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Screening for microbial markers in Miocene sediment exposed during open-cast brown coal mining

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Abstract

Viable microorganisms were found in Miocene lacustrine clays of the cypris formation excavated from 200-m below the surface as spoil during open-cast brown coal mining (Sokolov Brown Coal Basin, North-Western Bohemia, Czech Republic). Both saprotrophic microfungi of the genera *Penicillium*, *Verticillium*, *Cladosporium* and *Aspergillus* as well as heterotrophic bacteria were isolated from an intact sediment cores. Heterotrophic bacteria were classified by the MIS Sherlock System as representatives of genera *Nocardiopsis*, *Arthrobacter*, *Micrococcus*, *Kocuria*, *Rothia*, *Clavibacter*, *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Microbacterium*, *Acinetobacter* and *Pseudomonas*. A bacterium found among the strains had an atypical fatty acids profile enriched by branched fatty acids and polyunsaturated fatty acid (18:3ω6) and gave no MIS Sherlock match. Phospholipid fatty acids analysis indicates a relatively high (535 pmol g^{-1}) but inhomogeneously distributed viable microbial biomass. Fatty acids analyses of non-fractioned lipids (representing viable, storage and dead biomass; 8390 pmol g^{-1}) detected rich and homogenous profiles with fungal, bacterial and actinomycetal markers but no protozoan and algal fatty acids markers.

Introduction

Soil formation plays a principal role in the reconstruction of ecosystems disturbed by coal mining. Microorganisms are the first biota colonising the excaved spoil. Soil microbial community succession during pedogenesis has been studied in post-mining sites (0–40 years scale) after open-cast brown coal mining near Sokolov, North-Western Bohemia, Czech Republic (Chroňáková et al. 2004). During this study an abundant community of heterotrophic microorganisms was found in the freshly heaped clay spoil. These sediments are lacustrine clay sediments of the cypris formation deposited during the Miocene. They have now been brought to the surface from 200-m depth as spoil from brown coal mining. The results of Chroňáková et al. (2004) indicate either (1) an active microflora in Miocene sediments, and/or (2) that the pioneer heterotrophic microflora that colonise the spoil substrate during transport and heaping can easily use geogenic organic matter present in the spoil. It was decided to conduct a pilot search for an active pristine microbial community in Miocene substrate to determine which of the two hypotheses were correct and to

characterise the pioneer microbial community colonising the substrate.

Viable microbial communities have been found at considerable depths below the surface of the earth in several studies (for reviews see Balkwill et al. 1997; Brockman and Murray 1997; Pedersen 1997; Crocker et al. 2000). Microbial community character is controlled by the geology and hydrology of the subsurface environment (Colwell et al. 1997). A high concentration of organic carbon and low-permeability make clay-rich lacustrine Miocene sediments a suitable environment for long-term microbial survival (Frederickson et al. 1995). Microorganisms detected in such environments could be derived from the microbial community deposited with the sediments several-millions-years ago.

Material and methods

Sampling

Sediment (pH/H_2O 8.1, Ctot 4.5%, Ntot 0.2%; see Kříbek et al. 1998 for a full description) was sampled as an intact solid block of clay-stone (cca 10 kg; cca $0.5 \times 0.3 \times 0.3$ m) with help of open cast mining machinery from 200 m depth in the open-cast mine 'Jiří' (Sokolov coal mining district, Czech Republic lat 50°12′42″ N, long 12°41′00″ E). After excavation this material has the character of solid clay-stone which takes several years of weathering to disintegrate into small fragments. The block of clay-stone was transported in a sterile plastic bag to the laboratory. The block's surface was exposed to UV rays twice for 1 h on different sides and then further sterilised with a flame burner in a microbiological flow box (Lamin Air, Holten, Denmark). A 30-mm-surface layer was removed, the block was again seared with a burner and after that aseptically cut open. Material for analysis was sampled from the core of the cut block. Three mixed samples (each 50 g) were acquired from the core and processed immediately for microbiological analysis.

Fatty acids analyses

Phospholipid fatty acids analysis (PLFA) was used to characterise and quantify the complex viable community. Procedures described by Oravecz et al. (2004) were applied (with modification in the final detection step) using the MIS automatic identification system (Agilent 6850, FID, TSBA50, MIDI, Inc., Newark, DE). The same procedures, minus the lipids fractionation step by solid phase extraction on LiChrosolv column Si 60 (Merck, Germany), were applied to obtain total fatty acid profiles (TLFA). The TLFA gives general information about the viable, stored and dead biomass in sediment. At each individual step in the procedure blank samples were run in parallel with samples from the sediment. Fatty acids were designated as Oravecz et al. (2004) describe.

Microscopic and cultivable microbial counts

Direct bacterial counts were performed using 4′,6′-diamidino-2-phenylindole (DAPI) staining (Bloem 1995) and epifluorescence microscopy (dilution 10^1–10^4). Colony-forming units (CFUs) of bacteria and microfungi grew on R2A agar and trypticase soy broth agar (TSB) (Atlas 1993) at pH 7.2 and were counted after 7 days cultivation at 20 °C in the dark.

Characterisation of isolated strains

R2A and TSB media were used to isolate microfungi and heterotrophic bacteria. Bacterial isolates were identified by comparing their cellular fatty acids (FA) profile with the Sherlock Aerobic bacterial and Actinomycetal database (TSBA50, Actino) using the MIS Sherlock automatic identification System (MIDI, Inc., Newark, DE). Details of this procedure were described by Greenblatt et al. (1999). Microfungi were initially classified to the genus and subgenus level by the micromorphological characteristics of colonies growing on isolation media. We will identify of the species of the pure cultures in the future.

Results and discussion

A PLFA analysis of the viable microbial community, found only nine FAs in two samples, FAs in the third sample did not exceed the background level (Table 1). Non-specific saturated and

monounsaturated FAs, which occur in most microorganisms, dominated PLFA profiles. The only specific biomarker was the 10Me16:0 characteristic for actinomycetes which was also found in bacterial isolate-B14 identified as *Nocardiopsis dassonvillei* (Table 2). Although PLFA profiles gave poor results, the amount of PLFAs found (Table 1) indicated a relatively high viable biomass in the analysed lacustrine sediments. Several studies in have found PLFA biomass in different deep subsurface environments to be on average one-two orders lower than the results we measured (Colwell et al. 1997; Ringelberg et al. 1997; Brockman et al. 1998). The highest previously reported PLFA level from a similar environment (45 pmol PLFA g^{-1}) was detected by Frederickson et al. (1995) also in a lacustrine sediment.

Table 1. Molar percentage and total amount of PLFA (viable microbial biomass) and TLFA (total microbial biomass) recovered from Miocene subsurface clay sediment core (AV ± SD; $n=3$).

Fatty acids	PLFA mol%	TLFA mol%
10:0	nd	1.5 ± 0.2
i-3OH-11:0	nd	6.0 ± 3.1
12:0	2.5 ± 1.7	0.9 ± 0.1
3OH-11:0	nd	3.9 ± 0.3
i-13:0	nd	5.4 ± 0.3
13:0	nd	3.3 ± 0.9
14:0	bl	6.0 ± 0.4
15:1ω6c	7.2 ± 4.4	12.5 ± 1.5
i-3OH-14:0	nd	2.7 ± 0.1
2OH-14:0	4.7 ± 3.3	0.7 ± 0.1
16:17alc	nd	2.6 ± 0.4
i-16:0	nd	2.0 ± 2.8
16:1ω7c	20.6 ± 9.5	3.1 ± 2.2
16:0	30.0 ± 17.12	3.3 ± 1.4
i-3OH-15:0	nd	1.4 ± 0.9
10Me 16:0	10.4 ± 5.9	1.4 ± 1.9
a-17:0	nd	8.8 ± 0.9
10Me 17:0	nd	0.2 ± 0.3
i-18:0	nd	2.3 ± 1.8
18:2ω6	nd	13.5 ± 0.7
i-18:1	11.2 ± 7.9	0.6 ± 0.5
18:1ω9c	6.9 ± 1.9	2.4 ± 3.3
18:0	4.0 ± 2.9	2.0 ± 0.4
10Me 18:0	nd	9.4 ± 6.8
i-20:0	nd	2.1 ± 1.6
Total amount	pmol g^{-1} dw	pmol g^{-1} dw
	534.5 ± 471.2	8390.0 ± 613.1

The background level of procedual controls was 159.4 ± 98.7 pmol g^{-1} dw (AV ± SD; $n=6$). nd: Not detected. bl: Under the background level.

TLFA gave rich homogeneous profiles of 25 FAs (Table 1), including all of the 9 FAs detected in PLFA profile. Each from these 9 FAs only comprised a small fraction (molar percentage) of the TLFA profile, with the exception of 15:1ω6. Total PLFA, (which is a measure of viable biomass), represented approximately 6% of the total TLFA amount. This means that our TLFA results predominantly represent the dead and stored biomass. They have a lot of fungal marker (18:2ω6) and a rich spectrum of branched bacterial and specific actinomycetal markers (10Me 16:0, 10Me 17:0, 10Me 18:0). Polyunsaturated fatty acids typical for protozoa and algae were absent. The absence of protozoan and especially algal markers is noteworthy, as Kříbek et al. (1998) found that in Sokolov Basin sediments a large part of fossil organic matter was algal. The relatively high TLFA concentration in the sediment might serve as easy available substrate for heterotrophic bacteria and explain the relatively high heterotrophic growth of bacteria colonising the freshly heaped substrate (Chroňáková et al. 2004).

Table 2. Bacteria isolated from Miocene deep subsurface sediment core.

Design	Closest extant organism match on FA profile	Similarity index
B-5	*Acinetobacter calcoaceticus*	0.764
B-11	*Arthrobacter oxydans*	0.535
B-13	*Bacillus megaterium* GC subgr. A	0.919
S3-1	*Brevibacillus choshinensis*	0.623
B-8	*Clavibacter michiganensis nebraskensis*	0.121
S2-1	*Kocuria erythromyxa*	0.341
S1-2	*Kocuria erythromyxa*	0.721
B-3	*Microbacterium esteraromaticum*	0.597
S1-3	*Micrococcus luteus* GC subgr. C	0.307
B-6	*Micrococcus lylae* GC subgr. B	0.439
B-14	*Nocardiopsis dassonvillei*	0.160
B-12	*Paenibacillus alginolyticus*	0.656
B-10	*Pseudomonas putida* biotype A	0.452
B-1	*Rothia dentocariosa*	0.382
B-6	No matches found	G+
B-2*	No matches found	G+

Similarity index >0.5 indicates good matches. G+: Fatty acids profile enriched by branched fatty acids typical of Gram positive bacteria. *FA profile contained 18:3ω6c.

No viable bacterial cells were observed by epifluorescence microscopy, which may be due to binding of the fluorescein probe to clay particles (Bloem 1995; Bloem et al. 1995) and/or a very clustered cell distribution in the substrate. CFU bacterial counts showed inconsistent results along dilution series; however they were detectable in dilution 10^{-6} (i.e. 10^6 cells g^{-1} dw). This is comparable with data found for marine sediments (10^6–10^8 total cells cm^{-3}) or lacustrine sediments (10^5–10^7 total cells g^{-1}) by Parkes et al. (1994) and Fredrickson et al. (1995) respectively. It will take detailed study which takes into account spatial distribution and strong microbial–clay interaction to accurately estimate the viable biomass.

The average number of microfungi CFU was determined as 71.4×10^4 g^{-1} dry substrate (7.4–123.0×10^4) on R2A agar and 150.4×10^4 (65.4–265.0×10^4) on TSB agar, respectively. Raghlukumar et al. (2004) recovered 69–2493 CFU g^{-1} dry weight sediment culturable fungi from India Ocean deep-sea sediments that were 0.18–0.43 million years old. There is a question as to whether the microfungi in the screened sediments were present in viable or dormant form. Our PLFA results did not contain the typical fungal marker 18:2ω6 although it's precursor 18:1ω9, also typical for fungi, was present. Brockman et al. (1998) detected 18:2ω6 as well as 18:1ω9 in the PLFA profile of a deep vadose zone sediment. They did not immediately detect culturable fungi in analysed samples, but after 21–224 days of incubation propagule numbers typically increased to 100–10,000 g^{-1} dry weight substrate. Microfungi CFU-counts measured in our study were comparable to or higher than the CFU-counts (1.2–73.0×10^4 g^{-1}) described by Nováková (2001) for Sokolov post-mining dumps chronosequence (5–67 years). Isolated subsurface saprotrophic microfungi, determined as *Penicillium* subg. *Furcatum*, *Penicillium* subg. *Biverticillium*, *Verticillium* spp., *Cladosporium* spp., *Aspergillus* spp., represented common and frequently distributed soil microfungi. These findings indicate that these genera have persisted a long time in the Sokolov Basin sediment, which probably serves as a rich and suitable reservoir for microorganisms.

Sixteen bacterial isolates (out of the 30 isolated), which survived the purification conditions and MIS cultivation protocol procedures, were compared with the MIS Sherlock species databases, and fourteen of them showed a match (Table 2). Fourteen of the isolates were Gram positive, only two were Gram negative species. The results of this pilot screening of bacteria in the Sokolov Basin sediment correspond well to other studies of deep subsurface phylogenetic bacterial diversity. Studies of Balkwill et al. (1997); Chandler et al. (1998); Crocker et al. (2000) document the most frequently occurring strains as the Gram-positive *Arthrobacter*, *Bacillus*, *Kocuria*, *Micrococcus*, *Streptococcus*, *Clavibacter* and *Nocardiodes*, whereas *Acinetobacter*, *Pseudomonas*, *Comamonas*, *Sphingomonas*, *Variovorax*, *Bulkholderia* are the most frequently encountered Gram-negative genera.

The FA profile of one bacterial strain is also noteworthy. The B2-strain, forming orange colonies, contained polyunsaturated fatty acids 18:3ω6c (in portion 1.4% of total FAs). Its profile consists of 16:0, i-13:0, a-13:0, 16:0alc,18:0, 14:0, i-12:0, 12:0, i-15:0, i-17:0, a-17:1, 18:3ω6c, i-11:0, 17:0, 16:1ω11c, i-14:0, a-15:0 in descending order. *Acinetobacter calcoaceticus* was also found among our isolates. PUFA production has been found in this species (Yakimov et al. 2004), but no PUFA were recorded in the FA profile of our isolate. Polyunsaturated fatty acids in bacteria were long ignored by researchers; this is changing as there is interest in PUFA-producing bacteria for their ecological role and biotechnological application (Russell and Nichols 1999). The most noted PUFA-bacterial producers have been found in deep-sea sediment and sea ice and are often associated with halophilic and psychrophilic environments (Nichols and McMeekin 2002). Not much is known about bacteria that produce PUFA in the terrestrial subsurface environments, although particularly saline lacustrine sediments may represent similar environment to the deep-sea sediments.

Conclusion

The occurrence of a viable pristine microbial community in a Miocene lacustrine sediment exposed during open cast mining and heaped in post-mining sites was confirmed. The relatively high content of viable biomass and spectrum of saprotrophic fungi and heterotrophic bacteria showed that the clay rich sediment from Sokolov Basin is a microbially rich geological medium in which fungi and bacteria can survive for a long time. The study indicates that

these terrestrial deep subsurface lacustrine sediments could be similar to deep-sea sediments in that bacterial-PUFA producers occur in both.

The high content and rich spectrum of fatty acids in non-viable microbial biomass may be an easily available carbon source for the pioneer heterotrophic community that colonises the heaped mine spoil. The ecological contribution of the pristine Miocene microbial community to succession of soil microbial community developed during pedogenetic process requires further study.

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