*. The existence of gentamicin and kanamycin resistance cassettes in the mutants was confirmed by PCR using specific primers (data not shown). The inactivation of *copA1* was verified using PCR amplification with primers DS_COPA1_R2464 and GENF37, which were specific for the 3' region of this gene and for gentamicin cassette respectively. The PCR products were sequenced for further confirmation of the mutants. The primers used to confirm the genotype of the mutants are listed in Table 2. Mutants were designated as *Mc. capsulatus* Δ*copA1*. A schematic representation of the strategy used for constructing *M. capsulatus* Δ*copA*1 outlined in Fig. 1.

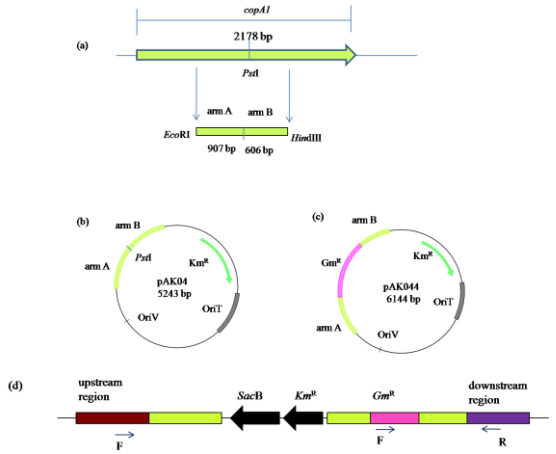

Fig. 1. Schematic representation of the strategy for constructing *Mc. capsulatus*Δ*copA1*(a) the wild-type gene (*copA1*) and the target region is highlighted by arrows; (b) The intermediate plasmid construct pAK04 with *copA1* fragment, restriction sites *Eco*R1 and *Hin*dIII were introduced by PCR to facilitate cloning; (c) The suicide plasmid construct pAK044 used to inactivate *copA3* and (d) Δ*copA1* following single homologous recombination of pK18*mobsacB*. Small/horizontal arrows indicate the primers used to check the genotype of the mutants.

2) Determination of Minimum Inhibitory Concentrations (MIC) for copper and silver

Metal sensitivity of the Δ*copA*1 and wild-type strains was determined by testing the ability of cells to grow on NMS plates supplemented with varying concentrations of copper (10-120 μM) or silver (1-7 μM). Copper was added as filter-sterilized $CuSo_4.5H_2O$ while silver was added as

$AgNO_3$. To ensure the strains compared were physiologically similar, they were grown in NMS with no-added copper to late exponential phase (OD_{540}~ 0.5) which were then diluted 100 times and 20 µl were spread on NMS agar plates (in triplicates). After 7 days of incubation at 45 ℃ in the presence of methane, the MIC of copper or silver was recorded as the minimum concentrations tested at which no colony formation was observed.

3) Growth of M. capsulatus at different copper concentrations

Growth patterns of Δ*copA*1 and wild-type strains growing on NMS medium supplemented with 0, 10, 30 and 50 µM copper was monitored by measuring the OD_{540}. Growth experiments were done in triplicates.

4) Determination of intracellular copper concentrations

The effect of *copA* mutagenesis on the intracellular copper accumulation of the *copA*1mutant compared to the wild-type organism was investigated. Cultures were grown on NSM medium with added 30 µM copper, at 45 ℃ in the presence of methane. Cells were centrifuged at 7,000 x g for 10 min and cell pellets were dried and dissolved in 3 ml trace metal-free grade nitric acid (Sigma). Samples were analyzed for ^{63}Cu content using a 7500 series inductively coupled plasma mass spectrometer (Agilent Technologies, USA) equipped with a cross-flow nebulizer and a quartz spray chamber. Calibration was achieved using external copper ICP-MS standards (Sigma, UK) and ^{166}Er as an internal standard. Each sample was measured in triplicate.

5) Naphthalene oxidation assay for sMMO activity

To study the effect of the *copA* mutagenesis on the sMMO expression, *M. capsulatus* wild-type and the Δ*copA*1 were tested for their ability to oxidise naphthalene using methods described previously [17]. Strains were grown on NMS medium supplemented with either 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 or 5.0 µM copper (as copper sulfate). Colonies were tested by adding tetrazotized-o-dianisidine solution, which when develop a purple colour indicate sMMO expression and activity.

6) Determination of whole-cell cytochrome oxidase activity

To investigate the role of CopA1 in the activity of cytochrome oxidase activity, cellular oxidase activity was tested in mutant and wild-type strains at two different copper regimes. Whole cells cytochrome oxidase activity was assayed for according to the method of Frangipani and Haas [18]. The molar extinction coefficient with a 1 cm path length for TMPD was 6.1 mM^{-1} cm^{-1} [19]. The activity was expressed in pmol TMPD oxidized min^{-1} $(mg\ dw)^{-1}$.

Statistical analysis

Differences between two means were tested using a t-test. All data tested to 95% significance value.

Bioinformatic analyses of CopA1 protein from *M. capsulatus*

CopA1 amino acid sequences from *M. capsulatus* and representatives of well-characterized metal-ion-transporting ATPases; *Enterococcus hirae* CopA, *Ent. hirae* CopB [2], *E. coli* CopA, *Synechococcus elongates* PacS and *Synechococcus* sp. CtaA [20] were retrieved via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using PRALINEPSI strategy of the freely available PRALINE http://www.ibi.vu.nl/programs/pralinewww/ [21].

7) CopA1 protein topology

The total number of the transmembrane helices of *M. capsulatus* CopA1 was carried out using Tied Mixture Hidden Markov Model (TMHMM), http://www.cbs.dtu.dk/services/TMHMM/.

III. Results

A. Disruption of copA1

To determine the function of *copA*, insertional inactivation mutagenesis was carried out and the resulting mutant was designated *M. capsulatus* Δ*copA1*.

1) Minimum Inhibitory Concentrations (MIC) for copper and silver

Δ*copA1* mutant strain was more resistant to elevated copper concentrations than the wild-type organism. Δ*copA*31 was found to grow at 100 µM, while the wild-type organism grew only at 80 µM added copper (Table II). Both the wild type and the mutant strains could grow on NMS plates supplemented with copper concentration up to 70 µM while neither of them grew at above 110 µM (data not shown). These results suggested that CopA of *Mc. capsulatus* might have potential roles in copper homeostasis in *Mc. capsulatus*.

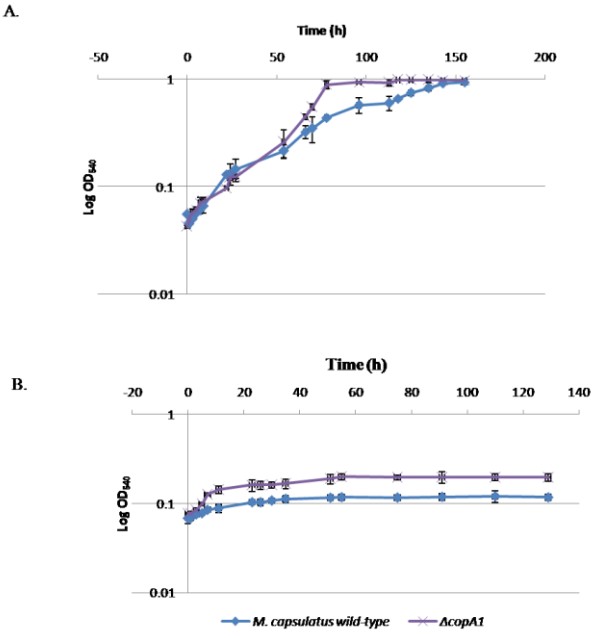

Fig. 2. Growth of *M. capsulatus* wild-type and Δ*copA*1 mutant strains on NMS amended with A, 30 µM and B, 50 µM added copper. All data points represent the mean of three replicates and error bars indicate the standard deviation.

No obvious phenotypic difference between the wild-type and mutant strains was observed related to silver. Both grew on NMS plates supplemented with silver concentration up to 4 µM but neither of them grew at above 5 µM (Table II). Also, it was observed that as the concentration of the added silver

increased, the growth of both the Δ*cop1* mutant and the wild-type decreased indicating that the *mutagenesis* of *copA1* of *Mc. capsulatus* had no effect on silver resistance or sensitivity

2) Growth of M. capsulatus at different copper concentrations

The results obtained in Fig. 2 showed Δ*copA1* seemed to grow relatively well at high copper concentrations (30 and 50 µM copper). Specific growth rates (µ) were 0.035 h^{-1} and 0.005 h^{-1} respectively. Nevertheless, wild-type strain struggled to grow at 50 µM (Fig. 2B) and exhibited significant differences in specific growth rate doubling time (P <0.001) (Table 2). No significant differences in growth patterns between Δ*copA*1 and wild-type grown at no added-copper at 10 µM copper (data not shown). These results were in general consistent with MIC data and the intracellular copper measurements.

3) Determination of intracellular copper

The intracellular copper concentration of Δ*copA*1 mutant strain was lower than that of the wild-type (Table 2). The mutant was accumulated half the amount of copper (51 ng (mg drywt biomass)$^{-1}$) when compared with the wild-type (99 ng (mg drywt biomass)$^{-1}$) (P < 0.001). The background copper concentration of the NMS medium with no-added copper is about 0.8 µM.

4) Naphthalene oxidation assay for sMMO activity

There was no difference between ΔcopA1 and the wild-type in terms of sMMO activity using the naphthalene assay, as can be seen in Table 2. At less than 2.0 µM added copper, both strains gave a purple colour upon addition of the diazonium salt, indicating that they expressed the sMMO enzyme. None of the strains expressed sMMO during growth under high copper concentrations (above 2.0 µM) as shown by no colour change with the naphthalene assay.

5) Cytochrome oxidase

There was no significant difference between Δ*copA1* strain and the wild-type oxidase activity under the copper conditions tested (no-added and 10 µM copper). These results indicated that inactivation of *copA*1 of *Mc. capsulatus* might not affect the cytochrome oxidase activity.

6) Bioinformatic analyses and protein topology of CopA protein from M. capsulatus

The amino acid identities between CopA and known P-type ATPases; *Enterococcus hirae* CopA, *Ent. hirae* CopB [2], Sequence alignment analyses revealed high homology of CopA1 protein from *M. capsulatus* to the well-characterized metal-ion-transporting ATPases from other organisms.

E. coli CopA, *Synechococcus elongates* PacS and *Synechococcus* sp. CtaA [20] ranged from 37 – 46%. Furthermore, eight membrane-spanning helices were predicted in *M. capsulatus* CopA. These proteins contained a heavy-metal-binding motif (CXXC) in which cysteine residues are invariant (Fig. 3A) and a transmembrane metal-binding binding site, a cysteine-proline-cysteine (CPC) motif (Fig. 3C). *M. capsulatus* CopA contained also a conserved TGES motif (Fig. 3B), an invariant phosphorylation site (DKTGTL) (Fig. 3D) and (6) ATP binding domain (GDGINDAP) (Fig. 3E).

TABLE II: DIFFERENCES BETWEEN *M. CAPSULATUS* WILD-TYPE AND Δ*COPA*1 MUTANT STRAIN.

Strain	*M. capsulatus* wild-type	Δ*copA*1
MIC for copper (µM)	80 ±5	100±5
Specific growth rate (h^{-1}) at 30 µM	0.016±0.001	0.035 ±0.005
Doubling time (h) at 30 µM	41±0.9	19.8 ±0.04
Specific growth rate (h^{-1}) at 50 µM	0.007 ±0.0004	0.005 ±0.0006
Doubling time (h) at 50 µM	99 ±5	86 ±3
Intracellular copper concentration (ng copper (mg drywt biomass)$^{-1}$)	99±5	51±4
Naphthalene assay for sMMO activity at 0, 0.5, 1.0, 1.5 10 µM added copper	positive	positive
Naphthalene assay for sMMO activity at 2.0, 2.5, 3.0, 3.5, 4.0 or 5.0 10 µM added copper	negative	negative
Cytochrome oxidase (no added copper) pmol TMPD oxidized min^{-1} (mg dw)$^{-1}$	144±29	149±7
Cytochrome oxidase (10 µM added copper) pmol TMPD oxidized min^{-1} (mg dw)$^{-1}$	109 ±15	113±8

MIC, Minimal Inhibitory Concentrations

IV. DISCUSSION

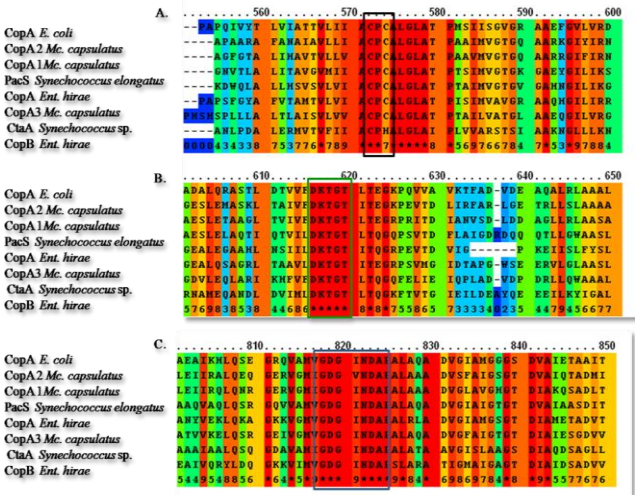

Fig. 3. Sequence alignment of *M. capsulatus* CopA1 (*accession* no. YP113215), CopA2 (accession no. YP113305) and CopA3 (accession no. YP114502) *Enterococcus hirae* CopA (*accession* no. AAA61835), *Ent. hirae* CopB (*accession* no. AAA61836), *E. coli* CopA (accession no. Q59385), *Synechococcus elongatus* PacS (*accession* no. BAA03907), *Synechococcus* sp. CtaA (*accession* no. AAB8202) proteins indicating: A, conserved CXXC motif; B, TGES motif; C, CPC motif; D, conserved DKTGTL motif and E, GDGINDAP motif . (unconserved 0 1 2 3 4 5 6 7 8 9 10 conserved).

Sequence alignment analyses CopA1, from *M. capsulatus* and those from well-characterized CopA from other bacteria, suggested *M. capsulatus* CopA1 is a P_{1B}-type ATPases. CopA share the characteristic features of this group of ATPases; eight transmembrane regions; N-terminal heavy-metal-binding motifs; a highly conserved phosphorylation site and ATP binding domain [22].

Furthermore, we disrupted *copA*1 and the resulting Δ*copA*1 strain was more resistant to elevated copper concentrations compared to the wild-type, suggesting that this ATPase plays a role in copper homeostasis in *M. capsulatus*. Our results were consistent with those obtained in *Escherichia coli*, CopA. These results are in line with the results obtained for *Ent*. *hirae* CopA, which was assumed to import copper into the cells [19]. Although a null in CopA of *Ent*. *hirae* showed no change in copper resistance, unlike the wild-type, it could not grow after two generations in copper-limited growth conditions [23]. These results suggested that CopA of *M*. *capsulatus* might have potential roles in copper homeostasis in *M*. *capsulatus*.

No obvious phenotypic difference between the Δ*copA*1 and the *M*. *capsulatus* wild-type was observed related to growth on NMS supplemented with different silver concentrations. This indicated that, unlike copper, the disruption of *copA1* did not confer silver resistance or sensitivity. Such findings coincide with those obtained from copper-resistant *Synechococcus* Δ*ctaA* mutant strain which showed similar response to silver compared to the wild-type [24]. However, such similarity in phenotype between the *M*. *capsulatus* Δ*copA* and parent strain with respect to silver, does not necessarily mean that CopA does not transport silver. In support of that, *Ent. hirae* CopB which did not confer silver resistance to cells, could transport copper and silver in *vivo* with similar rate [2], [20] . This is also indicated that some P-type ATPases share substrate specificity for copper and silver due to chemical similarities between these metals [5]

Δ*copA1* mutant accumulated only half the copper concentration of the wild-type. This could be attributed to partial impairment of copper trafficking of the cells. These results are in harmony with those of growth patterns at elevated copper conditions. Cells of Δ*copA1* were more resistant to higher levels of copper than the wild-type. Similar results were obtained by Fitch *et al*. [25] who noticed that *Ms*. *trichosporium* mutants accumulated lower intracellular copper levels than the wild-type. They explained the mutant phenotype by the possibility of defects in copper uptake [26]. Furthermore, Lewinson *et al*. [27] reported a copper-importing P-type ATPase, HmtA (heavy metal transporter A), in *P*. *aeruginosa* Q9I147 and confirmed their claim by many *in vivo* assays including, intracellular metal measurements. These results supported the proposal that *Mc*. *capsulatus* CopA have roles in copper homeostasis.

The results obtained from the naphthalene oxidation assay highlighted that the CopA1 is not involved in MMO regulation as indicated by the same response to an sMMO assay. Generation of a constitutively expressing sMMO mutant might need inactivation of more than one copper transporter.

There was no significant difference between Δ*copA1* strain and the wild-type oxidase activity under the copper conditions tested. Contrary to this observation, disruption of *Rubrivivax gelatinosus ctpA*, a gene encoding a copper-translocating P1B-type ATPase, showed a drastic decrease in both cbb_3 and caa_3 oxidase activities but no copper-sensitive phenotype was obtained. This result indicated that *R. gelatinosus* the CtpA has a role in cytochrome oxidase assembly but is not essential for copper resistance [28]. These findings suggest that *M. capsulatus* CopA1 might not have a role in cellular cytochrome oxidase assembly and/or activity.

Two copper-uptake systems have been observed in *M. capsulatus*, one is through methanobactin; a copper-binding, siderophore-like, chromopeptide that is produced a number of methanotrophs [29]-[32] and the other via the outer membrane protein; MopE [33], [34] Both of them presumably contribute to copper homeostasis in *M. capsulatus*, however, their interaction with CopA has yet to be investigated. To our knowledge this is the first study investigating the disruption of a P-type ATPase from *M. capsulatus*. The data described herein suggest *M. capsulatus* CopA1 has a vital role in copper homeostasis.

ACKNOWLEDGMENTS

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