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Sublithic bacteria associated with Antarctic quartz stones

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Abstract: Quartz stone sublithic cyanobacterial communities are common throughout the Vestfold Hills, Eastern Antarctica (68°S 78°E) contributing biomass in areas otherwise devoid of any type of vegetation. In this study, the sublithic microbial community and underlying soil was investigated using a variety of traditional and molecular methods. Although direct epifluorescent counts of the sublithic growth (average 1.1 x 10^9 cells g^{-1} dry weight) and underlying soil (0.5 x 10^9 cells g^{-1} dry weight) were similar, sublith viable counts (2.1 x 10^7 cfu g^{-1} dry weight) were on average 3-orders of magnitude higher in the subliths. Enrichment and molecular analyses revealed the predominate cyanobacteria were non-halophilic, able to grow optimally at 15–20°C, and were related to the *Phormidium* subgroup with several distinct morphotypes and phylotypes present. Sublithic heterotrophic bacterial populations and those of underlying soils included mostly psychrotolerant taxa typical of Antarctic soil. However, psychrophilic and halophilic bacteria, mostly members of the alpha subdivision of the Proteobacteria and the order Cytophagales, were abundant in the sublithic growth film (20–40% of the viable count and about 50% of isolated individual taxa) but absent from underlying soils. It is suggested that quartz stone subliths might constitute a "refuge" for psychrophilic bacteria.

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Introduction

Various environmental factors, in particular moisture, temperature and availability of organic matter, dictate the prevalence of bacteria in soil and lithic ecosystems in Antarctica. Bacteria in feldfield, rocky Antarctic soils and in cryptoendolithic environments (Broady 1986) tend to be robust and nutritionally versatile species, which are able to grow at low temperature and have a degree of tolerance to freezing and high osmotic pressure (Vincent 1988). Antarctic soil bacteria (Line 1988, Vishniac 1993, Siebert *et al.* 1996) are often cosmopolitan species and have also been isolated from soils in other parts of the world.

Sublithic (or hypolithic) communities found beneath translucent stones such as quartz, flint and limestone are features of various extreme, xeric environments (Berner & Evenari 1978). Sublithic growth is the growth which occurs on the base and sides of stones completely buried in soil (to a depth of 60 mm), with growth constrained to areas of the stone which get sufficient light. Quartz stones are abundant in the Vestfold Hills, with growth occurring on the undersides of stones on even the driest of soils. The growth appears as a bright green film less than 1 mm thick tenaciously bound to the quartz stone. The bulk of the biomass of this sublithic growth film is comprised of cyanobacteria (Broady 1981, 1986). Black-body heating warms the sublithic environment to temperatures 5–15°C above the ambient air temperature (Broady 1981). This elevated temperature coupled with the moderating effect of the stone mass suppresses the frequency of diurnal freezing and thawing. The stones also readily trap moisture with underlying soils having higher water contents than that of exposed surrounding soils even if the adjacent soils are already moist (Broady 1981). It can be hypothesized that this more moderate environment may support a distinct bacterial community different, or more complex than, bacterial colonizers of adjacent soils. In this study the microbial community of the quartz stone sublithic environment from the Vestfold Hills, Eastern Antarctica was investigated using both traditional and molecular methods, and its characteristics compared with soils located below the quartz stones.

Methods

Samples

Quartz rocks were collected from areas of the Vestfold Hills within 300 m of the shoreline along the Long Peninsula and also from islands off Broad Peninsula (close to Davis Station). Only stones with extensive sublithic growth were collected and were 13–20 cm long, 10–14 cm wide and 7–15 cm thick. The stones were taken from areas with little if any epilithic or edaphic vegetation present. Stones and soil samples from 0–50 mm beneath the stones were placed immediately into sterile plastic bags and stored at -20°C until examined further. Soil temperature was measured using a Fluke 51 K/J thermometer. Soil moisture was determined by comparing oven-dried soils (180°C for 16 h) with the original sample. Water activity was estimated from suspensions of soil in an equal volume of distilled water, using an Aqua Lab CX-2

water activity meter. The device measures water activity by determining the dewpoint of the sample with distilled water acting as a reference (a_w = 1.00). pH was measured using an Orion model 250A pH meter.

Enumeration and isolation of heterotrophic bacteria and cyanobacteria

Soil which was loosely adhering to the base of each stone was carefully brushed off with a sterile wire brush. The sublithic organic matter adhered very tightly to the stone surface, in the same manner as paint, and had to be scraped off, using a sterile scalpel; 100 mg quantities were placed in 10 ml of 2% (w/v) NaCl. The suspensions were mixed thoroughly by vortexing and then ground with mortar and pestle to a fine suspension. Soil from beneath the stones was also suspended and vortexed in 2% (w/v) NaCl. Suspensions were stained with 10 mg ml^{-1} DAPI (4,6-diamidino-2-phenylindole) for 30 min, diluted and then filtered through 0.2 mm black nucleopore membranes (Millipore). Filters were observed using epifluorescence with a Leitz DBRME microscope fitted with a synthetic fluorite objective and a standard bandpass blue filter (excitation 350 nm, emission 450 nm). At least 20 microscopic fields were counted for each sample assessed. To gain a heterotrophic bacterial count, cyanobacterial and algal cells (filamentous, rod-like and coccoidal cells much larger than bacterial cells and which also fluoresced orange–red under UV light) were not included in the count. Serial dilutions of the suspensions were plated onto various types of media including trypticase-soya (TSA) agar (containing 0.3% tryptone, 0.1% yeast extract and 1.5% Bacto-agar) (Line 1988); 10% strength TSA; 10% strength TSA amended with 2% NaCl and with 5% NaCl. Plates were incubated at 10°C for up to eight weeks. Bacterial colonies appearing on plates were enumerated and the morphotypes of different colonies assessed. Colonies with distinct morphotypes were subcultured to fresh media for purification. These plates were used as a source of bacterial isolates with selected colonies transferred and maintained on the media from which they were isolated.

Cyanobacteria were enriched under constant illumination (~200–300 lux) and rotation at 10°C. Suspensions were used to inoculate both liquid broth and media solidified with 1% agar noble (Difco Laboratories, Detroit, USA) for isolation of cyanobacteria using BG11 (a general enrichment/growth medium for cyanobacteria) and MN (a medium for marine cyanobacteria) mineral salts media (Castenholtz & Waterbury 1989). In addition BG11 lacking added sodium nitrate was used in attempt to enrich N_2-fixing cyanobacteria. Both BG11 and nitrogen-free BG11 were also amended with 2% and 5% NaCl. Positive cyanobacterial enrichments (appearing after 4–6 weeks incubation) were transferred to fresh BG11 or MN-media where appropriate and in addition were plated onto agar media for purification.

Preliminary characterization

Gram stained preparations of heterotrophic bacterial isolates were assessed by light microscopy. Smears were pre-treated with 2% glacial acetic acid before staining to avoid hypotonic shock (Dussalt 1955). Strains were assessed for growth on agar media with different levels of NaCl (0–10%) and at different temperatures (0–42°C).

16S rRNA sequencing of isolates

Methods for DNA extraction, PCR of 16S rRNA gene sequences and sequencing of DNA have been previously described (Bowman *et al.* 1997). In this study isolates were pre-screened using RLFP (restriction fragment length polymorphism) analysis. PCR-amplified 16S rDNA amplicons were digested separately with *RsaI*, *MspI* and *HinfI* (NEB, Beverley, USA) for 3–4 h at 37°C. Digests were then separated on a 3% (w/v) agarose gel, stained with ethidium bromide and photographed for evaluation. Each enzyme generated 5–12 bands. Strains were then selected on the basis of their unique RLFP patterns.

DNA extraction of sublithic material

Extraction of DNA used a modified version of the procedure of Fuhrman *et al.* (1988). Approximately 500 mg of material suspended in saline-tris-EDTA buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.4) containing 1% sodium dodecyl sulphate was placed into a sterile mortar and ground to a fine suspension. The suspension was transferred to sterile 15 ml centrifuge tubes using a wide bore pipette to reduce DNA shearing and 90 ml of 10 mg ml^{-1} lysozyme solution was added. The mixture was incubated at 60°C for 1 h and then boiled at 100°C for 2 min. The lysate was precipitated at -20°C with 2 vol. of 100% ethanol and 0.2 volumes of 10.5 M ammonium acetate. DNA was collected by centrifugation at 10000 g for 15 min and mixed gently with 0.5 ml 10 mM Tris-1 mM Na_2EDTA (pH 7.4) buffer and 0.5 ml Tris-saturated phenol. After centrifugation the aqueous layer was further purified by extracting with 0.6 ml 3:1 Tris-saturated phenol:chloroform and then with 0.6 ml chloroform. The aqueous layer was then precipitated using 0.12 ml 100% ethanol and 10.5 M ammonium acetate, centrifuged, dried using a vacuum dryer and re-dissolved in 50 µl of sterile distilled water. Nucleic acid yield was about 1 mg ml^{-1} with an A_{260}/A_{280} ratio of 1.6–1.7.

16S rRNA clone library construction

16S rRNA was amplified using 100–200 ng of QSSC DNA extracts, 50 pmol forward primer 530f (5'-GTG CCA GCM GCC GCG G-3') and reverse primer 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), 1.25 mM deoxynucleotides, Klentaq polymerase mix and buffer (Clontech, Palo Alto,

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USA). PCR was performed in a thermosequencer FTS-960 (Corbett Research, Sydney, Australia) with the following protocol: 1 cycle of 95°C for 3 min, 30 cycles of 94°C for 3 min, 50°C for 2 min and then 72°C for 6 min and a final cycle at 72°C for 6 min. Controls containing no DNA were also used to determine whether contaminants were being amplified. The amplified fragment was purified using the Prep-A-Gene kit (BioRad, Hercules, USA). The fragment was cloned using blunt-end ligation by employing the pGEM-T vector system I kit (Promega, Madison, USA) according to the manufacturer's instructions and using Epicurian coli XL ultracompetent cells (Stratagene, La Jolla, USA) for the transformations. Transformants were screened by blue-white selection on Luria agar treated with X-Gal/IPTG and containing 100 mg ml^{-1} ampicillin. White colonies were then transferred to fresh plates and re-incubated overnight. Clones were rapidly prescreened by suspending a loopful of cells and lysing the suspension with a final concentration of 2% SDS. The lysate was extracted with an equal volume of chloroform-isoamyl alcohol (24:1). A sample from the resultant aqueous layer was electrophoresed in a 1% (w/v) agarose gel and compared with a molecular weight marker. Positive clones were then cultivated overnight on plates and plasmid extracted using the Bres-A-Spin miniplasmid extraction kit (GeneWorks, Adelaide, Australia). To distinguish clone sequences RFLP analysis was performed as described above. For each distinct RLFP pattern obtained, two-three clones (if available) were sequenced. Sequence reactions used the Thermosequenase v. 2.0 ready reaction dideoxy cycle sequencing kit (Nycomed-Amersham, Little Chalfont, UK) and M13 reverse and forward primers and subsequent electrophoresis and analysis was then performed using a A377 automated sequencer (PE BioSystems, Foster City, USA). In most cases, 16S rDNA clones were completely sequenced with the sequences spanning nucleotide positions of 519 to 1507 (*E. coli* equivalent).

Phylogenetic analyses

Clone sequences were compared to the GenBank nucleotide data library using Gapped-BLAST searches (at http://www.ncbi.nlm.nih.gov/blast/blast.cgi) (Altschul *et al.* 1997) to determine their closest phylogenetic neighbours. The clone sequences were aligned manually to selected complete or near complete sequences downloaded from GenBank. Evolutionary distance was determined using the maximum likelihood algorithm employing the program DNADIST which is part of the PHYLIP v. 3.57c software package (Felsenstein 1993). Phylogenetic trees were generated by using neighbor-joining (NEIGHBOR program). Bootstrap analysis, using maximum parsimony (DNAPARS) and maximum likelihood analysis, was completed using the programs SEQBOOT and CONSENSE, using 250 replicate analyses. The RDP Chimera-CHECK program was used to detect PCR amplified hybrid sequences. In addition potential chimerism was also determined by detection of inconsistencies in branching-order and secondary structure by comparisons of sequences within the dataset and with GenBank sequences. Sequences generated in this study were deposited in the GenBank nucleotide library under accession numbers AF170731 to AF170793 and AF205366 to AF205372.

Results

Quartz stone sample characteristics

Sublithic growth appeared as a bright green film approximately 0.2–0.5 mm thick and tenaciously bound to the surface. The sublithic growth spread over either the bases of the stones or, for thicker stones (> 100 mm thick), around the circumference. Surface growth was not visible on rock surfaces exposed to the air but only occurred on rock surfaces buried in soil at depths of 10–50 mm. Maximum photosynthetically active radiation (PAR) reaching sublithic growth was estimated to range from 7–50 $\mu E\ m^{-2}\ s^{-1}$ assuming days of maximum insolation (cf. 1500 $\mu E\ m^{-2} s^{-1}$). Soil from beneath the stones had a pH of 7.4–7.7 whilst moisture content was 6–14%. Surrounding soils only possessed 0.5–2% moisture. Water activity measurements indicated the underlying soils were slightly saline (a_w 0.98–0.985). Temperature beneath the stones at the time of sampling (a period of maximum insolation on a clear sunny day) ranged from 3–7°C, about 10°C above the ambient air temperature.

Quartz stone total and viable counts

Total direct and viable counts of bacterial cells from nine quartz stones and seven underlying soil samples are shown in Fig. 1. Epifluorescent DAPI counts (which do not include cyanobacterial cells) for both sublithic growth (mean 1.1×10^9 cells g^{-1} dry weight) and soils were similar (mean 0.5×10^9 cells g-1 dry weight). Viable counts within the sublithic material (mean 2.1×10^7 cfu g^{-1}) were several orders of magnitude higher than those of the underlying soil (mean 2.3×10^4 cfu g^{-1}) as determined by direct plating onto 10% strength TSA+2% NaCl (Fig. 1). It was found that a better recovery was obtained when using this medium compared to other less saline and/or higher nutrient media. Recovery levels are low on agar owing to the considerable environmental differences between the quartz stone habitat compared to artificial media. Distinct bacterial colonial morphotypes (about 60% pigmented) were selected from the highest dilution plates (dilution 10^{-5} to 10^{-7} plates for sublithic growth and dilution 10^{-3} plates for soils) and used for viable count estimations agar plates for further study.

Quartz stone cyanobacteria

Light-microscopic observations of sublithic material from a number of samples indicated the presence of oscillatorian cyanobacteria and coccoidal cells (3–5 mm in diameter) morphologically similar to *Chroococcidiopsis*. Suspensions

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of material also revealed the presence of *Desmococcus*-like algae, *Navicula*-like diatoms and some mites and nematodes.

Although cyanobacteria were readily grown from quartz stone growth film using BG11 media and BG11 + 2% NaCl, enrichments using nitrogen-free BG11 and BG11 + 5% NaCl media were negative. Two filamentous cyanobacterial morphotypes were observed dominating the biomass enriched in BG11 and BG11 + 2% NaCl media. The first morphotype formed thinly sheathed trichomes (diameter 2–3 mm) with narrow transverse septa. The second morphotype formed thicker sheaths and trichomes (diameter 3–4 mm) containing irregular shaped coccoidal to rod-like cells with broad transverse septa. The latter strains formed gliding hormogonia on agar media. The isolates did not require Na^+ ions for growth but could tolerate up to 3% NaCl. The strains grew between 2 to 20°C but no growth occurred at 25°C. 16S rDNA sequence analysis of the filamentous isolates indicated they all clustered within the *Phormidium* subgroup (Turner 1997) (Fig. 2a).

Chroococcidiopsis-like and *Synechococcus*-like cells were also enriched in BG11 and MN media, respectively although the creation of axenic and/or stable xenic culture was unsuccessful. Enriched cells could be separated from contaminating non-cyanobacteria using Percoll density gradient centrifugation (Starink *et al.* 1994) allowing molecular identification. Spherical cells (diameter 4–5 mm), represented by enrichment 3-B, grouped within the cyanobacteria as an isolated lineage (Fig. 2a) with *Lyngbya* sp. PCC 7419 being amongst the closest relatives (sequence similarity < 0.91) (Fig. 2a). The salt-requiring *Synechococcus*-like cells, represented by enrichment 5-B, were closely related to *Synechococcus* sp. PCC 7335 (similarity 0.985) (Fig. 2a).

Heterotrophic bacteria

Isolates were obtained from plates used to calculate viable bacterial numbers and in most cases were present at population levels greater than 10^6 cells g^{-1} dry weight. Gram-negative and Gram-positive bacteria occurred at roughly the same incidence, though in some samples (particularly in stones 1 and 9) Gram-negative bacteria predominated. Individual strains representing different colony morphotypes were identified by using RLFP and 16S rDNA sequence analysis. Only representative strains showing distinctly different RLFP patterns (data not shown) were identified by sequencing. 16S rRNA analysis revealed heterotrophic bacterial isolates fell into three groups, the class Proteobacteria (alpha and gamma subdivisions) (Fig. 2b), the order Cytophagales (Fig. 2c) and the class Actinobacteria (Fig. 2d). The distribution of isolates between the various quartz stones and underlying soil is shown in Fig. 3. Most strains examined were identified as species of known bacterial genera, however many isolates represented taxa novel at the species level. From 20–40% of the viable count consisted of strains that were stenothermic displaying psychrophilic growth as defined by Morita (1975). Thus, these strains exhibited

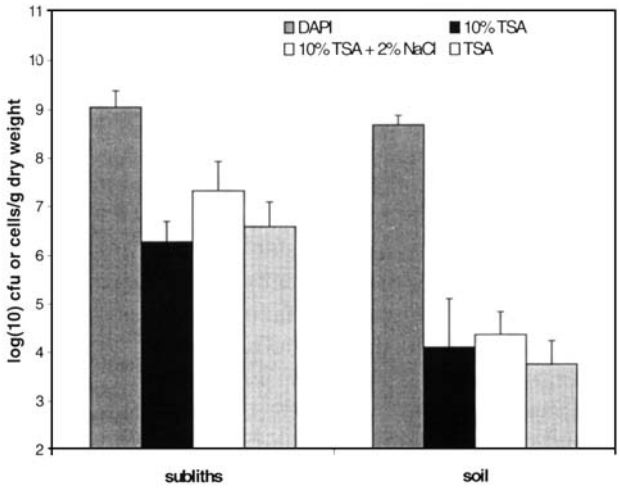

Fig. 1. Total direct bacterial counts (using DAPI as the epifluorescent stain) and viable bacterial counts (using 10% TSA, 10% TSA+2% NaCl and TSA as the growth media) within sublithic material and underlying soil.

best growth on agar at 20°C or less but did not grow at 25°C or higher. Most of these isolates also grew optimally with 1.5–3% NaCl, with several isolates unable to grow without at least 1% NaCl present in the media. The remaining isolates were mostly non-halophilic and psychrotolerant, able to grow at 0–4°C but with best growth occurring at 25–35°C. Certain taxa were ubiquitous in the samples. These included species of *Pseudomonas*, *Psychrobacter*, *Stenotrophomonas*, *Arthrobacter*, *Achromobacter*, *Micrococcus*, *Rhodococcus*, *Janibacter* and *Gelidibacter*, with the last genus consisting of psychrophilic and halophilic isolates. Some other isolates were also common but have no closely related (similarity < 0.92) cultivated relatives and include strains 9-9 (Fig 2b), 7-1, 9-14 (Fig. 2c) and 9-20 (Fig, 2d). Other taxa were more erratically distributed and were not isolated from all quartz stone sublithic material. Most heterotrophic bacteria isolated from the quartz stone sublithic material were also isolated from soils underneath the quartz stone, however the soils lacked cyanobacteria, alpha proteobacteria and flavobacteria (members of the order Cytophagales) (Fig. 3). These groups, which included most of the psychrophiles (Fig. 2b & c), thus appear to be strictly limited to the sublithic growth. Most of the psychrophilic bacteria had marine bacteria (on the basis of 16S rDNA sequences) as their closest relatives.

Direct molecular analysis of the quartz stone sublithic growth film

16S rRNA gene-based clone library analysis (Hugenholtz *et al.* 1998) was also used to study quartz stone sublithic growth. This method is able to detect bacteria which otherwise may not grow on the media employed in this study. Clone libraries were created from two representative sublithic samples and about 100 clones were screened for each library using RFLP analysis. Each RFLP pattern, consisting of one or more

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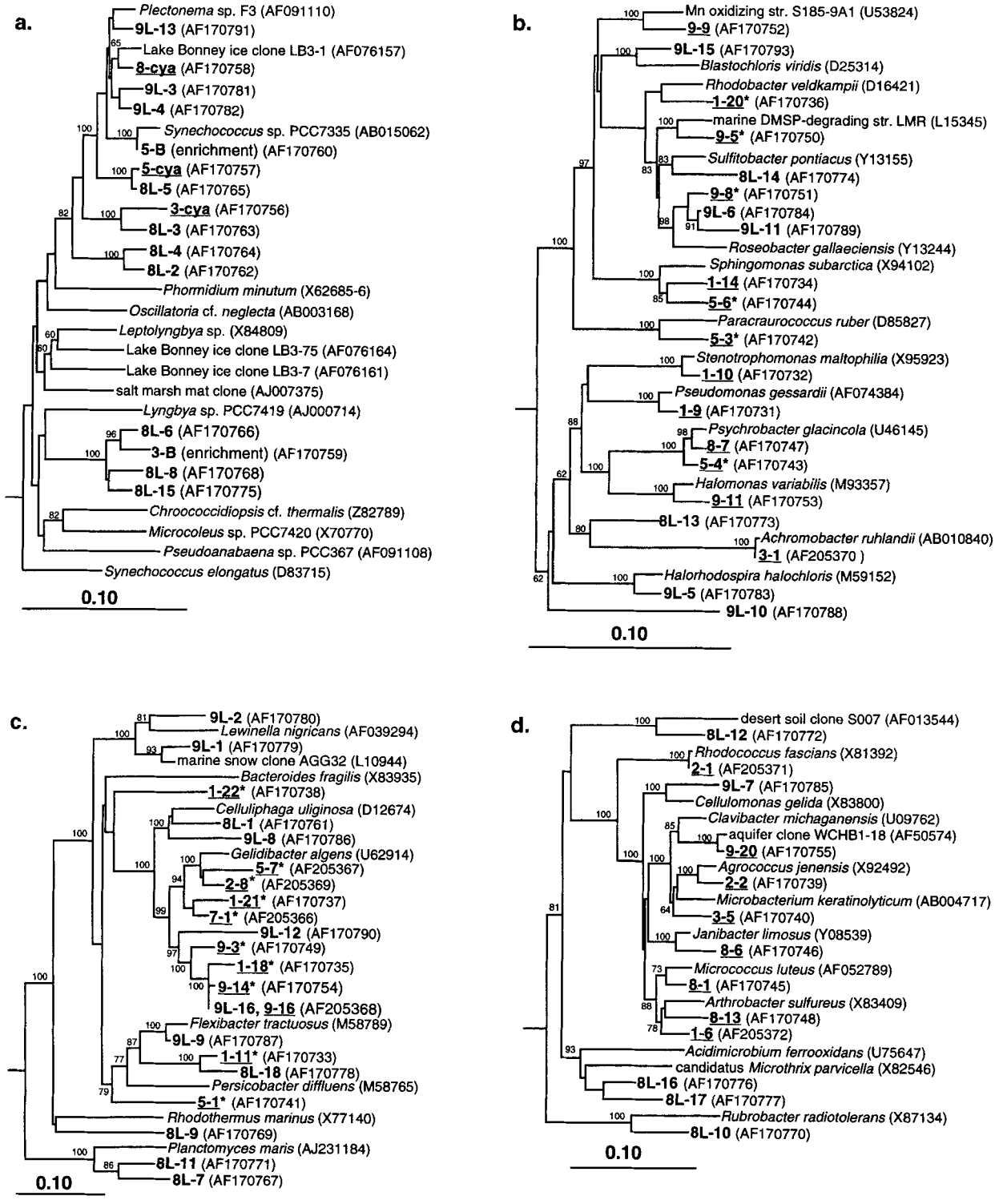

Fig. 2. Phylogenetic trees based on 16S rRNA gene sequences including both cultivated strains and clones derived from quartz stone subliths. Trees are divided by phylogenetic group: **a.** cyanobacteria, **b.** class Proteobacteria, **c.** order Cytophagales and **d.** class Actinobacteria. Names in bold and underlined are cultivated strains while names just in bold are clones. Names flagged by an asterisk are psychrophilic isolates. The numbers in parentheses after the names are GenBank accession numbers. The numbers at branch nodes are bootstrap values (only values over 60% are shown). The bar is equivalent to an evolutionary distance of 0.1 as determined by maximum likelihood analysis.

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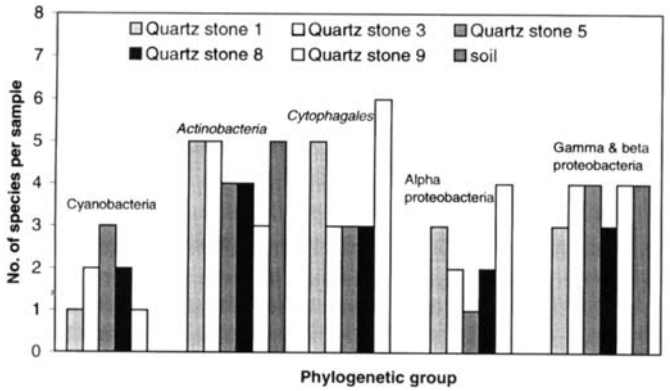

Fig. 3. Phylogenetic distribution of all cultivated taxa isolated from different quartz stone subliths and from underlying soil.

clones formed a phylotype. Only phylotypes showing greater than 2% difference in sequence dissimilarity are shown in the phylogenetic trees (Fig. 2). The distribution of clones within the different phylogenetic groups between the two quartz stones examined is shown in Fig. 4. Cyanobacterial clones dominated the libraries (71% of clones for stone 8 and 76% for stone 9) (Fig. 4) and consisted of two major groups of phylotypes. The first of these were the most frequently sampled (total of 112 clones) clustered amongst various quartz stone filamentous isolates (Fig. 2a) described above. A second cluster, only detected in abundance under stone 8, formed a common lineage with *Chroococcidiopsis*-like cells of enrichment 3-B (Fig. 2a). Other bacterial clones grouped with the alpha and gamma subdivisions of the Proteobacteria (Fig. 2b), the order Cytophagales (Fig. 2c), the order Planctomycetales (Fig. 2c) and the class Actinobacteria (Fig. 2d) at varying levels of incidence (Fig. 4). Only a single 18S rRNA clone was detected, clustering with members of the class Ulvophyceae (data not shown). The low recovery of *Eukarya* in the clone libraries was probably due to nucleotide mismatches between universal primer 1492r (P. Hugenholtz personal communication 1999) and the equivalent primer binding site on eukaryotic 18S rRNA. No archaeal clones were detected.

Discussion

Quartz stone cyanobacteria

The quartz stone sublithic growth film examined consisted mainly of oscillatorian cyanobacteria, with morphology typical of the *Lyngbya/Phormidium/Plectonema* group, and of spherical *Chroococcidiopsis*-like cells (Castenholz & Waterbury 1989). The predominance of these cyanobacteria was previously reported by Broady (1981) from studies also performed at the Vestfold Hills. The pattern of growth on the stones, PAR, temperature and moisture content of the underlying soil is also typical of that reported previously by Broady (1981, 1986). Available PAR restricted cyanobacterial growth below the rock to a maximum depth of about 50 mm (Broady 1981, 1986).

Cyanobacterial 16S rDNA clones were obtained which closely matched some of the isolates, with overall biodiversity of cyanobacterial genotypes in the sublithic growth examined appearing to be relatively narrow. However, other cyanobacteria are present including salt-requiring *Synechococcus*-like cells and *Pleurocapsa*-like cells which were not detected in the clone libraries. These, and possibly other cyanobacterial species, appear to be minor members of the community.

The resilience of cyanobacteria to the extremes of polar climate, including ability to survive freezing and desiccation, makes this group the single largest contributor to biomass on the Antarctic continent (Vincent & James 1996). Filamentous and spherical cyanobacteria, like those found in the sublithic growth film, have colonized a wide variety of Antarctic habitats including lake and pond mats, and epilithic and chasmolithic growth on ice free zone rocks, in ice shelf ponds and in glacial cryconite holes (Broady 1989, Wharton *et al.* 1981, Nienow & Friedmann 1993, Vincent *et al.* 1993). To date little data is available on the definitive identities of these cyanobacteria (e.g. 16S rDNA sequences), with identifications made only on the basis of morphology, a trait which does not correlate well with the phylogeny of the cyanobacteria (Turner 1997). Several sublithic clones were, however, similar to clones detected in the ice layers of Lake Bonney and Lake

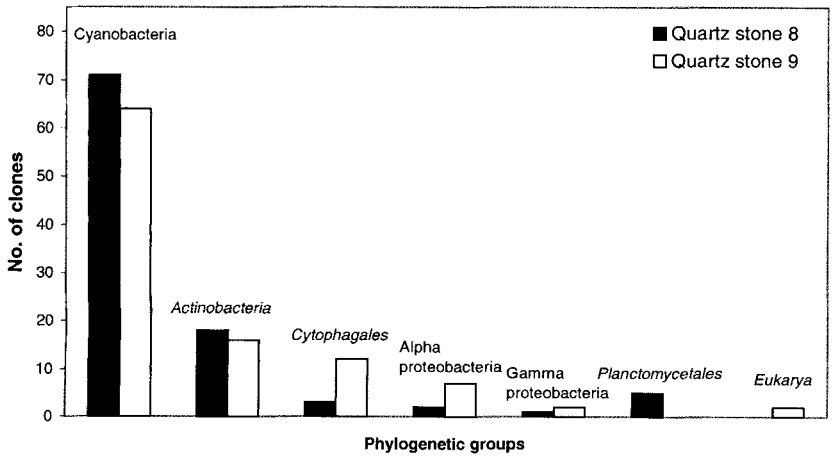

Fig. 4. Phylogenetic distribution of 16S rRNA gene clones detected in two different quartz stone subliths.

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Vanda (Priscu *et al.* 1998) (Fig. 2a) suggesting these and related species could be widespread in the Antarctic environment. Further analysis of cyanobacteria from other Antarctic sites using 16S rDNA sequencing or more general methods such as denaturing gradient gel electrophoresis (Muyzer & Smalla 1998) would be useful, providing information on endemism, and the extent of dispersal and colonization of various cyanobacterial types in different Antarctic environments.

Heterotrophic bacterial population structure

To determine heterotrophic community structure in the quartz stone habitat two different methods, traditional cultivation and 16S rDNA clone library analysis, were employed. Though each method has certain limitations, it was hoped that using both of these methods would offer a better perspective of the community structure.

Viable heterotrophic bacterial populations of sublithic growth film were considerably higher than is typical for the non-impacted Antarctic soil environment. Various studies of feldfield and glacially-derived Antarctic soils and material from sandstone cryptoendoliths only possess low levels of cultivable populations (Boyd *et al.* 1966, Line 1988, Vishniac 1993, Bolter 1993, Nienow & Friedmann 1993, Siebert *et al.* 1996). Using various media from 0.2% to 10% of the total population was recovered. By comparison the recovery of viable bacteria from underlying soils was less than 0.01% (Fig. 1). This difference in recovery was significant and suggests heterotrophic bacteria in the soils may be mostly dead or inactive. The relatively high recovery level of viable bacteria from the samples suggests the presence of a relatively healthy or active microbial community, possibly enhanced by better access to moisture, moderated temperatures and greater access to nutrients derived from cyanobacterial growth. On the other hand, it is likely that many bacteria present in the samples were not able to grow on the media used in the study. More experiments are needed to better compare sublithic growth and the soil, including direct measurements of *in situ* growth rates and metabolic activity using radiolabelled substrates or microelectrodes.

Only a few heterotrophic bacteria cultured from sublithic growth film were detected in the clone libraries. Often cultivation based biodiversity surveys reveals a different and sometimes greater diversity of bacteria, as certain community members, though low in population, are very readily cultured (Chandler *et al.* 1997). The clone libraries in this study revealed several taxa which were not cultured including most noticeably deep branching actinobacterial lineages including the *Rubrobacter*, *Microthrix* and *Acidimicrobium* lineages (Fig. 2d). This group represented the next most commonly detected phylotype after the cyanobacteria (9% of all clones) (Fig. 4.) and has been detected in a broad range of soil types (Rheims *et al.* 1996). Most of the other clones, in particular proteobacterial and Cytophagales-related clones, grouped with marine taxa. This was in accordance with cultivation data which also indicated many isolates had marine taxa as their closest relatives.

Psychrophilic bacteria within quartz stone sublithic growth

The quartz stones investigated harboured a surprisingly high population of psychrophilic and halophilic bacteria. Most of these were present at populations of $> 10^6$ cfu g^{-1} in given samples with species related to the genus *Gelidibacter* (Fig. 2c) the most numerous and found in all samples examined. The combination of psychrophily and halophily are ecophysiological characteristics more typical of marine bacteria, particularly those found in sea-ice algal assemblages (Bowman *et al.* 1997) and other permanently cold marine environments. Most of the sublithic Cytophagales isolates and clones cluster near filamentous gliding species, so far only isolated from seawater, surface marine sediment and sea-ice, including *Flexibacter tractuosus*, *Gelidibacter algens*, *Celluliphaga uliginosa* and *Lewinella* spp. (Bowman *et al.* 1997, Bowman unpublished data). Alpha proteobacteria found in the sublithic growth clustered mostly within the Rhodobacter branch in which numerous heterotrophic seawater bacterial taxa cluster, though none of the isolates closely matched named species. Overall, these taxa seem quite alien to typical Antarctic feldfield soils which surround quartz stones in the Vestfold Hills. None of the psychrophilic isolates survive for long at temperatures over 25°C, a temperature to which surface soils can be potentially heated by solar insolation (Vishniac 1993). Survival would be possible deeper in the soil but only if moisture was sufficient. Psychrophiles have not been definitively isolated from Antarctic soil to date, though some psychrophilic yeast and bacteria have been isolated from sandstone cryptoendoliths (Nienow & Friedmann 1993, Siebert *et al.* 1996). As no psychrophiles were isolated from the soil underlying the sublithic growth film, it is questionable if they exist in other more exposed areas of soil. Quartz stones could therefore be acting as a "refuge" for psychrophilic and possibly marine bacteria. Many saline Vestfold Hills lakes have also been identified as refuges for marine microbiota both psychrophilic and psychrotolerant (Franzmann 1996).

Assuming psychrophiles use quartz stones as a specific habitat: what is their origin, how did they colonize it and why do they concentrate there? In terms of colonization it is possible they are just marine bacteria blown to shore in sea spray which at some stage eventually colonize suitable sublithic environments. This seems on the surface a reasonable hypothesis as the stones studied were all collected from within 300 m of the shoreline. It will be interesting to determine whether quartz stones examined much further from the shoreline also harbour marine-like and psychrophilic bacterial populations. Likewise, the presence of cyanobacteria may be responsible for the high psychrophilic populations as overall higher populations of viable bacteria were recovered from the

sublithic growth film compared with adjacent soils. The higher population hypothetically could be due to greater substrate availability derived from cyanobacterial activity. It has been hypothesized that higher nutrient availability enhances the competitive edge of psychrophiles over psychrotolerant bacteria (e.g. in sea-ice communities, Bowman *et al.* 1997) by making their growth more efficient (Nedwell *et al.* 1999) at very low temperatures. However, much more research is needed to prove this. In this respect, an examination of the contribution of cyanobacterial photosynthetic activity to heterotrophic bacterial growth in the quartz stone sublithic environment is clearly needed.

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References

ALTSCHUL, S.F., MADDEN, T.L., SCHAFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

BERNER, T. & EVENARI, M. 1978. The influence of temperature and light penetration on the abundance of the hypolithic algae in the Negev Desert of Israel. *Oecologia*, **33**, 255–260.

BOLTER, M. 1993. Effects of carbohydrates and leucine on growth of bacteria from Antarctic soils (Casey station, Wilkes Land). *Polar Biology*, **13**, 297–306.

BOWMAN, J.P., MCCAMMON, S.A., BROWN, M.V., NICHOLS, D.S. & MCMEEKIN, T.A. 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Applied and Environmental Microbiology*, **63**, 3068–3078.

BOYD, W.L., STALEY, J.T. & BOYD, J.W. 1966. Ecology of soil microorganisms in Antarctica. *Antarctic Research Series*, **8**, 125–159.

BROADY, P.A. 1981. The ecology of sublithic terrestrial algae at the Vestfold Hills, Antarctica. *British Phycology Journal*, **16**, 231–240.

BROADY, P.A. 1986. Ecology and taxonomy of the terrestrial algae of the Vestfold Hills. *In* PICKARD, J., *ed. Antarctic oasis: terrestrial environments and history of the Vestfold Hills*. Melbourne: Academic Press, 165–202.

BROADY, P.A. 1989. Broadscale patterns in the distribution of aquatic and terrestrial vegetation at three ice-free regions on Ross Island, Antarctica. *Hydrobiologia*, **172**, 77–95.

CASTENHOLZ, R.W. & WATERBURY, J.B. 1989. Group I. Cyanobacteria. *In* STALEY, J.T., BRYANT, M.P. PFENNIG, N. & HOLT, J.G., *eds. Bergey's manual of systematic bacteriology*. Baltimore: The Williams and Wilkins Co, 1710–1728.

CHANDLER, D.P., LI, S.M., SPADONI, C.M., DRAKE, G.R., BALKWILL, D.L., FREDRICKSON, J.K. & BROCKMAN, F.J. 1997. A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. *FEMS Microbiology Ecology*, **23**, 131–144.

DUSSALT, H.P. 1955. An improved technique for staining red halophilic bacteria. *Journal of Bacteriology*, **70**, 484–485.

FELSENSTEIN, J. 1993. *PHYLIP v. 3.57c (phylogenetic inference program package)*. University of Washington, Seattle, USA.

FRANZMANN, P.D. 1996. Examination of Antarctic prokaryotic diversity through molecular comparisons. *Biodiversity and Conservation*, **5**, 1295–1305.

FUHRMAN, J.D., CCOMEAU, D.E., HAGSTROM, A. & CHAN, A.M. 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. *Applied and Environmental Microbiology*, **54**, 1426–1429.

HUGENHOLTZ, P., GOEBEL, B.N. & PACE, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, **180**, 4765–4774.

LINE, M.A. 1988. Microbial flora of some soils of Mawson Base and the Vestfold Hills, Antarctica. *Polar Biology*, **8**, 421–427.

MORITA, R.Y. 1975. Psychrophilic bacteria. *Bacteriological Reviews*, **39**, 144–167.

MUYZER, G. & SMALLA, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek International Journal of General & Molecular Microbiology*, **73**, 127–141.

NEDWELL, D.B. 1999. Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. *FEMS Microbiology Ecology*, **30**, 101–111.

NIENOW, J.A. & FRIEDMANN, E.I. 1993. Terrestrial lithophytic (rock) communities. *In* FRIEDMANN, E.I., *ed. Antarctic microbiology*. New York: Wiley-Liss, 343–412

PRISCU, J.C., FRITSEN, C.H., ADAMS, E.E., GIAVONNONI, S.J., PAERL, H.W., MCKAY, C.P., DORAN, P.T., GORDON, D.A., LANOIL, B.D. & PINCKNEY, J.L. 1998. Perennial Antarctic lake ice – an oasis for life in a polar desert. *Science*, **280**, 2095–2098.

RHEIMS, H., SPROER, C., RAINEY, F.A. & STACKEBRANDT, E. 1996. Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. *Microbiology*, **142**, 2863–2870.

SIEBERT, J., HIRSCH, P., HOFFMANN, B., GLIESCHE, C.G., PEISSL, K. & JENDRACH, M. 1996. Cryptoendolithic microorganisms from Antarctic sandstone of Linnaeus Terrace (Asgard Range) – diversity, properties and interactions. *Biodiversity and Conservation*, **5**, 1337–1363.

STARINK, M., BARGILISSEN, M.J., BAK, R.P.M. & CAPPENBERG, T.E. 1994. Quantitative centrifugation to extract benthic protozoa from freshwater sediments. *Applied and Environmental Microbiology*, **60**, 167–173.

TURNER, S. 1997. Molecular systematics of oxygenic photosynthetic bacteria. *Plant Systematics and Evolution*, **S11**, 13–52.

VINCENT, W.F. 1988. *Microbial ecosystems of Antarctica*. Cambridge: Cambridge University Press, 304 pp.

VINCENT, W.F., DOWNES, M.T., CASTENHOLZ, R.W. & HOWARD-WILLIAMS, C. 1993. Community structure and pigment organisation of cyanobacteria-dominated microbial mats in Antarctica. *European Journal of Phycology*, **28**, 213–221.

VINCENT, W.F. & JAMES, M.R. 1996. Biodiversity in extreme aquatic environments – lakes, ponds and streams of the Ross Sea sector, Antarctica. *Biodiversity and Conservation*, **5**, 1451–1471.

VISHNIAC, H.S. 1993. The microbiology of Antarctica soils. *In* FRIEDMANN, E.I., *ed. Antarctic microbiology*. New York: Wiley-Liss, 297–341.

WHARTON, R.A., VINYARD, W.C., PARKER, B.C., SIMMONS, G.M. & SEABURG, K. 1981. Algae in cryconite holes on Canada Glacier in southern Victoria Land, Antarctica. *Phycologia*, **22**, 355–365.