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Biogeochemical Activity of Anaerobic Microorganisms From Buried Permafrost Sediments

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Permafrost sediment samples, ranging in age from 7 thousand to 2 million years, from the northeastern region of Russian Arctic were analyzed for evidence of reducing conditions, viable populations of anaerobic bacteria and their metabolic end products. Field analyses of samples showed that all sediments were reduced with a redox potential ranging from +40 to −256 mV. Ferrous iron, acid-soluble sulfide, and methane were detected in the frozen sediments. Direct bacterial counts were 10^7 to 10^8 cells/g sediments as determined by epifluorescence microscopy using acridine orange. Denitrifiers and (H_2 + CO_2)-utilizing methanogens were detected in all samples, and acetoclastic methanogens, sulfate reducers, and Fe(III) reducers were detected in some samples and at much lower numbers. [^{35}S]Sulfide production from [^{35}S]sulfate was detected in soils incubated anaerobically at 4°C for 6 months. Thus anaerobic metabolic activity was present at temperatures near freezing. These results suggest that viable anaerobic bacteria reside in aged, reduced permafrost sediments. Future investigation should focus on detecting activity in the frozen state.

Keywords anaerobic, microbial activity, permafrost

Viable microorganisms have been recovered previously from buried Siberian permafrost soils and sediments (Gilichinsky et al. 1989; Zvyagintsev et al. 1990). Their abundance and diversity depended on the type of soil and age since burial below the zone of annual melting (Zvyagintsev et al. 1985). The largest numbers of viable cells were found in relatively young layers, 7–10 thousand years before present (BP), and in older soils and sediments

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with higher concentrations of organic matter. However, these earlier investigations were generally limited to determining the number of viable cells of aerobic microorganisms.

The sediments studied here are from geological suites that are derived from alluvial, lake, and marine origin. This origin suggests that some of these suites could have been formed under anoxic conditions (Zigert 1987). Hence, it is reasonable that viable anaerobic microorganisms may still exist in these permafrost sediments, especially those in a reduced state. The objective of this study was to determine if anaerobic bacteria and their metabolic end products could be detected in permafrost sediments varying in age from 7 thousand to 2 million years.

Materials and Methods

Sampling and Site

The sediments under investigation were obtained by slow rotary drilling so as not to melt the sediments, and without the use of any solutions, drilling muds, or chemicals. The temperature of extracted cores was never higher than −7°C. Surfaces of the extracted frozen core were trimmed away with a sterile knife, and the remaining core was immediately placed in presterilized aluminum tins, sealed, and kept frozen during storage and transport. When ready to use in the laboratory, the frozen core was fractured with a sterile knife and only internal segments of the core were taken by sterile forceps for microbial assays. The methods of sampling, storage, transport, and contamination control were detailed previously (Khlebnikova et al. 1990). These authors also showed that *Serratia marcescens* could not be detected in internal segments of cores that had purposely received surface contamination with this organism in the field.

The samples were taken from the tundra zone of the Kolyma-Indigirka Lowland (152–162°E and 68–72°N), which is in northeastern Siberia. Permafrost occurs throughout the area to depths of ~600–800 m. In this study, we consider the upper 100 m of the late Cenozoic permafrost. These layers are syngenetically frozen (sedimentation occurred concurrently with freezing, in which case the age of permafrost was taken as equal to the age of the sediments). Evidence suggests that buried permafrost in this region has remained continuously frozen since its period of deposition (Gilichinsky et al. 1995). The current average temperature of the sediments ranges from −9 to −12°C.

Samples of different age were obtained from two drilling wells (sites A and B) as well as the surface soils at the two sites, a sandy loam and peat soil, respectively (Table 1). All other samples were mineral soils except for sample 188, which was from a peaty horizon.

Site A (well number 4/91) is located directly on the coast of the Arctic Ocean. Permafrost deposits of marine origin were obtained at depths from 5 to 22 m and consisted of loam and loamy sands of the middle Pleistocene (100 thousand years BP) age. Site B (well number 3/92) is located 100 km inland and consists of 2 terrestrial suites: (1) an 8-m-thick Holocene deposit (7–10 thousand years BP), representing different lake or swamp deposits, and (2) a 40-m-thick late Pliocene–early Pleistocene (0.6–2.0 Myr BP) deposit of alluvial sandy loam.

Soil Analyses

Immediately after sampling, the quantity of methane and the redox potential were determined in sections of the permafrost cores. Methane concentration was determined after the samples were melted in sealed jars, by a field gas chromatograph equipped with a flame ionization detector. The redox potential was determined immediately after melting, using

TABLE 1 Geochemical characteristics of buried permafrost sediments

Sample number	Depth (m)	Age ($\times 10^4$ years)	E_h (mV)	CH_4 (ml/kg)	Fe(II) (g/100 g wet soil)	S^{2-} (g/100 g wet soil)
Site A						
195	0.09–0.18	Modern	nd	0	0.006	0
1332	7	10	nd	5.7	0.245	0.012
2411	18	10	nd	7.4	0.103	0.016
2581	20.7	10	nd	10.3	0.157	0.022
Site B						
8	0.05–010	Modern	−80	6.8	0.012	0
323	2.2	0.7–1.0	−100	7.0	0.008	0
188	4.4	0.7–1.0	+40	6.0	0.027	0
101	7.2	60–200	−100	2.0	0.156	0.035
399	13.5	60–200	+20	2.4	0.232	0.012
269	19.8	60–200	−120	4.0	0.284	0.014
352	24.8	60–200	−135	6.5	0.169	0.015
256	34.3	60–200	−256	4.0	0.240	0.022

Note. nd, not determined.

a potentiometer with a platinum electrode and silver chloride electrode as reference. In the laboratory, ferrous iron was determined by the method of Sorensen (1982), and sulfide was determined by a modification of Pachmayr's method as described by Volkov and Zhabina (1980).

Microbial Methods

Direct microscopic counts of bacteria in the soil were accomplished in the laboratory using the method of Hopkins et al. (1991). After melting and sample dispersion, suspended material was concentrated by centrifugation (8000 g for 20 min). A 0.2-ml volume of 0.015% acridine orange (AO) solution was added to 0.01 ml of the sedimented material and suspended in 1.0 ml H_2O (Kuznetsov and Dubinina 1989). The samples were incubated for 2 min, after which the stained bacteria were collected by filtration on a 0.2-μm pore size black polycarbonate membrane filters (Nuclepore Corp., Pleasanton, CA). The fluorescing cells were counted using an epifluorescent microscope (OPTIHOT, Nikon).

Viable counts of different groups of anaerobic bacteria were determined by the most probable number (MPN) technique, 5 tubes per dilution, using six 10-fold dilutions made in sealed anaerobic tubes. The soil suspension was prepared by diluting 1–2 g soil in 50 ml of the following salts solution: NaCl (1.0 g/L); $MgCl_2 \cdot 6H_2O$ (0.4 g/L); $CaCl_2 \cdot 2H_2O$ (0.15 g/L); NH_4Cl (0.25 g/L). The dilution and incubation media were prepared using strict anaerobic techniques (Hungate 1969). All media were incubated at 15°C for 1.5 months before scoring for growth. The data are means from two replicate soil samples.

Acetoclastic methanogens were assayed in a carbonate-buffered mineral medium with 10 mM acetate as the sole carbon and energy source (Widdel 1986). Hydrogenotrophic methanogens were measured in the same mineral medium but with H_2/CO_2 (80/20 v/v) as the primary substrate and 0.1 mM acetate to aid cell growth. Tubes were considered positive for methanogens when >100 ppm CH_4 was detected by gas chromatography. Denitrifier populations were measured in mineral medium with 5 mM nitrate, 20 mM citrate, and N_2 in the headspace of MPN tubes (Tiedje 1982). Tubes were scored positive when nitrate

was not detected by diphenylamine. Sulfate-reducing bacterial populations were assayed in a medium of 10 mM sulfate and 20 mM lactate (Widdel and Pfenning 1984). Sulfate consumption was determined by ion chromatography (Dionex −2000i/sp). The tubes were scored positive when the sulfate concentration was reduced by ≥30%. Iron reducing bacterial populations were assayed by the method of Lovley and Phillips (1986). The freshwater medium contained 0.05 g/L yeast extract and amorphous ferric oxyhydroxide (FeOOH). Bacterial activity was measured as an increase of Fe(II) in the medium (Sorensen 1982).

Sulfate-reducing activity of the sediment at 4°C was determined by measuring $^{35}S^{2-}$ production from $^{35}SO_4^{2-}$ (Ivanov et al. 1976). The sulfate reducer medium already described plus 1 ml lactate was added to each of 3 replicate 20-ml tubes containing 1 g of soil amended with 7 μCi solution of $Na_2{}^{35}SO_4$ (1.38 mCi/mmol specific activity). The soil was incubated anaerobically for 6 months. Formation of sulfide was measured by stripping the acidified sediment with N_2, trapping the evolved H_2S as ZnS, and counting the trapped fraction in a liquid scintillation counter.

Results

All soils from both sites showed evidence of reducing conditions. All samples from site B, including the surface soil, had a low redox potential, +40 to −256 mV (Table 1). Similar amounts of methane were detected in all samples with the exception of one sample from site A, number 195. Ferrous iron also was detected in all samples from both wells. Sulfide was found in all samples ≥100,000 years old.

Direct counts of AO stained cells yielded high numbers of bacteria, 10^7–10^8 cells/g. Similar numbers were obtained by DAPI staining of samples of similar age (Tiedje et al. 1994).

MPN incubations showed evidence of viable denitrifiers, acetoclastic methanogens, hydrogenotrophic methanogens, Fe(III) reducers, and sulfate reducers in some of the aged frozen soils (Table 2). The denitrifiers and hydrogenotrophic methanogens were found in higher numbers and in the oldest layers. Acetoclastic methanogens and sulfate reducers were found in low numbers, near the detection limit, and not in all samples. A high number of acetoclastic methanogens (10^6 cells/g soil) was detected in the modern soil (sample 8), for which the methane concentration in the tubes was 10,000 ppm. In the other samples the methane concentration was not more than 400 ppm when produced from either acetate or $CO_2 + H_2$. The background level of methane in noninoculated tubes was 50 ppm. Iron-reducing bacteria were only found in three samples of moderate age (from modern to 10,000 years). Sulfate-reducing bacteria were detected in half of the samples without a specific pattern.

Sediment samples from site B incubated with radioactive sulfate showed considerable sulfate reduction to sulfide (Table 2). After 6 months of incubation at 4°C, the fraction of radioactive label found in sulfide was as high as 27.0%. At site A only minor sulfate-reducing activity was detected.

Discussion

The results demonstrate that facultative and strictly anaerobic bacteria retained their viability at temperatures in the range of −7 to −12°C for very long periods of time, from several thousand to 2 million years. This may require some unique adaptive mechanism(s) to survive these stresses. The anaerobic microorganisms were generally evenly distributed throughout the geological section. This pattern is unlike that found previously for aerobic microorganisms, which were present in higher numbers only in the peaty, organogenic horizons rather than the mineral horizons (Gilichinsky et al. 1992).

TABLE 2 MPN estimates of anaerobic bacteria in buried permafrost soils, and sulfate-reducing activity of these sediments

Sample number	Direct count ($\times 10^8$/g)	Cultured organisms/g[a]					SO_4^{2-} reducing activity[b], $^{35}S^{2-}/^{35}SO_4^{2-}$
		Denitrifiers	Acetoclastic methanogens	Hydrogenotrophic methanogens[c]	Fe(III) reducers	Sulfate reducers	
Site A							
195	2.4	1.2×10^5	0	1.2×10^7	0	0	0.1
1332	0.6	2.5×10^5	0	2.5×10^7	0	0	0
2411	2.2	2.3×10^3	0	2.3×10^7	0	2.3×10^2	0
2681	1.3	2.0×10^6	0	2.0×10^7	0	0	0
Site B							
8	3.7	2.0×10^7	2.0×10^6	2.0×10^7	2.0×10^3	2.0×10^2	0.3
323	1.5	2.5×10^5	0	2.5×10^7	2.5×10^3	0	0
188	1.4	2.5×10^6	0	2.5×10^7	2.5×10^3	0	0.3
101	3.2	2.0×10^6	2.0×10^2	2.0×10^7	0	2.0×10^2	19.5
399	0.4	2.0×10^4	2.0×10^3	2.5×10^7	0	2.5×10^2	27.0
269	0.5	2.5×10^3	0	2.0×10^7	0	2.0×10^2	10.3
352	0.2	2.0×10^6	0	2.0×10^4	0	0	25.6
256	0.2	2.5×10^7	0	2.5×10^7	0	2.0×10^2	0.1

[a]Incubated at 15°C for 1.5 months under anaerobic conditions. Methanogen MPN tubes were scored again after 5.5 months with essentially the same results.
[b]Ratio of ^{35}S after incubation at 4°C for 6 months under anaerobic conditions.
[c]Methane production much lower than for known methanogens in this assay.

The capacity of methanogens to retain viability in permafrost conditions is important not only as an interesting biological phenomenon but as a potential source of greenhouse gas. It is well known that modern hydromorphic tundra soils produce methane with a net CH_4 flux to the atmosphere (Cicerone and Oremland 1988; Torn and Chapin 1993). Our previous investigation and this work have revealed that the methane content in permafrost soils is indistinguishable from that in modern tundra soils. This is important because global warming could both release methane from the modern permafrost soils and stimulate methanogens in the upper layer of the permafrost. Our previous studies revealed discrete pockets of methane as well as the absence of methane within some permafrost layers (Rivkina et al. 1992). It is extremely unlikely that such a discontinuous distribution could have arisen solely from the passive diffusion of methane from deeper horizons. Rather, the observed methane distribution argues for methane formation in situ. Finding viable methanogens in the aged permafrost provides an explanation for local production. The low methane production in the MPN tubes is noteworthy, since known methanogens would produce much more methane under these conditions, as was observed for one of the surface soils studied here. Permafrost conditions, however, may not have selected typical methanogens. Whatever the responsible organisms, the low rates of production can have geologic and atmospheric significance. Undoubtedly most of the methane was formed within these sediments before they were frozen, but the presence of viable methanogens in permafrost sediments raises the question as to whether these methanogens are active at native permafrost temperatures.

Research in this region by Zigert (1987) has revealed, in relatively young permafrost sediments (up to 40,000 years BP), acid-soluble sulfide minerals that were identified as greigite and mackinawite. These early diagenetic minerals are usually abundant in modern freshwater and marine bottom sediments (Lein 1978; Samarkin et al. 1992). They are metastable and, depending on the conditions, either turn into pyrite or are oxidized at later diagenetic steps. The long-term conservation of authigenic sulfides and the activity of sulfate-reducing bacteria at 4°C leads to the hypothesis that these minerals as well as methane were formed in situ within the permafrost. The long-term conservation of sulfides also argues for the existence of reducing conditions within these sediments, which may be more favorable for preservation of anaerobic bacteria.

Future investigation of permafrost sediments should focus on whether these bacteria are metabolically active, producing geochemically important products at in situ temperatures −9 to −12°C. The present exploratory work was done at temperatures above freezing but is suitable for known psychrophiles. It will be important in future work to solve the challenging problem of measuring activity in the frozen state.

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