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Thermophilic, anaerobic bacteria isolated from a deep borehole in granite in Sweden

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ABSTRACT **A borehole drilled to a total depth of 6779 m in granitic rock in Gravberg, Sweden, was sampled and examined for the presence of anaerobic, thermophilic, fermenting bacteria and sulfate-reducing bacteria. Growth in enrichment cultures was obtained only from water samples collected from a specific sampling depth in the borehole (3500 m). The hole was cased down to a depth of 5278 m and open to the formation below that level. All the water below 2000 m in depth standing in the borehole at the time of sampling must have entered at the 5278-m level or below, during a prior pumping operation. A strong salinity stratification certifies that no major amount of vertical mixing had taken place. The depth from which bacteria could be enriched was that of a pronounced local minimum of salinity. Pure cultures of thermophilic, anaerobic, fermenting bacteria were obtained with the following substrates: glucose, starch, xylan, ethanol, and lactate. The morphology and physiology of the glucose- and starch-degrading strains indicate a relationship to *Thermoanaerobacter* and *Thermoanaerobium* species. All but one of the newly isolated strains differ however from those by lacking acetate as a fermentation product. The glucose-degrading strain Gluc1 is phylogenetically related to *Clostridium thermohydrosulfuricum*, with an evolutionary distance based upon rRNA sequence comparisons of 3%. No sulfate-reducing or methanogenic bacteria were found.**

The examination of indigenous groundwater microorganisms has mostly been restricted to studies at the first few hundred meters below the surface level (1–3), where both aerobic and anaerobic bacteria were found to be present. Their metabolic activities in groundwaters have been examined extensively (3–5), partly for their importance for degradation of xenobiotics in polluted soil and groundwater (6, 7). Most of these bacteriological examinations of groundwater were done with water from sediment formations. Much less information is available about the diversity and metabolic activity of microorganisms in groundwater bodies enclosed in deep solid rock formations. Recently one study presented data about microbial populations in deep granite groundwater to a maximum depth of 860 m below surface level (8). This microbial community consisted of heterotrophic aerobic bacteria, but methanogens and sulfate-reducing bacteria could also be detected. However, in all these studies the temperature of the examined groundwater was below 20°C, and therefore mostly mesophilic bacteria were isolated. By entering deeper geological layers, the temperature will continuously increase, and at levels deeper than 2000–3000 m, temperatures above 60°C–70°C may be reached. Microbial populations that might live in water bodies at or below this depth should therefore differ from populations in higher groundwater and consist only of thermophilic or extreme-thermophilic bacteria. These specialized microbial communities have so far been studied only in hot springs, hydrothermal vents, or solfataras (9, 10). However, a spore-forming, thermophilic, sulfate-reducing bacterium, *Desulfotomaculum geothermicum*, has been isolated from anoxic geothermal groundwater from a depth of 2500 m with an *in situ* temperature of 58°C (11).

In the present study, groundwater from a borehole in granitic rock with a maximum depth of 6780 m was examined microbiologically. The deepest water sample accessible from this borehole was at a depth of 4100 m. Samples from the water column in the borehole were examined for the presence of thermophilic, fermenting bacteria and sulfate-reducing bacteria. Bacteria could be enriched only in samples from a distinct depth (3500 m), and these were fermentative; no sulfate-reducing bacteria were found.

MATERIALS AND METHODS

Sampling Technique. The investigated borehole was Gravberg 1 in the interior of the Siljan Ring, an ancient meteorite impact site in central Sweden. The hole was drilled in granite, in the complete absence of any sediments, to a final depth of 6779 m (true vertical depth). Samples from the borehole water were obtained after the drilling had been stopped for 4 months and the pump had been removed. The water standing in the borehole below the pump and its packer, at a depth a little shallower than 4000 m, had all entered from the formation. The hole was securely cased from a depth of 2000 m down to 5278 m, below which there was perforated (production) pipe and open hole down to the total depth. Any water that was sampled below the position of the pump and its packer must have entered from the formation at 5278 m or below. Between the surface and 2000 m depth, there may have been an accidental perforation in the casing, but if any water had entered there it could not have mixed with the water standing below 4000 m. When the packer was released and the pump was pulled out, the borehole was full of water almost to the top. For sampling of the borehole water, a polyamide hose with an inner diameter of 6 mm and a check valve at the lower end was introduced into the well with the valve in an open position. Pieces of 200 m in length were connected with brass couplings. The final depth of 4100 m for the sampling was reached after 8 hr, and the hose was left in the well overnight. After closing the check valve, the hose was taken up in 600-m pieces and the contents of each piece were pressed out by use of nitrogen and placed in sterile 1-liter glass bottles pregased with nitrogen. These glass bottles were directly transported to the laboratory, where they were stored at room temperature. A total of 90 samples were obtained, each representing a 48-m interval of the hose. Samples 11, 23, 28, 44, 57, 83, and 88 contained only 200–700 ml of borehole water, representing shorter intervals of the hose. The numbering of the samples started with the deepest part of the hose.

Media and Growth Conditions. For enrichment of fermenting bacteria from the water samples, three different media

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were used. (*i*) Water from the respective sample was placed in glass tubes (volume of 13 ml) with plastic screw caps and supplemented with the different substrates (see below), 0.1% yeast extract, and 0.1 g of hematite. Then the closed tubes were incubated at 60°C. (*ii*) One milliliter of each sample was added to a CO_2-buffered, sulfide-reduced saltwater medium. The medium was essentially the same as described (12). Trace element solution SL10 (13), selenite/tungstate solution (14), 0.1% yeast extract, and the respective substrates were added from sterile stock solutions after autoclaving. Hematite (0.1 g per tube) was added to each tube separately. (*iii*) For enrichment of alkaliphilic, halophilic bacteria, a medium was developed according to the chemical composition of the borehole water (for a depth of more than 4000 m). This medium contained NaCl (100 g/liter), KCl (2 g/liter), NH_4Cl (1 g/liter), $MgCl_2 \cdot 6H_2O$ (20.1 g/liter), KH_2PO_4 (0.5 g/liter), $CaCl_2 \cdot 2H_2O$ (0.24 g/liter), and $NaHCO_3$ (0.2 g/liter). After autoclaving, the medium was cooled under a gas mixture of N_2/CO_2 (9:1, vol/vol), and the pH was adjusted to 8.5–8.9. The medium was then placed in tubes with screw caps; supplemented with 0.1% yeast extract, the respective substrates, and 0.1 g hematite; and inoculated with 1 ml of sample water. All enrichment cultures were incubated at 60°C in the dark, and all were done in triplicate.

Substrates added to the enrichment cultures were formate (20 mM), acetate (20 mM), glucose (5 mM), fructose (5 mM), sucrose (5 mM), starch (0.1%), xylan (0.1%), pectin (0.1%), chitin (some particles per tube), lactate (20 mM), ethanol (30 mM), methanol (20 mM), glycerol (20 mM), gallate (5 mM), benzoate (5 mM), toluene (10 μl/10 ml), hexadecane (10 μl/10 ml), and gelatin (0.1%). The enrichment cultures with lactate, gelatin, benzoate, and acetate were carried out both with and without 20 mM Na_2SO_4 as possible electron acceptor for sulfate-reducing bacteria. The enrichment cultures were checked for growth visually and microscopically. The deep agar culture technique (15) was applied to isolate bacteria from enrichment cultures that showed growth.

Chemical Analysis. The conductivity of the water samples was measured with a CDM-3 conductivity meter (Radiometer, Copenhagen). The sulfide concentration was determined by the method described by Cord-Ruwisch (16).

RESULTS

Chemical Analysis of the Samples. The optical appearance of the samples varied among the samples and even changed during storage in the laboratory. The samples from the deepest collection points in the borehole (numbers 1–10, corresponding to 4100–3600 m in depth) were clear, light yellow, and contained only few black sediment particles. These particles reacted to a magnet and therefore are assumed to be mostly magnetite. Samples 11–16 (corresponding to 3500–3370 m in depth) were clear yellowish in the beginning but turned more black after several days of storage in the laboratory. After this change in color, a smell of hydrogen sulfide was noticed in the samples, but a chemical analysis showed that the concentration was below 1 mM. The black sediment in samples 11–16 reacted only partly to a magnet and is therefore assumed to be a mixture of magnetite and iron sulfide (FeS). Samples 17–90 (3400 m to surface level) were more or less yellowish and contained varying amounts of brown sediment. None of these sediments reacted to a magnet or changed color during storage.

The pH, measured after receiving the samples, ranged from 5.5 in the deepest to 6.6 in the upper samples. The conductivity of the samples was measured, and the results are shown in Fig. 1. The highest values for the conductivity were found in samples 1–11, whereafter a rapid drop occurred to reach a local minimum in samples 13 and 14. With increasing height, the conductivity also increased and reached a local maximum

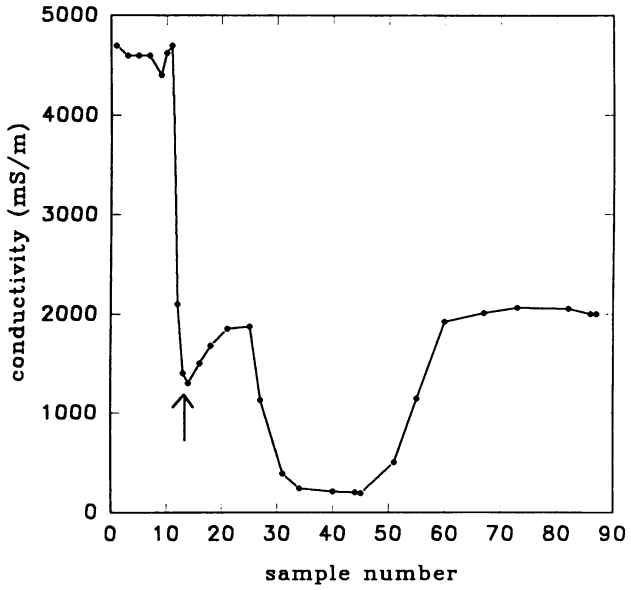

FIG. 1. Conductivity measured in water samples from the borehole. The numbering of the samples starts with one at the deepest part of about 4100 m and ends with number 90 at the surface. Each sample contained ≈1 liter of borehole water, which corresponded to a depth interval of about 48 m. The two local minima in the conductivity profile of the borehole water are interpreted as regions where freshwater from the surrounding rock had accumulated. The freshwater at about 3500 m sampling depth, from which bacteria were isolated (sample numbers 13 and 14; arrow), is thought to originate from 5278 m or below because the housing in the borehole was completely cased above this level. The other freshwater peak may have derived from a small leak in the casing at ≈2000 m.

at samples 21–25, with a following decrease and consistent low values for samples 31–45. After this minimum, the values increased again and at sample 60 reached the same range as those in samples 21–25. These values were stable up to surface level. The two local minima in the conductivity profile of the borehole are interpreted as regions where freshwater from the surrounding rock had accumulated. The water at about 3500-m sampling depth is thought to have originated from 5278-m depth or below. The freshwater peak at shallower levels may have derived from the small leak in the casing at ≈2000 m. However, the stratification seen in the salinity record makes it clear that no water from shallow levels mixed with water below the pump after its removal. All samples had a strong smell of oil, but small droplets of oil were visible only in the upper samples.

Enrichment Cultures. For the enrichment cultures, two synthetic saltwater media with low and high salinity were used as well as water from the samples, which was enriched with different added substrates. The cultures were checked for growth by measurement of turbidity and microscopic examination. Already 2–3 hr after inoculation of the sulfide-reduced medium with the borehole water, a strong precipitation was observed in all cultures. Microscopic control revealed, however, that the turbidity was caused by inorganic crystals. After 3–5 days, an increase in turbidity due to bacterial growth was observed in all parallel enrichment cultures with glucose from samples 13 and 14, but only in the sulfide-reduced medium and in the sample water. During the following weeks, growth was also observed with the following substrates: ethanol, lactate, gelatin, starch, xylan, and benzoate. For these substances, growth occurred in the enrichment cultures with original sample water from samples 13 and 14 but not in the synthetic media. Growth with gelatin and benzoate was very weak, but clearly better than in the controls with only yeast extract. No growth was found in the

enrichment cultures from the other samples and on the other substrates tested even after 4 months of incubation. It is noteworthy that neither sulfate-reducing nor methanogenic bacteria could be enriched. One main substrate for methanogenic and sulfate-reducing bacteria, H_2/CO_2, was, however, not tested.

The bacteria from the enrichment cultures were subsequently transferred to new tubes with the sulfide-reduced medium. The enrichment cultures growing on ethanol, lactate, starch, and xylan could be established in this medium, whereas the cultures on gelatin and benzoate could not.

In the enrichment cultures growing on glucose, a change in color was observed in the hematite, which turned black in the surface layer of the sediment. The black particles were not attracted by a magnet and may be iron sulfide.

Pure Cultures. From the transferred enrichment cultures, pure cultures were obtained by applying the deep agar culture technique for glucose, starch and, recently, xylan. The bacteria growing on ethanol or lactate, respectively, form colonies in the deep agar cultures, but all attempts to transfer these bacteria to liquid medium have so far not been successful.

Further characterization of the glucose- and starch-degrading strains revealed a temperature optimum around 65°C, the lowest and highest temperature supporting growth were 45°C and 75°C, respectively. All four strains were dependent on yeast extract. The salt tolerance of the strains was quite broad and ranged from 0.1% to 5% NaCl. All strains were rod-shaped with a tendency to form filaments and spheroplasts during late logarithmic phase. Spore formation was never observed for any of the strains under any of the conditions tested. Fermentation products from glucose or starch were ethanol, lactate, CO_2, and hydrogen. Only one of the glucose-fermenting strains, Gluc2, produced acetate. Species descriptions of the different strains are under preparation.

The 16S rRNA of strain Gluc1 was kindly sequenced and identified phylogenetically by the Ribosome Database Project at the University of Illinois, indicating a close relationship to *Clostridium thermohydrosulfuricum* with an evolutionary distance of 3%.

DISCUSSION

The water samples from the borehole in Gravberg 1 showed a great range of salinity values. The deepest local minimum of salinity is represented by the sampling depth of about 3500 m (samples 13 and 14; Fig. 1). This water, which is thought to originate from a depth of 5278 m or below, would have come from a formation whose temperature was between 65°C and 75°C. Interestingly these two water samples were the only ones from which thermophilic bacteria could be enriched and isolated. Since the problem of contamination during operation and sampling in a deep borehole is obvious and critical, the fact of finding thermophilic bacteria only in water deriving from rock in the same temperature range as that in which they could be cultured is a good indication that these bacteria represent members of the autochthonous flora. Nutrients brought in with the drilling fluid may have caused an enrichment of locally resident bacteria (zymogenous flora).

During the last decade, many new species of anaerobic, thermophilic bacteria have been described. For the enrichment of thermophilic organisms, two main sources have been used. (*i*) Soil, sewage sludge, and sediments from surface waters contained mostly members of the spore-forming genera *Clostridium* and *Bacillus* (17, 18). (*ii*) Hydrothermal vents and hot springs harbor populations of thermophilic and extreme-thermophilic Archaea and Bacteria belonging mostly to genera that have been newly defined.

The physiological data obtained for the newly isolated strains were compared with those of already described strains. The new strains isolated on glucose and starch shared most characteristics with members of the genera *Thermoanaerobacter* (19, 20), *Thermoanaerobium* (21, 22), and some *Clostridium* species. These genera as well as some thermophilic Clostridia were recently reclassified (23). Several members of these genera were isolated from hot springs or similar environments. However, strain Gluc1 differs markedly from all these genera by lack of acetate as a fermentation product. The two strains isolated on starch differ for the same reason, no acetate as end product, from the so far described bacteria. Thermophilic bacteria growing on ethanol or lactate have, to our knowledge, not been described yet.

So far it is not possible to draw any further conclusions on the ecology of the populations in the rock formation reached by the borehole. Indeed, conclusive evidence regarding the physiological significance of microorganisms in this environment will require *in situ* studies, which are not now possible. Our study concentrated on the enrichment and isolation of a small part of the microbial community—namely, thermophilic, fermenting bacteria growing at atmospheric pressure. The question about the carbon sources for these bacteria in the deep groundwaters remains open. The application of drilling aids renders it possible that the isolated bacteria represent members of the zymogenous flora and therefore only a portion of the indigenous flora. As possible carbon sources for the bacteria at the basis of the undisturbed food chain, volcanic gases (H_2, CO, CO_2, CH_4) might be considered, some of which were found during the drilling process (24) and would allow growth of methylotrophic, carboxydotrophic (25), and other autotrophic bacteria (methanogenic, homoacetogenic). Even more complex organic substances like hydrocarbons have been found in these deep formations (24). For further studies including these substrates, high pressure conditions, which more adequately reproduce the conditions at depth, must be included.

Fine-grained magnetite was found in large amounts in the borehole: some 12 tons were pumped up (24). The formation of such small magnetite grains has previously been associated with the action of bacteria, reducing hematite with hydrocarbons as the electron acceptor (26, 27). In our enrichment cultures, the reduction of hematite was observed, but the end product of this reaction under our enrichment conditions was not magnetite but possibly iron sulfide. None of the so far isolated pure cultures was, however, able to carry out the reaction from hematite to magnetite. No chemical reduction of hematite occurred in the uninoculated control cultures. The finding of thermophilic bacteria in deep rock formations indicates the existence of so-far unknown habitats for such organisms (28). The common occurrence of thermophiles in the near-surface environment may have a relation to a widespread deep subcrustal life.

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