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Original article

Anaerobic Eury- and Crenarchaeota inhabit ectomycorrhizas of boreal forest Scots pine

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ABSTRACT

Members of the euryarchaeotal genera *Methanolobus* and *Halobacterium* as well as group 1.1c Crenarchaeota were enriched from ectomycorrhizal samples and cultured under anaerobic conditions. 16S rRNA gene sequences of *Methanolobus* were obtained in a $H_2 + CO_2$ atmosphere and autofluorescent putatively methanogenic microbial cells were detected by epifluorescence microscopy of the anaerobic methane-producing enrichment cultures. *Halobacterium* and group 1.1c Crenarchaeota grew anaerobically when either H_2 or CH_4 was added to the atmosphere. Group 1.1c Crenarchaeota were also enriched under aerobic conditions on mineral media, but only when methane or methanol was added as carbon sources. The 16S rRNA gene sequences of 1.1c Crenarchaeota grown under both anaerobic and aerobic conditions were highly similar. Our study demonstrates the growth of group 1.1c Crenarchaeota and Halobacteria derived from non-extreme soil environment in non-saline enrichments under anaerobic conditions. The results suggest that 1.1c Crenarchaeota may play a role in the cycling of C-1 substrates in the boreal forest soil ecosystem.

1. Introduction

One gram of soil may harbour up to 10 billion microorganisms belonging to thousands of species [44]. Microorganisms in nature are generally difficult to isolate in pure cultures and it has been estimated that only about 1% of the microbes in soil grow on conventional laboratory media [54]. Therefore, very little is known about microbial functional ecology although the activity of the microbial community in boreal forest soil is high and has a great impact on soil respiration [14]. More than half of the microbial respiration in boreal forest soils relies on 'fast' carbon compounds such as photosynthates secreted into the soil and mycorrhizosphere by local trees [15,28]. Mycorrhizal symbioses are of critical importance to the soil microbial community as they are responsible for the amount and quality of compounds released by tree roots [28,52].

The mycorrhizosphere comprises a rich variety of microenvironments suitable for many different types of organisms [40]. Microbial activity modifies local environments to create anaerobic and microaerobic conditions [29]. Oxygen-depleted pockets can be formed within the compact structure of mycorrhizas and fungal external mycelia. These pockets, which have a constant supply of nutrients, could provide suitable microenvironments for strictly anaerobic methanogens.

Euryarchaeotal 16S rRNA gene sequences closely resembling those of the *Halobacterium* have recently been reported from the boreal forest tree mycorrhizosphere [4,5]. Methanogenic consortia [24] and lineages of the Thermoplasmatales [41] have been detected in the litter and topsoil of temperate deciduous forests. In the boreal region, Thermoplasmatales- and Methanomicrobiales-related Euryarchaeota have been found in lake water [19] and methanogens in peat lands [10,48] but not yet in boreal forest soil. Nevertheless, boreal forests have been shown to be, depending on circumstances, either methane sources [47], methane neutral [57], or atmospheric methane sinks [31,32]. Methane is a significant greenhouse gas and its concentration in the atmosphere has increased 150% since the beginning of industrial revolution [39]. Thus, a better understanding of the microorganisms involved in methane cycling is of great importance.

Crenarchaeota of the group 1.1c are the predominant type of Archaea found in boreal forest soil and mycorrhizospheres. They appear to be associated with ectomycorrhizal fungi [3–5] and are

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usually found in forest soils with a pH below 5 [2,13,17,21,37,58]. Although methanogenic archaea (e.g. [45]) and aquatic ammonia oxidising group 1.1a Crenarchaeota [23] have been isolated and soil group 1.1b Crenarchaeota [46] have been enriched from environmental samples, the ecological roles of the Eury- and Crenarchaeota inhabiting boreal forest soil ecosystems are yet to be clarified.

The aim of this study was to investigate both anaerobic and aerobic archaea of pine mycorrhizas by using enrichment cultures with special focus on the C-1 compounds of the carbon cycle. We investigated the methane production potential of the microbial communities of Scots pine mycorrhizas and bulk humus. In addition, the enrichment cultures obtained from mycorrhizas were characterized with molecular methods.

2. Methods

2.1. Sampling and processing of material

Three Scots pine seedlings of approximately three years of age were chosen from a 70-year-old Scots pine forest in southern Finland (60°28′N, 23°45′E), which has been described earlier [53]. The sampling was done in September 2005. The seedlings were collected with a soil core (15 cm diameter, 10 cm deep) through the humus layer. Cores with attached seedlings were immediately placed in anaerobic jars (WP0011 Oxoid, Oxoid Ltd., Hampshire, UK) equipped with a BBL GasPak Pouch® System (Franklin Lakes, NJ, USA) to remove oxygen from the atmosphere of the jar. The sealed jars were transferred to the laboratory where they were kept at +4 °C overnight. Humus cores were processed in an anaerobic chamber (FORMA 1025, Thermo Electron Corporation, Waltham, MA, USA). A composite sample of ten indigenous mature mycorrhizas (Mm), 0.02 g fresh weight (fw) was collected from each core. The mycorrhizal samples contained mixed pink *Tomentellopsis* type and yellow *Piloderma* type mycorrhizas. In the anaerobic chamber, mycorrhizal samples were homogenized in microcentrifuge tubes with sterile quartz sand before each sample was transferred to a sterile anaerobically prepared test tube with a butyl rubber stopper and containing 9 ml of anaerobic water. A bulk humus sample with no visible hyphae or roots (1 g fw) was also taken from each core and mixed with 50 ml anaerobic water. Microbial inocula of the mycorrhizal and humus samples were prepared by vortexing the vials; 3 × 1 min.

Enrichment cultures were prepared by aseptically removing 0.1 ml of the sample suspension with a sterile and N_2 flushed syringe and needle through the rubber septum and immediately injecting it into 120-ml infusion flasks containing 50 ml of liquid growth medium and an atmosphere of 80% N_2 and 20% CO_2.

Each of the three mycorrhizal samples was tested with seven different treatments as follows. A yeast extract (YE) based medium (L^{-1} 2.0 g yeast extract, 0.40 g KH_2PO_4, 0.53 g $Na_2HPO_4 \cdot 2H_2O$, 1 mg resazurin, 2.0 mg $FeCl_2 \cdot 4H_2O$, 0.05 mg H_3BO_3, 0.05 mg $ZnCl_2$, 0.03 mg $CuCl_2$, 0.05 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 mg $AlCl_3$, 0.05 mg $CoCl_2 \cdot 6H_2O$, 0.05 mg $NiCl_2$, 0.5 mg EDTA, 1 μl concentrated HCl, 2.0 μg biotin, 2.0 μg folic acid, 10 μg pyridoxine, 5.0 μg each of thiamine, riboflavin, nicotinamide, cyanocobalamine, p-aminobenzoic acid, lipoic acid, pantothenic acid, 0.3 g NH_4Cl, 0.3 g NaCl, 110 mg $CaCl_2 \cdot 2H_2O$, 100 mg $MgCl_2 \cdot 6H_2O$, 4.0 g $NaHCO_3$, 0.24 g $Na_2S \cdot 9H_2O$, 0.5 g L-cysteine hydrochloride monohydrate) was prepared anaerobically with the gas phase supplemented with either 40 ml $H_2 + CO_2$ (80% H_2 and 20% CO_2) or 7 ml CH_4 (100%) (Table 1). A mineral medium (BCCM medium number 72, M72, http://bccm.belspo.be/) was prepared anaerobically and also supplemented with the same amounts of H_2 or CH_4 as above (Table 1). In addition, the M72 medium was prepared aerobically and supplemented with either 40 ml $H_2 + CO_2$, 7 ml CH_4 or 0.5 ml CH_3OH (Table 1). The pH in all treatments was 6.8. The YE medium was only used in anaerobic enrichments. Resazurin (0.01%) was used as redox indicator in the anaerobic cultures. The sealed enrichment cultures were incubated for 93 days at 18 °C (150 rpm), during which time, samples were aseptically taken with a nitrogen flushed syringe and needle.

Table 1
The culture conditions and numbers of identified archaeal OTUs.

Culture condition	Medium	Treatment	OTUs tot	Identified OTUs types
Anaerobic	YE	1.33 mmol H_2 0.9 mmol CO_2	14	8 1.1c Crenarchaeota 1 Methanolobus 5 unidentified[b]
			22[a]	1 Methanolobus 3 Halobacterium 8 Bacteria 10 unidentified[b]
	M72	1.33 mmol H_2 0.9 mmol CO_2	0	0
	YE	0.29 mmol CH_4 0.58 mmol CO_2	11	4 1.1c Crenarchaeota 4 Halobacterium 3 unidentified[b]
	M72	0.29 mmol CH_4 0.58 mmol CO_2	3	2 1.1c Crenarchaeota 1 unidentified[b]
Aerobic	M72	13% O_2 0.29 mmol CH_4 0.25 mmol CO_2	25	11 1.1c Crenarchaeota 14 unidentified[b]
	M72	Ambient O_2 and CO_2 0.12 mol CH_3OH	16	7 1.1c Crenarchaeota 9 unidentified[b]
	M72	1.33 mmol H_2 0.9 mmol CO_2	0	0

[a] Results by the relatively unspecific primer combination 2.
[b] Sequences for these OTUs were not obtained due to sequencing failure.

For DGGE sampling, 1 ml of liquid from each of the mycorrhizal enrichment cultures was taken at 9 time points (day 0, 1, 2, 3, 7, 10, 14, 35 and 93). The enrichment samples were pelleted in a microcentrifuge (Heraeus, Heraeus Holding GmbH, Hanau, Germany), for 10 min at 13000 rpm. The supernatant was discarded and the pellet was resuspended in 50 μl sterile water (Orion Pharma, Espoo, Finland) and heated in a QBT block heater (Grant Instruments, Chelmsford, UK) for 10 min at 105 °C. The samples were then placed on ice for 5 min and subsequently stored frozen until used as PCR templates. Additional 1 ml samples were obtained on days 0, 7, 14 and 35 from the anaerobic YE + H_2 flasks for DNA extraction with MoBio UltraClean Soil DNA kit (MoBio laboratories, Carlsbad, CA, USA).

Methanogenic properties of the humus were tested in anaerobic YE + H_2 media. Three replicates of four different dilutions (10^{-1} to 10^{-4}) were prepared from the original samples containing 1 g humus suspended in 50 ml anaerobic water. The dilution 10^{-3} contained the equivalent amount of sample as the undiluted mycorrhizal samples.

2.2. PCR, DGGE and sequencing

To detect archaeal 16S rRNA genes in the mycorrhizal enrichments, a nested PCR was performed on an Eppendorf MasterCycler Gradient (Eppendorf, NY, USA). Two different primer combinations were used. In primer combination 1, primers 3f and 9r [17] were used in the primary, and primers A109a and A934b [12] in the secondary PCR. This primer combination was used on all samples. In primer combination 2, primers 8f and 1512r [56] in the primary, and primers A109a and A934b in the secondary PCR were used for the anaerobic YE + H_2 samples which were subjected to DNA extraction with the MoBio UltraClean Soil DNA kit. The presence of ammonia oxidising Crenarchaeota was additionally tested with primers CrenamoA23f and CrenamoA616r targeting the ammonia