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Full Length Research Paper

Detection of methanotrophic endosymbionts in *Sphagnum* sp. originating from Moszne peat bog (East Poland)

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Wetlands are one of the major sources of atmospheric methane. However, not all methane produced by methanogens reaches the atmosphere as a substantial amount of CH_4 (1 to 90%) is recycled in soil aerobic zone by methanotrophic bacteria. It was found that methane oxidation is also conducted by methanotrophic bacteria endosymbionts of *Sphagnum* mosses. The aims of the study were to examine *Sphagnum*-involved CH_4 oxidation in transition moor in Eastern Poland and the molecular studies of methanotrophic communities inhabiting *Sphagnum recurvum*. The identification of endosymbiotic methanotrophs was performed by polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) techniques. The methanotrophic activity of submerged and non-submerged *Sphagnum* mosses was determined at the level of 7.6±0.1 and 2.5±0.1 $\mu M\ CH_4 g^{-1} DW day^{-1}$ for complete plants from pool and lawn mosses, respectively. The highest activity was found in the top plants parts. On the basis of genomic analyses, it was found that endosymbionts belonged to methanotrophs of type I and II. The spread of *Sphagnum* species in peat bogs and the contribution of methanotrophic endosymbionts to methane recycling, (very important in relation to expected climate warming), could be greatly important in predicting the methane fluxes from wetlands.

Key words: Methane oxidation, methanotrophs, *Sphagnum* moss, symbiosis.

INTRODUCTION

Methane is one of the most important greenhouse gases. Because of its relatively low mixing ratio (c.a. 1774 ppb) (IPCC, 2001), CH_4 is considered to contribute to about 20% of global warming effect (EPA, 2006). Although, it was confirmed that global methane budget is strongly influenced by anthropogenic emissions (IPCC, 2001, 2007; Wuebbles, 2002), still the largest methane part (c.a. 37 %) entering the atmosphere annually originates from wetlands (IPCC 2001, 2007). However, another way of the wetlands impact on the composition of the atmosphere and, in particular on methane mixing ratio is their ability to act as an active CH_4 sink. It was confirmed, that substantial amount of CH_4 (1 to 90%) is recycled in aerobic zone by methanotrophic bacteria and does not reach the atmosphere (Segers, 1998; Marani and Alvalá, 2006; Freeman et al., 2002; Dinsmore et al., 2009). It is hard to estimate the overall pool of methane formed in anaerobic zones of wetland ecosystems. The participation of wetland CH_4 in global methane emissions makes understanding and assessment of CH_4 sink-source relationships in peatlands crucial for global warming processes (Freeman et al., 2002).

The flux of CH_4 from wetlands is governed by the balance between rates of CH_4 production (methanogenesis) and oxidation (methanotrophy). Various physico-chemical factors such as temperature, pH and levels of

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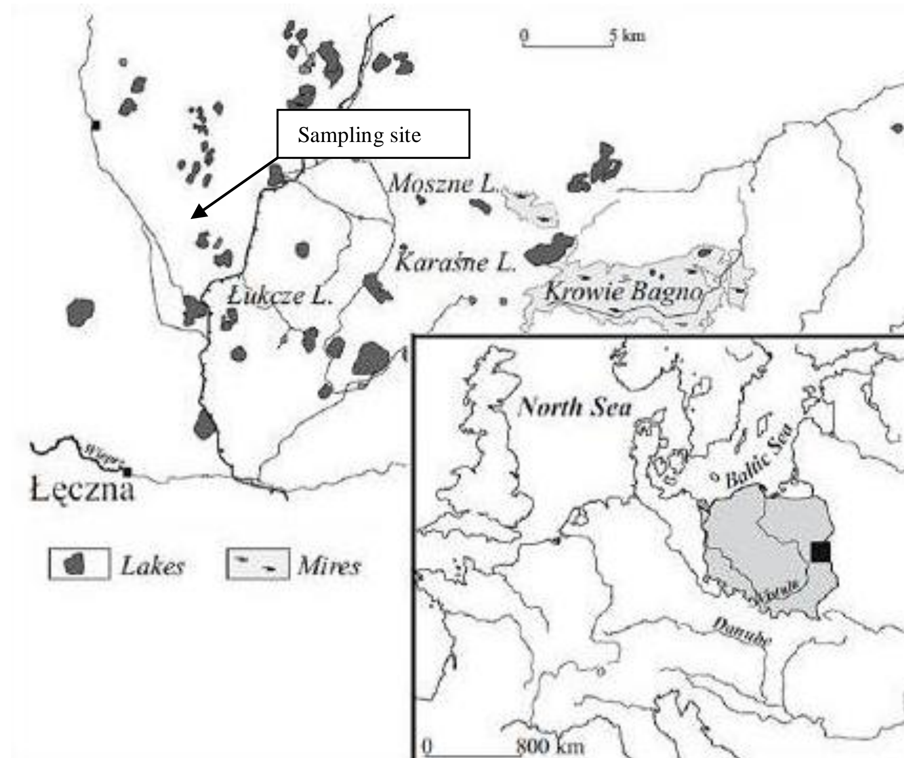

Figure 1. Localization of the Łęczyńsko-Włodawski lake District and the sampling site (from Bałaga, 2007; modified).

nutrients have been described as the main controlling factors of CH_4 emission from wetlands. Much attention has been paid to the effects of fluctuating water tables. However, recently it was found that methane oxidation is conducted by methanotrophic endosymbiotic bacteria of *Sphagnum sp.* which were found in the hyaline *Sphagnum sp.* cells (Raghoebarsing et al., 2005). This means that more knowledge on methane cycling and methanotrophs present in bogs is urgently required for the understanding and predicting effects of anthropogenic changes in wetland ecosystems and to prevent from enhanced methane emissions.

The primary aim of this study was to examine *Sphagnum*-dependant CH_4 oxidation in transition moor of Eastern Poland to explain the role of *Sphagnum*-methanotrophs symbiosis in diminuation of methane emission from peat soils. The second goal was the recognition of methanotrophic endosymbionts of the moss.

MATERIALS AND METHODS

Site description

The investigated *S. recurvum* plants were collected from both lawns (non-submerged) and pools (submerged) of an acidic peat bog (pH of 3.2 to 4.1), situated near Moszne lake, (Poleski National Park; 51° 23' N, 23° 63' E). The Moszne lake is a transition moor placed in the western part of the Łęczyńsko-Włodawski lake District, eastern Poland (51.51°N, 23.19°E). Moszne peat bog is a unique territory, being a miniature of tundra at its extremely southwestern European location (Figure 1). The average air temperatures of January and July are -4.1 and 17.9°C, respectively, and the average annual total rainfall is 551 mm (Kaszewski, 2002). The vegetation is dominated by graminoids such as *Eriophorum vaginatum* (L.), *Carex acutiformis*, *Carex gracilis*, *Sphagnum angustifolium*, *S. cuspidatum*, *S. flexuosum*, *S. magellanicum*, and *Polytrichum sp.* (Mieczan, 2009).

Determination of methane oxidation

The original plant samples were transported to the laboratory, carefully washed and immediately incubated under an atmosphere enriched with 1% v/v methane. The methanotrophic activity of bacteria connected with *S. recurvum* plants was determined under oxic conditions at ambient temperature (~20°C) and normal light conditions (with respect to daily changes) for complete plants as well as for their different parts (top, middle and bottom). The experiments were carried out with both submerged and non-submerged *Sphagnum* mosses. The plant samples were placed in glass bottles (120 cm^3), closed with rubber septa, capped with an aluminum cap and sealed with paraffin. Incubations representing each set of experimental conditions were performed in triplicate. The headspace concentrations of gases CH_4, CO_2, O_2 (100µl) during incubation were measured by gas chromatograph (3800 GC Varian, USA) equipped with flame ionization (FID) and thermal conductivity (TCD) detectors. Methanotrophic activity of the plant samples (µM $CH_4^{-1}DWday^{-1}$) was calculated from the slope of the linear regression of CH_4 concentration vs. time ($r^2 \geq 0.95$). Mosses indicating the greatest capacity for methane oxidation (submerged plants) were chosen for molecular investigations.

Table 1. The primers used in the present study.

Primer	Sequence (5'-3')	Target of gene	Reference
typIf	ATGCTTACCACATGCAAGTCGAACG	16S rRNA of type I methanotrophs	Chen et al. (2007)
typIr	CCACTGGTGTTCCTTCMGAT	16S rRNA of type I methanotrophs	Chen et al. (2007)
typIIf	GGGAMGATAATGACGGTACCWGGA	16S rRNA of type II methanotrophs	Chen et al. (2007)
typIIr	GTCAARAGCTGGTAAGGTTC	16S rRNA of type II methanotrophs	Chen et al. (2007)
A189f	GGNGACTGGGACTTCTGG	*pmo*A	Holmes et al. (1995)
mb661	CCGGMGCAACGTCYTTACC	*pmo*A	Bourne et al. (2001)

Methanotrophs cultivation

Methanotrophic endosymbiotic bacteria were enriched in a liquid NMS medium (Whittenbury et al., 1970), containing (in grams per liter of distilled water) 1 g $MgSO_4 \cdot 7H_20$; 0.17 g $Na_2HPO_4 \cdot 12H_2O$; 1 g KNO3; 0.272 g $KH_2PO_4 \cdot 12H_2O$; 2 g $CaCl_2 \cdot 6H_2O$; 0.005 g FeEDTA, with the addition of 0.1% (by volume) of trace elements stock solution containing (in grams per liter) 0.5 g Na_2EDTA; 0.2 g $FeSO_4 \cdot 7H_2O$; 0.03 g H_3BO_4; 0.02 g $CoCl_2 \cdot 6H_2O$; 0.002 g $NiCl_2 \cdot 6H_2O$; 0.03 g $CuSO_4 \cdot 5H_2O$; 0.1 g $ZnSO_4$; 0.003 g $MnCl_2 \cdot 4H_2O$ and 0.003 g $Na_2MoO_4 \cdot 2H_2O$. The inoculation was done using homogenate prepared from plants (250 µl) exhibiting the highest methanotrophic activity. The homogenates were obtained by rinsing the plants with 75% ethanol and sterile water followed by disruption using a mortar and pestle. Incubation was carried out for 5 to 7 days at 30°C under a 10:90 methane-air mixture with continuous agitation at 120 rpm. Control incubations were run parallelly under the same conditions but without methane. All steps in the enrichment procedure and subsequent molecular investigations were performed in a laminar flow chamber to reduce the risk of sample contamination.

DNA extraction

Total DNA was extracted from enrichment cultures where visual turbidity developed, using the method described previously by Sambrook and Russel, (2001) with some modifications. Cells from 10 ml samples of late-exponential cultures were collected by centrifugation. The pellet was suspended in 250 µl of TE buffer containing 50 mM Tris-HCl (pH=8.0), and 50 mM EDTA (pH=8.0). To achieve complete lysis of the cells, 1 ml of GES buffer (pH=8.0), containing 5 M guanidine thiocyanate, 100 mM EDTA, 0.5% sarkosyl was added. The mixture was incubated at room temperature for 10 min, then "crude lysates" were cooled on ice. After addition of 125 µl of ammonium acetate (7.5M), the samples were mixed and further incubated on ice. DNA was purified with 250 µl of chloroform – isoamyl alcohol (24:1) mixture, then precipitated with isopropanol, washed with cold ethanol, and dissolved in 50 µl of sterile distilled water.

PCR amplification

Primers specific for functional genes and 16S rRNA of the methanotrophic bacteria used in the current study are listed in Table 1. All oligonucleotides were synthesized at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw (Poland). Polymerase chain reactions (PCR) were run in a programmable thermal cycler (MJmini, Bio-Rad). The reaction mixture (50 µl) consisted of 1 x PCR MIX: PCR amplification buffer, 0.05 U/µl Taq DNA polymerase, 0.4 mM of each dNTP, 4 mM $MgCl_2$ (FERMENTAS), forward and reverse primers at 0.1 mM and 3.5 µl of template DNA. The reaction conditions were as follows: initial denaturation at 96°C for 4 min 30 cycles of 94°C for 2 min, primer annealing at 56°C for type I, 55°C for type II and 53°C for pmoA each for 1 min, and elongation at 72°C for 1 min. Final elongation were performed at 72°C for 3 min. The amplification products were analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Fluorescent *in situ* hybridization (FISH)

Fixation procedure

Cells growing in the logarithmic phase were harvested by centrifugation and resuspended in 0.5 ml of phosphate-buffered saline (PBS). The suspensions were then mixed with 1 ml of 4% (w/v) freshly prepared paraformaldehyde solution and fixed for 1 h at room temperature. The fixed cells were collected by centrifugation (6000•g for 1 min) and washed twice with PBS to ensure removal of paraformaldehyde. The resulting pellet was resuspended in 0.3 ml of 99.9% (v/v) ethanol and stored at 20°C until use. For use in FISH, the probe MA-221*Cy5 (5'-GGACGCGGGCCGATCTTTCG-3') was labeled with indocarbocyanine dye (Dedysh et al., 2001). Oligonucleotide probes were purchased from Integrated DNA Technologies (Germany).

Hybridization

Hybridization was done on 75% ethanol-rinsed and dried slides with eight wells for independent positioning of the samples. Approximately 4 µl of the fixed cell suspension was spread on each well, air dried, and dehydrated by successive passages through an ethanol series (50, 75, and 99.9% (v/v)) for 3 min each. As a hybridization chamber was used a 50 ml polypropylene Falcon tube containing a slip of filter paper soaked in hybridization buffer, as described by Stahl and Amann (2001). Hybridization buffer (10 µl) containing 1 M Tris-HCl (pH=8), 5 M NaCl, 10% sodium dodecyl sulfate (SDS) and 20% formamide was placed on each spot of the fixed cells and then 1 µl of fluorescent probe solution was added. The chamber was incubated for at 60 min at the hybridization temperature. Then, the slides were washed at the hybridization temperature for 15 min in washing buffer (1 M Tris-HCl, 0.5M EDTA, and 5 M NaCl) and rinsed twice with distilled water. The slides were air dried, stained with 4,6- diamidino-2-phenylindole (DAPI; 2 M) for 10 min in the dark, rinsed again with distilled water, and finally air dried.

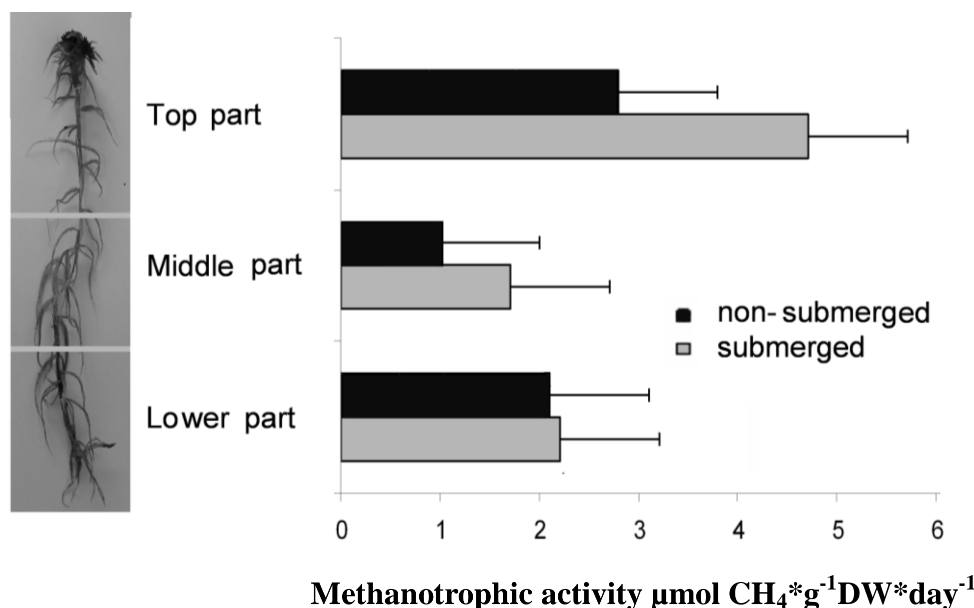

Figure 2. The potential of methane oxidation by different parts of submerged and non-submered *Sphagnum sp.* from Moszne peatbog at ambient temperature (20°C) and 1% methane. Error bars indicate standard deviations of at least three independent experiments.

Microscopy

The slides were analyzed by confocal microscope techniques (LSMS Pascal, Carl Zeiss, Jena, Germany) fitted with a UV laser. AxioVision software was used for image acquisition and processing.

Scanning electron microscopy

Tested plants were fixed in glutaraldehyde phosphate buffer (1%, 0,1M, pH=7.2). After removal of the fixative, samples were dehydrated, dried at critical point and coated with sputtered gold before. Preparations were analyzed using scanning electron microscopy (LEO 1430VP, Carl Zeiss, Germany).

Confocal laser scanning microscopy

The fragments of *Sphagnum* sp. were observed using a LSMS Pascal confocal laser scanning microscopic system (Carl Zeiss, Jena). Confocal stocks were acquired with a Ziess Plan Apochromat Oil DIC and as a fluorescent dyes were used: SYTO®9 (green-fluorescent) and propidium iodide (red-fluorescent) (Live/Dead ® BacLight™ kit, INVITROGEN). These stains differ in their ability to penetrate healthy bacterial cells, in the case of SYTO® 9 both live and dead bacteria were dyed. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes were used. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red.

Statistical analyses

Pairwise comparisons were made for methane oxidation rates of different Sphagnum sp. using ANOVA (n=3, Tukey's test). Statistical analyses of the data were performed in STATISTICA 10.0.

RESULTS

Methane oxidation

The methane oxidation rates in submerged *S. recurvum* plants indicates three times higher activity compared to non-submerged (7.6 ± 0.1 and 2.5 ± 0.1 µmol $CH_4g^{-1}DWday^{-1}$) samples of complete plants (as well for pool as lawn sites). The distribution tendency of methanotrophic activity along a single *S. recurvum* plant was similar in both locations plants (Figure 2). On the base of statistical analysis of methanotrophic activity, the significant differences between submerged and non-submerged *S. recurvum* plants was confirmed (Tukey's test, P = 0.0002). The highest methane oxidation rates were found in the top parts of both submerged and non-submerged mosses whereas the lowest were noted in the middle parts, what was confirmed by statistical analysis between top and middle parts (Tukey's test, P = 0.022), between bottom and middle parts no signification differences were noted (Tukey's test, P = 0.147 and P = 0.342).

Detection and observation of methanotrophic endosymbionts

The cultured endosymbiont cells observed by FISH technique were rod-shaped and about 2 to 3 µm long (Figure 3). Confocal microscopic observations showed very frequently the presence of living cells (green-fluorescent), as well as aggregates of endosymbiont's type II methanotrophic bacteria, oxidizing methane, belonging to type II of methanotrophic bacteria (Figures 3 and 4). Impressions

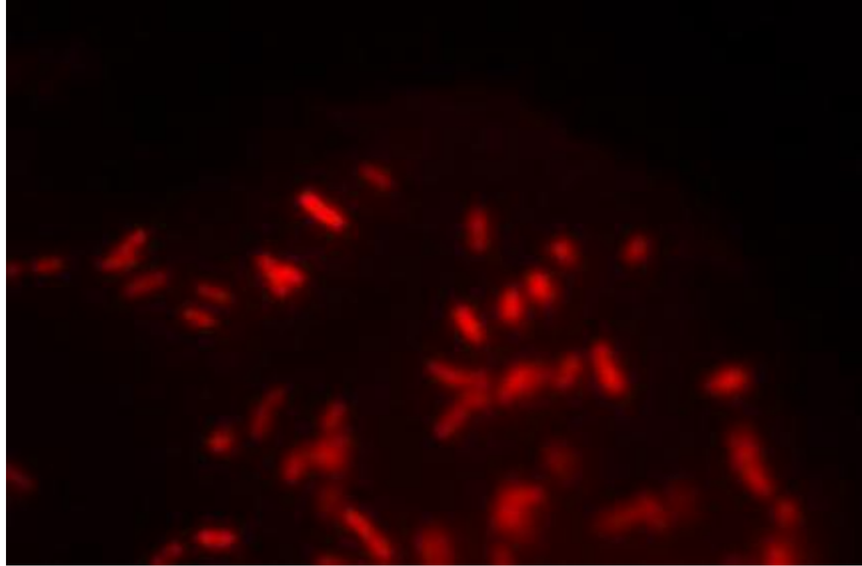

Figure 3. Fluorescent in situ hybrydisation of *Sphagnum sp.* isolates after hybrydization with MA-221*Cy5 probes (UV light)

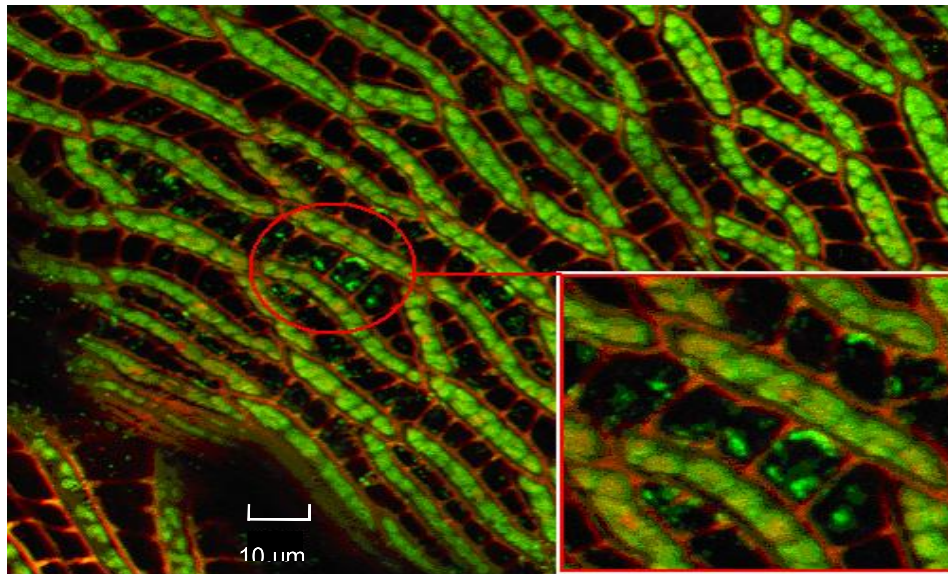

Figure 4. Confocal microscopy of gametophytes *Sphagnum* sp. using the Live/Dead®BacLight™ kit (Invitrogen).

of the specific microenvironment were obtained by analyzing gametophytes by scanning electron microscopy. The existence of chlorocyts and hyaline cells with the large circular pores, of the diameter about 5 µm and with the size the corresponding methanotrophic of 2.5 µm x 2 µm in size bacteria methanotrophic was observed by scanning electron microscopy. Total genomic DNA from cultured methanotrophs originated from submerged *S. recurvum* was isolated and bacterial 16S rRNA (methanotrophs type I and II specific) was amplified. Obtained products by lengths: 673 and 525 pb for type I and type II methanotrophs, respectively were found (lane 2 and 3) (Figure 5). Additionally, *pmo*A gene in *S. recurvum* was successfully amplified and obtained product showed the desired length of 510 bp (lane 4) (Figure 5).

DISCUSSION

Similar investigations on methane oxidation by plants were perform earlier by Dedysh et al. (1998) and Raghoebrasing et al. (2005). The first acidophilic methanotrophs were isolated from *Sphagnum* peat bogs in East

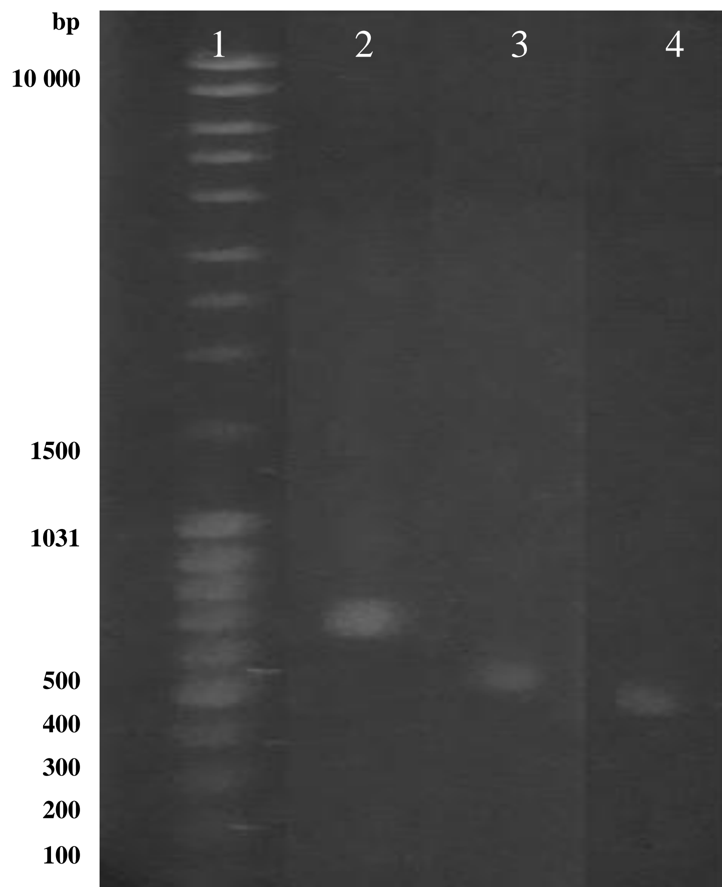

Figure 5. Gel electrophoresis of *Sphagnum* sp. isolates obtained with type II (lane 2), type I (lane 3) and *pmoA* (lane 4) (lane 1- MassRuler™ DNA Ladder Mix 100-10 000 bp, Fermentas).

of Siberia and North Russia boreal forests were qualified as the type of *Methylocella* sp. and *Methylocapsa* sp. (Dedysh et al., 1998 a,b, 2004). One of the first described species of an acidophilic methanotroph, *Methylocapsa acidiphila*, was isolated from peat bog in Siberia (Dedysh et al., 2002). The studies conducted on forest soils in Germany resulted in isolation of the *Methylocella silvestris* (Dunfield et al., 2003) and one year later the strain *Methylocella tundrae*, isolated from acid peat bog tundra in North part of Euro-Asia and North America was described (Dedysh et al., 2004). In 2005, Raghoebrasing and co-workers showed the presence of first methanotrophic bacteria inhabiting the hyaline cells and on the steam leaves of *Sphagnum* sp. originating from Dutch peat bogs. In our study, significant rates of methane oxidation by all samples of *S. recurvum* mosses from Poleski National Park (Poland) were demonstrated. Kip and co-workers (2011) suggested, that all *Sphagnum*-dominated wetlands across the globe showed methanotrophic activity. Potential of methane oxidation depends on water saturation of peats (submerged, non-submerged) and other environmental factors such requirements as temperature, and methane availability. The highest rates of methane oxidation was found in mosses sampled from northern Siberia, where they reached even 80 µmol $CH_4 g^{-1}$DWday^{-1} while for *Sphagnum* moss samples from Finland (Lakkasuo mire), the methane oxidation rates was in the range from 0 to 62 µmol $CH_4 g^{-1}$ DWday^{-1} (Larmola et al., 2010). In our tested plants, methanotrophic activity was noted at a level of 7.6 ±0.1 and 2.5±0.1 µmol $CH_4 g^{-1}$DWday^{-1} for submerged and non-submerged *S. recurvum* The values of methane oxidation by methanotrophs were connected with submerged and non-submerged conditions of *Sphagnum* plants and Raghoebarsing et al. (2005) found methane oxidation rates in the investigations of range 0.5 to 30 µmol $CH_4 g^{-1}$DWday^{-1}. In order to define the localization of methanotrophic bacteria, confocal microscopy and scanning electron microscopy were used. The observation provided with Live/Dead®BacLight™ kit indicated the presence of live bacteria inside cell plants (metabolically active, stained green). The confirmation of the presence of the methanotrophic bacteria was based on a positive result of gene *pmo*A, been the part of monooxygenase structure. The presence of type I and type II

methanotrophic bacteria were recognized, although previously, only bacteria of the type II were described (Raghoebrasing et al., 2005). The observation of gametophytes of *Sphagnum* sp. by scanning electron microscopy made it possible to characterize ecological niche of methanotrophs. Figure 4 shows that inside the leaves from *S. recurvum* tissue, a regular pattern of alternating green – chlorocytes and hyaline cells were formed. Each hyalocyst had an empty lumen, spiral thickenings on the inner wall surface, and one or more pores (Figures 4 and 5) of the size 4 to 20 µm (Lewis, 1988) usually filled with water, and regulating a water level inside the cells (Ligrone and Duckett, 1998). All these features allow the inhabitation of the hyaline cells by acidophilic methanotrophic bacteria (pH=3.4 of peat bog).

The results of the present study confirm the statement of Raghoebarsing et al. (2005) that a greater emphasis should be put on the role of plants (especially mosses) and their environmental requirements for CH_4 oxidation in peat profile. Studies on the isolation and identification of methanotrophic endosymbionts, as well as on the control of this association, could be very useful in the recognization of their new biotechnology perspectives.

ACKNOWLEDGMENTS

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