Photoautotrophs assimilate oxidized carbon obtained from one of two sources: dissolved or atmospheric. Despite its size, the pool of lithospheric carbonate is not known to be a direct source for autotrophy. Yet, the mechanism that euendolithic cyanobacteria use to excavate solid carbonates suggests that minerals could directly supply CO$_2$ for autotrophy. Here, we use stable isotopes and NanoSIMS to show that the cyanobacterium *Mastigocoleus testarum* derives most of its carbon from the mineral it excavates, growing preferentially as an endolith when lacking dissolved CO$_2$. Furthermore, natural endolithic communities from intertidal marine carbonate outcrops present carbon isotopic signatures consistent with mineral-sourced autotrophy. These data demonstrate a direct geomicrobial link between mineral carbonate pools and reduced organic carbon, which, given the geographical extent of carbonate outcrops, is likely of global relevance. The ancient fossil record of euendolithic cyanobacteria suggests that biological fixation of solid carbonate could have been relevant since the mid-Proterozoic.
The biological reduction of inorganic to organic carbon, or carbon (C) fixation, is arguably the most important biogeochemical transformation on Earth, bridging the geological and biological realms, and sustaining all global biomass. The three existing inorganic C pools are directly linked through abiotic processes\(^1\), but only dissolved C from the bulk aqueous medium or gaseous pools from the atmosphere can serve as substrate for autotrophs\(^2, 3\). At some \(10^{24}–10^{25}\) GT of C, lithospheric carbonates represent, by far, the largest reservoir in the global carbon cycle\(^4, 5\) and a practically inexhaustible potential C source. Euendolithic cyanobacteria are widespread photoautotrophs that thrive in intimate contact with carbonate substrates\(^6\), boring into the exposed mineral surface\(^7\). Recent studies using the model euendolith Mastigocoleus testarum strain BC008 have helped unravel the physiological mechanisms of boring, an otherwise geochemically paradoxical process\(^8\). The current physiological model proposes that carbonate dissolution occurs via \(\text{Ca}^2+\) removal from the boring front, followed by cell-to-cell transport and eventual ion extrusion at the substrate surface\(^9, 10\). During this process, protons are counter-transported towards the boring front, an action that likely results in the localized formation of dissolved \(\text{CO}_2\), from \(\text{CO}_3^{2-}\) released during carbonate dissolution in the interstitial space between cell and mineral inside of the solid. This would theoretically allow for fixation of mineral-sourced C by the excavating organism. Thus, the hypothesis that euendolithic cyanobacteria may be fixing the carbonate released during excavation of their own habitat is attractive for the following reasons: on one hand, it completes the geomicrobial action on the substrate, and on the other, it provides endoliths with a competitive advantage over photosynthetic epiliths, which may suffer from dissolved inorganic C (DIC) limitation as their biofilms thicken\(^11, 12\). Because carbonates have varying \(\delta^{13}\)C signatures, often distinct from that of their local bulk seawater DIC\(^13\), one can then use stable isotope analyses to track C sources in euendoliths. We searched for evidence for the use of mineral sources of C in endolithic autotrophy, and for a role of external C limitation in this process. By showing that mineral substrate isotopic signature is mirrored in the isotopic signature of biomass we provide compelling evidence for direct fixation of mineral derived carbon into endolithic biomass, in culture and nature. We also show that external DIC limitation enhances the propensity with which our laboratory model strain bores into calcite.

**Results**

**DIC limitation enhances endolithic infestation.** *M. testarum* BC008 can grow in either boring mode, producing endolithic biomass, or in a non-boring mode (producing planktonic or suspended biomass)\(^14\). Endolithic filaments can also grow out into the liquid medium, still attached to the substrate, as what we call benthic biomass. We reasoned that if BC008 fixed mineral carbon, cultures subjected to prolonged DIC limitation would show higher endolithic biomass yields than cultures with no imposed limitation. We tested this directly, growing cultures for 4 months in hermetically sealed vessels (DIC-limited, containing only 1.05 mg DIC), compared with controls grown open to the atmosphere. Cultures were inoculated using either planktonic biomass or with calcite chips seeded with incipient endolithic biomass, to assess the “choice” to initiate boring under limitation vs. the “choice” to leave the substrate in the absence of DIC. Expectedly, non-limited cultures grew more than DIC-limited cultures (8.4 ± 1.2 vs. 5.1 ± 0.8 mg). But in the latter, yields exceeded the theoretical yield from the available 1.05 mg of DIC (2.6 mg of dry biomass), indicating that calcite C had to have been the additional source. Non-limited cultures yielded less endolithic and more benthic biomass (Fig. 1a, b, f). Planktonic biomass from DIC-limited cultures displayed pigment bleaching, symptomatic of physiological stress, but not that from controls (Fig. 1c, d). Yet, endolithic and benthic biomass remained unbleached in all cultures (Fig. 1b), indicating that access to solid carbonates sufficed to relieve DIC limitation symptoms. Highest

![Diagram](image_url)
Fig. 2 Stable isotopic evidence for carbonate C fixation. a Natural δ13C abundance in endolithic, benthic and planktonic cultured biomass of *M. testarum* strain BC008 obtained via CF-IRMS (Red bars indicate means of at least five independent cultures). All means were significantly different (ANOVA, *p* < 0.06) from one another. The δ13C of potential sources of C (medium DIC and calcite substrate) are red closed diamonds. b, c correspond to cultures incubated with 13C-DIC enriched medium, and started with very lightly bored inocula (Supplementary Fig. 2), so that the virtual totality of the biomass grew in the presence of the tracer. b Exemplary NanoSIMS images of cells analyzed for isotopic composition of organic C and obtained from different positions in a boring bed (scale = 2 μm), with full quantitative data obtained from such images as a function of depth shown in c (Deep 13C image—black arrowhead indicates a single cell of several observable in this image). Also included in c are control cells grown in medium without tracer (triangles), and cells grown after removal of the tracer (squares); the gray box represents δ13C values indistinguishable from background using this technique. Error bars equal one s.d.

**Mineral derived carbon is fixed in endolithic cells.** If strain BC008 were indeed fixing mineral-sourced carbon, the isotopic signature of the mineral should be detectable in its biomass. Figure 2a shows δ13C values of endolithic, benthic and planktonic biomass fractions from long-term BC008 cultures, as well as those of the two potential sources. Mean δ13C for each biomass type differed significantly (*p* < 0.06). Planktonic biomass fractionated its DIC source from −4.2 ± 0.5‰ to a level typical of most planktonic autotrophs (δ13C = −22.5 ± 1.1‰) 15. If the sole C source for endolithic biomass had been DIC, one would have expected a δ13C similar to that of planktonic biomass, or even slightly more negative, due to diffusive fractionation 16. If the C source of endolithic biomass was mineral C, one would expect the δ13C of endolithic biomass to be more positive (by ~ 5.6 ± 1.2‰) than that of planktonic biomass. Indeed, the difference (8.8 ± 2.4‰), while slightly larger than expected, is consistent with this hypothesis. Benthic biomass gave an intermediate δ13C (Fig. 2a) indicating that it may have incorporated carbon from both sources, or perhaps exchanged organic photosynthetic from endolithic biomass while fixing DIC. To clarify the potential sources of C, particularly their relative contributions, we complemented our experiments with NanoSIMS imaging. For this, *Mastigocoleus* was incubated in closed cultures with 13C enriched (5479‰) DIC to follow the incorporation of this tracer vs. that of the mineral source (1.4‰). Control cultures incubated with tracer but no calcite substrate expectedly yielded very heavy biomass (~4089 ± 6.7‰).

Qualitative observations show clear differences in cellular δ13C as a function of the position of the cells in the boring bed (Fig. 2b). A quantitative survey across the boring bed (Fig. 2c) conspicuously shows a steep decrease in biomass δ13C with depth. Cells just outside the solid surface showed some tracer signal, suggestive of some 12.5% of their C originating in DIC, and endolithic cells close to the surface were at around 5% of control biomass C, but the DIC influence faded quickly with depth, so that just 15 μm below the surface (a few cells deep), δ13C become indistinguishable from that of the mineral (or that of control endoliths grown without tracer). Most endolithic biomass below the very surficial layer had no measurable contribution from external DIC. Hence, virtually all endolithic biomass C originated from mineral C.

**Natural endolithic communities also fix mineral carbon.** If laboratory results were generalizable to natural settings, one could predict that the δ13C of euendolithic microbial communities may align closer to that of their mineral substrate than to that of surrounding seawater DIC. To test this we analyzed endolithic biomass and mineral substrates in a variety of samples collected around the intertidal of Isla de Mona, Puerto Rico (Fig. 3a), selecting sites showing discordant δ13C values. Due to biological fractionation, biomass δ13C was always more negative than any potential source. It was, however, not invariant, but rather a direct and linear function (*R*² = 0.88) of the substrate δ13C. This is consistent with the notion that organic C (as in our model system) stems from the lithospheric pool and reflects its mineral isotopic variability. These are complex communities 17, which often show fractionation intensities less than those of pure cultures. In our case, they were around 9.58 ± 1.93‰, and were also a weak direct function of substrate δ13C (Δδ13C vs. δ13C, *R*² = 0.62), such that limestone endoliths, already quite depleted...
were obtained from six separate localities along the shore, collected within the bioerosional notch of the intertidal zone. Rock samples containing endolithic biomass, verified post hoc using a digital field microscope, were broken from large boulders and rock walls using a standard geological hammer. Samples were allowed to dry and then shipped to the laboratory.

Cultivation. Cultures of the euendolithic Mastigocoleus testarum strain BC008 were grown in standard, vented-cap, tissue culture flasks containing 15 ml of filter (0.22 μm) sterilized enriched seawater medium^2^ medium. Briefly, the medium composition is as follows: KNO₃, 300 mM; KH₂PO₄, 30 mM; neutralized sodium silicate, 300 μM; 1.0 ml/liter of a trace element solution containing per liter, 1.0 g FeCl₃.6H₂O, 620 mg MnSO₄. H₂O, 250 mg ZnSO₄.7 H₂O, 130 mg Na₂MoO₄.2H₂O, 4 mg CoCl₂.6 H₂O, 4 mg CuSO₄.5H₂O, and 6.0 g of disodium EDTA (sea water was obtained off the coast of Puerto Peñasco, Mexico, and filtered through a 0.22 μm filter. Vitamin stock solution was added to 2x final concentration). Cultures were incubated on a slowly rocking platform illuminated with a 16 h light/8 h dark cycle, at a light intensity of 2μmol photon m⁻² s⁻¹ from white fluorescent tubes. Boring cultures were obtained by adding ethanol-sterilized calcite chips (obtained from commercial blocky calcite, Ward’s Scientific) of small size (< 150 mm² surface area) to mineral-free liquid cultures, until the chips were colonized. Cultures containing 50 ml of medium with an initial gas headspace of 100 ml of 6 months. The Saturation Index for calcite in our media (based on seawater composition and taking final pH into account) was 0.83. In closed incubations (below), the Si was much higher (around 1.4) because of significant increases in pH.

Dissolved C limitation culture experiments. Calcite chip fragments used in these experiments were chosen so as to provide equivalent surface area for colonization (141 ± 2 mm²). We used two types of biomass as inoculum: mineral-free biomass (or “planktonic”) and biomass grown inside of calcite chips (or “endolithic”). Planktonic inoculum was obtained from cultures containing actively bored chips by harvesting biomass not associated with the mineral, which was re-suspended in fresh medium. The inoculum was homogenized by repeated passage through a 24 gauge sterile needle attached to a 3 ml syringe. Seed chips used for boring inoculum were obtained by incubating size-selected chips, without shaking, in 35 x 10 mm tissue culture plates, sealed with Parafilm®, containing 3 ml of homogenized inoculum (obtained as explained above). Initiation of chip infestation was then monitored by optical microscopy, and as soon as radial boring colonies (Supplementary Fig. 1) were evident (about 3 weeks), chips containing between 15 and 20 radial colonies were collected. These too were washed with sterile water, then brushed with sterile toothbrushes in sterile media and used as boring inoculum for the experiments.Triplicate independent cultures were grown for each type of inoculum (endolithic and planktonic), and under each of two conditions: open or closed to the atmosphere, so as to achieve different C limitation. All cultures were set up in 150 ml borosilicate glass serum bottles containing 50 ml of medium with an initial gas headspace of 100 ml of Inoculum consisted of either 100 μl of the planktonic homogenate together with three size-selected sterile naive chips, or three seed chips. Cultures open to the atmosphere were not sealed but capped with sterile cotton-packed gauze. Closed cultures were hermetically sealed with aluminum and Teflon crimp caps. Head-space in sealed bottles was flushed with N₂. Sterile, degassed Milli-Q water was added to open cultures, as needed, to keep volume at 50 ml, and make up for evaporative loss. Cultures were incubated for 4 months. Upon completion, planktonic biomass was collected from each bottle, rinsed three times in degassed Milli-Q water, pelleted, and dried at 60 °C for 72 h. Chips were removed from the cultures and gently rinsed with degassed sterile Milli-Q water in petri dishes, then brushed lightly with sterile toothbrushes to remove and harvest attached (but not endolithic) biomass (i.e., “benthic”) biomass. Calcite chips were then placed in a 15 ml Falcon tube and dissolved with 5 ml 1 N HCl to liberate endolithic biomass. After dissolution, tubes were spun, the supernatant discarded and the pelleted biomass rinsed with degassed sterile Milli-Q water then pelleted again. Endolithic biomass was then dried as described above. All dry biomass yields were determined gravimetrically. To gauge the depth of penetration in euendolithic growth, one chip from each culture was cracked into segments and the maximal penetration depth of the visible boring bed on the freshly opened face was recorded using the stage micrometer of a stereomicroscope, with measurements on six surfaces (n = 6).

Stable isotope analysis of biomass carbon. Organic C and its isotopic composition were measured by continuous flow isotope ratio mass spectrometry (CF-IRMS) in biomass samples from either field or cultures, after carbonate dissolution in acid as explained above, using a Costech Elemental Analyzer, Thermo Condò II, and Thermo Delta plus Advantage mass spectrometers. Biomass samples were encased in analytical tin capsules, combusted at 1020 °C in a reactor packed with chromous oxide and silvered cobaltous oxide, with flash combustion occurred on injection of a pulse of oxygen at the time of sample drop. Under these conditions, the tin capsule ignites, raising the sample temperature to 1800 °C. Combustion products were sent into a copper reduction reactor, where incomplete combustion products (NOₓ, CO, etc.) were reduced and excess O₂ is removed. Water is then chemically scrubbed from the helium. The final product gas (CO₂) was separated on a 3-meter Porapak-Q packed gas chromatography column. Sample analysis was interspersed with replicates of three different laboratory standards to ensure

**Methods**

**Field samples.** Marine coastal endolithic communities were sampled from Isla de Mona (18.0867° N, 67.8894° W), a small, predominantly carbonate (Mona Dolomite and Lirio Limestone) island located 40 miles West of Puerto Rico. Samples
instrument precision. These laboratory standards had previously been calibrated against NIST Standard Reference Materials (USGS40 and USGS514). The long-term standard deviation was 0.02 mg C in 10 mg C. All final δ13C/δ12C values are expressed in permil (%), calculated using Eq. 1, where values are expressed relative to the international standard V-PDB (Vienna Pee Dee Belemnite). All permil error is expressed as sample s.d.

\[
\delta^{13}C_{\text{PDB}} (\text{permil}) = 1000 \left( \frac{R_{\text{sample}}}{R_{\text{PDB}}} - 1 \right)
\]

where \( R_{\text{PDB}} \) is the ratio of 13C/12C of the sample and \( R_{\text{PDB}} \) is the ratio of 13C/12C of Pee Dee Belemnite (0.0112372).

Stable isotope analysis of inorganic carbon. The δ13C content of all inorganic carbon (mineral and dissolved inorganic carbon) was determined after conversion to CO2 through acidic (H2SO4:H2O2; 20:1) digestion by addition of excess of phosphoric acid in He-flushed hermetically sealed vials, and incubation at 60 °C in a multiprep heating block. All sample inorganic carbon ends up in the headspace, where the CO2 δ13C is equivalent to that of the carbonate or dissolved CO2 from the headspace sampled using a Thermo GasBench, and δ13C values obtained in an interfaced MAT 253 mass spectrometer.

NanoSIMS imaging experiments. Two small (3 x 2 x 2 mm) calcite chips where incubated for one month with planktonic biomass to initiate light infestation (<5% of the surface area infested), then collected, brushed with sterile toothbrushes, and rinsed in sterile media two times, to remove “benthic” outgrowth, and then incubated again for 5 days to allow broken filaments to self-repair. One chip was then poiting in 5 ml hermetically sealed vials containing 4.5 ml of δ13C-enriched seawater medium (5479‰) and the other in another vial containing non-enriched medium. Two sets of two cultures were concurrently incubated as controls: the first set contained no inoculum, one vial with a chip and one vial without, the second set contained a planktonic inoculum with no chips, one in enriched medium and the other in non-enriched medium. Cultures were incubated under standard conditions for 31 days, after which headspace gas and liquid medium aliquots were analyzed by isotopy ratio mass spectrometry (IRMS) as described above. In addition, each chip (or planktonic biomass in the case of the control cultures) was removed, rinsed twice in degassed sterile Milli-Q water, then twice in sterile medium. The biomass in the chips at the end of the incubation typically covered 80% of the surface at a much higher population density than initially (around 3.4%). Because of this we calculate that the probability of having imaged a cell that was present in the inoculum before the addition of tracer was <1/1000, and that the probability of encountering four such cells successively at random (as we finally shown include the 1/1000) is the probability of encountering four such cells successively at random (as we finally shown include the 1/1000).

Statistics. To test significant differences of biomass fraction yields between differing dissolved Ci limitation treatments, multivariable analysis of variation (ANOVA) was used. Post-hoc comparisons among dissolved C, treatment and inoculation schemes was done by means of Tukey’s HSD for each dissolved C, and inoculum level. One-way ANOVA analysis was run for the comparisons of biomass fraction isotopic content. Statistical analyses were performed using IBM SPSS Statistics version 21 software.

Data availability. All data are available upon request from the authors in digital format.

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Additional information

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