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Julia Kirtzel,[1] Nico Ueberschaar,[2]
Tanja Deckert-Gaudig,[3] Katrin Krause,[1]
Volker Deckert [ID],[3,4] Geoffrey Michael Gadd [ID][5] and
Erika Kothe [ID][1]*

[1]*Friedrich Schiller University, Institute of Microbiology, Microbial Communication, Neugasse 25, D-07743 Jena, Germany.*
[2]*Friedrich Schiller University, Mass Spectrometry Platform, Humboldtstr. 8, D-07743 Jena, Germany.*
[3]*Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Str. 9, D-07745 Jena, Germany.*
[4]*Friedrich Schiller University, Institute of Physical Chemistry and Abbe Centre of Photonics, Helmholtzweg 4, D-07743 Jena, Germany.*
[5]*University of Dundee, Geomicrobiology Group, School of Life Sciences, Dundee DD1 5EH, Scotland, UK.*

Summary

Although many fungi are known to be able to perform bioweathering of rocks and minerals, little information is available concerning the role of basidiomycetes in this process. The wood-rotting basidiomycete *Schizophyllum commune* was investigated for its ability to degrade black slate, a rock rich in organic carbon. Mechanical pressure of hyphae and extracellular polymeric substances was investigated for biophysical weathering. A mixed ß1-3/ß1-6 glucan, likely schizophyllan that is well known from *S. commune*, could be identified on black slate surfaces. Secretion of siderophores and organic acids as biochemical weathering agents was shown. Both may contribute to biochemical weathering in addition to enzymatic functions. Previously, the exoenzyme laccase was believed to attack organic the matter within the black slate, thereby releasing metals from the rock. Here, overexpression of laccase showed enhanced dissolution of quartz phases by etching and pitting. At the same time, the formation of a new secondary mineral phase, whewellite, could be demonstrated. Hence, a more comprehensive understanding of biophysical as well as biochemical weathering by *S. commune* could be reached and unexpected mechanisms like quartz dissolution linked to shale degradation.

Introduction

Black slates are sedimentary low-grade metamorphic rocks that contain more than 0.5 wt.% organic carbon (Fischer *et al*., 2007). Due to the formation conditions of this sedimentary rock material, it contains high amounts of organic matter, metals and minerals like pyrite (Fischer *et al*., 2007; Marynowski *et al*., 2011). When exposed to oxygen, black slate undergoes weathering processes that result in the degradation of organic matter and the release of organic chemical compounds (Fischer *et al*., 2007). Such oxidative weathering of black slate contributes to the global cycling of carbon (Berner, 2003; Petsch *et al*., 2005). The organic carbon release is estimated to correspond to ~12% of the direct annual CO_2 flux from oxidative weathering of sedimentary rocks (Jaffe *et al*., 2002). During weathering, redox-sensitive metals are mobilized and enter groundwater, sometimes in potentially toxic concentrations, where they can lead to serious problems for the environment and human health (Fouad and El-Rakaiby, 2009). In addition to natural weathering processes, human activity contributes significantly to oxidative weathering of rocks and minerals (Fischer *et al*., 2009). Mining activity and the resulting mining waste heaps increase the exposure of huge amounts of rock material to atmospheric oxygen, which causes further degradation of organic matter and the oxidation of pyrite (Lottermoser, 2003).

Microorganisms are able to bioweather a wide range of rocks and the involved strategies can be classified into biomechanical and biochemical mechanisms (Lee and Parsons, 1999; Gadd, 2017*)*. Direct mechanical weathering occurs when hyphae penetrate mineral grain boundaries, widen preexisting fissures or cause the formation of new cracks. Indirect mechanical processes include the action of extracellular polymeric substances (EPS) that can exert mechanical pressure on minerals, especially through wetting-drying cycles,

resulting in their erosion or abrasion (Gadd, 2007, 2010). *Schizophyllum commune* is a well-known producer of the EPS schizophyllan (Grisel and Muller, 1997). Biochemical weathering occurs through mechanisms that include acidolysis, complexolysis and redoxolysis (Gadd, 2007). Acidolysis and complexolysis are believed to be the most important mechanisms, and both result from excretion of organic acids (e.g., oxalic acid, citric acid) and inorganic acids (respiratory CO_2). For complexolysis, additionally, the secretion of chelating compounds (e.g., siderophores) is important (Daghino *et al.*, 2010). In addition, the accumulation of metals into biomass can increase weathering rates (Burgstaller and Schinner, 1993). Redoxolysis can involve attack of mineral surfaces by redox-active metabolites, including organic acids or extracellular enzymes, such as oxidizing enzymes (Włodarczyk *et al.*, 2016). Due to such biomechanical and biochemical weathering processes, minerals and rocks undergo biodeterioration, changes in surface topography and chemical transformations (Ortega-Morales *et al.*, 2016). These effects can include alterations such as etching, pitting, mineral dissolution, biomineralization and detachment ('spalling') of outer layers (Chen *et al.*, 2000; Gadd, 2016, 2017). However, the particular mechanisms involved in bioweathering and their relative significance depend on the particular rocks and minerals, and on the participating microorganisms.

The filamentous basidiomycete *S. commune* is a white-rot fungus that degrades complex plant biomass through the production of many exoenzymes such as laccases and glycoside hydrolases (Ohm *et al.*, 2010). This fungus has been shown to attack black slate, and the enzymes that are involved in wood decay are hypothesized to degrade the organic fractions of this rock (Wengel *et al.*, 2006). The gene *lcc2* coding for a laccase is expressed with and without the addition of black slate (Kirtzel *et al.*, 2018a). Since enzymatic biodegradation represents only a small proportion of possible biodeterioration mechanisms, we investigated additional mechanisms of fungal black slate degradation using complementary chemical and morphological methods at the micro- and nanoscale. We thus aimed at identification of complementary biophysical and biochemical contribution to bioweathering with this basidiomycete (Supporting Information Fig. S1).

Results

Elemental and mineralogical composition of black slate

The composition of the typical, low metamorphic black copper slate found, e.g., throughout the Thuringian Slate Mountains, Germany, was determined using X-ray fluorescence spectroscopy. The dominance of Si as major element and the presence of smaller proportions of Al, Fe, S, K, Mg and Ti could be confirmed (Table 1). Other

elements like Ca, Na and P were also detected but were less abundant.

X-ray diffraction analysis revealed the presence of quartz, kaolinite and muscovite (Fig. 1). Quantification by Rietveld curve fitting suggested the presence of 23.5% amorphous or poorly crystalline material. Quartz (SiO_2: 72.0%) was identified as a major component and kaolinite ($Al_2Si_2O_5(OH)_4$: 1.6%) and muscovite [$KAl_2(Si_3Al)O_{10}(OH,F)_2$: 2.8%] as additional minor phases.

Glucan production in interactions with black slate

The genetically tractable model basidiomycete *S. commune* with ubiquitous distribution worldwide shows the typical white rot on wood, and hence was a good choice to evaluate the impact of laccase on the shale samples. Mycelium of *S. commune* was observed in liquid culture in the presence of black slate. In the control without black slate, hyphae formed ample EPS, which connected and spread out between the hyphae, forming a net in the dried state after scanning electron microscopy preparation (Fig. 2). Occurrence of EPS was visibly reduced in the samples that were grown with black shale. Still, EPS was associated with the hyphae, where they established a connection with the rock particles. The hyphal network enclosed black slate grains and, especially for larger particles, hyphal growth could be seen on the rock grain surfaces.

To identify EPS, more than 800 tip-enhanced Raman spectra were recorded for 10 different areas on the tangled network

Table 1. Quantitative elemental composition of black slate.

| Compound	Value (%)
SiO_2	89.337
Al_2O_3	5.455
Fe_2O_3	0.974
SO_3	0.938
K_2O	0.842
MgO	0.281
TiO_2	0.214
CaO	0.079
V	0.059
Na_2O	0.035
P_2O_5	0.035
Ba	0.032
Cl	0.022
Ce	0.020
Pb	0.007
Zn	0.005
Zr	0.005
MnO	0.005
Rb	0.005
Ni	0.004
Cu	0.004
Y	0.003
Mo	0.003
Sr	0.002
Ga	0.002
Br	0.001
Se	0.001
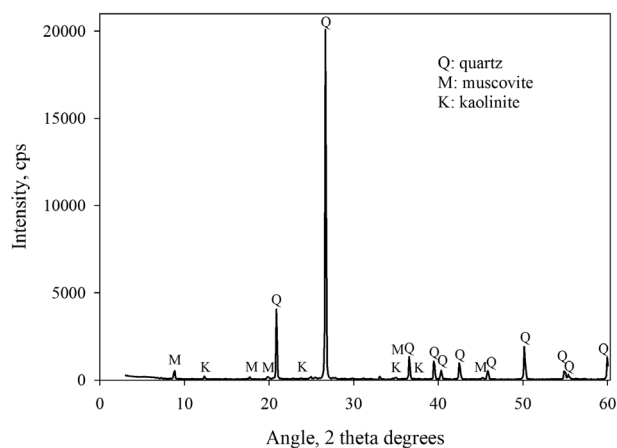
Fig. 1. X-ray diffractogram of the ground black slate material shows the presence of the minerals quartz, muscovite and kaolinite.

structure (Fig. 3). By comparing the data with spectra obtained from commercially available schizophyllan, the spectral region between 800 and 1200 cm^{-1} could be selected for identification. This spectral region is typical for carbohydrates (υC-O-C, υC-O, υC-C, δC-H) and does not overlap with features of other biorelevant components. Using eight marker bands, mixed ß1,3/ß1,6 glucan, most likely schizophyllan, could be assigned when at least two of the existing signals coincided with the reference data in the EPS measurements. Not all marker bands were present in all spectra, which may result from the extreme resolution effects and molecule orientation dependence that is well known in tip-enhanced Raman spectrometry (TERS; see Supporting Information Figs. S2 and S3; Deckert-Gaudig *et al*., 2009; Duan *et al*., 2016; Latorre *et al*., 2016). In some spectra, the presence of protein (amide I bands detection) was seen. In a further experimental series, arbitrarily selected sections located directly on four hyphae were studied, where 89% of the spectra showed a contribution from proteins. At these hyphae, only 7% of the spectra showed clear indication for schizophyllan, while contributions of other cell wall components like melanin (7%) and chitin (3%) were also identified. Thus, the presence of a schizophyllan-like glucan in the EPS network was confirmed at least four times more often than on hyphae. In scanning electron microscopy, the height of the hypha was determined to be around 700 nm, while the height of dried EPS still was measured with 20 nm, showing a large proportion of the glucan layer to overall biomass present. Thus, their contribution to biophysical weathering needs to be considered.

Contribution of biomineral formation and penetration of black slate

An additional feature was observed with *S. commune* grown with black slate in that biominerals attached to the hyphae, which were often covered with EPS, were found
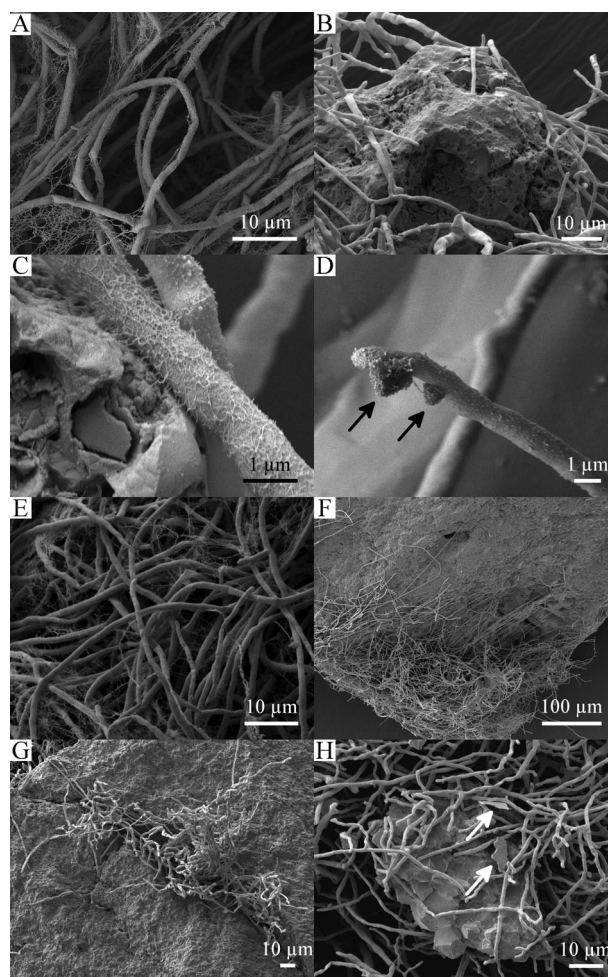

Fig. 2. Scanning electron micrographs images of *S. commune* grown in liquid culture. Images of empty vector control transformant *S. commune* EVC without (A) and with (B–D) black slate with extracellular polymeric substances adhering to a black slate grain (C) and the formation of biominerals (D, arrows) were compared with images of laccase overexpressing *S. commune* lcc2-OE without (E) and with (F–H) black slate (showing chasmoendolithic growth (G) and a possible splitting-off of small rock pieces (H, arrows). Typical images are shown from several examinations.

(Fig. 4). The elements C and Ca were associated with these secondary phases. Si, which was also detected, originated from the underlying rock material (quartz). Raman spectroscopy showed that the newly formed mineral was the calcium oxalate monohydrate, whewellite. Bitumen and quartz, which were additionally detected, are naturally present in black slate.

A strong effect of laccase was suggested using *S. commune* lcc2-OE, a laccase overexpressing strain with 15,000-fold laccase gene expression (Kirtzel *et al*., 2018b). This strain could be shown to penetrate black slate particles and to grow through cracks in the particle (Fig. 2G). Furthermore, the fungus probably caused a biodeterioration of the rock, which resulted in splitting-off of smaller particles (compare Fig. 2H).
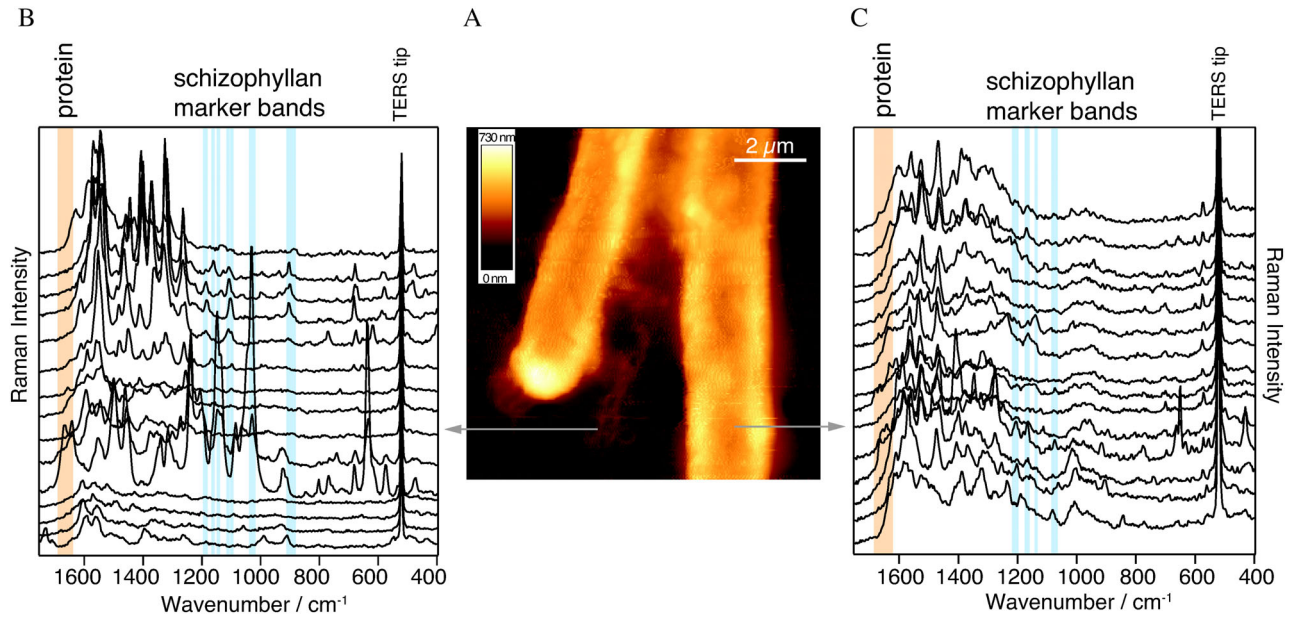
Fig. 3. Tip-enhanced Raman spectrometry on a hyphae-EPS sample at λ = 532 nm, p = 660 μW, t_{acq} = 1 s. AFM topography of *S. commune* lcc2-OE hyphae with tangled EPS (A); exemplary spectra recorded at equidistant points along a line on the EPS (B) or on the hyphae (C). Blue lines indicate schizophyllan marker bands. The orange line indicates amide I (protein) band region. Typical spectra are shown from several determinations.
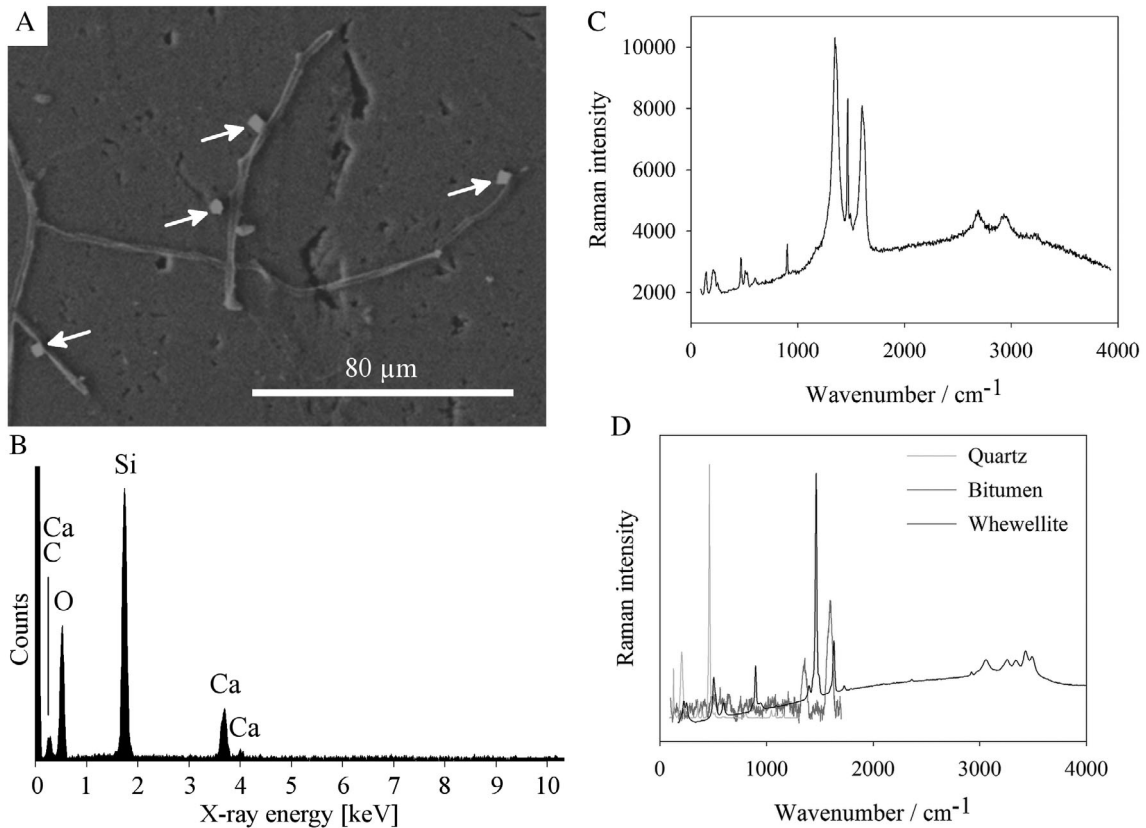
Fig. 4. Identification of biominerals. Biominerals produced on the hyphae of *S. commune lcc2*-OE (A, arrows) and analysis of the biominerals through EDX analysis (B). Raman spectrum of a biomineral (C) and reference spectra for bitumen, quartz and whewellite (D). Typical data are shown from several determinations.

Fungal interactions with polished black slate surfaces

For a more detailed analysis of fungus–rock interactions, rock samples were left intact instead of being provided as ground material to the fungus. Polished black slate pieces that had been overgrown with *S. commune* showed the first surface changes after 6 weeks of incubation. Locally, the upper rock layer was dissolved, which resulted in narrow, hyphal-shaped etching and pitting. After a longer incubation period of 6 months, the etch pits exhibited broader dimensions of 3–5 μm, which coincided with the approximate diameter of the hyphae (Fig. 5).

Again, overexpression of laccase showed a strong effect. While the empty vector control *S. commune* EVC caused only slight imprints, the transformant *S. commune* lcc2-OE induced deeper etch pits that were found at several locations on the rock surface.

To differentiate the minerals to which hyphae adhere, energy-dispersive X-ray (EDX) analysis was performed. This revealed that the etchings were found only on rock components composed of Si and O, probably quartz, whereas no such structures were found on carbonaceous surfaces.

Biodeterioration through release of organic acids and siderophores

Organic acid production by fungi is believed to lead to the dissolution of silicates (Huang and Keller, 1972; Fenoglio *et al*., 2000; Sterflinger, 2000). To investigate the ability of *S. commune* to produce organic acids, the fungus was grown with and without black slate and the liquid medium analysed. The relative concentrations of secreted acids differed between the investigated strains and by the addition of black slate (Fig. 6 and Supporting Information Table S1). In the latter case, a significant increase in ascorbic and tartaric acid was detected for all strains, whereas significantly less malic, maleic, citric, succinic, fumaric and epoxysuccinic acids were found for some of the tested strains. The excretion of oxalic acid was induced only in the dikaryon by black slate addition to the growth medium.

In order to test whether there was adsorption of organic acids with the rock material, black slate was incubated with organic acid standards. An association was demonstrated (Supporting Information Fig. S4), which implies that the sample analysis had underestimated the released organic acid fractions. The effect of pure organic acids in deterioration of the rock material was confirmed by spectrophotometric analysis that showed a higher absorbance at 450 nm due to suspended black slate particles (Supporting Information Fig. S5).

It is well known that dicarboxylic acids can complex metals and contribute to mineral dissolution through such complexation and protonolysis (Sterflinger, 2000; Jarosz-Wilkolazka and Gadd, 2003). Additionally, formation of siderophores was examined. All *S. commune* strains caused either a bleaching of blue chrome azurol S (CAS) agar or a pink discoloration of the medium (Supporting Information Fig. S6) indicating the production of siderophores.

Discussion

In this study, we have investigated the ability of the basidiomycete fungus *S. commune* to bioweather black slate, a sedimentary rock rich in organic matter. The colonization of rock particles and the growth of hyphae along cracks indicated some direct physical bioweathering of the black slate grains. Broader and preexisting cracks might be enlarged due to the effect of fungal turgor pressure, and such pressure of 4–18 bar could cause a breaking up of the black slate into smaller pieces (compare Adebayo *et al*., 1971). In addition, hyphae colonizing depressions, pores and mineral cleavage planes could begin to actively split the substrate and cause the formation of new cracks (Hoffland *et al*., 2004). The observed splitting of rock particles might be due to perfect cleavage
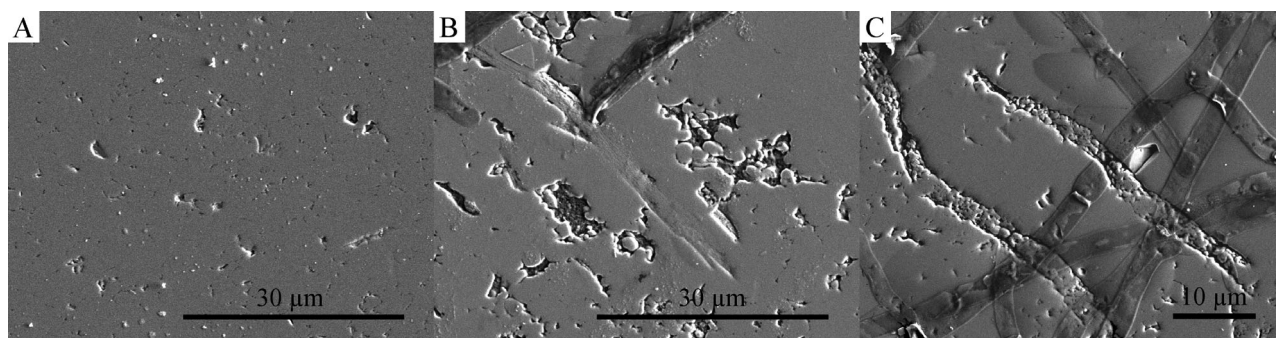
Fig. 5. Polished black slate pieces with a non-inoculated control showing naturally occurring depressions of the rock topography (A) and overgrown black slate samples showing etch pits of hyphae produced by *S. commune* EVC (B) and *S. commune* lcc2-OE (C). Typical images are shown.
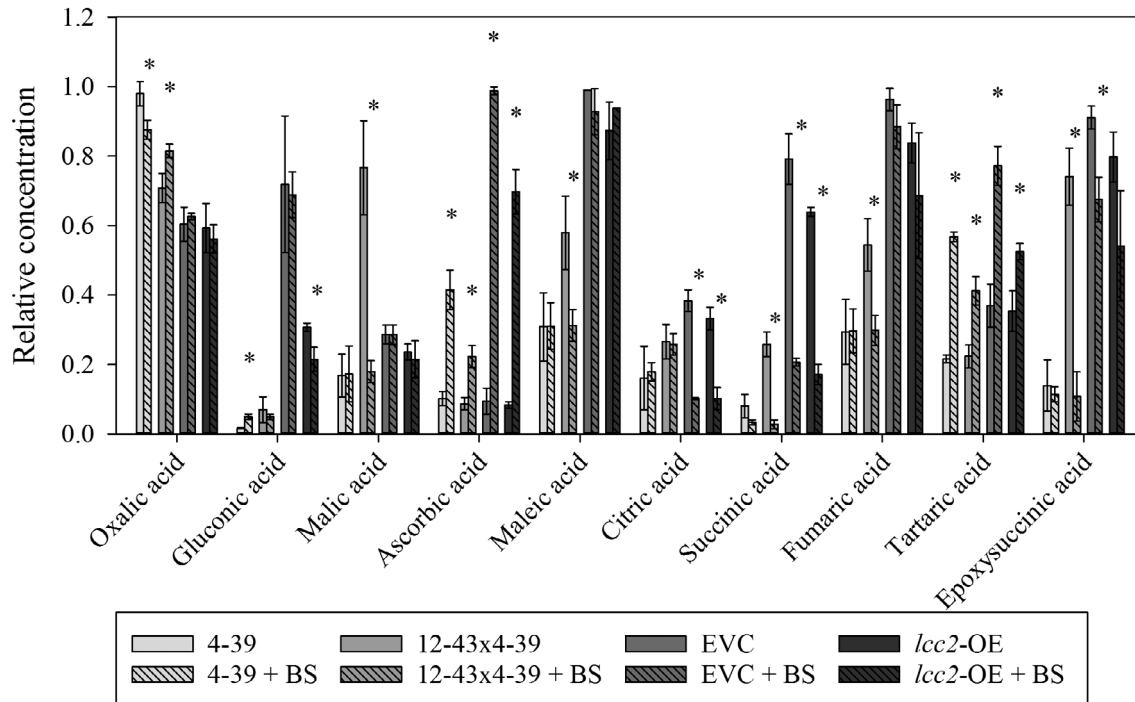
Fig. 6. Organic acids production and black slate-induced changes (BS) by different strains, the unmated monokaryon *S. commune* 4–39, a mated dikaryon *S. commune* dikaryon 12-43 × 4-39, empty vector control transformant *S. commune* EVC and laccase overexpressing *S. commune* lcc2-OE. Error bars indicate standard deviation; *$p < 0.03$, paired, two-tailed *t*-test.

panes in phyllosilicate minerals in the rock. However, some disintegration of the rock due to the shaking culture conditions cannot be excluded.

Our results have shown that the fungus not only dissolved parts of the rock material, but mediated crystallization of a new biomineral phase (whewellite, as shown by Raman identification), which is also reported for other fungi on limestone surfaces (Moron-Rios *et al.*, 2017). That would ultimately lead to changed surface mineralogy in weathered rocks. The formation of biominerals will also lead to expansion of cracks upon induced mineralization thereby contributing another biophysical weathering force. Indirect biomechanical weathering through the formation of EPS further exerts physical forces on the rock and *S. commune* is known to produce exopolysaccharides (Hao *et al.*, 2010). In the EPS, a schizophyllan-like glucan was identified located between the hyphae. This glucan is expected to contribute to attachment of the fungus to the black slate (Fomina *et al.*, 2006). The ability of EPS, including schizphyllan, to provide additional force in cracks may contribute to biophysical weathering (Grisel and Muller, 1997). However, the glucan was less visible in the presence of black slate, which might be indicative of regulatory functions after release of metals from the stone with the possibility of inhibition of growth or induction of a metabolic shift. Nevertheless, glucans like schizophyllan will contribute to biophysical weathering (see Grisel and Muller, 1997) in addition to turgor pressure and mineralization pressure.

In biochemical weathering of minerals, acidolysis by fungi is well known to play a fundamental role (Gadd, 2007). The mineral dissolution often is discussed for an increased availability of phosphorus and other nutrients necessary for fungal growth. When *S. commune* was grown with black slate, an altered profile of organic acid production was observed, which fits well with microarray analyses that have shown enhanced transcription of enzymes involved in organic acid production during the black slate treatment (Kirtzel *et al.*, 2017). Metabolites of the citric acid cycle, e.g., citric, succinic, fumaric and malic acid, were shown to be excreted and would cause a further acidification, in addition to the production of CO_2 through enhanced respiration (Sterflinger, 2000; Gadd, 2016). The contribution of organic acids to bioweathering so far has often been underestimated, which is also seen with *S. commune* that not only had been shown to produce oxalic acid dependent on carbonate in the medium and maleic acid (Takao, 1965), but many more acids could be shown to be produced with this study. Organic acids included ascorbic, gluconic, maleic, itaconic and tartaric acids, and an elevated concentration of ascorbic and tartaric acid was determined for *S. commune* in the presence of black slate. Ascorbic and tartaric acid are strong solubilizing agents of silicate minerals (Huang and Keller, 1972; Fenoglio *et al.*, 2000) and, therefore, could contribute to the dissolution of quartz, kaolinite and muscovite, the most abundant minerals in black slate. Furthermore,

oxalic acid is also a strong solubilizing agent of silicate minerals (Sterflinger, 2000) and has been shown to increase dissolution of quartz with the strongest impact at pH 7 (Bennett *et al*., 1988). Thus, the unexpected quartz etching can be explained by biochemical weathering through organic acid secretion. This suggests that a variety of other rocks might be weathered by *S. commune* or white-rot fungi in general, since quartz is one of the most common minerals in rocks.

Organic acids have metal complexing effects and play an important role in mineral dissolution as well as postulated roles in metal tolerance and detoxification (Urzì and Krumbein, 1994; Rauser, 1999; Sterflinger, 2000; Clemens, 2001; Gadd *et al*., 2014). For example, oxalic, citric and malic acid are strong chelators of trivalent metals such as Al^{3+} and Fe^{3+} (Jones, 1998), and the production of different organic acids is known to depend on the carbon source and limiting nutrients (Gadd, 1999; Sterflinger, 2000). Furthermore, different siderophores were produced as shown by the differing coloured reactions on the selective medium (Milagres *et al*., 1999). While siderophores are primarily concerned with Fe(III) coordination, they can also complex other metals, e.g., Cd, Cu, Pb and Zn (Kraemer *et al*., 1999; Neubauer *et al*., 2000; Dimkpa *et al*., 2009). By metal removal into the chelate, the dissolution of the mineral may be promoted. The additional metal binding abilities of siderophores might therefore contribute to bioweathering of the black slate.

In addition, enzymatic functions can contribute to weathering. In an earlier study, D-aspartate oxidase induction with black slate had been described (Kirtzel *et al*., 2017). This enzyme leads to the generation of hydrogen peroxide that in turn can oxidize and dissolve minerals releasing metal ions from ore surfaces (Nooshabadi and Rao, 2014). Thus, a role for hydrogen peroxide might be important in black slate dissolution.

Laccases are believed to play a role in the biodeterioration of rocks containing high amounts of organic matter (Willmann and Fakoussa, 1997; Włodarczyk *et al*., 2016). We initially hypothesized that laccase could contribute to biodeterioration, since the native substrate, lignin is similar to the kerogen fraction of the organic carbon in the black slate material. Thus, laccase action on the organic fraction seemed possible. However, here we found association of hyphae predominantly with quartz fractions in the rock. Hence, an alternative mechanism needs to be considered. As hydrogen peroxide is released in the reaction of laccase, this might explain the effects of laccase overexpression observed on black slate deterioration, with deeper and more efficient etching of quartz surfaces. The generation of reactive radicals by laccases might also be directly connected with rock bioweathering through redox reactions performed on the minerals. A role of laccase production might be associated with its protection against oxidative stress (Jaszek *et al*., 2006; Giardina *et al*., 2010), thereby protecting the fungus from released metals. In conclusion, this work adds substantial new evidence and insight into the potential role of basidiomycetes in bioweathering through biophysical as well as biochemical mechanisms; see Fig. 7. In comparison to the well-investigated ascomycetes, bacteria and lichens (Banfield *et al*., 1999; Gadd, 2010), we showed that *S. commune* possesses similar degradation strategies. This highlights the potential of basidiomycete fungi in rock decay and emphasizes the need to include them into research dealing with biological weathering.

Experimental procedures

Organism and culture conditions

To investigate fungus–rock interactions, *S. commune* 4-39 as a haploid monokaryon and *S. commune* 12-43 × 4-39 as a mated dikaryon were grown. The dikaryon had been shown to produce more laccase (Madhavan *et al*., 2014). To observe possible effects of laccase on black slate biodeterioration, a strain overexpressing laccase, *S. commune lcc2*-OE, and an empty vector control *S. commune* EVC were included in the study (for details, Kirtzel *et al*., 2018b). The empty vector does not contain the laccase gene, but otherwise, all elements on the vector that might be influencing the expression of native laccase. Hence, this was used as the relevant control, rather than a non-transformed wildtype strain of *S. commune*. Overexpression of the laccase gene *lcc2* had been shown before. As determined by qRT-PCR, a 15,000-fold expression compared to the wild-type strain and EVC was achieved with *S. commune lcc2*-OE (Kirtzel *et al*., 2018b). The fungi were inoculated on solid and liquid half-diluted minimal medium (½ MM containing 10 g glucose, 1 g aspartic acid, 0.5 g K_2HPO_4, 0.25 g KH_2PO_4, 0.25 g $MgSO_4$, 60 µg thiamine hydrochloride adjusts pH to 6.3 using 5 N NaOH; Raper and Hoffman, 1974) containing additional 11.2 g l^{-1} uracil for transformants. For liquid cultures, five pieces of 1 cm^2 inoculated agar blocks were transferred from a pregrown culture on solid medium into 200 ml liquid ½ MM with and without 3 g powdered black slate (0.5 g < 63 µm, 2.5 g 63 µm-1 mm) and incubated at 28 °C at 130 rpm. All experiments were performed using three biological replicates.

Solid medium (18 g l^{-1} agar) was inoculated with *S. commune* transformants and four sterile, polished black slate samples (~1 cm^2) were placed around the central inoculum. To allow the fungus to completely cover the rock samples and interact with its surface, agar plates were sealed with parafilm after 2 weeks of incubation and stored at 28 °C in the dark for up to 6 months. For microscopic analysis of unweathered

A.

B
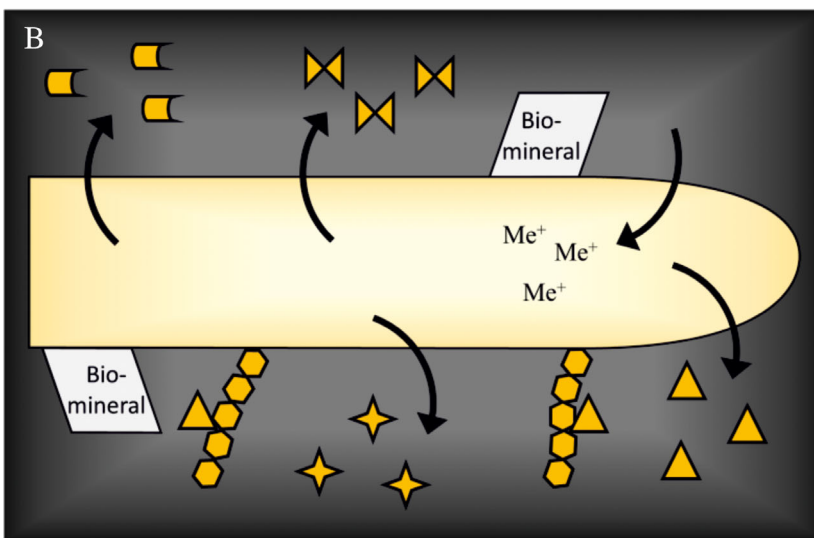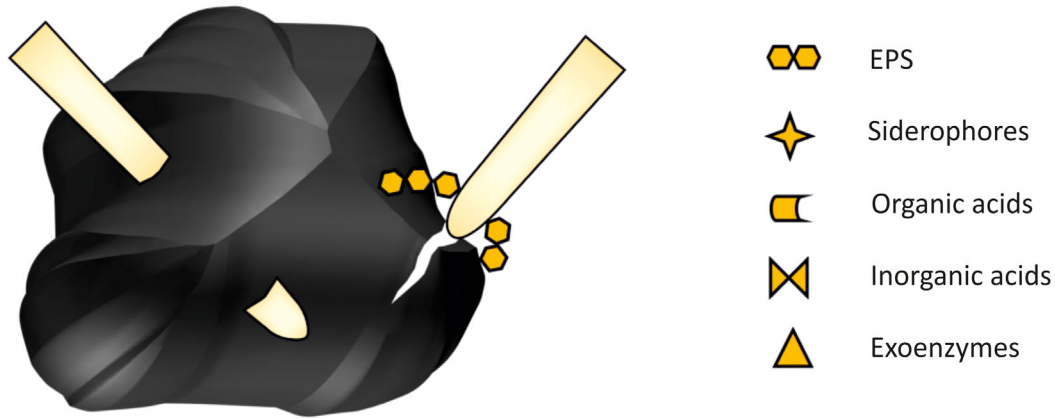
C
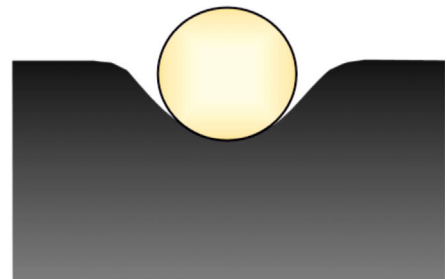
Fig. 7. Proposed scheme of mechanical and chemical bioweathering mechanisms for *S. commune*.
A. EPS (schizophyllan), turgor and mineralization pressure cause cracks in black slate and a split-off of mineral constituents.
B. Secretion of siderophores, exoenzymes as well as organic and inorganic acids causes a biochemical degradation and dissolution of black slate. EPS contain proteins and establish an enhanced adhesion to the rock. Accumulation of released metal cations (Me+) can cause a further rock decomposition. Consequences of biochemical weathering are visible as biominerals (whewellite) and etch pits (C).

black slate samples, controls without fungal inoculum were incubated as well.

For the incubation with black slate, we used minimal medium throughout instead of water. This allows for physiological processes to continue. Adsorption of metals to hyphae incubated for prolonged phases in tap water had shown earlier that adsorption of metals was possible (Merten *et al*., 2004).

X-ray diffraction and X-ray fluorescence spectroscopy of black slate

The mineralogy of powdered black slate (< 63 μm) was determined (XRD; HiltonBrooks, Crewe, UK). Samples

were analysed within the range of 3–60° 2θ in 0.1° increments. To quantitatively estimate mineral abundances, a Rietveld curve fitting, using corundum as an internal standard, was performed. The qualitative and quantitative elemental composition of black slate was determined (XRF; Philips PW2424). For all principal components, the main elements were expressed as oxides.

Interactions between S. commune *and black slate*

Mycelium from liquid cultures supplemented with or without black slate was harvested 6 weeks after incubation and CO_2 critical point dried to preserve the morphology of the fungal hyphae. For this, 70% ethanol was added to

the mycelium and replaced by a graded ethanol series of 70%, 80%, 90%, 96%, and 100% (v/v$_{aq}$), followed by three washing steps with acetone (30 min each) and the liquid carbon dioxide replacement (CPD 030, Bal-Tec, Balzers, Liechtenstein).

Alternatively, the hyphal mat was removed from the black slate blocks after 6 weeks and 6 months to achieve a closer examination of the interaction zone. The elemental composition of secondary minerals was determined using EDX analysis at an accelerating voltage of 20 kV.

Black slate blocks and liquid culture samples were sputter-coated with carbon or gold/palladium, respectively (30 nm; sputter coater 208HR, Cressington Scientific Instruments, Watford, UK), and examined using a field emission scanning electron microscope (Quanta 3D FEG and Jeol JSM-7400F) operating at an accelerating voltage of 5 kV.

TERS for characterization of EPS

It is well known that *S. commune* produces schizophyllan when cultured in liquid medium. So far, identification has required isolation of the compound from the culture medium and direct *in vitro* characterization was not possible. In this work, we intended to chemically characterize the EPS located around the hyphae at the nanoscale. For this attempt, *S. commune* was grown in ½ MM for 6 weeks, critical point dried as described above, and examined with near-field spectroscopy, namely TERS (Stöckle *et al.*, 2000; Deckert-Gaudig *et al.*, 2017). Further details on this emerging technique are provided in the Supporting Information and Supporting Information Fig. S2.

For the TERS experiments, cover slips were cleaned with a HNO$_3$/H$_2$O$_2$ (3:1) mixture for 2.5 h, and after thoroughly rinsing with deionized water, they were dried and stored in a desiccator at ambient temperature.

Schizophyllan (InvivoGen, France) of 1 mg was suspended in 100 μl water and 10 μl from this solution was placed on a cover slip and left to dry providing a standard sample.

For TERS measurements on *in vitro* samples, 20 μl ethanol was applied to a cover slip and mycelium and associated EPS was physically placed on the ethanol-wetted substrate and left to dry.

The TERS setup comprised an atomic force microscope (AFM, Nanowizard III, JPK Instruments AG, Berlin, Germany) mounted on an inverted microscope (Olympus IX71, Olympus, Hamburg, Germany). For irradiation, a laser (λ = 532 nm, P = 660 μW, Cobolt Samba, Cobolt AB, Sweden) was focused through a 60× (NA = 1.45) oil immersion objective (Olympus, Germany) on the sample. Spectra were acquired in back reflection geometry with a confocal spectrometer (SP2750A, Acton Advanced, Princeton Instruments) and a CCD camera (PIXIS

256 Princeton Instruments, Trenton). The acquisition time for all measurements was 1 s. After scanning the sample topography, spectra were recorded on regular grids (dimension 0.4–1 μm^2) with a point-to-point step size between 7 and 27 nm (pure schizophyllan), 13 and 33 nm (EPS) and 27 and 67 nm (hyphae). TERS tips were prepared by evaporating 25 nm silver on AFM tips (Tap190Al-G, Budget Sensors, NanoAndMore GmbH, Wetzlar, Germany). Tips were stored under argon prior to use.

Identification of secondary minerals

Raman spectroscopy (WITEC alpha300R with a UTHS300 spectrometer) was used to identify biogenically produced minerals on polished black slate samples incubated with *S. commune*. The measurements were conducted by means of a Raman microscope setup with a laser excitation wavelength of 532 nm and a laser power on the sample of 5 mW. The integration time per spectrum was 20 s. Reference spectra were obtained from the databases of RRUFF and Pigments Checker.

Organic acid production

The production of extracellular organic acids was determined using ultrahigh performance liquid chromatography (UHPLC). Medium from liquid cultures with and without black slate was analysed 7 days after inoculation (dpi). All samples were filtered through 0.22 μm polyethersulfone syringe filters (Roth, Karlsruhe, Germany) before analysis. To test whether black slate interacts with secreted acids, 1 mM or 10 mM organic acid standards were incubated with and without black slate for 7 days and measured using HPLC. In addition, any effects of these acids on black slate deterioration were evaluated by incubating a mixture of 0.5 mM organic acids with and without the rock material for 7 days, followed by centrifugation for 5 min at 6080*g*, and measuring the absorbance of the supernatant at 450 nm.

UHPLC coupled with high-resolution mass spectrometry (HRMS) was carried out using an UltiMate HPG-3400 RS binary pump (Thermo Fisher, Karlsruhe, Germany), WPS-3000 auto sampler set to 10 °C, and equipped with a 25 μl injection syringe and a 100 μl sample loop. The column was kept at 25 °C within the column compartment TCC-3200. A C-18 chromatography column was used (Thermo Accucore C-18 RP, 100 × 2.1 mm; 2.6 μm) with the gradient (Supporting Information Table S2) at a constant flow rate of 0.4 ml min^{-1}. Eluant A was water, with 2% acetonitrile and 0.1% formic acid. Eluant B was pure acetonitrile.

Mass spectra were recorded with a Thermo QExactive plus orbitrap mass spectrometer coupled with a heated electrospray source. Column flow was switched at

0.5 min from waste to the MS and at 11.5 min again back to the waste to prevent source contamination. For monitoring, two full scan modes were selected with the following parameters: polarity: positive; scan range: 80 to 1200 m/z; resolution: 70,000; AGC target: 3×106; maximum IT: 200 ms. General settings were: sheath gas flow rate: 60; auxiliary gas flow rate 20; sweep gas flow rate: 5; spray voltage: 3.0 kV; capillary temperature: 360 °C; S-lens RF level: 50; auxiliary gas heater temperature: 400 °C; acquisition time frame: 0.5–11.5 min. For negative mode, all values were kept instead of the spray voltage, which was set to 3.3 kV.

Standards were purchased from the Sigma-Aldrich (Darmstadt, Germany) mass spectrometry metabolite library used at a concentration of 0.5 μg on the column using the retention time standards p-fluorophenylalanine, p-fluorobenzoic acid and decanoic acid D-19.

Compounds were quantified based on their $[M - H]^-$ ion in negative mode. Peak detection and integration were carried out using the Thermo Xcalibur™ 3.0.63 Quan Browser software with the following settings: mass tolerance 15 ppm; mass precision 4 decimals; retention time window 30 s; signal from full scan experiment for peak detection algorithm ICIS (Smoothing 1); peak detection method highest peak. Area ratios of each analyte were determined and normalized relative to the highest concentration in the whole sample set which was set at 1.

Siderophore production

The ability of *S. commune* to secrete siderophores was tested using a CAS assay. MM plates were cut in half and filled with CAS agar according to Milagres *et al*. (1999). *S. commune* was placed on the medium side about 1.5 cm away from the CAS medium and colour changes observed after incubation for 23 days at 28 °C.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary Information.