Molecular characterization of endolithic cyanobacteria inhabiting exposed dolomite in central Switzerland

William V. Sigler,[1] Reinhard Bachofen[2] and Josef Zeyer[1]

[1]*Soil Biology, Institute for Terrestrial Ecology, Swiss Federal Institute of Technology (ETH), 8952 Schlieren, Switzerland.*
[2]*Institute for Plant Biology, University of Zurich, 8008 Zurich, Switzerland.*

Summary

The phototrophic microbial community inhabiting exposed dolomite in the alpine Piora Valley (Switzerland) forms a distinct endolithic bilayer that features adjacent red dolomite (exterior) and green dolomite (interior) layers that are *c.* 0.5–1 mm below the rock surface. Characterization of the community, with an emphasis on cyanobacteria, was conducted with culture-dependent and -independent approaches. Direct microscopy of green dolomite revealed four distinct morphotypes consistent with Chlorophyta genera *Chlorella* and *Stichococcus* and the Cyanobacterial genera *Nostoc* and *Calothrix*, whereas only *Stichococcus* and *Nostoc* were observed in the red dolomite. Enrichment in BG-11 media resulted in the growth of *Chlorella* and *Stichococcus*. Denaturing gradient gel electrophoresis (DGGE) analysis of DNA extracted from the enrichment revealed two dominant phylotypes with sequence similarity to *Chlorella osrokiniana* chloroplast and the cyanobacteria genus *Leptolyngbya*. 16S rRNA gene-based DGGE analysis of DNA extracted directly from both layers indicated that although both layers harboured phylotypes most similar to the Cyanobacterial genera *Nostoc*, *Chroococcidiopsis*, and *Microcoleus*, and the Chlorophyte *Stichococcus bacillaris*, the two layers also harboured unique genera such as *Scytonema*, and *Symploca (*red, external layer of dolomite) and *Chlorella* (green, internal layer of dolomite). The unique community structure of each layer suggests a selection process directed by the pressures of the endolithic environment. We conclude that the overall composition of the phototrophic community closely resembles that of endolithic communities located in extreme habitats, suggesting that a cosmopolitan community inhabits this defined niche.

Introduction

Since the first description of endolithic algae in dolomite rock (Diels, 1914), endolithic organisms have been observed in a variety of extreme terrestrial ecosystems including warm and cold deserts (Nienow *et al.*, 1988; Johnston and Vestal, 1991; reviewed by Bell, 1993), and Polar regions (reviewed by Vincent, 2000). More recently, the global distribution of endolithic organisms has been highlighted by communities detected in temperate environments such as the Niagara Escarpment, Canada (Gerrath *et al.*, 1995; 2000; Matthes-Sears *et al.*, 1997). Additionally, endoliths have been observed inhabiting a variety of rock types ranging from hard granite (Schultz *et al.*, 2000) to porous rocks such as gypsum, limestone or sandstone (Bell, 1993). Regardless of geographic location and rock type, each of these habitats is characterized by a surface microclimate that prevents microbial growth (Bell, 1993). Because of this extreme habitat, endolithic organisms maximize their survival by inhabiting existing cracks and fissures (known as chasmoendolithic growth), internal pores (cryptoendolithic), or by actively burrowing into the rock (euendolithic). It has even been suggested that escape into the endolithic environment is driven not by hostile surface conditions, but by decreased competition for nutrients and space beneath the rock surface (Matthes-Sears *et al.*, 1997). Although the ability of an organism to access shelter within the lithic interior appears to demonstrate a competitive advantage, the protection provided by the habitat is limited. Organisms native to the endolithic environment must possess adaptive ability and tolerance to severe stresses including temperature extremes, oligotrophy (Vestal, 1988a), desiccation (Lange *et al.*, 1994), and high UV flux (Garcia-Pichel and Castenholz, 1993).

Communities of microorganisms that inhabit endolithic environments include heterotrophic bacteria (Siebert *et al.*, 1996), fungi (Hirsch *et al.*, 1995), eukaryotic algae, cyanobacteria and fungal/cyanobacterial symbioses (lichens) (Gerrath *et al.*, 1995). So far, the most widespread of these groups are cyanobacteria and eukaryotic algae, which have been detected in significant abundance

in almost every endolithic ecosystem (Bell *et al.*, 1986; Matthes-Sears *et al.*, 1997). The ability to fix carbon dioxide, and in some cases atmospheric dinitrogen (N_2), gives the cyanobacteria, in particular, a clear competitive advantage over heterotrophic bacteria in colonizing the exterior few millimetres of exposed rocks. In fact, phototrophs, especially those growing endolithically, represent some of the only primary producers present in the extreme environment of the Antarctic desert (Johnston and Vestal, 1991). Light quality and intensity appear to be the main determinant of the maximum depth to which growth occurs in endolithic phototrophic communities (Nienow *et al.*, 1988). It has also been reported that sensitivity to intense light determines the minimum depth of cyanobacteria beneath the surface, as was suggested by a decrease in cyanobacterial photosynthesis during periods of high light intensity (Bell, 1986). As a result of interplay between incoming light intensity and attenuation by the rock strata, the presence of endolithic phototrophs is often characterized by a band(s) of coloured biomass (usually green) varying from less than one to several mm in thickness that forms at a given depth beneath the rock surface.

Most previous investigations of endolithic communities utilized culture-dependent techniques through which standard morphological characteristics were used to identify community members (Castenholz and Waterbury, 1989). The identification of cultured cyanobacteria is particularly problematic because (i) the morphology of laboratory cultures may not always represent the native form of the bacteria (Ward *et al.*, 1997), (ii) cyanobacteria are typically difficult to establish *in vitro*, and (iii) cyanobacteria may exhibit resistance to culture depending on the choice of solid or liquid media (Evans *et al.*, 1976; Sivonen *et al.*, 1989; Ward *et al.*, 1997). Throughout the past decade, culture independent techniques including the direct extraction of DNA has greatly facilitated community analysis of environmental samples. Following DNA extraction, several lines of investigation are possible, including sequencing of clone libraries, and genetic fingerprinting of both whole communities and bacterial isolates. Despite the advantages of a culture independent approach, few molecular-based studies of endolithic community structure have been performed. However, the utility of the molecular approach to investigate cyanobacterial communities has been demonstrated recently in desert soil crusts (Garcia-Pichel *et al.*, 2001; Redfield *et al.*, 2002), a system comparable to that of the endolithic environment. In these studies, genetic fingerprinting and the retrieval of 16S rRNA gene sequences revealed the consistent dominance of soil crust genera observed previously in endolithic environments (*Nostoc*, *Phormodium*, *Scytonema* and *Chroococcidiopsis*) as well as the dominant presence of the genus *Microcoleus*, which remains undetected in a true (non-soil crust) endolithic habitat.

The Piora endolithic environment features two distinct layers of biomass; a red layer (hereafter referred to as 'red dolomite') approximately 0.5–1 mm below the rock surface, and a green layer ('green dolomite') directly adjacent to and beneath the red dolomite (Fig. 1). Our preliminary investigations of the endolithic communities inhabiting the dolomite revealed a complex assemblage of cyanobacteria. However, questions still remain concerning the community structure, identity, and ecological interactions of the cyanobacteria inhabiting this poorly understood habitat, especially with regard to the bilayered system. The objective of the present study was to combine direct microscopic examination, molecular- and culture-dependent techniques (Fig. 1) to characterize the community structure of the endolithic cyanobacteria in the Piora Valley, Switzerland.

Results

Biological and chemical site parameters

The estimation of total cell counts in the red and green dolomite, as well as pH, bulk dolomite concentrations of organic C, total N, and other nutrients are summarized in Table 1.

Direct microscopic examination of phototrophic communities

Direct microscopic examination of dolomite wet mounts and enrichments in BG-11 revealed few major morphotypes of phototrophs, including the cyanobacterial genera *Nostoc* and *Calothrix*, as well as the algal genera *Chlorella* and *Stichococcus* (Fig. 2). Descriptions of the morphotypes, culture conditions, and putative identifications per the identification keys of Herdman *et al.* (2001)

Table 1. Selected biological and chemical parameters of the dolomite investigated in this study. Parameters were measured in bulk dolomite according to Fig. 1 unless otherwise noted.

Parameter	Measured value
Total cell count: red dolomite	4.0×10^9 (± 1.1)[a]
green dolomite	3.7×10^9 (± 0.4)[a]
pH (in water)	9.5
pH (in $CaCl_2$)	8.5
C_{org}	1.90%
N_{tot}	0.02%
Ca	20.15%
Mg	11.67%
K	0.19%
Fe	0.19%
P	<0.002%
S	156 μg g^{-1}
Cl	95 μg g^{-1}

a. Calculated as the number of DAPI stained cells g^{-1} dry dolomite according to the method of Zarda *et al.* (1997).
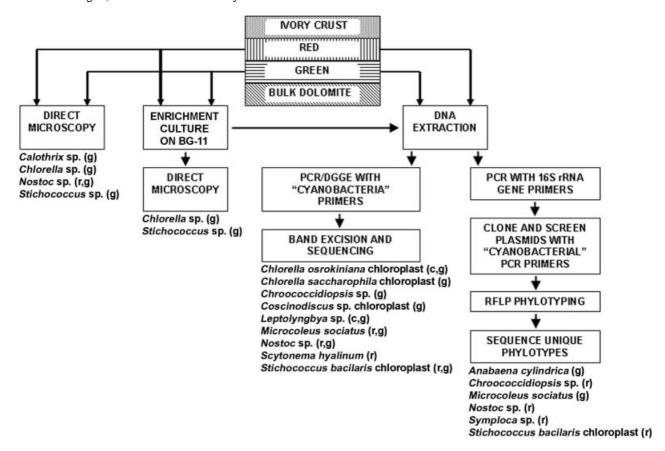
Fig. 1. Overview of the experimental procedures. The putative identification of each detected phototroph is listed below the procedural endpoints as well as a reference to the environment in which each was detected (r, red dolomite; g, green dolomite; c, culture enrichment on BG-11).

and Margulis and Schwartz (1998) are summarized in Table 2.

Following an incubation period of either seven- or 16 weeks in BG-11 liquid medium, abundant bacterial growth was observed in cultures inoculated with green dolomite. Although bacterial growth was also evident in cultures inoculated with red dolomite, microscopic inspection suggested an absence of phototroph growth. To confirm this conclusion we compared the red dolomite communities (microscopically) enriched on BG-11 following incubation under both light and dark conditions. Because both cultures contained visually similar communities, it was concluded that the red dolomite culture was not enriched with phototrophic bacteria.

*DGGE of phototrophic communities enriched in
BG-11 medium*

Polymerase chain reaction amplification of DNA extracted from BG-11 cultures using the cyanobacteria-specific primers of Nübel *et al.* (1997) revealed a product only from those cultures inoculated with green dolomite. Denaturing gradient gel electrophoresis (DGGE) analysis revealed

similar phylotypes regardless of incubation time (seven weeks versus 16 weeks) and sequence analysis of the excised DGGE bands revealed phylotypes most similar to the cyanobacteria *Leptolyngbya* sp. PCC 9221 (band C1) and the *Chlorella osrokiniana* chloroplast (band C2) (Table 3, Fig. 3A). Interestingly, only *Chlorella* sp. was observed visually in the BG-11 enrichment and wet mounts of dolomite whereas no cells matching the morphology of *Leptolyngbya* sp. were observed.

*DGGE of phototrophic communities in red and
green dolomite*

Denaturing gradient gel electrophoresis analysis of DNA directly extracted from the red and green dolomite revealed that multiple phototrophic phylotypes comprised the two layers. Furthermore, each layer, while harbouring several common phylotypes and similar band richness, displayed a distinct fingerprint (Fig. 3B). In the red dolomite, eight phylotypes (bands) were visualized, whereas 10 were observed in the green dolomite. Six phylotypes were shared between the two layers, whereas two and four phylotypes were unique to the red and green dolomite
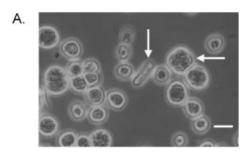
Fig. 2. Diversity of phototrophic morphotypes in green dolomite harvested from the Piora Valley, Switzerland.
A. *Chlorella*, including dividing (horizontal arrow) and non-dividing cells. The elongated cell (vertical arrow) was putatively identified as *Stichococcus* sp.
B. Young *Nostoc* colony: note terminal heterocysts.
C. Trichome of *Calothrix* featuring a basal heterocyst. All photographs were taken at 1000× magnification. Bar: 10 μm.

respectively. Sequence analysis of bands excised from the DGGE gel of the red dolomite community revealed highest similarity to three cyanobacteria sequences: *Nostoc* sp. PCC7120 (band 3), *Scytonema hyalinum* (band 4), and *Microcoleus sociatus* (band 7); and one chloroplast sequence derived from *Stichococcus bacillaris* (band 1 and 2). The eight bands derived from the green dolomite exhibited highest similarity to three cyanobacterial sequences: *Nostoc* sp. PCC7120 (band 14), *Chroococcidiopsis* sp. BB96.1 (band 15) and *Microcoleus sociatus* (band 17); and three chloroplast sequences: *Coscinodiscus* sp. JD 2001 (band 9), *Stichococcus bacillaris* (bands 10, 11 and 12), and *Chlorella saccharophila* (band 13). Sequence analysis determined that four of the six phylotypes that appeared to be common to both layers, based on co-migration of DGGE bands, were identical as deter-

mined by sequence analysis (*Stichococcus bacillaris* [bands 1 and 10, 2 and 12], *Nostoc* sp. PCC7120 [bands 3 and 14], and *Microcoleus sociatus* [bands 7 and 17]). Despite efforts to further process the other two co-migrating bands (bands 6 and 16, and bands 8 and 18), reliable sequences could not be determined.

Phylogenetic analysis of 16S rDNA sequences generated from directly cloned DNA

Of the 40 clones generated after directly cloning DNA extracted from red dolomite, 12 'cyanobacteria-positive' clones (27%) were detected after screening with cyanobacteria PCR primers. Following RFLP analysis, four different patterns (phylotypes) were identified. Of the 60 clones generated from green dolomite DNA, seven (12%) screened positive with cyanobacterial primers, with two distinct phylotypes identified by RFLP analysis. Plasmids harvested from each of the 'cyanobacteria-positive' clones were initially screened with PCR-DGGE analysis, which revealed that clone analysis and PCR-DGGE of directly extracted dolomite DNA resulted in bands of similar migration behaviour (compare Fig. 3B and C). Sequence analysis of representatives of the four red dolomite phylotypes revealed similarity to three cyanobacteria: *Nostoc* sp. PCC7120, *Symploca* sp. VP624c, and *Chroococcidiopsis* sp. BB96.1 and one chloroplast derived from *Stichococcus bacillaris* (Table 3). The two green dolomite phylotypes were most similar to *Microcoleus sociatus* and *Nostoc* sp. PCC7120 (Table 3).

Discussion

Although endolithic algal communities were first described in alpine environments (Diels, 1914), the investigations of the past decades have centred on the extreme habitats of warm and cold deserts found in the western United States and Antarctica respectively. More recent studies have given attention to temperate environments such as those found in the Niagara Escarpment in Canada. In each of these environments, the predominance of similar organisms including the genera *Gloeocapsa, Chroococcidiopsis, Nostoc* and *Scytonema* (Diels, 1914; Friedmann and Ocampo-Friedmann, 1984; Broady, 1986; 1989; Friedmann *et al.*, 1988; Büdel and Wessels, 1991; Gerrath *et al.*, 1995; Ferris and Lowson, 1997; Banerjee *et al.*, 2000) suggests that stresses common to endolithic environments worldwide have selected for a niche-specific assemblage of tolerant organisms. An example of the specificity of this selection within the cyanobacteria was provided by Nienow and Friedmann (1993) who showed through DNA–DNA hybridization, that two *Chroococcidiopsis* species isolated separately from hot and cold deserts were indeed the same. Whereas no previous detailed

Table 2. Morphological characterization of cyanobacterial and algal cells observed following examination of enrichment cultures and endolithic layers.

Morphotype (primary characteristics)	Relative abundance[a]			Taxonomic assignment[c]
	Enrichment[b]	Red dolomite	Green dolomite	
Unicellular, non-motile, spherical, 8–10 μm diameter; parietal chloroplasts; pigment characteristic of chlorophyll –a and –b combination; remains of cell wall debris suggested endospore formation followed by cell bursting	++ (Fig. 2A)	–	++	*Chlorella* sp.
Unicellular, elongated (1 × 3 μm in lithic samples) with rounded ends; larger cells (3 × 12 μm) were visible in the culture enrichment	+ (Fig. 2A)	++	++	*Stichococcus* sp.
Colonial mass of 8–10 spherical cells of 9–10 μm diameter enclosed in an elongated envelope (25–30 × 45–60 μm); two parietal interheterocysts; morphology consistent with late aseriate stage of *Nostoc*; phycocyanin degradation was apparent	–	+	+ (Fig. 2B)	*Nostoc* sp.
Short filaments (10–12 × 40–50 μm diameter) containing a chain of 5–6 spherical cells of 8 μm diameter; terminal heterocyst present at a broad base; sheath tapered to a pronounced apex at the non-heterocystous end	–	–	+ (Fig. 2C)	*Calothrix* sp.

a. ++, dominant; +, present; –, not detected.
b. Results represent growth from green dolomite BG-11 enrichment, see *Results* section for explanation.
c. Assignments per Herdman *et al.* (2001) and Margulis and Schwartz (1998).

study of endolithic community structure has relied on molecular analysis, investigation of cyanobacterial communities in arid soil crusts on the Colorado Plateau revealed sequences similar to those of several unidentified cyanobacteria as well as the genera *Oscillatoria*, *Chroococcidiopsis*, *Scytonema*, *Leptolyngbya*, *Microcoleus, and Phormidium* (Garcia-Pichel *et al.*, 2001; Redfield *et al.*, 2002). Therefore, it is no surprise that the majority of the organisms detected in the current study following 16S rRNA gene sequence analysis (Table 3) are most similar to those observed previously in environments characterized by similar selective pressures such as nutrient availability, and osmotic- and UV intensity-related stresses. We are aware that community analysis based on 16S rRNA gene sequences is limited by the lack of physiological information describing activities of the micro-

Table 3. Phylogenetic characteristics of clones generated from Piora Valley dolomite.

Clone (GenBank accession number)	Method	Endolithic layer	Length (bp)	Closest identified relative	
				Taxon (GenBank accession number)	% identity
Band C1 (AY153448)	Culture/DGGE[a]	Green	424	*Leptolyngbya* sp. PCC9221 (AF317507)	93
Band C2 (AY153449)	Culture/DGGE	Green	423	*Chlorella osrokiniana* chloroplast (X65689)	96
Band 1 (AY153450)	DGGE[b]	Red	424	*Stichococcus bacillaris* chloroplast (AF278751)	96
Band 2 (AY153451)	DGGE	Red	424	*Stichococcus bacillaris* chloroplast (AF278751)	96
Band 3 (AY153452)	DGGE	Red	425	*Nostoc* sp. PCC7120 (AP003598)	92
Band 4 (AY153453)	DGGE	Red	424	*Scytonema hyalinum* (AF334699)	92
Band 7 (AY153454)	DGGE	Red	425	*Microcoleus sociatus* (AF284809)	93
Band 9 (AY153455)	DGGE	Green	425	*Coscinodiscus* sp. chloroplast JD 2001 (AJ319823)	92
Band 10[c]	DGGE	Green	424	*Stichococcus bacillaris* chloroplast (AF278751)	96
Band 11 (AY153456)	DGGE	Green	424	*Stichococcus bacillaris* chloroplast (AF278751)	96
Band 12	DGGE	Green	424	*Stichococcus bacillaris* chloroplast (AF278751)	96
Band 13 (AY153457)	DGGE	Green	380	*Chlorella saccharaphila* chloroplast (D11349)	99
Band 14	DGGE	Green	425	*Nostoc* sp. PCC7120 (AP003598)	92
Band 15 (AY153458)	DGGE	Green	425	*Chroococcidiopsis* sp. (AF279107)	91
Band 17	DGGE	Green	425	*Microcoleus sociatus* (AF284809)	93
P3-2 (AY153459)	Direct[d]	Red	816	*Nostoc* sp. PCC7120 (AP003598)	90
P3-6 (AY153460)	Direct	Red	824	*Symploca* sp. VP624c (AY032934)	86
P3-10 (AY153461)	Direct	Red	816	*Chroococcidiopsis* sp. BB96.1 (AJ344555)	88
P3-14 (AY153462)	Direct	Red	824	*Stichococcus bacillaris* chloroplast(AF278751)	91
P5-33 (AY153463)	Direct	Green	835	*Microcoleus sociatus* (AF284809)	88
P5-41 (AY153464)	Direct	Green	827	*Anabaena cylindrica* (AF091150)	92

a. Culture/DGGE, sequencing of excised band following PCR/DGGE of DNA extracted from BG-11 enrichment.
b. DGGE, sequencing of excised band following PCR/DGGE of DNA extracted from either the red- or green dolomite.
c. In cases where bands contained DNA of identical sequence (e.g. Bands 1 and 10), only one sequence was submitted to GenBank.
d. Direct, sequencing of plasmids following PCR and cloning of DNA extracted from either the red- or green dolomite.
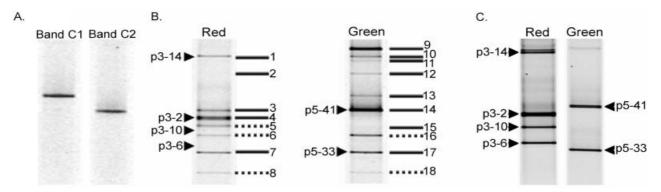
Fig. 3. Cyanobacterial PCR/DGGE analysis of endolithic phototroph communities.
A. Bands C1 and C2, which resulted from PCR/DGGE analysis of DNA extracted from BG-11 enrichment.
B. PCR/DGGE analysis of DNA extracted from either the red- or green dolomite. Solid lines represent bands excised and sequenced as described in *Experimental procedures*. Dashed lines represent bands detected in the DGGE analysis of the community, but not processed further, as described in *Results*.
C. DGGE analysis of 'cyanobacteria positive' plasmids that were generated from cloning of DNA isolated from red or green dolomite. For B and C arrowheads refer to the position of bands resulting from DGGE analysis of cloned cyanobacterial sequences (plasmids) that were reamplified with cyanobacteria-specific primers. All numbering refers to the band or clone number as described in the text.

bial community members. Although one must be cautious when interpreting the identity of and interactions between the described organisms, thoughtful speculation surrounding the interactions between the communities and their environment can be helpful.

Despite the fact that the Piora dolomite contained almost 2% organic carbon (w/w), it is likely that only a small portion of this pool is actually utilized by phototrophic organisms. However, liberated carbonate can be used as a secondary carbon source and is usually available because of organic acid-driven carbonate dissolution by heterotrophic bacteria that live in association with cyanobacteria (Vestal, 1988b). Nitrogen limitation also is likely non-existent in many endolithic environments due to the combination of slow bacterial growth and the percolation and diffusion of nutrients such as nitrate, particularly if overlying detritus is present (Friedmann and Kibler, 1980). However, we measured only 0.02% (w/w) total nitrogen in the Piora dolomite. Therefore, nitrogen represents a limiting nutrient and it is not surprising that some of the cyanobacterial phylotypes observed in the Piora dolomite are related to known diazotrophs, including *Anabaena*, *Calothrix*, *Scytonema* and *Nostoc* (Waterbury, 1991).

Regardless of geographic location, a characteristic common to most terrestrial endolithic environments is transient water availability (Vestal, 1993). Although the Piora Valley cannot be considered a desert environment, the area does experience episodes of hot and dry weather that desiccate the dolomite surface (Kirchofer, 2000). Cyanobacteria native to endolithic environments have evolved remarkable strategies to cope with water-related stress (Scherer *et al.*, 1984). Three dominant genera putatively detected in this study; *Nostoc*, *Microcoleus* and

Chroococcidiopsis, have been previously noted for their outstanding tolerance to dry conditions. In particular, after long periods of desiccation, *Microcoleus* and *Chroococcidiopsis* possess the ability to regain photosynthetic capacity within minutes following rewetting (Hawes *et al.*, 1992; Lange *et al.*, 1994). Additionally, *Nostoc* has been shown *in vitro* to resist water loss at potentials of −400 MPa (Potts, 1994).

Because primary production is an important role of cyanobacteria in the endolithic environment, light-attenuation is a vital limiting factor for phototrophic life. Analogous situations exist in microbial mats or planktonic communities, where decreased light intensity and quality with increasing depth drives a vertical stratification of species by selecting for organisms with the ability to adapt to decreasing photosynthetically active radiation. In the endolithic environment, the opposite situation is also significant, whereby high light intensity in the exterior few millimetres of exposed rock inhibits colonization by microbial communities. Of particular interest to this topic is the recent study of Bowker *et al.* (2002), which highlighted the enhanced mortality of the soil crust cyanobacteria *Microcoleous vaginatus* in the absence of an overlying layer of UV-shielding cells. It was hypothesized that a layer of highly pigmented cell types including *Nostoc* and *Scytonema* shielded the underlying *Microcoleous* population from the harmful impact of UVB radiation. A similar phylotype distribution was observed in the Piora dolomite. We detected organisms similar to both *Nostoc* (microscopically, DGGE band 3 and clone P3-2) and *Scytonema* (DGGE band 4) in the red dolomite, where photosynthetically active radiation was presumed to be strongest, whereas dominant populations of *Microcoleous*-type cells were detected in the green dolomite by DGGE (band 17,

and also less visually intense in the red dolomite [band 7]) and direct DNA sequencing (clone number P5-33). It is understood that some cyanobacteria contain compounds that play a role in protecting the cell from photooxidative damage. Specifically, *Nostoc* and *Scytonema* have been shown to contain multiple UVB-protective compounds such as scytonemins (Garcia-Pichel and Castenholz, 1993), mycosporine-like amino acids (Böhm *et al.*, 1995), carotenoids, and other uncharacterized pigments (Kumar *et al.*, 1996). Scytonemin and mycosporine-like amino acids absorb light in the UVA, -B, and -C wavelengths, the combination of which appears to provide adequate protection for the underlying *Microcoleus* from UV radiation. This combination, along with *Nostoc*-derived carotenoids, might also contribute to the characteristic red colouration of the dolomite. To the best of our knowledge, our observation of *Microcoleus*-type cells in the Piora system represents the first documentation of the genus in an endolithic environment, although it is commonly found in soil crust habitats (Mazor *et al.*, 1996; Garcia-Pichel *et al.*, 2001; Redfield *et al.*, 2002). It is plausible that the absence of *Microcoleus* from previous endolith inventories was due to studies reliant on culture-based methodology. Nevertheless, one must not discount the possibility that an obstacle to the frequent detection of *Microcoleus* is a function of its distribution in a highly specific niche within the endolithic environment.

Although commonly used for cyanobacteria culture, the selection of BG-11 as our medium could have favoured the faster-growing cyanobacteria and chlorophytes present in the Piora system. Therefore, the dominance of the genus *Chlorella* in our cultures was predictable, as it is competitive and can attain a relatively high growth rate (Elliot *et al.*, 2002). Future attempts at isolating cyanobacterial and algal cells from endolithic samples should include the use of various media types, as it is clear that enrichment in a single medium greatly underestimated the true cellular diversity. At the same time, however, each analytical approach uncovered a different subset of the phototrophic community confirming the necessity of a polyphasic approach. Based on the number of phylotypes detected among the methods used in this study, DGGE analysis proved to be the most descriptive method, as it revealed a higher complexity of the phototrophic community than the other approaches. The analyses revealed that red and green dolomite each harboured common phylotypes with several dominant types in each layer (as determined by relative abundance microscopically and visual intensity of DGGE bands). Additionally, each layer exhibited unique morphotypes/phylotypes and thus distinct community structure. Of the 12 different phototrophic morphotypes/phylotypes putatively identified in this study, six were detected by two or more methods (*Chlorella*, *Chroococcidiopsis*, *Stichococcus*, *Nostoc* and *Microco-*

leus), suggesting that these commonly detected phylotypes might be the most abundant phototrophs at this site.

Our direct DNA cloning procedure involved an initial PCR amplification with *Bacteria* domain primers followed by traditional cloning procedures. Selected clones were screened for the presence of cyanobacteria sequences by subjecting clones to a cyanobacteria-specific PCR reaction as described in *Experimental procedures*. The purpose of this methodology was twofold: first, because a larger portion of the 16S rRNA gene could be analysed, this approach resulted in more phylogenetic information than would have been available using the cyanobacterial primers alone, resulting in a more confident identification of the endolithic communities. Second, the approach supports the option for future studies of other taxa, as clones screened as 'non-cyanobacteria' were abundant and also collected for future investigation.

Experimental procedures

Site description

The Piora Valley in southern Switzerland (E 8°43′ 6′, N 46°33′ 0′) runs east–west for 8 km and lies between 1800 and 2000 m above sea level. The area experiences a mean annual temperature of between 0 and 5°C, and approximately 150 cm of precipitation per year (Spreafico *et al.*, 1992). The Piora Valley also experiences 1800–1900 h of sunlight per year with an average intensity of 150 W m^{-2} (Kirchofer, 2000). The dolomitic limestone (Ca:Mg = 2.8:1, molar basis) substratum is weathered, porous, and exhibits a semipermanently moist subsurface. The area is mostly soil-covered, but in many places bare dolomite escarpments are present beneath an overlying layer of vegetation and accumulated organic matter. Preliminary studies revealed a thin (2–3 mm thick) layer of green biomass (referred to as green dolomite) located 2–3 mm below the rock surface. An additional feature of the Piora endolith system is an occasionally occurring red dolomite layer (referred to as red dolomite), 1–2 mm thick, located between the green dolomite and the rock surface, resulting in a bilayered profile.

Sample preparation and determination of environmental parameters

Samples of dolomite were dislodged from several random locations using a hammer and chisel, and placed into sterile tubes. Upon arrival at the laboratory, a sterile probe was used to separate the rock into two fractions, corresponding to the colour of the endolithic biomass band. Specifically, a very thin (0.5–1 mm) crust of ivory-coloured rock that comprised the surface of the dolomite was removed to reveal an underlying band of red biomass. Although several attempts were made to harvest 'pure' crust samples free of red biomass, complete separation of the crust from the red dolomite was impossible. Red dolomite (1–2 mm thick) was carefully scraped and collected in a 1.5-ml microcentrifuge tube. The same procedure

was followed for the green dolomite (2–3 mm thick). Because one objective of this study was to assess differences in the community structure of the two endolithic layers, care was taken during colour-based separation not to contaminate a particular fraction by co-harvesting small, attached portions of dissimilar-coloured dolomite. This quality control limited the harvest of non-contaminated material to only several mg per rock sample. Thus, multiple samples harvested from differing locations in the Piora system were subjected to the collection process and like samples were pooled. A schematic of the experimental procedure is shown in Fig. 1.

Because of the limited sample size of each rock fraction, pH measurement and elemental analysis were performed on bulk samples (free of biomass layers) of crushed dolomite (Table 1). The pH of the dolomite was assessed in both water and 0.01 M $CaCl_2$, and elemental analysis was performed using an X-Laboratory 2000 X-ray fluorescence analyzer (Spectro, Germany). Organic carbon and total nitrogen (Kjeldahl) were commercially measured (Bachema, Switzerland).

Direct microscopic examination of dolomite layers and enrichment cultures

For microscopic examination, separate samples (*c.* 50 mg) of red and green dolomite were combined with 50 µl of 5 mM sodium phosphate buffer (pH 8) and mixed with varying force. Minimum force amounted to simply inverting the tubes several times while maximum force involved vortexing the contents for several seconds. The intent of this procedure was to resuspend as many morphotypes as possible for direct observation, keeping in mind that not all cells would exhibit similar detachment characteristics or resistance to breakage. The suspension was allowed to settle for 10 min, and then 10 µl of supernatant was observed in a wet mount using bright field- and phase contrast microscopy (1000×). Microscopic examination of both supernatant and settled sediment revealed that our 'maximum force' treatment adequately resuspended the bacteria as less than 10% of the bacteria remained attached to particle surfaces.

Phototrophs were cultured by inoculating 50 ml of BG-11 media (ATTC media 616) with powdered dolomite (separately as red or green dolomite fractions). Cultures were incubated with shaking (140 r.p.m.) in ambient light and at room temperature. Following the initiation of growth (approximately seven weeks) as well as after 16 weeks, a portion of each culture was analysed microscopically as described above. Additionally, 25 ml of each culture was centrifuged to pellet the cells followed by DNA extraction, PCR amplification with cyanobacteria/chloroplast-specific primers, denaturing gradient gel electrophoresis (DGGE), and sequencing as described below.

Total DNA isolation from dolomite

DNA was extracted in duplicate from 50 mg (red dolomite), or 100 mg (green dolomite) of dolomite as described previously (Sigler and Zeyer, 2002) except that one µl of 1 M dithiothreitol was added to the samples followed by incubation at room temperature for 30 min before bead beating. The resulting DNA samples were pooled, the concentration of the extracted DNA was determined by measuring absorbance at 260 nm and purity was estimated from the ratio of A_{260}:A_{280}.

Cyanobacteria and chloroplast 16S rRNA gene PCR and DGGE

Approximately 420 bp of the cyanobacterial (and chloroplast) 16S rRNA gene was amplified from each DNA sample using primers CYAN 359-f-GC (5′-GC-clamp-GGG GAA T C/T T TCC GCA ATG GG-3′) and CYAN 781-r (5′-GAC TAC T/A GG GGT ATC TAA TCC C A/T TT-3′) according to the method of Nübel *et al.* (1997). Denaturing gradient gel electrophoresis (DGGE) was performed using the D-Code Universal Mutation Detection System (Bio-Rad) as previously described (Nübel *et al.*, 1997) except that our samples electrophoresed for 14 h at 70 V. Following electrophoresis, gels were stained with a 1:10 000 dilution of Gel Star nucleic acid stain (Bio-Whittacker) and visualized using a Gel-Doc image analysis system (Bio-Rad). In order to assign putative identifications to the phylotypes detected by DGGE, most of the bands in the DGGE gels were excised and processed as described previously (Garcia-Pichel *et al.*, 2001), and commercially sequenced. The most closely related phylotype (or closely related chloroplast sequence) to each of the sequenced DNA/band samples was identified through a BLAST search of the GenBank database (Altschul *et al.*, 1990; Table 3). In cases where the two dolomite layers shared co-migrating bands, both bands were excised for sequencing in order to verify that the same phylotype was responsible for the common bands.

Rock fraction clone library preparation

Partial Bacteria domain-specific 16S rRNA gene fragments were amplified from each rock fraction for clone library preparation with primers p8-f (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Edwards *et al.*, 1989) and Uni-b-rev (5′-GAC GGG CGG TGT GT A/G CAA-3′) (modified from Amann *et al.*, 1995) in 50 µl PCR reactions containing approximately 50 ng DNA, 1X reaction buffer (Invitrogen), 2 mM $MgCl_2$, 1.5 mg ml^{-1} BSA, 0.2 mM dNTPs (Invitrogen), 0.3 µM each primer and 2 U of Invitrogen Taq DNA polymerase. PCR conditions were as follows: 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C with a final extension step of 6 min at 72°C. The reactions yielded a fragment of approximately 1400 bp. PCR products were purified, cloned, and plasmid inserts were screened for the presence of cyanobacterial sequences by picking 40 random clones (red dolomite) and 60 clones (green dolomite) with a sterile toothpick for use in PCR reactions with primers CYAN 358-f (without a GC-clamp) and CYAN 781-r as described above. Colonies yielding a PCR amplicon of a length consistent for a cyanobacterial insert (as assessed by gel electrophoresis) were reamplified using M13-f and M13-r primers. M13-amplified inserts were digested overnight with the combination of *Taq*I and *Hae*III restriction endonucleases (Promega). Cloned inserts were clustered based on their restriction fragment length polymorphism (RFLP) patterns, representative clones from each cluster were grown in overnight cultures,

and plasmids were isolated as described above. Partial sequences of plasmid inserts were determined in both directions using the ABI Prism Big Dye Cycle Sequencing kit and an ABI Prism 310 DNA sequencer (Applied Biosystems). Sequence analysis was performed as described above. Plasmids were also screened with PCR-DGGE analysis as described above to determine if the clone analysis and PCR-DGGE of directly extracted dolomite DNA would result in bands of similar migration behaviour, thus providing an indication of overlap in the phylotypes detected by differing methods.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences generated in this study were deposited in the EMBL/GenBank/DDBJ under accession numbers AY153448-AY153464.

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