High-Molecular-Mass Multi-c-Heme Cytochromes from *Methylococcus capsulatus* Bath†

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The polypeptide and structural gene for a high-molecular-mass c-type cytochrome, cytochrome $c_{553O}$, was isolated from the methanotroph *Methylococcus capsulatus* Bath. Cytochrome $c_{553O}$ is a homodimer with a subunit molecular mass of 124,350 Da and an isoelectric point of 6.0. The heme c concentration was estimated to be $8.2 \pm 0.4$ mol of heme c per subunit. The electron paramagnetic resonance spectrum showed the presence of multiple low-spin, $S = 1/2$, hemes. A degenerate oligonucleotide probe synthesized based on the N-terminal amino acid sequence of cytochrome $c_{553O}$, was used to identify a DNA fragment from *M. capsulatus* Bath that contains occ, the gene encoding cytochrome $c_{553O}$. occ is part of a gene cluster which contains three other open reading frames (ORFs). ORF1 encodes a putative periplasmic c-type cytochrome with a molecular mass of 118,620 Da that shows approximately 40% amino acid sequence identity with occ and contains nine c-heme-binding motifs. ORF3 encodes a putative periplasmic c-type cytochrome with a molecular mass of 94,000 Da and contains seven c-heme-binding motifs but shows no sequence homology to occ or ORF1. ORF4 encodes a putative 11,100-Da protein. The four ORFs have no apparent similarity to any proteins in the GenBank database. The subunit molecular masses, arrangement and number of hemes, and amino acid sequences demonstrate that cytochrome $c_{553O}$ and the gene products of ORF1 and ORF3 constitute a new class of c-type cytochrome.

*Methylococcus capsulatus* Bath is an obligate methylotroph that utilizes methane as its sole energy and carbon source. As for most other methanotrophs, methane and methanol are the only known growth substrates (6, 30). In methanotrophs, methane is oxidized via a series of two electron steps, with methanol, formaldehyde, and formate as intermediates (6, 30). The reductant for the first, energy-dependent, step is supplied by either NADH or by the respiratory chain, depending on which methane monoxygenase (MMO) is expressed (6, 15, 25, 30, 58). Formaldehyde is either assimilated via the NAD-linked formaldehyde dehydrogenase or by a tetrahydro-methanopterin-methanofuran-mediated pathway (6, 13, 30, 55, 62). Lastly, formate is oxidized to carbon dioxide by an NAD$^+$-linked formate dehydrogenase (34). With the possible exception of an electron donor to the membrane-associated methane monoxygenase (pMMO), c-type cytochromes are known to be involved only in the methanol oxidation step (6, 7, 38).

In contrast to the limited role of c-type cytochromes in the oxidation of growth substrates, methanotrophs show complex cytochrome c patterns similar to that observed in the facultative methylotrophs (6, 7, 11, 18, 30, 38, 61–65). For example, seven c-type cytochromes have been purified (5, 63–65), and the structural genes for two additional multiheme cytochromes have been identified (this study) in *M. capsulatus* Bath. Two of the seven have enzymatic activity; cytochrome c-peroxidase (65) and cytochrome P460 (10, 63), while the remaining five appear to function in electron transfer (5, 61, 63, 64). The complexity of the respiratory systems in methanotrophs provides suggestive evidence that the current biochemical models for methanotrophs underestimate the biochemical capabilities of these organisms. In addition to the known growth substrates, methanotrophs will oxidize or co-oxidize a variety of compounds, depending on the form of MMO expressed (14, 15, 20, 39, 52, 54, 59). Cells expressing the soluble MMO will oxidize straight-chain or branched-chain alkanes or alkenes up to eight carbons long as well as cyclic and aromatic compounds (14, 30, 51, 59). Cells expressing the pMMO will oxidize alkanes and alkenes up to five carbons long but will not oxidize cyclic or aromatic compounds (19, 30, 39, 52). With the exception of methane and, in some cases, methanol, the oxidation of other substrates does not support growth and has been termed co-oxidation. Implicit in the use of the term co-oxidation is that the oxidation provides no metabolic energy. However, some cosubstrates may generate metabolic energy. For example, both MMOs catalyze the energy-dependent oxidation of ammonia to hydroxylamine (16, 47, 63). In *M. capsulatus* Bath, cytochrome P460 catalyzes the four-electron oxidation of hydroxylamine to nitrite (63). This two-step oxidation of ammonia to nitrite is identical to that observed in nitrifying bacteria, although the enzymes catalyzing the steps have been shown to differ (9, 10, 63). The similar mechanisms of oxidation of ammonia in both groups of bacteria suggest that metabolic energy is obtained during ammonia oxidation in methanotrophs.

Whether the oxidation of hydroxylamine also provides reductant for ammonia (or methane) oxidation or whether all four electrons are transferred to the terminal oxidases (21, 62) via cytochrome $c'$ (64) and cytochrome $c_{554}$ (5) has not been determined for methanotrophs. In the nitrifying bacterium *Nitrosomonas europaea*, the four electrons from the oxidation of hydroxylamine are transferred to the tetraheme cytochrome, cytochrome $c_{554}$, which acts as a redox mediator from hydrox-
yiamine oxidoreductase to both the ammonia monooxygenase and the terminal oxidase (17, 33). In the current study, we present the isolation of an octyl-heme cytochrome, cytochrome c_{553O}. The structural gene for cytochrome c_{553O} was part of a gene cluster containing two other putative high-molecular-mass multiheme cytochromes. The physiological role for these proteins is still unknown. However, one or more of these high-molecular-mass cytochromes appears to be induced by ammonia (10) and may function like cytochrome c_{553O} in *N. europaea*.

### MATERIALS AND METHODS

#### Culture conditions

Culture conditions for *N. europaea*, *M. capsulatus* Bath, *Methylobacterium trichosporium* OB3b, *Methylocystis parvus* OBBP, *Methyllobacterium rubrum* Ad55, *Methylorubrobacter albus* BGS, and *Methylophomonas* sp. strains MN and MM2 were described previously (10, 18, 19, 61).

#### Isolation of cytochrome c_{553O}

All procedures were performed at 4°C. Cell lysis and initial separation of cytochrome c_{553O} from other e-type cytochromes was described by Zahn et al. (65). Following the Sephadex B-75 gel-filtration step, the sample was collected and brought to 20% saturation with a concentrated solution of ammonium sulfate. The sample was loaded on a phenyl Sepharose CL-4B column (2.5 by 21 cm) previously equilibrated in 1.24 M ammonium sulfate and 20 mM Tris (pH 8). The column was washed with a sequential order of 1.5 column volumes each of buffers containing 20 mM Tris (pH 8) plus 0.50 M ammonium sulfate. The cytochrome fraction remained bound to the column during the washing procedure and was eluted with 2 column volumes of a buffer containing 20 mM Tris (pH 8) plus 3% saturation ammonium sulfate. The fraction was dialyzed by ultrafiltration into 40 mM Tris (pH 9) and concentrated with a YM-10 ultrafiltration membrane. The column was washed in a sequential order with 1.5 column volumes of each buffer containing 20 mM Tris (pH 8) plus 1.24 M ammonium sulfate and 20 mM Tris (pH 8) plus 0.50 M ammonium sulfate. The cytochrome fraction remained bound to the column during the washing procedure and was eluted with 2 column volumes of a buffer containing 20 mM Tris (pH 8) plus 3% saturation ammonium sulfate. The fraction was dialyzed by ultrafiltration into 40 mM Tris (pH 9) and concentrated with a YM-10 ultrafiltration membrane. The fraction was loaded on a Q-Sepharose fast-flow column (1.25 by 14 cm) equilibrated in 40 mM Tris (pH 9), and the column was developed with a linear gradient of 0 to 200 mM KCl at 40 mM Tris (pH 9). Purified cytochrome c_{553O} eluted at a salt concentration of approximately 160 mM KCl. The cytochrome had a diethanolamine-reduced a-betam absorption maxima at 553 nm and an oxidized absorbance (A_{553O}/A_{420}) ratio of 4.3.

#### Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was carried out with the Laemmli method on 10 to 16% polyacrylamide gels (35). Gels were stained for total protein with Coomasie brilliant blue R. Proteins with peroxidase activity in SDS-polyacrylamide gels were stained by the dianisidine benzidine method (41). Preparative isoelectric focusing in a granulated gel matrix was performed with a Pharmacia Multiphor 1 system at 4°C with Ultrodeg and 2% ampholine (pH, 3 to 10) as described by the manufacturer.

#### Analytical ultracentrifugation

Sedimentation velocity experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge equipped with a Beckman AN-60 rotor. Samples of cytochrome c_{553O} were dialyzed against three changes of buffer containing 50 mM phosphate (pH 7) or 25 mM Tris-HCl (pH 8.0) plus 150 mM KCl. The sample and reference cell assemblies were monitored with a wavelength of 410 nm. Separate sedimentation velocity experiments were performed with rotors of 30,000 and 150,000 rpm. Solution density was maintained at 20°C during sedimentation experiments. Partial specific volume (v) of *M. capsulatus* Bath cytochrome c_{553O} was calculated from the amino acid composition by the method of Cohn and Edsall. Solution density values for cytochrome c_{553O} were estimated by using the total protein values determined by SDS-polyacrylamide gel electrophoresis, with the terminal oxidase (17, 33). In the current study, we present the isolation of an octyl-heme cytochrome, cytochrome c_{553O}. The structural gene for cytochrome c_{553O} was part of a gene cluster containing two other putative high-molecular-mass multiheme cytochromes. The physiological role for these proteins is still unknown. However, one or more of these high-molecular-mass cytochromes appears to be induced by ammonia (10) and may function like cytochrome c_{553O} in *N. europaea*.

#### Spectroscopy

Optical absorption spectroscopy was performed with an SLM 4101A spectrophotometer in the split-beam mode. Electron paramagnetic resonance (EPR) spectra were recorded at X band on a Bruker ER 200D EPR spectrometer equipped with an Oxford Instruments ESR-900 liquid helium cryostat. Operating parameters were as listed in the figure legends. Samples were maintained at 8K during spectral acquisition.

#### Heme, metal, and protein determination

The optical extinction coefficient values for cytochrome c_{553O} were estimated by using the total protein values derived from the amino acid analysis and a subunit molecular mass of 124,350 Da. Heme composition was determined by the pyridine ferrohemochrome method (18, 26). The acetic acid method was used to determine the terminal oxidase (17, 33). The protein was analyzed with the Pharmacia Multiphor 1 system at 4°C with Ultrodeg and 2% ampholine (pH, 3 to 10) as described by the manufacturer.

#### Amino acid analysis and sequence analysis

### RESULTS

### Purification of cytochrome c_{553O}

The purification of cytochrome c_{553O} from *M. capsulatus* Bath cultured in nitrate mineral salts medium was performed as described in Materials and Methods. The initial purification step involved separation of cytochrome c_{553O}, which migrates in the void volume from methanol dehydrogenase (MeDH) (approximate molecular mass, 120,000 Da) and other lower-molecular-mass e-type cytochromes (65), by using a 5 by 96 cm Sephadex G-75 column. The separation of cytochrome c_{553O} from MeDH was well beyond the normal separation capacity of Sephadex G-75. However, this separation was obtained if the resin was degassed before the resin was poured. If the resin was not degassed, cytochrome c_{553O} and MeDH comigrated in the void volume.

#### Molecular mass

In SDS-polyacrylamide gels, cytochrome c_{553O} migrated as a single band corresponding to a molecular mass of 142,000 Da (Fig. 1). The sample required both β-mercaptoethanol and heat treatments before being loaded on SDS-polyacrylamide gels for complete unfolding of the polypeptide chain, indicating the presence of interpeptide disulfide bonding (Fig. 1). Comparison of the subunit mass, as determined by SDS-polyacrylamide gel electrophoresis, with the subunit mass plus eight hemes c predicted by the gene sequence (124,350 Da) shows a discrepancy of approximately 12%. The high-charge density of the eight covalently bound hemes may be responsible for this discrepancy.

The molecular mass of native cytochrome c_{553O} from *M. capsulatus* Bath was estimated to be 202,577 ± 2,765 Da by analytical ultracentrifugation (Table 1). The geometrical shape of ferricytochrome c_{553O} was assigned based on the prolate ellipsoid model by using the values calculated for the partial specific volume and sedimentation values determined by sedimentation velocity experiments. The axial ratio value of approximately 15:1 (Table 1) indicates that the cytochrome has a nonuniform, highly elongated shape. The unique hydrodynamic property of cytochrome c_{553O} is probably the reason for the large difference in the holoenzyme mass determined by
sedimentation velocity (202,580 Da) and the proposed α, dimer estimated by the translated gene sequence plus 16 hemes c (248,700 Da). The results suggest that cytochrome c₅₅₃O consists of a dimer composed of two identical subunits.

**Heme and metal components.** The prosthetic groups of cytochrome c₅₅₃O, were identified as ε types by the acid acetone method and ferrohemochromogen spectra. Assuming a molecular mass of 124,350 Da and protein concentrations determined by amino acid analysis, cytochrome c₅₅₃O was determined to contain 8.2 ± 0.4 hemes.

Elemental analysis showed the absence of nonheme iron or other transition metals in cytochrome c₅₅₃O.

**Spectral properties.** Purified preparations of cytochrome c₅₅₃O exhibited a γ band/280-nm absorbance intensity ratio (411 nm/280 nm = 4.3) that fell within the range of other purified c-heme-containing cytochromes (γ band/280 nm = 4.2 to 5.6; Fig. 2) (28, 29, 65). The γ band of cytochrome c₅₅₃O exhibited a broad linewidth, a feature commonly observed with other multiheme cytochromes (37). Analysis of spectra of the ferrocytochrome in the near infrared region provided no evidence for the presence of a high-spin (HS) heme (≈630 nm), and there was no evidence that methionine was an axial ligand (≈695 nm) for hemes present in the cytochrome. Neither the ferrocytochrome nor the ferrocytochrome was observed to react or bind the ligands carbon monoxide, cyanide, or nitric oxide.

The low-temperature X band EPR spectrum of ferricytochrome c₅₅₃O is shown in Fig. 3. The spectrum is complex, with at least two major low-spin (LS) ferric heme centers, designated LS species 1 (LS 1): γₗ = 3.66, γ₁₂ = 1.8, γ₈ = <0.7 and LS 2: γₗ = 2.97, γ₁₂ = 2.26, γ₈ = 1.49, a minor population of an HS species (γ = 6.0), ferric iron in a rhombic environment (g = 4.27), and a free-radical signal at g = 2.00. The signal at g = 4.27 has been assigned to adventitiously bound ferric iron. While no structural assignment could be deduced for the free-radical g = 2 signal, the linewidth is identical to enzymes that employ a free radical located on an intrinsic amino acid residue as a cofactor (45). The free-radical signal appeared unrelated to the EPR signals associated with the hemes as demonstrated in the differences in power saturation characteristics (Fig. 3). At higher-microwave-power intensity, the free-radical signal was easily saturated (≈50 mW), while signals associated with the LS hemes remained unsaturated at high-microwave powers. The fast-relaxing behavior of the LS heme centers of cytochrome c₅₅₃O has also been observed in the 50-kDa multiheme cytochrome from Desulfurobacter acetooxidans (50). However, this property is uncommon in LS c-heme cytochromes.

Integration of LS signals originating from LS 1 and LS 2 accounted for only six of the eight hemes present per subunit. Based on a subunit molecular mass predicted from the gene sequence plus heme groups (124,350 Da), EPR spin quantitation experiments indicate that the stoichiometry of heme species is 5 mol/subunit associated with LS 2, 1 mol/subunit associated with LS 1, and less than 0.1 mol/subunit associated with the HS species (g = 6) present in cytochrome c₅₅₃O. The two hemes not directly accounted for by spin quantitation methods may be due to unassigned resonances present in the cyto-
Cloning and sequencing the occ gene cluster of *M. capsulatus* Bath. The N-terminal amino acid sequence of cytochrome c<sub>553O</sub> from *M. capsulatus* Bath was ASVSGSAKLDAGLGKV SVKGTAGLAPG. This sequence was used to synthesize a degenerate oligonucleotide probe with the sequence 5'-AA (A/G)-G(A/G)-AA(A/G)-ACI-GCI-GGI-(T/C)TI-GCI-GC-3', where I represents inosine. The probe was used to screen 2,300 clones of a cosmid library of *M. capsulatus* Bath genomic DNA, which identified a single positive clone containing a 3,477-bp open reading frame (ORF, occ, encoding cytochrome c<sub>553O</sub> (Fig. 4 and 5). A second ORF, ORF1, 3,241 bp long, was located 484 bp upstream of occ, and a third, ORF3, 3,985 bp long, was 435 bp downstream from occ. A fourth ORF, ORF4, encoding an 11,100-Da putative protein, was located 22 bp downstream of ORF3. Probable ρ-independent transcription termination sequences are located 44 bp downstream of ORF1 and 59 bp downstream of occ (Fig. 4). No transcription termination sequence was observed between ORF3 and ORF4.

Primer extension analysis indicates that ORF1 has transcription start sites at bases 97, 118, and 119 (Fig. 6). Consensus -35 and -10 σ<sup>70</sup> RNA polymerase promoter sequences are located upstream of the first transcription start site, while no consensus promoter sequences are upstream of the latter two sites. Another primer extension experiment indicated that occ has transcription start sites at bases 3712 and 3825 (Fig. 6). The first transcription start site is associated with -35 and -10 consensus σ<sup>70</sup> promoter sequences, while the second is associated with consensus σ<sup>70</sup> RNA polymerase promoter sequences. A third primer extension experiment indicated that ORF3 has a transcriptional start site at base 6782 associated with a -35 and -10 consensus σ<sup>70</sup> promoter sequence.

The physiological roles of cytochrome c<sub>553O</sub> and the gene products of ORF1, ORF3, and ORF4 have not been determined. However, a possible role in nitrogen metabolism is suggested by the two promoter sequences upstream of occ: an upstream promoter similar to canonical -35 and -10 sequences and a downstream promoter similar to NtrA-dependent -24 and -12 promoters in the *Enterobacteriaceae* (23). The results would be consistent with the earlier observation that at least one high-molecular-mass cytochrome is induced following the addition of ammonia to early-log-phase cultures of *M. capsulatus* Bath (10).

The nascent polypeptide encoded by occ containing the N-terminal amino acid sequence of cytochrome c<sub>553O</sub> (ASVSG SAKLDAGLGKV SVKGTAGLAPG) was preceded by a 33-residue signal peptide (Fig. 5). The occ polypeptide contains eight c-heme-binding motifs (CXXCH), consistent with the heme quantitation data that estimates 8.2 hemes per subunit. The processed c<sub>553O</sub> apocytochrome is predicted to have a mass of 119,408 Da, while the holocytochrome is predicted to have a mass of approximately 124,350 Da, somewhat less than the estimate of subunit mass by SDS-polyacrylamide gel electrophoresis (Fig. 1).

The ORF1 and ORF3 gene products are predicted to begin after the first transcription start site. The ORF1 and ORF3 gene products are predicted to begin after the first transcription start site.
with putative signal peptide sequences 36 and 26 residues long, respectively. The holocytochromes encoded by ORF1 and ORF3 are predicted to have molecular masses of approximately 118,620 and 94,000 Da, respectively. The holocytochromes encoded by ORF1 and ORF3 are predicted to contain nine and seven \(c\)-heme-binding site motifs, respectively (Fig. 5 and 7).

The occ and ORF1 polypeptides contain extensive regions of homology to each other, and the amino acid sequences of the

FIG. 5. Amino acid sequence of the occ, ORF1, ORF3, and ORF4 gene cluster. Putative signal peptides are italicized; \(c\)-heme-binding motifs (CXXCH) are underlined; cysteine residues outside of \(c\)-heme-binding motifs are in bold.

FIG. 6. Primer extension mapping of the 5' ends of the occ, ORF1, and ORF3 transcripts with primers THICA (A), THICB (B), and TDW2 (C). Sequencing reactions with plasmid DNA template are shown on the left, with primer extension products on the right. The autoradiographs were exposed for 2 to 4 days at 22°C.

FIG. 7. Primer extension mapping of the 5' ends of the occ, ORF1, and ORF3 transcripts with primers THICA (A), THICB (B), and TDW2 (C). Sequencing reactions with plasmid DNA template are shown on the left, with primer extension products on the right. The autoradiographs were exposed for 2 to 4 days at 22°C.
two nascent polypeptides are 38.38% identical. However, a search of GenBank with the tFasta program (Genetics Computer Group, Madison, Wis.) produced no putative proteins homologous to either occ or ORF1. Eight of the nine c-heme-binding motifs in the ORF1 polypeptide are conserved in the occ polypeptide, while the second c-heme-binding motif in ORF1, CYGCH, is lacking in occ (Fig. 5 and 7). Both the occ and ORF1 polypeptides contain several cysteine residues outside of c-heme-binding site motifs (Fig. 5 and 7). The polypeptide encoded by ORF3 also has cysteine residues outside of typical c-heme-binding motifs (Fig. 5 and 7).

**Southern blots.** A 3.50-kbp EcoRI-BglII fragment containing the occ gene of *M. capsulatus* Bath was used to probe restriction digests of genomic DNA from *M. trichosporium* OB3b, *M. parvus* OBBP, *M. marinus* A45, *M. albus* BG8, and *Methylomonas* sp. strains MN and MM2. In addition to hybridization with *M. capsulatus* restriction fragments, relatively strong hybridization was observed to restriction fragments of *M. parvus* OBBP DNA and *M. trichosporium* OB3b DNA (results not shown). No hybridization of the *M. capsulatus* Bath occ probe to DNA from other methanotroph species was observed. No hybridization to other species of methanotrophs was observed with a 1.5-kb BglII fragment of ORF3.

**DISCUSSION**

Both amino acid sequence and biochemical data indicate that cytochrome c<sub>553O</sub> belongs to a novel class of c cytochromes. The size of the polypeptide, the number and location of hemes, and the presence of cysteine residues outside of c-heme-binding motifs place cytochrome c<sub>553O</sub> as well as the gene products of ORF1 and ORF3, outside of Ambler’s classification of type II cytochromes (2–4). The size, sequence, and interheme distances distinguish cytochrome c<sub>553O</sub> from Ambler’s class III multimethine cytochromes as well as from other high-molecular-mass multimethine cytochromes (11, 31, 42, 44–46, 56, 60). In addition, these high-molecular-mass cytochromes show no similarities to the class IE cytochromes, which are characterized by non-heme-associated cysteine residues.

The role of cytochrome c<sub>553O</sub> remains unclear. Although redox titrations of cytochrome c<sub>553O</sub> were not performed, the fact that the cytochrome was not reduced by ascorbate suggests that all the hemes of cytochrome c<sub>553O</sub> have relatively low midpoint potentials. The fact that cytochrome c<sub>553O</sub> may be induced by ammonia indicates that cytochrome c<sub>553O</sub> may have a role in nitrogen metabolism. A role in nitrogen metabolism is also suggested by the two promoter sequences upstream of occ, an upstream promoter similar to consensus −35 and −10 sequences and a downstream promoter similar to NtrA-dependent −24 and −12 promoters in the *Enterobacteriaceae* (23).

The presence of both −35 and −10 promoter sequences as well as −24 and −12 promoter sequences was observed in the *glmA* gene, which encodes glucamine synthetase, an enzyme involved in ammonia assimilation in *M. capsulatus* Bath (12). Although no enzymatic activity has been assigned to cytochrome c<sub>553O</sub>, the presence of a stable free-radical signal (Fig. 3; g = 2.00) indicates that the cytochrome may have catalytic properties (45). Stable protein radicals, such as tyrosyl radicals, are usually associated with active sites of enzymes (45).

Nucleic acid sequence data indicate that there are two other high-molecular-weight, multi-heme c cytochromes in *M. capsulatus* Bath, the gene products of ORF1 and ORF3. The ORF1 gene product has considerable homology with cytochrome c<sub>553O</sub> yet the difference in its sequence is sufficient to indicate that it is not merely an isoenzyme. An additional c-heme-binding site motif, ORF3, has no sequence homology with occ or ORF1 but shares the structural properties of multiple heme-binding motifs, long distances between heme-binding motifs, and the cysteine residues not associated with c-heme-binding motifs (Fig. 7).

Gene probing with occ indicated that cytochromes similar to that from *M. capsulatus* Bath may be present in the type II methanotrophs, *M. trichosporium* OB3b and *M. parvus* OBBP, but not in the type I methanotrophs, *M. marinus* A45, *M. albus* BG8, and *Methylomonas* sp. strains MN and MM2. Probing results were consistent with gene probing with cyp, the structural gene for cytochrome P460 (10), but not with the phylogenetic relationships with ribosomal RNA or pMMO gene sequence data (30, 32). No hybridization to the ORF3 gene probe was observed with any of the methanotrophs or nitrifier tested. At present, it is uncertain if this class of c cytochromes is found in type I methanotrophs, since DNA from these methanotrophs does not hybridize to the *M. capsulatus* Bath occ or ORF3 gene probes.

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