Microbial excavation of solid carbonates powered by P-type ATPase-mediated transcellular Ca^{2+} transport

Ferran Garcia-Pichel¹, Edgardo Ramírez-Reinat, and Qunjie Gao

School of Life Sciences, Arizona State University, Tempe, AZ 85284

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Some microbes, among them a few species of cyanobacteria, are able to excavate carbonate minerals, from limestone to biogenic carbonates, including coral reefs, in a bioerosive activity that directly links biological and geological parts of the global carbon cycle. The physiological mechanisms that enable such endolithic cyanobacteria to bore, however, remain unknown. In fact, their boring constitutes a geochemical paradox, in that photoautotrophic metabolism will tend to precipitate carbonates, not dissolve them. We developed a stable microbe/mineral boring system based on a cyanobacterial isolate, strain BC008, with which to study the process of microbial excavation directly in the laboratory. Measurements of boring into calcite under different light regimes, and an analysis of photopigment content and photosynthetic rates along boring filaments, helped us reject mechanisms based on the spatial or temporal separation of alkali versus Acid-generating metabolism (i.e., photosynthesis and respiration). Instead, extracellular Ca^{2+} imaging of boring cultures in vivo showed that BC008 was able to take up Ca^{2+} at the excavation front, decreasing the local extracellular ion activity product of calcium carbonate enough to promote spontaneous dissolution there. Intracellular Ca^{2+} was then transported away along the multicellular cyanobacterial trichomes and excreted at the distal borehole opening into the external medium. Inhibition assays and gene expression analyses indicate that the uptake and transport was driven by P-type Ca^{2+} -ATPases. We believe such a chemically simple and biologically sophisticated mechanism for boring to be unparalleled among bacteria.

bioerosion | calcium metabolism | endoliths | microbialites

Endolithic cyanobacteria that bore their way into carbonate minerals, known as euendoliths, or true endoliths (1), constitute a major erosive force contributing to the morphogenesis of coastal (2) and terrestrial (3) limestones, the destruction of coral reefs and other biological carbonates (4, 5), and the obliteration of carbonate sands (6). They also represent a pest of commercial relevance for bivalve aquaculture (7). Despite their environmental relevance, the mechanism that enables them to dissolve carbonates, even in waters supersaturated with respect to calcite and aragonite, has remained elusive (8–13). It is in fact a geochemical paradox subject to speculation, in that cyanobacterial autotrophy will tend to increase pH and precipitate carbonates, not dissolve them (10). In the absence of moving or hard parts, etching must be clearly chemical. At circumneutral pH the dissolution reaction takes the following form: $\text{CaCO}_3(\text{s}) + \text{H}^+ \leftrightarrow \text{Ca}^{2+} + \text{HCO}_3^-$. The reaction shows that one can promote dissolution through acidification, which was long taken to be the basis of a potential excavation mechanism (9, 14), even though it cannot work well for cyanobacteria. Alternative mechanisms to explain the paradox of boring phototrophs have more recently been proposed (10). Some put forward an alternative way of acid generation that does not collide with acid-consuming CO_2 fixation. One postulates the agency of heterotrophic bacteria hosted within the borehole. Others call for the separation of photosynthesis and boring activities in space or time. A mechanism based on shifting the dissolution equilibrium by lowering Ca^{2+} concentration, obvious upon inspection of the aforementioned dissolution reaction, has also been proposed (10). The lack of cultured cyanobacteria that could

bore in a sustained fashion under laboratory conditions effectively prevented the experimental assessment of any of these models (9, 10). Only a few species of cyanobacteria are euendoliths, mainly in the morphogenera *Hyella*, *Solentia*, *Plectonema*, and *Mastigocoleus*, but the exact phylogenetic position of these cyanobacteria has not been resolved. We developed a stable microbe/mineral boring system based on a cyanobacterial isolate, strain BC008 (15). It likely represents one of the most common cyanobacterial euendoliths, the filamentous, true-branching, heterocystous *Mastigocoleus testarum* (14). We used this system to conduct growth experiments, as well as in vivo, real-time microscopic examination of the microbe/mineral system in action. Complementing the study with a molecular genetic investigation of the capabilities of BC008, we were able to gain significant insights into the question of how cyanobacteria bore.

Results and Discussion

BC008 (Fig. 1A) could bore into calcite chips in the laboratory and could routinely be grown on millimeter-sized, sterilized, illuminated crystalline blocky calcite chips immersed in liquid medium, which allowed for real-time microscopic observation of boring (Fig. 1B), and simple experimentation. The strain was demonstrably axenic (free of contaminants; *Materials and Methods* describes related tests). Thus, one can simply reject the alleged implication of heterotrophic bacteria in the boring process. To test for an excavation mechanism based on temporal separation between photosynthesis (in the daytime) and boring (at night, as a result of acidification caused by respiration of glycogen reserves), we measured BC008's rates of growth and excavation as a function of the duration of illumination period during growth (Fig. 2A). Although boring rates were optimal in the presence of a short daily dark phase, excavation could proceed unimpeded under constant illumination. A night period and the temporal cessation of photosynthesis was thus not a requirement for boring. A possible spatial separation of boring and photosynthesis along the cyanobacterial trichomes could alternatively be at play. In this scenario, cells at the boring front would function heterotrophically, producing net acidification of the medium by releasing CO_2 , at the expense of photosynthate transported from the cells at the lagging end. Precedent for such metabolic segregation can be found in heterocysts, specialized cells of some filamentous cyanobacteria. Heterocysts lose autotrophy and the ability to evolve oxygen, turning into a photoheterotrophic metabolism sustained with carbon fixed in neighboring cells, to optimize nitrogen fixation in anoxic environment (16). We tested the prediction that if apical cells were photosynthetically inactive, they should display pigmentation changes similar to those found in heterocysts (i.e., lowered levels of photosystem II and phycobiliproteins) or an

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¹To whom correspondence should be addressed. E-mail: ferran@asu.edu.

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Fig. 1. Strain BC008 and its ability to bore. (A) Photomicrograph of culture grown in liquid. (B) Close-up of a heavily infested calcite chip (infestation started from top). Single green filaments can be observed deep in the crystal matrix. Notice the reprecipitation of a yellow, light-scattering micrite layer close to the surface.

overall decrease in all photosynthetic pigments. By using laser scanning confocal fluorescence microscopy (LSCM), we probed

for such changes in actively boring filaments, but could not detect any (Fig. 2B). This speaks against a boring mechanism based on spatial separation of photosynthesis and heterotrophy. We also tested the prediction that if apical, boring cells were not photosynthetic, we should be able to detect directly a relative decrease in photosynthetic rates between populations of cells proximal to the boring front versus those that are distal to it. For this we “disinterred” boring BC008 populations by an artificial dissolution of the solid calcite chip using an EDTA perfusion method (17). As we dissolved the calcite chip, boring filaments of BC008 became exposed, and could be sequentially harvested into three discrete populations (surface, middle, and deep). Specific photosynthetic rates were not significantly different from those of free-living controls, indicating that the procedure did not affect photosynthesis. We could detect neither a significant difference among any of these fractions nor any decreasing trends with depth (Fig. 2C), thus invalidating the prediction. We then turned to testing a mechanism based on Ca^{2+} transport, whereby Ca^{2+} could be taken up by the boring cells to lower the extracellular ion activity product of CaCO_3 below that of calcite saturation, promoting localized, spontaneous mineral dissolution. Ca^{2+} must then be transported internally across cells and excreted at the lagging end of the filament into the external liquid medium. Here, one can make two predictions. First, Ca^{2+} should accumulate above saturation in the liquid medium close to the solid’s surface as carbonates are bored, and second, Ca^{2+} should be depleted below saturation equilibrium deep in the borehole, in the extracellular space close to the boring front. We could in fact detect and quantify such departures from equilibrium, when boring BC008 was imaged in real time under the LSCM in the presence of a fluorescent Ca^{2+} -sensitive reporter dye (Fig. 3A; *Materials and Methods*). At the boundary layer between solid and liquid, Ca^{2+} reached concentrations several fold that of (calcite) saturation. Active Ca^{2+} transport into this layer is required to maintain a steady supersaturation against diffusive losses. No such accumulations occurred in sterile chips, or in BC008-infested chips that had been bleach-killed before imaging, proving that transport was mediated by the cyanobacteria. Ca^{2+} buildup, in fact, required illumination of the sample, the magnitude of supersaturation being a function of the intensity of illumination

Fig. 2. Evidence against separation between photosynthetic and boring activities in strain BC008. (A) growth rate (as doubling time, right axis) in liquid culture, and the intensity of infestation on blocky calcite chips attained at 5 wk of incubation (left axis), both as a function of the length of the illumination period when grown on a day/night cycle. (B) Cell-specific photosynthetic pigment content measured *in situ* during boring using fluorescence emission spectroscopy of confocal optical sections with excitation at 488 nm. Lines show average ($n = 5$) emission spectra of single cells for cells close to the boring front (proximal) compared with cells close to the surface of the solid (distal). *Inset:* Chlorophyll *a*-specific emission at 685 nm (error bars are 1 SD); differences are not significant (t test; $P = 0.234$). (C) Photosynthetic rates of boring BC008 cells retrieved from layers close to the surface of chips compared with those in the middle and deep zones and those from nonboring cultures (control). No significant differences were found ($P > 0.33$ in all t tests; $n = 3$).

volvement of channels is consistent with the moderately negative effect of specific inhibitors on boring. Counter transported protons (two per Ca^{2+}) promote the conversion of carbonate ions released from calcite into CO_2 , most likely in concert with the carbon-concentrating mechanism (as shown). This CO_2 can then be used in photosynthesis. Although the participation of P-type Ca^{2+} ATPases and the transcellular calcium transport have been demonstrated, the exact cellular distribution and the specific nature of some of the transporters remain to be determined with certainty.

The mechanism delineated here is attractive because it can explain several geological phenomena associated with modern and fossil bioeroded substrates, in that the extreme local supersaturation that occurs close to the lagging end of a boring filament can account for the reprecipitation of carbonates as poorly crystalline phases (the micrite “rind” in Fig. 1) (23–25), or as alternative polymorphs such as aragonite or vaterite (26). This may also explain how reprecipitation at the lagging end may cause microbial borers to act as cements agent in sedimentary structures when growth allows them to cross between mineral grains (27). Our model also opens biological questions as to how boring cyanobacteria might cope with altered intracellular Ca^{2+} , which is otherwise kept low and tightly regulated (22). Because a lowering of ocean calcium carbonate saturation state driven by anthropogenic CO_2 emissions (28) will lower the bioenergetic cost of boring, a global increase in cyanobacterial bioerosion rates in shallow waters can be logically predicted, an outcome for which some experimental evidence is already available (29), likely affecting already impacted (30) coral reefs, as well as the bivalve fisheries industry (7).

Materials and Methods

Strain BC008 (15) was isolated from a marine snail shell in Cabo Rojo, Puerto Rico (latitude N 17° 56' 1.89", longitude W 67° 11' 32.64"). Cultures were kept boring on small calcite chips, submerged in liquid sterile medium [Provasoli enriched seawater (PES)] (31) containing 30 g L^{-1} of Instant Ocean salts (Spectrum Brands) at pH 8.3. BC008 was obtained here in axenic (pure) culture by micromanipulation of individual filaments under the dissecting microscope. Axenicity was eventually verified by meeting the following criteria: (i) absence of heterotrophic colonies on old-growth BC008 agar plates; (ii) absence of colony growth after 3 d of plating filaments of BC008 in PES agar supplemented with peptone, glucose, and yeast extract; (iii) absence of visible bacteria, other than BC008, in mature liquid cultures when observed under 1,000-fold magnification, phase contrast, in wet mounts; and (iv) presence of a single high-quality sequence after PCR amplification of a 1,100-bp-long fragment of the 16S rRNA gene using universal bacterial primers. Forward and reverse Sanger sequences were aligned with MEGA 4.0 to obtain a final sequence of 1,094 bp, which expectedly blasted to the heterocystous cyanobacteria. Photosynthetic rates were determined as O_2 evolution of dilute cell suspensions illuminated at a light intensity of 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ in a liquid-phase chamber (Hansatech), in which partial O_2 pressure was monitored with a Clark-type electrode, calibrated using air-saturated PES medium and a sodium-dithionite solution (zero O_2). For each value reported, the average of three consecutive rate determinations was used. Rates are expressed per unit mass of chlorophyll a, the concentration of which was determined spectrophotometrically in each cell suspension after centrifugation and pellet extraction in 90% acetone. For microscopy experiments, calcite chips of appropriate size were cleaved manually from commercial blocky calcite, sterilized by immersion in ethanol, added into tissue-culture bottles containing minimal PES medium, and inoculated with BC008. After 3 to 4 wk of growth in the light at room temperature, when the infestation of the chips was apparent but not massive, chips were retrieved. Surface growth outside of the chip was removed by gently brushing under sterile conditions, and incubation overnight was performed in fresh

medium. Boring biomass per unit area could be assessed sacrificially by extracting lipid-soluble pigments in acetone, and subsequent spectrophotometric determination of chlorophyll a. For most microscopy-based experiments, clean chips were placed in a custom-made incubation chamber built on a microscopic slide and allowed to incubate for at least 2 h before experimentation. Confocal laser microscopy allowed to optically section the areas around the calcite chip surface without physically disturbing any gradients that had formed. To take the image in Fig. 3, after a long period of growth of several months, calcite chips were cleaved once more along the direction of boring, to expose the boring filaments lengthwise. The chip was then placed on the stage chamber with the freshly cleaved surface facing up, so that filaments and boreholes close to the new surface, but still within the solid, could be imaged optimally. Single-plane horizontal fluorescent images were stored in two separate channels: green fluorescence for calcium and red autofluorescence for chlorophyll a. Three-dimensional images or vertical optical sections were reconstructed from stored data as needed. In all cases, 1 μm calcium green-5N (Molecular Probes) was used as fluorophore. Fluorescence intensity in the green was converted into absolute Ca^{2+} concentrations using a two point-calibration, whereby the signal inside the solid chip corresponds to zero Ca^{2+} and the signal far away from the chip corresponds to the medium in equilibrium with calcite (10 mM Ca^{2+}). Concentration versus fluorescence curves for calcium green in PES medium were run separately in a spectrofluorometer (Turner Designs). In PES medium, at pH 8.3, the dissociation constant for the dye was approximately 5 mM, and a linear approximation described the relationship as well as a hyperbolic one in the measured range. The linear relationship was used for extrapolation into the supersaturated region, as it will bias by defect (i.e., it is a conservative concentration estimator). The saturation kinetics of the dye was pH-dependent, so that potentially, pH excursions of the medium may have influenced the measurement of Ca^{2+} . Potential pH changes (an alkalization of the medium by either photosynthesis, or by the uptake of protons), if present, could have only resulted in an underestimation of Ca^{2+} concentration.

Forty gene sequences of cyanobacterial P-type ATPases from the National Center for Biotechnology Information database and Cyanobase were identified by having both Ca^{2+} and ATP-binding motifs. The sequence alignments at both amino acid and nucleotide level were analyzed. Group-specific sequence alignments were used to design degenerate primers matching conserved sequence regions corresponding to the phosphorylation and ATP-binding sites. These primers were used to clone putative Ca^{2+} P-type ATPases from BC008 through PCR amplification of its genomic DNA. PCR products were cloned into pCR4 vector through the TOPO TA cloning system (Invitrogen). Primers for the specific housekeeping genes, 16S rRNA gene and *rnpB* (the RNA component of RNase P), were also designed. High quality, DNA-free RNA extracts (20) from each sample were used to synthesize cDNA, primed using random hexamers (BioRad). Primers specific to each putative gene obtained in cloning efforts, as well as to the housekeeping genes, were designed and used to amplify approximately 0.15-kbp fragments from the cDNA in each extract using a real-time quantitative PCR (qPCR) procedure. All samples and controls are listed in *SI Text*. To evaluate differential gene expression, we used REST software, which addresses the measurement of uncertainty in expression ratios by using randomization and bootstrapping techniques (32). The quantification strategy is based on the expression ratio of a target gene versus reference gene(s). The difference in qPCR signal between control (nonboring culture) and sample is the numerator. In the denominator, one uses the difference in qPCR signal between control and sample for the reference gene. We used two reference genes: *rnpB* and 16S rRNA. Results using either one were consistent, but the statistics were more robust when using a combination of both. The data we present are based on the geometric mean between the two corresponding differences. We used bootstrapping with 5,000 iterations to provide 95% confidence intervals for expression ratios.

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