doi: 10.26114/sir.iung.2015.46.12

STUDIA I RAPORTY IUNG-PIB

ZESZYT 46(20): 217-241 **2015**

Agnieszka Wolińska, Zofia Stępniewska, Anna Pytlak

The John Paul II Catholic University of Lublin, Poland

METHANOTROPHIC BACTERIA FROM COAL MINE ROCK – CHARACTERISTICS, FUNCTIONS AND NUTRIENTS UPTAKE*

Keywords: methanotrophic bacteria, coal mine rocks, trace elements, NMS medium

Introduction

Methane (CH_4) is one of the principal energy sources available for bacteria in underground environments (37). Microbiological research of deep and ultra-deep subsurface oil-field groundwater's, basaltic and granitic aquifers as well as dolomite formations has confirmed the presence of phylogenetically diverse methanotrophic populations (22, 37).

Aerobic methane-oxidizing bacteria could play an important role in counteracting the increase of methane into the atmosphere. The aerobic methanotrophs live in oxic environments, where they use CH_4 as a sole source of carbon and energy. They transform CH_4 to CO_2, with methanol, formaldehyde and formate as intermediates (34). In the field of biotechnology, methanotrophs are a valuable biological resource because they can degrade the greenhouse gas methane (34, 37, 39). Thus, the activity of methanotrophic bacteria (MB) is dependent on the availability of CH_4 substrates and oxygen and on the environmental conditions like: pH, temperature, moisture, grain distribution and presence of inhibitors (39).

In spite of these reports the recognition of methanotrophs associated with coal-bearing environments is very limited. Analogically, little is known about nutrients uptake by MB during its culture under laboratory conditions.

Some of our previous studies (36, 39) demonstrated CH_4 oxidation by coalbed rocks in different parts of the Lublin Coal Basin (LCB), one of the major coal basins in Poland, which is characterized by very low CH_4 content. LCB is in the very early stage of exploration with only one active coal mine – Lubelski Węgiel "Bogdanka" SA (37). The investigated part of the LCB, carboniferous stratum is located at the depth of about

---

[*] Problematyka prezentowana w trakcie XII Sympozjum "*Trace elements in the environment*", zorganizowanego w ramach zadania 1.2 w programie wieloletnim IUNG-PIB.

700 m.b.s (meters below surface) below thick and water-saturated Jurassic Cretaceous sediments (37). Coalbed gas in the area is present in very small amounts and consists mainly of (21): CH_4 (14.4–76.9%), CO_2 (3.9–7.4%) and N_2 (18.7–76.2%).

The general aim of the present study was to gather new information about microbial activity in the LCB Carboniferous stratum through specific research goals which included:

- identification of methane-oxidizing bacteria inhabiting coalbed rocks using molecular biology methods (FISH, PCR),
- estimation of methanotrophic activity (MA) in LCB rock material,
- description of nutrients uptake by MB during standard procedure of growth and under salt stress conditions.

Characteristic of methanotrophic bacteria

Extermophilic microorganisms – like MB, belongs to the *Proteobacteria* (*Alphaproteobacteria*, *Gammaproteobacteria*) and might be seen as an example for understanding the various survival strategies under high or low temperatures, extreme pressure, low moisture and drastic salt concentrations (32). This group of bacteria was firstly identified in 1906 by Söhngen (32). Based on 16S ribosomal DNA analysis MB is subdivided into: Type I - belonging to the *Gamma*-subdivision of the *Proteobacteria* (i.e. *Methylobacter*, *Methylococcus*, *Methylomicrobium*, *Methylomonas*), using the ribulose monophosphate (RuMP) pathway for assimilation of formaldehyde, and Type II - belonging to the *Alpha*-subdivision (i.e. *Methylocystis*, *Methylosinus* strains) using the serine pathway for formaldehyde incorporation (15, 32, 42). Next investigations on MB group resulted in describing 16 new genera, with 12 in the *Gammaproteobacteria* and 4 in the *Alphaproteobacteria*. New genera within the *Gammaproteobacteria* include *Methylocaldum*, *Methylohalobius*, *Methtyhlothermus*, *Methylosarcina*, *Methylosoma* and *Methylosphaera* (29, 32). Then, more “unusual” filamentous methanotrophs have been discovered within the genera *Clonothrix* and *Crenothrix* (44), but these are also considered to be Type I methanotrophs as they are phylogenetic subsets of the *Methylococcaceae* family (32). Additionally, Type I methanotrophs contain subdivision, Type X, which is phylogenetically affiliated to *Gammaproteobacteria* but has some metabolic attributes of Type II (38). The current phylogenetic distribution of known methanotrophic genera based on 16S rRNA gene sequences is shown in fig. 1.

The pathway of methane oxidation is always initiated by the MMO (methane monooxygenase), which exists in two different forms (4):

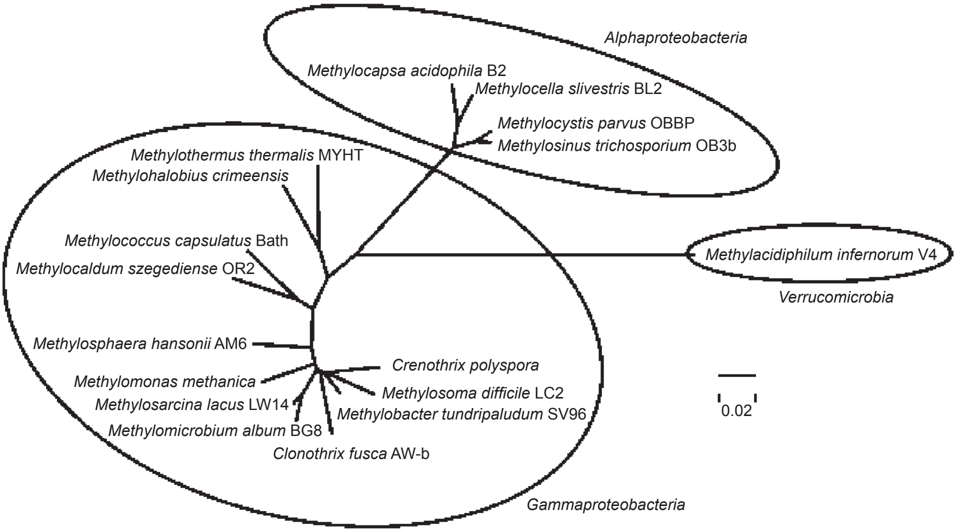

Fig. 1. Phylogenetic tree of methanotrophs based on 16S rRNA sequences

Source: Semrau et al., 2010 (32)

- the membrane-bound enzyme form pMMO (particulate MMO) – present in nearly all MB with the possible exception of the *Methylocella* cluster and is thought to be regulated by the amount of available copper (Cu),
- the soluble form of MMO (sMMO) - occurring in Type II at low concentrations of Cu (42).

Laboratories studying the pMMO agree that the pMMO is a Cu-containing enzyme, composed of three polypeptides with approximate molecular masses of 45 000 Da (6, 32). In the crystal structures from *M. capsulatus* Bath and *M. trichosporium* OB3b, each *Alfa, Beta, Gamma* monomer was modelled to contain either a dinuclear or mononuclear Cu centre as well as Zn site as shown in fig. 2.

Biotechnological potential of methanotrophic bacteria

In the field of biotechnology, MB are a valuable biological resource as they can degrade the greenhouse gas methane, and co-metabolize various organic compounds (32, 34). They are highly diverse and found in wide range of environment, i.e. tundra soils (7) peat bogs (7, 41, 42), landfills (44), rice field soils (16), permafrost soil (47).

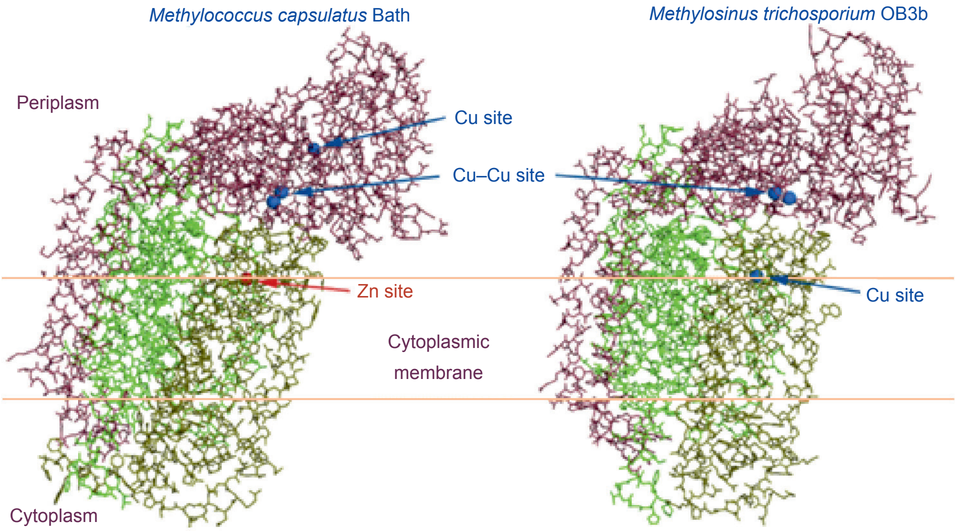

Fig. 2. Crystal structure of pMMO from *M. capsulatus* Bath and *M. trichosporium* OB3b with marked Cu and Zn site

Source: Hakemian et al., 2008 (14) and Semrau et al., 2010 (32)

Some of our previous studies (36, 37) demonstrated that MB also inhabited extremely difficult environment like the sedimentary rocks from the LCB and (UPCB) Upper Silesian Coal Basin (38), characterizing by low humidity, low trace elements content and high level of salt. Mentioned findings are especially important for the mining industry. Since, methane is regarded to be a major source of danger in the coal mine industry and one of the most harmful for the environment side product of coal excavation, in our opinion it was important to gain knowledge about distribution and factors influencing the MB naturally occurring in the Carboniferous sediments (38). We found that, MB inhabiting LCB coalbed rocks might have been supplied with nutrients, water and dissolved oxygen from overlying water-rich Jurassic and Cretaceous sediments enabling them utilization of the methane generated from underlying coal seams (37). Moreover, we demonstrated that gas samples collected in LCB coal mine contained 14.4–76.9% CH_4 with 3.9–7.4% of accompanying CO_2 whereas average CH_4 concentration in formation of gases collected in USCB coal mines was more than 90%, with 1% CO_2 (37). Thus, the presence of methanotrophic microflora in LCB coal-bearing stratum, suggest that MB may be more frequent constituents of the Carboniferous formations than previously thought and that hydro-geological conditions play a crucial role in maintaining their activity (37). Methanotrophic presence should, therefore, also be considered when planning a drilling for coal bed methane recovery.

Nutrients important for methanotrophic bacteria growth

It is known, that nutrition is the single most important factor controlling bacterial population dynamics in any environment. Different organisms need different complements of nutrients, and not all nutrients are required in the same amounts. Some nutrients, called macronutrients, are required in large amounts, while others, called micronutrients, are required in just trace amounts. Nutrient limitation may result in retardation of microbial growth and possible elimination from the environment. Microorganisms are able to adapt to changing nutrient conditions (11). The growth of MB which are gram-negative group of bacteria does not only depend on the availability of carbon but also on different other macro- and micronutrients.

Potassium (K) is required for the activity of several enzymes (i.e. phosphatases, ATP-ase, adenosine), whereas magnesium (Mg) functions is most of all to stabilize ribosomes, membranes, and nucleic acids and then is also required for the activity of many enzymes. Calcium (Ca), despite the fact that is not required by all microbial cells but can play a role in helping to stabilize microbial cell walls, and it plays a key role in the heat stability of endospores. Sodium (Na) is required by some, but not all, microorganisms, and it requirement is typically a reflection of the habitat. Iron (Fe) and manganese (Mn), play a major role in cellular respiration and are also a key component of cytochromes. It has been well-known for over 30 years that Cu plays a key role in the physiology and activity of MB (fig. 2), especially in regards to CH_4 oxidation by the particulate CH_4 monooxygenase (31). Many other metals are required or otherwise metabolized by microorganisms. Like, iron, these micronutrients are called trace elements or trace metals. Micronutrients typically play a role as cofactors for enzymes (11). Table 1 lists the major micronutrients and examples of enzymes in which each plays a role in the cell.

Typical microbial media, however, are designed to optimize growth and often have metal precipitates, poorly understood metal complexation, and ill-defined equilibrium conditions that prevent the assessment of metal bioavailability (25).

Previous studies largely focused on abiotic factors such as oxygen, nutrients, moisture, and temperature, etc. to enhance methanotrophic activity (15, 28, 32, 41). Recent studies are conducted in the direction that methanotrophs interact significantly with other bacteria in different ways (34). Anyhow, until recently remarkable less attention has been paid on nutritional requirements of MB cultured under laboratory conditions. Most popular studies in this subject were concentrated only on Cu (6, 25, 32, 35) and Ni (8, 9, 20). Consequently, the current study was realized in order to present a knowledge about nutrients depletion from the culture media by MB during standard culture growth on NMS medium and under salt stress conditions.

Table 1

Trace elements needed by microorganisms

Trace element	Cellular function
Chromium (Cr)	Possible but not proven component for glucose metabolism
Copper (Cu)	In respiration, cytochrome *c* oxidase; in photosynthesis, plastocyanin, some superoxide dismutases
Iron (Fe)	Cytochromes; catalases; peroxidases; iron–sulfur proteins; oxygenases; all nitrogenises, superoxide dismutase
Manganese (Mn)	Activator of many enzymes; component of certain superoxide dismutases and of the water-splitting enzyme in oxygenic phototrophs (photosystem II)
Nickiel (Ni)	Most hydrogenases; coenzyme F_{430} of methanogens; carbon monoxide dehydrogenase; urease, methyl reductase
Zinc (Zn)	Carbonic anhydrase; alcohol dehydrogenase; RNA and DNA polymerases; and many DNA-binding proteins

Source: Diekert et al., 1981 (8), with own modification

Methanotrophic bacteria culturing

Growth of Methanotrophic Bacteria Consortium (MBC), colonizing LCB rocks under laboratory conditions should be performed on nitrate minimal salt (NMS) medium as recommended by Whittenbury (45). Standard content of NMS is presented in table 2.

Table 2

NMS medium content

Solution	Components	Concentration in the medium ($mg\cdot l^{-1}$)
10 x NMS salts	KNO_3	988.04
	$MgSO_4\cdot 6H_2O$	988.04
	$CaCl_{2\ \text{(anhydrous)}}$	197.61
Ferrous (II) EDTA	Fe EDTA	3.37
Sodium molybdate	$Na_2MoO_4\cdot 2H_2O$	0.26
Trace elements	$CuSo_4\cdot 5H_2O$	0.2
	$FeSO_4\cdot 7H_2O$	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
	$MnCl_2\cdot 4H_2O$	$19.76\cdot 10^{-3}$
	$NiCl_2\cdot 6H_2O$	$9.88\cdot 10^{-3}$
Phosphate buffer	KH_2PO_4	256.89
	$Na_2HPO_4\cdot 12H_2O$	707.44

Source: Whittenbury, 1970 (45)

Described by us culture procedure were performed in the bioreactors BIOSTAT A plus (Sartorius), fig. 3, at following conditions: 10% CH_4, 30°C, 160 rpm by 45 hours.

Fig. 3. Bioreactors used for MBC culture on NMS medium (photo by Wolińska A., 2014)

During bacterial culturing optical density (OD_{600}) should be monitored, as this factor is considered to be a measure of the concentration of bacteria in a suspension (48).

Optical density is routinely used to identify the stage of growth for culture induction, inoculation and harvest. It is also presumed that OD correlates directly with biomass so that cell concentration can be monitored without having to conduct any procedures for measuring the cell dry weight or plating for colony forming units (CFU/mL).

Knowledge of OD_{600} value is particularly important in situation when we want to use cells that are in their mid-log phase of growth (48).

Estimated OD_{600} of MBC, colonizing LCB rocks reached maximal value (0.335) in 21 hours of experiment (fig. 4). Then, in 25 and 45 hours OD remained on similar level 0.168 and 0.161, respectively.

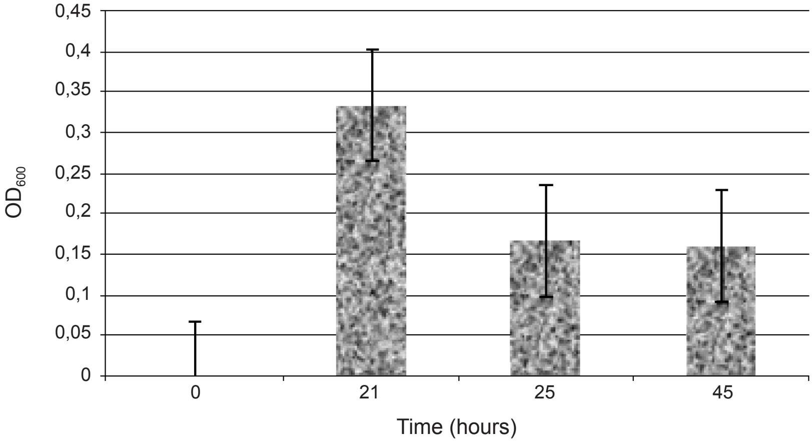

Fig. 4. Optical density changes during 45 hours of MBC growth on NMS medium (mean values of three replicates with standard error)

Source: Stępniewska et al., 2014 (40)

Nutrients depletion from NMS medium

Freshly prepared NMS was analyzed as regards the content of: Mn, Cu, Fe, Zn, Ni, K, Mg, Ca, Na by flame atomic absorption spectrometry (FAAS) technique (Z-8200 Hitachi, Japan). AAS analyses were later repeated after 21, 25 and 45 hours of MBC growth, in order to find trend of elements download from NMS. For AAS analyses we used supernatant, receiving after bacteria centrifugation. At the same time, in the second bioreactor MBC growth on NMS with 2% NaCl addition – as a hypo-osmotic stress factor was prepared. AAS analyses were analogically repeated as described above. Each analysis were performed in triplicate. Additionally concentration of phosphorus was determined, using an AutoAnalyzer3 (Bran & Luebbe, Germany). Concentration of phosphorus amounted from 6.61 to 5.08 $mg \cdot l^{-1}$ at the beginning and after 45 hours, respectively.

Nutrients depletion as a effect of MBC culturing on NMS medium registered during 45 hours standard growth (without NaCl stress factor) are presented in fig. 5.

Performed AAS analysis demonstrated that NMS medium include the highest amount of such macronutrients as: K (ca. 104 $mg \cdot l^{-1}$), Mg (c.a. 13 $mg \cdot l^{-1}$), Ca (c.a. 4.5 $mg \cdot l^{-1}$) and Ni (c.a. 1.5 $mg \cdot l^{-1}$). Other trace elements content in NMS not exceeded the level of 1 $mg \cdot l^{-1}$ (i.e. Na) or were even more limited to the level of <0.5 $mg \cdot l^{-1}$ (i.e. Mn), <0.3 $mg \cdot l^{-1}$ (i.e. Fe), <0.2 $mg \cdot l^{-1}$ (i.e. Cu) or <0.1 $mg \cdot l^{-1}$ (i.e. Zn).

During experiment time a slight loss of K (from 103.6 to 79.4 $mg \cdot l^{-1}$), Mg (from 12.99 to 9.27 $mg \cdot l^{-1}$), Ca (from 4.53 to 4.06 $mg \cdot l^{-1}$), Na (from 0.92 to 0.74 $mg \cdot l^{-1}$) and Zn (from 0.068 to 0.049 $mg \cdot l^{-1}$) were registered.

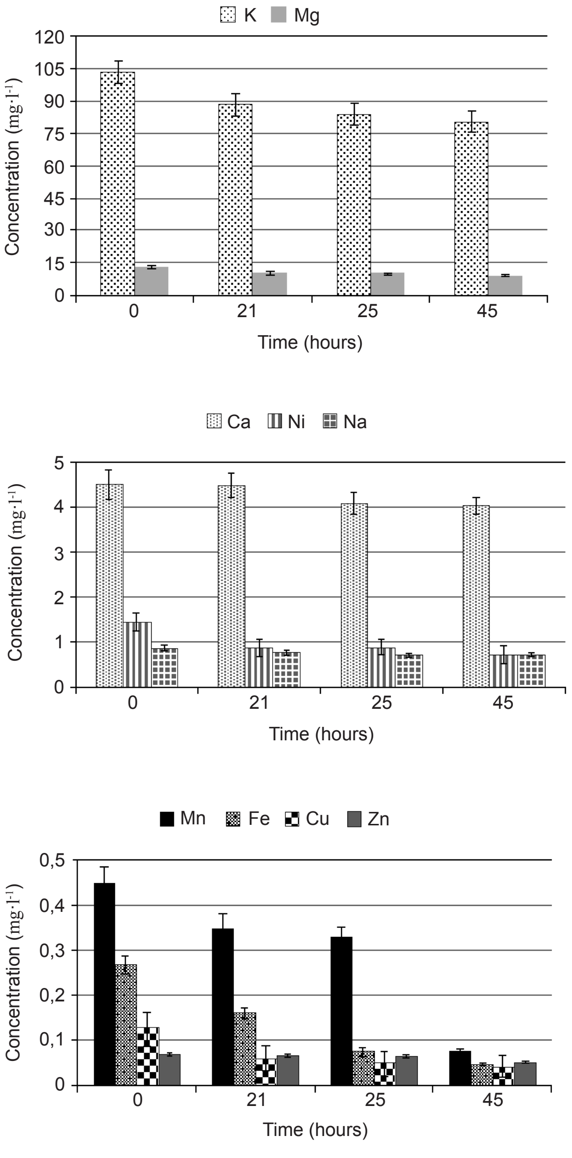

Fig. 5. Nutrients uptake by MBC during 45 hours standard growth on NMS medium (mean values of three replicates with standard error)

Source: Stępniewska et al., 2014 (40)

In the case of trace elements like Fe and Mn, at the end of experiment its concentration was by 83% lower in relation to the study beginning. The most noticeable consumption of Cu by MBC was noted during the first 21 hours, when its concentration was reduced from 0.13 mg·l^{-1} to 0.06 mg·l^{-1}. Afterwards (between 25–45 hours) Cu content remained on similar level (0.05-0.04 mg·l^{-1}). Almost 50% decrease of Ni content in NMS medium was stated after 45 hours bacterial growth, as it concentration dropped from 1.44 mg·l^{-1} to 0.73 mg·l^{-1}, respectively at the first and last hour of the experiment.

As is demonstrated in fig. 5 standard culture of MBC resulted on rather slow and proportional consumption of trace elements from the medium. What is interesting not dominated in the medium elements were consumed firstly and in the greatest quantities by MBC. Taking into account time of the experiment, we found that the highest trace elements consumption (exceeding 80%) by MBC took a place in relation to Fe and Mn, when its concentrations were reduced from 0.27 to 0.046 mg·l^{-1} and 0.45-0.078 mg·l^{-1}, respectively.

In the second place Cu loss by 69.23% was registered. Third position in terms of element consumption by MBC coincided with 50% Ni loss. Then, Mg decrease (on average 28%) with respect to its initial value was noted. On the similar level (27.94%) consumption of Zn by methanotrophs cultured on NMS was ranked.

Potassium, dominated macroelement in NMS decreased after 45 hours by 23.3% compared to baseline levels. Nearly 20% decrease was recorded in regards to Na, whereas Ca concentration was diminished by 10.37%. A study by Boisen et al. (1) has revealed that the kinetic characteristics of methanotrophs may be significantly influenced by different mineral nutrients. In particular, Fe and ammonia concentrations were important substrate components which increased the maximum methane utilization rate.

On the other hand, relatively high levels of both ammonia (70 mg·l^{-1}) and copper (0.3 mg·l^{-1}) inhibited the methane degradation, probably due to the toxic effect of copper-amine complexes (1). It is well known, that Cu is essential element to the health of plants and animals to build up proteins and plays an important role in functioning of metalloenzymes, i.e. oxygenases, peroxydases and reductases (19) such as methane monooxygenase, first enzyme in methane oxidation reaction chain (23), what might be the reason of demonstrated in current study strong (almost 70%) Cu consumption by MB. However, one should mention that at high concentration Cu can be toxic for organisms. Although different plants and microorganisms have different levels for copper toxicity, in general, a concentration above 20 mg·kg^{-1} leads to first symptoms in plants like browning of leaves or less growth, whereas its content over 0.1 mg l^{-1} in nutrient solutions can lower the activity of microorganisms (25). Nevertheless, the interpretation of our results has been challenging because of the lack of enough publications in available literature concerning the nutritional requirements of methanotrophs cultured on NMS medium under laboratory conditions.

Depletion of nutrients by MBC registered during 45 hours growth under salt stress condition are presented in fig. 6. In comparison with standard MBC growth described above, salt stress was the reason of significant differences in the nutrient uptake by methanotrophs.

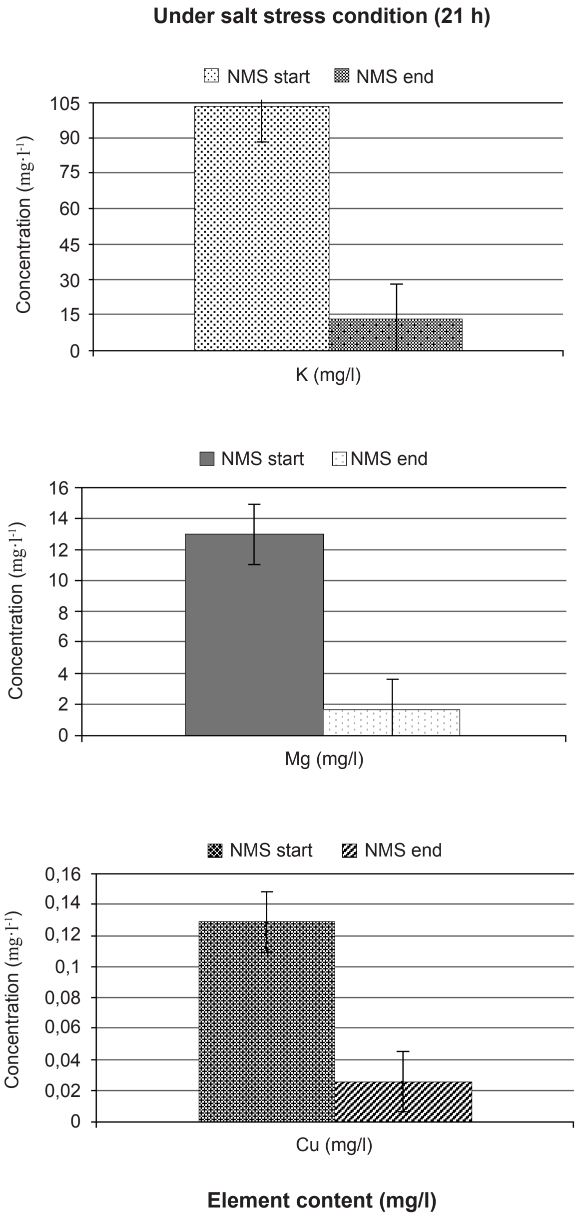

Fig. 6. Nutrients uptake by MBC after 21 hours of salt stress conditions (mean values of three replicates with standard error)

Source: Stępniewska et al., 2014 (40)

After 21 hours from 2% NaCl addition a sharp decrease of three among investigated elements K (from 103.6 to 13.072 $mg \cdot l^{-1}$), Mg (from 12.99 to 1.64 $mg \cdot l^{-1}$) and Ca (from 0.129 to 0.026 $mg \cdot l^{-1}$) was noted.

We found that K and Mg content was rapidly lowered even by 87.4%, whereas Cu concentration was reduced by 79.8%, in relation to its concentration in the initial medium. In the case of the other trace elements in the salinity conditions there was no difference in their uptake by MBC, in comparison with standard procedure of NMS culture growth.

Based on literature findings it is known that some methanotrophs isolated from marine waters, estuaries, arctic soil, groundwater, and soda lakes are halotolerant, i.e., capable of growth at salt concentrations between 0.15% and 4% (32). Heyer et al. (17) reported that *Methylohalobius crimeensis*, isolated from hypersaline lakes (salt concentrations ranging between 23% and 26%) has optimal growth at salt concentrations of c.a. 6-9%. Also, *M. hansonii*, isolated from Antarctic meromictic lakes with marine salinity, grows best in NMS medium amended with sea water, containing 3.5% salt (3). However, besides the fact that some of methanotrophs are halotolerant, there is lack of any information in literature database about differentiation in nutritional requirements of this group of bacteria under salt stress condition in relation to its standard culture on NMS.

Our study clearly demonstrated that under salt stress conditions methanotrophs in order to defend against stress factor first of all begin to download from NMS medium K and Mg. These two macronutrients allow them to survive the stress associated with salinity. The third element being used during osmotic stress is Cu, a key enzyme involved in the CH_4 oxidation.

As suggested by Csonka (5) the primary response of bacteria to exposure to a high osmotic environment is the accumulation of certain solutes, most of all: K, glutamate, trehalose, proline, and glycinebetaine, at concentrations that are proportional to the osmolarity of the medium.

The supposed function of these solutes is to maintain the osmolarity of the cytoplasm at a value greater than the osmolarity of the medium and thus provide turgor pressure within the cells. It is also known, that in many bacteria, an increase in the intracellular K concentration is seen as a response to increases in the external NaCl concentration. The importance of K in cellular homeostasis is also revealed by the finding that bacteria usually express multiple specific uptake systems, which allow these organisms to maintain high intracellular K concentrations against concentration gradients. In general, bacteria are capable of accumulating intracellular K at concentrations 100 times greater than that in the surrounding solution, and *Staphylococcus aureus* maintains a high intracellular concentration of 0.5 to 1.5M even if the extracellular concentration is in the low millimolar range (12, 13). Mentioned fact might be also an adequate explanation for noted in the current study strong accumulation of K by MBC cultured on NMS with NaCl addition. It is often assumed that Mg is essential element in biological systems, present in every cell type in every organisms (33). Standard example might be ATP (adenosine triphosphate), the main source of energy in cells, which must be bound to

Mg ion in order to maintain biological activity (often is coded as Mg-ATP). In single-cell organisms, such as bacteria, low levels of Mg manifests in greatly reduced growth rates. In Mg transport knockout strains of bacteria, healthy rates are maintained only with exposure to very high external concentrations of the ion (18). However, to our knowledge, this is the first study exploring the effect of significant (c.a. 87.5%) and comparable with K, Mg uptake from NMS by methanotrophs in the salinity stress conditions.

In our study we presented group of MBC inhabiting coal mine rocks which are halotolerant and are able for growth under salt stress conditions (2% NaCl in the medium).

Coalbed rock – environment for methanotrophic bacteria living

As was indicated by Pytlak et al. (28) carboniferous stratum in the LCB is located at the depth of c.a. 700 m.b.s (meters below surface), where the thickness of coal bodies ranges from 0.05 to 3.8 m. Most of the coal seams are located in the Upper Carboniferous, Westfalian B strata and are of limnic-fluvial origin (21). Described rock was collected from the area of seam 382 (c.a. 914 m below surface) and hammered manually, from the surrounding of a freshly excavated wall. Under laboratory condition rock sample was crushed into pieces and ground in a mill (Testchem Poland) to 2 mm maximum grain diameter. Physico-chemical rock characteristic is presented in table 3.

Table 3

Fresh coalbed rock characteristics (values of three replicates with standard deviation)

Factor	Seam location
	382
Lithology	Claystone
Depth (m.b.s.)	-914.4
Moisture (%)	0.74±0.16
pH	8.93±0.11
Eh (mV)	417.77±17.90
TC (%)	1.11
N-NO_3 ($mg \cdot kg^{-1}$)	0.78±0.01
N-NO_2 ($mg \cdot kg^{-1}$)	0.39±0.02
N-NH_4 ($mg \cdot kg^{-1}$)	39.45±4.47
P-PO_4 ($mg \cdot kg^{-1}$)	6.61±0.23

Source: Stępniewska et al., 2013 (37), with own modification

Results presented in table 3 indicated on convenient conditions for MB living in LCB coalbed rock (i.e. low moisture and carbon content, alkaline pH). The specific requirements of MB with an indication on various environmental factors are described below.

Environmental factors affecting methanotrophic activity

Microbial methane oxidation is a key process in the global methane cycle (27, 37, 39). Because of the crucial role of MB in the reduction of CH_4 emissions determination of methanotrophic activity in the investigated samples is very important. However, it should be remembered that the level of MA depends from several environmental factors.

One study in rice paddies, areas of CH_4 fluxes similar to that in landfills, indicated that Type II methanotrophs predominated over Type I methanotrophs as CH_4 concentrations increased (32). Other studies, however, have found that both types of methanotrophs were active and both contributed to CH_4 oxidation at high CH_4 concentrations (i.e. 10 000 p.p.m.v.), although Type I methanotrophs dominated at 1000 p.p.m.v. CH_4 (16, 32).

Also moisture content and temperature have been shown to affect MA (27, 36-39, 41, 42). Specifically, high moisture contents have been shown to limit MA, likely due to limited diffusion of CH_4 and air (4, 16, 27, 41). In contrary, at low moisture contents where diffusion is not the rate-limiting step on CH_4 availability, MA has also been observed to be inhibited, likely due to increased osmotic stress and/or desiccation (4, 16, 41, 42). From the limited number of phylogenetic studies done to date on the effect of temperature on methanotrophic communities it appears that Type I methanotrophs dominate at low temperatures (3, 24, 32). Moreover it was reported that at temperature extremes, however, i.e., <10 and >40°C, CH_4 oxidation is significantly limited in forest and landfill cover soil samples, likely due to inhibition of mesophilic methanotrophs (15, 32).

Another parameter that has been shown to have a range of effects on MA is the availability of nitrogen (32, 47). It was assumed that ammonium salts typically reduces the uptake of CH_4 by methanotrophs cells, either due to competition for binding sites in the methane monooxygenase or through product toxicity (7, 32). Other researchers noted that ammonia added as NH_4Cl more significantly inhibited *in situ* CH_4 oxidation in forest soils than did an equimolar amount of ammonia added as $(NH_4)_2SO_4$, possibly due to increased sorption (and thus reduced bioavailability) of ammonium on soils by sulfate (32).

Also effect of Cu on MA, specifically on the relative expression of sMMO and pMMO has been well known for some time (15, 19, 23, 27, 35). In cells expressing pMMO, Cu has also been shown to control expression up to 55-fold and to alter substrate affinity and specificity (16, 28, 32, 35).

Taking into account all factors described above and our experience in MB investigations we assumed that incubations, aiming to determine the methanotrophic activity (MA) in the rock material should be performed at oxic conditions at a temperature of 30°C (36). Suggested lab conditions for incubation of the coalbed rock material were previously confirmed as optimum for induction of MA by Stępniewska et al. (39), who found that further increase of substrate concentrations (CH_4 above 10%) did not result in elevated consumption rates. The same, temperature variant in experimental treatments was chosen based on preliminary experiments (39). The headspace concentrations of gases (CH_4, CO_2, O_2) might be determined using a gas chromatograph equipped with flame ionisation (FID) and thermal conductivity (TCD) detectors. MA expressed as ($\mu M\ CH_4\ g^{-1} \cdot day^{-1}$) is calculated from the slope of the regression line of the measured CH_4 molar amounts vs. time (28, 36).

In order to confirm that methane uptake in the investigated coalbed rock sample has biological character and is a consequence of MB activity the dynamics of gases (CH_4, O_2 and CO_2) both in the fresh and autoclaved samples were determined. Comparison of receiving results revealed that CH_4 uptake undoubtedly is due to activity of methanotrophic bacteria (36). Gases concentration dynamics of the coal-bed rock investigated is shown in fig. 7.

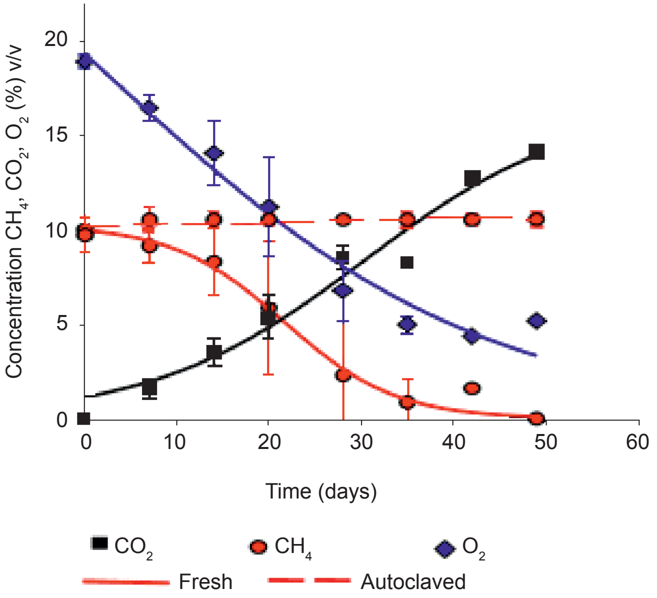

Fig. 6. Concentration dynamics of CH_4, O_2 and CO_2 in incubations of the coal-bed rock. Data points represent an average value and bars standard deviation of 3 experimental replicates

Source: Pytlak et al., 2012 (28)

In the case of fresh rock sample lag phase of methane was rapidly utilised with the use of molecular oxygen, which was confirmed by the parallel drop in the content of those gases phase and simultaneous growth of CO_2 mixing ratio (28). Opposing, methane concentration in the autoclaved samples remained on nearly unchanged level during the whole measured period. Calculated MA of the rock sample was 0.906 μM

CH_4 $g^{-1}\cdot day^{-1}$. The available literature show that MA noted in peat sample was almost 2 times higher than in coal-bed rock and amounted c.a. 1.656 μM CH_4 $g^{-1}\cdot day^{-1}$ (7) whilst MA calculated in lake sediment was much lower (in regards to rock material) and amounted 0.11 μM CH_4 $g^{-1}\cdot day^{-1}$ (28).

Mentioned values confirmed the fact that coal-bed rock samples are environment of relatively high MA. What is more, biological methane oxidation might have been the reason for present day low CH_4 levels in the LCB formations.

Identification procedures dedicated to methanotrophic bacteria

For DNA extraction from MBC we recommend the method of Sambrook et al. (31), with small modifications. Firstly, microbial cultures should be harvested by centrifugation. Then, the pellets need to be suspended in TE buffer and subjected to lyses using GES solution (5M *g*uanidine thiocyanate, 100 mM EDTA, 0.5% sarcosyl). After that, DNA should be purified using ice cold solution of ammonium acetate (7.5M) and subsequently mixture of chloroform: isoamyl (24:1) and cell debris removed by centrifugation (27). DNA precipitated at –20°C with isopropanol for two hours, pelleted by centrifugation at 17 500 x g for 30 min, rinsed 5 times with 70% v/v ethanol and resuspended in 30 ml of ultrapure, DNAse free water.

Polymerase chain reaction (PCR) should be performed using primer set recommended by Liebner et al. (24) for the detection of the *pmo*A gene of the methanotrophs. The primer sequences are as follows: A189f: GGNGACTGGGACTTCTGG and mb661r: CCGGMGCAACGTCYTTACC. Amplification will be effective if will be carried out as follows: initial denaturation (96°C for 4 minutes), followed by 30 cycles of denaturation (94°C for 1 min), primer annealing (54.5°C for 55 s), and elongation (72°C for 55 s). Final elongations at 72°C should be performed for 3 minutes (31).

Then, amplification products need to be checked for size and purity on 1% w/v agarose gels. Products of the correct size and purified using PCR Purification Kit (Qiagen) are proper for sequencing with the use of vector specific flanking primers (M13f and M13r). Nucleotide-nucleotide Blast (BlastN) are suggested to search the GeneBank for the nearest relative sequence. Original *pmoA* sequences achieved for the coalbed methanotrophs and selected public domain sequences are aligned and the phylogenetic tree is built using the neighbourhood-joining method (30) and MEGA 5.1 software. The evolutionary distances were computed using the Nei-Gojobori method (26).

Fluorescence *in situ* hybridization (FISH) should be performed according to Eller et al. (10) with suggested minor modifications. The cultured cells need to be harvested by centrifugation at 13.000×g for 5 min and resuspended in 100 μl of phosphate-buffered saline (PBS, pH 7.0). Fixation should be performed at room temperature for 3 h after addition of 300 of µl 4% paraformaldehyde (in PBS). The hybridization might be carried out on 10-well coated slides where 1–2 µl of cell suspensions are transferred and left to dry at room temperature for 2 h. Dehydratation is performed by washing slides in 50, 80, and 96% ethanol for 3 min each. Then, wells should be covered with 10 µl of hybridization buffer (Tris 2.4 $g·l^{-1}$, SDS 2.0 $g·l^{-1}$, EDTA 2.0 $g·l^{-1}$, NaCl 0.9 M, pH 7.4, 20% formamide) and 1 µl of the probe solution (50 ng $µl^{-1}$) should be added to each well. Hybridization in a water saturated atmosphere chamber (Memmert, Germany) should be carried out for 3 h at 46°C. Specificity of the probes applied are shown in table 4.

Table 4

Fluorescent-labelled probes targeting Type I and Type II of methanotrophic bacteria

Probe	Mg 705	Mg 84	Ma450
Methanotrophic bacteria	Type I	Type I	Type II
Sequence 5’ - 3’	CTGGTGTCCTTCAGATC	CCACTCGTCAGCGCCCGA	ATCCAGGTACCGTCATTATC
Target gen	16S rRNA	16S rRNA	16S rRNA
Fluorescent dye 5’	Fluoresceine	Cy3	Cy5

Source Stępniewska et al., 2014 (38)

Unbound oligonucleotides need to be removed by rinsing the slides with 20 ml washing buffer (Tris 2.4 $g·l^{-1}$, SDS 2.0 $g·l^{-1}$, EDTA 2.0 $g·l^{-1}$, NaCl 26.3 $g·l^{-1}$, pH7.4) pre-warmed to 48°C. Subsequently, the slides are incubated in the remaining washing buffer for another 20 min at 48°C, air-dried and mounted with Vectashield Mounting medium containing DNA-staining DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, USA). The slides migh be analyzed using fluorescence with a research microscope. The pictures are usually taken with camera and processed with the software provided by the manufacturer. The images should be captured in three channels (blue-nuclear counter stain, bright red (shown as green) – Cy3 and red – Cy5) and superimposed using camera software.

Molecular identification of methanotrophic bacteria consortium

In order to perform molecular identification of methanotrophs inhabiting coal rock from LCB amplification (PCR) was realized and resulted in receiving of product of c.a. 510 bp. Then PCR product were subsequently ligated into pGem®-T Easy vector, cloned and sequenced (28). Constructed phylogenetic tree is presented in fig. 8.

It was reported that received sequences were closely related (92-97%) to representatives of genera *Methylocaldum, Methylosinus, Methylobacter* and *Methylocystis*. Representatives of *Methylocystis* were earlier stated to be present in paddy soil and landfill land cover (fig. 8).

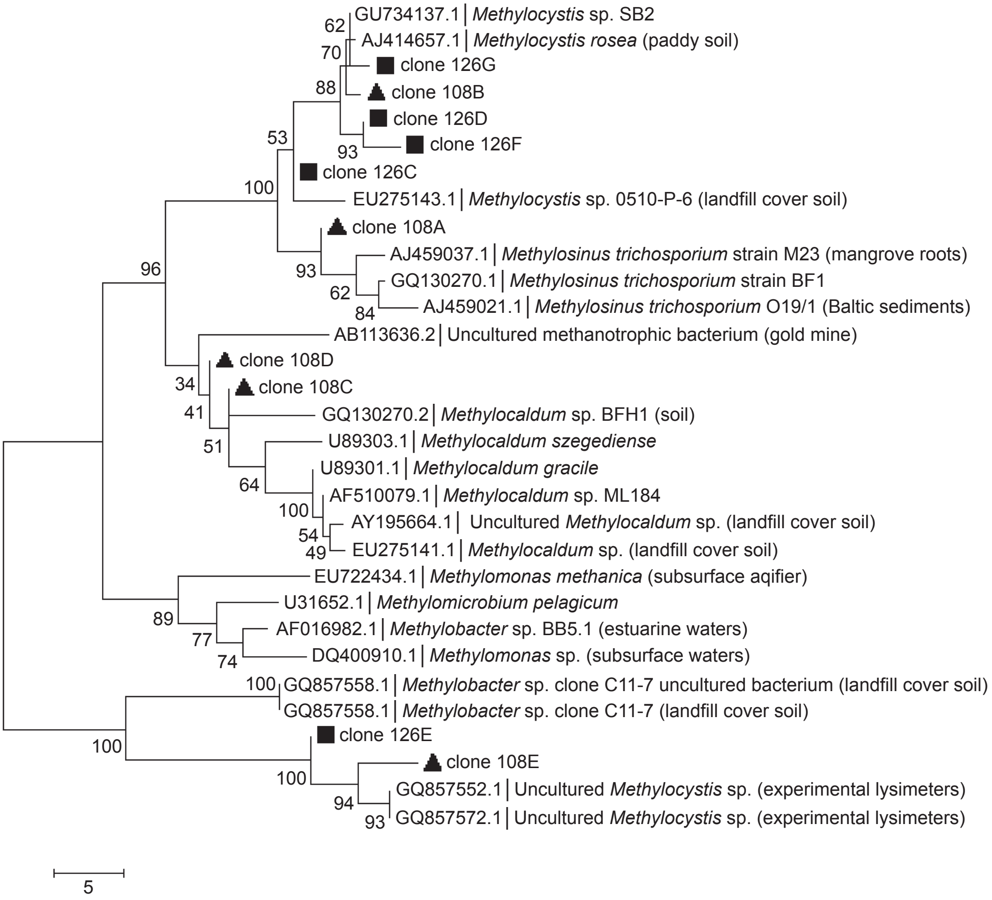

Fig. 8. Phylogenetic tree of bacterial amplicons

Source: Pytlak et al., 2012 (28)

Methylosinus trichosporium was noted in mangrove roots and Baltic sediments, whereas *Methylocaldum* bacteria are typical for soil environment. *Methylobacter* has representatives in such habitats as: landfill cover soil and estuarine waters.

Moreover, presented results revealed that cultivation-susceptible methanotrophic community of the LCB coal-bed rocks consists mostly of bacteria capable of resting stage formation, which could be a logical explanation of their presence in the severe, moisture and oxygen-depleted conditions of the coal-bed (28). However, so far the majority of studies were limited to aquatic habitats, e.g. Kotelnikova and

Pedersen (22) noted *Methylosinus* and *Methylocystis* species at the depth of 400 m, in igneous granitic rock aquifer at Äspö (Sweden), and representatives of the same genera were enriched from groundwater sandy aquifers in U.S. by Bowman et al. (2).

Further identification of MBC towards two methanotrophic types were realized by FISH analyzes. The combination of oligonucleotide probes Mg84 and Mg705 (coupled to Cy3) with Ma450 (marked with Cy5) allowed parallel detection of Type I and Type II of methanotrophs in enrichment cultures (fig. 9).

Type I cells were represented by short rods, 1–2 µm long and 0.7–1 µm wide, quite uniform in shape and size. Type II, less abundant, displayed two morphology shapes: straight (1 µm×2 µm) and curved bacilli (0.5×2 µm).

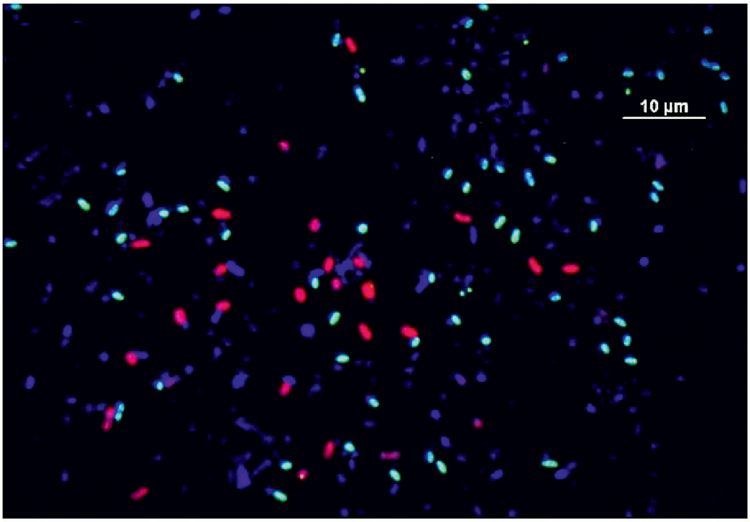

Fig. 9. Type I (green colour) and Type II (red colour) of methanothropic bacteria in a coal mine rocks
Source: Stępniewska et al., 2013 (37)

Indicated differences in MBC morphology undoubtedly result from the pleomorphic character of the observed bacteria. As reported by Trotsenko et al. (43) pleomorphism is a common feature of many methanotrophic species, e.g., belonging to genera *Methylocaldum* and *Methylocystis* or may result from the environment in which the bacteria are grown.

Summary

Methanotrophic bacteria are ubiquitous and present wherever stable methane emissions take place it should come as no surprise that such microorganisms were detected in the coal-bed rocks. Consequently, results presented in the current work indicate that subsurface environments should receive much more attention since their microbial inhabitants may play an important role in biogeochemical carbon cycling. MB inhabiting the coalbed rocks may be one of the factors responsible for CH_4 removal from Carboniferous formations.

It was demonstrated that coal-bed rock surrounding seams 382 in the Lublin Coal Basin are habitats of methanotrophic bacteria. Methanotrophic activity of the coal-bed rocks amounted 0.906 µM CH_4 $g^{-1} \cdot day^{-1}$. Detected MB, revealing high similarity of *pmoA* sequences to the genera of *Methylocistis*, *Methylosinus* and *Methylocaldum*, can be enriched from the coal-bed.

Presented results provide also a new knowledge about nutrient uptake by MBC under both conventional culture on solid medium as during salt stress. Standard microbial growth on NMS medium resulted on rather slow consumption of trace elements from the medium. What is interesting not dominated in the medium elements were consumed firstly and in the greatest quantities by MBC. Taking into account time of the experiment, we found that the highest trace elements consumption (exceeding 80%) by MBC took a place in relation to Fe and Mn, when its concentrations were reduced from 0.27 to 0.046 $mg \cdot l^{-1}$ and 0.45-0.078 $mg \cdot l^{-1}$, respectively. After that, Cu loss by 69.23% was registered. Third position in terms of element consumption by MBC coincided with 50% Ni loss. Then, Mg decrease (on average 28%) with respect to its initial value was noted. Analogically, consumption of Zn (27.94%) was stated. Potassium, dominated macroelement in NMS decreased after 45 hours by 23.3% compared to baseline levels. Similarly, by 23.14% decline of phosphorus was noted. Nearly 20% decrease was recorded in regards to Na, whereas Ca concentration was diminished by 10.37%.

It was also proved that presence of 2% NaCl in the medium modified the nutritional requirements of methanotrophic bacteria. Under salt stress condition much greater uptake (in relation to standard growth) of potassium in the amount of 90.51 $mg \cdot l^{-1}$, magnesium on the level of 11.35 $mg \cdot l^{-1}$ and copper on the level of 0.103 $mg \cdot l^{-1}$ were noted. In the case of the other trace elements in the salinity conditions there was no difference in their uptake by methanotrophic bacteria in comparison with standard procedure of culture growth on NMS.

Acknowledgments

Authors would like to thank our technical assistants: Renata Matczuk and Anna Sochaczewska for assistance in laboratory and control of MBC growth in bioreactors.

METHANOTROPHIC BACTERIA FROM COAL MINE ROCK – CHARACTERISTICS, FUNCTIONS AND NUTRIENTS UPTAKE

Abstract

Methanotrophic bacteria are ubiquitous in any environment where methane is present thus it is no surprise that this group of microorganisms inhabit the coal mine rocks. Results presented in the current paper clearly indicate that the underground environment (including mines) should deserve much more attention because its colonizing microorganisms play a significant role in the biogeochemical carbon cycle.

The Carboniferous sedimentary rock (clay-stone gray, taken form a depth of 914.4 m below ground level) originating from Lublin Coal Basin (LCB) was investigated. Growth of Methanotrophic Bacteria Consortium (MBC), colonizing LCB rocks under laboratory conditions were performed on nitrate minimal salt (NMS), recommended for the growth of this microorganisms group, in bioreactors BIOSTAT A Plus (Sartorius) at following conditions: 10% CH4, 30°C, 160 rpm by 45 hours.

At the beginning of the experiment, freshly prepared NMS was analyzed as regards the content of: Mn, Cu, Fe, Zn, Ni, K, Mg, Ca, Na by flame atomic absorption spectrometry (FAAS) technique (Z-8200 Hitachi, Japan). FAAS analyses were later repeated after 21, 25 and 45 hours of MBC growth, in order to find trend of elements download from NMS. At the same time, in the second bioreactor MBC growth on NMS with 2% NaCl addition – as a hypo-osmotic stress factor was prepared. FAAS analyses were analogically repeated as described above.

In order to be absolutely sure, that investigated bacteria belong to MBC methanotrophic activity (MA) of the coal material was determined. Then DNA isolation and PCR using primers specific for methanotrophs and based on diversity of *pmoA* gene were performed. Finally, received PCR products were sequenced.

Our results, clearly demonstrated that Carboniferous sedimentary rock is inhabited by MBC what was confirmed by high AM level amounting 0.906 μM CH_4 $g^{-1}\cdot day^{-1}$. It was also reported that received sequences were closely related (92-97%) to representatives of genera *Methylocaldum* (Type I)*,* *Methylosinus* and *Methylocystis* (Type II).

Presented results provide also a new knowledge about nutrient uptake by MBC under both conventional culture on solid medium as during salt stress. Standard microbial growth procedure on NMS medium resulted on rather slow consumption of trace elements from the medium. What is interesting not dominated in the medium elements were consumed firstly and in the greatest quantities by MBC. We found that the highest trace elements consumption (exceeding 80%) by MBC took a place in relation to Fe and Mn, when its concentrations were reduced from 0.27 to 0.046 mg· l^{-1} and 0.45-0.078 mg· l^{-1}, respectively. Later, Cu loss by 69.23% was registered. Third position in terms of element consumption by MBC coincided with 50% Ni loss. Then, Mg and Zn decrease (on average 28%) with respect to their

initial values were noted. Potassium, dominated macroelement in NMS decreased by 23.3% compared to baseline levels. Analogically, by 23.14% decline of phosphorus was observed.

It was also proved that presence of 2% NaCl in the medium strongly modified the nutritional requirements of MBC. Under salt stress condition much greater uptake (in relation to standard growth) of K in the amount of 90.51 mg· l^{-1}, Mg on the level of 11.35 mg· l^{-1} and Cu on the level of 0.103 copper were noted. In the case of the other trace elements in the salinity conditions there was no significant difference in their uptake by methanotrophic bacteria in comparison with standard procedure of culture growth on NMS.

In the current paper also literature findings about characteristics of methanotrophic bacteria, its biotechnological potential and trace elements important for methanotrophic bacteria growth are described.

References

1. Boisen A., Arvin E., Broholm K.: Effect of mineral nutrients on the kinetics of methane utilization by methanotrophs. Biodegradation, 1993, **4:** 163-170.
2. Bowman J., Jimenez L., Rosario I., Hazen T., Sayler G.: Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol., 1993, **59:** 2380-2387.
3. Bowman J., McCammon S., Skerratt J.: *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine salinity, meromictic lakes. Microbiol., 1997, **143**: 1451-1459.
4. Conrad R.: Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev., 1996, **60**: 609-640.
5. Csonka L.: Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. Mar., 1989, **53**: 121-147.
6. Dalton H.: The Leeuwenhoek lecture 2000. The natural and unnatural history of methane--oxidizing bacteria. Philos. T. R. Soc. B, 2005, **360**: 1207-1222.
7. Dedysh S., Dunfield P., Derakshani M., Stubner S., Heyer J., Liesack W.: Differential detection of type II methanotrophic bacteria in acidic peatlands using newly developed 16S rRNA-targeted fluorescent oligonucleotide probes. FEMS Microbiol. Ecol., 2003, **43**: 299-308.
8. Diekert G., Konheiser U., Piechulla K., Thauerr K.: Nickel requirement and Factor F_{430} content of methanogenic bacteria. J. Bacteriol., 1981, **148**: 459-464.
9. Ellefson W., Whitman W., Wolfer S.: Nickel containing factor FdZ0: chromophore of the methylreductase of *Methanobacterium.* Proc. Natl. Acad. Sci., 1982, **79**: 3707-3710.
10. Eller G., Stubner S., Frenzel P.: Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence in situ hybridisation. FEMS Microbiol. Lett., 2001, **198**: 91-97.
11. Fierer N., Schimel J., Holden P.: Influence of drying-rewetting frequency on soil bacterial community structure. Microb. Ecol., 2003, **45**: 63-71.

12. Graham J., Wilkinson B.: *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. J. Bacteriol., 1992, **174**: 2711-2716.
13. Gründling A.: Potassium uptake systems in *Staphylococcus aureus*: New stories about ancient systems. mBio, 2013**, 4:** doi:10.1128/mBio.00784-13.
14. Hakemian A., Kondapalli K., Telser J., Hoffman B., Stemmler T., Rosenzweig A.: The metal centers of the particulate methane monooxygenase from *Methylosinus trichosporium* OB3b. Biochem., 2008, **47**: 6793-6801.
15. Hanson R., Hanson T.: Methanotrophic bacteria. Microbiol. Mol. Biol. R., 1996, **60**: 439-471.
16. Henckel T., Friedrich M., Conrad R.: Molecular analyses of the methane-oxidizing microbial community in rice filed soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol., 1999, **60**: 1980-1990.
17. Heyer J., Berger U., Hardt M., Dunfield P.: *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int. J. Syst. Evol. Micr., 2005, **55**: 1817-1826.
18. Hmiel S., Snavely M., Florer J., Maguire M., Miller C.: Magnesium transport in *Salmonella typhimurium:* genetic characterization and cloning of three magnesium transport loci. J. Bacteriol., 1989, **171**: 4742-4751.
19. Jacobson F., Pistorius A., Ferkas D., DeGrip W., Hansson W., Sjolin L., Neutze R.: pH dependence of copper geometry, reduction potential and nitrate affinity to nitrite reductase. J. Biol. Chem., 2007, **282**: 6347-6355.
20. Jarrel K., Kalmokoff M.: Nutritional requirements of methanogenic archaebacteria. Can. J. Microbiol., 1988, **34**: 557-576.
21. Kotarba M., Clayton J.: A stable carbon isotope and biological marker study of Polish bituminous coals and carbonaceous shales. Int. J. Coal Geol., 2003, **55**: 73-94.
22. Kotelnikova S., Pedersen K.: Evidence for methanogenic *Archaea* and homoacetogenic Bacteria in deep granitic rock aquifers. FEMS Microbiol. Rev., 1997, **20**: 327-339.
23. Lieberman R., Rosenzweig A.: Biological methane oxidation: regulation, biochemistry and active site structure of particulate methane monooxygenase. Crit. Rev. Biochem. Mol. Biol., 2004, **39**: 147-164.
24. Liebner S., Rublack K., Stuehrmann T., Wagner D.: Diversity of aerobic methanotrophic bacteria in a permafrost active layer soil of the Lena Delta, Siberia. Microb. Ecol., 2009, **57**: 25-35.
25. Morton J., Hayes K., Semrau J.: Effect of copper speciation on whole-cell soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. Appl. Environ. Saf., 2000, **66**: 1730-1733.
26. Nei M., Gojobori T.: Simple methods for estimating the numbers of synonymous and nonsynony-mous nucleotide substitutions. Mol. Biol. Evol., 1986, **3**: 418-426.
27. Pytlak A.: Aktywność metanotroficzna i identyfikacja metanotrofów zasiedlających skały przywęglowe w różnych wyrobiskach górniczych kopalni Bogdanka. PhD thesis, The John Paul II Catholic University of Lublin, Poland, 2011.
28. Pytlak A., Kuźniar A., Stępniewska Z.: *pmo*A based detection of methanotrophic bacteria in coal-bed rocks of the Lublin Coal Basin. Acta Agrop., 2012, **19**: 403-413.

29. Rahalkar M., Bussmann I., Schink B.: *Methylosoma difficile* gen. nov., sp. nov., a novel methanotroph enriched from gradient cultivation from littoral sediment of Lake Constance. Int. J. Syst. Evol. Micr., 2007, **57**: 1073-1080.
30. Saitou N., Nei M.: The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 1987, **4:** 406-425.
31. Sambrook J., Fritsch E.F., Maniatis T.: A Laboratory Manual in Molecular Cloning. Cold Spring Harbor Laboratory Press, 1989, NY.
32. Semrau J., DiSpirito A., Yoon S.: Methanotrophs and copper. FEMS Microbiol. Rev., 2010, **34**: 496-531.
33. Shaul O.: Magnesium transport and function in plants: the tip of the iceberg. BioMetals, 2002, **15**: 309-323.
34. So-Yeon J., Kyung-Suk C., Tae K.: Density-dependent enhancement of methane oxidation activity and growth of *Methylocystis* sp. by a non-methanotrophic bacterium *Sphingopyxis* sp. Biotechnol. Rep., 2014, **4**: 128-133.
35. Stanley S., Prior S., Leak D., Dalton H.: Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnol. Lett., 1983, **5**: 487-492.
36. Stępniewska Z., Pytlak A.: Distribution of methanotrophic activity of carbonaceous rocks from different seams of Bogdanka coal mine. Pol. J. Environ. Stud., 2008, **17**: 550-554.
37. Stępniewska Z., Pytlak A., Kuźniar A.: Methanotrophic activity in Carboniferous coalbed rocks. Int. J. Coal Geol., 2013, **106**: 1-10.
38. Stępniewska Z., Pytlak A., Kuźniar A.: Distribution of the methanotrophic bacteria in Western part of the Upper Silesian Coal Basin (Borynia-Zofiówka and Budryk coal mines). Int. J. Coal Geol., 2014, **130**: 70-78.
39. Stępniewska Z., Szmagara A., Stefaniak E.: Optimization of environmental parameters conductive to methane oxidation with coal mine dump rock. Environ. Prot. Eng., 2004, **30**: 133-137.
40. Stępniewska Z., Wolińska A., Sochaczewska A., Matczuk R.: Nutritional requirements of methanotrophic bacteria from coal mine rocks. XII International Symposium Trace elements in the environment. 17-19.11.2014, Puławy, Poland. Book of abstracts, pp. 124-125.
41. Szafranek-Nakonieczna A., Bennicelli R.: Ability of peat soil to oxidize methane and affect temperature and layer deposition. Pol. J. Environ. Stud., 2009, **19**: 805-810.
42. Szafranek-Nakonieczna A., Stępniewska Z., Wołoszyn A., Ciepielski J.: Methanotrophs responsible for methane oxidation in natural peats from Polesie Lubelskie Region. Acta Agrop., 2012, **19**: 181-193.
43. Trotsenko Y., Medvedkova K., Khmilnina V., Eshinimayev B.: Thermophilic and thermotolerant aerobic methanotrophs. Microbiology, 2009, **78**: 387-401.
44. Uz I., Rasche M., Townsend T., Ogram A., Lindner A.: Characterization of methanogenic and methanotrophic assemblages in landfill samples. Proc. Royal Soc. London B. 2003, **270**: 202-205.
45. Whittenbury R., Davies S., Davey J.: Exospores and cysts formed by methane-utilizing bacteria. J. Gen. Microbiol., 1970, **61**: 219-226.

46. Vigliotta G., Nutricati E., Carata E., Tredici S., De Stefano M., Pontieri P., Massardo D., Prati M., De Bellis L., Alifano P.: *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic *g-Proteobacterium*. Appl. Environ. Microbiol., 2007, **73**: 3556-3565.
47. Zhang X., Xu S., Li C., Zhao L., Feng H., Yue G., Ren Z., Cheng G.: The soil/carbon nitro gen ratio and moisture effect microbial community structures in alkaline permafrost –affected soils with different vegetation types on the Tibetan plateau. Res. Microbiol., 2014, **2**: 128-139.
48. Zhang Z., Gentile T., Migdall A., Datla R.: Transmitance measurements for filters of optical density between one and ten. Appl. Opt., 1997, **36**: 8889-8895.

Adres do korespondencji:

dr Agnieszka Wolińska
Katedra Biochemii i Chemii Środowiska
Katolicki Uniwersytet Lubelski Jana Pawła II
ul. Konstantynów 1 I
20-708 Lublin
tel.: (81) 45 45 460
e-mail: awolin@kul.pl