Identification of a bacterial di-haem cytochrome c peroxidase from *Methylomicrobium album* BG8

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The nucleotide sequence of an open reading frame (*corB*) downstream of the copper-repressible CorA-encoding gene of the methanotrophic bacterium *Methylomicrobium album* BG8 was obtained by restriction enzyme digestion and inverse PCR. The amino acid sequence deduced from this gene showed significant sequence similarity to the surface-associated di-haem cytochrome c peroxidase (SACCP) previously isolated from *Methyllococcus capsulatus* (Bath), including both c-type haem-binding motifs. Homology analysis placed this protein, phylogenetically, within the subfamily containing the *M. capsulatus* SACCP of the bacterial di-haem cytochrome c peroxidase (BCCP) family of proteins. Immunospecific recognition confirmed synthesis of the *M. album* CorB as a protein non-covalently associated with the outer membrane and exposed to the periplasm. *corB* expression is regulated by the availability of copper ions during growth and the protein is most abundant in *M. album* when grown at a low copper-to-biomass ratio, indicating an important physiological role of CorB under these growth conditions. *corB* was co-transcribed with the gene encoding CorA, constituting a copper-responding operon, which appears to be under the control of a σ^54^-dependent promoter. *M. album* CorB is the second isolated member of the recently described subfamily of the BCCP family of proteins. So far, these proteins have only been described in methanotrophic bacteria.

INTRODUCTION

A surface-associated cytochrome c peroxidase (SACCP) isolated from the methanotrophic bacterium *Methylococcus capsulatus* (Bath) has recently been described (Karlsen et al., 2005). This protein shares significant sequence similarity with the members of the bacterial di-haem cytochrome c peroxidase family of proteins (BCCP, pfam03150) and appears to have a core structure resembling the resolved structures available from this family (Karlsen et al., 2005). However, the *M. capsulatus* SACCP can be distinguished from the other members of the BCCP family by being a much larger protein when compared to both the di-haem cytochrome c peroxidases and the MauG proteins, which constitute the two subfamilies (Chistoserdov et al., 1994; Ronnberg & Ellfolk, 1979). Furthermore, the two c-type haem-binding motifs of the *M. capsulatus* SACCP have, in their primary sequence, an increased inter-haem distance.

However, structural analyses (Karlsen et al., 2005) strongly suggests that these haem groups in the native protein are structurally located in close proximity to each other, as previously demonstrated for other di-haem cytochrome c peroxidases (Fulop et al., 1995; Shimizu et al., 2001). We have suggested that the *M. capsulatus* SACCP protein is included in a third, novel subfamily of the BCCP family of proteins (Karlsen et al., 2005). In contrast to the di-haem cytochrome c peroxidases and the MauGs, which are localized in the periplasm (Goodhew et al., 1990; Wang et al., 2003), the *M. capsulatus* SACCP was isolated from the bacterial cell surface (Karlsen et al., 2005). Copper-regulated multiple c-type cytochromes of the cytochrome *c*~553~ family have also been found on the *M. capsulatus* surface (Karlsen et al., 2008). Taken together, these findings imply that important redox processes also take place at this location in methanotrophic bacteria.

*tBlastn* searches with the *M. capsulatus* SACCP as the query have revealed significant sequence similarity to a partially sequenced putative open reading frame (*orf3*) of the methanotroph *Methylomicrobium album* BG8 (Berson & Lidstrom, 1997; Karlsen et al., 2005). However, since the fragment present in the databases terminates within the putative ORF, the sequence similarity is restricted to an N-terminal region of the *M. capsulatus* SACCP. The sequence similarity between *orf3* and SACCP can be increased in the N-terminal region *in silico* by introducing a frame-shift.
mutation in orf5′, indicating that a DNA sequencing-error is present in the published fragment. Furthermore, the fragment also includes the gene encoding the copper-repressible CorA protein (Berson & Lidstrom, 1997). CorA is the only protein present in the databases that shares significant sequence similarity to the MopE protein isolated from M. capsulatus (Fjellbirkeland et al., 2001). In M. capsulatus, the SACCp-encoding gene is located upstream of the gene encoding MopE and bioinformatic analyses strongly suggest that these genes form an operon (Karlsen et al., 2005; Ward et al., 2004). Similar to CorA, MopE is a copper-repressible protein produced in significant amounts on the cell surface during low copper-to-biomass growth (unpublished results for CorA) (Karlsen et al., 2003). Recently, the structure of the N-terminal truncated part of MopE (MopE*) that is secreted and exposed to the growth medium was resolved. The crystal structure revealed that MopE* is a copper-binding protein that coordinates copper ions, in a hitherto unique manner, with two histidines and the tryptophan metabolite kynurenine, formed by oxidative cleavage of the tryptophan indole ring and subsequent hydrolysis of the CD1 carbon (Helland et al., 2008).

In the present study, we explored further the homology between the M. capsulatus MopE/SACCp and the putative M. album CorA/orf5′ systems. The region immediately downstream of corA was sequenced after restriction enzyme digestion of genomic DNA and inverse PCR. A complete ORF that encodes a protein with a very high sequence similarity to the M. capsulatus SACCp was identified. We further show that the novel protein (CorB) is a c-type haem protein that is non-covalently associated with the outer membranes exposed to the periplasmic space. Furthermore, the expression of corB is regulated by the availability of copper during growth, and is most abundant during a low copper-to-biomass ratio. The gene encoding CorB was co-transcribed with corA, as part of a copper-responding operon transcribed during low-copper conditions. Homology analyses revealed that the CorB protein is the second described member of the c-type cytochromes, constituting the recently established subfamily encompassing the M. capsulatus SACCp in the BCCP family of proteins.

**METHODS**

**Growth of Methylomicrobium album BG8.** M. album BG8 (NCIMB 11123) was grown in batch cultures at 30°C, while shaking, with an atmosphere of methane and oxygen (50:50, v/v), in nitrate mineral salt medium as described by Whittenbury et al., (1970). Cells were grown either at a low copper-to-biomass ratio with no added copper in the growth medium, or with copper added to a final concentration in the medium of either 0.8 or 5 µM copper.

**Cell fractionation.** Cells were harvested by centrifugation at 5000 g for 10 min. Fractions enriched in soluble proteins, inner-membrane proteins or outer-membrane proteins were obtained as described previously (Fjellbirkeland et al., 1997).

**Extraction of cell-surface proteins and outer-membrane-associated proteins.** Cell-surface proteins were extracted from whole cells as described previously (Karlsen et al., 2005). Outer-membrane-associated proteins were extracted from an isolated M. album outer-membrane fraction by incubation with 20 mM Tris/HCl pH 7.4, 1 mM CaCl2 and 1 M NaCl, with rotation for 3 h at 4°C. The outer membranes were subsequently collected by centrifugation at 100,000 g at 4°C; the resulting supernatant contained the 1 M NaCl extracted proteins.

**SDS-PAGE and protein immunoblotting.** SDS-PAGE was performed as described by Laemmli (1970). Protein immunoblotting was carried out as described previously (Karlsen et al., 2005). Based on hydropathy plots, the hydrophilic internal peptide TGAKWLA-KGTKKV was chosen for the production of peptide-specific antibodies against CorB (Fig. 1). Immunization of rabbits followed by ELISA titre tests and affinity purification of the antibodies were performed by Sigma Genosys. In our Western blot analyses, the peptide-specific antibodies against CorB showed very little cross-reactivity towards other polypeptides present in M. album (Supplementary Fig. S1, available with the online version of this paper). Horseradish-peroxidase-linked goat anti-rabbit serum was purchased from Bio-Rad. Protein immunoblots were developed using Enhanced Chemi-Luminescence (ECL, GE Healthcare).

**c-Type haem staining.** Proteins were separated by non-reducing SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. Immobilized proteins were stained for c-type haem with the ECL assay as described previously (Dorward, 1993; Vargas et al., 1993).

**Reverse transcriptase PCR (RT-PCR).** First-strand cDNA synthesis was carried out using 36 ng total RNA and the PrimeScript RT-PCR kit from Takara, with random hexamer primers provided with the kit, according to the manufacturer’s protocol. PCR was performed using 5 µl cDNA with reagents provided with the PrimeScript RT-PCR kit from Takara and the primers corA-F (5′-CGGGCGCGGTCCC-GGCAGC-3′) and corB-R (5′-GATCCGGCATGAAGAAATGGCAC-3′) spanning the corA and corB intergenic region. The resulting PCR product was purified with a Qiagen PCR purification kit and sequenced using a Big-Dye sequencing kit and an ABI 3700 sequencer by the DNA Sequencing Service at the University of Bergen. Copper-dependent expression of corA was shown by PCR, as above, with the primer set corA-F2 (5′-CGGAATTCAGCCTTGTCACCC-3′) and corA-R (5′-GGCACACCCTGCCATCACC-3′).

**Total RNA isolation.** About 106 cells of M. album were harvested by centrifugation and resuspended in 100 µl TE with 2 mg lysozyme ml−1 prior to RNA isolation. Total RNA isolation was carried out using the Promega SV Total RNA Isolation System kit in accordance with the manufacturer’s instructions. Total RNA was eluted with 100 µl RNase-free water and stored at −80°C until further use. The amount and quality of the RNA were determined spectrophotometrically using a NanoDrop ND1000 from Thermo Scientific.

**Inverse PCR.** To obtain the 3′ nucleotide sequence of orf5′/corB, M. album genomic DNA was digested with KpnI or SpfI. DNA fragments of 1500 to 3000 bp were excised from agarose gels and purified with a Qiagen gel extraction kit. The purified DNA fragments were re-ligated using T4 DNA ligase. PCR was initiated with 2 µl template, 5 µl 10× Taq polymerase buffer (Takara), 1 µl 10 mM dNTPs, 0.5 µl 50 pmol µl−1 each of primers corB-1109F and corB-R, 0.25 µl Ex Taq polymerase (Takara), and water to a total volume of 50 µl. A 30-cycle PCR amplification was performed in a BioEngine thermal cycler (Bio-Rad) with the following programme: denaturation, 95°C for 30 s; annealing, 50°C for 30 s; polymerization, 72°C for 4 min. The resulting PCR products were purified and sequenced as stated above.
Fig. 1. Physical map, genomic orientation and nucleotide and amino acid sequence of CorA and the novel downstream ORF. (a) An updated physical map of the 5.4 kb M. album chromosomal region containing orf1, orf2 and corA with the corrected reading frame for corB. Putative recognition sites for integration host factor (IHF) and ς54 are indicated with boxes. The directions of transcription are indicated with shaded arrows. orf4 and orf5′ postulated by Berson & Lidstrom (1997) are shown with open arrows. The inverse-PCR fragments covering the complete corB gene are given below the ORFs. (b) The amino acids are indicated below the nucleotide sequence. The putative ς54 promoter predicted with the Promscan software is underlined upstream of the corA gene. The predicted ribosome-binding sites upstream of corA and corB are enlarged in the nucleotide sequence. The signal peptide as previously determined in corA, and the signal peptide predicted by SignalP in corB, are highlighted in red. The two c-type haem-binding sites revealed by the ScanProsite software are highlighted in yellow. Black arrows indicate a putative termination loop predicted by the RNA secondary structure prediction program. Half arrows indicate the forward and reverse primers used in the RT-PCR analyses. The amino acid sequence used for construction of the polyclonal antibody is boxed.
Bioinformatic tools. Scanprosite was used when searching the CorB sequence for conserved motifs (Gattiker et al., 2002). Sequence alignments were constructed with CLUSTAL_X using standard alignment parameters (Thompson et al., 1997) and visualized with the Genedoc software. SignalP and PSORT were used for topology analysis (Bendtsen et al., 2004; Gardy et al., 2005). Promoter prediction was performed with the Promscan software (http://www.promscan.uklinux.net). Homology analysis was performed by using the BLAST resources at NCBI (http://www.ncbi.nlm.nih.gov/). An RNA secondary structure prediction program was used for terminator analysis (Brodsy et al., 1995).

RESULTS

Sequencing of the M. album corB gene and bioinformatic analyses

Restriction enzyme digestion of genomic DNA and inverse PCR were used to obtain the DNA sequence downstream of the corA gene (see Methods). By combining the overlapping nucleotide sequences of the SphI and KpnI restriction enzyme fragments, a complete ORF of 2139 bp was revealed downstream of corA, encoding a putative protein consisting of 713 amino acids (Fig. 1a, b). A ribosome-binding site could be predicted immediately upstream of its potential start codon. Bioinformatic analysis of the deduced amino acid sequence, using SignalP and PSORT, predicted a leader peptide with a putative cleavage site between amino acids 33 and 34 (Fig. 1b). N-terminal processing would lead to a mature protein of 680 amino acids with a theoretical molecular mass of 72,919 Da. The putative protein contained two regions that match the c-type cytochrome superfamily profiles (PS51007) as revealed by a PROSITE database search of protein families and domains. Both of these regions contained the c-type cytochrome motif (CxxCH), suggesting the covalent binding of two haem groups to the mature polypeptide (Fig. 1). The deduced amino acid sequence of this ORF was used in homology searches with BLASTP, which revealed the highest sequence similarity to the M. capsulatus SACCP protein. A pairwise alignment between the M. capsulatus SACCP protein and the novel gene product of M. album demonstrated a significant conservation of several amino acids among these proteins, revealing a sequence identity and sequence similarity of 42% and 54%, respectively (Fig. 2). The amino acids conserved between these two proteins also included the two c-type haem-binding sites and the residues that possibly coordinate calcium ions, as demonstrated in the resolved structures of the bacterial di-haem cytochrome c peroxidases (Fig. 2) (Fulop et al., 1995; Shimizu et al., 2001). The novel ORF was denoted corB. When corB-encoded full-length protein was included in the multiple alignment with members of the BCCP family of proteins (not shown), it was evident that the primary structure of this protein differs significantly from the di-haem cytochrome c peroxidases and the MauG proteins, and clusters within the novel subfamily of the BCCPs that includes the M. capsulatus SACCP protein (Karlsen et al., 2005).

Subcellular localization and identification of the mature M. album CorB protein

M. album cells grown at a low copper-to-biomass ratio were separated into fractions of cytoplasmic/periplasmic...
proteins (soluble fraction), inner-membrane proteins (Triton X-100-soluble membrane protein fraction) and outer-membrane proteins (Triton X-100-insoluble membrane fraction) using the procedure described previously (Fjellbirkeland et al., 1997). The resulting cell fractions were then assessed by SDS-PAGE (Fig. 3a). The most abundant polypeptide, of approximately 60 kDa, in the soluble fraction (Fig. 3a) most likely represents the large subunit of methanol dehydrogenase. When a corresponding protein immunoblot was probed with a polyclonal antibody against a short oligopeptide derived from the M. album CorB sequence, an immunoreactive polypeptide of approximately 80 kDa was found to co-fractionate with the outer membrane proteins (Fig. 3b, Supplementary Fig. S1). The molecular mass of approximately 80 kDa of this polypeptide is close to the mass predicted for the mature CorB protein (~74.5 kDa, including two c-type haems). A subcellular localization in the outer membrane is in accordance with the presence of an N-terminal leader sequence in the precursor protein. To further establish the subcellular localization of this protein, whole cells were treated with a high-salt buffer (see Methods) to extract proteins that are non-covalently associated with the cell surface (Fig. 4a). Protein immunoblots (Fig. 4b) demonstrated that the CorB protein was not extracted from intact cells when treated with 1 M NaCl. An outer-membrane fraction was then incubated with the same high-salt buffer and analysed by protein immunoblotting. These analyses revealed that CorB can be extracted from isolated outer membranes (Fig. 4c, d). Taken together, these results strongly suggest that the M. album CorB protein has a cellular localization in the periplasmic space and is non-covalently associated with the outer membrane.

**Detection of c-type haem in CorB**

Bioinformatic analysis identified two c-type haem-binding motifs (CxxCH) in the M. album CorB primary structure (Fig. 2). In order to confirm the covalent binding of haem, non-reducing SDS-PAGE-separated outer-membrane fractions from cultures of M. album grown in both high and low copper-to-biomass ratios were transferred to a nitrocellulose membrane and directly stained for haem using enhanced chemiluminescence (ECL) (Fig. 5). An evident signal of approximately 90 kDa was visualized in

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**Fig. 3.** SDS-PAGE (a) and protein immunoblot analysis (b) of proteins obtained during the fractionation of M. album. Samples of each fractionation step were collected and comparable amounts were analysed. (a) A 12.5 % polyacrylamide gel stained with Coomassie Brilliant Blue (CBB) R-250. (a, b) Lane 1, whole cells; lane 2, soluble fraction; lane 3, total membrane fraction; lane 4, Triton X-100 soluble membranes (enriched inner-membrane fraction); lane 5, Triton X-100 insoluble membranes (enriched outer-membrane fraction). (b) Protein immunoblot of (a), using CorB-specific rabbit antibody (see Methods). Molecular mass markers are indicated to the left of both panels. The full immunoblot corresponding to the section presented in (b) is shown in Supplementary Fig S1.

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**Fig. 4.** SDS-PAGE and protein immunoblot analyses of protein fractions obtained during the NaCl extraction of whole cells (a, b) and the NaCl extraction of the isolated outer membrane fraction (c, d). (a, c) Polyacrylamide gels (12.5 %) stained with CBB R-250. (b, d) Protein immunoblots of (a) and (c), respectively, using CorB-specific rabbit antibody. (a, b) Lane 1, M. album cells resuspended in buffer containing low ionic strength (20 mM Tris/HCl); lane 2, 20 mM Tris/HCl wash (supernatant after centrifugation of resuspended cells); lane 3, 1 M NaCl extraction of whole cells; lane 4, whole cells after treatment with 1 M NaCl. (c, d) Lane 1, M. album outer-membrane fraction; lane 2, 1 M NaCl extract of the outer-membrane fraction; lane 3, outer-membrane fraction after treatment with 1 M NaCl. Molecular mass markers are indicated to the left of all panels.
the outer-membrane fraction of cells grown at a low copper-to-biomass ratio (Fig. 5a). A protein immunoblot using antibodies directed against SACCP on the same nitrocellulose membrane and stained for c-type haem peroxidase activity, (b). Protein immunoblot of the membrane corresponding to (a) using the anti-CorA serum. Molecular mass markers are indicated to the left of (a).

**Synthesis of the* M. album *CorB protein**

The *M. album* *corB* gene is located 77 bp downstream of the copper-repressible *corA* gene. Neither a putative terminator stem–loop nor a promoter region could be predicted with high significance in their intergenic region, indicating that these genes are organized as a single transcriptional unit. The production of CorA is regulated by the availability of copper, CorA being most abundant when cells are grown in a low copper-to-biomass ratio. In order to explore whether, as expected, the expression of *corB* would respond to the availability of copper if concomitantly expressed with *corA*, *M. album* cells were grown at three different copper levels and analysed for the presence of CorB protein. SDS-PAGE profiles of the cells grown with different copper levels revealed significant changes in the overall protein pattern, including a decrease in the abundance of a polypeptide migrating with the apparent molecular mass of the PmoA subunit of the particulate methane monooxygenase, with decreasing copper concentrations (Fig. 6a). A corresponding protein immunoblot using the antiserum directed against the *M. album* CorB protein demonstrated that its synthesis was altered in response to different copper levels, being most abundant in cells grown in a low copper medium (Fig. 6b). Furthermore, RT-PCR analysis using specific primers (see Methods and Fig. 1) covering the region between the *corA* and *corB* genes produced a DNA fragment of 409 nt (Fig. 6c). This RT-PCR product was verified by DNA sequencing to cover the DNA region linking the *corA* and *corB* genes, hence confirming the formation of a polycistronic mRNA. The differential expression of *corB* in cells cultured under high- or low-copper conditions was also demonstrated at the RNA level, using primers recognizing the *corA/corB*

**Fig. 5.** c-Type haem staining of CorB. (a, b) Lanes 1 and 2, *M. album* outer-membrane fractions of cells grown at a high and low copper-to-biomass ratio, respectively. (a) Protein samples separated by non-reducing SDS-PAGE immobilized onto a nitrocellulose membrane and stained for c-type haem peroxidase activity. (b). Protein immunoblot of the membrane corresponding to (a) using the anti-CorA serum. Molecular mass markers are indicated to the left of (a).

**Fig. 6.** Copper regulation analyses of CorB. (a) SDS-PAGE analysis of *M. album* cells grown with 5 μM (lane 1), 0.8 μM (lane 2) and no copper (lane 3) added to the growth medium. The 10 % polyacrylamide gel was stained with CBB R-250. The putative pMMOA subunit is indicated in lane 1. (b) Protein immunoblot of (a) using the anti-CorB serum. Molecular mass markers are indicated to the right of both (a) and (b). (c) RT-PCR analysis of the *corA–corB* transcript prepared from the same cultures as used in (a) and (b). Lane 1, RT-PCR product with primers (Fig. 1) covering the intergenic region between *corA* and *corB* from the *M. album* culture grown with no added copper; lane 2, DNA size ladder. Lanes 3, 4 and 5 show the copper-dependent regulation of *corA–corB* (see Methods) from *M. album* cells grown with no copper, 0.8 μM and 5 μM copper in the growth medium, respectively.
transcript (Fig. 6c). A putative $\sigma^{34}$ ($\sigma^H$) promoter sequence – TGGCAT-N$_5$-CTGCCTA – could be predicted 120 nt upstream of the corA start codon using the Promscan software (Fig. 1). This $\sigma^{34}$ promoter showed high sequence similarity to the consensus $\sigma^{34}$ promoter identified by Barrios et al. (1999), and also to the known $\sigma^{34}$ dependent gene, mmoX, encoding the large subunit of soluble methane monoxygenase (Nakamura et al., 2007). Further upstream, an integration host factor (IHF)-binding site was also recognized (consensus sequence WATCAAN$_4$TTR), which is typically found upstream of $\sigma^{34}$ promoters (Fig. 1) (Collado-Vides et al., 1991). An IHF-binding site was also found upstream of the mmoX gene in M. capsulatus (Csaki et al., 2003). The sequencing of the $\psi$hl restriction fragment further revealed a 279 nt novel sequence downstream of the corB stop codon. However, no additional ORF, or partial ORF, with significant sequence similarity to any proteins present in the databases was revealed by BLASTX searches with this DNA sequence. A putative stem–loop with an estimated free energy of $\pm 76.15$ kJ mol$^{-1}$ could be predicted downstream of corB by the RNA secondary structure prediction program, suggesting the presence of a Rhodobacter independent terminator (Fig. 1b).

**DISCUSSION**

The complete nucleotide sequence of a novel ORF (corB) downstream of the corA gene has been obtained by inverse PCR and subsequent DNA sequencing. The resequencing of parts of the published SalI fragment (Berson & Lidstrom, 1997) revealed minor sequencing errors, and led to corrected versions of orf4 and orf5 (corB) of M. album (Fig. 1, GenBank accession no. GU319974). Homology analysis with the deduced amino acid sequence of corB revealed significant homology to the SACCP protein isolated from the methanotroph M. capsulatus (Fig. 2), including the two $c$-type haem-binding motifs and a putative calcium-binding site. BLASTP searches with CorB, against all sequenced genomes available, also revealed significant sequence similarity to hypothetical proteins from Photobacterium profundum, Nitrosomonas sp., Pseudomonas fluorescens AL212, Sorangium cellulosum, Polaromonas naphthalenivorans and Nostoc punctiforme, in which several of these amino acid sequences have previously been shown to share sequence similarity to SACCP (Karlsen et al., 2005). This unusual group of dihaem cytochrome $c$ peroxidase-like sequences include members from several genera of prokaryotes ($\beta$- and $\delta$-proteobacteria and cyanobacteria), demonstrating that the corB/SACCP-like genes are not specific solely to methanotrophic bacteria. Furthermore, the sequence similarity and sequence identity values among the proteins within this group are between 40–55% and 29–41%, respectively, indicating a rather distant phylogenetic relationship between these proteins. However, when their amino acid sequences are structurally aligned with the resolved structures of cytochrome $c$ peroxidases (CCPs) from Nitrosomonas europaea and Pseudomonas aeruginosa, the most conserved segments, including the $c$-type haem binding motifs, are located in the core of the CCP structure, indicating a similar fold in these proteins that brings the two $c$-type haem groups in close proximity to each other (Karlsen et al., 2005). Since CorB differs significantly from the MauGs and other CCPs of the BCCP family of proteins, it should be included in this novel group of di-haem cytochrome $c$ peroxidases within the BCCPs (Karlsen et al., 2005).

The genome sequencing of M. capsulatus revealed the SACCP gene immediately upstream of mopE (Ward et al., 2004). Furthermore, the MopE and SACCP proteins are both copper repressible, and previous bioinformatic analyses have strongly indicated that these proteins form a single transcriptional unit (Karlsen et al., 2003, 2005). The two proteins are also co-localized on the M. capsulatus bacterial surface and appear to be functionally related. Homologous to the SACCP/MopE system, corB is clustered together with the corA gene in the M. album genome. However, the genomic organization of these genes is different from that of M. capsulatus, in that corA is situated upstream of corB. The CorA protein is at present the only protein in the databases that shares significant sequence similarity with MopE. The determination of the crystal structure of MopE* revealed an unusual copper-binding site where copper was coordinated in a tetrahedral arrangement involving two histidines and a kynurenine. Kynurenine is a tryptophan metabolite formed by oxidation of Trp130 (Helland et al., 2008). The residues involved in the binding of copper are conserved among CorA and MopE, including this particular tryptophan (Helland et al., 2008). However, whether or not the specific tryptophan residue of CorA is also post-translationally modified and involved in copper binding is still an open issue. Unlike the di-haem cytochrome $c$ peroxidases that have been assigned a role in detoxification, i.e. H$_2$O$_2$ removal in the periplasm (Goodhew et al., 1990), knockout mutants of MauG proteins have revealed the involvement of these proteins in the formation of the tryptophan-tryptophyl quinone prosthetic group of the methylamine dehydrogenase (MADH) present in facultative methylotrophs (Pearson et al., 2004; Wang et al., 2003). There has been great progress towards understanding the mechanistic involvement of MauGs in the formation of the tryptophan-tryptophyl quinone cofactor (Li et al., 2006, 2008; Wilmot & Davison, 2009). This endogenous post-translational modification involves two oxidation reactions and one covalent cross-linking reaction of two tryptophan groups. Since Trp130 was found to be oxidized to kynurenine in MopE, and conceivably also in CorA, it is striking that the M. capsulatus SACCP and the M. album CorB share significant sequence similarity to the MauG proteins. Thus, it is tempting to speculate that these proteins have an equivalent role in methanotrophs in the formation of kynurenine, by carrying out appropriate electron-transfer reactions. On the other hand, methylamine dehydrogenase
activity is not detected in *M. capsulatus* (Zahn et al., 1997), and homologues of the genes in the *mau* gene cluster involved in methylamine dehydrogenase activity are not found in the *M. capsulatus* genome (Ward et al., 2004). It is therefore unlikely that MopE/SACCP proteins are involved in methylamine dehydrogenase activity and thus, by inference, neither are CorA/CorB in *M. album* BG8.

Unlike the *M. capsulatus* SACCP, which was found to be associated with the cellular surface, CorB appears to have a periplasmic location. This subcellular location is in line with that of the CCPs and the MauGs, and with the general view that in bacteria the c-type cytochromes function in the periplasm, where they are involved in electron-transport activities, e.g. mediating electron transfer to and from a variety of dehydrogenases and reductases (Allen et al., 2005; Ferguson, 2001). Since CorA is a surface-associated protein (unpublished results) any direct protein–protein interaction with CorA would have to take place in the periplasm prior to the export of CorA through the outer membrane.

The CorA/CorB system of *M. album* and the MopE/SACCP system of *M. capsulatus* appear to be strikingly similar. In addition to their sequence similarity, both systems appear to form copper-responding operons that are expressed during low copper-to-biomass conditions, thereby strengthening the assumption that they play a role in copper homeostasis. At present, the exact function of these systems is not known. Since *M. album* BG8 possesses genes encoding only particulate methane monoxygenase (Lloyd et al., 1999), their function would appear not to be related per se to the pMMO/sMMO switch that takes place in *M. capsulatus* under lower copper-to-biomass conditions (Nielsen et al., 1997). Studying knockout mutants and double-knockout mutants of SACCP/CorB and CorA/ MopE may reveal the specific roles of these proteins in the methanotrophic copper homeostasis; such work is currently in progress. However, preliminary work on such mutations in *M. capsulatus* indicates that this approach may not give clear phenotypes, i.e. growth rates in batch cultures and protein composition on the cell surface. The multitude of various c-type cytochromes found in *M. capsulatus* (Karlsen et al., 2008; Ward et al., 2004), many of which are copper regulated, may serve as substitutes for each other (Otten et al., 2001).

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