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Original Research

Identification of Methanotrophic Bacteria Community in the Jastrzebie-Moszczenica Coal Mine by Fluorescence *in situ* Hybridization and PCR Techniques

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Abstract

The aim of our study was to identify the methanotrophic group of bacteria inhabiting coal mine rocks at the Jastrzębie-Moszczenica (Jas-Mos) coal mine, by application of molecular techniques: polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH). In the first stage of the experiment, methanotrophic activity (AM) of the rock material at different temperatures (20 and 30ºC) and methane (CH_4) concentrations (1, 5, 10, 20%) was determined. AM noted both in 20 and 30ºC reached a similar level equaling c.a. 0.2 μM CH_4 $g^{-1} \cdot day^{-1}$. The PCR reactions were performed using primers specific for *pmoA* gene (encoding a key subunit of particulate methane monooxygenase) – A189f and mb661, whereas FISH was realized with Mg705, Mg84 (Type I of methanotrophs), and Ma450 (Type II of methanotrophs) probes. Sequence analysis of *pmoA* gene demonstrated that microorganisms, being present in the investigated rocks material were similar with 95-100% identity to the following methanotrophs genera: *Methylosinus*, *Methylobacter*, *Methylocystis*. Concentrations of CH_4 between 1-10% did not affect on bacterial variability, but in combination with 20% of CH_4, significant dominance of methanotrophic bacteria Type I was stated.

Keywords: methanotrophic bacteria, FISH technique, coal mine rocks

Introduction

Methane oxidation can occur in both aerobic and anaerobic environments; however, there are completely different processes involving different groups of prokaryotes [1]. The presented study focuses on the aerobic oxidation of methane and the aerobic community of methanotrophs, which belong to a unique group of gram-negative methylotrophic bacteria, utilizing methane as a sole carbon and energy source [1-3]. Due to this fact, methanotrophs play an important role in the oxidation of methane in the natural environment, oxidizing biologically produced methane in anaerobic environments and thereby reducing the amount of methane released to the atmosphere [1, 4].

The methanotrophic group of bacteria appears to be present in many environments and has been found in mud, swamps, rivers, rice paddies, streams, oceans, ponds, meadow soils, sediments, deciduous woods, and sewage sludge

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[4, 5]. Aerobic methanotrophs are found within 14 recognized genera, comprising two major phylogenetic groups: Type I methanotrophs belong to the *Gammaproteobacteria*, while Type II belongs to the *Alphaproteobacteria* [2, 5]. It is known that the Type I group of methanotrophs includes the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylosarcina*, *Methylohalobius*, *Methylosoma*, and *Methylococcus*, which belong to the gamma subdivision of the *Proteobacteria*, whereas the Type II group of methanotrophs is represented by *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa* genera, belonging to the alpha subdivision of the *Proteobacteria* [3].

The oxidation of methane to CO_2 is started from its conversion to methanol by the methane monooxygenase enzyme, existing in two forms: a particulate membrane-bound type (pMMO) and a soluble cytoplasmic form (sMMO). The pMMO has been determined in all methanotrophs except for the genus *Methylocella* [6], whereas the sMMO subunit is present only in certain methanotroph strains [7].

Nowadays, the most popular molecular tools used to detect methanotrophs in environmental samples include the retrieval of 16S rRNA gene and functional genes, encoding key enzymes of methane oxidation, such as *pmoA* and *mmoX*, being the active site subunits of particulate (pMMO), and soluble methane monooxygenases (sMMO), respectively [1, 4, 8]. Fluorescence *in situ* hybridization with oligonucleotide probes targeted at 16S rRNA also is among the most powerful tools developed in modern microbial ecology for direct specific recognition of target populations in their natural environment [9, 10]. In FISH, fluorescently labelled oligonucleotide probes target and bind to rRNA, providing an effective means of identification and qualitative and/or quantitative microbial population analysis in natural and engineered environments [11, 12].

Methanotrophs can be isolated from a wide variety of environments, but coal-bed rock is a new and still poorly recognized habitat of methanotrophs, which so far has not been considered as taking part in methane transformation in coal deposits. To our knowledge the amount of studies regarding the methanotrophic group of bacteria is still limited. Thus, the current study concentrated on application of the latest molecular techniques for identification of methanotrophic bacteria inhabiting coal-bed rocks.

Experimental Procedures

Description of Rock Materials

The coal-bed rocks used in the current study originate from Jastrzębie-Moszczenica coal mine, which belongs to the largest coal basin in Europe – the Upper Silesian coal Basin (USCB) in southern Poland and the Ostrava-Karvina region in the Czech Republic.

Rock material signed as JM-4 was collected from the surroundings of seam 510/2 from a depth of c.a. 475 m below the surface. The relevant characteristics of the rock material are reported in Table 1.

Table 1. The selected properties of the investigated rock material (JM-4).

Parameter	Value
Humidity [%]	0.674±0.147
Density ($g \cdot cm^{-3}$)	1.374±0.0177
pH [H_2O]	7.575±0.003
Eh [mV]	37.100±0.100
N-NO_3 [$mg \cdot kg^{-1}$]	0.647±0.010
N-NO_2 [$mg\ kg^{-1}$]	0.226±0.005
N-NH_4 [$mg\ kg^{-1}$]	18.432±0.097
P-PO_4 [$mg\ kg^{-1}$]	1.211±0.065
IC [%]	<0.01±0.000
TC [%]	7.110±0.001
TOC [%]	7.110

Methanotrophic Activity Determination

Incubations aimed at determining the MA of the investigated rocks commenced within 5 days from collecting the samples. Prior to incubations, rocks were crushed into small pieces and ground in a mill (Testchem, Poland) to a 2 mm maximum grain diameter.

The MA of bacteria inhabiting coal bed rocks was determined under aerobic conditions at two temperatures: 20 and 30ºC (Heraeus Instrument). Triplicate samples of the material (15 g) were placed in dark bottles (60 ml), filled with deionized water to obtain sample moisture adequate to 100% of total water capacity, then closed with rubber septa, capped with an aluminum cap, and sealed with paraffin. An appropriate concentration of CH_4 (1-20% v/v CH_4) in experimental treatments was obtained by replacing an appropriate volume of air with high purity (99.99%) methane (Praxair, Poland), using a gastight syringe (5 ml, SGE, Australia).

The headspace concentrations of gases were determined using a gas chromatograph (3800 GC Varian, USA), equipped with flame ionization (FID) and thermal conductivity (TCD) detectors. Gases were separated on Molecular Sieve 5A, 0.53 mm ID, 30 m length and Poraplot Q, 0.53 mm ID, 25 m length columns (Varian, USA), using helium as the carrier gas. The analyses were carried out under the following conditions: injector temperature 120ºC, oven temperature 40ºC, temperature of detectors: 120ºC and 200ºC for TCD and FID, respectively. Value of MA was expressed as $\mu M\ CH_4\ g^{-1} \cdot day^{-1}$.

Methanotrophic Bacteria Cultivation

Methanotrophically active rock material was used for preparing bacterial cultivation on liquid medium NMS (10 ml), and incubated for 7 days at 20 and 30ºC, with contin-

Table 2. Composition of NMS media (pH 6.8) used in the current experiment.

Components	Content ($mg \cdot l^{-1}$)
KNO_3	988.04
$MgSO_4 \cdot 6H_2O$	988.04
$CaCl_2$ (anhydrous)	197.61
Fe EDTA	3.37
$Na_2MoO_4 \cdot 2H_2O$	0.26
$CuSo_4 \cdot 5H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.49
$ZnSO_4 \cdot 7H_2O$	0.39
H_3BO_3	$11.66 \cdot 10^{-3}$
$CoCl_2 \cdot 6H_2O$	$49.40 \cdot 10^{-3}$
EDTA	0.25
$MnCl_2 \cdot 4H_2O$	$19.76 \cdot 10^{-3}$
$NiCl_2 \cdot 6H2O$	$9.88 \cdot 10^{-3}$
KH_2PO_4	256.89
$Na_2HPO_4 \cdot 12H_2O$	707.44

uous agitation of 175 rpm (Incubator Shaker Series, Innova 42), with the addition of various methane mixing ratios (1-20% v/v). Detailed composition of NMS medium, prepared according to Whittenbury et al. protocol [13] is shown in Table 2.

Fluorescence *in situ* Hybridization (FISH)

FISH analyses were done according to Eller and coworkers [22] protocol, with minor changes. The following probes were used in this study: Ma450 (5'ATCCAGGTACCGTCATTATC3'), Mg84 (5'CCACTCGTCAGCGCCCGA3'), and Mg705 (5CTGGTGTTCCTTCAGATC3'). The probes were synthesized and labeled with fluorochromes (CY5, CY3) by MWG Biotech (Ebersberg, Germany).

Cells (harvested by centrifuging at 13,000 × g for 5 min) were resuspended in 100 µl phosphate-buffered saline (PBS, pH 7.0). The fixation stage was performed at room temperature for 3 h after the addition of 300 µl 4% paraformaldehyde (in PBS). Until the hybridization rocks and cells were stored at -20ºC in 50% ethanol in PBS.

The hybridization was carried out on 10 well-coated slides where 1-2 µl of fixed rocks or cell suspensions were transferred and left to dry at room temperature for 2 h. Dehydration was performed by washing slides in 50, 80, and 96% ethanol for 3 min each. Then, wells were covered with 10 µl of hybridization buffer (Tris 2.4 $g \cdot l^{-1}$, SDS 2.0 $g \cdot l^{-1}$, EDTA 2.0 $g \cdot l^{-1}$, NaCl 0.9 M, pH 7.4, 20% formamide) and 1 µl of the probe solution (50 $ng \cdot \mu l^{-1}$) was added. Hybridization was carried out for 3 h at 46ºC in a water-saturated atmosphere chamber in a water bath (Memmert, Germany). Unbound oligonucleotides were removed by rinsing the slides with 20 ml washing buffer (Tris 2.4 $g \cdot l^{-1}$, SDS 2.0 $g \cdot l^{-1}$, EDTA 2.0 $g \cdot l^{-1}$, NaCl 26.3 $g \cdot l^{-1}$, pH 7.4) prewarmed to 48ºC. Subsequently, the slides were incubated in the remaining washing buffer for another 20 min at 48ºC, air-dried, and mounted with Vectashield Mounting medium containing DNA-staining DAPI (4'6-diamidino-2-phenylindole) (Vector Laboratories, USA).

The slides were analyzed using fluorescence with a Nikon Eclipse 80i research microscope. The pictures were taken with a Digital Sight camera (Nikon) and processed using the software provided by the manufacturer. The images were captured in three channels (blue-nuclear counterstain, bright red (shown as green) – Cy3 and red – Cy5) and superimposed using the camera software.

DNA Isolation and PCR

For molecular probing, total cellular DNA was extracted from cultured bacteria as described by Sambrook and Russel [14]. The PCR reactions were performed in 25 µl (total volume) using PCR master Mix (Fermentas), with 2.5 µl of template DNA, and the following starters with high specificity for methanotrophic gene [1]: A189f (5'GGNGACTGGGACTTCTGG3') and mb661r (5'CCGGMGCAACGTCYTTACC3'). Amplification was performed in a Professional Basic thermal cycler (Biometer) in 30 cycles of 80 s at 94ºC, 60 s at 55ºC, and 80 s at 72ºC. Negative controls were included in all experiments by replacing the DNA template with 2 µl of sterile water. PCR products were analyzed by 1% agarose gel electrophoresis and purified with a QIAquick PCR Purification Kit (Qiagen, USA). PCR products were visualized with ethidium bromide in Rad apparatus (ALPHA INNOTECH).

The sequencing processes were performed on the purified product immediately after the PCR reaction in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics Warsaw, Poland). The obtained sequences were compared to the closest relatives in the NCBI Gen-Bank database using the BLAST program.

Results and Discussion

Methanotrophic Activity in the Rock Material

The value of MA in the investigated rock material (JM-4) was expressed as µM of oxygenated CH_4 per one gram of rock material per day. CH_4 oxidation was studied at two combinations of temperatures (20 and 30ºC), and at different CH_4 concentrations: 1, 5, 10, and 20%. The received results are presented in Fig. 1.

At 20ºC an increasing linear trend for MA in the range of 1-10% CH_4 addition was noted, with its maximum equaling 0.309 (±0.179) µM CH_4 $g^{-1} \cdot day^{-1}$. The increase of substrate concentration to 20% CH_4 resulted in a 19.74%

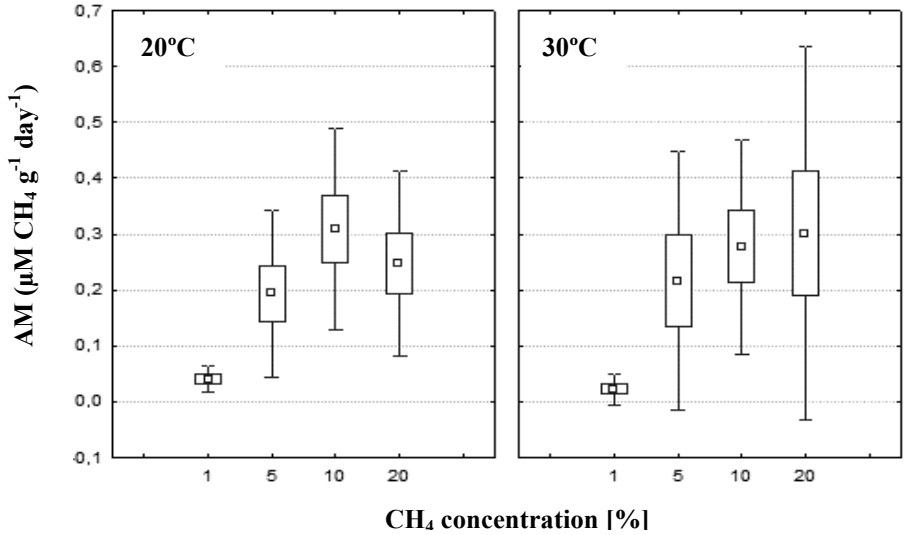

Fig. 1. Methanotrophic activity in JM-4 rock estimated at two temperatures (20 and 30ºC) and different CH_4 concentrations (1, 5, 10, 20%).

reduction of MA, in relation to activity noted at 10% CH_4 supplementation. Estimated MA at 20ºC reached the following values: 0.04 (±0.02), 0.194 (±0.15), 0.309 (±0.18) and 0.248 (±0.166) µM CH_4 g^{-1}·day^{-1}, for: 1, 5, 10 and 20% CH_4, respectively.

At 30ºC the rate of CH_4 oxidation increased with the increase of substrate content at the whole range applied (1-20% CH_4), reaching the highest MA on the level of 0.302 (±0.333) µM CH_4 g^{-1}·day^{-1} at 20% CH_4 addition. Noted MA at 30ºC with 1, 5, and 10% CH_4 supplementation equaled values as follows: 0.022 (±0.027), 0.216 (±0.23), and 0.277 (±0.192) µM CH_4 g^{-1}·day^{-1}.

Comparison of determined MA levels in JM-4 rocks at 20 and 30ºC are shown in Fig. 2. We found that at 20ºC the average value of MA equaled 0.198 (±0.17) µM CH_4 g^{-1}·day^{-1}, whereas at 30ºC it was 2.94% higher and amounted to 0.204 (±0.242) µM CH_4 g^{-1}·day^{-1}; however mentioned values did not significantly differ from each other (p=0.351).

It is often assumed that methanotrophic bacteria are present wherever stable methane emission takes place. Several studies have confirmed the presence of methane utilizing microbial consortia both in the surface [3] and subsurface [15] environments; therefore, the occurrence of methane-oxidizing bacteria accompanying coal seams should not come as a surprise. A comparison of our data with earlier studies by Stępniewska et al. [16] and Stępniewska and Pytlak [17, 18] demonstrates little lower level of MA in coalbed rock from Jas-Mos in relation to the Bogdanka coal mine (southeastern Poland). The mentioned authors reported that the average value of methane oxidation rates stated at 30ºC equaled 0.231 µM CH_4 g^{-1}·day^{-1} in the rock taken from the middle of the seam, and this value was 11.68% higher from MA noted in the Jas-Mos coal mine. However, a study of Beckmann et al. [19] performed on the German coal mine rocks indicated the highest CH_4 formation rates (almost 0.13 µM CH_4 g^{-1}·day^{-1}), which was 36.27% lower in comparison to MA received in the frame of the current work. It is worth noting that the effectiveness of coal dump rock in oxidizing CH_4 creates new possibilities of their disposal, which could help to reduce the amounts of the rock material collected in the dumping areas, which has disturbed the countryside of many industrial areas [18].

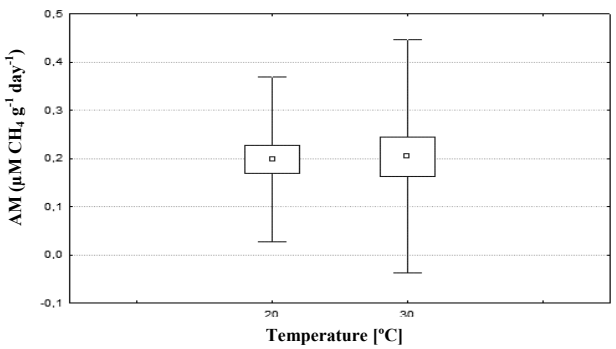

Fig. 2. Comparison of methanotrophic activity in JM-4 rock at 20 and 30ºC.

Identification of Methanotrophs by Fluorescence *in situ* Hybridization

The results of FISH analyses on JM-4 rock material are demonstrated in Fig. 3. FISH was realized with the following oligonucleotide probes: Mg84 and Mg705 specified to Type I methanotrophs (marked in green color) and Ma450 specified to Type II methanotrophs (marked in red color). The blue signed microbial cells (signed with DAPI) accompanied the bacteria community in 0.6-0.8 µm length. Type I and II of methanotrophic bacteria were represented by coccus- or coccobacillus-shaped microorganisms in c.a. 1 µm length.

We found that an increase in CH_4 concentrations from 1 to 10% did not influence significantly bacterial variability (Fig. 3 A-C). However, a significant effect of substrate con-

centration on microbial content was noted only in combination with 20% CH_4 and resulted in significant domination of Type I methanotrophic bacteria (Fig. 3D). Also, at increasing concentrations of CH_4 a systematical reduction of accompanying bacteria abundance in rock material was observed.

The available literature shows that FISH is potentially very useful for studies of methanotroph ecology [9, 11], and a number of 16S rRNA-targeted oligonucleotide probes have been developed for specific detection of Type I and Type II methanotrophs [9, 20]. McDonald et al. [1] and Dedysh et al. [9] demonstrated that the Ma450 probe possesses a wide scope and perfectly matches almost all 16SrRNA sequences of *Methylosinus* and *Methylocystis* strains deposited in public databases, which also was confirmed in our study. In contrast, probes Mg84 and Mg705, which target most known genera of Type I methanotrophs (as suggested by Dedysh et al. [9]) failed to detect any numerically significant population of these organisms in acidic peat. But our findings indicated that the same probes used for rock samples seemed to be useful tools in Type I methanotrophs detection.

Identification of Methanotrophs by PCR (*pmoA* Gene)

DNA isolation was realized both from rock samples incubated at 20 and 30ºC. PCR and sequencing of amplified products were performed with primers A189f and mb661, specifically for *pmoA* gene, encoding a key subunit of particulate methane monooxygenase (*pmoA*). The positive result of PCR was achieved in each JM-4 sample incubated in the range of 1-20% CH_4 concentrations, which is presented on an electrophoregram (Fig. 4). Comparative analyses of the *pmoA* gene sequences revealed the phylogenetic types that are constituted by the methanotrophic bacteria community. Similarity of percentage based on the *pmoA* gene sequences to the closest relatives in the NCBI nucleotide sequences database are summarized in Table 3.

Generally, the received sequences of *pmoA* gene with identity of 95-100%, revealing that the genera of *Methylosinus*, *Methylobacter*, and *Methylocystis* are common methanotrophic groups inhabiting JM-4 rock. 100% sequence identity was achieved for rock samples incubated at 20ºC with supplements of 5, 10, and 20% CH_4, where

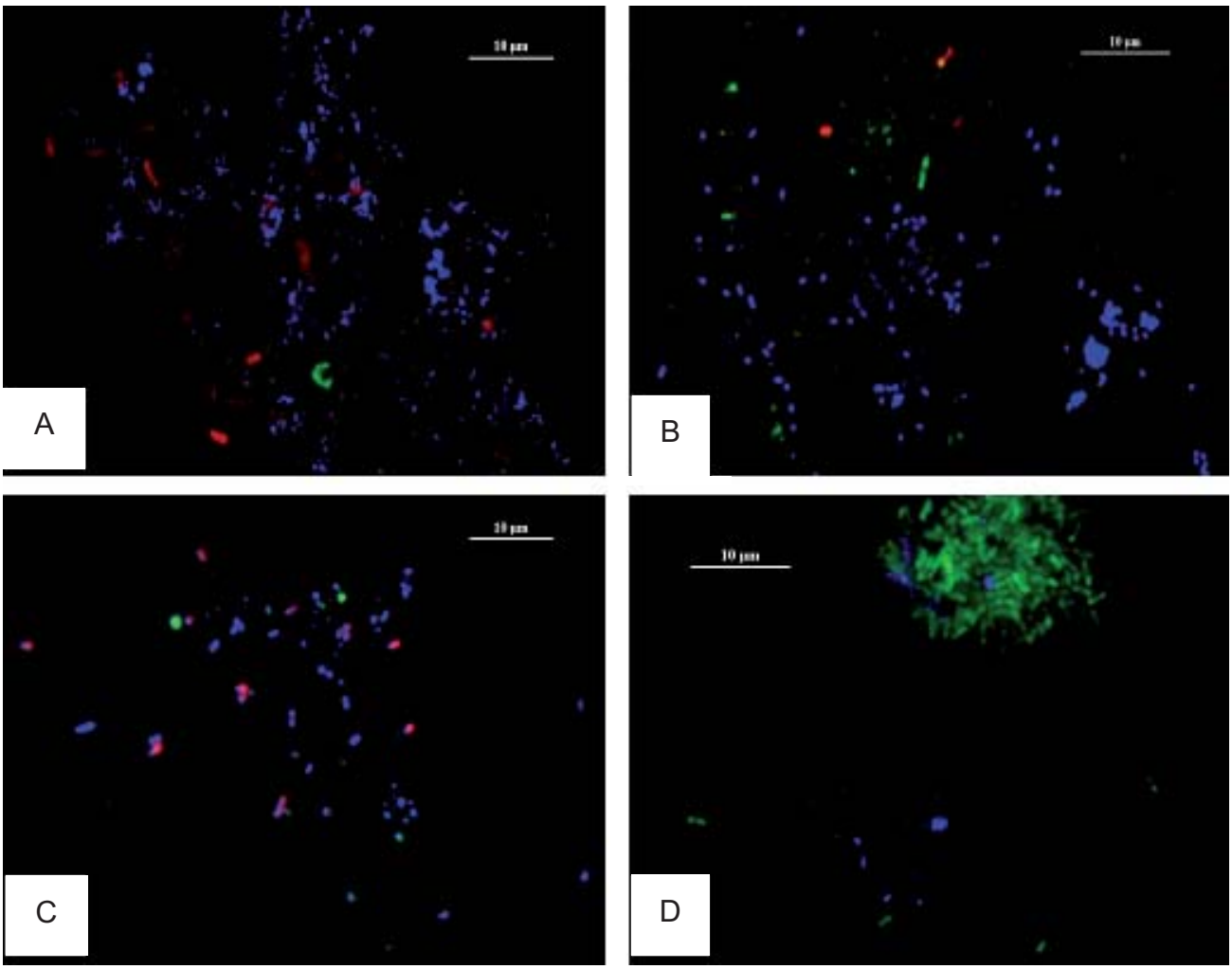

Fig. 3. Methanotrophic bacteria inhabiting JM-4 coal rock. FISH with Mg705 and Mg84 probes (Type I) – green colored, and Ma450 probe (Type II) – red colored. Pictures arranged according to growing gradient of methane: A – 1% CH_4, B – 5% CH_4, C – 10% CH_4, and D – 20% CH_4.

Table 3. Percentage similarity based on the pmoA gene sequences to the closest relatives in the NCBI nucleotide sequence database.

Sample	Concentration of CH_4 (%)	Closest relative in NCBI database	Accession number	Identity (%)
JM-4 20ºC	1	Methanotrophic isolate 2	AF182482.1	99
		Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	95
		Methylobacter sp. BB5.1	AF016982.1	95
	5	Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	100
		Methylobacter sp. BB5.1	AF016982.1	99
		Methylobacter sp. clone C11-7	GQ857558.1	99
		Methanotrophic isolate 5hm22	AY236085.1	99
	10	Bacterial clone ZW141	HQ909064.1	100
		Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	100
		Methylobacter sp. BB5.1	AF016982.1	100
		Methanotrophic isolate 5hm22	AY236085.1	99
		Methylobacter sp. clone C11-7	GQ857558.1	99
	20	Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	100
		DGGE isolate – PMOB-C	EU292152.1	99
		Methylobacter sp. BB5.1	AF016982.1	99
JM-4 30ºC	1	DGGE isolate – PMOB-B	EU292166.1	99
		Eubacteria pAMC524	AF150769.1	99
		Methanotroph isolate 2	AF182482.1	99
		Methanotroph isolate 1	AF182481.1	99
		Methanotrophic isolate 5hm8	AY236084.1	99
	5	*Methylocaldum* sp. 5FB	AJ868403.2	97
		DGGE isolate – E56A-B	AJ579665.1	97
		Methylocaldum sp.	AY195657.1	97
		Methylocaldum tepidum	U89304.1	97
		Clone HH-pmoA-MNP-89	FR799416.1	97
		Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	100
		Bacterial clone ZW141	HQ909064.1	99
		Methylobacter sp. BB5.1	AF016982.1	99
		Methylobacter sp. clone C11-7	GQ857558.1	99
		Methylosinus trichosporium	AJ459037.1	99
		Methanotroph C9	EU359001.1	97
		Methanotroph type 7	AF182489.1	95
	10	*Methylocystis* sp. SC2	AJ584611.1	99
		Methylocystis sp. B2/7	AJ459025.1	99
		Methylocystis sp.	AJ431386.1	99
		Bacterial clone WP1AN23	FN395338.1	99
	20	*Methylosinus trichosporium*	AJ459037.1	99
		Methanotroph C9	EU359001.1	99
		Methanotroph type 7	AF182489.1	95
		DGGE isolate – PMOB-A	EU292151.1	99
		Type I of methanotrophic bacteria – clone 05A-M40-85L	EU275111.1	100
		Methylobacter sp. clone C11-7	GQ857558.1	99
		Methylobacter sp. BB5.1	AF016982.1	100

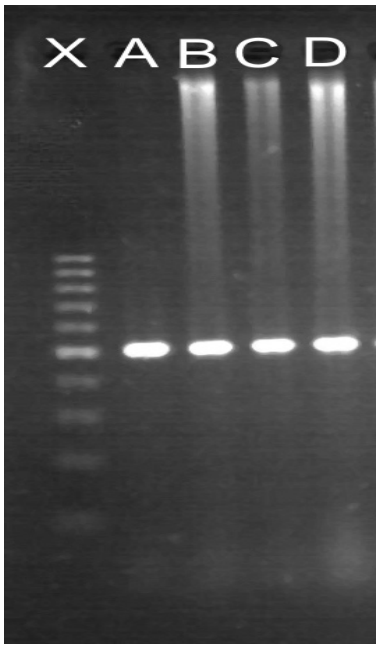

Fig. 4. Electrophoregram of PCR products, X: MassRuler™ DNA Ladder, A: DNA fragment isolated from JM-4 with 1% CH_4, B: DNA from JM-4 with 5% CH_4, C: DNA from JM-4 with 10% CH_4, D: DNA from JM-4 with 20% CH_4.

Type I of methanotrophs, *Methylobacter* sp. BB5.1, and bacterial clone ZW141 were identified. In the case of rock materials incubated at 30ºC, methanotrophs of Type I were found with 100% sequence identity only in the case of 5 and 20% CH_4 concentrations. The bacterial genera *Methylocaldum* sp. 5FB, *Methylocaldum tepidum,* and *Methylocaldum* sp. with 97% identity were noted in samples incubated at 30ºC with 5% CH_4. Genera like *Methylocystis* sp. SC2, *Methylocystis* sp. B2/7, and *Methylocystis* sp. with 99% identity have been present in rock samples incubated at 30ºC with 10% CH_4. At the same temperature, *Methylocystis trichosporium* was found with 99% identity in the rock material incubated at 5 and 20% of CH_4 concentrations. Environments from which the mentioned methanotrophs were earlier isolated are mostly represented by water basins and dump layers.

The majority of known aerobic methanotrophic bacteria are restricted to two phylogenetic groups, the *Methylococcaceae* (Type I methanotrophs), expressing the ribulose monophosphate pathway for the fixation of C1-compounds, and the *Methylosinus-Methylocystis* group (Type II methanotrophs), which use the serine pathway [21]. Therefore, 16S rRNA gene probes and primers are now available, which covers the majority of known methanotroph diversity [1]. Similar to our findings, genera of methanotrophic bacteria were noted by Han et al. [4] in an alkaline soil environment, where a diverse range of bacteria capable of utilizing methane, including the Type II methanotrophs (*Methylosinus/Methylocystis*), the Type I methanotrophs (*Methylobacter/Methylosomas*, and *Methylococcus*) were described, and by Eller and Frenzel [22] in an Italian rice field.

Methanotrophs have been recognized as a group of microorganisms that occupy a specific niche in ecosystems. Due to their unique ability to metabolize methane aerobically, methanotrophs play an important role in the regulation of global methane emissions and in maintaining the ecological balance [23]. The presence of methanotrophic bacteria in coal mine rocks can reduce methane levels in mines, organic waste deposits, and other locations from which methane is volatized to the atmosphere [24]. Thus, studies concentrating on coalbed rocks as a new, still poorly recognized habitat of a fascinating group of methanotrophic bacteria are highly postulated.

Conclusions

Our laboratory study revealed that AM in investigated rock materials (JM-4) did not depend significantly on temperature in the range between 20-30ºC, as in 30ºC AM reached the level of c.a. 0.204 μM CH_4 $g^{-1}\cdot day^{-1}$, and this value was only about 3% higher in comparison to AM stated at 20ºC (c.a. 0.198 μM CH_4 $g^{-1}\cdot day^{-1}$). The demonstrated AM levels in JM-4 rock material are considered to have the potential for playing the role as an effective methane biofilter.

The growth of bacterial culture on NMS medium was observed at each combination of CH_4 concentrations (1, 5, 10, and 20%), and confirmed the high range of bacterial affinity to the carbon substrate.

The significant impact of CH_4 concentration on bacterial flora variability was reported at 20% CH_4, where total domination of Type I methanotrophic bacteria was found.

Finally, we stated that bacteria inhabiting the investigated coal mine rocks, with 95-100% identity for *pmoA* gene, are similar to the following genera: *Methylosinus*, *Methylobacter*, and *Methylocystis*.

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