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Heat-Tolerant Methanotrophic Bacteria from the Hot Water Effluent of a Natural Gas Field

LEVENTE BODROSSY,[1] J. COLIN MURRELL,[2] HOWARD DALTON,[2] MIKLOS KALMAN,[3] LASZLO G. PUSKAS,[4] AND KORNEL L. KOVACS[1]*

Institute of Biophysics,[1] Institute of Genetics,[3] and Institute of Plant Biology,[4] Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary, and Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom[2]

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Methanotrophic bacteria were isolated from a natural environment potentially favorable to heat-tolerant methanotrophs. An improved colony plate assay was developed and used to identify putative methanotrophic colonies with high confidence. Fourteen new isolates were purified and partially characterized. These new isolates exhibit a DNA sequence homology of up to 97% with the conserved regions in the *mmoX* and *mmoC* genes of the soluble methane monooxygenase (MMO)-coding gene cluster of *Methylococcus capsulatus* Bath. The copper regulation of soluble MMO expression in the same isolates, however, differs from that of *M. capsulatus* Bath, as the new isolates can tolerate up to 0.8 μM copper without loss of MMO activity while a drastic reduction of MMO activity occurs already at 0.1 μM copper in *M. capsulatus* Bath. The isolates can be cultivated and utilized at elevated temperatures, and their copper- and heat-tolerant MMO activity makes these bacteria ideal candidates for future biotechnological use.

Methanotrophs are obligate methane-oxidizing bacteria capable of utilization of methane as a sole source of cellular carbon and energy (33). Methanotrophs are widespread in natural habitats and play an important role in carbon recycling (8, 9). Methane has become one of the most important greenhouse gases partly because its concentration in the atmosphere is increasing at the alarming rate of about 1% per year (4). The largest biological sinks for methane operate via methane-oxidizing bacteria. The initial enzyme in the pathway for aerobic methane catabolism, methane monooxygenase (MMO), has a broad substrate specificity. Therefore, methanotrophic bacteria can initiate the degradation of a variety of environmental pollutants, including particularly recalcitrant chlorinated hydrocarbons, e.g., the widespread groundwater toxic contaminant and potential carcinogenic trichloroethylene (11, 13, 14, 22, 35).

Methanotrophs may possess two types of MMO, a soluble enzyme (sMMO) and a membrane-bound one (pMMO). sMMO appears considerably more effective than pMMO in the biodegradation of chlorinated hydrocarbons. According to several earlier observations, the inducible sMMO is widespread in certain subgroups, i.e., in all type II and type X methanotrophs (13, 30, 31), while among the type I methanotrophs sMMO was found only in *Methylomonas methanica* 68-1 (12). sMMO activity is usually found in methanotrophs growing in copper-free environments (18), except for some mutant strains of *Methylosinus trichosporium* OB3b (5, 24), which are Cu tolerant. A low copper-to-biomass ratio has generally been considered a key factor in determining sMMO expression (25).

Because of the potential significance of methanotrophs in a variety of environmental biotechnology applications, *Methylococcus capsulatus* Bath and *M. trichosporium* OB3b have been extensively studied and the biochemical and molecular properties of their sMMO have been characterized (for reviews, see references 13, 19, and 20).

Methods to isolate and identify methanotrophs on the basis of their sMMO activity (1a, 7, 32) as well as on the sequence homologies among sMMO genes of various origins have been devised (16, 29). A diagnostic colorimetric assay is based on the observation that monooxygenases catalyze the conversion of naphthalene to 1-naphthol, and 1-naphthol formation can be visualized or monitored colorimetrically by the addition of aromatic diazo compounds to the reaction mixture (23). This reaction has been used to test the trichloroethylene degradation potential of methanotrophs (1a). *o*-Dianisidine, which turns purple in the presence of naphthol, was the choice as a coloring agent and was applied in a colorimetric plate assay of methanotrophic colonies (7).

The cultivation of thermotolerant microorganisms for industrial processes is desirable, because the costs of bacteriological process control can be significantly reduced and conversion rates can be substantially increased by using thermophilic microorganisms (15, 28). In addition to these general advantages in a variety of potential bioconversion applications, heat-tolerant methanotrophs would be particularly effective in microbial methanol-producing systems (6) where the inhibitory reaction product can be removed from the reactor continuously by simple condensation if the reaction temperature is raised to 60 to 65°C.

Here we describe improved procedures to screen bacteria for sMMO expression when grown on solid media at elevated temperatures. The colony MMO activity detection method is a modification of a color test developed by Wackett and Gibson (32). In addition, MMO-coding DNA sequence homologies are identified by using PCR as described by McDonald and coworkers (16). The results of our search for thermotolerant methanotrophs and the characterization of the isolated heat-tolerant strains are also reported. Each of the 14 new isolates appears to be equipped with a copper-resistant sMMO activity, which may be of particular interest for further research on the regulation of sMMO expression and for potential practical applications.

* Corresponding author. Mailing address: Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary. Phone: (36 62) 432 232. Fax: (36 62) 433 133. Electronic mail address: Kornel@everx.szbk.u-szeged.hu.

MATERIALS AND METHODS

Cultivation of bacteria. Unless otherwise indicated, the isolates were grown in a copper-deficient mineral salts medium supplemented with potassium nitrate (NMS) (33, 34). The copper-deficient, or copper-minus, medium contained no deliberately added copper; however, no special effort was made to remove fortuitous traces of copper contamination introduced by the mineral salts components. Copper-plus, also called copper-containing, NMS contained 0.4 μM $CuSO_4$. To solidify media, 1.5% (wt/vol) Bacto agar (Difco Laboratories) was routinely added. Liquid cultures were grown in 50-ml Erlenmeyer flasks containing 10 ml of medium in a water bath shaker at 200 rpm. The gas-to-liquid ratio was about 4:1 in the stoppered flasks. The headspace was filled with methane-air in a 1:1 gas mixture. Plates were incubated in anaerobic jars under a 1:1 methane-air atmosphere at 37 or 45°C for 10 to 15 days. The gas phase was replaced every 2 to 3 days with a fresh methane-air mixture.

Storage of the bacteria. Pure isolates were stored at −70°C after the following treatment: cells of a 5-ml 1-day-old liquid culture were collected by centrifugation at 10,000 × *g* for 10 min and were resuspended in sterile potassium phosphate buffer (pH 7.0, 20 mM). The cell suspensions were immediately frozen at −70°C.

In situ sMMO assay. (i) Routine assay. Ten- to fifteen-day-old plates containing well-developed colonies were first covered with copper-minus NMS supplemented with 0.8% (wt/vol) agar at 45°C. When the top agar solidified, the plates were inverted, and a few naphthalene crystals were sprinkled in the lids of the plates. The plates were incubated at 45°C for 30 min before 1 ml of 5-mg/ml *o*-dianisidine (tetrazotized, zinc chloride complex; Sigma Chemicals Ltd.) was layered on the surface of the top agar. The plates were then further incubated at 45°C for 10 min. The purple color of sMMO-positive colonies remained stable at room temperature for a few hours or, when stored at 4°C, for several weeks. In comparative studies, the sMMO plate assay method as described elsewhere (7) was used.

(ii) Assay with heat shock. In assays employing heat shock, the top agar contained 1.5% (wt/vol) agar and was spread hot, i.e., 92 to 97°C. In addition, the plates were incubated at 60°C for 10 min prior to sprinkling of the naphthalene crystals into the lids.

(iii) Assay on cell extracts. Cells were suspended in MOPS (morpholinepropanesulfonic acid) buffer containing 25 mM MOPS (Calbiochem), 1 mM benzamidine (Aldrich), and 5 mM sodium thioglycolate (Fluka) at pH 7.0, before disruption with a French press (20,000 lb/in^2, minimum of two times) and by centrifugation (30,000 × *g*, 4°C, 60 min). The pellet was resuspended in the same volume of MOPS buffer. Both the supernatant and pellet fractions were assayed for naphthalene oxidation activity. Before the assay, NADH (Sigma) was added at a final concentration of 0.5 mM. Incubations were done as described above for the routine assay. Color development was carried out with 0.2-mg/ml *o*-dianisidine (tetrazotized; zinc chloride complex; Sigma Chemicals Ltd.). The reaction product was observed immediately after mixing, as the color tended to fade with time.

(iv) Inhibition of the sMMO assay. Acetylene has been identified as a potent and selective inhibitor of MMO activity (25). Therefore, negative controls for the in situ plate sMMO assay were parallel plates treated with acetylene; such plates were routinely included in every experiment.

In vivo heat stability assay of the sMMO. Ten- to fifteen-day-old plates were used and treated as described above for the in situ sMMO activity assay, except that 1.5% (wt/vol) agar was used and the plates were incubated at higher temperatures (45 to 65°C) for 30 to 60 min before addition of the naphthalene crystals.

Purification of chromosomal DNA. Chromosomal DNA from methanotrophs was prepared as described by Oakley and Murrell (21).

Hybridization. DNA fragments separated in a 1.5% (wt/vol) agarose gel were Southern blotted by the capillary transfer technique onto Hybond-N filters (Amersham). Prehybridization was done in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and 5× Denhardt's solution at 65°C for at least 30 min (as detailed in reference 26). Hybridization was carried out under the same conditions for 4 to 6 h. The filters were washed with 2× SSC plus 0.1% (wt/vol) SDS at 58°C before exposing them for 14 h on Phosphorimager plates (Molecular Dynamics).

PCR primers and amplification. PCR primers *mmoC*m1 and *mmoC*m2 were designed by selecting the most-conserved sequence motifs from the DNA sequences encoding protein C of sMMO in the two MMO clusters (2, 3, 16). The two primers are about 550 bp apart; their relative positions within the *mmo* gene cluster are indicated in Fig. 1. *mmoC*m1 and *mmoC*m2 were synthesized by the standard phosphoramidite method on solid support (Biotronik D-100). A deoxyinosine residue (I) was incorporated at the sites where the two sequences differed in the third positions of some codons. The primers used were as follows: *mmoC*m1, 5′-GGCTGIGCIACCTGCAAGGC-3′; *mmoC*m2, 5′-CCIGTGC CGCCGGCIACGAA-3′. The synthesis and design of other methanotroph-specific PCR primers have been published elsewhere (16).

PCRs were performed in 0.5-ml Eppendorf tubes under a layer of 50 μl of mineral oil (Sigma Chemical Co.). The total reaction volume was 50 μl, including 5 μl of 10× PCR amplification buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer, 1 μl of 20-mg/ml bovine serum albumin (Boehringer Mannheim), and 200 μM (each) deoxynucleotide triphosphate. The template DNA was 10 μl of either sonicated bacterial suspension (optical density at 520 nm, approximately 3.0 before sonication) or 50 pg of purified chromosomal DNA per ml. *Taq* DNA polymerase (1.0 U; Promega) was added after the hot start. The amplification was carried out by using a DNA thermal cycler (Combi Thermal Reactor, Hybaid). The PCR profiles were as follows: 94°C for 5 min before *Taq* polymerase was added; then 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final elongation step at 72°C for 10 min. The PCR products were separated by 1% agarose gel (1% [wt/vol]) electrophoresis (26).

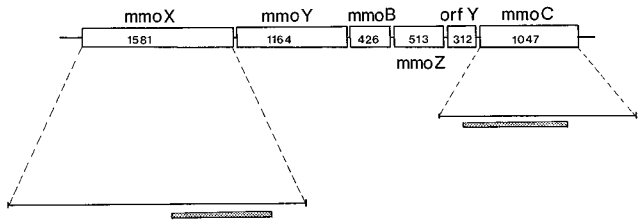

FIG. 1. Organization of *mmo* genes in *M. capsulatus* Bath and the positions of diagnostic PCR products of methanotrophs. The amplified regions in the *mmoX* and *mmoC* genes are indicated by the hatched bars.

Cloning of the PCR products. Products of PCR amplification performed with phosphorylated primers *mmoC*m1 and *mmoC*m2 were cloned into *Sma*I-digested and calf intestine phosphatase-treated pBS vector (Stratagene) via blunt-end ligation, and the ligated product was cloned into *Escherichia coli* DH5α competent cells. Some PCR products were cloned into pCRII vector as described in the TA-cloning kit, version 2.2 (Invitrogen Corp.). Preparation of competent cells and transformation were performed as described by Inoue et al. (10). Screening for sMMO-positive clones was done by α complementation as described by Sambrook et al. (26) and by colony hybridization with PCR primers *mmoC*m1 and *mmoC*m2 as probes.

DNA sequencing. DNA for double-strand sequencing was prepared by using the method of Saunders and Burke (27) and sequenced by using the dideoxynucleotide chain termination method (Sequenase, version 2.0; USB).

RESULTS

Methanotrophic colony assay. The primary selection during isolation was based on the naphthalene oxidation capacity of the cells. The use of an agar overlay improved the sensitivity and selectivity in two ways. The dye could be spread on top of the agar overlay without washing off cells from the solid growth medium surface by the reagent and thereby blurring colony borderlines. Also, this alteration of the original method (7) decreased the diffusion of the reaction product naphthol from the colonies, thus sharpening the colony contours and making the developed color more pronounced. An enhanced resolution was very useful when methanotrophic colonies were to be identified among many nonmethanotrophic ones during the early stages of strain purification.

Isolation and cultivation of new methanotrophs. Water samples were collected from different points of a greenhouse heating system fed directly from a hot spring connected to a natural gas field near Szeged, Hungary. The temperature at the sampling points varied between 30 and 55°C. Water samples were concentrated by centrifugation at 12,000 rpm (23,000 × *g*) (Sorvall RC5C centrifuge, SS34 rotor) for 20 min and suspended in sterile copper-deficient NMS. The volumes of the original samples were thus reduced 500- to 1,000-fold. Portions of the samples were diluted 10^2- to 10^4-fold, and 100-μl aliquots were spread onto copper-free NMS and incubated at 37°C, while the rest of the samples were stored at −20°C.

Some colonies, later proving to be nonmethanotrophic, appeared after 1 day of growth and reached their maximum size in 3 to 4 days, in line with the observations of Whittenbury et al. (34). Methanotrophic colonies reached a diameter of 0.5 to 1.5 mm, which seemed to be essential for proper color development, within 10 to 15 days (Fig. 2).

On the basis of the sMMO colorimetric colony assay, 12

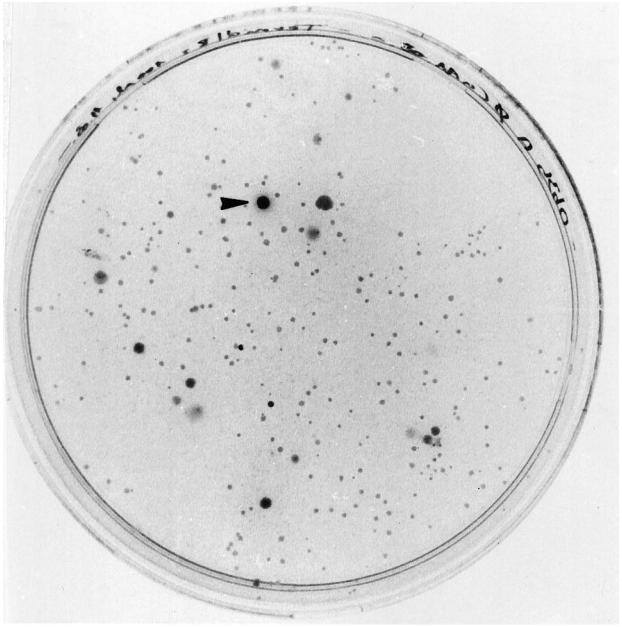

FIG. 2. Partially purified methanotroph culture stained for colony sMMO activity. The purple colonies stain positive for sMMO (arrowhead); these colonies were recovered after the assay and plated again on copper-free NMS medium under methane for further purification.

isolates apparently exhibited a positive sMMO reaction. The final color of the sMMO-positive colonies varied from pink to dark purple. All of them grew in liquid culture on copper-deficient NMS under an atmosphere of methane-air mixture, whereas none of them showed growth in the absence of methane. Also, none of the isolates grew on NMS plates (either copper-plus or copper-minus) in the absence of methane.

Purification of the isolates was carried out by serial dilution of resuspended single colonies and screening on copper-deficient NMS plates. Of the 12 isolates, 2 proved to be mixed methanotrophic cultures displaying various colony morphologies. After further purification, a total of 14 pure methanotrophic isolates were obtained. All 14 new putative methanotrophic isolates were able to grow at 45°C without a significant decrease in their growth rate, compared with the 37°C-grown controls. Above 45°C, the growth became gradually impaired, and no growth on copper-deficient NMS plates or in copper-minus liquid NMS was seen above 55°C. Further cultivation was carried out at 45°C in order to prevent the isolates from losing their ability to grow at elevated temperatures. Microscopic examination revealed that all the isolates were invariably coccoid bacteria, like *M. capsulatus* Bath, with a tendency to form diplococcoid and tetracoccoid aggregates in exponential-growth phase. Based on the differences in their MMO activity and MMO stability, as well as other growth properties, the 14 isolated pure cultures could be divided into two groups. Representatives of these groups, designated BL4 and BL13, were studied in further detail.

Restoration of the colony sMMO activity by heat shock. After being subcultured 10 to 15 times, the originally strongly sMMO-positive isolates gradually began losing their ability to develop color in the naphthalene oxidation assay. This apparent loss of sMMO activity could be alleviated by the application of a heat shock (60°C for 10 min) prior to the color development assay (Fig. 3). Controls, including parallel plates of the same isolates that had been exposed to acetylene before the sMMO activity test as well as *M. capsulatus* Bath plates, failed to show any activity in the assay. It is to be noted that *M. capsulatus* Bath develops very small colonies on copper-deficient NMS plates and that the sMMO plate assay gives equivocal results with small colonies under any conditions.

Equally remarkable was that the heat shock did not decrease the viability of the colonies significantly; therefore, it was possible to subculture the strains following heat shock at 60°C for 10 min and the color development reaction.

sMMO heat stability in vivo. As the viability of the putative new methanotrophs and their in vivo sMMO activity appeared to withstand the heat shock, they were further tested for in vivo sMMO heat stability at 65°C for up to 1 h. No detectable loss of color-forming activity, determined either at 45 or at 65°C in the modified plate assay, was found. Again, acetylene-treated controls displayed no color development in the colony assay, indicating that the reaction was indeed sMMO specific (25). Although the isolates did not show significant growth at 65°C, as presented above, the purple colonies which tested positive in the in vivo sMMO test after heat treatment at 65°C for 1 h exhibited normal growth at 45°C. These results suggest that the in vivo sMMO activity as well as the general viability of the new isolates has a considerable heat tolerance despite their inability to grow habitually at temperatures above 50 to 55°C. The in vivo sMMO stability shows substantial variations beyond heat treatment at 65°C for 1 h, depending on the isolate and its cultivation history. *M. capsulatus* Bath, which has an optimum growth temperature of 45°C, also developed normal colonies on copper-containing NMS plates at this temperature following heat treatment at 65°C for 1 h. However, the colonies

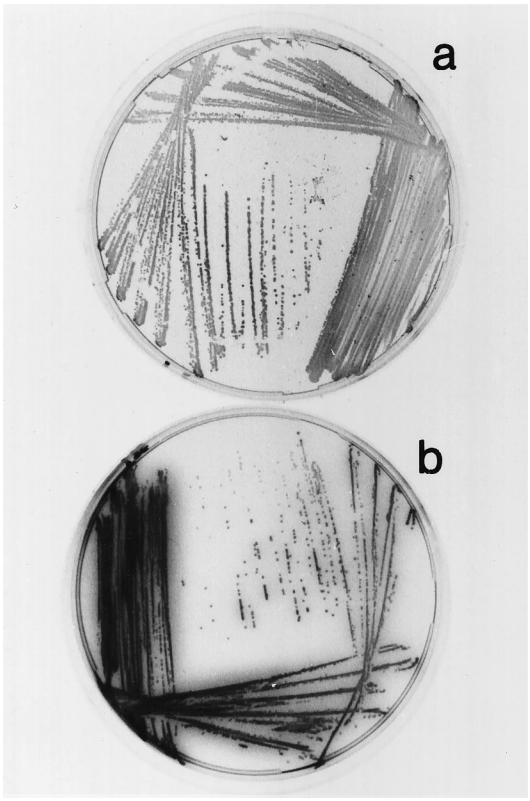

FIG. 3. The effect of heat shock on restoration of colony sMMO activity. A pure methanotroph culture that had been maintained at 45°C shows gradually weakening color response in the colony assay (a), which was restored by a heat shock applied to a parallel plate (b).

tested negative in the colony plate assay, in line with earlier findings at 45°C (25).

Isolates grown in the presence of Cu^{2+} display sMMO activity. Since copper deficiency is the induction signal for sMMO synthesis, one would expect positive colony activity assay results only, if any, on copper-deficient plates. Contrary to this prediction, colonies of all of our new methanotrophic isolates show equally strong MMO activity in the presence and in the absence of copper in the growth medium with or without heat shock. Acetylene, a suicide substrate and active-site probe for MMO, inhibits this activity, and no naphthalene-oxidizing activity was detected in a variety of known nonmethanotrophic strains. In the control experiments with *M. capsulatus* Bath, the growth rate as well as MMO activity was strictly regulated by copper, corroborating earlier observations (25). Therefore, we concluded that a copper-independent MMO activity was demonstrated in the newly isolated methanotrophic strains.

Cellular localization of MMO activity. Naphthalene oxidation activity was found in the supernatant of broken cell suspensions of each new isolate as well as in those of well-characterized control strains. The membrane fraction of the same cells showed no apparent sign of naphthalene-oxidizing activity. This may be due to the selective inhibitory effect of thioglycolic acid on pMMO activity (4a).

Growth on methanol. All new putative methanotrophs were able to grow overnight in 0.1% methyl alcohol (MeOH)-NMS liquid culture at 37 to 45°C, when inoculated with liquid cultures grown under methane. However, trials to maintain MeOH-NMS cultures of the same isolates by repeated inoculation onto MeOH-NMS plates at 37 or 45°C were unsuccessful. Colonies were obtained from MeOH-containing liquid cultures of the BL13-type new isolates as well as from those of *M. capsulatus* Bath on 0.02% MeOH-NMS plates at 37°C, but only when a relatively large inoculum was used. Elevation of the incubation temperature to 45°C inhibited MeOH-dependent growth of the BL13-type isolates on MeOH-NMS plates, while good growth was seen on NMS plates under CH_4 at the same temperature. No growth of BL4 could be achieved in the presence of 0.02 to 0.1% MeOH on MeOH-NMS plates at any temperature. The switch back from MeOH as the sole carbon source to CH_4 worked with one type of isolate (BL13) on NMS plates incubated under CH_4 but failed with the other one (BL4).

For comparison, *M. trichosporium* OB3b did grow well in the presence of 0.02% MeOH on plates at 30°C. Nevertheless, substituting the carbon source MeOH with methane improves the growth characteristics of both *M. capsulatus* Bath and *M. trichosporium* OB3b.

Hybridization. Southern hybridization with an 11.9-kb probe containing the entire, approximately 7.9-kb-long sMMO gene cluster from *M. capsulatus* Bath (17, 18) was carried out in order to test the similarities and differences of the sMMO-coding DNA regions of the new isolates to *M. capsulatus* Bath.

As illustrated in Fig. 4, *Bam*HI, *Eco*RI, *Pst*I, and *Sal*I digests of BL4, BL5, BL13, and *M. capsulatus* Bath chromosomal DNA were hybridized with an 11.9-kb DNA fragment harboring the entire MMO gene cluster of *M. capsulatus* Bath. Results show that the distribution of hybridizing fragments of BL13 and BL5, one of the BL13-type new isolates, is very similar to that of the *M. capsulatus* Bath sMMO genes. The hybridization pattern similarity with BL4-type methanotrophs was weak.

PCR amplification products. PCR primers specific to the structural gene *mmoX* encoding the α subunit of protein A as well as primers corresponding to *mmoC* coding for the protein C product of sMMO were used (see Materials and Methods).

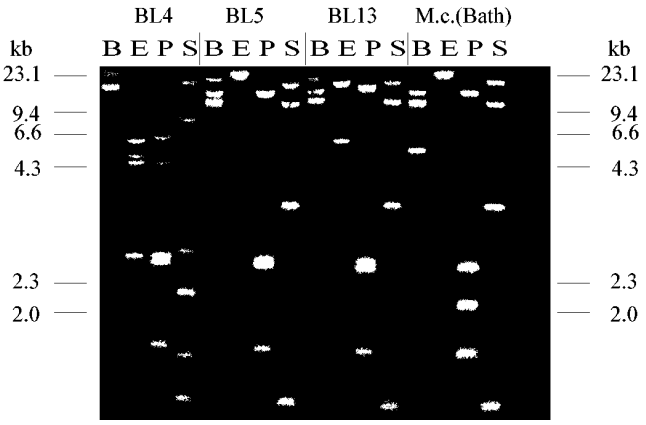

FIG. 4. Southern hybridization on *Bam*HI-, *Eco*RI-, *Pst*I-, and *Sal*I-digested (B, E, P, and S, respectively) total DNA from new methanotrophic isolates BL4, BL5, and BL13 and *M. capsulatus* Bath. The digests were probed with the *mmo* gene cluster of *M. capsulatus* Bath.

The locations of these primers are indicated in Fig. 1. The results of PCR amplification of the *mmoC* fragment from the 14 separate new methanotrophic isolates and control microorganisms are shown in Fig. 5. A very similar picture was obtained for the PCR employing *mmoX*-specific primers (data not shown). The PCR of *mmoX* is a positive control for the results shown in Fig. 5, as the *mmoX* sequence is more conserved in methanotrophs than the *mmoC* is (19). Therefore, in strains containing the *mmoC* sequence, the *mmoC* gene is very likely to be present as well.

The PCR fragments were cloned and sequenced. The sequences are very homologous to the corresponding DNA sequences in the *M. capsulatus* Bath sMMO cluster (Fig. 6). The type strains BL4 and BL13 show strong homologies with the conserved *M. capsulatus* Bath *mmoX* (97.2% for both strains) and *mmoC* (96.4 and 97.6%, respectively) genes.

DISCUSSION

Methanotrophs have been recognized as a group of microorganisms which occupy a specific niche in certain ecosystems.

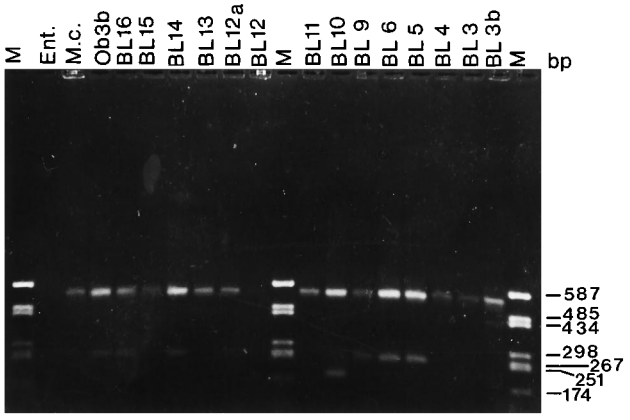

FIG. 5. PCR products amplified with *mmoC*-specific primers *mmoC*m1 and *mmoC*m2. Total genomic DNAs from the new methanotrophic isolates were used as templates. Positive control templates included DNA from *M. trichosporium* OB3b and *M. capsulatus* Bath (OB3b and M.c., respectively). A negative control template contained total DNA from a nonmethanotrophic fermentative bacterium, *Enterobacter cloaceae* (Ent.). The lambda DNA marker lane is indicated (M).

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                    .         .         .         .         .
M.c.(Bath)    1 GCATCKALCSEGDYDLKGCSVQALPPEEEEEGLVLLCRTYPKTDLEIELP 50
BL4           1 GWATCKALCKEGDYDLKGCSVQALPPEEEEDGLVLLCRTYPKTDLEVELP 50
BL13          1 GWATCKALCSEGDYDLKGCSVQALPPEEEEEGLVLLCRTYPKTDLEIELP 50

                    .         .         .         .         .
M.c.(Bath)   51 YTHCRISFGEVGSFEAEVVGLNWVSSNTVQFLLQKRPDECGNRGVKFEPG 100
BL4          51 YTHCRISYGEVGSFEAEVVGLNWVSSNTVQFLLQKRPDECGNRGVKFEPG 100
BL13         51 YTHCRISYGEVGSFEAEVVGLNWVSSNTVQFLLQKRPDECGNHGVKFEPG 100

                    .         .         .         .         .
M.c.(Bath)  101 QFMDLTIPGTDVSRSYSPANLPNPEGRLEFLIRVLPEGRFSDYLRNDARV 150
BL4         101 QFMDLTIPGTDVSRSYSPANLPNPDGRLEFLIRVLPEGRFSDYLRNDARV 150
BL13        101 QFMDLTIPGTDVSRSYSPANLPNPDGRLEFLIRVLPEGRFSDYLRNDARV 150

                    .         .         .
M.c.(Bath)  151 GQVLSVKGPLGVFGLKERGMAPRYFVAGGTG 181
BL4         151 GQVLSVKGPLGVFGLKELGMAPRYFVAGGTG 181
BL13        151 GQVLSVKGPLGVFGLKEHGMAPRYFVAGGTG 181
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FIG. 6. Sequence comparison of some translated putative protein fragments of the PCR products shown in Fig. 5. The variations from the corresponding sequence from *M. capsulatus* Bath are indicated by boldface letters.

With the use of their unique capability to metabolize methane through an oxidative chain of reactions, methanotrophs create a link between anaerobic, methane-producing, and aerobic layers of sediments, rice paddies, and peat bogs (17, 18). The same enzyme, MMO, enables these microorganisms to attack and decompose hazardous manmade chemicals, i.e., chlorinated hydrocarbons. Most of these compounds, such as the potentially dangerous carcinogen trichloroethylene, are notorious for being extremely resistant to microbiological degradation and for contaminating a significant portion of groundwater supplies.

Improved colony color screening. Because of their role in maintaining ecological balance and in regulating global methane emission, methanotrophs can be expected to be more widespread in nature than they are presently known to be. A likely explanation of why methanotrophs have been recognized as abundant microbes in relatively few ecosystems is largely based on the difficulty of identification of methanotrophic activity. A color development plate assay has been developed by Graham et al. (7), recently. The method exploits the naphthalene oxidation ability of sMMO (32). The test is fairly simple to carry out and suitable for screening a large number of independent samples. Color blurring, however, frequently takes place as the reagent is spread over the colonies and cells are washed away. This may lead to an increased number of false-positive results and thereby renders isolation of methanotrophs a challenge. Application of an agar overlay, as described here, preserved the colony contours and simplified the application of the color reagent. Taking advantage of the improved colony screening assay, we searched for heat-tolerant methanotrophs in the methane-saturated hot water environment of a natural gas field effluent system. The new isolates were thus first selected on the basis of their naphthalene-oxidizing activity. Since naphthalene oxidation may not be exclusively restricted to the presence of MMO activity in the cells of colonies which test positive for sMMO activity, a specific MMO inhibitor, acetylene, was used as a negative control (25).

The specificity of naphthalene oxidation by sMMO has been a fundamental presumption in these assays. The validity of this assumption in the case of the new isolates was checked in separate experiments. First, it was determined that naphthalene oxidation takes place in the cytoplasmic fraction of the new isolates. Second, this activity was inhibited by growing the cultures at a high copper concentration, which is inhibitory for sMMO synthesis. Third, SDS-polyacrylamide gel electrophoresis separation of crude cytoplasmic protein extracts of the new isolates showed that sMMO polypeptides are dominant components in the crude cytoplasmic extracts, as they are in other methanotrophs. The presence of these protein bands correlated with the naphthalene-oxidizing activity (data not shown). In principle, naphthalene oxidation can be carried out by pMMO as well, and we have detected pMMO homologous DNA sequences in the new isolates by PCR, recently (1). In the presence of thioglycolic acid, which is a stabilizing agent of sMMO activity and a specific inhibitor of pMMO, however, naphthalene oxidation activity was still detected in the cytoplasmic fraction of the new isolates. Taken together, the findings discussed above led to the conclusion that in the new isolates regulation of expression and/or activity of MMO operates similarly to that in the well-characterized methanotrophic strains, e.g., *M. capsulatus* Bath and *M. trichosporium* OB3b. Methanotrophic cells were viable after the colony assay and were thus easily picked and plated for further purification with methane as the sole carbon source. A major difficulty encountered was due to the slow growth rate of methanotrophs, as fast-growing heterotrophic contamination tended to dominate the population and was difficult to eliminate from the isolates.

Several rounds of purification were needed to make sure that a contamination-free, pure methanotrophic culture was established from the mixed population of the natural habitat.

Heat treatment affects MMO activity. After being successfully replated several times within a couple of weeks at 45°C, the sMMO-positive colonies gradually lost their naphthalene-oxidizing capacity. This apparent gradual loss of the naphthalene-oxidizing activity was completely eliminated by a heat shock (60°C, 10 min), indicating that a lack of accessibility of the reaction components took place at 45°C, i.e., the active enzyme and its substrate were available in the system, but the MMO could meet and oxidize naphthalene only after heat shock. The molecular mechanism is yet to be understood; a likely explanation based on microscopic observations involves a rearrangement of cell wall layers at 45°C during the extended incubation time. None of the 14 new methanotrophic isolates were able to grow at temperatures above 50 to 55°C. The heat shock experiments, however, suggested that this temperature limitation of growth was not due to a heat inactivation of their sMMO. sMMO activity and also the ability to grow at 45°C were preserved after heat treatment at 65°C for at least 1 h.

Molecular biology shows close relationship between the isolates and *M. capsulatus* Bath. In order to verify that the isolates

obtained from the colony plate assay are genuine methanotrophic strains and to establish how these isolates were related to known subgroups of methanotrophic bacteria, molecular biology tools were employed. The fundamental observation which made this analysis possible was that *mmo* gene sequences were found to be highly conserved in the two gene clusters sequenced to date (17, 18). Assuming that a similar sequence homology also existed in sMMO-coding DNA fragments in other methanotrophs, PCR-based methods to detect methanotrophic bacteria in environmental samples were developed (16). These studies pointed to the *mmoX* gene, the product of which is the α subunit of the hydroxylase component of sMMO, as the most suitable one with diagnostic value for PCR amplification of DNA of methanotrophic bacteria from environmental samples. *mmoX* was found to be more conserved than the *mmoC* gene; therefore, *mmoC*-based PCR was suggested to be useful for examining the diversity of methanotrophic sequences (16). In our present study, the PCR primer sets used in the studies by McDonald and coworkers (16) and an *mmoC*-specific primer set were employed in addition to hybridizations with sMMO-coding heterologous DNA fragments. Several conclusions could be drawn from the results. First, the molecular biology tests unequivocally corroborated that the pure cultures isolated with the colony plate assay were in fact methanotrophic strains containing sMMO-coding DNA regions. Second, the oligonucleotides, specific for *mmoX* and *mmoC* genes, were equally effective as PCR primers. Third, the DNA sequences amplified with the PCR using *mmoX*- and *mmoC*-derived primers exhibited the same high levels of identity (96 to 97%) to the corresponding sequences of *M. capsulatus* Bath. In light of the results, it is particularly remarkable that the sequenced portions of *mmoC* PCR products of the methanotrophs isolated in the present study were hardly distinguishable from the corresponding *mmoC* sequence of *M. capsulatus* Bath. On the basis of the high level of sequence identity, the new isolates are tentatively assigned to the type X subgroup of methanotrophic bacteria.

Nonidentity between *M. capsulatus* Bath and the new isolates. In spite of the high level of sequence identity between *M. capsulatus* Bath and the isolate BL4 or BL13 (Fig. 6), there were characteristic and reproducible differences at the DNA sequence level and significant physiological variations among the three strains. In this respect the following observations are of importance.

(i) All BL isolates possessed an sMMO activity that was stable at 65°C for at least 1 h, and the colonies contained viable cells after such treatment. No active sMMO was detectable after similar incubation in *M. capsulatus* Bath, although it was possible to recover growth at 45°C after the heat treatment.

(ii) All BL isolates displayed an apparently copper-independent MMO activity at all temperatures up to 65°C. These cultures grew well on NMS plates under methane both in the presence and in the absence of copper at 45°C, and there was no observable difference in the color development rate and intensity in the colony plate assay between the copper-containing and copper-deficient plates. In contrast, *M. capsulatus* Bath grew poorly on copper-deficient NMS plates and formed very small colonies. Normal-size colonies were obtained on copper-containing plates; however, these colonies were inactive in the plate assay as in previous studies (25).

The only published example of a non-copper-regulated sMMO expression was observed with some mutants of the type II methanotroph, *M. trichosporium* OB3b (5, 24). The fact that now there exist several cases for which sMMO expression and/or activity do not strictly depend on the copper level in their environment raises important questions about what role copper may play and at which level of molecular regulation it operates during the formation of a functionally active sMMO enzyme.

Alternatively, the new isolates may be much more efficient at scavenging the traces of Cu than *M. trichosporium* OB3b. In this case, alternative mechanisms in some methanotrophs for protection of sMMO to Cu inhibition can exist. Further studies of these strains are expected to reveal the fate and role(s) of copper in these complex processes.

(iii) All new methanotrophic isolates exhibited growth in 0.1% MeOH-containing liquid media at 45°C. The BL13-type isolates as well as *M. capsulatus* Bath did grow on 0.02% MeOH-NMS plates at 37°C, but replating on MeOH-NMS plates failed to yield any colonies. Growth of colonies from MeOH-NMS plates was retained only after plating BL13 and *M. capsulatus* Bath onto NMS plates and incubation under methane. The BL4-type isolates differed from the other ones and from *M. capsulatus* Bath by being extremely sensitive to MeOH on plates, although they grew in liquid MeOH-NMS cultures.

Biotechnological implications. The results of this study hold some significance for future biotechnological applications of methanotrophs. The methanotrophs, isolated with high certitude by using an improved colony screening test, apparently possess several properties that make them advantageous candidates for biotechnological use. Although the isolates grow at temperatures that are comparable to those of the well-characterized strain *M. capsulatus* Bath, their sMMO activity is heat stable in vivo, and thus a biomass cultivated, e.g., at 45°C can be utilized for performing bioconversion tasks at higher temperatures. Perhaps more important is the observed copper-resistant sMMO activity in the new isolates. This phenomenon is not only an exciting scientific curiosity but a pronounced advantage for practical use as well, since the milieu where the sMMO in these microorganisms has to perform biotechnological decontamination assignments (groundwater, aquifer, wastewater, etc.) is usually not copper free.

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