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Physical and Chemical Deterioration of Silicate and Carbonate Rocks by Meristematic Microcolonial Fungi and Endolithic Lichens (Chaetothyriomycetidae)


Abstract

Physicochemical deterioration processes driven by lithobiontic microcolonial fungi (MCF) and endolithic lichens (EL) are still mostly unresolved. Here, the millimetric penetration of MCF strains within silicate and carbonate lithotypes was quantified. The influence of petrographic features in determining hyphal passageways satisfies a model of physical penetration during the early stages of colonization, already described for EL. The MCF and EL secretion of iron-chelating metabolites accounts for iron mobilization in desert-varnish formation, often putatively related to fungal colonization. Increased dissolution of limestone by the model iron chelator desferrioxamine indicates the possible involvement of these MCF and EL secrete in pitting carbonates.

Keywords: desferrioxamine, hyphal penetration, MCF, siderophore, varnish

INTRODUCTION

Rock-dwelling meristematic microcolonial fungi (MCF) and endolithic lichens (EL), which mainly belong in Chaetothyriomycetidae (Eurotiomycetes, Ascomycota), are assigned a crucial role in the physical and chemical deterioration of rocks, including historical and culturally significant stone substrata (Caneva et al. 2008; Gueidan et al. 2008).

EL physical penetration pathways through internal discontinuities and along planes of weakness of rocks were described both in situ and in vitro by microscopically examining naturally colonized field samples and rock slabs incubated with the lichen aposymbionts, respectively (Pinna et al. 1998; Favero-Longo et al. 2009). Although a physical activity was repeatedly suggested also for meristematic mycelial MCF as well as yeast-like MCF and other rock-dwelling, non-lichenized fungi (Sterflinger 2000; Gorbushina 2007), only a few species were so far investigated, on a few lithotypes, both in situ and in vitro (Table 1).
Thin cross-sections of marbles naturally encrusted by dematiaceous non-lichenized fungal communities indicated that fungal growth is not restricted to the rock surface, single hyphae or bundles of hyphae penetrating within the intercrystalline space (Sterflinger and Krumbein 1997). The same penetration pattern was also demonstrated in vitro by incubating marble slabs with some microcolonial and non-microcolonial strains isolated from field samples (Diakumaku et al. 1995; Sterflinger and Krumbein1997). Penetration within Portland cement by several strains, including the MCF species Coniosporium uncinatum, through cracks generated with accelerated weathering of the matrix, was recently shown in laboratory tests (Wiktor et al. 2009).

Physical penetration by mechanical destructive forces was constantly recognized as a colonization mechanism for non-lichenized fungi (Diakumaku et al. 1995; Sterflinger and Krumbein 1997; Wiktor et al. 2009). However, hyphal penetration was either assessed qualitatively only or in point observations, mostly within carbonate rocks, in spite of widespread colonization of MCF on silicate rocks (Staley et al. 1982).

The cell-wall pigment melanin was suggested to play a significant role in the penetration of MCF within hard substrata, in analogy to the host surface penetration process by parasitic fungi (Sterflinger and Krumbein 1997; Sterflinger et al. 1999; Sterflinger2000). However, inhibition of melanin synthesis by tricyclazole was shown to poorly affect MCF ability to colonize marble

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**TABLE 1** Sets of rocks and dematiaceous non-lichenized fungi previously examined by observation of cross-sections of (a) field samples and (b) in vitro incubated rock slabs

<table>
<thead>
<tr>
<th>Reference</th>
<th>Lithotype</th>
<th>Mala morphology of rock</th>
<th>dwelling fungi</th>
<th>Species</th>
<th>Systematic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Sterflinger and Krumbein 1997</td>
<td>Marble</td>
<td>Black pigmented—myxellar</td>
<td>Monosicyte sp.</td>
<td>Microascales (Sordariomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—yeast-like</td>
<td>Cylindrosporum sp.</td>
<td>Chaetothyriales (Eurotiales)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—mieristic</td>
<td>Trinatotheca sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phaeoscylla sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Capnodotyella sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b Diakumaku et al. 1995</td>
<td>Marble</td>
<td>Black pigmented—myxellar</td>
<td>Alternana sp.</td>
<td>Pleosporales (Dothideomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nein-black pigmented—micellar</td>
<td>Phoma sp.</td>
<td>Mitosporic Ascomycetes</td>
<td></td>
</tr>
<tr>
<td>Sterflinger and Krumbein 1997</td>
<td>Marble</td>
<td>Black pigmented—myxellar</td>
<td>Monosicyte sp.</td>
<td>Microascales (Sordariomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—yeast-like</td>
<td>Exophiala sp.</td>
<td>Chaetothyriales (Eurotiales)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—mieristic</td>
<td>Trinatotheca sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phaeoscylla sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Capnodotyella sp.</td>
<td>Capnodiales (Dothideomycetes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wiktor et al. 2009</td>
<td>Portland cement</td>
<td>Black pigmented—myxellar</td>
<td>Alternana alternata</td>
<td>Pleosporales (Dothideomycetes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—yeast-like</td>
<td>Exophiala sp.</td>
<td>Chaetothyriales (Eurotiales)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black pigmented—mieristic</td>
<td>Coniosporium uncinatum</td>
<td>Chaetothyriales (Eurotiales)</td>
<td></td>
</tr>
</tbody>
</table>

*sensu De Leo and Urzi (2003) and Wiktor et al. (2009).*
Production of brownish pigments was also reported for some culture isolates of the mycobiont of Bagliettoa baldensis and B. marmorea (EL; Favero-Longo et al. 2009), which feature a PKS gene that in Chaetothyriales is involved in melanin biosynthesis (Gazzano 2010). However, the hyphae of these two lichen species do not exhibit brown-black colors when they grow inside rocks (Favero-Longo et al. 2009), and similar observations also apply to the majority of EL (Pinna et al. 1998), thus suggesting that the process underlying rock penetration does not rely on hyphal melanization. On the other hand, chemical deterioration of rocks by EL and MCF was repeatedly invoked, but poorly supported with experimental data.

With the exception of Verrucaria rubrocincta, EL were not found to secrete either oxalic acid (Pinna et al. 1998; Burgartz et al. 2004), or mycobiont polyketides (“lichen substances”) (Purvis et al. 1992) leading the biogeochemical action of most epilithic lichens (Adamo and Violante 2000; Gadd 2007). Unknown chemical pathway(s) is (are) thus hypothesized to explain the EL life-style within carbonate rocks and to yield the well known EL pitting effect.

Although MCF are major agents of biopitting on marbles, neither in their case was production of acids ever shown under either laboratory or field conditions (Diakumaku et al. 1995; Wollenzien et al. 1995; Sterflinger et al. 1999). Similarly, a role for carbon dioxide was suggested, but not investigated (Sterflinger 2000).

Extracellular polymeric substances (EPS) and/or chelating compounds released by MCF were putatively considered as responsible of desert varnishes of extremely hot and cold environments (Taylor-George et al. 1983; Sterflinger et al. 1999; Perry and Kolb 2003; Perry et al. 2004). However, no accepted explanation of the involvement of MCF in varnish formation is currently provided (Perry et al. 2007), although the capability of fungi to oxidize reduced iron and manganese is established (Krumbein and Jens 1988; Sterflinger et al. 1999) and the mobilization of ferric ions by hydroxamate siderophores produced by MCF was hypothesized on the basis of preliminary experiments (Adams et al. 1992).

Siderophores are low molecular weight organic molecules produced by many microorganisms, such as bacteria and fungi, to support their mineral nutrition under iron-deficient conditions (Winkelmann 2007). By chelating Fe3+, siderophores mediate its uptake by microbial cells from insoluble hydroxides or from iron adsorbed to solid surfaces (Gadd 2007). The secretion of siderophores (mainly hydroxamates) was widely recognized in symbiotic and saprotrophic fungi, including several species of Aspergillus, Penicillium and Paecilomyces (Renshaw et al. 2002), which belong in Eurotiomycetidae (Eurotiomycetes). However, it was not investigated in Chaetothyriomycetidae, i.e., the sister group of Eurotiomycetidae (Hibbett et al. 2007). Moreover, despite the well known siderophore overproduction under iron limitation (Winkelmann 2007), potential dissolution effects of siderophores on iron-poor mineral substrata, such as carbonate rocks, which are typically colonized by MCF and EL (Pinna et al. 1998; Sterflinger 2000; Caneva et al. 2008), was disregarded.

In this work, both physical and chemical interactions of MCF and EL with rocks are considered, to contribute new insights on their deterioration activity. The first aim is to assess the
penetration patterns, under controlled conditions, of three strains of meristematic MCF within a set of carbonate and silicate rocks commonly employed in the field of Cultural Heritage. Incubations of rock slabs were performed, supporting fungal growth with a nutrient-rich culture medium, and within-substratum colonization was quantified by serial measures on cross-sections following 4 months of incubation. The influence of structural characteristics and mineralogical composition of the different lithotypes on MCF penetration patterns during the early stages of colonization is discussed in comparison with previous studies on other lithobiontic microorganisms, particularly EL. The second aim of this paper is to assess the secretion of chelating siderophore-like compounds by the three strains of MFC and three strains of EL. A model test on the potential effect of siderophore-like molecules on the dissolution of carbonate rocks was also performed by incubating iron-poor limestone slabs in a solution of desferrioxamine, which is currently used in model systems as a representative of the group of hydroxamate siderophores (Neubauer et al. 2000).

**MATERIALS AND METHODS**

**Cultures**

Strains of the MCF Coniosporium perforans (CBS885.95), C. uncinatum (CBS100212) and Sarcinomyces petricola (CBS600.93) (Chaetothyriales, Chaetothyriomycetidae) were purchased from Centraalbureau voor Schimmelcultures (CBS, The Netherlands) and maintained on Malt Extract Agar (MEA) at 15°C. Samples of the EL Bagliettoa baldensis, B. marmorea (Verrucariales, Chaetothyriomycetidae) and Acrocordia conoidea (Pyrenulales, Chaetothyriomycetidae) were collected from calcareous outcrops located in the Classic Karst plateau (46VG abyss, Trieste, NE Italy). The mycobionts were isolated from the collected thalli by perithecia transplantation, according to Favero-Longo et al. (2009). Cultures on Bold's Basal Medium (BBM, Deason and Bold 1960) were incubated at 13–15°C in the light and daily checked for contamination by bacteria or fast-growing fungi by microscopic examination.

**Incubation of the MCF Strains on Rock Slabs**

The MCF strains were cultured on nutrient-rich (Malt Extract Agar, MEA) and nutrient-poor (Bold's Basal Medium, BBM, Deason and Bold 1960) media and used for in vitro incubation tests. Growth rates on the two media at 15°C in the light were monitored monthly (three plates/strain). Slabs (1 × w × h: 2 × 1.5 × 1.5 cm) of carbonate (travertine, limestone, marble) and silicate (granite, gneiss, sandstone) rocks were freshly cut with a diamond disk saw, without polishing the obtained rock faces. Petrographic features are summarized in Table 2 and also available at http://www.atlantepetro.unito.it. The slabs, after autoclave sterilization (20’ at 120°), were incubated on MEA plates on the top of colonies of the MCF (one slab/plate). Two plates per strain per lithotype were set up.
After four months, the slabs were cut perpendicularly to the most colonized surface along a transect completely covered by mycelium and the obtained cross-sections (four cross-sections for each strain-lithotype set) were stained using the periodic acid-Schiff method (PAS; Whitlach and Johnson 1974). Sections were observed under reflected light using an Olympus SZH10 stereomicroscope. Along the colonized transect, measuring points were established at every millimetre from the cross-section vertex; the maximum depth of hyphal penetration was then measured beneath each measuring point perpendicularly to the colonized transect. The results were statistically analyzed by means of ANOVA with the post-hoc Tukey's test.

Relationships between the colonization rates above the rock slabs, within rock slabs and on the nutrient-rich medium were examined for *Sarcinomycetes petricola* by quantifying colony area using image analysis through a color-based pixel classification (WinCAM Pro 2007d, Regent’s Instruments; see Gazzano et al. 2009 for technical details).

**Detection of Siderophore Production**

The MCF and EL production of siderophore-like compounds was detected using the universal chrome azurol S (CAS)-agar plate assay (Schwyn and Neilands 1987). This method is based on a competition for iron between the ferric complex of an indicator dye (CAS) and a chelator, or siderophore, produced by microorganisms. The secretion of siderophores is marked by a change in color of the agar from blue to yellow/orange. Three plates per strain were set up, incubated at 13–15°C in the light, and monitored for 24 months.

**Dissolution of Limestone Driven by Desferrioxamine**

Slabs of c. 3 mm in diameter of a Cretaceous biomicritic limestone from the Classic Karst (wt.% composition: CaO 54.2, MgO 0.6, CO₂ 45.2 according to EDS analysis on thin sections; the orthochemical component is made up of a micritic aphanite background mosaic with very scarce sparry-calcite recrystallization zones) were incubated in a water solution of desferrioxamine (DFX, 0.35 mM, liquid/solid ratio: 0.08 mg mL⁻¹; Sigma-Aldrich) and water, as a control. Ultrapure MilliQ water was used.

After 24 h, Ca release in the supernatant was measured by ICP-AES with an IRIS II Advantage/1000 Radial Plasma Spectrometer by Thermo-Jarrel Ash Corp. The optical system was sealed with inert gas, with no moving parts, high-resolution (ER/S) capable. The Echelle grating & dispersion prism monochromator

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**Table 2: Petrographic features of the examined lithotypes (a= magmatic, b= sedimentary, c= metamorphic rocks)**

<table>
<thead>
<tr>
<th>Lithotype</th>
<th>Code</th>
<th>Minerals</th>
<th>Microstructure</th>
<th>Grain size</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Granite</td>
<td>SN1</td>
<td>Pl, Qtz, Kfs, Bt</td>
<td>heterogeneous, isotropic, hypidiomorphic</td>
<td>up to centimetric</td>
</tr>
<tr>
<td>b Limestone</td>
<td>SN2</td>
<td>Cal (incl. bioclasts) Dol</td>
<td>heterocrystalline, matrix-supported, with micritic cement and local stylolitic surfaces</td>
<td>submillimetric</td>
</tr>
<tr>
<td>Qtz-Sandstone</td>
<td>SN3</td>
<td>Qtz, Fe-ox</td>
<td>heterocrystalline, slightly stratified</td>
<td>submillimetric</td>
</tr>
<tr>
<td>Travertine</td>
<td>SN4</td>
<td>Cal</td>
<td>heterogeneous, anisotrophic, with up to millimetric porosity</td>
<td>submillimetric</td>
</tr>
<tr>
<td>c Marble</td>
<td>SN5</td>
<td>Pl, Kfs, Bt, Qtz, Ep</td>
<td>heteroblastic (with micranoporite), foliated</td>
<td>submillimetric</td>
</tr>
<tr>
<td>Orthogneiss</td>
<td>SN6</td>
<td>Pl, Kfs, Bt, Qtz, Ep</td>
<td>heteroblastic (with micranoporite)</td>
<td>up to millimetric</td>
</tr>
</tbody>
</table>

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*Pl: plagioclase; Qtz: quartz; Kfs: K-feldspar; Bt: biotite; Cal: calcite; Dol: dolomite; Ep: epidote; Fe-ox: iron-oxhydroxides; Kfs: K-feldspar; Pl: plagioclase; Qtz: quartz (Kretz 1973).*

*Hypidiomorphic: a texture that is a mix of elongated (bounded by characteristic crystal-face forms), subhedral (partly euhedral), and anhedral (not bound by any characteristic crystal face) grains.*

*Stylolites: non-structural fractures developed along pressure solution surfaces, appearing as jagged discontinuities, often marked with insoluble clays, oxides and/or carbonates.*

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*ER/S: Echelle grating & dispersion prism monochromator.
range was extended between 165 and 800 nm, with an optical resolution of 0.007 nm (at 200 nm). The photo device was a CID Camera (Charge Injection Device) frozen to −50°C.

Supernatants were also analyzed to detect the formation of Fe- and Ca-complexes by a Ultimate 3000 (Dionex) HPLC system interfaced to LTQ-Orbitrap® (Thermo, Rodano, Italy) linear ion-trap high resolution mass spectrometer (resolving power ≥60,000). Chromatographic separation was obtained using a Pursuit RP18 column (150 × 2.0 mm × 3 μm), fitted with a guard column (Varian, Leini, Italy). The injection volume was 20 μl. Separation of the analytics of interest was achieved using the following binary gradient: 95/5 to 0/100 in 40 min. formic acid 0.05% in water/methanol at a flow rate of 200 μl/min. A LTQ Orbitrap mass spectrometer was equipped with an ESI ion source and an atmospheric pressure interface. The tuning parameters adopted for ESI source were: capillary voltage 28.00 V, tube lens 30 V. The source voltage was set to 4.1 kV. The heated capillary temperature was maintained at 275°C. Mass accuracy of recorded ions (vs. calculated) was ± 15 ppm (without internal calibration).

![Graph](image_url)

**FIG. 1** Growth of microcolonial fungi on MEA (black symbols) and BBM (grey symbols) after 1 and 4 months from inoculation. Diameters of colonies (means ± standard error) of Coniosporium uncinatum (squares, n = 3 on MEA, n = 3 on BBM), C. perforans (circles, n = 6 on MEA, n = 3 on BBM) and Sarcinomyces petricola (triangles, n = 6 on MEA, n = 3 on BBM) strains.

**RESULTS**

**MCF Growth and Penetration Pathways in vitro**

The different media used affected mycelial features and substratum exploration (expressed as colony diameter on the medium surface) of the three MCF strains. During the first month, 6–9 mm diameter colonies developed on MEA, exhibiting a moriform (sensu De Leo et al. 1999) and cerebriform meristematic habit for Coniosporium and Sarcinomyces isolates, respectively
Radial growth rate then decreased for all isolates during the following 3 months. Although the C. uncinatum and S. petricolacolonies featured a predominantly superficial growth, agar penetration was observed for C. perforans. On BBM, aerial mycelial production was reduced for all strains as compared to MEA (Figure 2). On the former medium, the C. perforans strain exhibited a significantly higher growth rate than the other two strains, as well than on MEA (Figure 1). On this substratum, although the meristematic habit was maintained, hyphae were almost unpigmented (Figure 2). By contrast, mycelia of the other two strains were consistently dematiaceous and exhibited a lower and more constant growth rate than on MEA (2.5 mm month⁻¹).

In the incubation tests, MCF developed both on the agar surface and on the surface of the rock slabs of all lithotypes. Although carbonate rocks always exhibited surface colonization on the bottom slab face, mycelium development on adjacent faces was only observed for the C. uncinatum strain on marble and limestone and the C. perforans strain on marble. The bottom and adjacent faces of silicate slabs were
widely colonized by all strains. However, average colonization of the most colonized faces was always approx. 40% for *S. petricola*, without significant differences between lithotypes (Figures 3 A,B).

**FIG. 3** MCF colonization of rock slab surfaces. *Sarcinomyces petricola* on the bottom face of a travertine slab: micro-photograph acquired in stereomicroscopy (A) and recognition of mycelium (black areas) by the image analysis software (B). Colony details of *Coniosporium uncinatum* on marble (C), *C. perforans* on gneiss (D) and granite (E). Scales: 0.5 mm.

On the lithic slabs, all strains exhibited different growth forms, ranging from scattered small colonies to a sparse diffuse mycelium (Figures 3 C-D). The *S. petricola* isolate did not form cerebroid colonies, but rather groups of adjacent, discrete small colonies. On highly colonized surfaces, *Coniosporium* isolates developed a thick, felt-like mycelium (Figure 3E), featuring lower adhesion to the substratum than the small colonies. Microscopic mycelial features on the surface of rock slabs were similar to those described on MEA for all strains, with toruloid dematiaceous hyphae.

After 4 months, dematiaceous hyphae penetrating within the examined lithotypes were observed for all MCF strains, with the exception of *S. petricola* incubated with marble.

Sandstone slabs exhibited a widespread aspecific PAS staining, hampering the quantification of hyphal penetration, which occurred sparsely within the abundant rock porosities through the entire slab thickness (Figure 4A).
The tested strains showed different penetration patterns in the different lithotypes, such patterns being however consistent for all strains in a given lithotype (Figures 4 B,F). Mycelia penetrated limestone along discontinuities in the stylolitic areas, which feature bigger grain size and, most likely, higher porosity. Colonization within travertine was localized in the millimetre-wide macroporosities. Growth in the fine-grained marble was exclusively intergranular, hyphae developing alongside crystal boundaries, while granite also featured intragranular penetration along the cleavage plains of feldpars. In gneiss, hyphae mainly developed along discontinuities that are arranged according to the foliation planes and mostly localized in finer-grained, biotite-bearing domains. Light pink halos were observed
around the areas penetrated by hyphae for all lithotypes (Figure 4B and 4F), suggesting occurrence of PAS-stained extracellular polymeric substances (Sterflinger 2000; Gazzano et al. 2009).

High variability in penetration depth was detected, with respect to both each species in the different lithotypes and the different species within each lithotype (Figure 5). Coniosporium isolates exhibited the deepest penetration within marble, limestone, and travertine, which were poorly or nonpenetrated by the S. petricola strain. C. uncinatum colonies exhibited hyphae down to average depths of 3.5 mm and 6.6 mm within marble and limestone, respectively, significantly deeper than those of C. perforans in the same lithotypes (1 and 3 mm) and in travertine (about 1.5 mm).

![Image](image.png)

**FIG. 5** Maximum hyphal penetration within rock coupons by Coniosporium uncinatum (white columns, Cu), C. perforans (light grey columns, Cp) and Sarcinomyces petricola (dark grey columns, Sp) strains. Data are shown as means ± standard errors (marble: Cu n = 30, Cp n = 33, Sp n = 51; limestone: Cu n = 19, Cp n = 45, Sp n = 41; travertine: Cu n = 25, Cp n = 27, Sp n = 29; granite: Cu 45 >, Cp 43 >, Sp n = 47; gneiss: Cu n = 52, Cp 25 >, Sp n = 38). According to Tukey’s test, columns that do not share at least one letter are statistically different (p < 0.05; capital letters: differences in a single lithotype between different species; small letters, differences of one species within different lithotypes; apices indicate a different series of statistical analyses). *: in the case of Coniosporium isolates, penetration depths were measured perpendicularly to the foliation, while in the case of S. petricola, the coupons were cut having the foliation perpendicular to the colonized surface, thus favoring the penetration.

On the other hand, S. petricola strains exhibited within granite the maximum penetration values (7.4 mm) and penetrated within gneiss slightly more (2.4 mm) than the other strains, which were possibly influenced by different orientation of schistosity. Penetration of Coniosporium isolates within these silicate rocks was similar, being around 4.5 and 1.5 mm of depth within granite and gneiss, respectively.
Siderophore Production

A positive reaction of the CAS test was observed upon transfer of all colonies of MCF and EL strains on CAS agar plates (Figure 6). Although MCF growth on the surface of the medium was initially slow, a small halo was already visible around the colony border after two weeks (Figures 6 A–C). After prolonged incubation (up to 18 months), all MCF colonies developed a dense aerial mycelium, but only C. perforans exhibited extensive growth directly on the culture medium. Discoloration areas wider than the growth areas of MCF mycelia were observed, indicating a particularly abundant release of iron-chelating molecules (Figures 6 D–F). EL mycobionts also grew poorly on the CAS-agar medium and caused discoloration below and around the colonies, which was observed after 9 months and expanded up to 24 months (Figures 6 G–I). Similarly to MCF colonies, some B. baldensis and A. conoidea colonies were completely covered by dark brownish pigments.

Dissolution of Limestone Driven by Desferrioxamine

Incubation tests showed that the equilibria of calcite dissolution were changed by desferrioxamine, which determined a 72% increase in calcium release with respect to pure water (115.5 ± 5.4 vs. 67.1 ± 8.5 mg/mg·L) after 1 day of incubation.

The presence of Ca-chelates was investigated by HPLC-mass spectroscopy. The basic components of complexation equilibria in a water solution are (a) the chelant (or ligand, L); (b) metal ions; (c) protons. It is worth mentioning that the ligating groups can display different protonation states (Harris 2002) that co-exist in solution according to the specific protonation and formation constants (e.g., Neubauer et al. 2000). In our test, the ligand desferrioxamine was detected in 3 forms: metal-free, iron- and calcium-bound, respectively (Figure 7).

The first one was fully protonated (LH$_3$; 561.36 u.m.a.). Iron-chelates were fully protonated (Fe-LH$_3$; 617.43 u.m.a. ≈ DFX molecular mass + Fe atomic mass ≈ 561.36 + 55.85), and fully deprotonated (FeL: 614.27 u.m.a. ≈ DFX molecular mass + Fe atomic mass – 3 H$^+$ ≈ 561.36 + 55.85 – 3.03). Calcium-chelates were fully protonated (Ca-LH$_3$; 601.35 u.m.a. ≈ DFX molecular mass + Ca atomic mass ≈ 561.36 + 40.03), without one proton (Ca-LH: 600.32 u.m.a. ≈ DFX molecular mass + Ca atomic mass – 1H$^+$ ≈ 561.36 + 40.03 – 1.01), and without two protons (Ca-LH: 599.32 u.m.a. ≈ DFX molecular mass + Ca atomic mass – 2H$^+$ ≈ 561.36 + 40.03 – 2.02). Integration of the signals identified the metal-free form as dominant (peak area of 1.15·10$^5$ a.u.), followed by iron and calcium complexes (peak areas of the different forms between 10$^5$ and 10$^6$ a.u.).

DISCUSSION

The physical and chemical activity of microorganisms determines their complex deterioration patterns and their sphere of influence on and within rocks (Barker and Banfield 1998; Banfield et al. 1999; Gazzano et al. 2009; Gorbushina and Broughton 2009). Such processes, however, were so far not completely resolved for MCF and EL. This paper first quantifies the MCF capability to penetrate down to millimetric depths within different carbonate and silicate rocks, similarly to EL physical penetration pathways (see Favero-Longo et al. 2009).

In addition, the discoloration of CAS culture medium testifies the release, by MCF and EL, of one or more unidentified iron-chelating compounds, i.e., the first reported metabolites, secreted by these fungi, which might be involved in their chemical deterioration of rocks. As the production of iron-chelating metabolites involved in iron nutrition, i.e., siderophores, was reported for several species of
the sister-taxon Eurotiomycetidae (Renshaw et al. 2002), the metabolites detected by the CAS tests in the MCF and EL Chaetothyriomycetidae strains tested in our work are here likely considered as siderophore-like compounds.

Physical Penetration Pathways

MCF growth within lithic substrata was previously investigated from a qualitative point of view, in assessing their process of penetration within carbonate rocks (Diakumaku et al. 1995; Sterflinger and Krumbein 1997) and in describing their endolithic life-style within silicate rocks in extreme desert environments (Perry and Kolb 2003; Selbmann et al. 2008). At the best of our knowledge, a recent estimation of 130 µm depth for Coniosporium uncinatum hyphae within a Portland cement slab, following inoculation of a mycelium suspension and incubation for 1 month, is the only quantitative measure concerning MCF within lithic substrata (Wiktor et al. 2009).

In this research, MCF inoculations were performed on the nutrient rich medium MEA, which supported the development of abundant biomasses of the three strains, featuring colony morphology strongly differing from the common habit of MCF in the field (Gorbushina and Broughton 2009). Interestingly, in the in vitro system, colony development was more extensive on the surface of the silicate rocks slabs, mostly colonized by a felt-like, thick mycelium, while the small scattered colonies observed on carbonate substrata were more reminiscent of the field morphology. Different habit and biomass on nutrient rich media, however, do not necessarily imply strongly different growing rates with respect to nutrient-poor conditions.

BBM oligotrophic conditions did not support the development of new fungal colonies (sensu Sterflinger and Krumbein 1997), but rather the growth of exploratory hyphae (sensu Sterflinger and Krumbein 1997): biomass production was strongly reduced, but this variation was not directly correlated with the radial growth of colonies, which (i) decreased (S. petricola strain: -45%), (ii) was not significantly different (C. uncinatum strain) or (iii) even increased (C. perforans strain: +20%) with respect to that measured on MEA. As growth variability with different nutrient availability is strain-related, the slab incubations on MEA likely indicate the hyphal exploration potential, which was found not to differ significantly from that observed in oligotrophic environments such as BBM. It is worth mentioning that some authors do not consider MCF as typical oligotrophs, as they can be easily grown either on nutrient-poor or rich media (Blackhurst et al. 2005; Gorbushina et al. 2005; Gorbushina and Broughton 2009).

Different penetration patterns were observed within the different lithotypes and significant differences were also detected between the three strains, what rules out the often purported common behaviour of MCF within rocks (Gorbushina and Broughton 2009). In all lithotypes, the two Coniosporium isolates differed in the penetration depth; S. petricola, which exhibited the deepest penetration within granite and gneiss, did not penetrate within marble, limestone and travertine, suggesting that the rock bulk chemistry can affect mycelium exploration potential. Fungal community structure on a weathered pegmatitic granite was shown to be driven by the chemical composition of mineral substratum, indicating selective pressure by individual chemical elements on fungal populations in situ (Gleeson et al. 2005).

The type of internal discontinuities or weakness planes mainly conditioned the distribution of hyphae within the different lithotypes, as previously discussed for lichen-forming fungi both in the field (Sanders et al. 1994; Favero-Longo et al. 2005; Câmara et al. 2008) and in vitro (Favero-Longo et al. 2009).
et al. 2009). The influence of foliation in supporting and orienting hyphal penetration, as in the case of gneiss, was previously discussed for lichen hyphae within siliceous schist (Sanders et al. 1994) and foliated serpentinite (Favero-Longo et al. 2005). Penetration within macro- and micro-porosities, such as the one described for travertine and sandstone, respectively, was observed for lichen hyphae within dolostones (Cámara et al. 2008) and EL mycobionts within several types of white marbles (Favero-Longo et al. 2009).

FIG. 6 Siderophore production by microcolonial (A-F) and endolithic lichen-forming fungi (G-I) yielding discoloration of CAS-blue agar, which changes to yellowish colors. Plates inoculated with the MCF for 2 weeks (A-C) and 18 months (D-F). A,D, Sarcinomyces petricola, B,E, Coniosporium uncinatum, C,F, C. perforans. Plates inoculated with the EL isolated mycobionts of Bagliettooa baldensis (G), B. marmorea (H), and Acrocordia conoidea (I) for 9 months (H) and 24 months (G, I). Plate diameter: 5.5 cm; scale G-H: 1 cm.
FIG. 7 HPLC separation of iron- and calcium complexes of desferrioxamine following incubation of iron-poor limestone slabs in desferrioxamine 0.35 mM. a, LH₃ (peak integration: 1.15E9 arbitrary units); b, Fe-L (3.03E7 a.u.); c, Fe-LHₓ (1.16E6 a.u.); d, Ca-LH (8.70E5 a.u.); e, Ca-LHₓ (1.97E5 a.u.); f, Ca-LH₃ (1.10E6 a.u.), on the basis of the detected masses (u.m.a., on the figure).

Cleavage planes of minerals are known to be planes of weakness which can be attacked by hyphae, as it was observed in feldspars of granite and previously described in lichen studies for several silicate minerals, including plagioclase (e.g., Prieto et al. 1997; Lee and Parsons 1999). Penetration along the grain boundaries of calcite was described in marbles, as assessed for EL in axenic slab incubations (Favero-Longo et al. 2009). Hyphal penetration within limestone appeared to be mainly related to stylolitic surfaces, where higher porosity most likely occurs (Dawson 1988; Raynaud and Carrioschaffhauser 1992). By contrast, penetration did not affect the rock matrix, as previously described for EL mycobionts following one-year incubation with limestone slabs, which were only penetrated through cracks (Favero-Longo et al. 2009).

The penetration depths observed in this work are comparable to those reported for lichenized fungi within different silicate and carbonate rocks, also ranging from few hundreds of microns to several
millimetres (e.g., 10 mm within granite and 16 mm within limestone: Favero-Longo et al. 2005 with refs. therein). Moreover, the penetration rates measured within rock slabs were only slightly lower than the growth rates evaluated on the agar surface, that were also in the order of several millimetres after 4 months, suggesting that development of hyphae in the rocks is poorly obstructed as it occurs within pre-existing discontinuities. It is worth noting that this physical penetration pathway is similar to the early stages of colonization described for EL on carbonate rocks, which also featured similar hyphal penetration depths (ranging from 100 to 1250 μm depending on lithotypes; Favero-Longo et al. 2009).

Chemical Activity

Siderophore-producing fungi from several lineages, including Eurotiomycetidae, were shown to pit, etch and dissolve iron-rich silicate minerals, such as olivine and crocidolite asbestos, upon in vitro incubation for up to 6 weeks (Callot et al. 1987; Martino et al. 2003). Similar effects were not detected by means of light microscopy observations on the silicate and carbonate rock slabs incubated with MCF for 4 months and on carbonate slabs incubated with EL isolated mycobionts for 12 months (Favero-Longo et al. 2009). The relatively limited discoloration halo observed in the CAS plates (ca. 3–5 mm from the colony border, see Figure 6) suggests that the absence of external dynamic factors (e.g., water), which mobilize the released chemicals in field conditions, may limit the in vitro chemical biodeteriogenic activity (Favero-Longo et al. 2007).

It is worth noting that some other lithobionts, such as cyanobacteria, rarely behave as chasmosoliths (inhabiting fissures; sensu Golubic et al. 1981) during the first stages of colonization, while they show a clear euendolithic growth (actively penetrating substrata; sensu Golubic et al. 1981) during subsequent stages (Ascaso et al. 2004).

Mobilization and concentration of ferric ions by rock dwelling fungi, possibly contributing to desert varnish formation, were investigated by Adams et al. (1992) by means of laboratory experiments on hydroxamate siderophores: a growth stimulation of a hydroxamate-auxotroph bacterium (Arthrobacter flavescens) was observed upon incubations with powdered rock varnishes bearing MCF (2 out of 2 samples yielding positive results), as well as with cultured MCF cells and paper disks amended with MCF culture fluids (2 out of 4 samples yielding positive results). However, MCF communities used for the experiments were not characterized, what possibly explains why these preliminary results have been surprisingly disregarded in more recent researches on causative relationships between desert varnish and fungi (Perry et al. 2007). In the present work, the pioneer hypothesis by Adams et al. (1992) on a siderophile-driven desert varnish formation is supported by the detection of iron chelators in all tested MCF strains. Moreover, EL, which are often associated to MCF in extreme environments, are also found to secrete iron chelators and may be thus similarly involved in varnish formation. Such an involvement was also previously suggested for lichen communities of cold and hot deserts, which were not characterized in terms of species composition, but were supposed to mobilize iron and manganese in rocks by secreting acidic compounds, including oxalic acid (Laudermilk 1931; Johnston and Vestal 1993).

On the other hand, our test on the dissolution of an iron-poor limestone upon incubation with the model siderophore DFX highlighted the strong effects of hydroxamate siderophores in mobilizing calcium under iron limitation. Predominant chelation of calcium rather than ferric ions at high pH values, which prevail in calcareous soils, was already predicted for the Rhizopus polyoxycarboxylate siderophore rhizoferrin, which is characterized by a relatively low apparent stability constant for ferric ions (Log $K_{\text{app}} = 19.1$) and a relatively high constant for calcium (Log $K_{\text{app}} = 6.0$) at pH 7.0 (Shenker et al. 1999).
DFX is well known as a highly specific iron chelator, because of the high stability constant of iron chelates (Log $K = 30.60$; Anderegg et al. 1963), but in our experiment it formed calcium chelates (Log $K = 2.64$; Anderegg et al. 1963), thus increasing by 72% calcium release from the limestone with respect to pure water.

Similarly, other highly specific iron chelators, which are overproduced by fungi when iron concentration is low (Winkelmann 2007), are likely to drive carbonate dissolution. When secreted by rock dwelling MCF and EL, iron chelators such as those detected by our CAS tests, are thus likely to represent the repeatedly invoked chemical agents of the biodeterioration effects, such as pitting and etching, which have been often reported on carbonate stonework.

**CONCLUSIONS**

Although meristematic MCF and EL are extremely disparate in terms of lifestyle (non-lichenized versus lichenized, respectively), they share a common ancestor, a preference for mineral substrata, extraordinary extremotolerance (Gueidan et al. 2008) and secretion of dark pigments (Favero-Longo et al. 2009). Their similarity is here further supported by the observation of a common pattern of physical penetration in the early stages of colonization and the secretion of iron chelator(s).

MCF incubation with freshly cut and sterilized slabs indicated that hyphal penetration down to millimetric depths within carbonate and silicate rocks can occur without previous deterioration by other organisms or physical-chemical agents, and that different penetration patterns depend on both MCF strains and lithotypes. The influence of rock structure and mineralogical composition in modulating inter- and intra-granular hyphal passageways satisfies the model of physical penetration suggested for MCF (with reference to qualitative analyses on marble slabs only; Sterflinger and Krumbein 1997). A similar model was described in vitro for the early stages of colonization of carbonate rocks by EL mycobionts, while chemical processes were invoked for later stages of colonization and the well known pitting effects (Favero-Longo et al. 2009).

Similarly, the observed rapid MCF penetration within rocks does not exclude the potential activation, during the later stages of colonization, of chemical processes. Our chemical tests suggest that chemical deterioration of both silicate and carbonate rocks by MCF and EL depends on the secretion of siderophore-like compounds. These may indeed account for iron mobilization in desert varnish formation on silicate rocks, but also for calcium scavenging from carbonates featuring low iron contents.

Other, nonmutually exclusive, carbonate dissolution processes were hypothesized for EL by Tretiach et al. (2008) based on indirect immunofluorescence and histochemical localization and in vivo activity tests of carbonic anhydrase. This enzyme increases the speed of the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$, thus accelerating the dissolution of calcium carbonate by accelerating the hydration of respiratory $\text{CO}_2$. This alternative scenario, which needs further experimental evidence, enlightens the complexity of phenomena involved in carbonate deterioration, which remains an interesting goal for further research, together with the chemical identification of the iron-chelating compounds detected in our work.
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Notes

*sensu De Leo and Urzì (2003) and Wiktor et al. (2009).

1Bt, biotite; Cal, calcite; Dol, dolomite; Ep, epidote; Fe-ox, iron-oxhydroxides; Kfs, K-feldspar; Pl, plagioclase; Qtz, quartz (Kretz 1973).

2Hypidiomorphic: a texture that is a mix of euhedral (bounded by characteristic crystal-face forms), subhedral (partly euhedral), and anhedral (not bounded by any characteristic crystal face) grains.

3Stylolites: non-structural fractures developed along pressure solution surfaces, appearing as jagged discontinuities, often marked with insoluble clays, oxides and/or carbonates.

REFERENCES


