

The gammaproteobacterium *Achromatium* forms intracellular amorphous calcium carbonate and not (crystalline) calcite

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Abstract

Achromatium is a long known uncultured giant gammaproteobacterium forming intracellular CaCO₃ that impacts C and S geochemical cycles functioning in some anoxic sediments and at oxic-anoxic boundaries. While intracellular CaCO₃ granules have first been described as Ca oxalate then colloidal CaCO₃ more than one century ago, they have often been referred to as crystalline solids and more specifically calcite over the last 25 years. Such a crystallographic distinction is important since the respective chemical reactivities of amorphous calcium carbonate (ACC) and calcite, hence their potential physiological role and conditions of formation, are significantly different. Here, we analyzed the intracellular CaCO₃ granules of *Achromatium* cells from Lake Pavin using a combination of Raman microspectroscopy and scanning electron microscopy. Granules in intact *Achromatium* cells were unequivocally composed of ACC. Moreover, ACC spontaneously transformed into calcite when irradiated at high laser irradiance during Raman analyses. Few ACC granules also transformed spontaneously into calcite in lysed cells upon cell death and/or sample preparation. Overall, the present study supports the original claims that intracellular Ca-carbonates in *Achromatium* are amorphous and not crystalline. In that sense, *Achromatium* is similar to a diverse group of Cyanobacteria and a recently discovered magnetotactic alphaproteobacterium, which all form intracellular ACC. The implications for the physiology and ecology of *Achromatium* are discussed. Whether the mechanisms responsible for the preservation of such unstable compounds in these bacteria are similar to those involved in numerous ACC-forming eukaryotes remains to be discovered. Last, we recommend to future studies addressing the crystallinity of CaCO₃ granules in *Achromatium* cells recovered from diverse environments all over the world to take care of the potential pitfalls evidenced by the present study.

KEYWORDS

ACC, intracellular biomineralization, micromanipulation, Raman

1 | INTRODUCTION

Achromatium oxaliferum has first been fully described by Schewiakoff (1893). Since then, it has been shown that the genus *Achromatium*

groups a diversity of giant colorless sulfur-oxidizing bacterial species which form intracellular elemental S⁰ and CaCO₃ granules (Gray & Head, 2014). They are widely distributed worldwide at the oxic-anoxic boundary in sediments of freshwater, brackish, and marine

environments (Ionescu et al., 2020; Salman et al., 2015). Since they can represent a relatively high biovolume, up to 90% of the total microbial volume in a sediment, they have received particular attention. No strain has been cultivated so far but culture-independent methods have been used to infer their physiology and ecology (e.g., Ionescu et al., 2017; Mansor et al., 2015). *Achromatium* cells have been reported to live in the sulfate reduction zone, sometimes in areas of low free sulfide activity, at least for freshwater representatives (Gray & Head, 2014). They oxidize sulfide to elemental sulfur using O_2 or possibly nitrates (Salman et al., 2015). Some populations have been reported to be autotroph for C while others might be organotroph (Gray et al., 1999). *Achromatium* cells may have a significant role in local geochemical cycles of Ca since they form large amounts of intracellular $CaCO_3$ granules. Moreover, they sometimes produce these granules in solutions undersaturated with all Ca-carbonate mineral phases. In these sediments, *Achromatium* cells are the sole carriers of Ca-carbonates (Gray & Head, 2014). Consistently, freshwater sediments from a wetland region close to Rydal Water (UK) have been reported to show a correlation between the solid-phase calcium content and *Achromatium* cell numbers (Head et al., 2000). Since the dynamics of the cells is associated with the dynamics of the oxic-anoxic boundary, it creates a unique connection between variations of redox conditions and carbonate formation.

The function of the intracellular $CaCO_3$ granules has been widely questioned. For example, it has been suggested that these intracellular Ca-carbonate granules may buffer intracellular pH variations associated with sulfur oxidation (e.g., Salman et al., 2015). This idea has been supported by the recent observations that these Ca-carbonate granules dissolved when cells were exposed to oxygen and sulfur oxidation was promoted (Yang et al., 2019). In contrast, Schorn et al. (2020) suggested that the $CaCO_3$ granules are located in the periplasmic space of *Achromatium* cells, therefore raising questions about this intracellular pH buffering function of $CaCO_3$. An alternative, suggested and also debated, function for $CaCO_3$ granules is that they may serve as ballasts in *Achromatium* and anchor cells to the sediment close to the oxic-anoxic boundary (Gray & Head, 2014).

All members of the *Achromatium* genus form intracellular Ca-carbonates, and it has long been thought that this biomineralization process was specific to this bacterial taxon. However, widespread and phylogenetically diverse cyanobacteria have been shown to be able to biomineralize intracellular Ca-carbonates as well (Benzerara et al., 2014; Couradeau et al., 2012). Li et al. (2016) showed that the intracellular Ca-carbonates are amorphous calcium carbonates (ACC) in all these cyanobacteria. Moreover, a magnetotactic bacterium affiliated to Alphaproteobacteria was recently shown to biomineralize intracellular ACC as well (Monteil et al., 2020). Last, many eukaryotes have also been shown to biomineralize ACC instead of crystalline calcite or aragonite, at least as a precursor phase (Addadi et al., 2003). Whether this capability to form intracellular $CaCO_3$ convergently appeared several times during evolution in these organisms or it involves homologous pathways (in connection with

e.g., C fixation) remains to be determined by future molecular biology approaches.

There are discrepancies in the literature about the nature of the intracellular Ca-carbonates in *Achromatium* (Table S1). These discrepancies started as early as *Achromatium* was discovered. Indeed, Schewiakoff (1893) followed by, for example, Virieux (1913) first suggested that intracellular granules were composed of calcium oxalate. This interpretation was based on the observation of recrystallized granules extracted from *Achromatium* cells by various reactants, which looked like the Ca-oxalates formed by plants. However, a few years later, West and Griffiths (1913), also using various chemical tests, concluded that the granules were calcium carbonates. At that time, they thought that they were analyzing cells belonging to a genus different from the one analyzed by Schewiakoff (1893), which they named *Hillhousia viirabilis*, but it was later shown to be *Achromatium* as well. Moreover, West and Griffiths (1913) mentioned that the $CaCO_3$ granules in the cells did not polarize and only crystallized when extracted from the cells. They therefore concluded that the calcium carbonate granules within the cells were in "a colloidal form" and different from the ones in dead organisms. Bersa (1926) reiterated similar experiments and again suggested that *Achromatium* granules were composed of amorphous $CaCO_3$. However, we noticed that authors have started to mention calcite instead of amorphous $CaCO_3$ at least since 1991 (e.g., Babenzien, 1991). Head et al. (1995) and Head et al. (2000) directly addressed the question of the crystallinity of *Achromatium* $CaCO_3$ granules by performing x-ray diffraction analyses of purified preparations of intact cells and suggested that the granules were calcite and not another structural form. Interestingly, Gray (2006) mentioned calcite but specified that this calcite may not be purely crystalline. More recently, Salman et al. (2015) analyzed Ca-carbonate granules in situ within *Achromatium* cells by Raman microspectroscopy and also concluded that they were calcite crystals. Consistently, most recent studies assumed that intracellular granules were calcite crystals based on the latest reports in the literature (e.g., Babenzien et al., 2015; Ionescu et al., 2017; Mansor et al., 2015; Schorn & Cypionka, 2018; Yang et al., 2019). Yet, the observations by West and Griffiths (1913) suggesting that *Achromatium* $CaCO_3$ granules are composed of ACC seem precisely documented. Moreover, the presence of intracellular ACC in cyanobacteria and the recently discovered magnetotactic alphaproteobacterium urges new efforts in characterizing the crystallinity of $CaCO_3$ granules in *Achromatium*. If there was a difference between *Achromatium* and other bacteria, this would suggest that specific mechanisms are involved in *Achromatium* and other bacteria in order to crystallize Ca-carbonate or preserve ACC from crystallization and they would need to be investigated in the future. Moreover, since ACC and calcite have different thermodynamic properties, they may hold different functional roles and/or the same role but with different efficiencies. Consequently, we re-investigated the nature of the $CaCO_3$ granules in *Achromatium* cells by conducting Raman analyses on *Achromatium* populations found in the sediments of Lake Pavin.

2 | MATERIAL & METHODS

2.1 | Site description and sample collection

Sediments were collected on the shore of Lake Pavin, Massif Central, France in November 2019. Sampling was achieved by filling one-liter glass bottles with 300–400 ml of sediments and 600–700 ml of water that overlaid the sediments. Air bubbles were excluded. Once in the laboratory, bottles were stored with their cap closed, under dim light and at room temperature (~25°C) from a few days up to a few months. The sampling location at Lake Pavin was 45.499107°N, 2.886273°E. No in situ measurement of O₂, sulfate, and sulfide profiles was conducted on these samples. However, a previous study showed that the oxic-anoxic boundary extended in the first five millimeters of the sediments (Monteil et al., 2020).

2.2 | Light microscopy observations, cell sorting and whole genome amplification (WGA)

Observation and micromanipulation were carried out on a 20- μ l drop of pore water harvested in the first millimeter below the interface water/sediments of a sample collected in Lake Pavin. Cell sorting was done with an InjectMan[®] NI2 micromanipulator and a CellTram[®] vario, hydraulic, manual microinjector from Eppendorf mounted on a Leica DM IL LED microscope equipped with a 63x/0.70 PH objective. The microscope and micromanipulator were placed inside a clean chamber exposed beforehand for 1 hr to ultraviolet germicidal irradiation (wavelength of the lamp: 254 nm). Single-cells with an ultrastructure and granules typical of *Achromatium* species were transferred two consecutive times with a sterile microcapillary into a 4- μ l drop of Lake Pavin water filtered at 0.2 μ m, before their final transfer into a 4- μ l drop of phosphate-buffered saline (PBS). This drop was stored at -20°C before WGA. To obtain sufficient gDNA for 16S rRNA gene sequencing, WGA was carried out using the multiple displacement amplification (MDA) technique with the REPLI-g Single-Cell Kit (QIAGEN) following the manufacturer's instructions.

2.3 | Cloning and sequencing of the 16S rRNA genes

The 16S rRNA gene was amplified using the Phusion[®] Hot Start Flex DNA Polymerase following the manufacturer's recommendations, a DNA template of 50–70 ng/ μ l and the 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGHTACCTTGTACGACTT-3' primers (Lane, 1991). Blunt-end fragments of 16S rRNA gene sequences were cloned using a Zero Blunt[®] TOPO[®] PCR Cloning Kit with One Shot[®] TOP10 chemically competent *E. coli* cells. The inserts were sent for sequencing (Eurofins Genomics Germany GmbH). Sequences were compared with data found in the NCBI nucleotide database using the Basic Local Alignment Search Tool. Sequences were deposited in the NCBI

Genbank database under the accession numbers MN990460 and MN990461.

2.4 | Phylogenetic analyses

The 16S rRNA gene sequences of representative type strains of all Thiotrichales (Gammaproteobacteria) families were downloaded from the public NCBI database (January 2020). This database was enriched with several sequences related to the *Achromatium* genus, some of the newly described candidate Thiotrichales species, and the sequences of the two Lake Pavin *Achromatium* OTUs (Pavin-1 and Pavin-2). The 52 sequences were aligned using MAFFT 7 (Katoh & Standley, 2013) and trimmed using Gblocks (Talavera & Castresana, 2007) to get a final alignment of 1,410 bp. A maximum-likelihood (ML) tree was built with RAxML 8.2.6 (Stamatakis, 2014) under the GAMMAI model of rate heterogeneity and the GTR substitution model. A total of 350 bootstrap replicates automatically determined by the MRE-based bootstopping criterion were conducted using the rapid bootstrapping algorithm, among which 100 were sampled to generate proportional support values. In this phylogeny, members of the Francisellaceae and Fastidiosibacteraceae families were used to root the tree based on previous phylogenies of the Thiotrichales order (Xiao et al., 2018).

2.5 | Preparation of cells for microscopy analyses

Lake Pavin *Achromatium* cells were sorted by micromanipulation and directly deposited on a glass coverslip (18 × 18 mm) coated with poly-L-lysine to improve cells' adhesion. Raman microspectroscopy analyses were conducted first with a systematic localization of the analyzed cells. The coverslip was then sputtered with carbon. Cells analyzed by Raman were relocated and further analyzed by scanning electron microscopy (SEM). We note that the assessment of CaCO₃ crystallinity was performed by Raman spectroscopy before C-sputtering, and therefore, we presently do not know if the later step may induce ACC crystallization. Moreover, air-drying of cells, exposition of cells to vacuum, and C-sputtering can cause cell damages, in particular flattening. However, in the present study only the features that were not affected (such as presence/absence of a cell wall) were investigated.

2.6 | Reference ACC synthesis and thermogravimetric analyses

ACC synthesis was carried out following the procedure described by Wang et al. (Wang et al., 2017). Fresh solutions of 100 ml of 50 mM CaCl₂ and 50 mM Na₂CO₃ (+0.5 g NaOH) were prepared with cold (4°C) MilliQ water. The CaCl₂ solution was placed in a 500 ml Teflon centrifuge bottle. A 30 × 30 cm², 75 mm thick polyethylene sheet was placed in the upper part of the centrifuge bottle with a steel ball

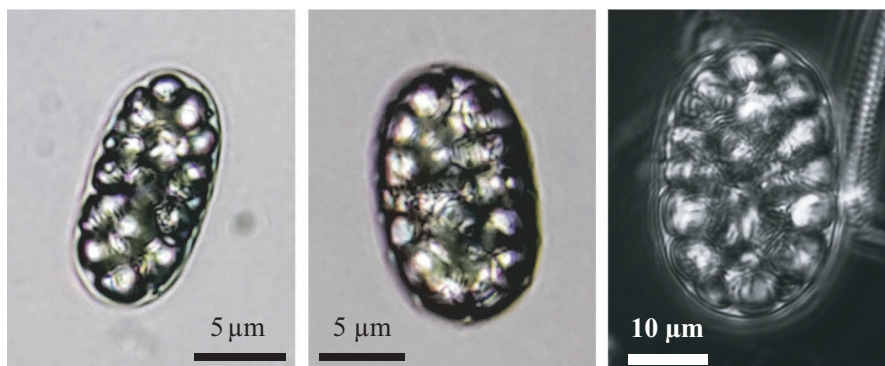


FIGURE 1 Light microscopy images of *Achromatium* cells isolated from Lake Pavin sediments

(1 cm in diameter, 5 g). The Na_2CO_3 -NaOH solution was poured into the polyethylene sheet, keeping both solutions separated. The bottle was then placed in a centrifuge (Beckman Coulter model Avanti J-20 XP) pre-cooled to 4°C, and it was spun at 3,656 g for 3 min. The force exerted by the ball on the polyethylene sheet breaks the sheet and throws the Na_2CO_3 -NaOH solution into the CaCl_2 solution extremely rapidly. The supernatant liquid was poured out, and the solid was rinsed with a few mL of dry cold (4°C) acetone. The suspension in acetone was filtered on a Buechner funnel, and the solid was recovered and dried at room temperature under vacuum for 4–5 hr.

Thermogravimetric analysis-differential scanning calorimetry (TGA-DSC) analyses of the reference ACC were performed using a METTLER TOLEDO® TGA/DSC3+. 8.6 mg of dry powdered sample was placed in an alumina crucible, and the sample was heated from 25 to 750°C under a 20 ml/min N_2 flow. The temperature scanning rate was set at 20°C/min. Noteworthy, TGA-DSC analyses could not be performed on natural samples due to limited amounts of sample.

2.7 | Raman microspectroscopy analyses

Raman spectra were recorded using a Renishaw InVia Reflex spectrometer equipped with a 532 nm Renishaw diode laser. The laser was focused on the sample using a DM2500 Leica microscope with a 100× objective (NA = 0.75) to obtain a planar resolution of $\sim 1 \mu\text{m}^2$. The maximum laser power was measured at ~ 90 mW under the microscope without the objective. The laser incident beam was polarized circularly by a quarter wavelength plate placed before the microscope. We selected several neutral density filters to acquire Raman spectra at varying laser powers from 0.1 up to 100% of the maximum laser power. Therefore, at 1%, the power delivered in a $1 \mu\text{m}^2$ spot was 900 μW and the irradiance was 9.10^8 W/m^2 . The signal was filtered by edge filters and dispersed by a diffraction grating with 2,400 grooves/mm, and the signal was analyzed with a RENCAM CCD (charge-coupled device) detector. Before each session, the spectrometer was calibrated in energy using a silicon standard. Spectra were collected using the software WIRE 4.3 provided by Renishaw.

It has been shown that Raman spectra of the different CaCO_3 crystalline polymorphs (calcite, aragonite, vaterite) are significantly different from each other and can therefore be used as unambiguous

signatures of these phases (Behrens et al., 1995). The ν_1 Raman bands of ACC and calcite were fitted with 2 Voigt functions using the FitViewer program, a Graphical User Interface (GUI) designed by Olivier Forni and Paolo Pilleri at the Institut de Recherche en Astrophysique et Planétologie (IRAP). The fit was performed with the MPFIT algorithm (Markwardt, 2009) which is based on the Levenberg-Marquardt method (Moré, 1978). As further described by (Vácz, 2014), the profile described by the Voigt function results from the convolution of a Lorentzian profile by a Gaussian function. The Gaussian profile was assumed to model the instrumental contribution, and its FWHM was fixed at 1.9 cm^{-1} , equal to the spectrometer bandpass as measured experimentally using the emission band of a Ne lamp. In turn, the Lorentzian function was assumed to model the Raman transitions, corrected from the instrumental response.

2.8 | Scanning Electron Microscopy

Scanning electron microscopy analyses were performed using a Zeiss ultra 55 field emission gun SEM. Secondary electron (SE2) images were acquired using an Everhart-Thornley detector at an accelerating voltage of 2.0 kV and a working distance of ~ 3.5 mm. Backscattered electron images were acquired for chemical mapping using an angle selective backscattered (AsB) detector at an accelerating voltage of 15 kV and a working distance of ~ 7.5 mm. Elemental maps were generated from hyperspectral images (HyperMap) by energy dispersive X-ray spectrometry (EDXS) using an EDS QUANTAX detector. EDXS data were analyzed using the ESPRIT software package (Bruker).

3 | RESULTS

Some pore water harvested from the first millimeters below the water/sediment interface was deposited on a glass slide and examined under the light microscope. Large cells were observed, measuring on average $22.3 \pm 6.7 \mu\text{m}$ in length and $14.2 \pm 4.0 \mu\text{m}$ in width (31 measurements), which corresponded to volumes between ~ 500 and $3,000 \mu\text{m}^3$ (Figure 1). They contained numerous intracellular inclusions measuring around $3 \pm 1.3 \mu\text{m}$ in diameter (18 measurements, 4 cells). Five cells were sorted, and the 16S rRNA gene of two of them

Bootstraps values

- 90%-100%
- 70%-89%
- 50%-69%

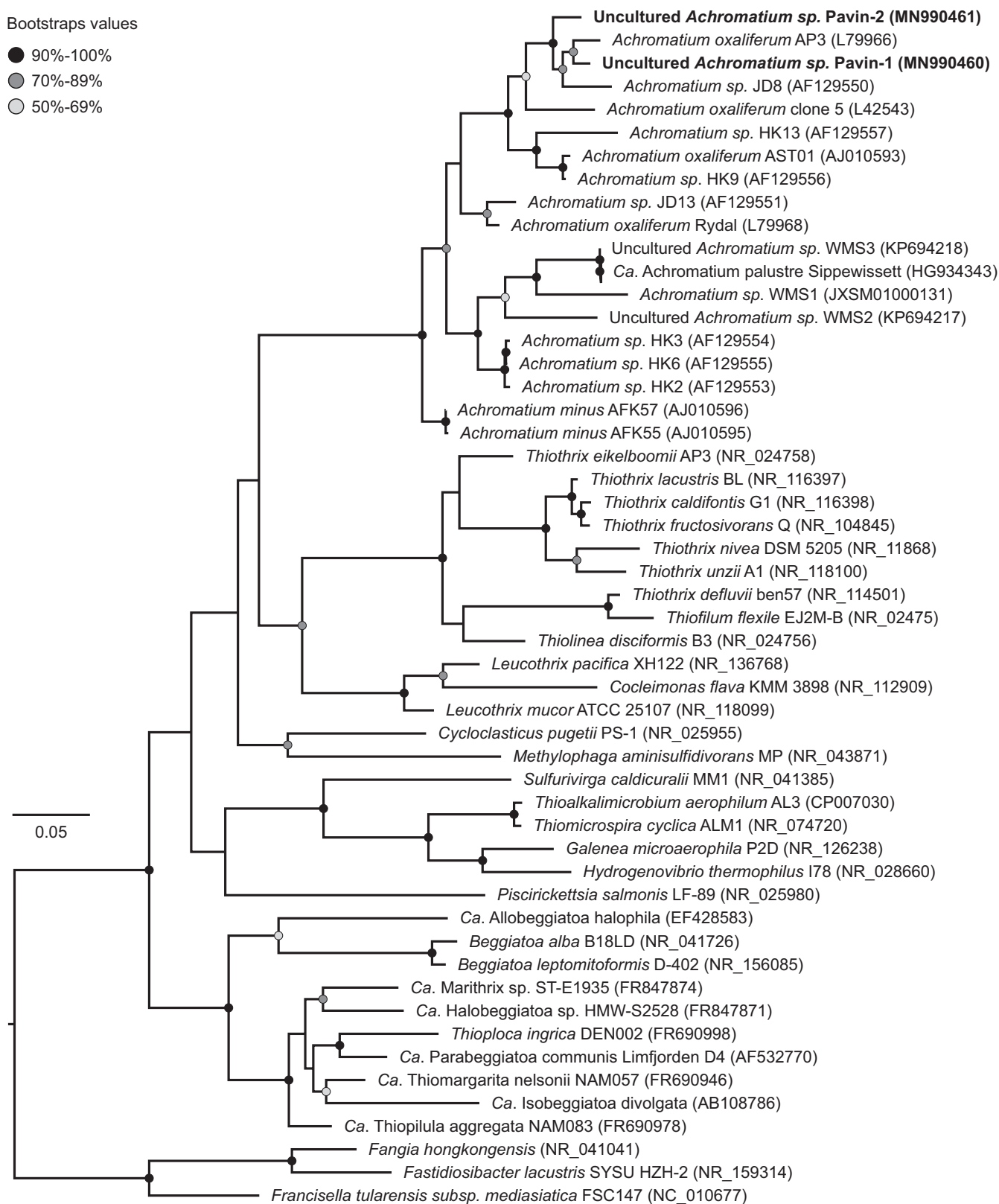


FIGURE 2 Maximum likelihood phylogenetic tree of the Thiotrichaceae and Piscirickettsiaceae families based on the 16S rRNA gene sequences showing the evolutionary relationships between the two CaCO_3 -accumulating isolates from Lake Pavin (Pavin-1 and Pavin-2) and their *Achromatium* relatives. The tree was built with the maximum likelihood method and the GTRGAMMA1 substitution model. *Fangia hongkongensis*, *Fastidiosibacter lacustris*, and *Francisella tularensis* subsp. *mediasiatica* were used as an outgroup. The tree was drawn to scale, and branches length represents the number of base substitutions per site. Only bootstrap values above 50% are shown and annotated to a circle

was successfully sequenced after whole genome amplification. Their 16S rRNA gene sequences clustered in the *Achromatium* cluster A (Figure 2) as defined by Mansor et al. (2015). They shared 96%–97% sequence identity with the *Achromatium* L79966 and AF129550 OTUs, sequenced from a wetland on the margins of Rydal Water and an upland tarn on Jenny Dam, respectively, both located in the United Kingdom and composed of freshwater as in Lake Pavin (Gray et al., 1999).

Moreover, *Achromatium* cells in Lake Pavin had a size range consistent with that of *Achromatium* cells previously observed in Rydal water (cell volumes between 1,000 and 30,000 μm^3) and the Jenny Dam (cell volumes between 150 and 26,000 μm^3).

Raman spectroscopy analyses were conducted on seven *Achromatium* cells sorted from the sediments by micromanipulation. Several spots were analyzed for each cell. Then, cells were systematically relocated and imaged by SEM and their elemental chemical composition mapped by SEM-EDXS.

One of the 7 cells showed a particular Raman spectrum with major peaks at 150.8, 219.7, and 473.5 cm^{-1} (Figure 3). This spectrum was identical to the spectrum referred to as biosulfur by Nims et al. (2019), consisting of elemental sulfur inclusions in bacteria. Consistently with what was reported by these authors, the three observed peaks were unambiguously assigned to asymmetric S-S bending, symmetric S-S bending and S-S bond stretching of a S_8 ring

structure, respectively. Consistently, SEM-EDXS mapping showed that this cell contained numerous S-rich inclusions (Figure S1).

On 5 cells out of 7, Raman spectra measured with 1% of the maximum laser power were similar to those shown in Figure 4. These spectra showed two sets of peaks: a first set comprised four peaks at 604, 749.4, 1,128, and 1,311.3 cm^{-1} . Based on Pätzold et al. (2008), these peaks were assigned to the ν_{24} , ν_{15} , ν_{22} , and ν_{21} modes of a porphyrin ring in cytochromes. Interestingly, these bands disappeared upon prolonged irradiation (Figure S2). The second set comprised a very broad peak, likely the convolution of two broad peaks, extending between 681 and 752 cm^{-1} and another broad peak centered, depending on the spectra, between 1,079.45 and 1,082.43 cm^{-1} . These peaks were identical to those of an ACC reference spectrum (Figure 4). The very broadband between 681 and 752 cm^{-1} was assigned to the in-plane bend (ν_4) of C-O bonds. The strong and broad peak at around 1,079.9 cm^{-1} corresponded to the symmetric stretching of C-O bonds in carbonate groups (ν_1 peak) in a CaCO_3 phase. This assignment to ACC was further supported by the absence of sharp peaks characteristic of lattice mode vibrations in crystalline CaCO_3 phases below 400 cm^{-1} . Instead, we observed a single broad bump between the cutoff at ~ 50 nm and ~ 400 nm (Figure 4). Accordingly, SEM-EDXS analyses showed that these cells were rich in Ca with a minor EDXS peak of Mg, the relative height of which varied from one cell to another (Figure S1).

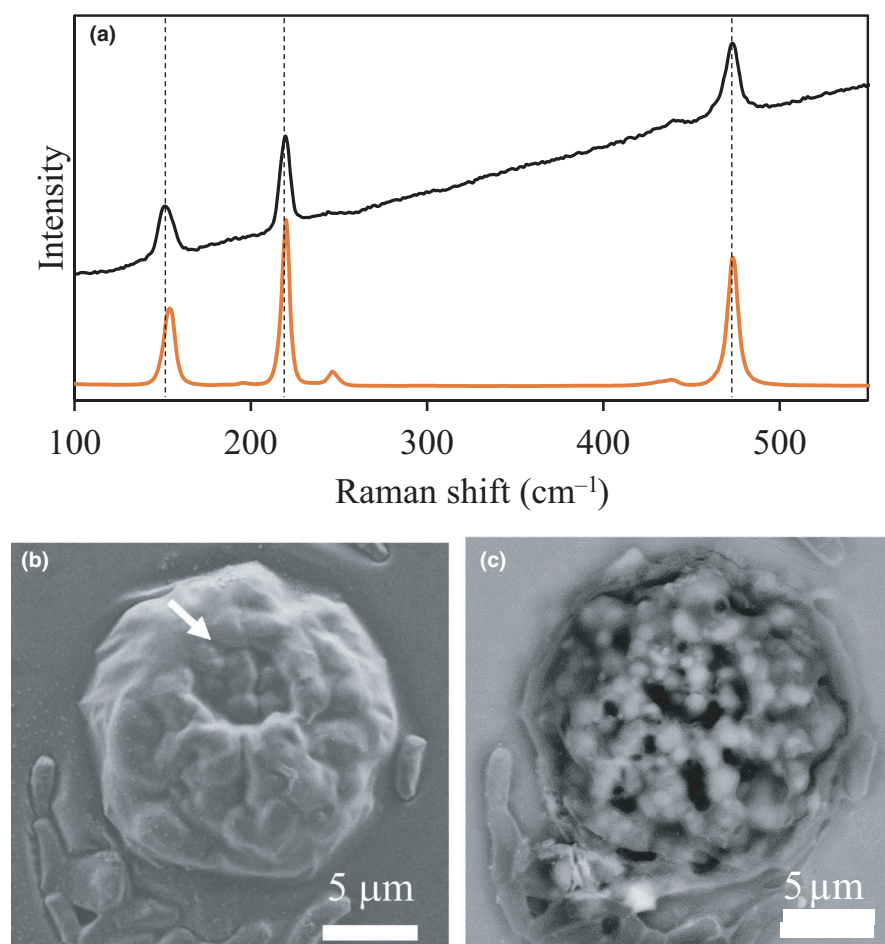


FIGURE 3 (a) Raman spectrum of an *Achromatium* cell with sulfur inclusions. The top black spectrum is obtained on the *Achromatium* cell shown in the SEM images. The bottom orange spectrum was issued from the RUFF database and was measured on elemental sulfur (#R040135). Vertical dashed lines indicate peaks at 150.8, 219.7, and 473.5 cm^{-1} . (b) SEM image of the analyzed *Achromatium* cell was measured in the secondary electron mode (SE2; left) with an accelerating voltage of 2 keV. The white arrow indicates the approximate location where a low power Raman analysis was performed. (c) SEM image of the same cell shown in (b), observed in the backscattered electron mode (ASB; right) with an accelerating voltage of 15 keV

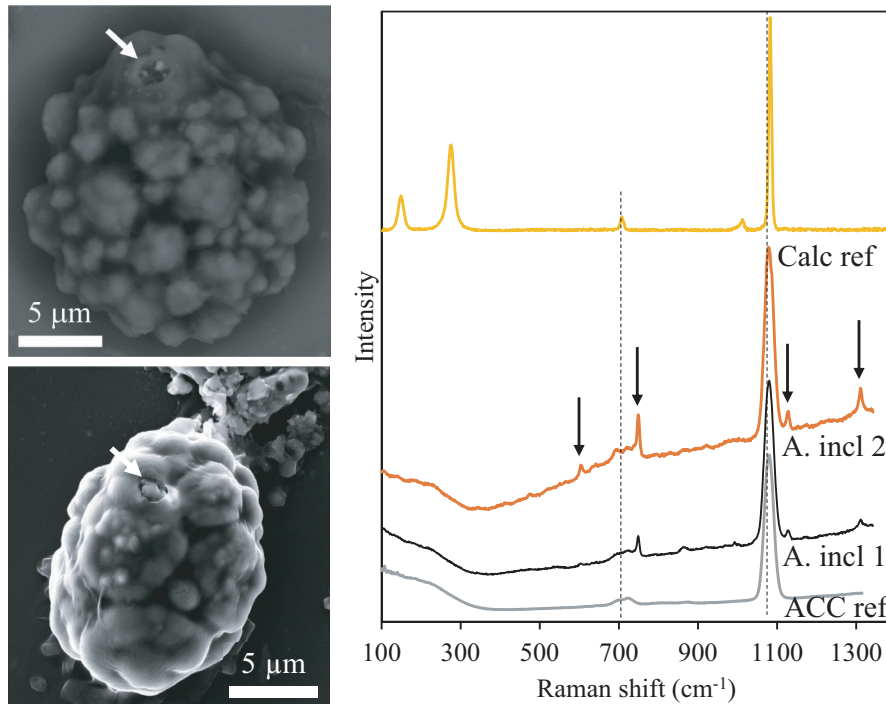
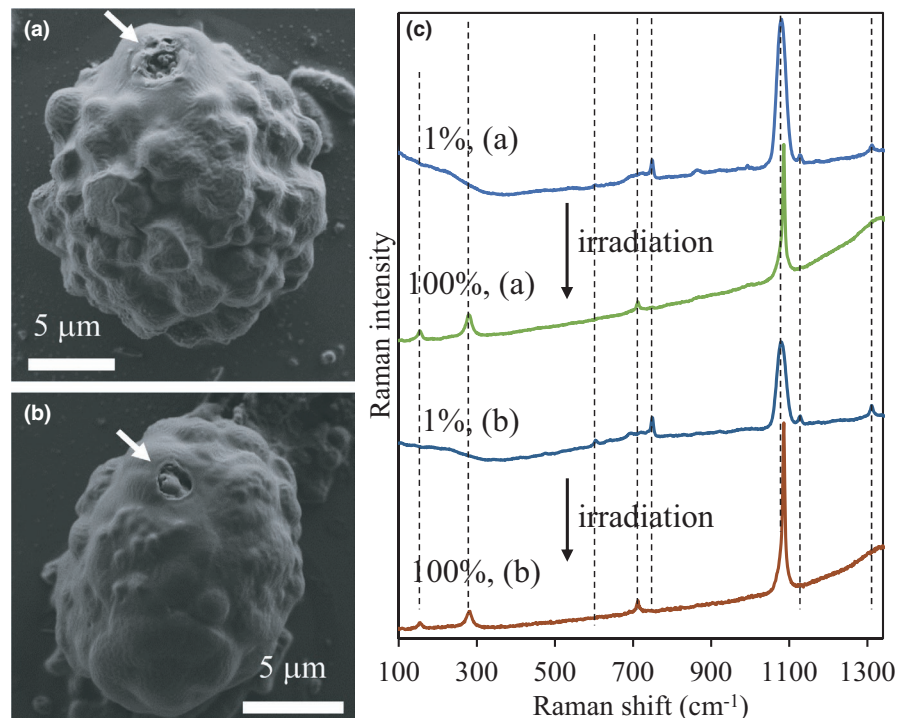


FIGURE 4 (Left) SEM images of the cells on which the Raman spectra were measured. White arrows indicate where full power Raman analyses were performed. (Right) Raman spectra. The two middle spectra (A. incl 1 and 2) are Raman spectra of CaCO_3 inclusions in *Achromatium* cells. The two dashed lines show (i) a broadband between 681 and 752 cm^{-1} , assigned to the in-plane bend (ν_4) of C-O bonds in ACC and (ii) a broadband around $1,079.9\text{ cm}^{-1}$ assigned to the symmetric stretching (ν_1) of C-O bonds in carbonate groups in ACC. Arrows show bands at 604.2 , 749.4 , $1,127.9$, and $1,311.3\text{ cm}^{-1}$, assigned to the ν_{24} , ν_{15} , ν_{22} , and ν_{21} modes of a porphyrin ring in cytochromes. The top spectrum (Calc ref) is a Raman spectrum of a reference calcite crystal retrieved from the RUFF database (#R040170). The bottom spectrum (ACC ref) is a Raman spectrum of a synthetic reference ACC particle

FIGURE 5 (a and b) SEM images acquired at 2 keV in the secondary electron mode showing the *Achromatium* cells analyzed by both Raman and SEM. Arrows show the irradiation spots where ACC transformed into calcite following full laser power irradiation. (c): Raman spectra obtained using 1% and 100% laser power on cells shown in (a) and (b). Dashed lines are located at ~ 154 , 280 , 604 , 712.2 , 749.4 , $1,079.9$, $1,127.9$, and $1,311.3\text{ cm}^{-1}$



When full laser power was used, the spectra measured on *Achromatium* intracellular granules changed and eventually showed very different spectral features (Figures 5 and 6). First, two Raman

bands appeared at ~ 154 and 280 cm^{-1} after irradiation. These bands were unambiguously assigned to the translation, and higher frequency libration modes in calcite and were due to collective

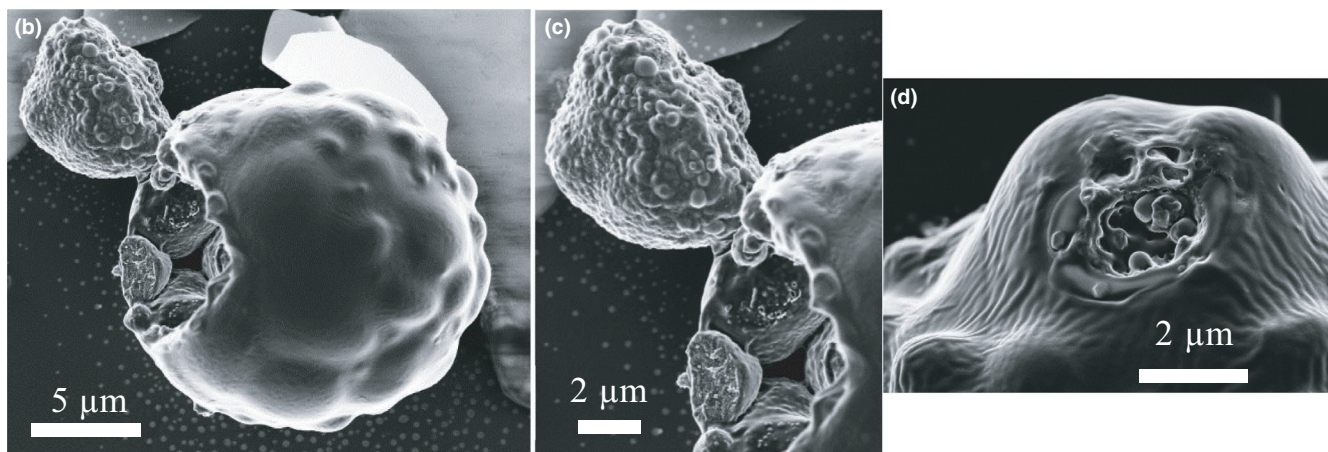
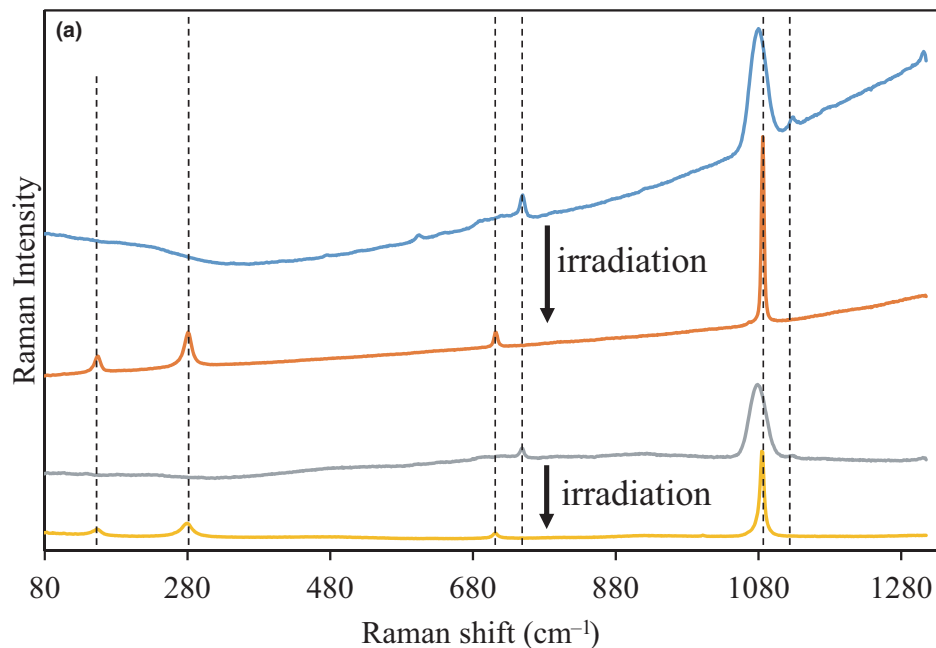


FIGURE 6 (a) Raman spectra of ACC granules in 2 *Achromatium* cells obtained by low laser power irradiation (blue and gray spectra), before transformation into calcite after full laser power irradiation (orange and yellow). Dashed vertical lines are located at ~ 154 , 280, 712.2, 749.4, 1,079.9, and 1,127.9 cm^{-1} . (b) SEM image obtained at 2 keV by the InLens detector. A fragment was partly detached from the cell on the top left after laser irradiation. (c) Close-up of the detached fragment showing the granular texture of calcite in the extruded fragment vs. smooth texture in ACC within the cells. (d) Close-up of the crater induced by laser in another *Achromatium* cell and showing the texture of the transformed area

synchronized vibrations of atoms around their equilibrium positions (Perrin et al., 2016). The very broad ν_4 band of ACC was replaced by a narrow band at 712.2 cm^{-1} that was consistently assigned to the ν_4 mode of calcite. The broadband at $\sim 1,079.9$ cm^{-1} of ACC was replaced by a narrower band at $\sim 1,086$ cm^{-1} , also consistently assigned to ν_1 in calcite. Last, the Raman bands at 604, 749.4, 1,128, and 1,311.3 cm^{-1} of cytochromes, associated with ACC, were absent from the calcite spectra. SEM pictures at low voltage of the cells where this transformation was observed showed that they were systematically enveloped by a cell wall, detected here as an envelope opaque to low energy electrons and therefore preventing the direct visualization of the granules inside the cells (Figures 4–6). In contrast, when the cell wall was disrupted, one could visualize directly the

intracellular CaCO_3 granules, as shown in Figure 6. Local damages induced by the laser spot could be observed as a crater measuring ~ 2 μm in diameter (Figure 5b). In one case, the cell partly disrupted and the irradiated calcite residue was extruded from the cell. The resulting byproduct showed a granular microtexture (Figure 6).

The ν_1 band of *Achromatium* CaCO_3 granules irradiated at low laser power (from 1% to 10% of the total power) and full laser power was systematically fitted. In most cases, a very good fit was obtained using one Voigt function but for several spectra measured after strong irradiation, the ν_1 band was clearly asymmetric and best fitted with two Voigt functions (Figure S3). When plotting the ν_1 band position vs. the full width at half maximum (FWHM) of the Lorentzian profile, a clear difference was observed between weakly

(0.1%–10% of the maximum laser power) vs. strongly (100% of the maximum laser power) irradiated granules, further highlighting the transformation of ACC into calcite upon irradiation (Figure 7). The FWHM for weakly irradiated synthetic and *Achromatium* ACC varied between 24.9 and 28.6 cm^{-1} and was positively correlated with the band position, which varied between $\sim 1,079.5$ and $1,082.4 \text{ cm}^{-1}$. In contrast, the FWHM for all strongly irradiated *Achromatium* ACC was between 21 down to 2.5 cm^{-1} which negatively correlated with the band positions, varying between $1,082.4$ and $1,086.3 \text{ cm}^{-1}$. The components with higher FWHM/lower position were minor fit components of asymmetric bands and were interpreted as poorly crystalline remnants mixed with more crystalline components in irradiated zones similarly to what was shown by Wehmeister et al. (2011) (Figure 7).

In the case of one *Achromatium* cell, the Raman spectrum measured with 1% of the maximum laser power was very similar to that of the most irradiated *Achromatium* granules and to that of calcite with bands at ~ 154 , 280, 712.2 and $1,086.2 \text{ cm}^{-1}$ (Figure 8). SEM observations performed at low voltage showed that this cell was devoid of a cell wall on the contrary to the cells containing ACC as mentioned before.

4 | DISCUSSION

4.1 | Precautions to be taken in order to assess the crystallinity of CaCO_3 in *Achromatium* cells

The present data clearly show that the CaCO_3 granules in Lake Pavin *Achromatium* intact cells are composed of ACC and not calcite. Only

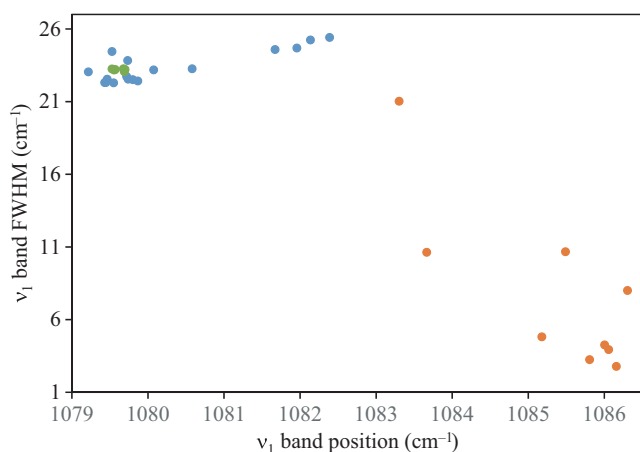


FIGURE 7 Scatter plot showing the full width at half maximum (FWHM) vs. the position for the ν_1 Raman band of the CaCO_3 phase. Blue dots correspond to Raman spectra measured on *Achromatium* CaCO_3 inclusions with reduced laser power (1 or 5%). Green dots correspond to Raman spectra measured on synthetic reference ACC grains with reduced laser power (1 or 5%). Orange dots correspond to Raman spectra measured on *Achromatium* CaCO_3 inclusions with full laser power and showing signs of crystallization

one cell contained calcite granules but SEM observations suggested that this cell was damaged since it did not show any cell wall as discussed below. Several studies have detailed how Raman spectroscopy could unambiguously discriminate between ACC and calcite (e.g., DeCarlo, 2018). This includes the presence (in calcite)/absence (in ACC) of lattice modes at ~ 154 and 280 cm^{-1} ; a sharp (calcite) vs. wide (ACC) ν_1 peak. The latter feature has been explained as the result of a high variability in the bonding environment around the carbonates in ACC which causes deviations in the lengths of C–O bonds. Last, a shift in the ν_1 peak position from lower (ACC) to higher (calcite) wavenumbers is observed. Wang et al. (2012) and Perrin et al. (2016) showed that variations in the Mg content of ACC and calcite could change in a correlated manner the FWHM and position of the ν_1 peak. Accordingly, we observed in our study some correlated variations in the FWHM and peak position of the ν_1 peak in ACC granules, with variation of FWHM in the 22.5 – 25.4 cm^{-1} range, which according to Wang et al. (2012) may be explained by variations in their Mg contents in the 0%–10% range. Perrin et al. (2016) also noticed that (a) the FWHM of calcite and ACC, whatever their Mg content, did not overlap, and (b) ν_1 wavenumbers lower than $1,086 \text{ cm}^{-1}$ and ν_1 FWHM greater than 22 cm^{-1} were two unambiguous criteria to determine the amorphous nature of calcium carbonate. Therefore, it can be confidently concluded that Raman spectra collected on most Lake Pavin *Achromatium* cells were characteristic of ACC.

This conclusion fully agrees with the early studies by West and Griffiths (1913) who suggested that intracellular granules in

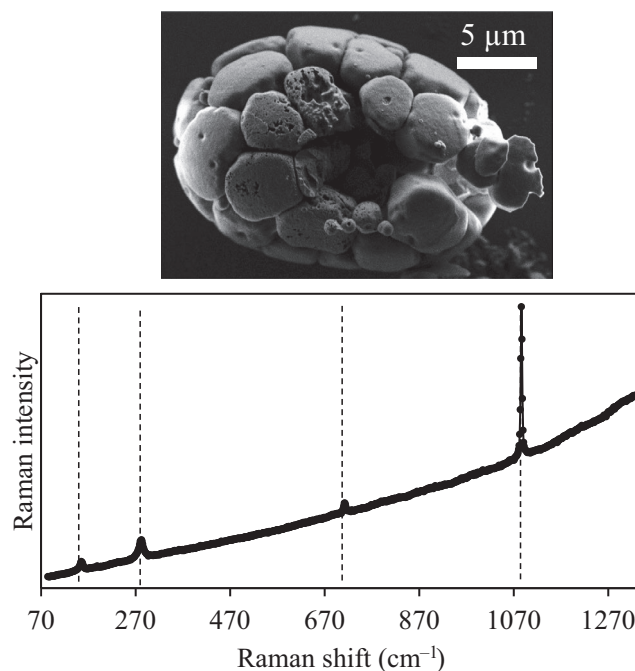


FIGURE 8 (a) SEM images acquired at 2 keV in the secondary electron mode showing an *Achromatium* cell analyzed by both Raman at 1% laser power and SEM. (b): Raman spectrum obtained using 1% laser power on the cell shown in (a). Dashed lines are located at ~ 154 , 280, 712.2, and $1,086.2 \text{ cm}^{-1}$ and underline bands that are characteristic of calcite

Achromatium were composed of a colloidal form of CaCO_3 and later on by Bersa (1926) who mentioned amorphous CaCO_3 ("amorphen (kolloidalen) Form" in their manuscript). It also better explains Yang et al.'s (2019) FIB observations of composite structure of the granules with submicrometer laminations, which would be difficult to explain within a single crystal, as mentioned by the authors themselves. Similar laminations in ACC granules, attributed to chemical variations, were observed by TEM by Martignier et al. (2017) and Cam et al. (2016).

We note that several artefacts may lead to an erroneous assessment of the crystallinity of *Achromatium* CaCO_3 granules. First, in a population of *Achromatium* cells, some ACC granules may transform into calcite because of, for example, cell death and/or sample preparation. Indeed, it has been emphasized by many authors (e.g., Gower, 2008; Politi et al., 2006) that ACC is usually unstable and often converts to a crystalline phase during sample preparation of biominerals for ex vivo examinations. Consistently, a spontaneous transformation of ACC into calcite was documented by the early observations by West and Griffiths (1913) and Bersa (1926) when they killed the cells by adding alcohol or drying. Schorn et al. (2020) observed dissolution of *Achromatium* ACC granules when they treated cells with acids. In the meantime, Schorn et al. (2020) sometimes evidenced some crystallization when killing them with no acidification of the solution. In the present study, while we observed that simple drying does not induce crystallization of ACC, we observed one cell containing calcite. On the contrary to other cells, this one lacked a cell wall. We cannot affirm whether the cell wall was already disrupted in the sample before micromanipulation or if it occurred upon sample preparation but as mentioned above, this explains why ACC granules transformed into calcite in this cell before Raman analyses.

Second, in addition to sample preparation, irradiation at too high laser power can also cause ACC crystallization as shown in the present study. This transformation may result from the local heating of ACC in the laser spot over $\sim 330^\circ\text{C}$, a temperature at which the solid–solid transformation of ACC into calcite is detected by thermogravimetric analyses (Figure S4). Such a local increase of T may result from a high absorption of light by, for example, cytochromes, which contribute to the Raman signal of *Achromatium* cells and efficiently absorb visible light at ~ 530 nm (Okada et al., 2012) and a slow dissipation of the heat in the material (Fau et al., 2019). As shown by Fau et al. (2019), a more precise assessment of the temperature increase induced by laser heating would require the knowledge of the optical penetration depth and thermal penetration depth in organics-containing ACC granules, which we do not know yet.

An alternative interpretation of these observations could consist in suggesting that only a surficial layer of the *Achromatium* granules was illuminated and therefore heated by the laser. This surficial layer would be an ACC layer covering an initially unseen calcite core. This initially unseen calcite core may then become apparent after volatilization of the ACC layer. However, this interpretation is unlikely for two reasons. First, this would mean that ACC is quite opaque. While we do not know the value of the optical penetration depth as mentioned above, Schmidt and Wagermaier (2017) reported that cystoliths in plants, composed of ACC and measuring $40\text{--}60$ μm

function as light scatterers due to their transparency to visible light. This suggests that green Raman light likely propagates throughout the $3\text{--}4$ μm wide ACC granules of *Achromatium*. Second, if a surficial layer of ACC was volatilized (after crystallization into calcite at $\sim 330^\circ\text{C}$), this would produce CaO, which we did not detect by Raman.

Overall, the high susceptibility of ACC to transform into calcite upon cell death and/or sample preparation and/or laser irradiation concurs to the assumption made by Gower (2008) that this explains why it took so long to discover ACC although it is common. This applies well to *Achromatium* too. Two studies produced data suggesting a calcitic nature of the CaCO_3 granules in *Achromatium* using bulk x-ray diffraction (Head et al., 2000) and Raman spectroscopy (Salman et al., 2015). However, XRD is much more sensitive to calcite than ACC and therefore even in a bulk sample where most of the cells contain ACC and only a few dead ones contain calcite, the latter signal would show up. Salman et al. (2015) analyzed *Achromatium* cells by Raman spectroscopy and obtained one spectrum typical of calcite. However, they did not mention the use of neutral density filters and may have acquired Raman spectra at full laser power. Alternatively, morphologically and genetically distinct subpopulations of *Achromatium* have been evidenced in several localities, although this idea was challenged by recent studies that instead suggested variations in gene expression across varying environments (Ionescu et al., 2020). One may postulate that some *Achromatium* populations may produce calcite instead of ACC. Moreover, the *Achromatium* populations studied by Salman et al. (2015) thrived in a salt marsh which is chemically very different from the freshwater of Lake Pavin. While it is not clear how salinity may directly impact the stability of ACC, it is possible that environmentally driven variations in ion homeostasis in cells result in different chemical environments around CaCO_3 granules. Similarly, one may wonder if *Achromatium minus* thriving in the more acidic lake Fuchskuhle (pH = 4.2–4.6) (Glöckner et al., 1999) may form calcite instead of ACC. Overall, only future systematic studies will be able to decipher if intracellular CaCO_3 granules in *Achromatium* living cells are always composed of ACC or sometimes calcite. The present study offers guidelines to avoid potential artifacts including the use of low Raman laser irradiation (preferably < 1 mW for a 532 nm laser) and then SEM to verify that the cell wall is still enveloping the cells. It is possible that air-drying induces some damages in some cases and that high-pressure freeze-substitution sample preparation may overcome this issue (Blondeau et al., 2018) but this will have to be tested by future studies.

4.2 | What makes bacteria producing intracellular ACC instead of intracellular calcite?

ACC is a notoriously unstable phase (e.g., Addadi et al., 2003; Cavanaugh et al., 2019). Therefore, it may appear surprising that *Achromatium*, a magnetotactic alphaproteobacterium and some cyanobacteria, manage to preserve CaCO_3 as ACC intracellularly and

that it does not transform into a crystalline phase such as calcite. In any case, the mechanism involved in this stabilization appears to no longer operate as soon as the cells lyse/die explaining why in this case ACC spontaneously crystallizes as reported here and by previous studies.

Laboratory biomimetic experiments showed that several chemical species can stabilize ACC at least transiently, such as magnesium ions, triphosphate ions, polymers such as diphosphate-substituted poly(ethylene glycol) and polyaspartate, polyphosphonates, some oligosaccharides, or amino acids (Addadi et al., 2003; Zou et al., 2020). It is clear that at least some of these chemical species are abundant within the cell environment and may, therefore, play a role in ACC stabilization. However, how they may degrade and hence stop from stabilizing ACC when the cells disrupt remains to be understood. Alternatively, the stabilization of ACC may well be explained by confinement as demonstrated by, for example, Zeng et al. (2018) and Cavanaugh et al. (2019). Based on microfluidics experiments, Cavanaugh et al. (2019) concluded that ACC is the expected byproduct of intracellular biomineralization occurring within a micrometer-sized compartment when the solution in this compartment is supersaturated with ACC. Moreover, they predict that ACC may remain stable for durations much longer than the lifetime of the organisms, that is, months before 1% of crystallization occurs in a $10 \mu\text{m}^3$ vesicle and hundred years before 99% of crystallization occurs in the same volume. According to Jin et al. (2018), confinement can be seen as a barrier to water diffusion, which hinders the dissolution of ACC and reprecipitation as calcite. In cyanobacteria forming intracellular ACC, it has been shown that this confinement may be provided by an organic envelope of a yet-unknown composition (Blondeau et al., 2018). We presently do not know the genes or proteins involved in the formation of this organic envelope. In *Achromatium* and the recently discovered magnetotactic alphaproteobacterium, it has been suggested that ACC granules are confined by lipid bilayers (Gray & Head, 2014; Monteil et al., 2020). It can be speculated that such envelopes may also control the maximum size of the granules as observed for example in magnetotactic bacteria and prevent their agglomeration but this will need further assessment in the future. Cavanaugh et al. (2019) noticed that when the confinement was broken, the exposition of ACC to heterogeneous nucleators induced rapid crystallization. Moreover, Liu et al. (2020) explained how this transformation of ACC into calcite by dissolution-reprecipitation could preserve the morphology of the granules by occurring without the need to nucleate a separate crystal. How the envelopes of ACC granules in cyanobacteria and *Achromatium* rupture will need to be further studied. For this purpose, TEM appears as the choice method since Raman microspectroscopy does not offer a spatial resolution high enough to detect such an organic envelope.

Overall, intracellular compartmentalization by lipid bilayers or proteins seems required to explain the formation and stabilization of ACC. This contradicts the recent suggestion by Schorn et al. (2020) that CaCO_3 in *Achromatium* cells would be in contact with the extracellular environment and form in pockets delimited by an

invagination of the cytoplasmic membrane and would, therefore, be periplasmic. We note that these conclusions by Schorn et al. (2020) were not derived from direct observations of the ACC granules within these pockets by, e.g., TEM tomography. Instead, they were postulated based on (a) the idea that the easy loss of CaCO_3 upon various physico-chemical treatments is consistent with the fact that they would not be membrane enclosed; and (b) the fact that fluorescein, a hydrophilic dye that does not penetrate membranes, did penetrate inside cavities hosting CaCO_3 . However, several other facts may oppose Schorn et al. (2020) conclusion. First, ACC granules in cyanobacteria are also easily and rapidly lost upon various physico-chemical treatments and yet they clearly form within intracellular enclosed compartments (Blondeau et al., 2018). The chemicals used by Blondeau et al. (2018), that is, ethanol which is membrane permeable, are not the same as those used by Schorn et al. (2020), that is, HCl, which is membrane impermeable. Yet both chemical may harm/stress the cells and induce intracellular changes. Second, Bentov et al. (2009) similarly observed that calcein, another membrane-impermeable dye as these authors underline it, did stain the truly intracellular CaCO_3 of foraminifera. Last, the suggestion that CaCO_3 in *Achromatium* would be in direct contact with the extracellular environment is inconsistent with the report of ACC formation by some *Achromatium* cells in environmental solutions undersaturated with ACC (Gray & Head, 2014).

4.3 | Implications of an ACC instead of a calcitic composition of intracellular granules

There are several important ecological, geological, and ultrastructural implications in reporting that *Achromatium* intracellular granules are composed of ACC and not calcite.

First, several studies have discussed the potential function(s) of *Achromatium* CaCO_3 granules (e.g., Gray & Head, 2014; Salman et al., 2015; Schorn et al., 2020; Yang et al., 2019). The dominant view emerging lately suggests that these granules buffer intracellular pH, which may otherwise vary due to the redox transformations of S species. It has been noted that for this purpose the cells need to quickly adjust their CaCO_3 content to rapidly changing environmental conditions, such as exposure to O_2 (Yang et al., 2019). We argue that this can be better explained by ACC than calcite. Addadi et al. (2003) and Weiner et al. (2005) consistently suggested that ACC is a widespread biomineral phase and holds a homeostatic function in eukaryotes which is facilitated by its metastability. Moreover, while the reactivity of calcite and ACC (e.g., the kinetics of precipitation or dissolution) depends on prevailing chemical conditions, it has been shown that under given conditions, ACC reactivity might be higher, partly because of a higher surface area (Lassin et al., 2018). Altogether, these arguments support the idea that ACC may respond more quickly to chemical changes than calcite and therefore fill such a buffering function with a higher efficiency.

A second implication results from the solubility difference between ACC and calcite. Although there remains some uncertainty

about the exact value of the solubility of ACC, it is much higher, at least one order of magnitude than that of calcite (Brečević & Nielsen, 1989; Carino et al., 2017; Purgstaller et al., 2019). Solubility of ACC is even higher when its Mg content increases (Purgstaller et al., 2019). This means that ACC precipitation requires a higher activity of Ca^{2+} and/or a higher activity of HCO_3^- and/or a higher pH within the compartment where it forms. It also means that ACC starts dissolving under conditions where calcite would still be stable. The chemical composition inside the compartments where granules form is not known but future modeling efforts aiming at inferring the chemical conditions prevailing in these compartments should take into account the fact that these granules are composed of ACC and not calcite.

As a third implication, Bolze et al. (2002) reported densities of 1.62 and 2.75 g/cm^3 for hydrated ACC and calcite, respectively. Fernandez-Martinez et al. (2013) measured a density of 2.18 g/cm^3 for dry ACC powder which remains significantly lower than that of calcite. This means that when ACC crystallizes to calcite, this represents a volume variation between 20% to 40%. This suggests that when ACC crystallizes to calcite in dying cells, one should expect a reduction in size of the granules, unless further *post-mortem* crystal growth occurs. Moreover, it has been hypothesized by some authors that intracellular granules in *Achromatium* are used to regulate buoyancy of the cells (Gray, 2006). While ACC-filled cells remain denser than ACC free cells, they are lighter than calcite-filled cells, and therefore, this role for increasing cell buoyant density would be less efficient with ACC than calcite. Last, Mansor et al. (2015) noted that the precipitation of CaCO_3 within cells generates H^+ and assumed that these protons may be used to generate ATP. They used for their calculations the density of calcite to estimate the average amount of Ca stored in cells which they equaled to the amount of CO_3^{2-} stored into the granules and from there inferred the equivalent amount of generated protons. Here, we note that these calculations should be revised; using ACC density will decrease these numbers by at least 20%–40%. We also note an additional difficulty for these calculations: first Mg substitution should be taken into account since it impacts the density of the ACC phase. Then, on the contrary to calcite, the $\text{Ca}:\text{CO}_3^{2-}$ stoichiometry is not always 1:1 in ACC as there can be some HCO_3^- molecules as well, the proportion of which may depend on pH and temperature conditions (Carino et al., 2017). Those should not be counted to infer the equivalent proton amount. The advent of future experimental approaches, for example, measuring intracellular ATP *in vivo* will be essential to test these calculations.

Last, as mentioned above, the stability of ACC is lower than that of calcite. Therefore, even if ACC granules remain confined after the death of the cells, one may not expect to find genuine ACC in the geological record. Indeed, Cavanaugh et al. (2019) showed that confined ACC granules measuring $\sim 1 \mu\text{m}^3$ would be preserved from crystallization up to about one million years. Therefore, a fossil of an ACC granule would be a crystalline phase, most likely calcite. In order to understand how to look for some potential traces of these

ACC granules in the geological record, it will be important to decipher in the future: (a) what conditions are prone to crystallization of ACC in calcite; (b) if the morphology of ACC granules is always preserved upon crystallization; and (c) if the crystallized granules replacing ACC store some organic molecules that might be present in ACC granules and/or if a particular trace element composition is preserved.

5 | CONCLUSION

We find robust evidence that cells of the giant gammaproteobacterium *Achromatium* sp. in Lake Pavin form intracellular ACC and not calcite. In contrast, previous reports of calcite may have been affected by the high instability of ACC, which transforms readily into calcite upon some sample treatments, which alter the integrity of the cells, or strong laser irradiation. Raman microspectroscopy seems to be the most appropriate tool to infer the ACC nature of these inclusions since analyses can be conducted under native conditions. However, special care has to be taken to minimize irradiation delivered to the cells and check that cells are still covered by a wall. Moreover, we advocate that future studies should prefer the term CaCO_3 to calcite or ACC if they did not characterize the crystallinity of the intracellular granules. Reporting that ACC instead of calcite is the constituent of CaCO_3 granules in *Achromatium* cells does not enlighten the mechanisms of formation of these granules. However, it sets new constraints on them such as the requirement of a confinement and the need for a higher saturation level of the solution in which CaCO_3 forms. It also modifies the quantitative impact of these biominerals on the physiology of the cells. Last, Jin et al. (2018) highlighted that ACC is widespread in biomineralizing eukaryotes. Here, we show that this is also the case in prokaryotes since the presently three known cases of intracellular biomineralization in bacteria, i.e. diverse cyanobacteria, some magnetotactic Alphaproteobacteria, and *Achromatium*, all form ACC.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in zenodo at <http://doi.org/10.5281/zenodo.4295976>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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