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1 **Detection of biogenic amorphous calcium carbonate (ACC) formed by bacteria using FTIR**
2 **spectroscopy**

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19 **Abstract:**

20 While the formation of intracellular amorphous calcium carbonate (ACC) by living organisms is
21 widespread, its detection in prokaryotes remains difficult owing to its susceptibility to transform
22 or dissolve upon sample preparation. Because of these challenges, a large number of ACC-
23 forming prokaryotes may have been undetected and their abundance in the natural environment
24 is possibly underestimated. This study identifies diagnostic spectral markers of ACC-forming
25 prokaryotes that facilitate their detection in the environment. Accordingly, ACC formed by
26 cyanobacteria was characterized using Fourier transform infrared (FTIR) spectroscopy in near-IR,
27 mid-IR, and far-IR spectral regions. Two characteristic FTIR vibrations of ACC, at $\sim 860\text{ cm}^{-1}$ and
28 $\sim 306\text{ cm}^{-1}$, were identified as reliable spectral probes to rapidly detect prokaryotic ACC. Using these
29 spectral probes, several *Microcystis* strains whose ACC-forming capability was unknown, were
30 tested. Four out of eight *Microcystis* strains were identified as possessing ACC-forming capability
31 and these findings were confirmed by scanning electron microscopy (SEM) observations. Overall,
32 our findings provide a systematic characterization of prokaryotic ACC that facilitate rapid detection
33 of ACC forming prokaryotes in the environment, a prerequisite to shed light on the role of ACC-
34 forming prokaryotes in the geochemical cycle of Ca in the environment.

35 **Keywords:** Amorphous calcium carbonate; FTIR; cyanobacteria; ACC; calcium

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38 **1. Introduction**

39 Compared to the crystalline polymorphs of calcium carbonate (CaCO_3) such as calcite,
40 vaterite and aragonite, amorphous calcium carbonate (ACC) is more soluble and therefore fairly
41 unstable except in several biotic cases¹. Biogenic ACC was first documented in eukaryotic
42 organisms such as mollusks, sea urchins, sponges, ascidians, and crustaceans, where ACC acts as
43 a precursor phase for the formation of calcite and aragonite minerals in skeletal architectures¹⁻⁶.
44 Several studies have shown that diverse prokaryotic organisms are also capable of forming ACC.
45 For example, the large uncultured sulfur bacterium *Achromatium oxaliferum*, widely distributed
46 at the oxic-anoxic boundary in sediments of freshwater, brackish, and marine environments, was
47 shown to form numerous intracellular ACC inclusions filling most of the cell volume⁷. Recently,
48 a magnetotactic bacterium affiliated to the Alphaproteobacteria was shown to form intracellular
49 ACC⁸. The repertoire of prokaryotes forming ACC also includes numerous phylogenetically
50 diverse cyanobacteria^{9,10}. Detection of biogenic ACC in cyanobacteria was particularly
51 interesting because they are the only known ACC-forming prokaryotes that are culturable in the
52 laboratory. These strains have the highest Ca demand among all cyanobacteria, hypothesized to
53 be driven by their need to form intracellular ACC, which suggests a biological function of ACC.
54 This role possibly consists in an intracellular pH buffer or a storage form of inorganic carbon
55 and/or Ca^{11,12}. The formation of ACC by bacteria has significant geochemical implications. First,
56 intracellular ACC inclusions formed by some strains were shown to be associated with their
57 capability to strongly accumulate radioactive alkaline earth elements such as ⁹⁰Sr and ²²⁶Ra,
58 offering some potential for remediating pollutions by these elements¹³. Moreover, some strains
59 of ACC-forming cyanobacteria are shown to sequester high concentrations of alkaline earth
60 elements such as Ba and Sr. This raises questions about the current understanding of the

61 biological role of Ba/Sr in cyanobacteria and, in reverse, the role of these cyanobacteria in the
62 geochemical cycling of Ba and Sr, especially in environments where they might be abundant^{11,14-}
63 ¹⁶. The biogeochemical implications of bacterial ACC are not only limited to cyanobacteria.
64 Owing to the exceptional size of *A.oxaliferum*, often greater than 17 μm in length and 10 μm in
65 width, ACC within *A. oxaliferum* cells can represent most of the solid calcium present in some
66 sediments¹⁷.

67 Despite the increasing number of reports about ACC-forming prokaryotes and their
68 biogeochemical significance, detection of ACC in prokaryotes remains a difficult task. Pure
69 abiogenic ACC is highly unstable, transforming within minutes to crystalline calcium carbonate
70 polymorphs such as calcite, or aragonite¹⁸. In contrast, ACC found in prokaryotes remains
71 relatively stable intracellularly with no obvious spontaneous transformation to crystalline phases
72 such as calcite. The mechanisms underlying such an unprecedented stability of biogenic ACC
73 remain unknown. Various organic or inorganic additives (Mg, P) and/or confinement of ACC
74 within small volumes, have been shown to stabilize ACC¹⁹⁻²². This stabilization process is
75 interrupted upon cell lysis, or cell death, which therefore induces ACC loss. Techniques such as
76 scanning electron microscope (SEM) with energy dispersive X-ray spectrometry (EDXS),
77 transmission electron microscope (TEM) with EDXS, and Raman microspectroscopy (but not for
78 ACC in autofluorescent cyanobacteria) have been successfully used to detect ACC in
79 bacteria^{23,24} (Table 1). While these techniques are interesting as they provide information at the
80 single-cell scale, their use is time-consuming, which can become prohibitive when several
81 samples (e.g., a screening of numerous bacterial strains) require a preliminary confirmation of
82 the presence or absence of ACC, and when a more global average view of the ACC content of a

83 sample is needed. Moreover, it has been shown that bacterial ACC can be lost and/or
 84 transformed upon some sample preparation protocols (Table 1)^{8,23}. Because of these challenges,
 85 a large number of ACC-forming prokaryotes may have remained undetected and their abundance
 86 in the natural environment possibly underestimated. Overall, there is thus a need for the use of
 87 techniques allowing efficient detection of ACC in these complex samples with minimal sample
 88 preparation.

Table 1: Summary of techniques used for characterization of ACC in bacteria and their limitations

Technique	Limitation
Scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDXS) ^[23]	ACC prone to loss upon fixation during sample preparation; time consuming to derive a global average view; no discrimination between crystalline and amorphous phases
Transmission electron microscopy (TEM-EDXS) ^{[23], [8]}	ACC prone to loss upon fixation and sample preparation; time consuming to derive a global average view
Raman microspectroscopy ^[24]	may induce ACC crystallization when using high-intensity lasers; Raman signal is overwhelmed by the auto-fluorescence of ACC forming cyanobacteria

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90 Fourier transform infrared (FTIR) spectroscopy is one of the most commonly used
 91 vibrational spectroscopies (e.g.²⁵⁻²⁸), capable of rapidly detecting CaCO₃ polymorphs *in situ*,
 92 non-destructively, and without complex sample preparation, making it an ideal approach for
 93 detection of ACC in prokaryotes at a bulk scale. FTIR spectroscopy has been a popular choice to
 94 study the kinetics of ACC crystallization in eukaryotic biominerals (e.g.^{2,29}) and abiotic
 95 carbonates (e.g.³⁰⁻³²). In the mid-IR region, crystalline carbonate phases such as calcite have
 96 distinct bands at ~714 cm⁻¹ (U₄), ~866 cm⁻¹ (U₂), ~1084 cm⁻¹ (U₁) and 1420-1470 cm⁻¹ (U₃).
 97 ACC shares U₁- U₃ vibrations with crystalline CaCO₃ but lacks the distinct vibrational band at
 98 ~714 cm⁻¹ and has an additional shoulder on the U₃ band³³. The lack of U₄ band in ACC along
 99 with the characteristic U₃ shoulder have been used extensively as a diagnostic feature to detect

100 biogenic ACC present in various eukaryotic carbonates such as spicules of ascidian ⁶, spicules of
101 sea urchin ²⁹, fish-gut carbonates ³⁴, and earthworm secreted carbonate granules ³⁵. In eukaryotic
102 carbonates, where crystalline CaCO₃ coexists with ACC, the U₃/ U₄ and U₂/ U₄ peak intensity
103 ratios have been shown to provide semi-quantitative information on the fraction of amorphous
104 and crystalline phases ^{18 35}. Unlike the mid-IR spectral range, the far-infrared (FIR) spectral
105 region (80-650 cm⁻¹) offers valuable insights about lower energy lattice vibrations that
106 correspond to vibrations among different carbonate units ³⁶. Brusentsova et al. compiled a
107 comprehensive dataset of FIR spectra measured on several reference crystalline carbonate
108 polymorphs, but no dataset exists for synthetic or biogenic ACC³⁷. While FTIR spectroscopy has
109 been widely used as a tool to detect ACC in eukaryotic carbonates, the application of FTIR
110 spectroscopy to the detection of prokaryotic ACC is limited and not straightforward³⁸. Unlike
111 eukaryotic carbonates that are typically composed of a large fraction of crystalline carbonate
112 with ACC and low organic content, prokaryotic ACC consists of a low ACC content mixed with
113 high organic content. Indeed, they are commonly found at low abundance in the environment and
114 surrounded by a complex assemblage of cellular biomolecules ^{38,39}. The high organic content
115 could mask the characteristic asymmetric vibration (U₃) band in the region 1500-1400 cm⁻¹ and
116 symmetric vibration band (U₄) at 714 cm⁻¹ due to overlapping bands from proteins (amide I and
117 II peaks) and rocking vibration of methylene group of long-chain alkanes ⁴⁰. Such interferences
118 may prevent the direct use of diagnostic indicators of ACC (e.g., absence of U₄ band),
119 previously established based on eukaryotes ³⁹⁻⁴¹. Mechanically separating the organics from
120 ACC in prokaryotes does not appear as a viable approach as this may result in the rapid
121 transformation of ACC outside the cell, as mentioned previously. Owing to these complications,

122 the question remains as to what IR spectral features of ACC are suitable for its detection in
123 systems dominated by prokaryotes?

124 Within this context, we conduct a comprehensive study of the key spectral features of ACC
125 across the near-IR, mid-IR and far-IR, spectral range in ACC-forming cyanobacteria and test
126 these features in a set of cyanobacterial strains whose ACC-forming capabilities remain
127 unknown.

128 **2. Materials and methods**

129 **2.1. Synthesis of abiotic ACC**

130 An abiotic amorphous calcium/magnesium carbonate (referred as ACC hereafter) was prepared
131 in-house following the protocol described in⁴². The sample was precipitated by mixing two 100
132 ml solutions: (i) an aqueous carbonate solution containing 0.05 M of K_2CO_3 (pH = 11.4); and (ii)
133 an aqueous calcium and magnesium solution containing 0.045 M of $CaCl_2 \cdot 2H_2O$ (Sigma-
134 Aldrich) and 0.053 M of $MgCl_2 \cdot 6H_2O$ (adjusted to pH = 8.6). The calcium- and magnesium-
135 containing solution was poured directly into the carbonate solution. The resulting precipitate was
136 isolated by centrifugation, washed twice with anhydrous ethanol, and dried at 37°C for 7 days.
137 Bulk mineralogy of the dried powder was analyzed using a Panalytical powder X-ray
138 diffractometer, equipped with a Co-anode X-ray source. Patterns were recorded between
139 $2\theta=3-70$ degrees, with 0.001 degree steps and 2 milliseconds/step. The characterization of
140 synthesized ACC using X-ray diffraction (XRD) showed only broad peaks, confirming that the
141 synthesis resulted in an amorphous phase (Fig. S1). Synthesized ACC was stored at room
142 temperature in a desiccator. The synthesized ACC remained amorphous for at least 1 year, as

143 confirmed by XRD (data not shown). Thermogravimetric analysis-differential scanning
144 calorimetry (TGA-DSC) analyses were performed using a SDT Q 600 TA. About 15 mg of
145 powder were placed in an aluminum crucible and the sample was heated up to 900 °C under a
146 100 ml-per-minute nitrogen flow. The temperature scan rate was set at 5 °C per minute. Mass
147 spectrometry analyses were coupled with the TGA-DSC analyses using a ThermoStare GSD 301
148 T3 (Pfeiffer Vacuum). The signal corresponding to 44 amu (CO₂) and 18 amu (H₂O) were
149 recorded.

150 **2.2. Culture conditions for cyanobacteria strains**

151 Here, we will refer to strains forming intracellular ACC as ACC+ strains and strains not forming
152 intracellular ACC as ACC- strains. Two reference strains, *Synechocystis* PCC 6803 (ACC-) and
153 *Cyanothece* PCC 7425 (ACC+) were cultured in the BG-11 medium, a standard growth medium
154 used for freshwater cyanobacteria and the chemical composition of which is provided in Stanier
155 et al.⁴³. Cultures were grown with agitation and continuous light (5-10 μmol m⁻² s⁻¹). When cell
156 suspensions reached an optical density of ~1, cells were harvested by centrifugation, rinsed, and
157 dried at 40 °C for 48 hours. In addition, *Microcystis* spp. strains from the Paris Museum
158 Collection (PMC) were included in this study as strains whose capacity to form intracellular
159 ACC was initially unknown. Eight different *Microcystis* strains were cultured to reflect some
160 phenotypic diversity. These strains were grown in the standard BG-11 medium, without
161 agitation, at 23 °C, with a 12:12 photoperiod and a light intensity of ~ 5 μmol m⁻² s⁻¹. The cells
162 were harvested via centrifugation and dried at 40 °C for 48 hours. The total Ca concentration of
163 the dried biomass was measured by acid digestion of ~5 mg of dry biomass and subsequent

164 analysis of the digestate using inductively coupled plasma - optical emission spectrometry (ICP-
165 OES).

166 **2.3 SEM analysis**

167 *Microcystis* cell suspensions were filtered on 0.22 μm polyethersulfone (PES) filters, then rinsed
168 three times with 10 mL MilliQ® water. The filters were dried at ambient temperature and
169 mounted on aluminum stubs using double-sided carbon tape. The samples were carbon coated
170 prior to SEM analyses. Scanning electron microscopy (SEM) observations were performed using
171 a Zeiss Ultra55 SEM at IMPMC. The electron accelerating voltage used was 10 or 15 kV with a
172 working distance of 7.5 mm and a 60 μm aperture. Images were collected with a backscattered
173 electron detector (AsB detector) and the maps of the different elements (Ca, P, K, Mg, Si, Fe)
174 were retrieved from energy dispersive X-ray spectrometry (EDXS) hyperspectral data
175 (HyperMap).

176 **2.4. FTIR spectroscopy analyses**

177 The abiotic ACC and dried biomass of the different strains were finely ground in an agate
178 mortar. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were
179 acquired over the mid-IR region (4000-600 cm^{-1}) using a NICOLET 6700 FTIR spectrometer
180 equipped with a diamond internal reflection element (IRE), a KBr beam splitter and a mercury
181 cadmium telluride (MCT) detector. The sample was mounted on the diamond IRE. Spectra were
182 accumulated by collecting 64-100 scans with a resolution of 1 cm^{-1} . Spectra processing including
183 baseline correction, normalization, and peak fit was carried out using the Origin
184 software™. Baseline corrected mid-IR spectra were fitted using a peak analyzer (based on the

185 Levenberg-Marquardt algorithm). All biomass spectra were fitted in the 945 ± 5 to 845 ± 5 cm^{-1}
186 frequency range. The initial peak position was determined by the local maximum method, which
187 finds the frequency corresponding to the local maximum within the 20-point range. The spectra
188 were fitted with linear combinations of Voigt lineshapes, the centroids of which were initially set
189 to values determined from the local maxima peak analysis and remained unfixed during the fit
190 unless noted otherwise. The baseline was fixed for the fit (cf. section 3.3). The area under the
191 spectrum and above the background was used for spectral normalization (cf. section 3.4).

192 For the near-infrared analyses, bi-directional reflectance spectra were obtained using the
193 SHADOWS instrument⁴⁴ in the 500-4000 nm (20000 - 2500 cm^{-1}) spectral range. Samples were
194 deposited in a cylindrical sample holder (13 mm in diameter, 5 mm in depth) and measured
195 under a standard observation geometry (incidence= 0° , emergence= 30° , phase= 30°). Spectra were
196 calibrated using the spectral reflectance spectralonTM and infragoldTM and corrected for the non-
197 lambertian behavior of spectralon. Last, for far infrared analysis, approximately 6 mg of dried
198 sample were mixed with 80 mg of polyethylene (PE). Pellets were formed in a vacuum press
199 under ten tons of pressure for ~ 5 min, and spectra were acquired in transmission mode using the
200 Bruker IFS 66v/s FTIR spectrometer in the 600 - 50 cm^{-1} spectral range, at a resolution of 4 cm^{-1}
201 and using a deuterated triglycine sulfate (DTGS) detector and a multilayer mylar beam splitter.
202 No baseline correction was performed because it was difficult to define and subtract a baseline
203 accurately.

204 **3. Results and Discussion**

205 **3.1 Spectral features of synthetic ACC across near-IR, mid-IR and far-IR**

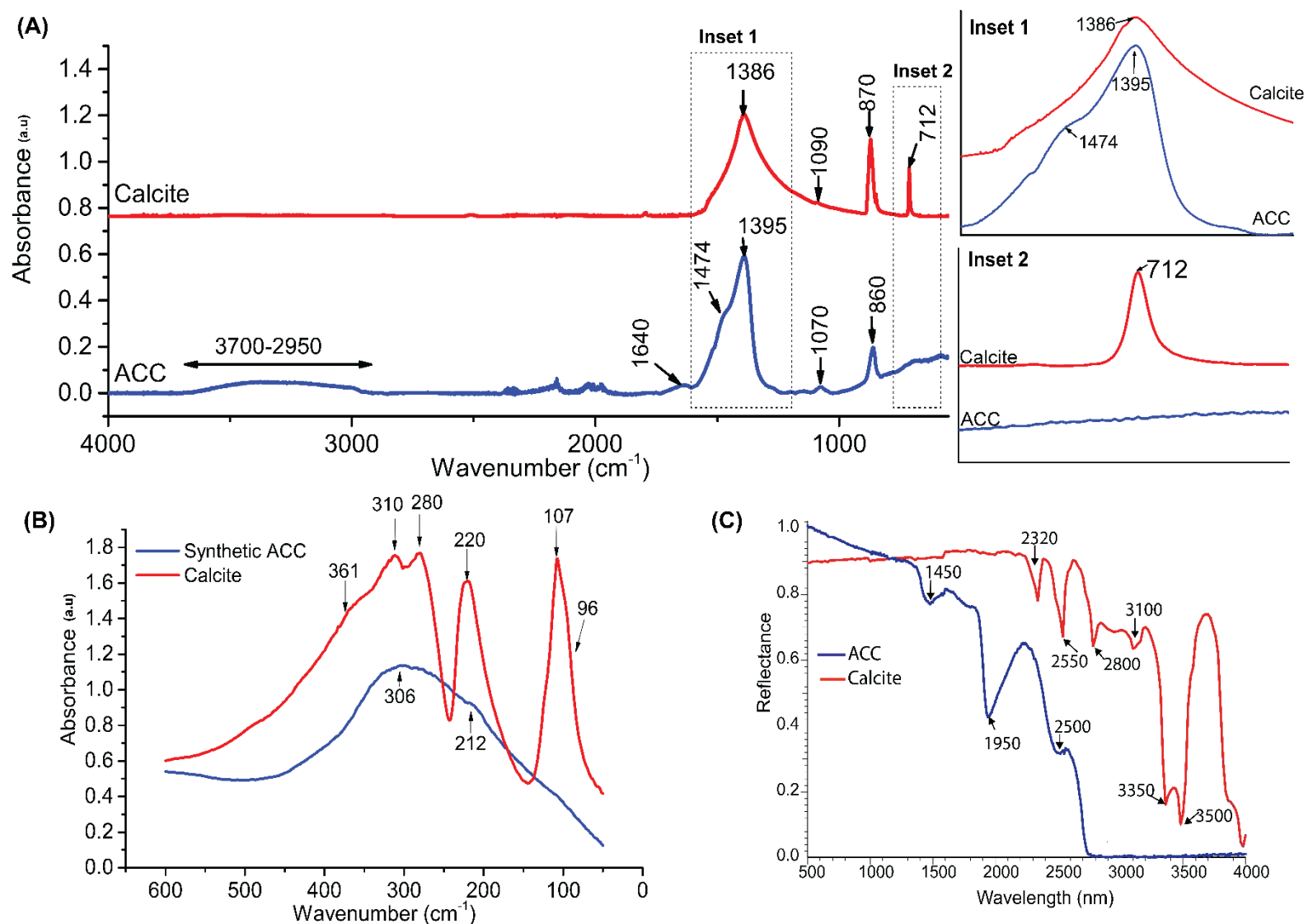


Figure 1: (A) ATR-FTIR spectra of synthetic ACC and calcite in the mid-IR spectral range. The arrows correspond to characteristic mid-IR active frequencies of ACC. The dashed rectangle envelops the zoomed regions of mid-IR ACC spectra shown in the inset 1 (1500–1200 cm^{-1}) and inset 2 (750–680 cm^{-1}). The bands between 2500–2000 cm^{-1} in ACC spectra are due to ambient CO_2 and insufficient purging of the sample chamber. (B) Far-IR spectra of synthetic ACC, and calcite. Arrows denote the characteristic features of ACC and calcite. (C) Near-IR spectra of synthetic ACC, and calcite. Details on the assignment of the vibrations of ACC across the entire region are listed in Table S1. The X-axis is in wavelength, where 500 nm corresponds to $2 \times 10^4 \text{ cm}^{-1}$ and 4000 nm corresponds to $2.5 \times 10^3 \text{ cm}^{-1}$.

207 The characteristic peaks of synthetic ACC across the spectral range of 20,000-50 cm^{-1} are shown
208 in Fig. 1 and summarized in Table S1. The band assignments of the IR peaks observed in ACC
209 are listed in Table S1. In the mid-IR spectral region, ACC is specifically indicated by the
210 presence of a shoulder at 1474 cm^{-1} and the absence of a peak at 712 cm^{-1} (Fig. 1, Inset 1-2)³².
211 This spectral range also showed for ACC a broad band between 2950 and 3700 cm^{-1} (O-H
212 stretching) and at 1650 cm^{-1} (O-H bending), both corresponding to structural water within
213 ACC³². Consistently, the water content in ACC was estimated to 17 wt% (corresponding to 0.94
214 mole of water per carbonate) based on TGA-DSC-MS analyses (Fig. S2). The out-of-plane
215 bending absorption (U_2) at $\sim 860 \text{ cm}^{-1}$ (FWHM=25 cm^{-1}) was observed. This band is sensitive to
216 differences in chemical environment changes from amorphous to crystalline forms³⁰. Indeed,
217 with the transformation of an amorphous phase to a crystalline phase, the U_2 band position shifts
218 from 860 cm^{-1} (ACC) to 870 cm^{-1} (calcite) (Fig. 1A). The U_2 band position is also sensitive to
219 the Mg content in ACC: the band shifts towards lower wavenumbers with increasing Mg content
220 in ACC and can vary between 860-864 cm^{-1} ^{20,27}. The remaining bands in the mid-IR spectral
221 range were characteristic vibrations of the carbonate ion and are common in both ACC and
222 crystalline polymorph³².

223 In the FIR region, ACC shows a broad peak around $\sim 306 \text{ cm}^{-1}$ and a weak shoulder on this
224 broad peak at $\sim 212 \text{ cm}^{-1}$ (Fig. 1B). For comparison, calcite FIR spectra show several sharp peaks
225 at 310 (with a shoulder at 361), 280, 220, 107 (shoulder at 96) cm^{-1} , consistent with previous
226 studies (e.g. ^{36,37}). These sharp bands are due to IR-active collective lattice vibrational modes that
227 involve relative translations of the calcium and carbonate ions. In contrast, ACC is rotationally
228 disordered which results in a broad envelope across the available frequencies⁴⁵. Lastly, in the

229 NIR region, the ACC spectra is dominated by broad absorption peaks at 1450 nm (6896 cm^{-1}),
230 1950 nm (5128 cm^{-1}), 2500 nm (4000 cm^{-1}), and the absence of absorption bands beyond ~2500
231 nm (Fig. 1C). The peaks <2500 nm are attributed to O-H stretching overtones originating from
232 structural and/or weakly bonded water⁴⁶. Above 2500 nm the reflectance is close to zero, because
233 of the saturation of the fundamental OH stretching mode. Moreover, the typical carbonate
234 vibrations in region >2500 nm (see calcite NIR spectra in Fig. 1C) are masked by strong water-
235 related absorption bands in ACC^{47,48 49}. Overall, due to the presence of water in ACC, the NIR
236 region offers no diagnostic spectral features of ACC.

237 Several key insights can be drawn from the above analyses. All bands of ACC in the NIR
238 region are masked by structural water. Almost all biological occurrences of ACC are known to
239 be hydrous, and thus, NIR is not a particularly useful IR fingerprinting region for ACC. In
240 contrast, mid-IR and far-IR regions offer promising diagnostic spectral features that we test
241 below to detect ACC in cyanobacteria.

242 **3.2 FTIR spectra of ACC+ and ACC- –cyanobacteria in the mid-IR and far-IR ranges**

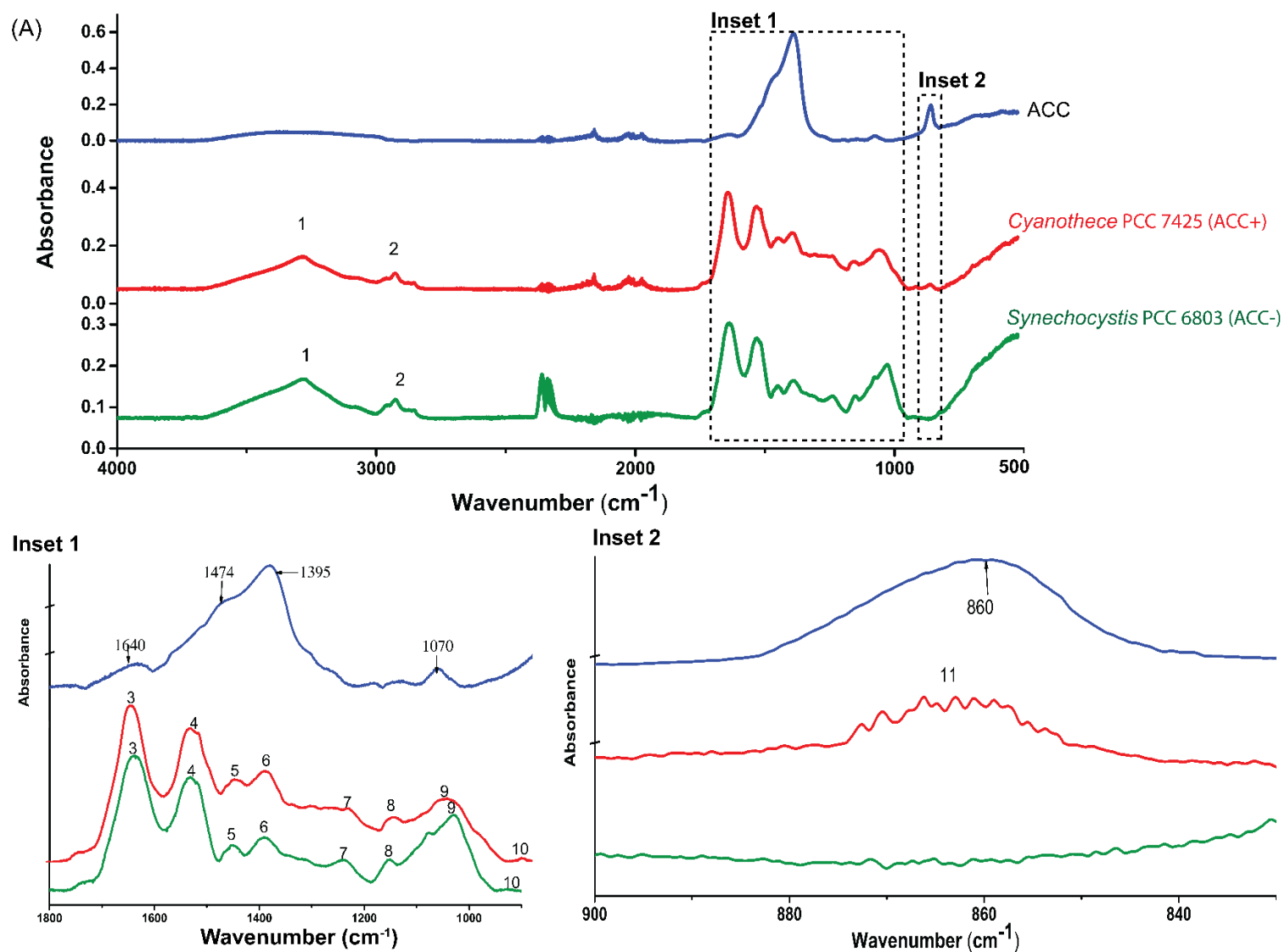


Figure 2: (A) ATR-FTIR spectra of the *Cyanothoece* PCC 7425 (ACC+), *Synechocystis* PCC 6803(ACC-), and synthetic ACC. The bands between 2500-2000 cm⁻¹ in the spectra are due to ambient CO₂ and H₂O resulting from insufficient purging of the sample chamber. The dashed rectangle envelops the zoomed regions of mid-IR ACC spectra shown in the inset 1 (1800-900 cm⁻¹) and inset 2 (900-820 cm⁻¹). Numbers on the spectra correspond to the band# in Table 2.

244 Several absorption bands were identified in the mid-IR spectra of *Cyanothece* PCC 7425
245 (ACC+) (Fig. 2). The spectral band assignment for the ACC+ and ACC- strains was achieved
246 based on a comparison and cross-referencing against existing FTIR literature about phototrophic
247 microorganisms (^{41,50-53}) and is summarized in Table 2. Bands [1-10] were present in both
248 *Cyanothece* PCC 7425 and *Synechocystis* PCC 6803 and were attributed to non-ACC
249 components. These components included O-H vibrations derived from water (band 1), methyl
250 and methylene group vibrations from lipids and fatty acids (band 2), amide I and amide II from
251 proteins (bands 3,4), asymmetric bending of methyl groups of lipids, and symmetric stretching
252 from C-O group in carboxylic acids (bands 5,6), a combination of asymmetric stretching from
253 P=O group of nucleic acids and/or polyphosphates and C-O stretching vibrations from
254 polysaccharides (bands 7, 8, 9, 10) ^{50,51,54}. The bands between 2500-2000 cm⁻¹ are due to
255 ambient CO₂ and insufficient purging of the sample chamber. *Cyanothece* PCC 7425 had an
256 additional absorption band present at ~860 cm⁻¹ (band 11) which was absent in *Synechocystis*
257 PCC 6803. The peak position of band 11 coincides with the ACC peak at 860 cm⁻¹ (Fig. 2).
258 Moreover, the FWHM of the fitted peak at 860 cm⁻¹ in *Cyanothece* PCC 7425 was 25.1 cm⁻¹,
259 matching that of the 860 cm⁻¹ peak of ACC. No absorption band was found around ~700 cm⁻¹
260 region in *Cyanothece* PCC 7425 suggesting the absence of crystalline carbonate polymorphs.
261 This is consistent with the under-saturation of the BG-11 growth medium with respect to
262 carbonate phases, which does not allow the precipitation of extracellular crystalline carbonates in
263 the growth solution (Fig. S3). Spectral features of ACC apart from the 860 cm⁻¹, namely 3600-
264 2700 cm⁻¹ (O-H stretching), 1650 cm⁻¹ (O-H bending), 1470-1420 cm⁻¹ (U₃) and 1084 cm⁻¹ (U₁)
265 had strong interference with the lipids and water bands (1, 2), protein bands (3, 4), carboxylic

266 acids bands (5,6) and nucleic acid or phosphorylated polysaccharides (7,8,9) as shown in Fig.
267 2B.

Table 2: Wavenumbers and band assignment for characteristics vibrations found in the mid IR spectra of *Cyanothece* PCC 7425 and *Synechocystis* PCC 6803. The band assignment was primarily based on the ^{50,51,54}.

Band Index	Wavenumbers (cm ⁻¹)	Main functional group
1	3279	water
2	2960-2856	methyl and methylene group vibrations from lipids and fatty acids
3	1641	amide I from proteins
4	1528	amide II from proteins
5	1444	methyl groups of lipids and C-O group in carboxylic acids
6	1391	methyl groups of lipids and C-O group in carboxylic acids
7	1241	Nucleic acids, phosphoryl group
8	1153	P=O group of nucleic acids and/or polyphosphates and C-O vibrations from polysaccharides
9	1058-1029	P=O group of nucleic acids and/or polyphosphates and C-O vibrations from polysaccharides
10	919	P=O group of nucleic acids and/or polyphosphates and C-O vibrations from polysaccharides
11	860	out-of-plane bending of carbonates in ACC (U ₂)

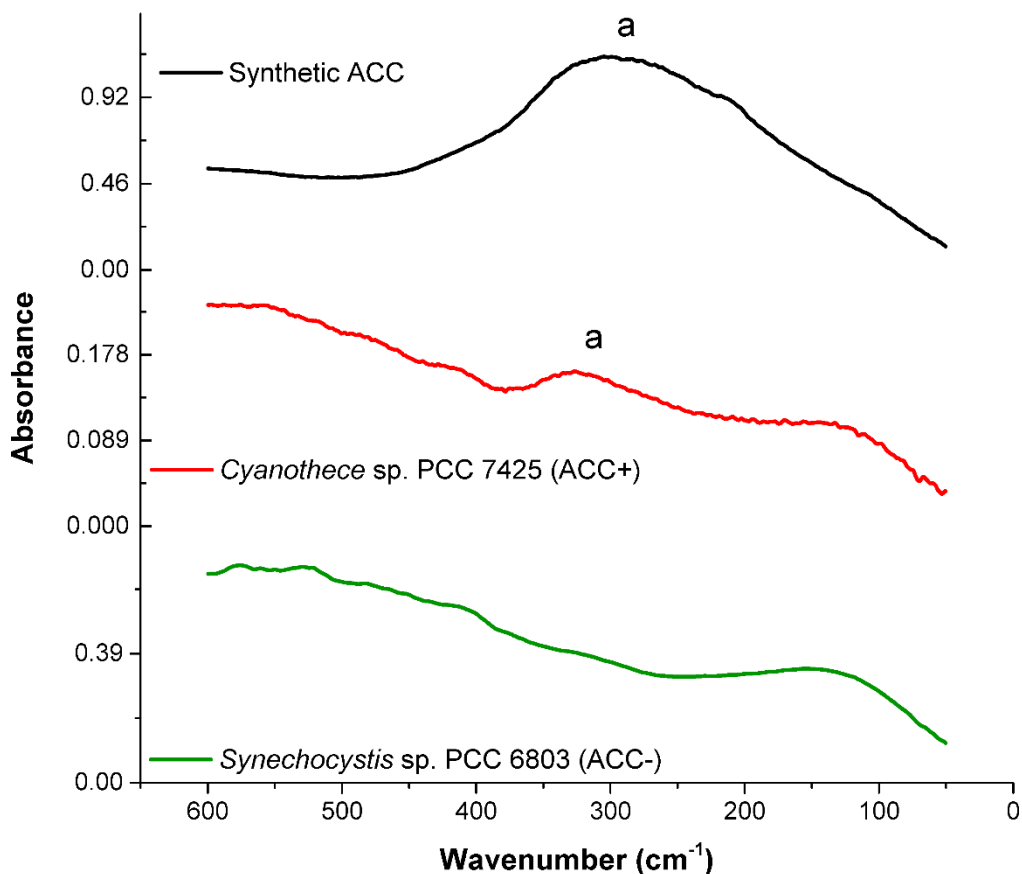


Figure 3: FIR spectra of the ACC+ and ACC- cyanobacteria. The peak labeled marked as “a” denotes the location of the characteristic features of ACC in FIR at $\sim 306\text{ cm}^{-1}$. This peak was at $\sim 320\text{ cm}^{-1}$ in *Cyanothece* PCC 7425 (ACC+).

270

271 In the NIR region, the spectra of *Cyanothece* PCC 7425 and *Synechocystis* PCC 6803 looked
 272 almost identical, which is not surprising based on the lack of ACC features in the NIR region due
 273 to superimposition with structural water (Fig. S4). The different peaks found in the NIR spectra
 274 of cyanobacteria were primarily attributed to lipids, proteins, and water (Fig. S5) in accordance
 275 to Vaidyanathan et al.⁵⁵.

276 In the FIR region, broad features around 150 cm^{-1} were observed in both ACC+ and ACC-
 277 cyanobacteria strains, possibly corresponding to torsional frequencies of primary amines^{56,57}.

278 Interestingly, FIR spectra of *Cyanothece* PCC 7425 showed a broad but strong peak around ~320
279 cm^{-1} , which was absent in *Synechocystis* PCC 6803 (Fig. 3). The asymmetric line shape is
280 characteristic for samples prepared with PE as filling material, and it is not found in the spectra
281 of samples prepared without PE⁵⁸. The position of this peak coincides with the broad peak
282 observed in FIR spectra of synthetic ACC (Fig. 1), suggesting that the peak might be due to ACC
283 in *Cyanothece* PCC 7425.

284 Comparing the mid-IR and far-IR spectra of ACC+ and ACC- cyanobacteria strains,
285 only the U_2 band at 860 cm^{-1} and the broad peak at $\sim 306 \text{ cm}^{-1}$ (characteristic vibrations of ACC)
286 are free from overlaps with spectral features attributed to organics. Consistently, they are present
287 in ACC+ cyanobacteria, while absent in ACC- cyanobacteria. Indeed, ACC features in spectral
288 region $>900 \text{ cm}^{-1}$ are masked by vibrations from organics, suggesting that spectral features of
289 ACC in the spectral region $> 900 \text{ cm}^{-1}$ are not reliable diagnostic indicator of presence of ACC
290 in a sample dominated by organics. Such complex combination between ACC bands and organic
291 bands is shown to be absent in organic-poor eukaryotic ACC, where the intense vibrational
292 bands from ACC mask the bands from the matrix biomolecules, making the ACC mid-IR bands
293 in regions $> 900 \text{ cm}^{-1}$ detectable⁵⁹. Based on these results, we argue that the use of spectral
294 features of ACC in region $> 900 \text{ cm}^{-1}$ to confirm ACC in an organic-rich matrix could result in
295 some misleading results. For instance, Enyedi et al. used the presence of bands at 1494 cm^{-1} and
296 at 1066 cm^{-1} in an organic-rich bacterial extra-polymer-substance (EPS) to infer the presence of
297 ACC in bacterial EPS³⁸. Fortunately, this interpretation was further supported by other
298 complementary techniques (electron diffraction and Raman spectroscopy). However, we note
299 that the interpretation of the FTIR data by Enyedi et al. is not accurate, since the characteristic

300 peaks of ACC at 1494 cm^{-1} and at 1066 cm^{-1} could be attributed to the vibrations from organic
301 components present in the samples, such as proteins, nucleic acid, and phosphorylated
302 polysaccharides.

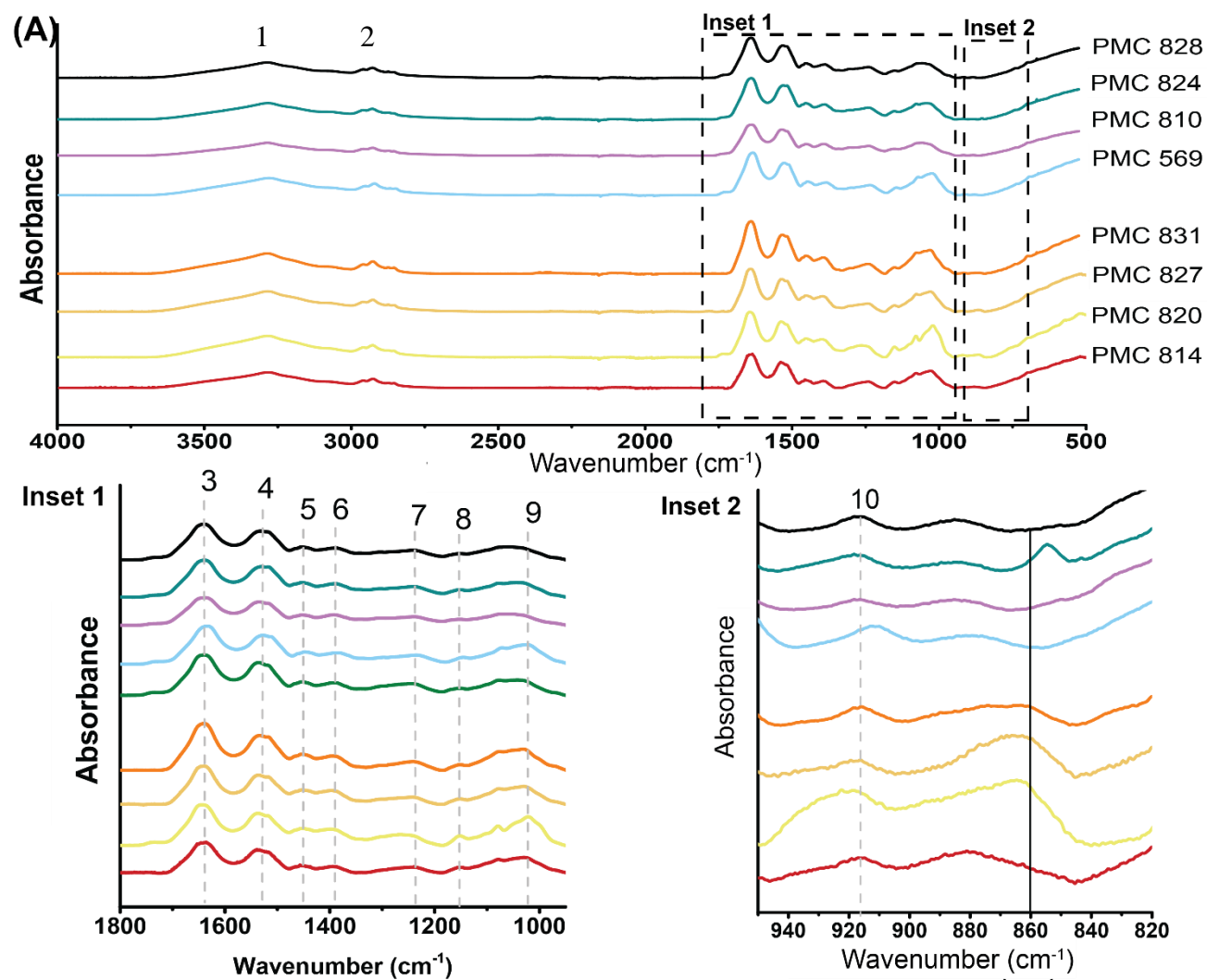
303 Whether these spectral features apply to a broader diversity of prokaryotes remains an open
304 question. The characteristic peaks of major biomolecules (e.g., proteins, polysaccharides) can be
305 expected to remain the same across different prokaryotic taxa, but some minor differences have
306 been reported due to solution chemistry, growth conditions and taxonomy^{41,60-62}. In the next
307 section, we assess the effect of physiological differences on the above-identified ACC spectral
308 features by surveying different cyanobacterial strains affiliated to the same *Microcystis* genus.

309 **3.3 Case study: Examining the capability of *Microcystis* strains to form ACC using FTIR** 310 **spectroscopy and SEM**

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Figure 4: (A) ATR-FTIR spectra of PMC *Microcystis* sp. strains in the mid IR range. The dashed rectangle envelops the zoomed regions of mid-IR ACC spectra shown in the inset 1 (1800-900 cm^{-1}) and inset 2 (950-820 cm^{-1}). Numbers on the spectra correspond to the band numbers and their assignment are provided in Table 1. The vertical gray dash lines in Inset 1 are guidelines to facilitate identification of the position of bands (1-10) in the different PMC *Microcystis* sp. strains. The solid black line in Inset 2 denotes the location of characteristic U_2 band of ACC ($\sim 860 \text{ cm}^{-1}$).

316 The cyanobacterial genus *Microcystis* has been intensely^(e.g.63–66) studied because it
317 comprises some strains producing hepato- and neuro-toxins, and blooming mostly in freshwater
318 and brackish water, causing significant environmental issues.

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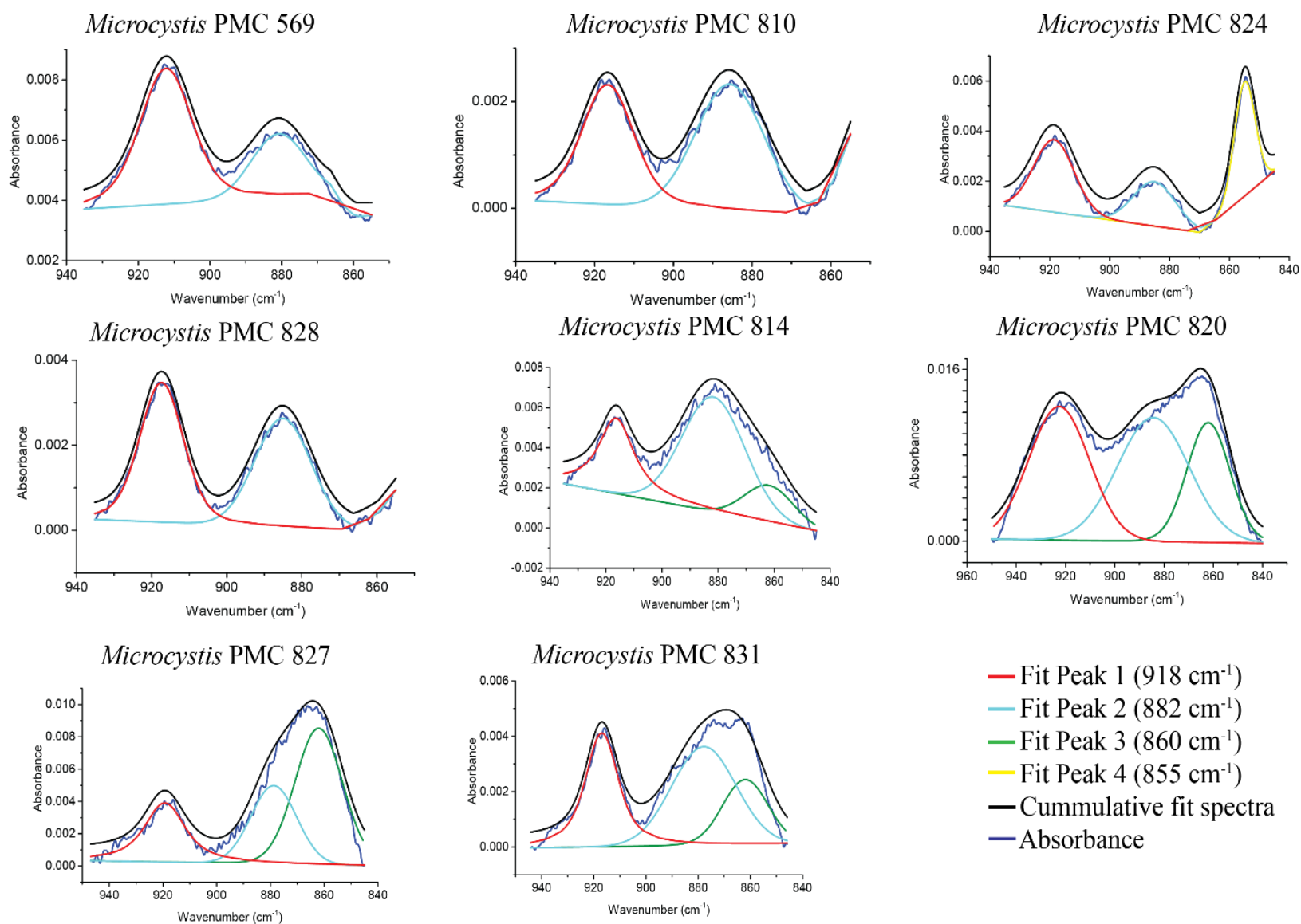


Figure 5: Peak fit of *Microcystis* strains in the frequency region 940-840 cm^{-1} . For strains PMC 569, PMC 810 and PMC 828, best fit was achieved by using 2 band components ($\sim 918 \text{ cm}^{-1}$, 882 cm^{-1}); for PMC 824, best fit was achieved by using 3 band components (918 cm^{-1} , 882 cm^{-1} and 855 cm^{-1}); for strains PMC 814, 820 and 831, 3 band components (918 cm^{-1} , 882 cm^{-1} and 860 cm^{-1}) yielded best fit. The peak positions of the fitted bands are noted in table 3 and fit quality summarized in the table S2

321 While ACC formation has been evidenced in a broad diversity of cyanobacteria¹⁰, *Microcystis*
322 has so far been poorly studied in this perspective. Here, we acquired FTIR spectra of several
323 different *Microcystis* strains in the mid-IR region to investigate if some of them may form ACC
324 (Fig. 4). Characteristic bands of proteins, lipids, polysaccharides, and phosphate groups (bands
325 1-10) were observed in the 4000-900 cm⁻¹ spectral region in all *Microcystis* strains, similarly to
326 what was observed in *Cyanothece* PCC 7425 and *Synechocystis* PCC 6803 (Fig. 4A). The band
327 location and assignment were in good agreement with the previously reported bands in
328 *Microcystis* in the 4000-1000 cm⁻¹ frequency range⁴¹. Visual inspection of *Microcystis* strains in
329 the 950-800 cm⁻¹ region reveals that all *Microcystis* spectra had a clearly defined band at ~918
330 cm⁻¹, labeled as band 10 in Fig. 4C. Additionally, a broad peak at ~880 cm⁻¹ was clearly
331 observed in *Microcystis* PMC 569, PMC 810, PMC 824, and PMC 828, while this band appeared
332 visually as an asymmetric broad band for the other *Microcystis* strains (PMC 814, PMC 820,
333 PMC 827 and PMC 831) (Fig. 4C). A sharp peak at ~855 cm⁻¹ was observed in *Microcystis*
334 PMC 824 only. In *Microcystis* PMC 820, PMC 827 and PMC 831, a band close to 860 cm⁻¹ was
335 observed. Unlike what was observed for *Cyanothece* PCC 7425, this band, possibly affiliated to
336 the 860 cm⁻¹ peak of ACC, was not well-defined and overlapped with other neighboring peaks in
337 the 950-800 cm⁻¹ spectral range (Fig. 4B).

338 To overcome the challenge of overlapping bands, we fitted the experimental spectra of all
339 *Microcystis* strains in the 950-800 cm⁻¹ frequency range with a combination of four (Voigt) peak
340 components: fit peak 1 at ~918 cm⁻¹ and fit peak 2 at ~880 cm⁻¹, fit peak 3 at ~860 cm⁻¹, and fit
341 peak 4 at ~855 cm⁻¹. Fig. 5 shows the best fits for different *Microcystis* strains, and the peak
342 properties are listed in table 3. The fit quality parameters are listed in Table S2. Based on the

343 curve fit, we find that fit peak 1 at $\sim 918\text{ cm}^{-1}$ and fit peak 2 at $\sim 880\text{ cm}^{-1}$ were present in all
344 *Microcystis* strains (Table 3). The fit peak 1 at $\sim 918\text{ cm}^{-1}$ was also present in *Synechocystis* PCC
345 6803 and *Cyanothece* PCC 7425 (peak labeled 10 in Fig. 2 and Fig. 4) and is attributed to a
346 combination of asymmetric stretching from the P=O group of nucleic acids and/or
347 polyphosphates and C-O stretching vibrations from polysaccharides. Intra-strain variations in the
348 intensity of fit peak 1 at $\sim 918\text{ cm}^{-1}$ could potentially be due to variations in the polyphosphate
349 and polysaccharide content of the cells. Polyphosphates and polysaccharides serve as carbon,
350 phosphorus and energy reserves, and their content is known to vary during the growth of the
351 cyanobacteria^{41,52}. The fit peak 2 at $\sim 880\text{ cm}^{-1}$ could match with vibrations of crystalline
352 carbonates. However, no peak at $\sim 714\text{ cm}^{-1}$ was found suggesting the absence of such phases.
353 Alternatively, fit peak 2 may be assigned to bicarbonate⁶⁷. Cyanobacteria are known to
354 accumulate high amount of intracellular bicarbonate owing to carbon concentrating
355 mechanisms⁶⁸. The bicarbonate band is generally weak and sensitive to the degree of hydration
356 of bicarbonate ions, i.e. the band is red shifted and becomes broader with increasing hydration
357⁶⁹. Unfortunately, it is not possible to further confirm this band assignment based on other bands
358 characteristic of bicarbonate since they overlap with carbonate vibrations and/or are masked by
359 vibrations of biomolecules. A third possible affiliation of fit peak 2 can be made as strong =C-H
360 out-of-the plane bending vibrations of alkenes⁵⁴. The other peaks characteristics of alkenes are
361 stretching =C-H vibrations at $3100\text{-}3000\text{ cm}^{-1}$ and C=C stretching vibrations at $1660\text{-}1500\text{ cm}^{-1}$,
362 both of which overlap with band #1 (attributed to water) and band #3, #4 (attributed to proteins)
363 in all *Microcystis* strains⁷⁰. Alkenes typically form part of the structure of fatty acids, and/or
364 carotenoid pigments (commonly present in photosynthetic cells and one of the main components

365 of the phytoaccumulation complex^{70,71}). Last, fit peak 2 at $\sim 880\text{ cm}^{-1}$ could be due to the P=O
366 group vibrations of polyphosphates as proposed by Wang et al.⁷². This assignment is also
367 consistent with the presence of other characteristic bands (bands 7, 8, 9, 10) of polyphosphates
368 observed in all the *Microcystis* strains (Table 2). As a summary, fit peak 2 may result from
369 vibrations in bicarbonates, alkenes and/or polyphosphates. Interestingly, fit peak 4 at 855 cm^{-1}
370 was only found in *Microcystis* PMC 824 and had a relatively narrow FWHM (8 cm^{-1}) in
371 comparison to the 860 cm^{-1} (U_2) band of ACC (FWHM= 25 cm^{-1}) suggesting that the band at
372 855 cm^{-1} is not linked to the 860 cm^{-1} band of ACC. Nitrates and chloride salts, contained by the
373 BG-11 growth medium are known to give rise to a sharp peak at $\sim 855\text{ cm}^{-1}$ ⁷³. Thus, the band at
374 $\sim 855\text{ cm}^{-1}$ could be assigned to nitrate salts from the growth medium and may result from in-
375 sufficient rinsing of the *Microcystis* PMC 824 cell suspension.

376 Last, peak fit of the *Microcystis* spectra revealed that fit peak 3 at 860 cm^{-1} was only
377 present in strains PMC 814, PMC 820, PMC 827, and PMC 831 (Fig. 5 and Table 3). The overall
378 quality of the fit for these strains was poorer when fit peak 3 was not included in the fit
379 components (Fig. S5 and Table S2). The FWHM of fit peak 3 at 860 cm^{-1} in *Microcystis* strains
380 was slightly narrower ($\sim 21\text{ cm}^{-1}$) but still comparable with the 860 cm^{-1} band of synthetic ACC
381 (25 cm^{-1}) (Table 3). By contrast, poor fit quality was obtained upon fitting a band at 860 cm^{-1} in
382 the experimental spectra of the PMC 824, PMC 569, PMC 810, and PMC 828 strains, suggesting
383 that the band at 860 cm^{-1} was not present in these spectra (Fig. S5 and Table S2). Thus, based on
384 the presence/absence of the 860 cm^{-1} band in *Microcystis* strains, we were able to screen four
385 (PMC 814, PMC 820, PMC 827 and PMC 831) out of eight strains as promising candidates to
386 possess ACC-forming capabilities. These findings were further reinforced by analyzing the FIR

387 region spectra of *Microcystis* strains. We compared the FIR spectra of two *Microcystis* strains:
388 *Microcystis* PMC 827 and *Microcystis* PMC 824. We found broad features around 150 cm^{-1} in
389 both PMC 827 and PMC 824 strains, similar to that observed in ACC+ and ACC- cyanobacterial
390 strains and possibly corresponding to torsional frequencies of primary amines^{56,57}. FIR spectra of
391 *Microcystis* PMC 827 showed a broad but strong peak around $\sim 320\text{ cm}^{-1}$, which was absent in
392 *Microcystis* PMC 824 (Fig. 6). The position of this peak coincides with the broad peak observed
393 in FIR spectra of synthetic ACC as well as in ACC+ forming cyanobacteria strain (Fig. 3),
394 confirming the presence of ACC in *Microcystis* PMC 827 and its absence in PMC 824. This is
395 consistent with observations on the ACC-forming potential of these strains derived from the mid-
396 IR spectra of these strains.

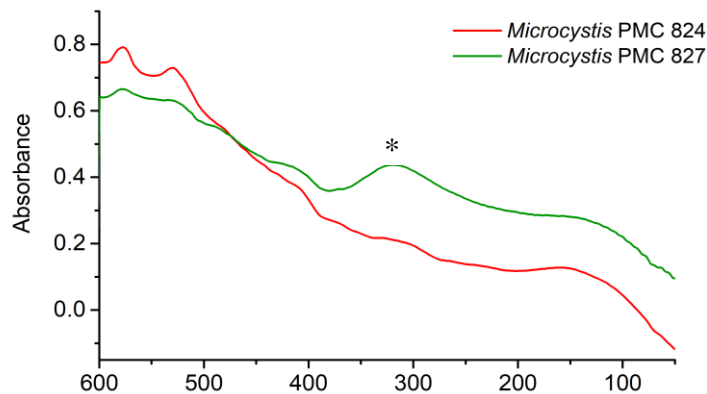


Figure 6: FIR spectra of *Microcystis* PMC 827 and *Microcystis* PMC 824. The peak labeled marked as “*” denotes the characteristic features of ACC in FIR at $\sim 300\text{-}320\text{ cm}^{-1}$.

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402 Table 3: Peak spectral properties of the fitted peaks in *Microcystis* strains corresponding to the best fit for
 403 each strain.

	Fitted Peak Centers (cm ⁻¹)				FWHM (cm ⁻¹)			
	Peak 1 [*]	Peak 2	Peak 3 ^{**}	Peak 4	Peak 1	Peak 2	Peak 3 ^{**}	Peak 4
<i>Microcystis</i> PMC 569	912 [†]	881±0.09	**	NA	18±0.19	17±0.11	**	**
<i>Microcystis</i> PMC 810	917 [†]	886±0.06	**	NA	16±0.16	20±0.73	**	**
<i>Microcystis</i> PMC 824	919 [†]	885±0.1	**	855±0.03	16±0.15	17±0.22	**	8±0.06
<i>Microcystis</i> PMC 828	917 [†]	885±0.04	**	**	14±0.08	18±0.09	**	**
<i>Microcystis</i> PMC 814	916 [†]	882±0.71	862±1.77	**	15±0.82	26±1.14	21±1.19	**
<i>Microcystis</i> PMC 820	923 [†]	884±1.19	862±0.3	**	29±1.14	34±4.4	21±1.45	**
<i>Microcystis</i> PMC 827	919 [†]	879±3.07	862±1.8	**	18±0.61	20±4.27	21±0.92	**
<i>Microcystis</i> PMC 831	917 [†]	878±5.88	862±2.6	**	15±0.91	29±8.4	21±1.78	**

*This fit band is same as band 10 in table 1

[†] This parameter was fixed

** Peak 3 center was constrained to vary between 861± 1 cm⁻¹

* The gaussian FWHM of peak 3 was set to be at least 20 cm⁻¹

** No peak fit at this location.

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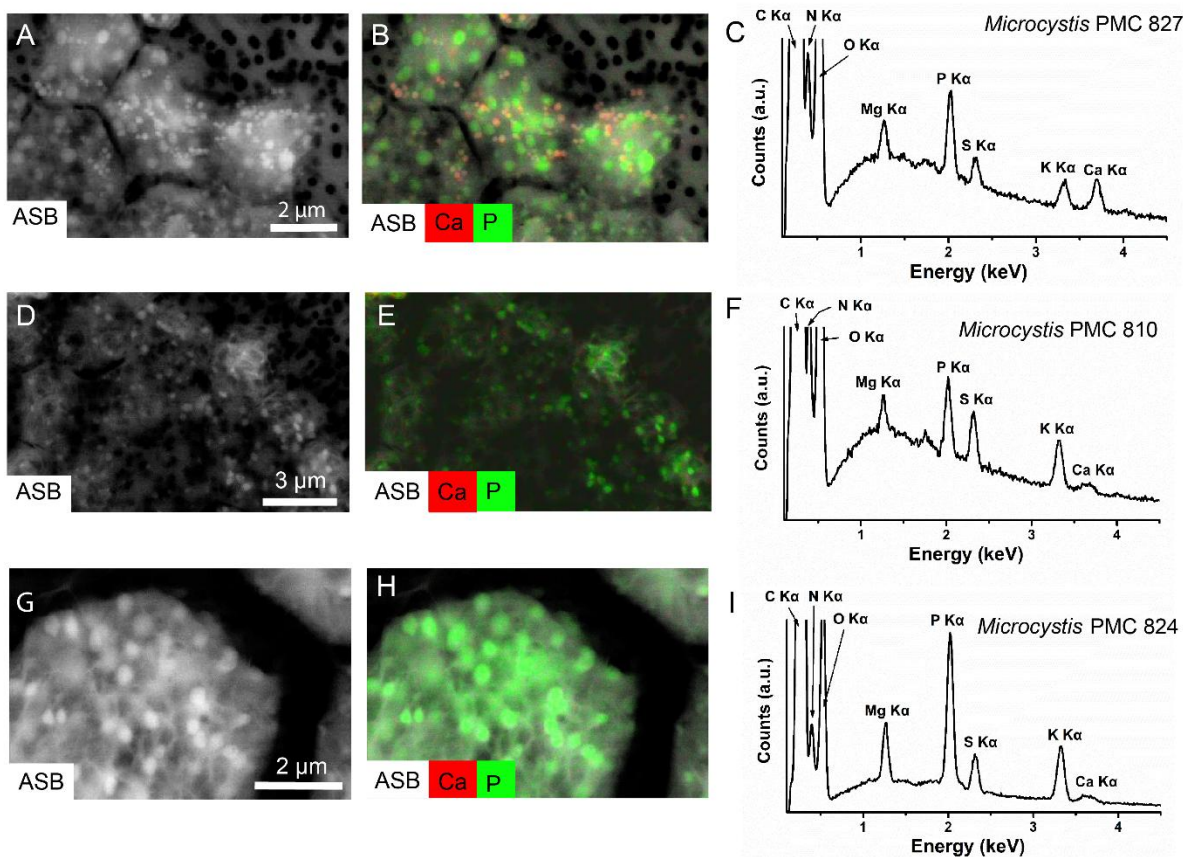


Figure 6: Representative SEM images of *Microcystis* strains found to be capable of forming ACC and not forming ACC based on FTIR. (A, D, G) SEM image of the *Microcystis* PMC 827 (ACC+), *Microcystis* PMC 810 (ACC-), *Microcystis* PMC 824 (ACC-) strains in AsB detection mode, respectively. (B, E, H) Corresponding overlay of AsB image with EDXS maps of Ca (red) and P (green) for *Microcystis* PMC 827 (ACC+), *Microcystis* PMC 810 (ACC-), *Microcystis* PMC 824 (ACC-) respectively. (C, F, I) SEM-EDXS spectra of *Microcystis* sp. PMC 827 (ACC+), *Microcystis* PMC 810 (ACC-), *Microcystis* PMC 824 (ACC-) respectively.

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406 To further confirm our findings on the capability of *Microcystis* strains to form ACC, we
 407 analyzed all the *Microcystis* strains by SEM-EDXS (Fig. 6 and Fig. S6). For each strain, several
 408 cell aggregates were randomly selected, and EDXS maps were obtained. Each cell had a variable
 409 number of inclusions, ranging from zero to a hundred. Based on EDXS elemental mapping, all
 410 *Microcystis* strains contained inclusions enriched in P, containing Mg, K, and Ca were
 411 interpreted as polyphosphate (polyP) granules. Several functions of polyP inclusions have been

412 inferred in bacteria, including the provision of an alternative source of energy under some
413 specific environmental conditions[10]. Observation of polyP inclusions in *Microcystis* is
414 consistent with the presence of polyP IR bands [8-10] in all *Microcystis* strains [Table 2, Fig. 4].
415 Qualitatively, the number of polyP inclusions vary among different *Microcystis* strains as
416 observed in SEM micrographs, which suggests that the amount of polyP content varies across
417 *Microcystis* strains (Fig. 6 and Fig. S6). The variation in polyP content across *Microcystis* strains
418 is also reflected in the differences observed in peak height of the polyP bands [8-10] present in
419 *Microcystis* strains (Fig. 4). In contrast to polyP inclusions, inclusions enriched in Ca with little
420 or no P were interpreted as ACC. They were only found in strains PMC 814, 820, 827, and 831,
421 which was perfectly consistent with FTIR results. Altogether, based on the presence/absence of
422 the mid-IR U_2 absorption band at 860 cm^{-1} and a broad band at $\sim 300\text{ cm}^{-1}$ in FIR region, both
423 characteristics of ACC, and using SEM-EDXS as a supporting tool, we identify four out of eight
424 *Microcystis* strains that are able to form ACC, whereas the rest of the *Microcystis* strains do not
425 form ACC, at least not in sufficient amount to be detected by FTIR spectroscopy and/or under
426 the growth conditions that were used in the present study.

427 **Semi-quantitative estimation of ACC in *Microcystis***

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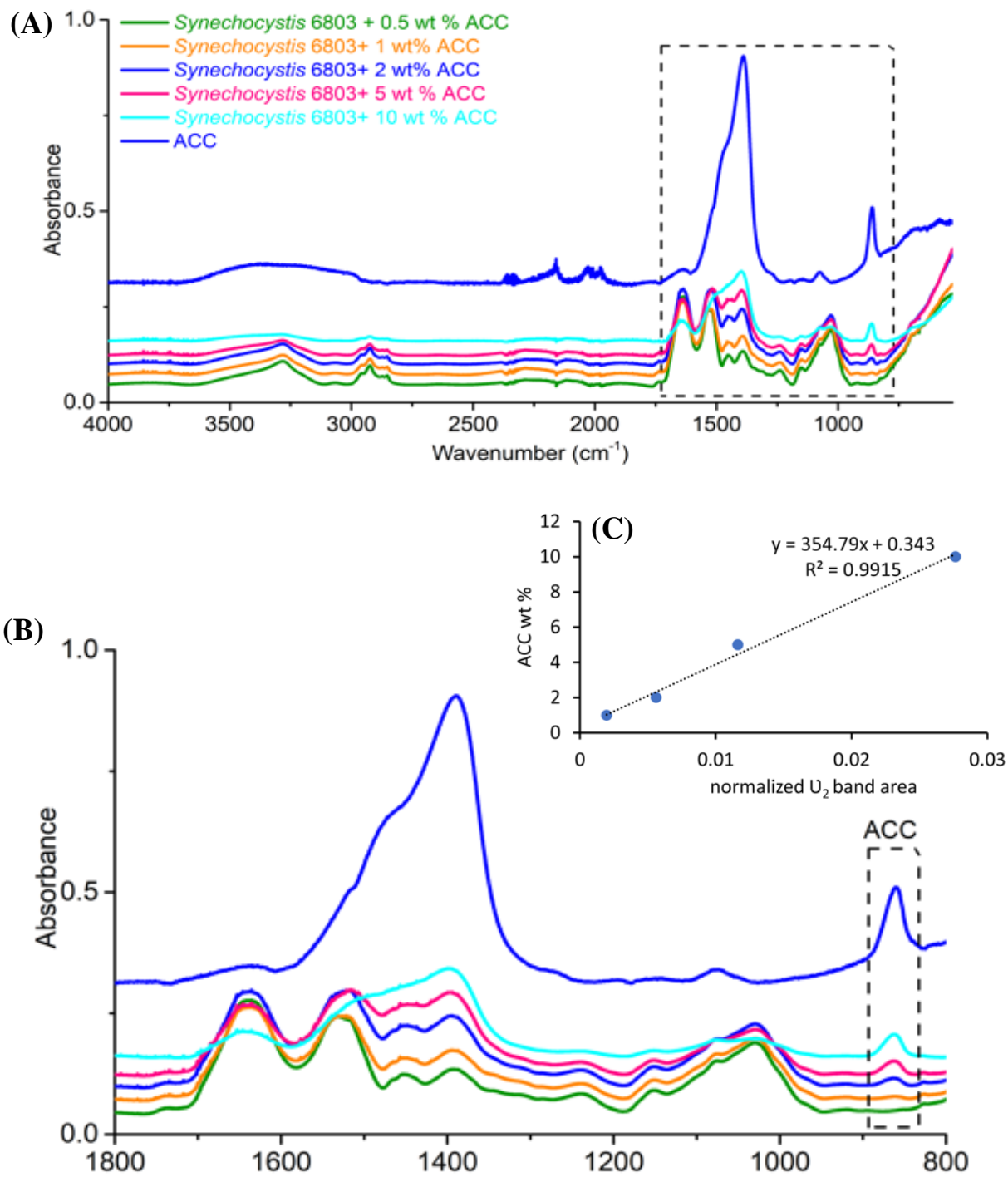


Figure 7: (A) ATR-FTIR spectra of *Synechocystis* PCC 6803-ACC mixtures. (B) Zoomed plot of the boxed region in (A). The peak intensity of U₂ band of ACC was normalized by the frequency region marked by dashed rectangle in (A). (C) The FTIR calibration curve of normalized peak area of U₂ band for different *Synechocystis* PCC 6803-ACC mixtures.

430 The relative intensity of FTIR spectral bands such as proteins, silicate, lipids have frequently
431 been used to correlate the chemically measured content of cellular biomolecules^{74,75}. In this
432 study, the intensity of the 860 cm⁻¹ band is used to compare the variation in ACC content in
433 *Microcystis* strains identified as ACC+ strains. To do so, we first measured a calibration curve to
434 assess the relationship between ACC wt % in a physical mixture of *Synechocystis* PCC 6803
435 (ACC-) +synthetic ACC and the normalized 860 cm⁻¹ band area. Mid-IR spectra of different
436 *Synechocystis* PCC 6803 +synthetic ACC mixtures are shown in Fig. 7A. The higher the ACC wt
437 % in the mixtures, the lower the contribution of the organics to the characteristic ACC bands at
438 1474 (U₃), 1070 (U₁), and 1640 (O-H bending) was observed (Fig. 7B). An increase of the ACC
439 content in the mixtures correlated with an increase of the 860 cm⁻¹ band (Fig. 7B). To correct for
440 possible variations in the path length (thickness of individual cyanobacterial mixture), and water
441 content, all spectra were area-normalized. In the literature, the integrated area under the amide
442 I/II band has been used for normalizing diverse peak areas of interest⁷⁶. However, for the
443 different mixtures of the present study, the relative absorption was not constant for both amide I
444 and II bands (Fig. 7B). Moreover, amide bands suffer from interferences with vibrations
445 associated with ACC structural water (O-H bending at 1650 cm⁻¹) and the U₃ band of ACC at
446 1474 cm⁻¹ (Fig. 7B). Therefore, in our study, it was more appropriate to use the integrated area
447 between 1800-832 cm⁻¹ frequencies, to normalize the area under the 860 cm⁻¹ band (between
448 880-832 cm⁻¹). The computed changes in the normalized area of the peak at 860 cm⁻¹ are plotted
449 against the ACC wt % in the mixtures (Fig. 7C). A strong linear correlation is found between the
450 normalized area of the 860 cm⁻¹ band and the amount of ACC present in the *Synechocystis* PCC
451 6803 + ACC mixtures. We applied this relationship to estimate the ACC content in ACC+

452 *Microcystis* strains. As shown in Table 4, the “calculated ACC” content in ACC+ *Microcystis*
453 strains varied between 0.5-3% wt ACC (Table3). This calculated ACC content was compared

Table 4: The calculated ACC (wt %) content in 4 *Microcystis* strains, using the calibration curve equations determined on artificial *Synechocystis* PCC 6803 -ACC mixtures.

Strain	Normalized U ₂ band area	Calculated ACC (wt%)	Derived ACC content*
PMC 814	4.00E-04	0.48	0.91
PMC 820	8.00E-03	3.18	4.23
PMC 827	2.38E-03	1.19	3.92
PMC 831	7.20E-04	0.60	

* Derived based on the assumption that the total Ca concentration measured on ICP-OES in the sample constitutes ACC reservoir.

454
455 with the ACC content estimated from the measured total Ca concentration in the dried biomass,
456 referred to as the “derived ACC content” in Table 4. Although the estimated absolute value of
457 derived and calculated ACC content in strains were different, the trends were similar, that is
458 *Microcystis* PMC 820 had the highest ACC content, followed by *Microcystis* PMC 827. Similar
459 variations in the ACC content of diverse strains have been observed before. For the known
460 ACC+ cyanobacteria strains, the maximum amount of ACC content can vary from 2-8 wt%¹¹.
461 Reasons for such variations in the ACC content are not clear and will need to be further assessed
462 by future studies. It could be linked with the varying physiological state of the cells, or a
463 different function of ACC in these strains^{10,11}.

464 **Conclusions**

465 This study probed intact cells of cyanobacteria forming intracellular ACC inclusions using FTIR
466 spectroscopy, to identify diagnostic IR spectral features of ACC that are suitable for its detection
467 in systems dominated by prokaryotes. ACC has several IR active bands that have been widely
468 used to discriminate ACC from other CaCO₃ polymorphs. While several of these bands were

469 found to overlap with characteristic bands of proteins, lipids, polysaccharides, and phosphate
470 groups, two ACC characteristic bands at 860 cm^{-1} and a broad peak at $\sim 306\text{ cm}^{-1}$ were free from
471 any overlap from biomolecules. Moreover, the bands at 860 cm^{-1} and a broad peak at $\sim 306\text{ cm}^{-1}$
472 were systematically present only in ACC+ cyanobacteria and absent in ACC- cyanobacteria,
473 suggesting that both bands could serve as a spectral marker to screen strains for their capability
474 of forming ACC. The reliability of these diagnostic features of ACC was tested on several
475 *Microcystis* strains whose ACC-forming capabilities were unknown. Using mid-IR and far-IR
476 ACC spectral features, we identified four out of eight *Microcystis* strains as ACC forming
477 strains. These findings were confirmed by SEM-EDXS observations. Lastly, we quantified the
478 ACC content of *Microcystis* strains using the variations in 860 cm^{-1} band intensity. While the
479 semi-quantification approach used here is an approximation, it provides some insights into
480 assessing the Ca accumulation potential of the ACC-forming *Microcystis* strains and other
481 prokaryotes and their relevance as a geochemical reservoir of Ca in the environment. Overall,
482 FTIR spectroscopy in the mid-IR and far-IR range appear as a very valuable and reliable tool to
483 diagnose the presence of ACC in bacteria and screen efficiently numerous samples before using
484 more time-consuming techniques such as electron microscopy. Whether these spectral features
485 are effective in detecting ACC-forming prokaryotes in a complex environmental sample
486 composed of different microbial populations as well as extracellular minerals remains an
487 interesting topic for future research.

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