A copper P-type ATPase in *Methylococcus capsulatus* (Bath) has a role in copper homeostasis

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Abstract*—Methylococcus capsulatus* (Bath) is a Gram-negative, spherical-shaped bacterium that utilizes methane, a potent greenhouse gas, as its carbon and energy sources thus alleviating global warming. Copper is an essential trace element for the physiology this bacterium, however, it is highly toxic at elevated levels. Therefore, copper homeostasis must be tightly regulated. Out of different proteins involved in copper homeostasis, CopA, a copper-translocating P-type ATPase (CopA), which is involved in copper transport across membranes of various organisms. Little work has been done on copper homeostasis in methanotrophs and in particular on *Methylococcus capsulatus*. The current study addressed the disruption of a CopA2 homologue (MCA0805; *copA2*) in *M. capsulatus* by insertional inactivation. The results showed that the phenotype of the resulting mutant, *M. capsulatus* Δ*copA2*, was copper sensitive; its growth was reduced at a copper concentration of 30 µM, and accumulated three-fold more copper intracellularly compared to the wild-type strain. No observed phenotypic difference between the mutant strain and wild-type related to growth at different silver concentrations. These findings indicate that *M. capsulatus* CopA2 has a pivotal role in copper homeostasis and confers intrinsic copper resistance.

***Keywords*—** CopA; Copper homeostasis; P-type ATPases; *Methylococcus capsulatus*.

I. INTRODUCTION

Methylococcus capsulatus Bath is an obligate methanotrophic bacterium that uses methane monooxygenase enzyme (MMO) to oxidize methane to methanol to gain its needs of energy and carbon sources [15], [30]. Although *Mc. capsulatus* has been studied extensively over the past 40 years and the significance of copper in its physiology, many aspects about copper homeostasis is still unclear. This bacterium possesses two forms of MMO; the particulate methane monooxygenase (pMMO) which is associated with intracytoplasmic membranes, and the soluble (cytoplasmic) methane monooxygenase (sMMO). the biosynthesis and activity of MMO is regulated by copper-to-biomass ratio [23]. pMMO is expressed at high copper to-biomass ratios growth conditions whereas sMMO is expressed at low copper-to-biomass ratios [36]. Furthermore, copper is the active center metal of pMMO [4], cofactors for many essential enzymes and enhances the synthesis of the of intracytoplasmic membranes network [9].

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Due to its extreme toxic effects at high concentrations [19], copper uptake, trafficking and the intracellular copper quota are required to strictly controlled. Many proteins are involved in copper handling in the cell in a highly coordinated manner until it is delivered to copper-containing proteins and sub-cellular compartments [34]. In addition to copper sensors and chaperons, copper-translocating P-type ATPase (CopA) plays a key in copper homeostasis and is involved in copper transport across membranes [27], [34], [24]. P-type ATPases are a big family of membrane proteins which are found in all living cells, 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 [1]. The heavy metal transporters, P_{1B}-type ATPases, are a subgroup of P-type ATPases [2].

A number of copper transport homologues have been identified in the genome of *M. capsulatus* [39]. One of these genes is a copper translocating P-type ATPase homologue; MCA0805 (*copA2*). It is of interest to explore whether CopA2 is involved in the copper trafficking in *M. capsulatus*. To this end, a targeted mutagenesis approach was used to generate a mutant in *M. capsulatus copA*2 and the resulting mutant strain, Δ*copA2*, was subsequently characterized and compared to wild-type *M. capsulatus*.

II. MATERIALS AND METHODS

Growth media and strains

M. capsulatus was grown on Nitrate mineral salt (NMS) medium [40]. 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. For liquid cultures, *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 and incubated at 30 ^{o}C. 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 1.

DNA manipulation

Extraction of genomic DNA of *M. capsulatus* was carried out using the method described previously [20]. 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.

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 ^{o}C for 1 min, 55 ^{o}C annealing temperature for 1 min and extension at 72 ^{o}C for 1 min per 1kb of DNA amplified, followed by a final extension step at 72 ^{o}C for 10 min.

Construction of *M. capsulatus* Δ*copA2*

copA2 was disrupted using insertional inactivation mutagenesis technique to determine the function of the gene (Fig 1). *copA2* DNA fragment was amplified using the primers COPA2F506-*Xba*1 and COPA2R51959-*Hin*dIII (Table 1). The purified DNA fragments were cloned into pCR2.1-TOPO to give the constructs pAK222. Then, *copA2* DNA fragments were cloned to plasmid vector, pK18mob*sacB* via *Xba*1and *Hin*dIII restriction sites, to give the constructs pAK02 (Fig 1). The gentamicin resistance cassette (*Gm*^{R}) was cloned via the *Pst*1 restriction site in the *copA2* to give the final constructs, pAK022 which were electroporated into *E. coli* strain S17.1 λpir [17]. Conjugation of plasmid from *E. coli* into *M. capsulatus* was based on the method of Martin and Murrell [21]. A schematic representation of the strategy used for constructing *M. capsulatus* Δ*copA* outlined in Fig. 1. 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 *copA2*. The existence of gentamicin and kanamycin resistance cassettes in the mutants was confirmed by PCR using specific primers (data not shown). Disruption of *copA2* was confirmed by PCR using the primer pair US_COPA2_F140 and GENR851, which targeted the region upstream from *copA2* and the 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 1. The resulting mutant was designated as *Mc. capsulatus* Δ*copA2*.

Table 1 Bacterial strains, plasmids and primers used in the study.

	Description	Source
Strains		
M. capsulatus (Bath)	Wild-type	University of Warwick Culture Collection
M.capsulatus ΔcopA2	ΔMCA0805 (*copA*); *Gm*^{R}	This study
E. coli S1 7.1 *λpir*	*recA1 thi pro hsdR*$^{-}$ RP4-2Tc,,Mu-Km,, Tn7*λpir*	[17]
Plasmids		
pAK222	pCR2.1–TOPO containing 1067 bp *copA2* fragment	
pAK02	*Km*^{R}, pK18mob*sac*B with 1,454 bp *copA2* fragment *Xba*I – *Hin*dIII insert	This study
pAK022	pAK02 with *Gm*^{R,} insert via *Pst*1site	This study
Primers		
COPA2F506-*Xba*1	5'-**TCT AGA** ATGCAGTCATGGCGGCCATC 3'	This study
COPA2R51959-*Hin*dIII	5' **AAGCTT**GAACACTGCCGCGAGCT TAC 3	This study
US_COPA2_F140	5' CGGAGCGTTGTTACCTCTTG 3'	This study
DS_COPA2_2363	5' CACGCCAGATTCTGATGGAC 3'	This study
GENF37	5' GACATAAGCCTGTTCGGTTC 3'	This study
GENR851	5' GCGGCGTTGTGACAATTTAC 3'	This study

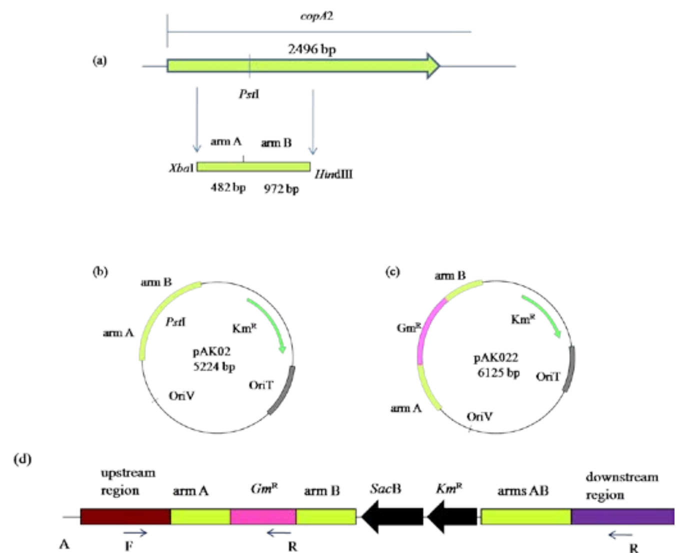

Fig. 1. Schematic representation of the strategy of constructing *Mc. capsulatus* Δ*copA2* (a) the wild-type gene (*copA2*) and the target region was highlighted by arrows; (b) The intermediate plasmid construct pAK02 with *copA2* fragment, restriction sites *Xba*I and *Hin*dIII were introduced by PCR to facilitate cloning; (c) The plasmid construct pAK022 used to inactivate *copA2* and (d) *Mc. capsulatus* Δ*copA2* following single homologous recombination of pK18*mobsacB*. The primers used to check the genotype of the mutants indicated by small horizontal arrows.

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

Minimum Inhibitory Concentrations (MIC) of the Δ*copA2* and wild-type strains were determined by testing the ability of cells to grow on NMS plates supplemented with different 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°C 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.

Growth of *M. capsulatus* at different copper concentrations

Δ*copA2* and wild-type strains were grown on NMS medium supplemented with 0, 10, 30 and 50 µM copper and growth patterns were monitored by measuring the OD_{540}. Growth experiments were done in triplicates.

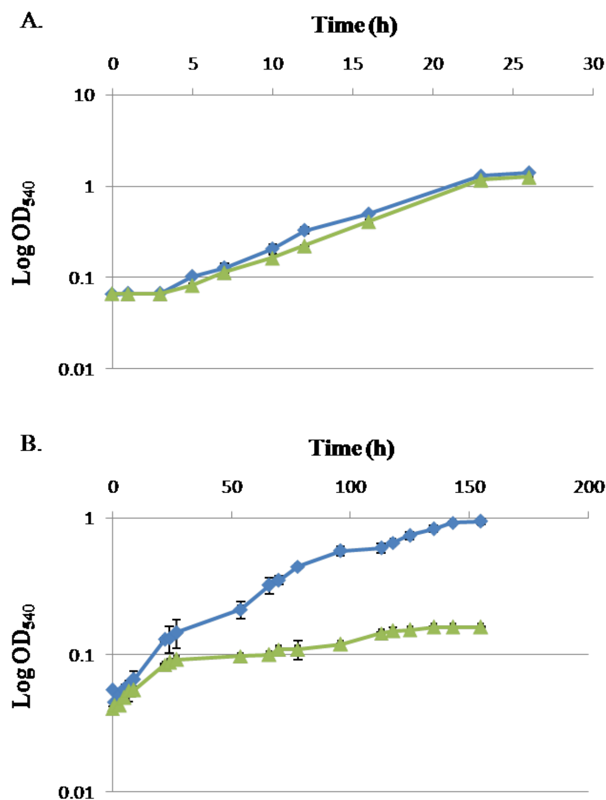

Fig.2. Growth of *M. capsulatus* wild-type (squares) and Δ*copA2* mutant strains (triangles) on NMS amended with A, 10 µM and B, 30 µM added copper (growth in triplicates).

Determination of intracellular copper concentrations

The effect of *copA2* disruption on the intracellular copper accumulation of the *copA2* mutant compared to the wild-type organism was investigated. Cultures were grown on NSM medium with added 30 µM copper, at 45 °C 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.

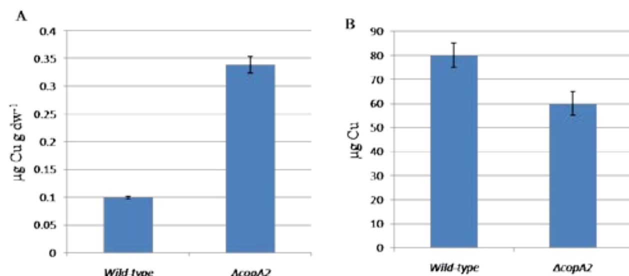

Fig. 3. Copper measurements for the *Mc. capsulatus* Δ*copA2* and the *Mc. capsulatus* wild-type strains grown on NMS with 30 µM added copper (A) and (B) and Minimum Inhibitory Concentrations (MIC) for copper (B). Error bars indicate the standard deviation of three replicate measurements.

Statistical analysis

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

Bioinformatic analyses of CopA protein from *M. capsulatus*

Amino acid sequences from *M. capsulatus* CopA2 and representatives of well-characterized metal-ion-transporting ATPases; *Enterococcus hirae* CopA, *Ent. hirae* CopB [34], *E. coli* CopA [27], *Synechococcus elongates* PacS [31] and *Synechococcus* sp. CtaA [8], [37], [38], 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/ [32].

CopA2 protein topology

The total number of the transmembrane helices and their in/out orientation relative to the membrane, of *M. capsulatus* CopA2 was carried out using Tied Mixture Hidden Markov Model (TMHMM), http,//www.cbs.dtu.dk/services/TMHMM/.

III. RESULTS

Disruption of *copA2*

To determine the function of *copA2*, insertional inactivation mutagenesis was carried out and the resulting mutant was designated *M. capsulatus* Δ*copA2*.

Minimum Inhibitory Concentrations (MIC) for copper and silver

Δ*copA2* strain was more sensitive to higher copper than the wild-type organism. Growth of Δ*copA2* cells was inhibited at a copper concentration of 65 µM while the wild type organism couldn't grow at 80 µM added copper (Fig 3B). Both the mutant and the wild type strains could grow on NMS plates supplemented with copper concentration up to 40 µM (data not shown).

No distinctive phenotype between the wild-type and mutant strains was observed in response to different levels of silver. Δ*copA2* and the wild-type strains could grow on NMS plates supplemented with silver concentration up to 4 µM but neither of them grew at above 5µM. It was also noticed that as the concentration of the added silver increased, the growth of both the Δ*copA2* and the wild-type decreased.

Growth of Δ*copA2* at different copper concentrations

Results showed no significant differences in growth patterns and growth rates between the *ΔcopA2* mutant strain and wild-type at 10 µM added copper (Fig. 2A). Nevertheless, *ΔcopA2* strain exhibited a significant difference in specific growth rate (0.005 h^{-1}) compared to the wild-type (0.02 h^{-1}) ($P < 0.001$) (Fig. 2B). The growth of Δ*copA2* strain was ceased at 65 µM added copper (data not shown). These results were in general consistent with MIC data and the intracellular copper measurements.

Determination of intracellular copper

The intracellular copper concentration of Δ*copA2* mutant strain was higher than that of the wild-type (Fig 3A). The mutant was accumulated three-fold more (0.3 µg (mg drywt biomass) $^{-1}$) copper than the wild-type (0.1 µg (mg drywt biomass)$^{-1}$) ($P < 0.001$). The background copper concentration of the NMS medium with no-added copper is about 0.8 µM.

Bioinformatic analyses and protein topology of CopA2 protein from *M. capsulatus*

Sequence alignment analyses revealed high homology of CopA2 protein from *M. capsulatus* to the well-characterized metal-ion-

transporting ATPases from other organisms. The amino acid identities between CopA2 and known P-type ATPases; *Enterococcus hirae* CopA, *Ent. hirae* CopB [34], *E. coli* CopA [27], *Synechococcus elongates* PacS [31] and *Synechococcus* sp. CtaA [8], [38] ranged from 37 – 46%. Furthermore, eight membrane-spanning helices were predicted in *M. capsulatus* CopA2. 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 TGES motif in the phosphatase domain (Fig. 3B), an invariant phosphorylation site (DKTGTL) (Fig. 3D) and (6) ATP binding domain (GDGINDAP) (Fig. 3E).

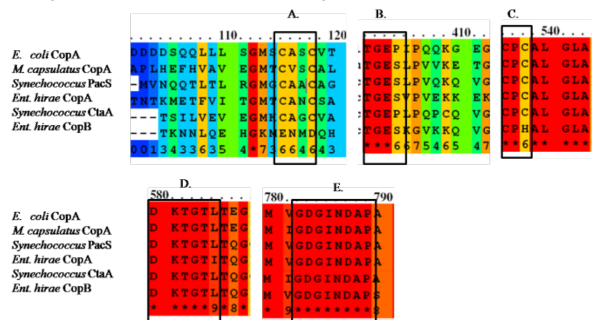

Fig. 4 Sequence alignment of *M. capsulatus* CopA2 (*accession* no. YP113305), *Enterococcus hirae* CopA (*accession* no. AAA61835), *Ent. hirae* CopB (*accession* no. AAA61836), *E. coli* CopA (accession no. Q59385), *Synechococcus elongatus* PacS (*accession* no. BAA03907) and *Synechococcus* sp. CtaA (*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)

IV. DISCUSSION

Sequence alignment analyses CopA2, from *M. capsulatus* and those from well-characterized CopA from other bacteria, suggested *M. capsulatus* CopA2 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 [2], [3].

Furthermore, we disrupted *copA2* and the resulting Δ*copA2* strain was more sensitive to elevated copper concentrations compared to the wild-type, suggesting that this ATPases is a copper-exporting pump. Our results were consistent with those obtained in *Escherichia coli*, CopA. Disruption of *E. coli copA* by insertion of a kanamycin cassette resulted in a mutant sensitive to 2.5 mM copper compared to 3.5 mM in the wild-type [27], [28] . A corresponding change in copper sensitivity was obtained in many other studies on different bacteria in which the copper ATPases were inactivated; *e.g.*, *Helicobacter pylori* [6] and *Ent. hirae* CopB [24].

No obvious phenotypic difference between the Δ*copA2* 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 *copA2* 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 [26]. In addition, deletion of *copA* in *E. coli* resulted in a copper sensitive but not silver sensitive [13]. However, such similarity in phenotype between the *M. capsulatus* Δ*copA2* 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 [24], [34]. This is also indicated that some P-type ATPases share substrate specificity for copper and silver due to chemical similarities between these metals [1]. The mutant was tested for sMMO expression using naphthalene assay [7] and whole cells cytochrome oxidase activity [12], [22] (Data not shown). No significant differences were observed indicating that *copA*2 of *Mc. capsulatus* is not involved in MMO regulation or in cytochrome oxidase functionality.

The accumulation of high concentrations of copper ions in cells of the Δ*copA2* compared to the wild-type highlights their inability to export copper; therefore, CopA2 is likely to function in maintaining tolerable intracellular copper quota. These results are congruent with those previously reported for disruption of other copper-ATPases associated with copper detoxification in *Pseudomonas aeruginosa* [29], [37], [14], *Salmonella typhimurium* [25] *Ent. hirae* [35] and in *Staphylococcus aureus* [33] .

Two copper-uptake systems have been observed in *M. capsulatus*, one is through methanobactin; a copper-binding, siderophore-like, chromopeptide that is produced and excreted by a number of methanotrophs [18], [10], [41], [5] and the other via the outer membrane protein; MopE [11], [16]. Both of them presumably contribute to copper homeostasis in *M. capsulatus*, however, their interaction with CopA2 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* CopA2 has a vital role in copper homeostasis and confers intrinsic copper resistance.

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