Carbon transformations by attached bacterial populations in granitic groundwater from deep crystalline bed-rock of the Stripa research mine

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This paper presents and compares the assimilation rates of CO₂ and lactate, and the lactate respiration rates, of attached bacterial populations growing in slowly flowing groundwater (1–3 mm s⁻¹) from deep crystalline bed-rock of the Stripa research mine, Sweden. The bacteria studied grew in anoxic, high-pH (9–10) and low-redox artesian groundwater flowing up through tubing from two levels of a borehole designated V2, 812–820 m and 970–1240 m below ground. Bacteria were allowed to attach to and grow on sterile glass microscope slides in laminar-flow reactors connected to the flowing groundwater. Total numbers of bacteria were counted by acridine orange direct counts. The bacteria grew slowly, with doubling times of 34 d at 10 °C for the 812–820 m population, 23 d for the 970–1240 m population at 10 °C and 16 d for this population at 20 °C. Numbers of attached bacteria reached between 10⁶ and 10⁷ bacteria cm⁻². The populations at the two levels of the borehole were different in physiology as well as in phylogeny and reflected the heterogeneity between the sampling levels. The earlier proposed presence of sulphate-reducing bacteria could not be confirmed. This is discussed in relation to results from 16S rRNA gene sequencing studies. The CO₂ assimilation rates (as mol CO₂ cm⁻² h⁻¹, using liquid scintillation techniques) increased with depth and temperature. The quotients calculated for inorganic/organic carbon utilization were between 0.07 and 0.25, indicating that autotrophy could not support the levels of growth observed and that heterotrophy was the dominant carbon transformation process for growth of the studied populations. The Stripa bacteria could further be seen not only to assimilate but also to catabolize lactate and release CO₂, which added to the indications of a heterotrophic dominance in the Stripa environment.

Keywords: deep groundwater, carbon dioxide assimilation, lactate respiration, attached bacteria, heterotrophy

INTRODUCTION

Radioactive waste from nuclear power plants has to be processed and safely deposited in repositories. The present Swedish concept is to encapsulate the waste in steel-copper canisters and surround them with bentonite in crystalline bed-rock, 500 m below ground (SKB, 1991). During 1987, a research project on microbial processes in deep groundwater systems was initiated at the department of General and Marine Microbiology at the University of Göteborg. Our results (Pedersen & Ekendahl, 1990,

1992a, b) and those of others (Erlich, 1990; Gold, 1992; Pedersen, 1993; Pedersen & Albinsson, 1992; Stevens *et al.*, 1993; West *et al.*, 1982, 1985) clearly demonstrate that microbes may be involved in many subterranean geochemical processes, such as diagenesis, weathering and precipitation, and in oxidation/reduction reactions of metals, carbon, nitrogen and sulphur – just as they are in most terranean environments. The aim of the project is to understand how subterranean bacteria interact with these processes during and after the construction of a nuclear waste repository, so as to achieve the safest possible disposal of high-level nuclear waste. The project comprises studies on several interacting microbial processes in groundwater that are important for the performance safety assessment of nuclear waste disposal. Bacteria are

Abbreviations: AODC, acridine orange direct counts; SRB, sulphate-reducing bacteria; TIC, total inorganic carbon.

inevitably interacting with the carbon cycle, thereby affecting pH, redox potential and the organic content of the groundwater. The proposed presence of sulphate-reducing bacteria in groundwater (Fontes *et al.*, 1989; Fritz *et al.*, 1989; Pedersen & Ekendahl, 1992a) would mean the possible risk of canister corrosion through their production of hydrogen sulphide. Growth of bacteria on fracture surfaces and their participation in the formation of fracture minerals, weathering and radionuclide sorption to the rock may also be influential.

Our field investigations have been focused on the Stripa research mine, a former iron ore mine in south central Sweden. We found active bacteria in the anoxic groundwater from the deep granite bed-rock of Stripa (Pedersen & Ekendahl, 1992a) that assimilated carbon dioxide and thus had an autotrophic potential for *in situ* production of organic carbon. They incorporated introduced formate, glucose and leucine; and the incorporation of lactate was especially high, demonstrating a heterotrophic potential. Bacteria attached to glass surfaces were more active than unattached bacteria in the water.

In this work we have measured the rates of CO₂ and lactate assimilation, lactate respiration and biofilm development on glass surfaces in slowly flowing anoxic groundwater from depths ranging from 800 to 1200 m in a borehole in the Stripa research mine. We compared the results both within and between the populations studied. Attempts were also made to determine whether sulphate reduction was performed by the attached bacteria. Possible effects of attached bacterial populations on the carbon cycle in groundwater from deep crystalline bed-rock are discussed, as well as possible bacterial groups.

METHODS

Study site. This study was performed in the Stripa research mine in Guldsmidshyttan, Västmanland, Sweden. The mine contained iron ore, a quartz-banded haematite occurring in a lepatite formation, which was mined out in 1976. The mine was then used for underground research, for both national and international (OECD/NEA) studies, until it was closed in 1991.

Experimental set-up. The borehole V2 (76 mm wide) was drilled subvertically through medium-grained granite adjacent to the lepatite from a mine drift 410 m below ground to 860 m in 1977 and continued to 1240 m in 1981. The borehole had an average of about two fractures per metre of rock. The flowing groundwater studied came from two levels of this borehole, 812–820 m and 970–1240 m. The levels were closed off with packers made of inflatable 76 mm rubber tubes. The artesian water from these levels has been continually flowing up to the drift through 6 mm teflon tubing since 1990 and its chemical composition has been well characterized (Nordström *et al.*, 1985). Laminar-flow reactors (Pedersen, 1982; Pedersen *et al.*, 1986) were connected to the slowly flowing anoxic groundwaters at flows of approximately $1 \times 10^{-3} \text{ m s}^{-1}$, as shown in Fig. 1. Each reactor contained 12 + 24 parallel hydrophilic microscope glass slides, $60 \times 24 \times 1$ and $40 \times 24 \times 1$ mm, respectively. The glass slides had been heated in a muffle furnace at 475 °C for 4 h. The waters were chilled to 10 °C when flowing through the tubing up to the drift. The 970–1240 m

Fig. 1. Experimental setup with laminar-flow reactors connected to flowing groundwater from the Stripa borehole V2.

groundwater was re-heated to 20 °C in a hut in the drift to imitate the *in situ* temperature in the borehole (Fig. 1). The flowing waters were collected from sterile 5 l glass bottles connected to the laminar-flow reactors. All water used for measurements and incubations was taken from these bottles unless otherwise noted.

Water analysis. The pH and the E_h were measured *in situ* in the mine with a PHM Autocal pH meter (Radiometer), a GK2421C combined pH electrode and redox electrode PK1401. Sulphate was measured turbidimetrically with BaCl₂ (Franson, 1985), sulphide by an iodometric method (Franson, 1985) and the total inorganic carbon (TIC) contents were measured with a coulometer (model 5011 CO₂ Coulometer) (Huffman, 1977). These procedures were repeated at a 30 d interval.

Oxygen was measured as follows. Winkler bottles for each level and temperature were filled with groundwater under N₂ atmosphere. The bottles were stored at +4 °C and a modified form of the common Winkler method was used for the analysis (Carritt & Carpenter, 1972). Measurements of other gases are reported in Pedersen *et al.* (1991).

Total numbers of unattached and attached bacteria. Acridine orange stained direct counts (AODC) were used to determine the total numbers of unattached bacteria, as described earlier (Pedersen & Ekendahl, 1990). Groundwater bacteria were allowed to attach to and grow on the glass slides in the laminar-flow reactors for up to 161 d for the 812–820 m level (January to June 1991) and 90 d for the 970–1240 m level (April to June 1991). The bacteria were stained in acridine orange, rinsed with distilled water and the total number of cells was counted.

Assimilation of CO₂. Incubations for assimilation measurements were done anaerobically since the groundwater was essentially anaerobic. Groundwater, sampled from the two borehole levels, was pressure filtered in 20 ml volumes under N₂ atmosphere

through Dynagard hollow-fibre syringe filters (pore size $0.2\ \mu\text{m}$), into sterile 50 ml polypropylene centrifuge tubes with lids (Nunc). Microscope slides from the laminar-flow reactors were transferred under nitrogen atmosphere to tubes with corresponding filtered groundwater, one slide per tube. Labelled carbonate, $\text{Na}_2^{14}\text{CO}_3$ (Amersham Sweden), was subsequently added in 1 ml oxygen-free portions to assay the CO_2 assimilation of the populations studied. The specific activity used was $2.06 \times 10^9\ \text{Bq mmol}^{-1}$ and the final concentration of labelled $[^{14}\text{C}]$ carbonate in the tubes was $20\ \mu\text{M}$. This chosen concentration was high so as to permit detection of assimilation by few bacteria having low activities and slow growth. The unlabelled TIC concentration in the groundwater was $50\ \mu\text{M}$ at the 812–820 m level, and $57\ \mu\text{M}$ at 970–1240 m (Table 1). Thus the summed TIC concentrations (labelled and unlabelled) in the tubes were 70 and $77\ \mu\text{M}$, respectively. One millilitre of an unlabelled oxygen-free lactate solution (pH 10, final concentration $10\ \mu\text{M}$) was added as an organic carbon source to one set of tubes. The microscope slides were incubated horizontally for 1–4 h at 10 or 20°C with slow shaking, after which formalin was added to a final concentration of 2% (v/v). Controls for abiotic adsorption of the labelled carbonate were achieved by addition of formalin (2%) to clean and sterile sample slides together with the labelled compound at sampling, and processing them as the other samples. Subsequently, each slide was rinsed with a surface rinse (Pedersen *et al.*, 1986) containing distilled water with 1% (w/v) NaCl (pH 3.0), cut into four pieces (Pedersen & Ekendahl, 1992b), placed in 20 ml scintillation cocktail (Ready Safe, Beckman) and radioactivity measured in a Beckman LS 3801 scintillation counter. Control counts were subtracted from sample counts.

Doubling the ^{14}C concentration in the tubes from 20 to $40\ \mu\text{M}$ resulted in a corresponding increase in ^{14}C assimilation, but the uptake rate was not affected by this concentration change. The isotope contents of the liquid in the tubes were measured just before the addition of formalin by taking out $0.1\ \text{ml}$ into 10 ml Ready Safe. The total radioactivity in each tube was calculated as the sum of the radioactivity in the liquid and on the glass slide. The sums were equal to the added amounts and had a standard deviation of between 2.4 and 6.2% of the mean value with $20\ \mu\text{M}$ and of 13% with $40\ \mu\text{M}$, showing that the added isotope did not disappear during the experiment and that the tubes had similar ^{14}C activities.

During sampling, pH and E_h were measured *in situ* in series parallel with the assimilation experiments to check that incubation conditions were constant. Tubes were prepared without slides but with unlabelled compounds added to the filtered groundwaters. pH in the tubes decreased somewhat (maximum 0.6 units) during incubation. The redox potentials were stable. Temperature had no detectable influence on either pH or E_h .

Calculations of CO_2 assimilation/total cell carbon ratios. The quotient between CO_2 assimilated and carbon demanded for growth was calculated as follows. The carbon content of mean-sized bacteria was calculated from their volumes. This was done by measuring the length and width of the bacteria with a ruler directly on slide projections with biofilm bacteria from the reactors. Two slides from each level and temperature were used, and 50 cells were measured on each of them. The volume per bacterium (V) was calculated according to Grigorova & Norris (1990):

$$V = (\pi/4) \cdot D^2 \cdot (L - D/3) \quad (1)$$

where D is width and L is length.

A conversion factor for the calculation of the carbon content of a mean-sized bacterium was determined by multiplying a 30% dry weight of a cell (Neidhardt *et al.*, 1990), a 50% dry weight carbon content (Neidhardt *et al.*, 1990; Stanier *et al.*, 1990) and an assumed bacterial density similar to water of $1 \times 10^{-12}\ \text{g}\ \mu\text{m}^{-3}$. The conversion factor, F , calculated in this way, was $1.5 \times 10^{-13}\ \text{g C}\ \mu\text{m}^{-3}$ or $0.125 \times 10^{-13}\ \text{mol C}\ \mu\text{m}^{-3}$. For comparison, Grigorova & Norris (1990) compiled several direct biomass estimations where a majority of the investigators had used epifluorescence microscopy for cell volume estimates and CHN analyses for cell carbon measurements. These conversion factors ranged from 0.5×10^{-13} to $6.5 \times 10^{-13}\ \text{g C}\ \mu\text{m}^{-3}$ and showed large coefficients of variation. Grigorova & Norris (1990) recommend the average conversion factor $3.1 \times 10^{-13}\ \text{g C per}\ \mu\text{m}^3$ of bacteria.

The measured amount of carbon assimilated per bacterium at a density of $1.8 \times 10^6\ \text{cells cm}^{-2}$ was divided by the carbon demanded for growth for one bacterium, taking into consideration the doubling time of the population. This quotient, here called Q , shows the autotrophic potential of the population. The following formulae were used:

$$Q = A/B \quad (2)$$

where A is assimilated carbon as mol C per bacterium per hour (results taken from Table 3) and B is carbon demanded for growth, and

$$B = (F \cdot V \cdot k) / (\log 2 \cdot 24) \quad (3)$$

where F is the conversion factor (see above) and V is cell volume, and k is the bacterial growth rate constant, determined from linear regressions during the growth period from day 27 onwards.

Lactate assimilation and respiration. Lactate assimilation of attached bacteria was measured with the same tube setup and methods as in the CO_2 incubations, i.e. with 20 ml filtered groundwater and one glass slide in each tube. The tubes contained 1 ml of 1.0 or $2.5\ \mu\text{M}$ [$\text{U-}^{14}\text{C}$]lactate, specific activity $5.69 \times 10^9\ \text{Bq mmol}^{-1}$, and were closed with rubber stoppers and incubated anaerobically as described before. Respiration was measured as shown in Fig. 2. To each tube with labelled lactate $0.8\ \text{ml}$ $0.3\ \text{M}$ HCl was added after incubation. The tubes were shaken, N_2 gas was bubbled through for 15 min and the released $^{14}\text{CO}_2$ originating from the respired lactate was precipitated in 20 ml serum bottles preceded by an empty safety bottle. The three traps each contained 10 ml $1.9\ \text{M}$ KOH. Two subsamples of 0.1 and 1 ml were taken from each trap and put

Fig. 2. Experimental setup for measuring lactate respiration and sulphate reduction.

Table 1. Major parameters of the Stripa borehole V2

Sampling depth (m)	pH	E_h (mV)	Temp.* (°C)	SO_4^{2-} (μM)	S^{2-} (μM)	TIC (μM)	O_2 (μM)	Conductivity (μS cm ⁻¹)	Flow rate (m s ⁻¹)
812–820	9.4	+199	18	1433	106	50	3.4	1640	2.8×10^{-3}
970–1240	10.2	–3	26	520	233	57	0	1180	0.5×10^{-3}

* At sampling depth in the borehole.

into glass vials with 10 ml scintillation cocktail (Hionic Fluor, Packard). The radioactivity was measured as d.p.m. in the scintillation counter. Background controls were made with 0.1 and 1 ml of the KOH solution in Hionic Fluor and subtracted from sample counts. Then the slides were cut as described for CO_2 assimilation. Also, duplicate controls showed that lactate did not release CO_2 when HCl was added. The closed tubes did not release any CO_2 . The total isotope content in each tube (radioactivity in the liquid and on the glass slide), was measured after the respiration measurements. The values had a standard deviation of between 3.5 and 8.4 % of the mean and showed that the added concentration in the tubes was reproducible and that the isotope did not disappear during the experiment.

Sulphate assimilation and reduction. Sulphate assimilation and reduction to sulphide were measured with a tube setup similar to that for lactate respiration (20 ml filtered groundwater, one slide and 1 ml oxygen-free isotope solution per tube) and the methods used for lactate assimilation and respiration measurements (see Fig. 2), but with the following changes. The isotope was $Na_2^{35}SO_4$ with a specific activity of 4×10^7 MBq mmol⁻¹, giving a final concentration of ^{35}S in the tubes of 2.8 or 4.2 nM. The tubes also contained 1 ml unlabelled lactate to a final concentration of 10 μM. The sulphate reduction was stopped by precipitating H_2S with 5 ml 20 % (w/v) zinc acetate (Fossing & Jørgensen, 1989) and the tubes were immediately frozen on dry ice. Acidification (giving a pH below 2) was done with 1.5 ml 5 M HCl. The traps contained 10 ml 5 % zinc acetate + 0.1 % acetic acid (Fossing & Jørgensen, 1989). N_2 was bubbled through for 30 min. From each of three traps 3 ml subsamples were mixed with 15 ml Hionic Fluor. Testing the method with sulphate reducers previously isolated from another groundwater (Pedersen & Ekendahl, 1990) and subcultured in the laboratory showed that the method worked very well.

RESULTS

Chemistry of the groundwaters

The results from the water analysis are shown in Table 1. These data differ between the sampling depths, indicating that the groundwaters were obtained from fracture systems with no close hydraulic connections.

Growth and total number of bacteria

The total numbers of unattached bacteria are presented in Table 2. There were more bacteria present at the deeper level; the variation in the numbers of bacteria was low between the sampling occasions. Fig. 3 shows total

Table 2. Total numbers of unattached bacteria of the Stripa borehole V2

Sampling date (1991)	N*	$10^{-5} \times \text{Bacteria ml}^{-1} \dagger$	
		812-820 m	970-1240 m
Groundwater sampling			
15 April	3	0.13 (59)	3.4 (21)
29 May	2	0.23 (14)	2.5 (11)
25 June	2	0.22 (49)	1.4 (79)

* N is the number of independent samples.

† SD (%) shown in parentheses.

Fig. 3. Total number of attached bacteria on surfaces exposed to flowing groundwater from the Stripa borehole V2 for different times. ●, 812–820 m, 10 °C; ■, 970–1240 m, 10 °C; ▲, 970–1240 m, 20 °C.

numbers of bacteria on slides after attachment and growth for up to 161 d. The bacteria appeared in micro-colonies on the surfaces, which indicates growth. This is shown on SEM images in Fig. 1 of Ekendahl *et al.* (1994). The total numbers after 43 d were between 10^6 and 10^7 bacteria cm⁻², as compared to total numbers in the liquid phase in the region of 10^4 ml⁻¹ for 812–820 m and 10^5 ml⁻¹ for

Fig. 4. *In situ* assimilation rates of CO_2 without and with the addition of $10 \mu\text{M}$ unlabelled lactate, assimilation rates of ^{14}C from $[\text{U-}^{14}\text{C}]\text{lactate}$, and respiration rates of assimilated $^{14}\text{C}[\text{lactate}]$, measured as $^{14}\text{CO}_2$ evolution, by attached bacteria in groundwater from the Stripa borehole V2. ●, 812–820 m; ■, 970–1240 m, 10 °C; ▲, 970–1240 m, 20 °C. The dashed line in each panel represents 1.8×10^6 bacteria cm^{-2} , and is used for calculations of CO_2 assimilation rates in Table 3.

970–1240 m. Linear regression from the 27th day onward gave doubling times for the populations; these are shown in Table 4. The correlation coefficients, r , and probabilities, p , for 812–820 m, 970–1240 m (10 and 20 °C), were 0.94 and 0.06, 0.96 and 0.19, 0.91 and 0.28, respectively.

Assimilation of CO_2 , lactate assimilation and respiration

The assimilation of CO_2 and lactate, and respiration of the added lactate to CO_2 was plotted against the incubation time (4 h, not shown). A detailed description of the calculation criteria is given by Pedersen & Ekendahl (1992b). Briefly, assuming that the assimilation was correlated with time, only data that in a linear regression had a correlation coefficient larger than 0.7 and a significance level larger than 95% were further examined. We assumed the slope of each line to reveal the *in situ* rate of assimilation and respiration, as $\text{mol cm}^{-2} \text{h}^{-1}$. These rates are summarized in Fig. 4, and presented in detail by Pedersen *et al.* (1991). Generally the rates were higher when there were more bacteria present. The bacteria did assimilate CO_2 and lactate and also respired lactate into CO_2 .

Table 3 compares rates of assimilation and respiration by an average attached bacterium at a density of $1.8 \times 10^6 \text{ cm}^{-2}$ in the different populations. The rates were higher in the population from 970–1240 m than in that from 812–820 m. Raised temperature gave higher assimilation rates.

CO_2 assimilation/total cell carbon ratios

The evaluation of the quotient Q between CO_2 assimilated and carbon demanded for growth gave the results shown in Table 4. For example, in the 812–820 m population, the doubling time was 34 d (assuming that growth from days 27–161 was linear). This gives the result that one bacterium demanded $1.42 \times 10^{-17} \text{ mol C}$ per hour to be built up. The CO_2 assimilation rate was $1.2 \times 10^{-18} \text{ mol C}$ per bacterium per day at a density of $1.8 \times 10^6 \text{ bacteria cm}^{-2}$ (Table 3). The quotient Q was 0.09 (Table 4). For the 970–1240 m population at 10 °C the corresponding quotient was 0.07 and for this population at

Table 3. *In situ* assimilation rates of CO_2 without and with the addition of $10 \mu\text{M}$ unlabelled lactate, assimilation rates of ^{14}C from $[\text{U-}^{14}\text{C}]\text{lactate}$, and respiration rates of assimilated $^{14}\text{C}[\text{lactate}]$, measured as $^{14}\text{CO}_2$ evolution, by an average attached bacterium at a density of 1.8×10^6 bacteria cm^{-2}

All assimilation and respiration rates are expressed as $\times 10^{-18} \text{ mol h}^{-1}$ per bacterium. ND, Not determined.

Depth (m)	Temp. (°C)	CO_2 assimilation	CO_2 assimilation with $10 \mu\text{M}$ lactate	Lactate assimilation	Lactate respiration
812–820	10	1.2	1.6	0.2	ND
970–1240	10	1.6	3.6	6.0	2.2
970–1240	20	12.9	9.5	9.1	5.4

Table 4. Data for and calculation of the autotrophic capability of the Stripa populations

For a detailed description, see text.

Level (m)	Temp. (°C)	Doubling time (d)	Cell volume, V (μm^3)	Carbon assimilated (A)*†	Carbon required for growth (B)†	'Autotrophy', Q (= A/B)
812–820	10	34	0.92	1.2	14.2	0.09
970–1240	10	23	1.01	1.6	23.1	0.07
970–1240	20	16	1.59	12.9	51.9	0.25

* From Table 3. † Expressed as $\times 10^{-18}$ mol C h⁻¹ per bacterium.

20 °C it was 0.25 (days 27–71). All quotients are well below 1, indicating that autotrophy is far from enough to supply these populations with carbon for growth.

Sulphate assimilation and reduction

Only a few of the slides showed sulphate assimilation that was more than 50 % higher than in the controls. There was no detectable production of sulphide in any of the tubes.

DISCUSSION

The laminar flow reactors have been shown to constitute a reproducible environment for the study of attachment and growth of bacteria (Pedersen, 1982). The experimental variance is very low, making the reactors well suited for comparisons between environments as were done here. Except for pressure and temperature, the reactor environments were believed to parallel the *in situ* chemical and physical environments reasonably well to make such a comparison valid. One of the most important factors governing attachment and growth of bacteria is the bacterial species itself (Pedersen *et al.*, 1986). Depending on species and strain, different attachment patterns are obtained. The opposite process, separation from the surface, occurs during multiplication of irreversibly attached bacteria and as desorption of reversibly attached bacteria in laminar flow systems (Escher & Characklis, 1990). Here, the reactors were used as a first approximation of a fracture surface system with the assumption that some of the dominant bacteria growing on fracture surfaces in the deep environment will detach, move with the flowing water, attach and grow again, eventually in the reactor environment imitating the *in situ* situation.

Both the chemical data and the bacterial numbers counted in the Stripa groundwater reflect a heterogeneity between the sampled levels. This heterogeneity was also observed when the salinity profile was studied (Nordström *et al.*, 1985). It indicates that the groundwaters came from fracture systems without close interconnections. The content of sulphide and trace amounts of oxygen in the

groundwater from the 812–820 m level indicates that this water might have been a mix of oxic and anoxic waters from different fractures. The study of the attached Stripa populations using 16S rRNA gene sequencing (Ekendahl *et al.*, 1994) revealed that there were different populations at each of the two levels studied in this borehole. Three dominant groups of Proteobacteria were found. One of them was found only at 812–820 m, where 63 % of the bacteria belonged to this group. A second existed almost exclusively and also dominating (83 % at 10 °C, 65 % at 20 °C) at 970–1240 m, and a third was equally distributed between the levels (17 % at 812–820 m, 4 % and 17 % at 970–1240 m, 10 and 20 °C, respectively). The assimilation rates of both CO₂ and lactate were faster at the deeper (970–1240 m) compared to the upper level (812–820 m). The increased rates of the 970–1240 m population at 20 °C compared to 10 °C were most likely an effect of temperature, as the enzyme activities in bacteria usually increase when temperature is raised. The population morphology also differed, according to SEM images, but the population structure was not altered to any great extent by the shift in temperature, as shown by our 16S rRNA studies (Ekendahl *et al.*, 1994). The ecosystems sampled thus contained very few species of bacteria showing different metabolic activities and it seems clear that the spatial distribution of bacteria in the rock studied was dependent on the groundwater environment. The 16S rRNA analysis supports the conclusion that the studied populations were different in composition.

The origin of the observed bacteria is difficult to determine. Stevens *et al.* (1993) reported a study on deep groundwater bacteria in drilled artesian wells in Washington, USA, and argued that the anaerobic, alkaline groundwaters favour the growth of aquifer organisms over growth of most contaminants being introduced from the surface. Likewise it can be speculated that the bacteria found were true inhabitants of the Stripa borehole V2. For a more detailed discussion of the origin of these bacterial populations, see Pedersen & Ekendahl (1992a).

Electron acceptors like oxygen, nitrate and nitrite were not available in amounts likely to dominate the metabolism of the Stripa bacteria. Methanogenic bacteria

have earlier been proposed to exist in the Stripa groundwater (Pedersen & Ekendahl, 1992a). They can use CO_2 as both carbon source and terminal electron acceptor to produce methane. Both CO_2 and methane were present in the waters (Pedersen *et al.*, 1991). However, since no Archaeobacteria were found in the 16S rRNA gene sequencing study (Ekendahl *et al.*, 1994), they should not be abundant, at least in the reactor environment.

It was proposed earlier that since the Stripa bacteria readily assimilated lactate anaerobically (Pedersen & Ekendahl, 1992a) the dominant species might be sulphate-reducing bacteria (SRB). Fritz *et al.* (1989) used carbon isotope fractionation to draw the conclusion that SRB were present in the Stripa groundwaters. In addition, Fontes *et al.* (1989) obtained stable isotope data that proved bacterially enhanced redox processes to play an important role in the control of the concentration and heavy isotope contents of aqueous sulphate in Stripa. But sulphate reduction could not be demonstrated, even though sulphate is the most abundant electron acceptor available. Neither could SRB be isolated from the groundwater or from the surfaces (Ekendahl *et al.*, 1994). In the biofilm populations the dominant bacteria belonged to the beta and gamma groups of the Proteobacteria, and not to the delta group, where all known SRB belong (Ekendahl *et al.*, 1994). Therefore the conclusion was made that no or very few SRB were present. This result does not necessarily contradict the stable isotope data. Dating with ^{14}C analysis indicates groundwater ages of 5000–20000 years (Nordström *et al.*, 1985), so the stable isotope profile may have been formed in the past. It is also possible that the sulphate-reducing activity occurred in other geological formations than that intersected by the borehole V2, perhaps during infiltration of surface waters rich in organic carbon.

By comparing the amount of CO_2 assimilated during a certain time and the amount of carbon needed to build up a bacterial cell growing during that time, the capability for autotrophy can be evaluated. The values of quotient Q calculated for carbon utilization were between 0.07 and 0.25 (Table 4), indicating that autotrophy could not support the levels of growth observed and that heterotrophy was the dominant carbon transformation process for growth of the studied populations. The CO_2 assimilation did not decrease consistently (Table 3) when lactate was added, suggesting that there were no autotrophs changing to a heterotrophic metabolism (Hallbeck & Pedersen, 1991) when organic carbon appeared. The measured CO_2 assimilation may thus have been due to heterotrophs using CO_2 for anaplerotic reactions to replace intermediates in the tricarboxylic acid cycle (Prescott *et al.*, 1993). It is important to use several different carbon sources for assimilation experiments, as shown in previous Stripa V2 studies (Pedersen & Ekendahl, 1992a). One reason is that different carbon sources are utilized to varying degrees by the same species. The earlier observed utilizations of added lactate and also glucose, leucine and some formate by parts of the populations (Pedersen & Ekendahl, 1992a) support the

presence of heterotrophs. The Stripa bacteria could further be seen not only to assimilate but also to catabolize lactate and release CO_2 (Table 3), which adds to the indications of a heterotrophic dominance in the Stripa environment.

With the laminar flow reactor as a model system, the amount of inorganic carbon (TIC) flowing through a reactor can be compared with the assimilation of CO_2 by the attached bacteria. The water flow rates were 66.3 ml min^{-1} (812–820 m) and 12.4 ml min^{-1} (970–1240 m). Multiplying the TIC values from Table 1 with the amount of water flowing through the reactor during 1 h, the TIC flow rates were $200 \mu\text{mol h}^{-1}$ (812–820 m) and $42 \mu\text{mol h}^{-1}$ (970–1240 m). Using $1.8 \times 10^6 \text{ bacteria cm}^{-2}$ as a common value for bacterial density (Table 3), there were 1.45×10^9 bacteria per reactor growing on a surface area of 806.4 cm^2 . The CO_2 assimilation data from Table 3 were then multiplied by the numbers of bacteria in the reactor to give the total assimilation during 1 h. These values of total assimilation in the reactor were divided by the TIC flow rate values; the results show that the bacteria assimilated between 10^{-5} and 4.4×10^{-4} of the TIC passing through with the water.

Moving from our reactor model system to a typical hard rock fracture, the water flow rate and the volume/surface area ratio will decrease. Since channelling of the groundwater has been suggested to dominate in bed-rock (Moreno *et al.*, 1988; Pedersen & Ekendahl, 1992a; Tsang *et al.*, 1988) we assumed a fracture width of 0.1 mm with sides of 1 cm^2 (Moreno *et al.*, 1988) and used TIC values from Table 1 and bacterial density and assimilation data from Table 3 in the following calculations. At the 812–820 m level, the flow rate was 2.8 mm s^{-1} (Table 1) and it would take 3.57 s for 0.01 ml of water to pass a channel area of 2.02 cm^2 . During that time $4.3 \times 10^{-15} \text{ mol CO}_2$ would be assimilated by the attached bacteria. The quotient between this calculated figure and the measured TIC (0.5 nmol in 0.01 ml of water, from Table 1) is 8.7×10^{-6} . Thus, during 3.57 s, one part out of approximately 115000 ($1/8.7 \times 10^{-6}$) of the TIC in the water would be utilized for each cm of bed-rock channel through which the groundwater flows. Considering a 'plug flow' (the water moves without mixing with new water), the TIC would be depleted after a flow distance of 1.15 km or after 115 h, provided no addition of carbonate from degradation of organic matter or dissolution of mineral occurred. The values for the 970–1240 m level (flow rate 0.5 mm s^{-1}) at 10°C and 20°C were 176 m and 98 h and 22 m and 12 h, respectively. It becomes obvious that the measured CO_2 assimilation must have been balanced by a respiratory activity, indicated by the autotrophy quotients (Table 4) to be 4–14 times larger than the CO_2 assimilation activity. Indeed, the biofilm bacteria studied respired added lactate and the CO_2 released represented 7×10^{-5} to 1.8×10^{-4} of the TIC flowing through the reactors, but this figure is lower than the CO_2 assimilation calculated above (10^{-5} to 4.4×10^{-4}). The biofilm bacteria obviously must have metabolized organic carbon compounds present in the groundwater

other than lactate if a steady state between assimilated and produced CO_2 is anticipated.

There was typically between 30 and 300 $\mu\text{mol l}^{-1}$ of dissolved organic carbon in the groundwaters studied and also methane. About 50% of this dissolved organic carbon was composed of complex humic and fulvic acids (Pettersson *et al.*, 1990), and a smaller part was organic substances with shorter carbon chains than glucose. The sources of this organic carbon are unexplored. It could be organic material percolating down from the ground surface, but as dating with ^{14}C analysis indicates groundwater ages of 5000–20000 years (Nordström *et al.*, 1985), this seems unlikely. The other possible source would be organic deposits in the rock or organic compounds migrating up from very deep layers of the earth (Gold, 1992). It will be an important task to investigate the sources and fluxes of organic carbon in the subterranean environment. The results presented here indicate that organic carbon is the main fuel for bacterial processes of importance to nuclear waste disposal and the fluxes will then determine the process rates. Investigations of bacterial carbon transformations and the ecology of important groups of deep granitic groundwater bacteria will be continued and are now concentrated on the environment around the Äspö Hard Rock Laboratory (Gustafsson *et al.*, 1988, 1989, 1991) now under construction on the Swedish east coast.

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