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EXPERIMENTAL ARTICLES

Primary Characterization of Dominant Cell Surface Proteins of Halotolerant Methanotroph *Methylomicrobium alcaliphilum* 20Z

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Abstract—Cell surface-associated proteins with molecular masses of 27 and 80 kDa found in *Methylomicrobium alcaliphilum* were studied. The *MEALZv2_1030034* and *MEALZv2_1030035* genes encoding these proteins were identified in the partially annotated genome of the methanotroph. Polypeptides *MEALZv2_1030034* and MEALZv2_1030035 showed high homology (>50% identity) with the surface proteins CorA and CorB of *Methylomicrobium album* BG8 expressed in conditions of low copper content in the growth medium. Electron microscopic analysis with antibodies revealed localization of the 27-kDa protein in the base of the cup-shaped structures of S-layers. The mutant with an insertion in the *MEALZv2_1030034* gene lost the ability to grow on the medium with methane, but grew in the presence of 0.2% methanol. In this case, the cup-shaped structures of S-layers were not bound to the cell wall but occurred as regular aggregates in the intercellular space. The 80-kDa protein was found mainly in the periplasm, had a peroxide-degrading activity, and was classified as a di-heme cytochrome *c* peroxidase. The mutant deficient in the *MEALZv2_1030035* gene grew on methane at a high rate and had higher activities of C_1 compound oxidation enzymes than did the parent strain. The role of the proteins MEALZv2_1030034 and MEALZv2_1030035 and distribution of their homologues in other methanotrophs are discussed.

Keywords: methanotrophs, *Methylomicrobium alcaliphilum* 20Z, surface proteins, di-heme cytochrome *c* peroxidase, S-layers.

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The bacterial cell envelope is an important factor of adaptation to changing environmental conditions. The cell envelope of aerobic methanotrophs has a structure typical of gram-negative bacteria and includes a cytoplasmic membrane, periplasm, peptidoglycan layer, and outer membrane. Importantly, the outer membrane of methanotrophs is different in chemical composition from those of other gram-negative bacteria, having significant levels of sterols and hopanoids. Since these lipids seldom occur in prokaryotes, their presence is probably associated with methane utilization [1, 2]. Moreover, the cell surface of halotolerant methanotrophs of the genus *Methylomicrobium*, namely *Mm. alcaliphilum* (5Z, 20Z, and S1), *Mm. buryatense* (5B, 5G, 6G, and 7G), and nonhalophilic *Mm. album* BG8, contains glycoprotein layers (S-layers) as a monolayer of cup-shaped structures, which are packed in hexagonal (*p*6) symmetry [3–6]. The S-layers characterized by tetragonal (*p*4) symmetry of arrangement of protein subunits are formed by the thermotolerant strains of *Methylococcus capsulatus* (Texas and 874) [7, 8].

The study of cell surface proteins and their encoding genes in methanotrophs is of interest for production of useful proteins by heterologous expression. It is promising due to the nonpathogenicity of methanotrophs, their low protease activity, and ability to reach high cell density on simple growth media. In addition, the study of surface proteins and their functions in methanotrophs with different physiological and biochemical peculiarities may lead to better understanding of the role of cell envelopes in the adaptation of these bacteria to natural fluctuations [9].

This work was aimed at identification and primary characterization of dominant surface proteins in the halotolerant methanotroph *Mm. alcaliphilum* 20Z.

MATERIALS AND METHODS

Cultivation of bacteria. *Mm. alcaliphilum* 20Z was grown in methane–air atmosphere (1 : 1) on P mineral medium at 29°C with addition of 3% NaCl [3]. *Escherichia coli* S17-1 was grown in liquid or on agarized LB

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medium at 37°C. Plasmid-transformed *E. coli* cells were grown in medium with kanamycin added to a final concentration of 50 μg/ml.

Identification of the major surface proteins. The suspension of *Mm. alcaliphilum* 20Z cells (1 ml) harvested in the exponential growth phase (OD_{600} = 1.0) was centrifuged for 5 min at 8000 *g*. The cells were resuspended in 100 μl of 100 mM Tris–acetate buffer, pH 8.2, followed by addition of 5 μl of 0.5% dansyl chloride solution, and incubated for 20 min at 50°C. Then the biomass was centrifuged at 13000 *g* for 5 min and washed from dansyl chloride with the same buffer. The pellet was resuspended in 100 μl of denaturing buffer (10% β-mercaptoethanol; 10% glycerol; 4% SDS; 500 mM Tris–HCl, pH 8.0; 0.02 M Na_2EDTA, pH 8.0; 10% bromophenol blue) and heated for 15 min at 95°C. Proteins were separated by electrophoresis in 12% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) [10]. Fluorescent protein bands were revealed in UV (λ_{240}) or after staining the gels with Coomassie G-250 [11].

Isolation and mass spectrometric analysis of biotinylated surface proteins. Surface proteins were biotinylated and then isolated using a Sileks-M kit (Moscow, Russia) according to the manufacturer's protocol. The procedure included the treatment of whole bacterial cells with a biotin probe, protein extraction with 3 M guanidine hydrochloride (GHCl), incubation with streptavidin-coated magnetic particles, washing of the magnetic particles/protein complex with 3 M and 5 M GHCl for removal of the nonspecifically bound and aggregated proteins, cleavage of the proteins from streptavidin–biotin, and precipitation with cooled acetone. The denaturing buffer was added to the resulting protein precipitate and heated for 5 min at 95°C. Proteins were separated by SDS-PAGE [10] and stained with Coomassie R-250 [11]; the protein bands were excised for mass spectrometry. Trypsinolysis of the proteins in the gel and extraction and mass spectrometric analysis of the peptides were carried out at the Institute of Biomedical Chemistry, Russian Academy of Sciences (Moscow).

Solubilization of the surface proteins. The cells of strain 20Z were separated from the medium by centrifugation and resuspended in ten volumes of 6 M LiCl. The obtained suspension was centrifuged for 1 h at 16000 *g*, and the supernatant was dialyzed against deionized water overnight at 4°C. The formed semitransparent protein flakes were precipitated by centrifugation (30 min, 16000 *g*) and used for electron microscopy or stored at –20°C for subsequent purification and preparation of polyclonal antibodies.

Immunoblotting. Cell surface proteins were obtained and separated by SDS-PAGE and transferred onto a Hybond nitrocellulose membrane (Amersham, United Kingdom) at 150 mA for 30 min [12]. Nitrocellulose filters with transferred proteins were incubated for 2 h at 26°C under stirring in PBS solution (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) with 5% of skim dry milk for elimination of nonspecific sorption. Then the filters were incubated for 2 h at 26°C in the same solution containing primary rabbit antibodies obtained to the purified proteins (1 : 2000). After hybridization, the filters were washed three times with 20–30 ml of WB solution (PBS, 5% skim dry milk, and 0.1% Tween-20). Then the filters were incubated with secondary goat anti-IgG conjugated with peroxidase (Stress Gene, United States) in a 1 : 300 dilution (26°C, 50 min). After washing with the WB solution, antigen bands were stained with diaminobenzidine in 50 mM Tris–HCl buffer (pH 7.6) containing 0.2% H_2O_2 and 0.03% $NiCI_2$ [12].

Electron microscopic immunocytochemistry. The cells of *Mm. alcaliphilum* 20Z were incubated overnight with polyclonal rabbit antibodies to the purified proteins. After washing with TBS buffer (10 mM Tris-HCl, pH 7.3, 0.15 M NaCl, 0.05% sodium azide), the biomass was resuspended in TBS buffer with colloidal gold-labeled protein A (particles 8.3 or 5 nm in diameter) (MP Biomedicals, United States). After incubation for 12 h and washing, the cells were analyzed in a JEM 100B electron microscope (Japan) as described [3]. Ultrathin cell sections were obtained as described [8]; the sections were hybridized with the respective antibodies by the procedure described above. For negative contrasting, the cells were treated with 0.1% aqueous solution of uranyl acetate.

Obtaining of knockout mutants. The nucleotide sequence of the inactivated gene was cloned in plasmid pK18mob, which is not replicated in *Mm. alcaliphilum* 20Z [13]. Then the nucleotide sequence of gentamycin resistance gene was cloned in the above gene at the SalI restriction site. The obtained construction was used to transform the competent cells of *E. coli* S17-1 [14] for further conjugative transfer into *Mm. alcaliphilum* 20Z [15]. The obtained mutants were selected by gentamycin resistance and kanamycin sensitivity.

Genomic DNA purification and PCR amplification. Chromosomal DNA from cells of *Mm. alcaliphilum* 20Z was purified by the modified method [16]. A fragment of the *mxaF* gene encoding the large subunit of methanol dehydrogenase (MDH) was amplified using the functional primers mxaF1003f/mxaF1561r [17] synthesized at Syntol (Moscow, Russia). The reaction mixture (30 μl) contained: 75 mM Tris–HCl buffer, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 2.0 mM $MgCl_2$, 0.25 μM of the each primer, and 0.5 μg of DNA. Each dNTP (0.2 μM) and *Taq* polymerase (1–2 U) (Sileks-M, Moscow, Russia) were added. PCR amplification was carried out in a Hybaid DNA ther-

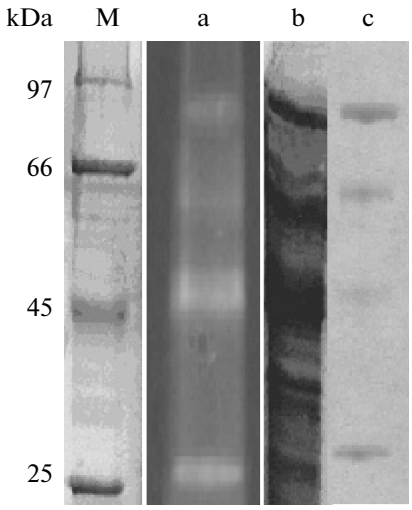

Fig. 1. SDS-PAGE of the major surface proteins of *M. alcaliphilum* 20Z: proteins from dansyl chloride-treated cells (visualization with UV at 260 nm) (a), total cell proteins stained with Coomassie G-250 (b), and proteins obtained by biotinylation (c). M indicates protein markers.

mocycler (United Kingdom) in the following mode: Hot-start, 3 min at 94°C; 30 cycles: 94°C for 30 sec; 72°C for 1.5 min; and the final cycle at 72°C for 5–10 min. Reaction products were separated by electrophoresis in 1% agarose gel (Gibco BRL, United Kingdom) and stained with ethidium bromide (5 μg/ml).

Cloning, expression and purification of cytochrome *c* peroxidase. The *MEALZv2_1030035* gene (https://www.genoscope.cns.fr/agc/mage) was amplified from the genomic DNA of *Mm. alcaliphilum* 20Z using the following primers: forward (5'-ATA**CATATG**TTTATTAGCGTTAAAAAAAC-3') and reverse (5'-ATA**AAGCTT**AGGTTGTAAAAAAC-3') carrying the recognition sites for restriction endonucleases *Nde*I and *Hind*III, respectively. The PCR product was purified in a Wizard column (Promega, United States), treated with site-specific endonucleases *NdeI* and *Hind*III, and ligated into the expression vector pET22b$^+$ pretreated with the same restriction endonucleases. The obtained plasmid was transformed in *E. coli* BL21 (DE3). Transformed *E. coli* cells were grown overnight at 37°C in the LB medium, transferred into fresh LB medium containing 100 μg/ml of ampicillin, and grown to OD_{600} of 0.6–0.7. Protein expression was induced by isopropyl-β-D-thiogalactopyranoside at the final concentration of 1 mM. After incubation at 37°C for 5 h, the cells were precipitated by centrifugation (6000 *g*, 20 min, 4°C) and resuspended in 20 mM Tris–HCl buffer, pH 8.0, containing 150 mM KCl, 20 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). *E. coli* cells were disrupted in an MSE disintegrator (United Kingdom) at 150 W and 10 Hz (3 × 0.5 min with 0.5-min intervals); the suspension was centrifuged for 20 min at 10000 *g* and 4°C. The supernatant was applied to a 0.5-ml column containing Ni^{2+} nitroacetate agarose (Superflow, Qiagen). After intensive washing with the lysis buffer, the protein was eluted with the lysis buffer containing 160 mM imidazole. The 0.5-ml fractions were collected, and 10 μl of a sample was analyzed for the presence of MEALZv2_1030035-His_6 by electrophoresis in 12% SDS-PAG. Peroxide-degrading protein activity was determined in the gel by oxidation of *o*-dianizidine in the presence of H_2O_2 [12].

Physiological and biochemical studies. The effect of pH of the medium was investigated by growing the initial and mutant *Mm. alcaliphilum* strains in 750-ml flasks under methane–air atmosphere (1 : 1). The respective pH value of the medium was found by adding the Na-carbonate or Tris–HCl buffers up to a final concentration of 50 mM. Enzymatic assays were carried out by the methods described previously [18].

Bioinformation analysis. Comparative analysis of the sequences of DNA and proteins was performed using PSI-BLAST available at [http://www.ncbi.nlm.nih.gov]. Analysis of nucleotide sequences and their translation into amino acid sequences and determination of restriction sites and ORF were carried out using the GeneRuner 3.00, VectorNTI®Advance v. 9.0, DNAStar, and Clone Manager 5 software packages. Amino acid sequences were aligned using the Clustal X (v1.62b) [19] and GeneDoc software packages.

RESULTS

Identification of the major surface proteins of *Mm. alcaliphilum* 20Z. The cells were treated with the fluorescent reagent dansyl chloride, which interacts with the free amino groups of amino acids [20]. SDS-PAGE of total proteins from dansyl chloride-treated cells (see Materials and Methods) showed in UV four protein bands with molecular masses of 27, 45, 60 and 80 kDa (Fig. 1a). Gel staining with Coomassie G-250 confirmed that these proteins were dominant in the spectrum of total cell proteins (Fig. 1b).

For isolation of surface proteins, the methanotroph cells were pretreated with a biotin probe. Biotinylated proteins were extracted with GHCl and collected onto streptavidin-coated magnetic particles. Subsequent SDS-PAGE of the proteins released from streptavidin–biotin also showed the presence of four major protein bands. Biotinylation of proteins with molecular masses of 27, 45, 60 and 80 kDa suggests their noncovalent binding with the outer cell membrane (Fig. 1c).

The isolated surface proteins were exposed to trypsinolysis in the gel, and the MALDI mass spectra of protein fragments were obtained. Their comparison with the peptide spectra in the Mascot database

showed similarity of the 60-kDa protein to the large subunit of methanol dehydrogenase of methanotrophic bacteria, since a sufficiently high Score 92 value and coincidence of eight peptides was obtained in most cases. A 550-bp DNA fragment from strain 20Z was amplified and sequenced using the mxaF1003f/mxaF1561r pair of primers constructed for the conservative region of the large MDH subunit [17]. In silico trypsin cleavage of translated amino acid sequences of the fragment in the relevant program at the ExPaSy site revealed 16 peptides with molecular masses of over 500 Da, which completely overlapped with the MALDI spectra of the peptides obtained in vitro. Thus, the 60-kDa protein was identified as a large subunit of MDH.

Detection of MDH at the outer cell wall surface of *Mm. alcaliphilum* 20Z was quite unexpected, as it is known, that in the thermotolerant *Mc. capsulatus* Bath, this enzyme is localized in the cytoplasmic membrane (CM) and the periplasm, as well as in the intracytoplasmic membranes (ICMs), where it forms supramolecular complexes with the particulate methane monooxygenase (pMMO), which oxidize methane to formaldehyde [21]. Surface localization of MDH was revealed also in *Mm. album* BG8 [22].

Comparison of the mass spectra of the peptides obtained by trypsinolysis of the 27-kDa protein revealed no similar proteins in the relevant database. However, MS/MSn analysis made it possible to identify two peptide sequences, VSVEVS and QFVVGGI, with m/z 1336.7 and 2315.0, respectively (Fig. 2a). An open reading frame (ORF) *MEALZv2_1030034* was found in the partially annotated genome of *Mm. alcaliphilum* 20Z (https://www.genoscope.cns.fr/agc/mage) using the Blast software (Fig. 2b). Its product is a polypeptide of 229 amino acids with a calculated molecular mass of 25.6 kDa. Analysis of the translated amino acid sequences within the protein MEALZv2_1030034 showed 22 strongly acidic, 19 strongly basic, 67 polar, and 73 hydrophobic amino acids. The isoelectric point (pI) was 6.2. N-terminal sequencing by the Edman method showed the presence of an ATTLNG sequence corresponding to amino acid positions from 27 to 32. The first 26 amino acids MKKTNKVKALTFAVATMGALTSLPSHA are believed to form a leader peptide, which is cleaved after protein transport across the cell membrane. Accordingly, the calculated mass of the mature protein is 22.8 kDa.

MEALZv2_1030034 of *Mm. alcaliphilum* 20Z shows high similarity of translated amino acid sequences with the cell surface-associated proteins CorA of *Mm. album* BG8 (52% identity, 68% similarity) [23] and MopE of *Mc. capsulatus* Bath (23% identity, 40% similarity) [24, 25]. However, analysis of the annotated genomes of *Methylosinus trichosporium* OB3b, *Methylocella silvestris* BL2, *Methylobacter tundripaludum*, and *Methylacidiphilum infernorum* V4 showed no homologues of the above protein in these methanotrophs.

A similar approach (protein trypsinolysis and analysis of the MALDI spectra of the trypsinolysis products) was used to identify the gene encoding the protein with a molecular mass of 80 kDa. The ORF of MEALZv2_1030035 encoding a polypeptide with the calculated molecular mass of 78.8 kDa was identified by the primary sequence of the fragments. The calculated amino acid sequences of this protein showed 59% identity (73% similarity) to the CorB protein from *Mm. album* BG8 [22] and 43% identity (57% similarity) with the Mca protein from *Mc. capsulatus* Bath [26]. The MEALZv2_1030035 protein (710 amino acids) contains 86 strongly acidic, 80 strongly basic, 173 polar, and 243 hydrophobic amino acids (pI = 6.41). Similarity was also shown for the structure of these proteins in *Mm. alcaliphilum* 20Z, *Mm. album* BG8, and *Mc. capsulatus* Bath, because their sequences contained two supposed heme-binding motifs (CxxCH).

The *MEALZv2_1030035* gene was cloned to obtain a homogeneous recombinant protein with 6 histidine residues at the C-terminus, which showed a peroxide-decomposing activity (Fig. 3). This protein may therefore be classified as a di-heme cytochrome *c* peroxidase [22, 26].

Cellular localization and protein expression conditions. The cell surface of the methanotroph *Mm. alcaliphilum* 20Z was covered with a monolayer of cup-shaped structures (Fig. 5a) of glycoprotein nature [3]. Surface proteins were solubilized by lithium chloride and dialyzed against water. Electron microscopy of the semitransparent protein flakes precipitated as a result of dialysis showed the presence of the S-layer cup-shaped structures (Fig. 4). SDS-PAGE of the obtained suspension showed the major protein band of ~27 kDa. Protein flakes were dissolved in 5 M GHCl and exposed to gel filtration on Sepharose CL-6B. Rabbit polyclonal antibodies to the purified protein preparation were used to specify its localization by the methods of immunocytochemistry and electron microscopy.

Whole cells, as well as their ultrathin sections, were treated with the obtained antibodies and the antibodies to rabbit proteins (colloidal gold-labeled protein A). Electron microscopic studies showed that gold particles were localized in the surface layer of the cells outside the cell wall (Fig. 5b) and near the bases of cup-shaped structures (Fig. 5d–5f). Gold-labeled antibodies were also revealed in separately lying fragments of the S-layer that did not contain outer membrane fragments (Fig. 5c).

Electron microscopy of the *Mm. alcaliphilum* 20Z cells treated with polyclonal antibodies to the 80-kDa protein and colloidal gold-labeled protein A showed

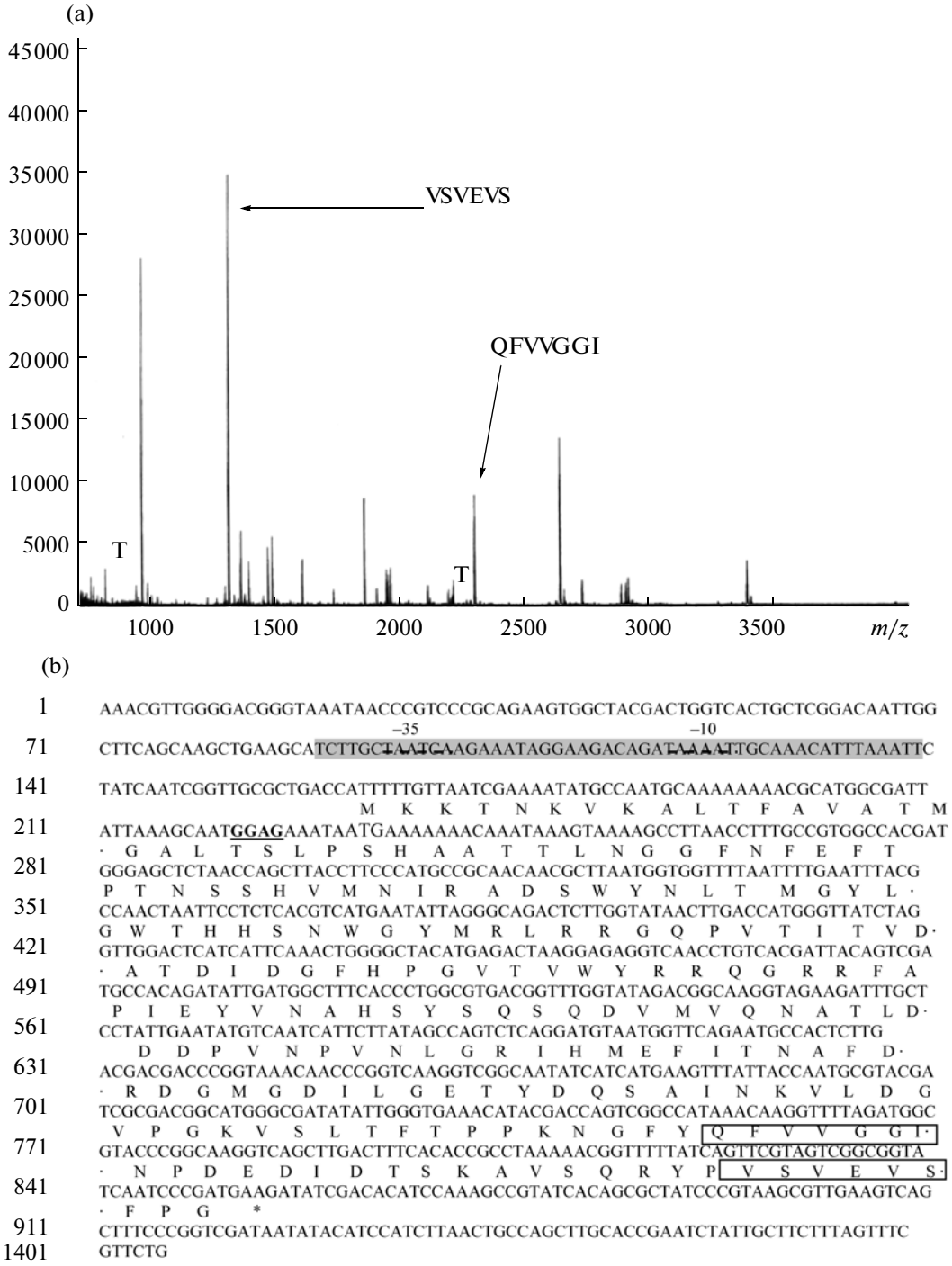

Fig. 2. Identification of the 27-kDa surface protein. Mass spectrometric analysis of polypeptide sequences obtained by trypsinolysis in gel (a) and the nucleotide sequence of the *MEALZv2_1030034* gene in *M. alcaliphilum* 20Z (b). Supposed Shine–Dalgarno sequences are underlined. The nucleotide sequence determined as a promoter region in the Neural Network Promoter Prediction (NNPP) with the probability of 0.85 is in gray. Dotted line indicates supposed −10 and −35 promoter regions. Sequences identified by MS/MSn fragmentation of the tryptic peptides are marked with frames.

that at least fragments of the protein MEALZv2_1030035 were exposed at the outer surface of the cell wall (Fig. 5g). Nonuniform labeling of the surface with gold probably results from shielding of this protein by the S-layers. The treatment of ultrathin sections with antibodies showed mainly periplasmic location of the 80-kDa protein (Fig. 5h).

Analysis of the relative content of the 27-kDa protein in the cells grown at different copper concentrations in the medium showed that its level increased

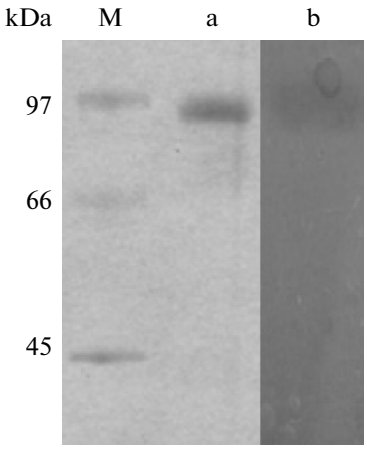

Fig. 3. SDS-PAGE of recombinant protein MEALZv2_1030035 (a) and detection of peroxide-degrading activity in the presence of *o*-dianizidine (b). M, protein markers.

when the cation concentration decreased (Fig. 6), as well as during the stationary growth phase. These data correlate with the important function of copper ions in the metabolism of methanotrophs possessing particulate MMO (pMMO), since copper is part of the active center of this enzyme [27].

Induction of the synthesis of the cell surface-associated proteins CorA and MopE in copper-limited growth medium was previously shown for the methanotrophs *Mm. album* BG8 and *Mc. capsulatus* Bath [23, 25]. The obtained *Mm. album* BG8 mutants in the CorA protein showed low viability; even an increase of copper content in the medium to 5 μM did not improve their growth [25].

For elucidation of the role of MEALZv2_1030034, we obtained a mutant of *Mm. alcaliphilum* with an insertion in the gene encoding this protein. The cells of the mutant strain lost the ability to grow on methane but grew in the presence of methanol (0.2%). Electron microscopy showed that, in most cells of the population of this variant, the S-layer cup-shaped structures (CSS) were chaotically located close to the cell surface (Fig. 7a) and were found in the intercellular space as a bilayer of "cups" with adjacent bases (Fig. 7b). A membrane-like zone (two electron-dense layers separated by an electron-transparent layer) was formed in the center of the bilayer, in the region of closure of CSS bases. Such structures are usually formed by polar phospholipid or protein molecules. The MEALZv2_1030034 protein is obviously involved in attachment of the S-layer cup-shaped structures to the outer membrane and is necessary for the growth of strain 20Z on methane.

The cells of *Mm. alcaliphilum* mutant defective in the *MEALZv2_1030035* gene grew on methane more rapidly than the cells of the original strain. Moreover, wild type cells did not grow at all at pH 11, while the mutant cells grew actively but with rapid acidification of the medium to pH 8. The cells of the mutant strains had S-layers of typical morphology and formed myelin-like structures (Fig. 7c). Enzymatic analysis of cell extracts showed higher activities of methanol, formaldehyde, and formate dehydrogenases for the mutant in protein MEALZv2_1030035 compared to the parent strain (table). Intense acidification of the medium probably results from the enhanced rate of oxidative processes.

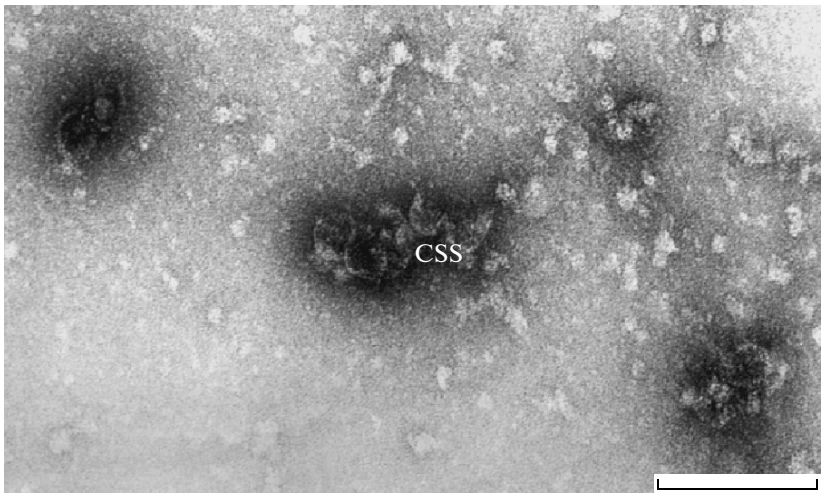

Fig. 4. Solubilization of protein structures from the cell surface by lithium chloride. CSS, cup-shaped structures. Bar scale: 0.1 μm.

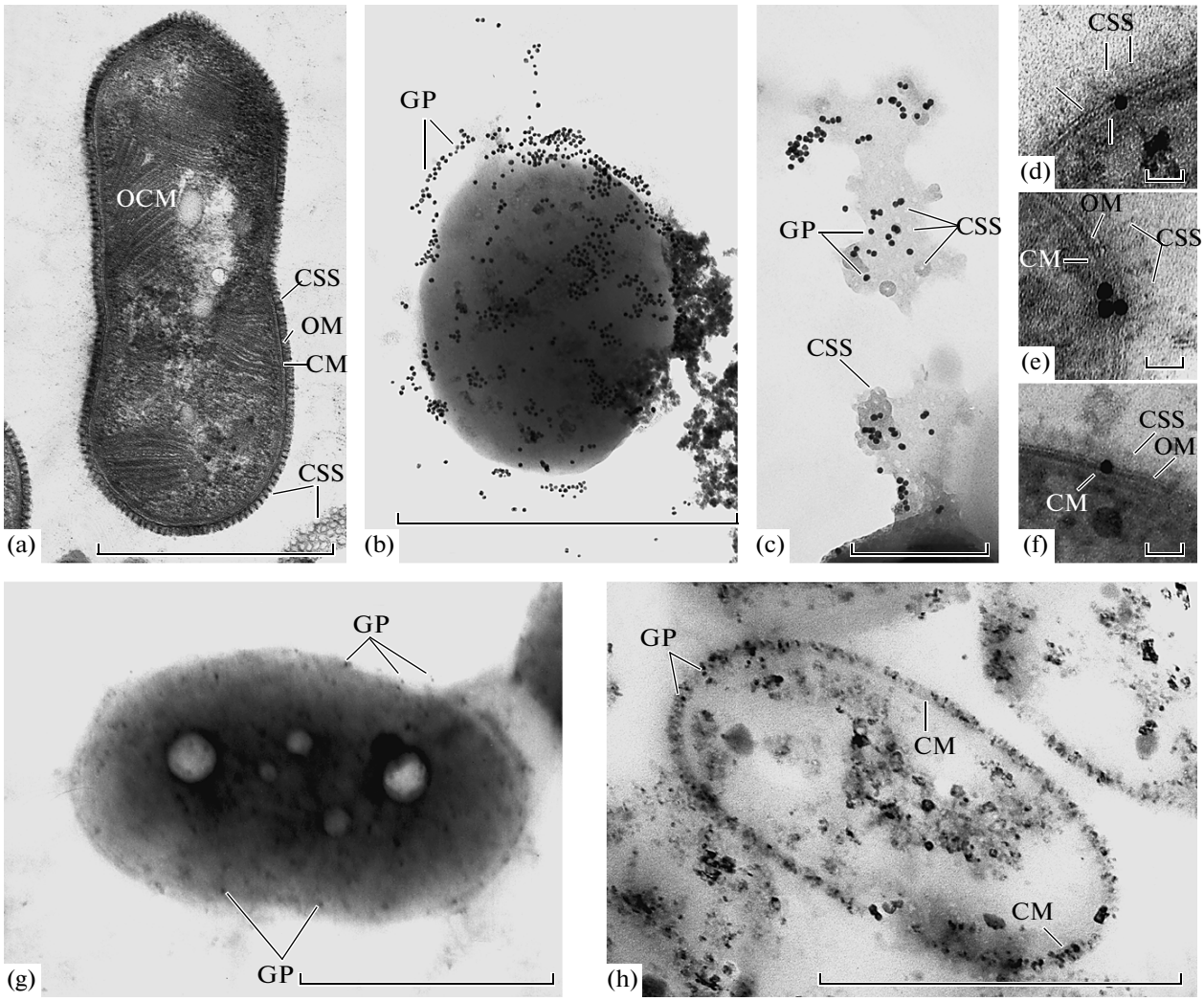

Fig. 5. Ultrastructure of an *Mm. alcaliphilum* 20Z cell (a, ultrathin section) and localization of proteins MEALZv2_1030034 (b–f) and MEALZv2_1030035 (g, h). Whole cells (b), S layer fragment (c) and ultrathin sections (d–f) were treated with antibodies to the MEALZv2_1030034 protein and colloidal gold with a particle size of 8.3 nm. Whole cells (g) and ultrathin sections (h) were treated with antibodies to the MEALZv2_1030035 protein and colloidal gold with a particle size of 5 nm. CSS, cup-shaped structures; GP, gold particles; OM, outer membrane; CM, cytoplasmic membrane. Bar scale: 1 (a, b, g, h), 0.2 (c), and 0.05 μm (d–f).

The attempt to identify the gene of the forth major 45-kDa protein by the peptide spectra from the Mascot database gave no definite results, although this protein was probably responsible of formation of the S-layer structures. Some difficulties with identification of the respective gene may be due to the absence or low homology of S-layer proteins in most bacteria [28].

DISCUSSION

Mm. alcaliphilum 20Z can grow under conditions of elevated salinity (up to 10% NaCl) and alkaline pH values (pH > 9) [3], unlike the neutrophilic nonhalophilic *Mm. album* BG8 (mesophile) and *Mc. capsulatus* Bath (thermotolerant). In spite of the differences in their physiological and biochemical properties, the major surface proteins of these methanotrophs are two proteins with the highest homology in two species of the genus *Methylomicrobium*. In *Mm. album* BG8, the genes encoding these proteins, *corA* and *corB*, are located in an operon, the transcription of which is repressed under excess of copper in the cultivation medium [22]. In the chromosome of *Mc. capsulatus* Bath, the genes encoding the homologues of these proteins are arranged in reverse order and compose a *mca-mopE* operon. In *Mm. alcaliphilum* 20Z, the respective proteins are products of the MEALZv2_1030034 and MEALZv2_1030035 ORF, which are also located at the same locus of the chromosome but are separated by a 412-bp fragment, with their cotranscription not improbable. Expression of the MEALZv2_1030034 protein under copper limitation indicates its involvement in homeostasis of this cation in the cells of *Mm. alcaliphilum* 20Z.

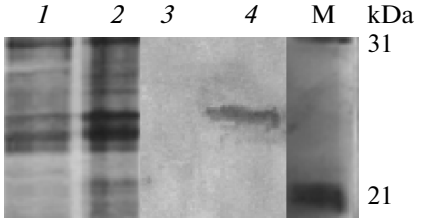

Fig. 6. Expression of MEALZv2_1030034 in *M. alcaliphilum* 20Z at different copper concentrations in the medium: 12% SDS-PAGE of total cell proteins (*1, 2*) and immunoblotting (*3, 4*). The cells were grown in the presence of 0.1 g/l $CuSO_4 \cdot 5H_2O$ (*1*, *3*) and in the medium without copper (*2, 4*).

High homology with the cell surface protein CorA (copper repressible) described in *Mm. album* BG8 in 1997 [23] makes it possible to use this designation for the MEALZv2_1030034 protein that we have identified. However, the CorA abbreviation (cobalt resistant) has been used since 1969 for the large family of transmembrane proteins functioning as Mg^{2+} transporters in a wide range of organisms including bacteria, archaea, animals, and humans [29, 30]. Proteins of this family participate also in the transport of Ni^{2+}, Co^{2+}, and Ca^{2+} and have an insignificant homology (<10%) with the copper-repressed CorA from *Mm. album* BG8, MopE* from *Mc. capsulatus* Bath, and MEALZv2_1030034 from *Mm. alcaliphilum* 20Z. It is interesting that the genomes of *Mm. alcaliphilum* 20Z, *Mm. album* BG8, *Methylosinus trichosporium* OB3b, *Methylocella* (*M.*) *silvestris* 20Z, *Mm. album* BG8, *Methylosinus trichosporium* OB3b, *Methylocella silvestris* BL2, and *Methylobacter tundripaludum* have been shown to carry the genes coding for magnesium transporters of the CorA family with ~50% identity of translated amino acid sequences. It should be noted that the gene of Mg^{2+} transporter, *corA*, has not been found in *Mc. capsulatus* Bath.

Copper ions play a key role in the metabolism of methanotrophs, since they are necessary for the synthesis and activity of particulate methane monooxygenase [27]. However, homologues of the MEALZv2_1030034 protein are not characteristic for all methanotrophs possessing pMMO, since the analysis of annotated genomes did not reveal respective genes in the psychrophile *Methylobacter tundripaludum* with pMMO, the mesophile *Methylosinus trichosporium* OB3b with both particulate and soluble MMO, the thermoacidophile *Methylacidiphilum infernorum* V4 with a phylogenetically distant pMMO, or the moderate acidophile *Methylocella silvestris* BL2 with only the soluble form of the enzyme.

The presence of CorA homologues in *Mm. alcaliphilum* 20Z, *Mm. album* BG8, and *Mc. capsulatus* correlates with the formation of the S layers in these bacteria. In *Mm. alcaliphilum* 20Z and *Mm. album* BG8, the S layers show three-dimensional organization as cup-shaped structures arranged with hexagonal

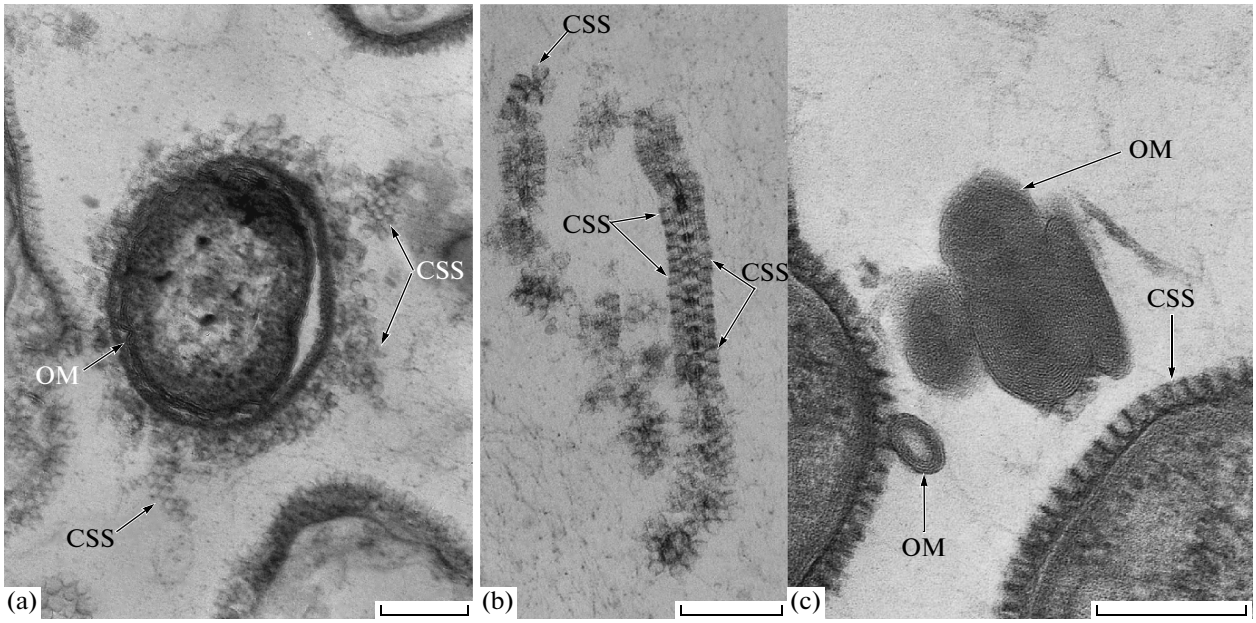

Fig. 7. Ultrathin sections of *M. alcaliphilum* 20Z mutant cells with inactivated *MEALZv2_1030034* (a) and *MEALZv2_1030035* (c) genes. Negative contrasting of S-layer fragment of the strain mutant in the *MEALZv2_1030034* gene (b). CSS, cup-shaped structures; OM, outer membrane; MS, myelin-like structures. Bar scale: 0.2 μm.

Enzyme activities in the cell extracts of *Mm. alcaliphilum* 20Z and the *MEALZv2_1030035*$^-$ mutant (nmol min^{-1} mg^{-1} protein)

Enzyme	Cofactor	*Mm. alcaliphilum* 20Z	*MEALZv2_1030035*$^-$ mutant
Methanol dehydrogenase	PMS	252	694
Formaldehyde dehydrogenase	NAD^+	6	11.5
	PMS	1.5	2.2
Formate dehydrogenase	NAD^+	26	180
	PMS	2.5	36.5

(*p*6) symmetry. While the formation of S-layers in *Mc. capsulatus* Bath has not been studied, by analogy with other strains of this species (Texas and 874), they may be represented by a monolayer of polypeptides packed with planar tetragonal (*p*4) symmetry [7, 8].

We have shown that the 27 kDa protein (MEALZv2_1030034) is necessary for attachment of the S layers to the outer membrane, because, in the mutant lacking this protein, these structures were not attached to the cell wall. Moreover, the cells with mutations in this protein could not grow on methane but actively grew on methanol, and the protein expression was intensified in response to the decrease of copper content in the growth medium. It is likely that MEALZv2_1030034 is a part of a specialized system for copper ion transport to pMMO. Coexpression of this protein with di-heme cytochrome *c* peroxidase indicates participation of this protein tandem in the transport of copper, which is necessary for growth on methane.

X-ray structure analysis of the MopE protein from *Mc. capsulatus* Bath [31] revealed a copper-binding site containing kynurenine (the product of Tryptophan-130 oxidation), in addition to histidine residues His-132 and His-203. The recombinant protein with non-oxidized tryptophan did not bind copper. It was suggested that Trp-130 oxidation in *Mc. capsulatus* Bath was an enzymatic process performed by di-heme cytochrome *c* peroxidase encoded by the *mca* gene. However, this hypothesis has not yet been confirmed by enzymatic and genetic studies.

In contrast to the MEALZv2_1030034 mutant, the *Mm. alcaliphilum* mutant in di-heme cytochrome *c* peroxidase did not lose the ability to grow on methane but had a higher growth rate and ability to grow at more alkaline pH values, exhibiting enhanced activity of C_1 oxidation enzymes. It is known that the intensity of oxidative processes increases when methanotrophs are cultivated in an alkaline medium and when their growth rate increases, which is accompanied by intense formation of reactive oxygen species (ROS) [32, 33]. In turn, high ROS levels may lead to nonenzymatic tryptophan oxidation in the relevant Cu^{2+} binding site, thereby providing the necessary modification of MEALZv2_1030034 in the absence of di-heme cytochrome *c* peroxidase. However, this suggestion needs further investigation.

The sequences homologous to the *MEALZv2_1030035* gene have been found in the genomes of a wide range of methanotrophs; however, these polypeptides show rather low homology. MEALZv2_1030035 has 11 and 5% identity (18 and 11% similarity) with those in *Mb. tundripaludum* and *Ms. trichosporium* OB3b, respectively. The homologues of di-heme cytochrome *c* peroxidase have not been found in acidophilic methanotrophs, which correlates with the absence of pMMO in *M. silvestris* BL2 and with a highly divergent enzyme form in the extreme acidophile *Ma. infenorum* V4.

To date the presence of two copper-repressed cell surface proteins has been shown in S layer-forming methanotrophs. It may be supposed that S layers screen copper transport by means of other carriers, e.g., the low-molecular-mass siderophore methanobactin [34]. Hence, an additional mechanism is required to maintain copper homeostasis in the cells. The reasons for the formation of S-layers in *Mm. alcaliphilum* 20Z, *Mm. album* BG8 and *Mc. capsulatus* are presently under investigation.

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