

Molecular Characterization of an Endolithic Microbial Community in Dolomite Rock in the Central Alps (Switzerland)

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Abstract Endolithic microorganisms colonize the pores in exposed dolomite rocks in the Piora Valley in the Swiss Alps. They appear as distinct grayish-green bands about 1–8 mm below the rock surface. Based on environmental small subunit ribosomal RNA gene sequences, a diverse community driven by photosynthesis has been found. *Cyanobacteria* (57 clones), especially the genus *Leptolyngbya*, form the functional basis for an endolithic community which contains a wide spectrum of so far not characterized species of chemotrophic *Bacteria* (64 clones) with mainly *Actinobacteria*, *Alpha-Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*, as well as a cluster within the *Chloroflexaceae*. Furthermore, a cluster within the *Crenarchaeotes* (40 clones) has been detected. Although the eukaryotic diversity was outside the scope of the study, an amoeba (39 clones), and several green algae (51 clones) have been observed. We conclude that the bacterial diversity in this endolithic habitat, especially of chemotrophic, nonpigmented organisms, is considerable and that *Archaea* are present as well.

Introduction

Microorganisms inhabiting rock were first observed and described 100 years ago [19, 45, 79], nevertheless, except for cyanobacteria, little is known about the community composition and the biodiversity of these microbial ecosystems. They are typical for hot and cold arid

environments where in the pores of the rock, they are partially sheltered from a number of physical stresses such as solar radiation, heat, cold, or desiccation. Various organisms settle on the surface and invade pores and cracks. Within the rock, they form a structured biofilm, a clearly defined organismic layer or band a few millimeters below the surface [15–17, 24–26, 35, 42, 44, 58, 65, 88–90]. Contrary to submerged biofilms, endolithic biofilms are patchier due to local inhomogeneities of rock structures and environments [42]. These communities contain bacteria, fungi, and eukaryotic microalgae [81, 82]. They form complex physiological networks tied to solid particles by extracellular polymeric substances (EPS). The synthesis of these polymers is controlled by different environmental stress factors (e.g. [77]). The organismic composition is governed by the hostile environment. Water is only periodically available in the form of rain, dew, or just atmospheric humidity. Therefore, EPS are most important for the endolithic population as they retain water and act as osmoprotectant and nutrient reservoirs. In the Alps, main nutrients are scarce, and the daily and seasonal temperatures oscillate widely. At high altitudes, the sunlight with a strong part in the UV is a further life threatening factor [95]. Habitats with such fluctuating environmental conditions pose a strong challenge to organisms, and life there may reach its limits at least in certain periods.

Endolithic microorganisms have gained interest in the past decades for several reasons: e.g., as possible analogs of extraterrestrial life, such as life on Mars [2, 24, 40, 43, 46, 59, 63, 73, 94, 95], for the study of the mechanisms of adaptation to extreme and hostile conditions [30, 36, 87, 96], to study the processes of weathering and mineral dissolution [12, 91] or for phylogenetic reasons [17, 29, 65, 86, 88, 89].

Endolithic microbial communities are found worldwide in dry and aquatic environments. The ones studied and

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described came from cliffs of the Niagara escarpment [32, 56, 57] from streams in the UK [68] and from gypsum cliffs in Nova Scotia [28]. They are found in hot and arid desert environments [5–7, 23], in travertine in Turkey [69], in arctic and antarctic locations [2, 26, 27, 44, 74, 93], in mountainous regions [42, 65, 88, 89], and in the marine littorals [92]. Most investigations have been based on traditional techniques, mainly light and electron microscopy, and on cultures. They have usually been focused on pigmented microorganisms, oxygenic phototrophs such as green algae and cyanobacteria as well as filaments of fungi as partners of lichen symbiosis. Cyanobacteria are important in the early stages of primary succession processes in soils, especially because many species are able to fix dinitrogen [47]. However, it must be assumed that a variety of heterotrophic organisms will rapidly follow the phototrophs after their invasion. So far, molecular methods have hardly been used. They have even been thought to be useless in studying endoliths [93]. However, molecular techniques are now successfully applied to characterize endolithic communities such as the cyanobacterial population in the dolomite rocks in Switzerland [82], the endolithic community in the McMurdo Dry Valleys in the Antarctica [17], or the microbial population in rocks of the Rocky Mountains [29, 65, 89].

The objective of the present study is to describe the broad genetic diversity of the endolithic bacterial populations present in the dolomite formations of the Swiss Alps by culture independent molecular methods. Dolomite rocks ($\text{CaMg}(\text{CO}_3)_2$) in the Piora Valley in southern Switzerland are often bare of vegetation and exposed to hostile conditions. Such weathered rocks harbor chasmoendolithic and cryptoendolithic (definitions, see [34]) phototrophic and heterotrophic microbial communities which become easily visible as grayish-green bands some millimeters below the surface. This hidden microbial ecosystem was first characterized by Diels [19] in the Italian Dolomites and has been studied in Piora dolomite by molecular [82], spectroscopical, and optical techniques [42]. At a depth of 2 to 8 mm, the phototrophic microorganisms still receive enough photosynthetic active radiation while they are protected from excessive sunlight with a high fraction in the UV range [42]. As most organisms of environmental samples cannot be cultured by standard methods yet, a description of the microbial diversity of this special microbial ecosystem has been obtained by sequence analyses of polymerase chain reaction (PCR) amplified fragments of the small subunit of the ribosomal ribonucleic acid gene (SSU rRNA gene). The knowledge of the composition of the microbial community will help to better understand the biogeochemical processes that occur in these habitats. Preliminary results have been presented earlier [41, 81].

Materials and Methods

Sampling Site

Dolomite rock material was collected in the Piora Valley in the southern part of the Swiss Alps at an elevation of 1,965 m above sea level in summer 2001 and 2003. The coordinates of the specific sampling site are 46°32'51" N, 8°43'05" E. Details of the site are given by Sigler et al. [82] and Horath et al. [42]. The geology of the Piora Valley, oriented east-west, is characterized by a dolomite trough, a few hundred meters wide, surrounded by crystalline rock formations. Due to erosion by wind and water, the dolomite is often exposed to the atmosphere, forming white cliffs. Such sites are sparsely covered with black epilithic cyanobacteria and lichens. Especially in slightly weathered dolomite, endolithic microorganisms are easily observed when the surface layer is removed. They form a grayish-green layer about 1–8 mm below the rock surface. Rock pieces of some millimeters or centimeters in size were cut off from the surface with an ethanol-flamed chisel and hammer, and samples with visible endolithic bands were kept in Falcon tubes in the dark at 4°C until DNA extraction in the laboratory.

DNA Extraction

DNA extraction was performed as described by Sigler et al. [82]. In brief, 0.5 to 0.6 g of rock samples of the green layer was scratched into a sterile empty Petri dish with sterilized tools, then put into 2-ml sterile microfuge tubes containing 1.0 ml of extraction buffer (50 mM NaCl, 50 mM ethylene diamine tetra acetic acid disodium salt dihydrate (EDTA; Fluka 03685), 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (TRIS-HCl; Fluka 93363) and 5% sodium dodecyl sulfate (SDS; Fluka 71729), final pH 8), 0.5 g glass beads (0.1 and 0.5 mm in diameter) and eventually 0.5 ml of a phenol–chloroform–isoamylalcohol-mixture (v/v/v=49.5/49.5/1, Fluka 77618). The tubes were sealed with Parafilm®, shaken in a bead beater (“FastPrep®”, BIO 101, La Jolla, CA, USA) at 5.5 m s⁻¹ for 30 s and centrifuged for 4 min at 10,000×g. Nucleic acids were isolated by standard phenol/chloroform extraction and ethanol precipitation [75]. The dry DNA pellet was redissolved in 50 µl distilled autoclaved water.

PCR Amplification of SSU rRNA Genes

The small subunit rRNA gene was amplified from genomic DNA by PCR with several pairs of primers (see Table 1). PCR was performed in 200-µl thin-walled tubes on a “Progene” or a “Genius” thermocycler respectively (Techno LTD, Duxford Cambridge, U.K) in a volume of 25 µl. The reaction mixture contained (final concentrations): the

Table 1 List of clone library names, sequences of primers, and numbers of different clones obtained (3% sequence difference level)

Library name	Primer	Primer sequence (5' to 3')	Reference	Obtained products	Total clones	Sequenced clones	Different clones
Dolo	27F	AGA GTT TGA TCM TGG CTC AG	[20, 50]	Bacteria/Chloroplasts	36	30	22
	1524r	AAG GAG GTG ATC CAR CCG	[50] Slightly modified				
DoAr	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	<i>Euamoeba</i> sp.	39	1	1
	1517r	ATC CAG CCG CAG RTT C	This paper				
ud	536f	CAG CMG CCG CGG TAA TWC	[49]	Bacteria/Crenarchaea	35	16	10
	1392r	ACG GGC GGT GTG TRC	[49]				
DA	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	<i>Chlorella</i> sp. (18S)	16	8	4
	1512uR	ACG GHT ACC TTG TTA CGA CTT	[50] Slightly modified 1492r				
DOS	89Fb	ACG GCT CAG TAA CRC	[10]	<i>Crenarchaea</i>	38	10	3
	915R	GTG CTC CCC CGC CAA TTC CT	[85]				
DOL	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	<i>Bryophyta</i> (18S)	28	5	1
	1512uR	ACG GHT ACC TTG TTA CGA CTT	[50] Slightly modified				
DoCY	CYA359F	GGG GAA TTT TCC GCA ATG GG	[66]	<i>Cyanobacteria</i>	23	9	6
	CYA1342R	GAC CTG CAA TTA CTA GCG	[78]				
Docu	CYA359F	GGG GAA TTT TCC GCA ATG GG	[66]	<i>Cyanobacteria</i>	36	17	6
	CYA1342R	GAC CTG CAA TTA CTA GCG	[78]	Chloroplasts			
Sequencing Primers (5' to 3')							
	M13 forward	GTA AAA CGA CGG CCA G	[60]				
	M13 reverse	CAG GAA ACA GCT ATG AC	[60]				
	519r	GWA TTA CCG CGG CKG CTG	[49]				
	536f	CAG CMG CCG CGG TAA TWC	[49]				
	1099r	GGG TTG CGC TCG TTR C	[50] Slightly modified				
	1114f	GYA ACG AGC GCA ACC C	[50] Slightly modified				

appropriate *Taq* buffer (1×), 1.5–2.0 mM MgCl₂, 0.1 mg ml⁻¹ bovine serum albumine, 0.2 mM dNTP's, 200 nM forward primer, 200 nM reverse primer, 40–100 U ml⁻¹ *Taq* Polymerase (Sigma, Promega, Invitrogen, or Pharmacia), and approximately 50–100 ng template DNA. PCR was run under the following conditions: initial denaturation at 94°C for 2 min, 10 cycles of 94°C for 20 s, 60–0.5°C/cycle for 30 s, 72°C for 60 to 90 s depending on the length of the product, 20 cycles of 94°C for 20 s, 50°C to 58°C for 30 s, depending on the annealing temperature of the primers, 72°C for 60 to 90 s. The products were checked on a 1% agarose gel in 0.5× TAE buffer [1×=40 mM Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol), 20 mM glacial acetic acid, 1 mM Na₂EDTA of pH 8.0].

Cloning

PCR-amplified products were cloned without purification with the TOPO TA cloning kit (Invitrogen) as specified by the manufacturer's manual.

Restriction Fragment Length Polymorphism

After plasmid DNA mini preparation with alkaline lysis [75] and the reamplification of the SSU rRNA gene with M13 primers, restriction was carried out with *Hinf* I and *Hae* III and the fragments analyzed on a Spreadex® EL 800 Wide Mini S-50 gel (Elchrom Scientific) run at 55°C for 1 h at 10 V cm⁻¹. The gels were stained with 10,000 times diluted 1% (*w/v*) ethidium bromide and viewed with 302 nm UV illumination.

DNA Sequencing

Reamplified plasmid inserts were purified by filtration (Amicon Microcon YM-100 filter, Millipore Corporation, Bedford, MA, USA), and 100 to 180 ng DNA (dissolved in 1 µl H₂O) were used for sequencing-PCR using 0.8 µl BigDye® Terminator v3.1 (Applied Biosystems), 1.5 µl sequencing buffer (5×), 6.8 µl of H₂O Milli Q, and 0.25 µl (5 µM) of one of the sequencing primers listed in Table 1. Before the automated loading into the polymers on the 48-capillary sequencer (Applied Biosystems 3730 DNA Analyzer), the PCR products were purified by centrifugation through Sephadex G50 (Amersham Pharmacia). The raw sequences were aligned and combined using the Gene Codes Sequencer software (www.genecodes.com).

Nucleotide Sequence Accession Numbers

The SSU rRNA gene sequences found have been deposited at the DNA Data Bank of Japan and can be retrieved under the accession numbers AB257629 to AB257698 and AB334273 to AB334298.

Phylogenetic Tools

Rarefaction curves were generated with the program “Analytic Rarefaction 1.3” provided by Steven M. Holland at “<http://www.uga.edu/~strata/software/Software.html>”. The newly obtained SSU rRNA gene sequences were compared with known sequences in the NCBI database (Genbank) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) by the use of the Basic Local Alignment Search Tool (BLAST) [1] to determine their approximate phylogenetic affiliation.

The EMBOSS Pairwise Alignment Tool at “<http://www.ebi.ac.uk/emboss/align/>” provided by the European Bioinformatics Institute was used to compare single sequences in the following mode: “Method: water”; “Gap Open: 10.0”; “Gap Extend: 10.0”; “Molecule: DNA”; “Matrix: DNAfull”.

The new SSU rRNA gene sequences were further added to the rRNA gene sequence database of the Technical University of Munich (*ssu_jan04_corr_opt.arb*, release February 2005) by the use of the program package ARB ([54], <http://www.arb-home.de>). The integrated tool ARB_ALIGN was used for automatic sequence alignment, which was then checked with a critical eye according to the secondary structure of the rRNA molecule, and corrected. If missing, the latest best fitting sequences found by NCBI-BLAST were added to the ARB database.

The final phylogenetic trees were derived from the basic phylogenetic tree of about 51,000 SSU rRNA sequences after adding the new sequences with appropriate filters, and the “Maximum Parsimony Method.” Bootstrap values were calculated from the sequences used in the final trees by using the “Phylip Parsimony Method”, integrated in ARB, compressing vertical gaps, running 100 bootstrap samples.

In order to plot a phylogenetic tree, many different algorithms are available today, which all lead to acceptable results if they are based on a proper sequence alignment [52]. Therefore, emphasis has been put on an accurate alignment. The trees presented are copies of the largest tree, namely “tree_1000_jan05” in the ARB database “*ssu_jan04_corr_opt.arb*”. After adding the new sequences to the existing tree containing more than 50,000 single SSU rRNA sequences, the new trees have been reduced to a convenient size for illustration. Bootstrap values have been calculated although they are not considered to be very important, since these values can be shifted by omitting closely branching sequences before calculation (Eichenberger, Ch., personal communication).

Bootstrapping has been introduced to provide confidence intervals in phylogenetic calculations [13, 21], because calculated trees are never fully true and require flexible interpretations. When using Maximum Parsimony, Distance Matrix (Neighbor Joining), or Maximum Likelihood, the result should not be overestimated because its variation

among different methods is a negligible indicator of the confidence interval [21]. Furthermore, the order of adding sequences to a calculation has an effect on the tree topology [e.g., 53]. Thus removing and rereading complete groups to a tree may rearrange its branching. In our case, it improved the congruence of the results of ARB and NCBI.

Results

In a previous study, we investigated endolithic bacterial communities in exposed weathered dolomite rocks by confocal laser scanning microscopy, pigment analysis, and reflectance spectroscopy [42]. Communities depending on photosynthesis usually harbor a sum of heterotrophic organisms which feed on exudates and lysed cells. As it is hardly possible to characterize the diversity of environmental microorganisms by cultivating them, we analyzed the endolithic heterotrophic community by cloning and sequencing their SSU rRNA genes.

SSU rRNA Gene Clone Libraries

Isolation of DNA from fine powdered rock material posed some difficulties as DNA tended to stick to and precipitate with the inorganic rock debris. Suitable amounts of DNA were obtained following the procedure of Sigler et al. [82]. To evaluate the diversity of the prokaryotic endolithic community, eight independent clone libraries with different combinations of universal and phylogenetic group-specific oligonucleotide primers were constructed, including two libraries with specific cyanobacterial primers (Table 1). In total, 254 clones were analyzed by restriction fragment length polymorphism (RFLP), 96 of which were sequenced. Assuming a threshold of a minimal 3% sequence difference between species [84], 53 sequences fell into distinctly related groups. From these 53 phylotypes, 45 belong to *Bacteria* (including three chloroplasts of two green algae and a moss), three to *Archaea*, and five to *Eukarya* (Table 2). Scanning the graphic alignment of the NCBI-BLAST analysis of the new sequences, no chimeras have been detected ([1], <http://www.ncbi.nlm.nih.gov/>).

A wide diversity was found in the clone libraries obtained with the bacterial primer pair 27f/1524r and the “universal” primer pair 536f/1392r. In the bacterial library, 22 out of 36, and in the “universal” library, nine out of 35 clones were different. The other primer pairs resulted in less diverse libraries. As an extreme, primer pairs 8aF/1517r (DoAr) and 8aF/1512uR (DOL) yielded 39 and 28 RFLP-identical clones, respectively (Table 2). Primer 1517r (Table 1) was originally designed to increase the number of *Archaea* clones but resulted in the detection of a so far unknown 18S rRNA gene sequence fragment

closely related to *Saccamoeba limax* (99.4%, clone DoAr09).

Rarefaction curves for all the eight clone libraries are shown in Fig. 1. The shapes of the curves “Dolo” and “ud” indicate that further sampling would increase the number of operational taxonomic units (OTUs, 3% difference level). In contrast, the other graphs, except for the summarized data, level off rapidly, a phenomenon for discussion.

A quarter of the obtained bacterial sequences (64 out of a total of 251 clones) originated from phototrophic oxygenic organisms. *Cyanobacteria* were numerous with 11 phylotypes, chloroplasts of green algae (Dolo-01, Dolo-34) or of bryophytes (Docu-30) with three different phylotypes (seven clones). Among the heterotrophic species, the representatives of the phylum *Actinobacteria* were the most numerous (15 clones, seven phylotypes), followed by *Alpha Proteobacteria* (14 clones, ten phylotypes), and *Bacteroidetes* (12 clones, two phylotypes). *Acidobacteria* (seven clones, two phylotypes), *Gamma Proteobacteria* (five clones, one phylotype), and *Gemmatimonadetes* (two clones, two phylotypes) were less frequent. Only one clone was found in each of the proposed divisions TM6 and TM7, as well as in the phylum *Planctomycetes* (Fig. 2 and Table 2). The green nonsulfur phototrophic bacteria group of the *Chloroflexi* yielded six clones (four phylotypes). The sum of bacterial phyla found in the dolomite of the Piora Valley covers ten of approximately 75 bacterial phyla known or postulated so far [51, 72, 76]. All the archaeal sequences found fell into the group of uncultured *Crenarchaeotes* (Table 2). Eukaryotic 18S rRNA gene sequences have been found in groups related to *Euamoebida*, *Bryophyta*, and *Chlorophyta* (83 out of a total of 251 clones, five phylotypes; Table 2). The phylogenetic trees give an overview of the distribution of the newly detected SSU rRNA gene sequences in the domains of *Bacteria*, *Archaea*, and *Eukarya* (Figs. 3a, b, 4, and 5).

Within all sequences analyzed, the percentages of sequence identity with SSU rRNA gene sequences available at GenBank (<http://www.ncbi.nlm.nih.gov/>) range between 85.2% and 99.7%. Clone “DOS_02”, on a length of 791 bp, was even 99.9% identical with the uncultured archaeon clone HL17 (AJ608203) in loam from a bank of the river Waal in the Netherlands, while clone “Dolo-07”, on a length of 1,425 bp, shows only an 83.8% similarity with the uncultured *Chloroflexus* clone pItb-vmat-61 (AB294962) from a microbial mat in a shallow submarine hot spring in Japan (Table 2). For some sequences, ARB or “EMBOSS Pairwise Alignment Algorithms” have found different closest relatives as compared to NCBI-BLAST, but then often with smaller sequence coverage. Among the 45 different bacterial phylotypes, 18 (40%) were less than 95% identical to the closest 16S rRNA gene in the nucleotide sequence database, 14 phylotypes (31%) were

Table 2 Phylogenetic affinities of SSU rRNA gene sequences obtained from dolomite in the Piora Valley, Central Alps

Clone	Frequency ^a	Phylogenetic affiliation	Closest NCBI-BLAST Match (accession no.)	% Identity	Accession no.
Dolo-26	1/36	<i>Acidobacteria</i>	Uncultured bacterium clone Amb_16S_1159 (EF018708)	96.8	AB257649
ud01	6/35	<i>Acidobacteria</i>	Uncultured bacterium clone Elev_16S_1031 (EF019528)	98.8	AB257683
ud02	6/35	<i>Actinobacteria</i>	Bacterium Ellin504 (AY960767)	96.4	AB257684
Dolo-16	1/36	<i>Actinobacteria</i>	<i>Goodfellowia coeruleoviolacea</i> , strain NRRL B-24058 (DQ093349)	94.1	AB257641
Dolo-39	1/36	<i>Actinobacteria</i>	<i>Micrococcineae</i> strain Ellin124 (AF408966)	93.9	AB257657
ud31	3/35	<i>Actinobacteria</i>	Uncultured actinobacterium clone FBP460 (AY250884)	99.1	AB257697
Dolo-10	1/36	<i>Actinobacteria</i>	Uncultured bacterium AT425_EubY10 (AY053479)	91.8	AB257636
ud17	2/35	<i>Actinobacteria</i>	Uncultured bacterium clone C-F-15 (AF443586)	94.6	AB257690
ud19	1/35	<i>Actinobacteria</i>	Uncultured organism clone DLE037 (EF127609)	92.7	AB257692
Dolo-28	2/36	<i>Alpha proteobacteria</i>	<i>Brevundimonas variabilis</i> (AJ227783)	98.8	AB257650
Dolo-09	1/36	<i>Alpha proteobacteria</i>	Marine alpha proteobacterium strain V4.MO.17 (AJ508754)	94.9	AB257635
Dolo-08	1/36	<i>Alpha proteobacteria</i>	<i>Sphingomonas asaccharolytica</i> , strain IFO 15499-T (Y09639)	96.7	AB257634
Dolo-14	1/36	<i>Alpha proteobacteria</i>	<i>Sphingomonas asaccharolytica</i> , strain IFO 15499-T (Y09639)	97.4	AB257639
Dolo-04	1/36	<i>Alpha proteobacteria</i>	Uncultured alpha proteobacterium clone OS-C38 (EF612400)	95.6	AB257630
Dolo-11	1/36	<i>Alpha proteobacteria</i>	Uncultured bacterium clone "Hot Creek 25" (AY168723)	91.7	AB257637
Dolo-24	1/36	<i>Alpha proteobacteria</i>	Uncultured bacterium clone JSC8-E1 (DQ532238)	97.9	AB257648
Dolo-22	3/36	<i>Alpha proteobacteria</i>	Uncultured proteobacterium 59H11 (AF245037)	98.5	AB257646
Dolo-05	2/36	<i>Alpha proteobacteria</i>	Uncultured soil bacterium clone PK_XIII (EF540444)	97.0	AB257631
Dolo-32	1/36	<i>Alpha proteobacteria</i>	Uncultured soil bacterium clone PK_XIII (EF540444)	93.0	AB257653
ud04	7/35	<i>Bacteroidetes</i>	Uncultured Bacteroidetes bacterium clone J35E6 (DQ365993)	96.5	AB257685
ud10	5/35	<i>Bacteroidetes</i>	Uncultured soil bacterium clone M52_Pitesti (DQ378268)	98.2	AB257688
Dolo-06	5/36	<i>Gamma proteobacteria</i>	Xanthomonas-like sp. V4.BO.41 (AJ244722)	97.3	AB257632
Dolo-19	1/36	<i>Gemmatimonadetes</i>	Uncultured bacterium clone 5-31 (DQ833469)	90.4	AB257644
Dolo-18	1/36	<i>Gemmatimonadetes</i>	Uncultured Gemmatimonadetes clone Skagen138 (DQ640715)	93.4	AB257643
Dolo-21	1/36	<i>Planctomycetes</i>	<i>Planctomyces</i> sp. (strain: Schlesner 658) (X81954)	96.7	AB257645
Dolo-31	1/36	TM6	Uncultured bacterium clone Ebpr8 (AF255643)	93.5	AB257652
ud08	1/35	TM7	Uncultured candidate division TM7 bacterium clone 71 (AF513102)	92.1	AB257687
DoCY-44	4/23	<i>Cyanobacteria</i>	<i>Gloeobacter violaceus</i> PCC 7421 (BA000045) / (AP006573)	95.7	AB334275
Docu-04	3/36	<i>Cyanobacteria</i>	<i>Leptolyngbya frigida</i> ANT.LH52.2 (AY493575)	95.0	AB334284
Docu-01	22/36	<i>Cyanobacteria</i>	<i>Leptolyngbya</i> sp. CENA 112 (EF088337)	96.9	AB334282
Docu-19	4/36	<i>Cyanobacteria</i>	<i>Leptolyngbya</i> sp. CNP1-B3-C9 (AY239600)	94.2	AB334292
Docu-28	3/36	<i>Cyanobacteria</i>	<i>Leptolyngbya</i> sp. Greenland_7 (DQ431002)	95.1	AB334294
DoCY-46	4/23	<i>Cyanobacteria</i>	<i>Nostoc</i> sp. 'Pannaria aff. leproloma cyanobiont' (EF174228)	98.9	AB334277
DoCY-45	1/23	<i>Cyanobacteria</i>	Uncultured cyanobacterium clone 100M1_F2 (DQ514011)	93.2	AB334276
DoCY-55	1/23	<i>Cyanobacteria</i>	Uncultured cyanobacterium clone 100M1_F2 (DQ514011)	96.7	AB334280
DoCY-42	8/23	<i>Cyanobacteria</i>	Uncultured cyanobacterium clone HAVOmat106 (EF032780)	94.0	AB334274
Docu-24	2/36	<i>Cyanobacteria</i>	Uncultured cyanobacterium clone HAVOmat31 (EF032786)	94.1	AB334293
DoCY-39	5/23	<i>Cyanobacteria</i>	Uncultured <i>Gloeobacter</i> sp. clone HAVOmat17 (EF032784)	95.8	AB334273
Dolo-23	1/36	uncultured Chloroflexi	Uncultured Chloroflexi bacterium clone AKYH1480 (AY922118)	96.0	AB257647
ud07	2/35	uncultured Chloroflexi	Uncultured Chloroflexi bacterium clone AKYH1521 (AY922125)	99.7	AB257686
Dolo-07	2/36	uncultured Chloroflexi	Uncultured Chloroflexus clone pltb-vmat-61 (AB294962)	83.8	AB257633
Dolo-17	1/36	uncultured Chloroflexi	Uncultured Chloroflexus clone pltb-vmat-61 (AB294962)	85.2	AB257642
DOS_21	7/38	<i>Crenarchaeota</i>	Uncultured archaeon clone DRV-A006 (AY923076)	98.2	AB257680
DOS_02	21/38	<i>Crenarchaeota</i>	Uncultured archaeon clone HL17 (AJ608203)	99.9	AB257674
DOS_05	10/38	<i>Crenarchaeota</i>	Uncultured archaeon clone FJF-WS-Arch07 (AJ867731)	99.6	AB257676
ud14	2/35	<i>Crenarchaeota</i>	unidentified archaeon SCA1150 (U62812)	99.4	AB257689
DOL_01	28/28	<i>Bryophyta</i>	<i>Blindia acuta</i> (AF023681)	99.7	AB257668
DA-01	7/16	<i>Chlorophyta</i>	<i>Pseudomuriella</i> sp. Itas 9/21 14-1d (AY195974)	92.3	AB257659
DA-04	6/16	<i>Chlorophyta</i>	<i>Stichococcus bacillaris</i> K4-4 (AB055866)	98.6	AB257661
DA-12	3/16	<i>Chlorophyta</i>	Uncultured <i>Dunaliellaceae</i> clone Amb_18S_930	94.6	AB257663

Table 2 (continued)

Clone	Frequency ^a	Phylogenetic affiliation	Closest NCBI-BLAST Match (accession no.)	% Identity	Accession no.
			(EF023670)		
Docu-30	2/36	Chloroplast	Chloroplast of <i>Hymenostylium recurvirostre</i> (DQ629553)	99.7	AB334295
Dolo-34	1/36	Chloroplast	Uncultured chlorophyte clone FQSS008 (EF522228)	96.9	AB257654
Dolo-01	4/36	Chloroplast	Uncultured chlorophyte clone FQSS008 (EF522228)	97.5	AB257629
DoAr-09	39/39	<i>Euamoebida</i>	<i>Saccamoeba limax</i> (AF293903)	99.5	AB257667

^aThe frequency of the clones is given as the number of clones of one sort of phylotype divided by the total number of clones in that library

in the range between 95% and 97% sequence identity, showing genus level relation [84], while 13 phylotypes (29%) were within the species level (more than 97% sequence identity).

Bacterial Community

The quantitative distribution of the different mostly heterotrophic phylotypes in the bacterial clone libraries “ud” and “Dolo” (excluding chloroplasts and the specific

cyanobacterial libraries “Docu” and “DoCY”) is diagrammed in Fig. 2. There are four predominant groups accounting for more than 80% of 64 clones: the *Actinobacteria* together with *Proteobacteria* (*alpha* and *gamma*), *Bacteroidetes*, and *Acidobacteria* are the most numerous. Looking separately at individual bacterial phylotypes, the five clones ud01, ud02, ud04, ud10, and Dolo-06 are the most numerous ones, all in all accounting for 45% of the non-oxygenic “ud” and “Dolo” clones. Based on NCBI-BLAST [1], these phylotypes represent *Bacteroidetes* (ud04=10.9%,

Figure 1 Rarefaction curves for the different libraries and for the sum of all clones obtained. The threshold is set at 3% sequence difference to distinguish between different OTUs. For clone names see, Table 1

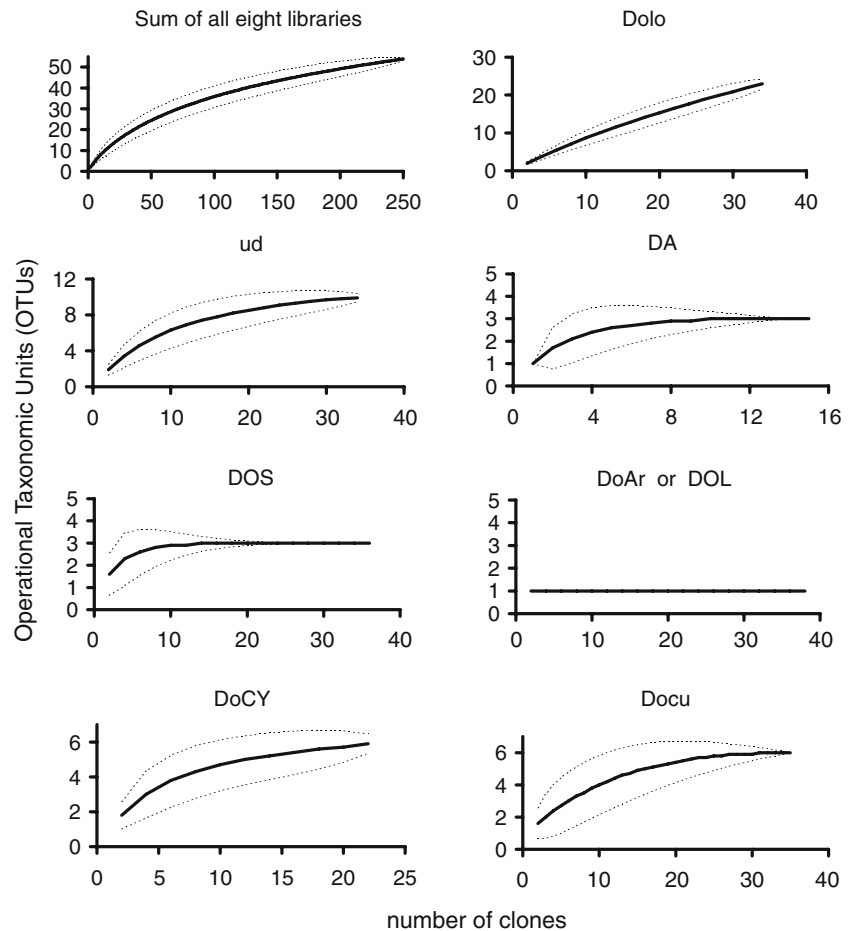
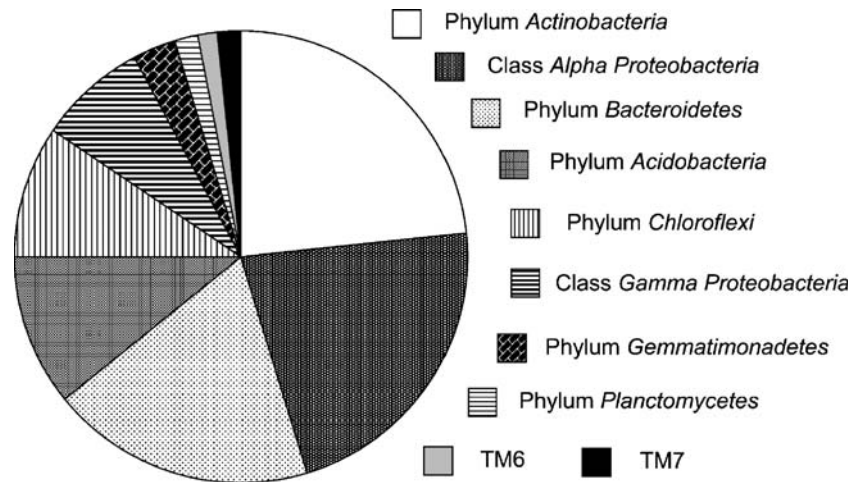


Figure 2 Distribution of phyla among the bacterial libraries “Dolo” and “ud”. The five groups *Actinobacteria*, *Proteobacteria* (mainly *Alphaproteobacteria*), *Bacteroidetes*, *Acidobacteria*, and *Chloroflexi* are predominant in terms of the number of OTUs with 3% level distinction



ud10=7.8%, each percentage referring to the sum of non-oxygenic “ud” and “Dolo” clones), *Acidobacteria* (ud01=9.4%), *Actinobacteria* (ud02=9.4%), and *Gamma Proteobacteria* (Dolo-06=7.8%; Table 2, and Fig. 2). None of these five most numerous sequences show a similarity to known SSU rRNA gene sequences of less than 95%. Several bacterial groups collectively account for a significant fraction of the total number of clones, while individual phylotypes are not particularly numerous. Nine phylotypes belong to the class *Alpha Proteobacteria* representing 22% of bacterial clones. Seven phylotypes belong to the phylum *Actinobacteria* and represent 23% of bacterial clones. Four phylotypes affiliate with uncultured *Chloroflexi*, accounting for 9.4% of the clones. Two phylotypes fall into the category of the phylum *Gemmatimonadetes* and consist of one clone each (3.1%). Phylotypes of *Planctomycetes*, of TM6 and of TM7 appear only once, each representing 1.6% of the bacterial clones. The phylogenetic position of the bacterial phylotypes is depicted in the trees in Fig. 3a and b.

Archaea

The archaeal library generated with the primer pairs 519f/1392r and 89Fb/915R (Table 1) resulted in three phylotypes—or four if ud14 and DOS_02 are counted as two separate phylotypes. They are 99.8% identical within their 420 bp fragment between positions 519 and 934 (*Escherichia coli* numbering). All the archaeal phylotypes found belong to the phylum *Crenarchaeota* and therein to the uncultured *Crenarchaeota* (Fig. 4). The phylotype of the clone DOS_02 amounts for the largest part of the crenarchaeal clones with 21 of 40 representatives (52.5%). It is followed by DOS_05 with ten clones (25%), DOS_21 with seven (17.5%), and ud14 with two (5%) out of 40 clones. All these clones show similarities of more than 98% with SSU rRNA gene

sequences from the public database, but for the time being, these are all uncultured archaeons. The closest named organism is *Cenarchaeum symbiosum*, an uncultured marine sponge symbiote [37], with similarities of 86% to ud14 and 81% to DOS_02, according to the EMBOSS Pairwise Alignment Tool provided by the EBI.

Eukaryotic Microorganisms

The primer combinations 8aF/1512uR and 8aF/1517r resulted in several eukaryotic sequences of SSU rRNA (Table 2). As the clones DA-04 and DA-15 are quite similar (97.2%), they are counted as one phylotype, likewise the clones DA-01 and DA-11, with 98.1% similarity. Hence, there are five different phylotypes, three of which belong to the class *Chlorophyta*: one to the order *Euamoebida* in the class *Lobosea*, and one to a moss in the division of the *Bryophyta* (Fig. 5). The phylotype of DoAr09 is 99.4% identical to *Saccamoeba limax* and the most numerous, with 39 out of a total of 83 eukaryotic clones (47%). Nevertheless, these numbers should not be overestimated, since they come from three combined clone libraries which were obtained under different conditions (chloroplasts not included). The moss represented by DOL_01 forms one third (33.7%) of the eukaryotic clones and is followed by the clones DA-01 (8.4%), DA-04 (7.2%), and DA-12 (3.6%), all belonging to the *Chlorophyta*. Two phylotypes, DA-01 and DA-12, have similarities of less than 95% to other sequences in public databases. DOL_01, DA-04, and DoAr-09 have NCBI-BLAST matches of more than 98%. Interestingly, DOL_01 (AB257668) is 99.2% identical to the *Hymenostylium recurvirostre* 18S rRNA (DQ629394), and Docu-30 (AB334295) is 99.7% identical to the *H. recurvirostre* chloroplast 16S rRNA (DQ629553), which suggests that protonemata of *Hymenostylium* prosper in the interstices of dolomite rock.



Figure 3 Phylogenetic tree with bacterial endolithic SSU rRNA gene sequences from alpine dolomite rock of the Piora Valley (in bold type) together with the closest relatives according to NCBI and ARB (tree calculated with ARB, Maximum Parsimony Method). The figures of

Bootstrap values are given in percent. *Saccharomyces cerevisiae* is used to root the tree. Accession numbers and the length of the sequences (nucleotides) are indicated after the names. **a** part 1, **b** part 2

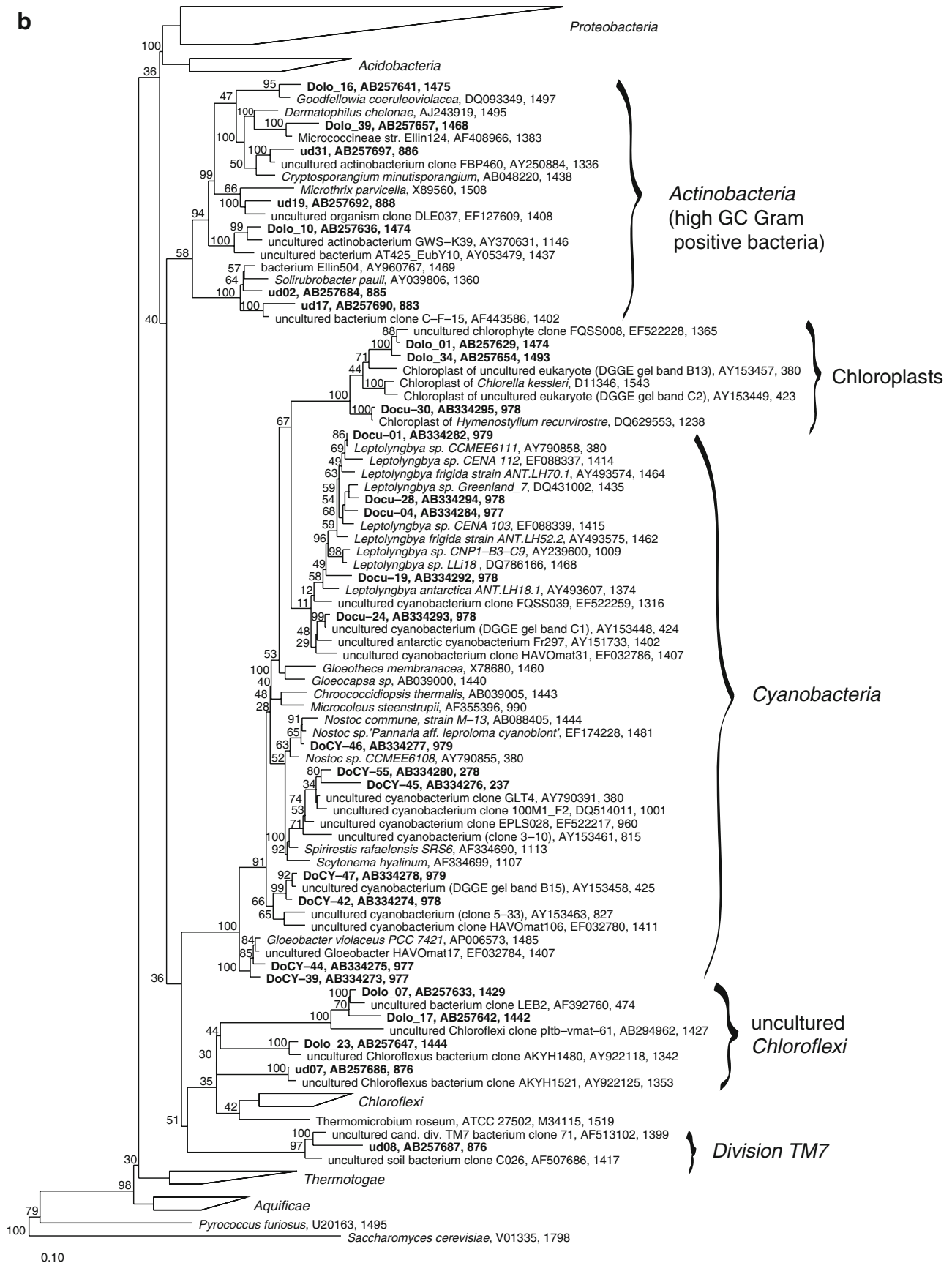
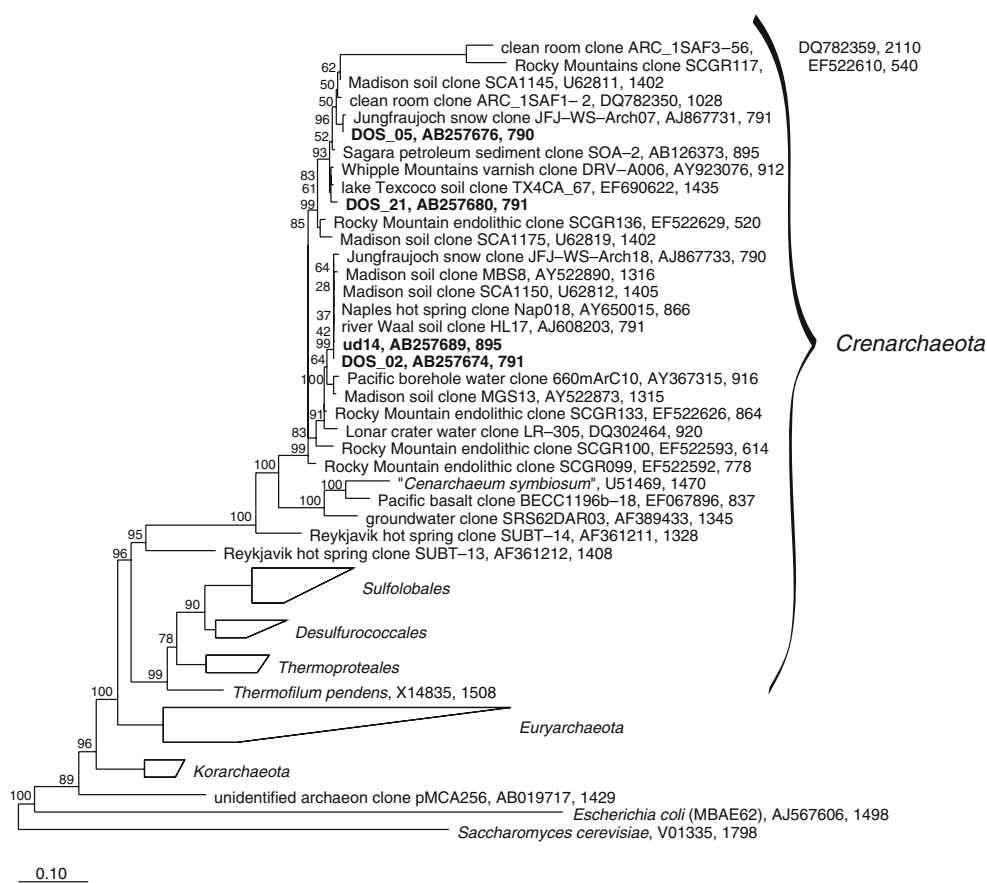


Figure 3 (continued)

Figure 4 Phylogenetic tree of archaeal endolithic SSU rRNA gene sequences obtained from alpine dolomite rock of the Piora Valley (in *bold type*) together with other sequences of *Archaea* (tree calculated with ARB, Maximum Parsimony Method). All sequences found fall into the group of uncultured Crenarchaeota. *E. coli* and *S. cerevisiae* are used as the out-group. The figures of Bootstrap values are given in percent. Accession numbers and the length of the sequences (nucleotides) are indicated after the names



Cyanobacterial Libraries

Two libraries were constructed with the specific primers CYA359F/CYA1342R. One came from a direct extraction of DNA from dolomite rock (DoCY) as described before, the other was obtained from an enrichment in a ten times diluted cyanobacterial BG11 medium seeded with rock material containing an endolithic band (Docu). 16S rRNA gene amplification, cloning, and sequencing yielded five different *Leptolyngbya* species (Docu-01, Docu-04, Docu-19, Docu-24, Docu-28) as well as a chloroplast of the moss *H. recurvirostre* (Docu-30, 99.7%). The DoCY cloning yielded six different phylotypes related to *Nostoc* (DoCY-46), *Gloeobacter* (DoCY-39 and DoCY-44), uncultured *Spirirestis* (DoCY-45 and DoCY-55), and an uncultured cyanobacterium (DoCY-42). The cyanobacterial sequences are included in the phylogenetic tree depicted in Fig. 3b.

Discussion

Many endolithic ecosystems were studied in the past century, focusing mainly on algal and cyanobacterial diversity, by use of culture techniques and microscopic morphotypes for identification [96]. As the various stress

factors present in endolithic sites may induce variations in size, color, and morphology, one cannot rely on morphological properties in situ or after cultivation. *Gloeocapsa sanguinea/alpina* changes its color from red (*G. sanguinea*) to blue (*G. alpina*), depending on the environmental pH level [45]. Morphological information alone may substantially mislead taxonomic identification [65]. Neither can pure culture techniques cover the full biodiversity, since in such a community culture, replication times of different species vary considerably, and mutualistic relations between species may get lost. Furthermore, it is questionable whether the better known epilithic microorganisms differ from the endolithic ones, which are thought to be restricted to the subsurface only. As it has, so far, hardly been possible to culture most environmental microorganisms, culture-independent molecular methods are suitable to obtain more information on the bacterial diversity. Walker and Pace suggest that, compared to other terrestrial ecosystems such as soil, endolithic communities in the Rocky Mountains, the Antarctica or the ones described here, are relatively simple systems with a rather restricted diversity. However, they also admit that molecular surveys do not completely sample the genetic diversity of a community [90].

Diels [19] and Jaag [45] found cyanobacteria in European Dolomite sites, Bell [7] in semi-arid regions and

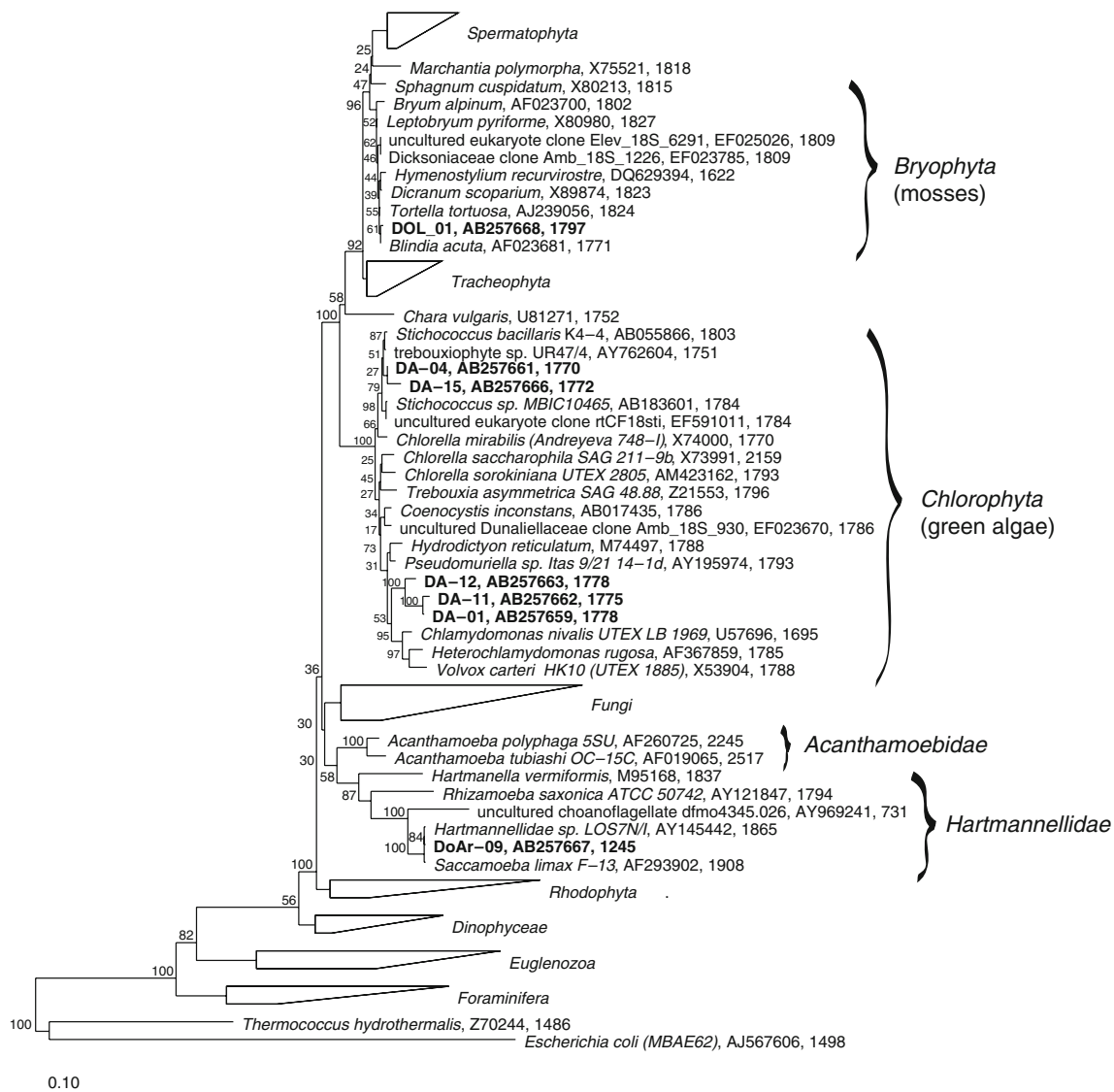


Figure 5 Phylogenetic tree of eukaryotic endolithic SSU rRNA gene sequences obtained from alpine dolomite rock of the Piora Valley (in *bold type*) together with other sequences of *Eukarya* (tree calculated with

ARB, Maximum Parsimony Method). Accession numbers and the length of the sequences are indicated after the names. *E. coli* is used to root the tree. The figures of Bootstrap values are given in percent

deserts in the southwest of the United States, Nienow and Friedmann [64] in the Antarctica, and Ferris and Lawson [22] as well as Gerrath et al. [31, 32] in limestone of the Niagara escarpment, all of which were classified by microscopy and culture techniques. Only a few of those genera have been confirmed with molecular methods. In endolithic habitats, cyanobacterial species related to *Plectonema* [17] and *Acaryochloris* [18] have been found as well as species related to *Anabaena*, *Chroococcidiopsis*, *Microcoleus*, *Nostoc*, and *Scytonema* [82]. The relationship between most of these sequences and the cultured strains is less than 96%. Up to now, Walker and Pace [89] have only found phylotypes “considerably different” from cultivated cyanobacteria. They have discovered two novel clades of specific endolithic cyanobacteria which are related to

cultivated strains with less than about 94% sequence similarity {Owl Canyon Sandstone clone OCSS038 (EF522486) as compared with *Spirirestis rafaensis* (AF334690)}. Lists of cultivated species and those of sequenced SSU rRNA genes hardly ever overlap, suggesting that species easy to cultivate may be the rare ones in nature. Norris and Castenholz [65] isolated endolithic phototrophs from rock material by culture techniques. Their list contains *Gloeocapsa*, very common in dolomite rock, as well as *Schizothrix*, *Nostoc*, and *Leptolyngbya*; all these genera were already mentioned by Jaag [45] or found with molecular methods by Sigler et al. [82]. However, about one third of the cultures listed by Norris and Castenholz have a similarity of less than 97% to the closest relatives known, and according to currently used criteria [84] may be

considered to be new species. This indicates that the bacterial diversity in most ecosystems must be larger than what has so far been detected by microscopy or cultivation as well as by sequencing.

By using specific cyanobacterial primers (CYA359F and CYA1342R), we found 11 phylotypes of cyanobacteria and three different sequences of chloroplasts of two green algae and one moss (Table 2). The cyanobacterial sequences indicated as closest cultivated relatives *Gloeobacter violaceus*, *Spirirestis rafaelsensis*, several *Leptolyngbya* sp., *Nostoc edaphicum*, and *Nostoc commune*. *Microcoleus steenstrupii* was found to be related to the clones DoCY-45 and DoCY-55, which were difficult to sequence and are only available as short sequences of about 200 bp. Sequences from the same sampling site, obtained earlier, suggest that *M. steenstrupii* as well as relatives of *Nostoc* PCC7120, of several *Chroococcidiopsis* sp., and of *Chlorella* sp. are also present there [82]. Sigler's DGGE band C1 obtained from an enrichment culture (AY153448) is now seen as the closest relative of our clone Docu-24. Both of them represent so far uncultivated cyanobacteria with 99.8% similarity between each other. The closest known cultivated strain to "band C1" is *Leptolyngbya* sp. PCC 9221 (94%), which confirms that there is still a gap in our knowledge as far as cultivated strains and collected environmental sequences are concerned. Sigler's sequence of band 15 (AY153458) now shows the closest similarity to clone DoCY-47 (AB334278) while bands 3 and 14 come closest to clone 46C-WNS (AB374402), which was gathered from a very similar environment in the Grisons, Switzerland. Interestingly, we also found a single chloroplast sequence, Docu-30 (AB334295), which corresponds 99.7% with a known chloroplast sequence of the moss *Hymenostylium recurvirostre* (DQ629553). This is affirmed by the presence of the 18S rRNA gene sequence of clone DOL_01 (AB257668) which is similar to the 18S rRNA gene sequence of *Blindia acuta* (AF023681) and of *H. recurvirostre* (DQ629394) by 99.5% and 99.2%, respectively.

Most environmental information on endolithic microorganisms is available on cyanobacteria. Clusters of *Leptolyngbya* are widely present in broad variations in all investigated ecosystems, in endolithic communities in the Rocky Mountains, in travertine of the Yellowstone National Park, in deep-sea basalt, and in alpine Piora dolomite [55, 65, 82, 89, and this paper]. *Nostoc* type filamentous organisms have been found in Piora and the Yellowstone, while relatives of coccoid *Gloeobacter* were observed in Piora and the Antarctica. *Gloeocapsa*, *Synechococcus*, *Synechocystis*, and *Chroococcidiopsis* are also present in all the above-mentioned systems but have not been detected in this study.

Little is known about the biodiversity of the heterotrophic bacterial communities accompanying the phototrophs. They were not dealt with in older studies for technical reasons. Sigler et al. [82] mentioned a large number of

"non-cyanobacterial" clones without giving details. The phylogenetic tree (Fig. 3a and b) shows that in spite of the hostile environment, the heterotrophic endolithic population is quite diverse and consists of many different species. The cloning yielded 31 different chemotrophic bacterial clones with only a few doublets. This and the rarefaction curves of the clone libraries "Dolo" and "ud" indicate that the inventory of new sequences is far from complete (Fig. 1). It contrasts with the organismic composition found in antarctic endolithic communities, where in communities with cyanobacteria as primary producers only two heterotrophic groups, the α -proteobacteria and the *Thermus-Deinococcus* group, were predominant besides the *Cyanobacteria*. The three groups together contributed to over 80% of the communities [17]. It remains to be tested whether it is possible to find more phylotypes in the McMurdo Dry Valleys or in the Piora dolomite by using different DNA extraction methods and different primers for the SSU rRNA gene. Using primer 1524r, for instance, instead of primer 1525r, with a difference of one base at the 3-prime end, already results in a strongly decreased number of detected cyanobacteria.

Most Piora sequences did not closely match with known sequences, and none of them were fully identical with a known sequence. The phylogenetic composition of the endolithic communities in Swiss dolomite was broader than the one in the Rocky mountains [89] with many phylotypes in the group of *Actinobacteria*, of *Alphaproteobacteria*, of *Bacteroidetes*, and of *Acidobacteria*. The group of *Actinobacteria* make up 23% of all phylotypes found in Piora dolomite, with a similar occurrence in the Rocky Mountains [89], on a wall in Fairy Cave, Glenwood Springs, CO, USA [3], and in rock varnish of the Whipple Mountains [48], but with 44%, they are more frequent in limestone of Ek Balam, Yucatan, Mexico [62] and with 65% predominant in rocks of the geothermal environment of the Yellowstone Park [88]. An explanation for the high fraction of *Actinobacteria* could be their strong cell wall and the capability of forming spores. Their high GC-content is also an advantage in extreme environments. In the dolomite of Central Switzerland, the overall sequence similarity of non-phototrophic prokaryotes was 94.9%; 40% of the bacterial clones and 45% of the chemotrophic ones showed a similarity of less than 95% to known SSU rRNA gene sequences. The highest similarity to cultured strains has been found in clones Dolo-40 and Dolo-28 with similarities of 99.4% and 98.8%, respectively, they are related to *Brevundimonas variabilis*, an α -proteobacterium. The lowest degree of similarity as compared with known 16S rRNA genes showed the clones Dolo-07, Dolo-17, and Dolo-29 with similarities of around 84%.

The observation of an in vivo absorption peak at about 720 nm in the pigments of the endolithic populations [42] suggests the presence of organisms from the branch of green

nonsulfur phototrophs. These organisms were originally thought to live only in extreme environments such as hot springs [9, 38, 39, 70, 71], but some time ago, they were also found in temperate and even cold environments, such as wastewater treatment systems [4, 8, 80], the deep ocean [33], endolithic systems [17, 67, 89], as well as subsurface soil (paleosol) at a depth of 188 m [14]. Our sequence data confirm the presence of several green nonsulfur strains in the dolomite rock of the Piora Valley.

As in Antarctic endolithic communities [17, 83], except for *Cyanobacteria* and *Actinobacteria*, many phylotypes appeared in low numbers or even just as one, suggesting that the diversity must be substantially larger than presented by the clone libraries. This contrasts with some of the rarefaction curves obtained (Fig. 1), which level off rapidly. We assume that this rapid flattening of some curves in Fig. 1 is due to technical limitations such as biased DNA extractions and/or insufficiently fitting amplification primers for the communities in question.

While Smith et al. [83], de la Torre et al. [17], and Sigler et al. [82] did not describe any *Archaea* in endolithic communities, *Crenarchaeota* phylotypes were found in the Rocky Mountains and in deep-sea basalt [55, 89]. In the phylogenetic tree with the archaeal branch (Fig. 4) the three sampling sites show a different distribution. Together with sequences from Australian marine stromatolites [67] and other uncultured *Crenarchaea*, samples from marine basalt {clone BECC1196b-18 (EF067896) as representative} group closely around *Cenarchaeum symbiosum*. On the other hand, the archaeal clones from the Rocky Mountains partially group around our clones ud14 and DOS_02 or form a slightly different group clustered around the clone “ARC_1-SAF3-56” (DQ782359) from a clean assembly room for NASA spacecraft [61] but are still closer to ud14 than to the basalt group. Interestingly, many other locations all over the world harbor *Crenarchaea* similar to the ones in the Piora dolomite, such as snow from Jungfraujoch in the Swiss Alps (AJ867733), for example, or soil from a rarely flooded plain by the river Waal in The Netherlands (AJ608203), or slit from a hot spring near Naples (Italy; AY650015), or the ODP 892 b borehole in the Pacific (AY367315), or soil in an agricultural research station in Madison (USA; U62812), or soil in the former Lake Texcoco close to Mexico City (EF690622), or in excavated material from a borehole, 200 m deep, of an oil drilling project in Japan (AB126373), or in the sediment of the Lonar Crater Lake in India (DQ302464).

On the whole, the archaeal sequences from the arid endolithic sites [present study and 89] are more related to each other than to endolithic organisms from aquatic sites [55]. A similar clustering has been observed in the group of the *Cyanobacteria*. The phylogenetic cluster formation of clones in similar habitats is more common than that of clones which live in different environments and are geographically

further apart from each other. This indicates that both, geographical distances of the habitats and site-specific environmental factors have an influence on the biogeography of the organisms.

Among the heterotrophs, phagotrophic protists, mainly ciliates and flagellates, play an important role in the nutrient cycle as consumers of bacteria in aquatic environments. It has recently been discovered that *Amoeba* feed on cyanobacteria [97]. It is, thus, of special interest to find such consumers also in dry endolithic environments, where cyanobacteria form a large part of the biomass.

Conclusion

The results presented in this paper demonstrate that the bacterial diversity in endolithic habitats, especially of chemotrophic, nonpigmented organisms, is considerable but has been hidden and, therefore, underestimated previously. As most of the sequences have only been found once or in low numbers, a much greater diversity than the one described here may be expected. The finding of some ribosomal sequences of the crenarchaeal branch demands for a more detailed study of the *Archaea*.

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