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The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath)

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Abstract The genes encoding the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Methylococcus capsulatus* (Bath) were localised to an 8.3-kb *Eco*RI fragment of the genome. Genes encoding the large subunit (*cbbL*), small subunit (*cbbS*) and putative regulatory gene (*cbbQ*) were shown to be located on one cluster. Surprisingly, *cbbO*, a second putative regulatory gene, was not located in the remaining 1.2-kb downstream (3′) of *cbbQ*. However, probing of the *M. capsulatus* (Bath) genome with *cbbO* from *Nitrosomonas europaea* demonstrated that a *cbbO* homologue was contained within a separate 3.0-kb *Eco*RI fragment. Instead of a *cbbR* ORF being located upstream (5′) of *cbbL*, there was a *moxR*-like ORF that was transcribed in the opposite direction to *cbbL*. There were three additional ORFs within the large 8.3-kb *Eco*RI fragment: a *pyrE*-like ORF, an *rnr*-like ORF and an incomplete ORF with no sequence similarity to any known protein. Phylogenetic analysis of *cbbL* from *M. capsulatus* (Bath) placed it within clade A of the green-type Form 1 Rubisco. *cbbL* was expressed in *M. capsulatus* (Bath) when grown with methane as a sole carbon and energy source under both copper-replete and copper-limited conditions. *M. capsulatus* (Bath) was capable of autotrophic growth on solid medium but not in liquid medium. Preliminarily investigations suggested that other methanotrophs may also be capable of autotrophic growth. Rubisco genes were also identified, by PCR, in *Methylococcus*-like strains and *Methylocaldum* species; however, no Rubisco genes were found in *Methylomicrobium album* BG8, *Methylomonas methanica* S1, *Methylomonas rubra*, *Methylosinus trichosporium OB3b* or *Methylocystis parvus* OBBP.

Keywords *Methylococcus* · Ribulose bisphosphate carboxylase/oxygenase · Methanotroph

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Introduction

Methanotrophs are a unique group within the Proteobacteria in being able to utilise methane as a sole source of carbon and energy (Hanson and Hanson 1996). Type I methanotrophs assimilate formaldehyde via the ribulose monophosphate pathway and have membranes arranged in bundles within the cytoplasm, while type II methanotrophs assimilate formaldehyde via the serine pathway, fix N_2 and have membranes aligning the periphery of the cell (Hanson and Hanson 1996). *Methylococcus capsulatus* (Bath) is an unusual type I methanotroph in that it assimilates formaldehyde using the ribulose monophosphate pathway but also possesses some enzymes of the serine pathway. It also fixes N_2 and its DNA has a relatively high G+C content, which is more characteristic of type II methanotrophs (Hanson and Hanson 1996).

Taylor (1977) demonstrated in *M. capsulatus* (Bath) the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Analysis of the partially purified enzyme by SDS-PAGE yielded two distinct polypeptides corresponding to the large subunit (48.0 kDa) and the small subunit (14.5 kDa) of Rubisco (Taylor 1977; Taylor et al. 1980). Taylor et al. (1980) estimated that the Ru-

bisco of *M. capsulatus* (Bath) had an $\alpha_6\beta_6$ structure which differed from the typical hexadecameric structure ($\alpha_8\beta_8$) of the Form I Rubisco found in Proteobacteria such as *Ralstonia eutropha* and *Acidithiobacillus ferrooxidans* and also in cyanobacteria and higher plants. However, the accuracy of this estimation was questioned by Tabita (1988), as no known Rubisco was included as a control.

M. capsulatus (Bath) uses both Rubisco and carboxylation of C_3-metabolites to fix CO_2 (Taylor et al. 1981). Under oxic conditions, *M. capsulatus* (Bath) exhibits both the carboxylation and oxidation reactions of Rubisco (Taylor et al. 1980). Despite the presence of a complete Calvin Benson Bassham (CBB) cycle, *M. capsulatus* (Bath) does not appear to grow autotrophically (Taylor et al. 1981; Stanley and Dalton 1982). Rubisco activity in *M. capsulatus* (Bath) is also reported to be energy-dependent (Taylor 1977; Taylor et al. 1981; Stanley and Dalton 1982). In the presence of methane, *M. capsulatus* (Bath) fixes $^{14}CO_2$, with the labelled carbon appearing in sugar phosphates (Stanley and Dalton 1982). In the absence of methane, but in the presence of H_2 and/or formate, Rubisco activity was recorded for 7 h, but there was an accumulation of labelled 3-phosphoglycerate and only a limited amount of labelled sugar phosphates was detected (Stanley and Dalton 1982). No further work has been undertaken to characterise the Rubisco genes in *M. capsulatus* (Bath) at the biochemical or molecular level.

The majority of genes encoding CBB pathway enzymes are generally located within one operon, although the order and number of genes within the *cbb* operon can differ between bacterial genera (Meijer et al. 1991; Shively et al. 1998). The large and small subunits of Rubisco are encoded by *cbbL* and *cbbS*, respectively. Transcriptional regulation of *cbb* genes, including the Form II Rubisco *cbbM*, is positively regulated by a LysR-like protein, CbbR (Viale et al. 1991; Windhövel and Bowien 1991; van den Bergh et al. 1993). *cbbR* can range in size from 927 bp in *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*; Shively 1999a) and *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*; Shively 1999b) to 1,002 bp in *Xanthobacter flavus* (van den Bergh et al. 1993). *cbbR* is usually located near Rubisco structural genes and is divergently transcribed from *cbbL* and *cbbS*. Mutagenesis of *cbbR* results in inhibition or reduction of Rubisco expression (Gibson and Tabita 1993; van den Bergh et al. 1993).

Hydrogenophilus thermoluteolus contains additional genes, *cbbQ* and *cbbO*, the products of which are thought to be involved in post-translational modification of Rubisco (Yokoyama et al. 1995; Hayashi et al. 1997). *cbbQ*, encoding a 29.6-kDa polypeptide, is located 3′ of *cbbLS* in the Form I Rubisco gene cluster (Yokoyama et al. 1995; Hayashi et al. 1999) and 3′ of *cbbM* in the Form II Rubisco gene cluster (Hayashi et al. 1999). c*bbQ* has 49% similarity to *nirQ* from *Pseudomonas aeruginosa* and 53% similarity to *nirQ* from *Pseudomonas stutzeri* (Yokoyama et al. 1995). *nirQ* is an independent transcriptional unit with a regulatory role in nitrate and nitric oxide reduction (Jüngst and Zumft 1992). When *cbbQ* from *H. thermoluteolus* is co-expressed with *cbbLS* in *Escherichia coli*, the V_{max} of Rubisco is increased two-fold (Hayashi et al. 1999). Similarly, when a second gene, *cbbO*, located 3′ of *cbbQ* in *H. thermoluteolus*, is co-expressed with *cbbLS* in *E. coli*, the same increase in the Rubisco V_{max} is observed (Hayashi et al. 1997). CbbO has no sequence identity with any known protein (Hayashi et al. 1997), but within the genome of *N. europaea* there is an ORF 3′ of a Rubisco gene cluster (http://spider.jgi-psf.org/JGI_microbial/html/nitrosomonas/nitro_homepage.html) encoding a putative polypeptide possessing 42% identity (57% similarity) to CbbO from *H. thermoluteolus.*

This work describes the molecular characterisation of the Rubisco gene cluster in *M. capsulatus* (Bath) and *cbbL* from other methanotrophs, examines the phylogeny of the *cbbL* gene from *M. capsulatus* (Bath) and investigates the potential of methanotrophs for autotrophic growth.

Materials and methods

Growth of bacterial cultures

Methanotrophs were grown in nitrate mineral salts (NMS) medium (Dalton and Whittenbury 1976) with methane as a sole carbon and energy source. All methanotrophs were incubated at 30 °C except *M. capsulatus* strains Aberdeen, Bath (NCMB11132), M and Texas, *Methylocaldum szegediense* OR2 and *Methylocaldum tepidum* LK6 which were grown at 45 °C. *M. capsulatus* (Bath) was also grown under copper-limited conditions as described by Nielsen et al. (1996). *E. coli* TOP10 (Invitrogen) was used as the host for the construction of gene libraries and was grown in Luria-Bertani broth (LB) at 37 °C. Fresh competent cells were prepared for each library using the $CaCl_2$ method (Sambrook et al. 1989). Ampicillin (50 μg ml^{-1}) was used to select for transformants.

Autotrophic and chemoorganoautotrophic growth of *Methylococcus capsulatus* (Bath) and other methanotrophs

All methanotrophs were grown in NMS medium containing 0.4 μM $CuSO_4$ and 7.5 μM $NiCl_2$. To solidify media, 0.5–1.5% (w/v) Bacto agar was added. Plates were incubated in anaerobic jars under a gas mixture of H_2:air:CO_2 (approximately 60:34:6) for 10–30 days. The gas phase was replaced every 4–5 days with fresh gas mixture. Attempts to grow methanotrophs autotrophically in liquid culture were made using the same medium and a gas mixture of the same composition. To avoid potential loss of H_2, 100-ml Hypovial vials containing 10 ml medium were sealed with butyl-rubber seals. All methanotrophs were incubated at 30 °C, with the exception of *M. capsulatus* (Bath), *M. szegediense* OR2, and *M. tepidum* LK6, which were incubated at 45 °C. Two sets of negative controls were used in which N_2 replaced CO_2 or H_2. For chemoorganoautotrophic growth, 2.5 mM formate was included in the medium and N_2 replaced H_2 in the headspace. Again, as for cultivation on solid medium, the gas phase was replaced every 4–5 days with fresh gas mixture to prevent oxygen depletion of the culture.

Preparation and probing of Southern blots

DNA was extracted from methanotrophs using the method of Oakley and Murrell (1988). Chromosomal DNA from *M. capsulatus* (Bath) was digested with *Bam*HI, *Eco*RI, *Pst*I, *Hin*dIII, *Sal*I, *Xba*I and *Eco*RI/*Hin*dIII (Gibco BRL). Chromosomal DNA from the type I methanotrophs *Methylomicrobium album* BG8, *Methylomonas methanica* S1 and *Methylomonas rubra*, and the type II

methanotrophs *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP were digested with *Bam*HI, *Eco*RI and *Hin*dIII (Gibco BRL). DNA fragments were separated on a 0.7% (w/v) agarose gel and transferred onto Hybond-N nylon membranes (Amersham). Southern blots were performed according to Sambrook et al. (1989) and all probes were prepared by random priming with ^{32}P-dGTP (Feinberg and Vogelstein 1983). The chromosome of *M. capsulatus* (Bath) was probed with a 1.2-kb *cbbL* PCR product from *Nitrosospira* sp. NpAV. Probing for *cbbO* was carried out with an internal PCR product of 1.2 kb from *cbbO* of *N. europaea*. Chromosomal DNA from type I and type II methanotrophs was probed with a 0.5-kb internal *cbbL* PCR product (bp 195–706) from *M. capsulatus* (Bath). All hybridisations were carried out at 55 °C in hybridisation solution (0.5 M Na_2HPO_4 and 0.5 M $NaHPO_4$, pH 6.8, 7% SDS, 5 mM EDTA, pH 8.0) and final washes were at 55 °C in 2×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate).

Construction and probing of a Rubisco gene library from *Methylococcus capsulatus* (Bath)

Chromosomal DNA was digested with either *Sal*I or *Eco*RI then size-fractionated, 0.5–2.0 kb for *Sal*I and 4.0–10.0 kb for *Eco*RI, by electrophoresis through a 0.7% (w/v) agarose gel, after which the fragments were excised from the gel and cleaned using Geneclean (Bio101). All fragments were cloned into pUC19 and the resulting plasmid library was transferred into *E. coli* using standard methods (Sambrook et al. 1989). The *Sal*I and the *Eco*RI libraries contained 700 and 587 clones, respectively, and were screened by pooling groups of ten clones, growing clones in LB and extracting plasmid DNA using the mini-prep method of Saunders and Burke (1990). Plasmid DNA was digested with either *Sal*I or *Eco*RI, electrophoresed through a 0.7% (w/v) agarose gel, transferred to a Hybond-N nylon membrane by Southern blotting and probed with the 1.2-kb *cbbL* PCR product from *Nitrosospira* sp. NpAV for the *Sal*I library and the 0.5-kb *cbbL* PCR product from *M. capsulatus* (Bath) for the *Eco*RI library. After identifying a positive pool of recombinant plasmids with the appropriate probe, individual clones from that pool were grown in 5 ml LB, plasmid DNA was mini-prepped, digested, electrophoresed and transferred to a Hybond-N nylon membrane and then probed with the appropriate gene. The *Sal*I library was probed with a ^{32}P-dGTP random-prime-labelled (Feinberg and Vogelstein 1983) 1.2-kb PCR product encoding the *cbbL* gene from *Nitrosospira* sp. NpAV, while the *Eco*RI library was probed with a similarly prepared 0.5-kb PCR product encoding *cbbL* from *M. capsulatus* (Bath).

Preparation of probes and PCR amplification of *cbbL* from other methanotrophs

All PCR reactions contained in 50 µl: 1×PCR buffer, 200 nM of each dNTP, 2.5 mM $MgCl_2$, 500 ng of each primer and 250 ng DNA. The 1.2-kb PCR product from *Nitrosospira* sp. NpAV was amplified using the primers RBCA 202F 5′-GTSGTSTGGACCGACCG-3′ (forward) and RBCA 1418R 5′-TCGGTSGRSGTGTAGTTGAAG-3′ (reverse). The 0.5-kb *cbbL* fragment was PCR-amplified from *M. capsulatus* (Bath) and other methanotrophs using primers McBCBBL 195F 5′-CTGCTGACCGACCTCGACTA-3′ (forward) and McBCBBL 706R 5′-GTCACGTTGAGGTAGTGGCC-3′ (reverse). The primers for amplifying the 1.0-kb *cbbL* fragment were CBB 88F 5′-CTGGCGGTCTTCAAGATCAC-3′ (forward) and CBB 1076R 5′-TGGTCGAAGAAGATGCCGCG-3′ (reverse). The primers for amplifying *cbbO* from *N. europaea* were *cbbO* 266F 5′-AGAAGTCGGTGACGATGTTG-3′ (forward) and *cbbO* 1501R 5′-TGAACAGTTGACCATGCTGG-3′ (reverse). The fragments were amplified as follows: 5 min denaturation followed by 35 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min with a final extension time of 10 min. The *cbbL* PCR products from methanotrophs screened for *cbbL* were cloned using the TOPO TA cloning kit (Invitrogen) and then analysed by RFLP using *Eco*RI before sequencing.

Sequencing and analysis of the Rubisco gene cluster

Custom-made oligonucleotides (Gibco BRL) were used for double-stranded sequencing of Rubisco genes using a *Taq* dye deoxy terminator cycle sequencing kit and a model 373A DNA sequencing system gel apparatus (Applied Biosystems). Sequences were analysed using the Genetics Computer Group Wisconsin Package, version 8. BLAST 2.0 similarity (Gish and States 1993) and ORF searches were performed using the Internet facility at the National Centre for Biotechnical Information (http://www.ncbi.nlm.nih.gov). Predicted polypeptide masses were calculated using the Internet facility at ExPASy Molecular Biology Server (http://expasy.nhri.org.tw/tools/peptide-mass.html). The sequences of Rubisco genes from *Methylococcus* contained on plasmid pNJB54 have been deposited in GenBank under accession number AF447860.

Phylogenetic analysis of *cbbL* from *Methylococcus capsulatus* (Bath)

Protein sequences were aligned using ClustalW (http://www.ebi.ac.uk) and corrected for spacing by eye. The corresponding DNA alignments were obtained by matching the codon to the aligned amino acid using the program PUTGAPS (Dr J.O. McInerney, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland). Phylogenetic analyses were done using PAUP* v4.0a4b (Swofford 2000). To infer distance trees, Minimum Evolution (ME) with LogDet (Lake 1994; Lockhart et al 1994) pairwise distances (LogDet/ME) was used. Since LogDet assumes that all sites can vary, on a maximum parsimony tree the fraction of invariant sites was estimated using maximum likelihood with a General Time Reversal model (GTR R matrix) and a two-rate model. The tree space was searched with random additions of taxa and Tree Bisection-Reconnection (TBR) branch swapping. For maximum parsimony (MP) analyses, 100 heuristic searches were carried out with random addition of taxa and TBR branch swapping. LogDet/ME and MP bootstrapping were carried out with 1,000 replicates and one random addition of taxa per bootstrap replicate. The proteobacterial and cyanobacterial CbbL amino acid sequences used in the phylogenetic analysis were as follows: *Ralstonia eutropha* H16 ATCC17707 (U20584), *Ralstonia eutropha* ATCC17699 megaplasmid pHGI (U20585), *Bradyrhizobium japonicum* (AF041820), *Allochromatium vinosum* ATCC17899 *rbcL* (D90204), *Allochromatium vinosum rbcA* ATCC17899 (M26396), *Hydrogenophaga pseudoflava* (U55037), *Hydrogenophilus thermoluteolus* (D30764), *Hydrogenovibrio marinus* MH-110 copy 1 (D43621), *Hydrogenovibrio marinus* MH-110 copy 2 (D43622), manganese-oxidising bacterium SI85–9A1 (L32182), *Nitrobacter vulgaris* (L22885), *Nitrobacter winogradskyi* IFO14297 (AF109915), *Alvinoconcha hessleri* symbiotic bacterium (M34536), *Ralstonia eutropha* (M17744), *Rhodobacter capsulatus* (L82000), *Rhodobacter sphaeroides* (M64624), *Sinorhizobium meliloti* (AF211846), *Thiobacillus denitrificans* (L42940), *Acidithiobacillus ferrooxidans* ATCC19859 (X70355), *Acidithiobacillus ferrooxidans* Fe1 (D90113), *Thiomonas intermedia* K12 (AF046933), *Halothiobacillus neapolitanus* (AF038430), *Xanthobacter flavus* (X17252), *Synechococcus* sp. strain PCC7002 (D13971), *Anabaena* sp. (J01540), *Synechococcus* sp. strain PCC6301 (X03220), *Prochlorothrix hollandica* (X57359), *Synechococcus* sp. (U46156), *Synechococcus* sp. strain A1 (D13539), *Synechocystis* sp. (X65960), *Nitrosomonas europaea* (http://spider.jgi-psf.org/JGI_microbial/html/nitrosomonas/nitro_homepage.html) and *Methylococcus capsulatus* (Bath) (this investigation).

RNA extraction and reverse transcriptase PCR of Rubisco in *Methylococcus capsulatus* (Bath)

The RNA extraction method was based on that of Nielsen et al. (1996). *M. capsulatus* (Bath) growing exponentially under both copper-limited and copper-replete conditions were harvested (OD_{540} 0.4–0.6) by centrifugation at 10,000×*g*. Cells were resus-

pended in 200 µl of a solution containing 0.3 M sucrose and 0.01 M sodium acetate, pH 4.5; 0.4 g glass beads (Sigma) and 400 µl of phenol saturated with 50 mM sodium acetate, pH 4.5, were added. The cells were vigorously shaken for 30 s and placed on ice for 30 s. This was repeated three times. The lysed cells were incubated at 65 °C for 2 min, vortexed for 1 min and centrifuged for 5 min at 12,000×*g*. The aqueous layer was removed to a clean tube and 400 µl phenol were added. The tubes were placed in an ice/ethanol bath for 30 s, centrifuged (12,000×*g*) for 5 min and the aqueous layer then removed to a fresh tube. To the aqueous layer, 400 µl phenol/cholorform (1:1) were added, vortexed for 1 min, then centrifuged for 5 min (12,000×*g*) and the aqueous layer removed to a fresh tube. The RNA was precipitated by adding 3 M sodium acetate (40 µl) and 96% ethanol (900 µl) and storing at –20 °C for 20 min. Following centrifugation for 20 min at 12,000×*g*, the RNA was washed in ice-cold 70% ethanol, air dried and resuspended in sterile distilled water. RT-PCR was carried out using expanded reverse transcriptase (Roche) according to the manufacturer's instructions. The reverse primer used was McBCBBL 706R 5′-GTCACGTTGAGGTAGTGGCC-3′.

Results and discussion

Orientation of the Rubisco genes in *M. capsulatus* (Bath) and neighbouring genes

Probing of the *M. capsulatus* (Bath) genome with a *cbbL* fragment from *Nitrosospira* sp. NpAV demonstrated that there was only one copy of *cbbL* in this methanotroph. Probing the genome of *M. capsulatus* (Bath) with *cbbM* from *Rhodobacter sphaeroides* indicated that *M. capsulatus* (Bath) did not contain a Form II Rubisco (Dewulf and Murrell, unpublished observation). Initially, a 1.2-kb *Sal*I fragment was cloned to begin the sequencing of *cbbL*, while a larger fragment containing the whole gene cluster was isolated. The first clone, pNJB55, a 1.2-kb *Sal*I fragment, contained the majority of *cbbL* (Fig. 1). This enabled PCR primers to be designed to amplify a discrete DNA fragment of *cbbL* from *M. capsulatus* (Bath) to use as a probe to locate the whole Rubisco gene cluster in *M. capsulatus*. Plasmid pNJB54, which contained an *Eco*RI fragment of 8,392 bp, was the second *cbbL*-containing clone identified.

There were seven ORFs on pNJB54 (Fig. 1), three of which encoded the Rubisco genes *cbbL* (1,422 bp), *cbbS* (357 bp) and *cbbQ* (801 bp) and which were transcribed in the same direction. A *moxR*-like ORF (Brune 1995; Pattaragulwanit et al. 1998) of 1,044 bp was located 964 bp 5′ of *cbbL* and divergently transcribed from *cbbL*. Transcribed in the same direction and 3′ of the *moxR*-like gene was a 552-bp ORF with significant identity to *pyrE*, which encodes an orotate phosphoribosyltransferase involved in the synthesis of purines and pyridines (Neuhard et al. 1985; MacGregor et al. 1996). An *rnr*-like ORF of 1,842 bp overlapped the *pyrE*-like ORF, but was transcribed in the same direction as *cbbL*. *rnr* encodes an exoribonuclease, Rnase R (Cheng et al. 1998), in *E. coli* (Burland et al. 1995) and *Vibrio cholerae* (Heidelberg et al. 2000). The seventh ORF of 927 bp was located 3′ of *cbbQ*. It had no significant sequence similarity to any known ORF and therefore was designated ORFX.

The –10 (Pribnow box) sequence TATAAT, which is identical to that of *E. coli* (Pribnow 1975), was found at –153 bp from the *cbbL* start codon. A similar distance of 153 bp from the Pribnow box to the start codon of *cbbL* was observed in the symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990). Fourteen bp upstream of this typical Pribnow box consensus sequence was a potential –35 sequence of GTAGCA. This deviates from the –35 sequence from *E. coli* (Harley and Reynolds 1987), which is not uncommon in gram-negative bacteria (Raibaud and Schwartz 1984). The GC content of the genes *cbbL*, *cbbS* and *cbbQ* of *M. capsulatus* (Bath) ranged from 61.3 to 66.1 mol %, and there was a bias towards G or C in the third codon position, which was consistent with codon usage patterns observed in methanotrophs (Murrell 1992).

cbbL

The *M. capsulatus* (Bath) *cbbL* ORF of 1,422 bp was initiated at bp 4,466 in pNJB54. A ribosome-binding site, AGGAGA, was located 5 bp 5′ of *cbbL*. The predicted polypeptide sequence of 474 amino acids had a theoretical molecular mass of 52,934 Da, which was larger than the 48 kDa observed by Taylor et al. (1980) using SDS-PAGE. The sequence of *cbbL* from *M. capsulatus* (Bath) was 87% identical to that of *cbbL* from the symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990),

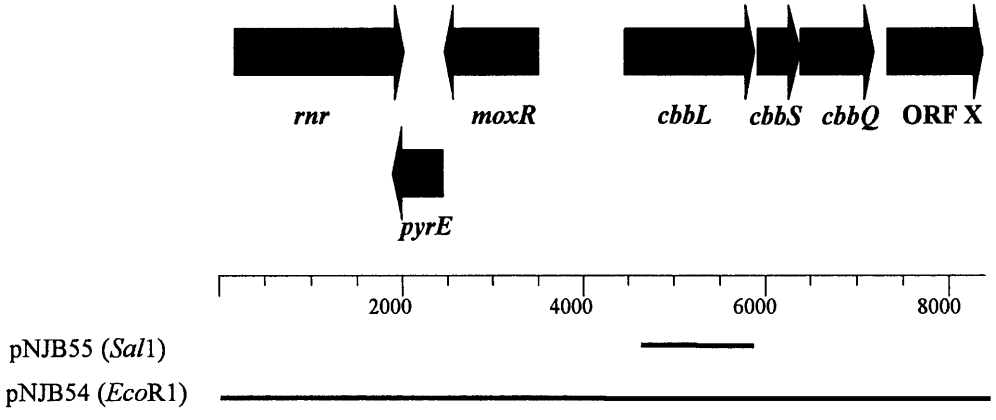

Fig. 1 The Rubisco gene cluster from *Methylococcus capsulatus* (Bath) contained *cbbL*, *cbbS* and *cbbQ*. A *moxR*-like gene was located upstream (5′) of *cbbL* together with a *pyrE*-like and *rnr*-like ORFs. An unidentified and incomplete ORFX was located downstream (3′) of the gene cluster

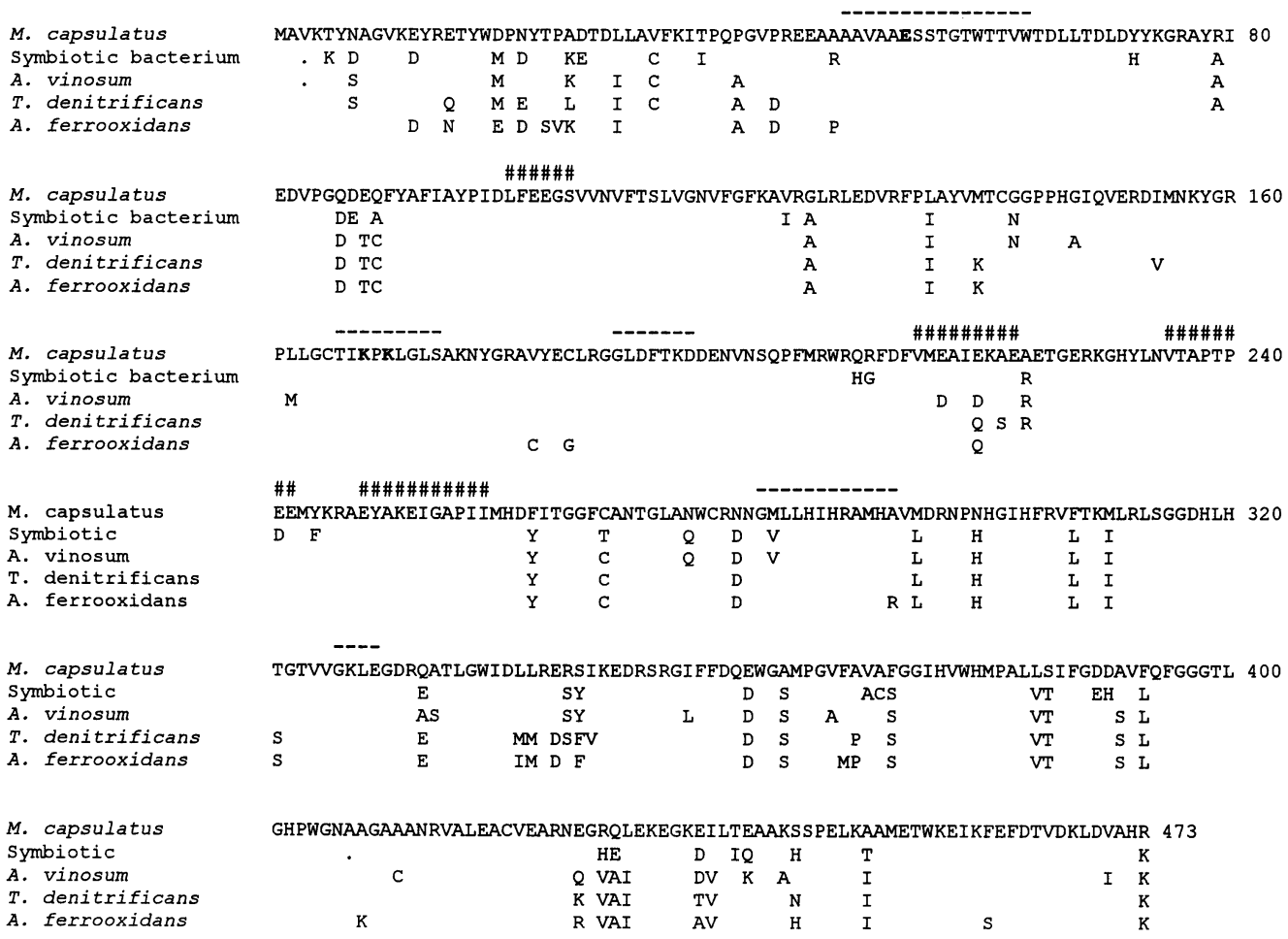

Fig. 2 Alignment of CbbL from *M. capsulatus* (Bath), symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990), *Thiobacillus denitrificans* (Hernandez et al. 1996), *Allochromatium vinosum* (Viale et al. 1989; Kobayashi et al. 1991) and *Acidithiobacillus ferrooxidans* (Pulgar et al. 1991). The amino acid residues which are thought to be important in catalysis (–) and enzyme structure (#) are indicated (Hwang and Tabita 1991; Pulgar et al. 1991). The conserved glutamate residue at position 53 and the conserved lysine residues at positions 168 and 170 are highlighted

Thiobacillus denitrificans (Hernandez et al. 1996), *Allochromatium vinosum* (Viale et al. 1989; Kobayashi et al. 1991) and *Hydrogenophaga pseudoflava* (Y.M. Kim and S.N. Lee, direct sequence submission to GenBank; accession number U55037). The sequence of CbbL from *M. capsulatus* (Bath) was 81% identical (86% similar) to that of the CbbL from the symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990), *T. denitrificans* (Hernandez et al. 1996), *Allochromatium vinosum* (Viale et al.1989; Kobayashi et al. 1991) and *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) (Pulgar et al. 1991).

Within CbbL from *M. capsulatus* (Bath) there were conserved residues (glutamate residue 53 and lysine residues 168 and 170) that are involved in catalysis (Hwang and Tabita 1991; Tabita 1988; Miziorko and Lorimer 1983). An alignment of CbbL sequences most closely related to CbbL from *M. capsulatus* (Bath) is shown in Fig. 2. Although CbbL from *M. capsulatus* (Bath) is most similar to CbbL from the sulfur-oxidising bacteria *T. denitrificans* and *A. ferrooxidans*, the symbiotic bacterium of *Alvinoconcha hessleri* and *A. vinosum*, the alignment highlights that CbbL from *M. capsulatus* (Bath) is distinct from this group. Within the alignment there are 15 amino acid residues, at positions 86, 125, 135, 262, 279, 294, 299, 307, 310, 360, 363, 384, 385, 393 and 473, which are conserved among the four sulfur-oxidising bacteria but which are not conserved in *M. capsulatus* (Bath). However, of the 15 residues, only three of the changes in *M. capsulatus* (Bath), occurring at positions 299, 307 and 393, were to functionally dissimilar amino acids. The significance of these changes, if any, remains to be determined.

cbbS

The intergenic region between *cbbL* and *cbbS* was 26 bp. *cbbS* (357 bp) was initiated at position 5,913 of pNJB54. A potential ribosome-binding site, AGGA, was located at –13 bp from the ATG start codon. CbbS (119 amino acids) had a theoretical molecular mass of 13,702 Da, which was

CbbS

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                                  ##########                                  ##########
M. capsulatus          MSDMQDYKSSLSDSGSRKFETFSYLPPMNPEKIRRQVEYIVSRGWNPAIEHTEPENAFDHYWYMWKLPMFGETDVDAILA 80
Symbiotic bacterium      EI   N  VS PS          ELGV    K       K     V
A. vinosum               E    S   E VN          R DADR  K       K                                 I T  K
T. denitrificans         EVM    R   PA          A  AAD  K    L  K           HLM S                 I R  G
A. ferrooxidans          EV     R   PA A    L    ALTADE  Q VA    K     V         GN                   T  K

                                 ########
M. capsulatus          EAEACHKAHPNNHVRLVGYDNFRQTQGAAMVIYRGPSV... 121
Symbiotic bacterium              SH    I    YA S QGT    F  PISAKC
A. vinosum                        I F  YA SK  E  V   KP ...
T. denitrificans               N            A S         KT ...
A. ferrooxidans          R   RN H    I      K S  TSL V   GKTV..
```

Fig. 3 Alignment of CbbS from *M. capsulatus* (Bath), symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990), *T. denitrificans* (Hernandez et al. 1996), *A. vinosum* (Viale et al. 1989; Kobayashi et al. 1991) and *A. ferrooxidans* (Pulgar et al. 1991). Amino acid residues important in enzyme assembly (Pulgar et al. 1990) are indicated (#)

slightly lower than the 14.5 kDa observed by Taylor et al. (1980) using SDS-PAGE. The nucleotide sequences of *cbbS* are not as conserved across genera as those of *cbbL*, particularly at the 5′ and 3′ ends. However, *cbbS* from *M. capsulatus* (Bath) was 87% identical to *cbbS* from the symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990) over 211 bp, 89% identical to *cbbS* from *A. vinosum* (Viale et al. 1989; Kobayashi et al. 1991) over 175 bp and 81% identical to *cbbS* from *T. denitrificans* (Hernandez et al. 1996) over 290 bp. The predicted amino acid sequence of *cbbS* from *M. capsulatus* (Bath) was 81% identical (92% similar) to CbbS from the symbiotic bacterium of *Alvinoconcha hessleri* (Stein et al. 1990); 78% identical (89% similar) to CbbS from *A. vinosum* (Viale et al. 1989; Kobayashi et al. 1991); 78% identical (86% similar) to CbbS from *T. denitrificans* (Henandez et al. 1996) and 71% identical (86% similar) to CbbS from *A. ferrooxidans* (Pulgar et al. 1991).

As with CbbL, there were amino acids normally conserved within the sulfur-oxidising bacteria that were different in CbbS from *M. capsulatus* (Bath), but both changes were for functionally similar amino acids (Fig. 3). The small subunit of Rubisco is not directly involved in catalysis, but is important in the structure of the enzyme (Tabita 1988). Within the sequence of CbbS, there are conserved residues (Fig. 3) involved in enzyme structure formation (putative holoenzyme-binding sites), residues 19–28, 61–70 and 94–99 (Pulgar et al. 1991).

cbbQ

cbbQ encodes a polypeptide with a role in the post-translational activation of Rubisco (Hayashi et al. 1997, 1999). There was no physiological or biochemical evidence for the existence of *cbbQ* within the genome of *M. capsulatus* (Bath). However, when the DNA sequence adjacent to *cbbS* was determined, a *cbbQ*-like ORF was located 118 bp 3′ of *cbbS* in *M. capsulatus* (Bath). The ORF for *cbbQ* (801 bp) had a potential ribosome-binding site, AGGAG, situated at –8 bp from the ATG start codon. The derived 266-amino-acid sequence had a theoretical molecular mass of 29,748 Da. *cbbQ* from *M. capsulatus* (Bath) was 82% identical to *cbbQ* from *Rhodobacter capsulatus* over 467 bp (Paoli et al. 1998), and 81% identical to the *p30* gene from *A. ferrooxidans* over 342 bp (N.S. Guiliani et al., direct sequence submission to Genbank; accession number AJ1333725). *p30* encodes a protein over-expressed by *A. ferrooxidans* when it is grown on iron which has 76% identity (86% similarity) to CbbQ from *H. thermoluteolus* (Yokoyama et al. 1995). Unexpectedly, *cbbQ* from *M. capsulatus* (Bath) also had 82% identity to the *cbbR* associated with the Form II Rubisco from *Thiobacillus intermedius* K12 over 330 bp (Shively and Soyer 1998). The number of database sequences available for *cbbQ* is limited, which may explain the relatively poor sequence similarity observed. The predicted amino acid sequence of CbbQ from *M. capsulatus* (Bath) was 78% identical (88% similar) to the sequence of CbbQ from *Acidithiobacillus ferrooxidans* (AF307091), 73% identical (82% similar) to CbbQ from *H. thermoluteolus* (Yokoyama et al. 1995) and 55% identical (69% similar) to NirQ from *Pseudomonas aeruginosa* (Arai et al. 1994).

cbbO

A fifth gene of the Rubisco gene cluster, *cbbO*, was predicted to be located downstream of *cbbQ*. In *N. europaea*, *cbbO* (http://spider.jgi-psf.org/JGI_microbial/html/nitrosomonas/nitro_homepage.html) was located 52 bp downstream of *cbbQ*. Also, 3′ of *cbbQ* on pNJB54 there were another 1,205 bp before the end of the *M. capsulatus* (Bath) DNA fragment. Within this sequence, no *cbbO*-like ORFs could be identified. However, probing the *M. capsulatus* (Bath) genome with *cbbO* from *N. europaea* revealed that a *cbbO* homologue was located on a separate *Eco*RI fragment of approximately 3.0 kb (data not shown).

moxR-like gene

An ORF of 1,044 bp, encoding a polypeptide of predicted molecular mass 38,448 Da, was located 959 bp 5′ from *cbbL* and divergently transcribed from *cbbL*. The sequence of this ORF had 80% identity (over 302 bp) with a

moxR homologue from *A. vinosum* (Brune 1995; Pattaragulwanit et al. 1998). The predicted polypeptide sequence of this ORF had 33% similarity with MoxR from *Paracoccus denitrificans* (van Spanning et al. 1991) and 31% identity (50% similarity) with MoxR from *Methylobacterium extorquens* (Amaratunga et al. 1997), both methylotrophs. *moxR* is a gene involved in regulating formation of active methanol dehydrogenase (van Spanning et al. 1991). The *moxR*-like ORF in *M. capsulatus* had no sequence similarity with any known *cbbR* gene, although the direction of transcription and the location of this gene 5′ of *cbbL* in *M. capsulatus* (Bath) is consistent with other known *cbbR* genes. However, the sequence length is longer and it is located further upstream (5′) of *cbbL* than any known *cbbR* gene. Whether the *moxR*-like gene is involved in the regulation of Rubisco expression in *M. capsulatus* (Bath) is an interesting question that warrants further investigation.

Neighbouring genes

Of the remaining three ORFs, two were located 5′ of *cbbL* and the third was observed 3′ of *cbbQ*. An ORF of 552 bp, which was transcribed divergently from *cbbL*, began at nucleotide 2,440 of pNJB54. This ORF encoded a polypeptide with 64% identity (78% similarity) to PyrE from *P. aeruginosa* (MacGregor et al. 1996) and 61% identity (77% similarity) to PyrE from *Salmonella typhimurium* (Neuhard et al. 1985). A second ORF of 1,842 bp, also 5′ of *cbbL* but transcribed in the same direction as *cbbL*, began at position 184 and overlapped the *pyrE*-like ORF by 136 bp. The derived polypeptide sequence was 55% identical (72% similar) to the *rnr* gene product from *E. coli* (Burland et al. 1995) and 56% identical (71% similar) to the *rnr* gene product from *V. cholerae* (Heidelburg et al. 2000). Initial evidence suggested that *rnr*, originally designated *vacB*, had a regulatory function in the virulence of *Shigella flexneri* (Tobe et al. 1992), but it is now believed to encode an exoribonuclease, RNase R (Cheng et al. 1998). In a *Burkholderia* strain, it was suggested that *rnr* was involved, together with other genes, in the establishment of a symbiotic relationship with the fungus *Gigaspora margarita* (Riuz-Lozano and Bonfante 2000). In both *Burkholderia* (Ruiz-Lozano and Bonfante 2000) and *E. coli* (Cheng et al. 1998) a rRNA/tRNA methyltransferase gene, *yifH*, was located 3′ of *rnr* and was thought to comprise part of a gene cluster. No *yifH*-like ORF was found on pNJB54. Methanotroph-like bacteria have been reported to be involved in a symbiotic relationship with deep-sea bivalves (Cavanaugh et al. 1987, 1992; Distel et al. 1995). The *rnr*-like gene could therefore be involved in establishing such methanotrophs in a symbiotic relationship. An incomplete ORF, designated ORFX, whose derived amino acid sequence shared 44% similarity with a putative secreted cellulase from *Streptomyces coelicolor* (Redenbach et al. 1996) was located 278 nucleotides 3′ from *cbbQ*.

Phylogenetic analysis of *cbbL* from *Methylococcus capsulatus* (Bath)

The phylogenetic position of *cbbL* from *M. capsulatus* (Bath) was determined using a broad collection of bacterial reference sequences (Fig. 4). Delwiche and Palmer (1996) divided the Form I Rubisco into two major types, green-type (clade A and B in Fig. 4) and red-type (clade C and D in Fig. 4). *M. capsulatus* (Bath) *cbbL* is located within clade A (97% bootstrap support) which contains *cbbL* from several α- β- and γ-Proteobacteria and also a cyanobacterium. The position of *M. capsulatus* (Bath) *cbbL* within this clade, however, is not well resolved, because bootstrap support for relationships within clade A is generally low, but the phylogeny of *cbbL* does appear to be incongruent with relationships for the Proteobacteria inferred from other genes (Delwiche and Palmer 1996; Watson and Tabita 1997; Tabita 1999). To explain the incongruence, it has been suggested that *cbbL* genes have undergone both horizontal gene transfer and gene duplication with incomplete sampling of paralogues (Delwiche and Palmer 1996). This, together with the lack of resolution of the position of *M. capsulatus* sequence in our phylogenetic analyses, makes it difficult to infer the exact origin of *M. capsulatus* (Bath) *cbbL* within clade A.

Expression of *cbbL* from *Methylococcus capsulatus* (Bath)

Although *M. capsulatus* (Bath) contains a complete cluster of Rubisco genes and the expressed enzyme had been purified (Taylor 1977; Taylor et al. 1980) attempts to grow *M. capsulatus* (Bath) autotrophically in liquid culture were unsuccessful (Taylor et al. 1981; Stanley and Dalton 1982). *M. capsulatus* (Bath) only grows on methane or methanol and is primarily grown on methane since methanol is toxic, even at low concentrations. *M. capsulatus* (Bath) possesses two methane monooxygenases which catalyse the first step in the oxidation of methane. The particulate methane monooxygenase (pMMO) is expressed under copper-replete growth conditions, whereas the soluble methane monooxygenase (sMMO) is expressed under copper-limited growth (reviewed by Murrell et al. 2000). This raised the question: is *cbbL* expressed by *M. capsulatus* (Bath) under both growth conditions? RT-PCR of RNA extracted from *M. capsulatus* (Bath) expressing both pMMO and sMMO, using primers specific for *cbbL* from *M. capsulatus* (Bath), showed that *cbbL* was expressed under both growth conditions (data not shown).

cbbL in methanotrophs other than *Methylococcus capsulatus* (Bath)

Two sets of PCR primers, yielding a 0.5-kb or 1.0-kb PCR product, were designed to amplify *cbbL* from *M. capsulatus* (Bath). These PCR primers were applied to other

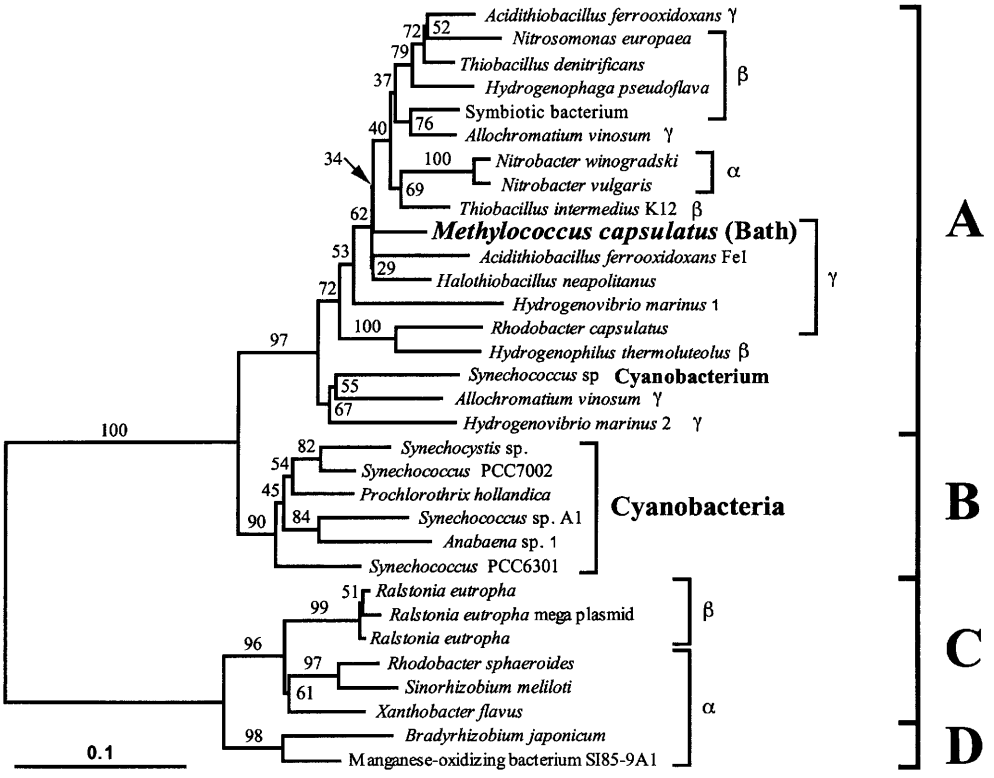

Fig. 4 Phylogenetic position of *M. capsulatus* (Bath) *cbbL*. The new nucleotide sequence was aligned to a collection of bacterial *cbbL* reference sequences using the protein alignment (459 unambiguously aligned residues) as a guide and its phylogenetic position inferred from codon positions 1 and 2 (918 positions). The tree shown is the majority consensus tree calculated from 1,000 bootstrap replicates of LogDet-minimum evolution analyses with 41.77% of sites estimated as invariant for the calculation of pairwise distances. The branch lengths of the consensus tree were estimated with the distance settings used for the bootstrap analysis. Bootstrap support values (%) are shown above the branches. The tree was rooted on the clade C and D according to the analysis of Delwiche and Palmer (1996). Proteobacterial and cyanobacterial taxonomic clades are indicated on the *right* (based on 16S rRNA analyses). *Bar* 10% estimated sequence divergence

Table 1 Comparison of the large subunit of Rubisco, CbbL, from methanotrophs with the large subunit of Rubisco, CbbL, of *Methylococcus capsulatus* (Bath)

Methanotroph	Number of nucleotides	Identity (%)	Number of amino acids	Identity (%)	Similarity (%)
Methylococcus capsulatus (Aberdeen)	511	97.4	171	99.4	100
Methylococcus capsulatus (M)	620	98.9	206	100	100
Methylococcus capsulatus (Texas)	620	98.9	206	100	100
Methylococcus strain WFM	511	84.8	170	94.7	98.2
Methylococcus strain F1-Diko	511	98.6	171	98.2	99.4
Methylocaldum szegediense OR2	511	84.8	170	94.1	97.6

methanotrophs to detect *cbbL*. *cbbL* was amplified from DNA from all *M. capsulatus* strains (M, Texas and Aberdeen), *M. capsulatus*-like isolates WFM and F1-Diko (DNA kindly donated by Dr. C. Miguez, Biotechnology Research Institute, National Research Council, Montreal) and *Methylocaldum szegediense* OR2 (Table 1). The sequences of *cbbL* from the other *M. capsulatus* strains were 97.4–98.9% identical to *cbbL* of *M. capsulatus* (Bath). The derived amino acid sequences of *M. capsulatus* (M) and *M. capsulatus* (Texas) were identical to CbbL from *M. capsulatus* (Bath) while *M. capsulatus* (Aberdeen) varied by one amino acid (Table 1). Interestingly the 1.0-kb *cbbL* PCR primers failed to amplify a product from *M. capsulatus* (Aberdeen) while the 0.5-kb primer set was successful. *cbbL* from *M. szegediense* OR2 was 84.4% identical to *cbbL* from *M. capsulatus* (Bath), and the derived amino acid sequence of CbbL from *M. szegediense* OR2 had 94.1% identity (97.6% similarity) to CbbL from *M. capsulatus* (Bath) (Table 1). A 1.0-kb PCR fragment of *cbbL* was amplified from *Methylocaldum tepidum* LK6 and partial sequencing also confirmed that the fragment was *cbbL*. Attempts to amplify *cbbL* from *Methylomonas rubra*, *Methylomonas methanica* S1, *Methylomicrobium album* BG8, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP were unsuccessful.

Southern blots containing DNA from all methanotrophs that were negative for the PCR amplification of *cbbL* were probed with *cbbL*. *M. methanica* S1 and *M. rubra* appeared to contain a *cbbL* homologue, and several unsuccessful attempts were made to clone these. Probing the genome of *M. album* BG8, *M. trichosporium* OB3b and *M. parvus* OBBP with a methanotroph *cbbL* gene probe failed to locate any *cbbL* homologues, suggesting that they lacked Rubisco. In previous studies (J.C. Murrell and P. DeWulf, unpublished data), a Form II Rubisco gene probe (*cbbM*) was used to probe the chromosome of all methanotrophs used in this study, but no *cbbM* homologues were detected.

Autotrophic and chemoorganoautotrophic growth of methanotrophs

Attempts to grow *M. capsulatus* (Bath) autotrophically in liquid medium were unsuccessful. Reproducible autotrophic growth of *M. capsulatus* (Bath) on H_2 and CO_2 was, however, achieved on solid medium although growth was significantly slower than on methane. Colonies appeared after 1 week of incubation, reaching a diameter of 0.5–1.0 mm after 3 weeks. Purity of cultures was confirmed by phase-contrast microscopy, growth on methane and absence of growth on heterotrophic media (*M. capsulatus* does not grow on nutrient-rich media). No growth was observed on plates with H_2 alone or CO_2 alone. *M. capsulatus* (Bath) was also capable of chemoorganoautotrophic growth with formate and CO_2. In the presence of only formate as a growth substrate, *M. capsulatus* (Bath) did not grow, suggesting that at high concentrations, CO_2 was used as carbon source with formate oxidation providing the energy and reducing power for autotrophic growth. It is not clear why *M. capsulatus* grew autotrophically only on plates and not in liquid culture. Appropriate controls were included to ensure that the organism was not growing on agar plates by scavenging trace amounts of carbon from the agar (otherwise they would have still grown in the presence of hydrogen alone). It is possible that some density-dependent phenomenon is responsible for growth on plates or that the trace organic compounds, undoubtedly present in the agar, may be required to "prime" the autotrophic growth process. The lack of growth on plates is not without precedence in the methanotrophy literature. For example, thermophilic methanotroph strain HB grows well on plates on methane but cannot grow to appreciable optical densities in liquid culture (Bodrossy et al. 1999). Conversely, some marine methanotrophs grow well in liquid culture but are very difficult to grow on all types of solid media including agar, agarose and silica-gel plates (Lees et al. 1991).

An initial screen of representative methanotrophs indicated that the capacity for autotrophic growth is not limited to *M. capsulatus* (Bath). *M. tepidum* LK6, *M. trichosporium* OB3b and *Methylocystis* strain M (but not *M. methanica* S1 or *M. szegediense* OR2) were capable of autotrophic growth on plates when both H_2 and CO_2 were used as growth substrates. The ability of *M. trichosporium* OB3b and *Methylocystis* strain M to grow, albeit poorly, on plates with H_2 and CO_2 was unexpected since no *cbbL* or *cbbM* homologues were detected in these bacteria. The failure of *M. szegediense* OR2 to grow autotrophically, despite having a *cbbL* gene, may be due to inappropriate growth conditions, or Rubisco may not be active in this methanotroph.

An evolutionary link between methylotrophic and autotrophic metabolism has been proposed (Quayle and Ferenci 1978; Colby et al. 1979). The fact that *M. capsulatus* (Bath) possesses the ribulose monophosphate pathway, Rubisco and some enzymes of the serine pathway certainly warrants further investigation at the biochemical and molecular levels. The exact role of Rubisco in *M. capsulatus* (Bath) and other methanotrophs is still uncertain and might be investigated by, for example, deleting *cbbL* by marker-exchange mutagenesis to assess the phenotype of such a mutant. Unfortunately, this may prove to be very difficult due to the restricted physiology of methanotrophs and associated problems with the lack of a good genetic system for manipulation of these bacteria.

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