



HAL
open science

Evidence of high Ca uptake by cyanobacteria forming intracellular CaCO₃ and impact on their growth

Alexis de Wever, Karim Benzerara, Margot Coutaud, Géraldine Caumes, Melanie Poinsoot, Fériel Skouri-panet, Thierry Laurent, Elodie Duprat, Muriel Gugger

► To cite this version:

Alexis de Wever, Karim Benzerara, Margot Coutaud, Géraldine Caumes, Melanie Poinsoot, et al.. Evidence of high Ca uptake by cyanobacteria forming intracellular CaCO₃ and impact on their growth. *Geobiology*, 2019, 10.1111/gbi.12358 . hal-02285144

HAL Id: hal-02285144

<https://hal.sorbonne-universite.fr/hal-02285144v1>

Submitted on 12 Sep 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Evidence of high Ca uptake by cyanobacteria forming intracellular CaCO₃**
2 **and impact on their growth**

3
4 Running title: Ca homeostasis in cyanobacteria

5
6 De Wever Alexis¹, Benzerara Karim^{1*}, Coutaud Margot¹, Caumes Géraldine¹, Poinso
7 Mélanie¹, Skouri-Panet Fériel¹, Laurent Thierry², Duprat Elodie¹, Gugger Muriel²

8
9 ¹Sorbonne Université, Muséum National d'Histoire Naturelle, UMR CNRS 7590, Institut de
10 Minéralogie, de Physique des Matériaux et de Cosmochimie, 4 place Jussieu, 75005 Paris,
11 France

12 ²Collection des Cyanobactéries, Institut Pasteur, 75724 Paris Cedex 15, France

13
14 **Keywords: calcium; intracellular biomineralization; cyanobacteria; ACC**

15
16 * Corresponding author

17 Tel.: +33(0)144277542

18 E-mail address: karim.benzerara@upmc.fr

19
20 Published in *Geobiology*

21
22
23
24
25
26
27
28
29
30
31
32

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57

Abstract

Several species of cyanobacteria biomineralizing intracellular amorphous calcium carbonates (ACC) were recently discovered. However, the mechanisms involved in this biomineralization process and the determinants discriminating species forming intracellular ACC from those not forming intracellular ACC, remain unknown. Recently, it was hypothesized that the intensity of Ca uptake (i.e., how much Ca was scavenged from the extracellular solution) might be a major parameter controlling the capability of a cyanobacterium to form intracellular ACC. Here, we tested this hypothesis by systematically measuring the Ca uptake by a set of 52 cyanobacterial strains cultured in the same growth medium. The results evidenced a dichotomy among cyanobacteria regarding Ca sequestration capabilities, with all strains forming intracellular ACC incorporating significantly more calcium than strains not forming ACC. Moreover, Ca provided at a concentration of 50 μM in BG-11 was shown to be limiting for the growth of some of the strains forming intracellular ACC, suggesting an overlooked quantitative role of Ca for these strains. All cyanobacteria forming intracellular ACC contained at least one gene coding for a mechanosensitive channel which might be involved in Ca in-flux as well as at least one gene coding for a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger and membrane proteins of the UPF0016 family which might be involved in active Ca transport either from the cytosol to the extracellular solution or the cytosol towards an intracellular compartment. Overall, massive Ca sequestration may have an indirect role by allowing the formation of intracellular ACC. The latter may be beneficial to the growth of the cells as a storage of inorganic C and/or a buffer of intracellular pH. Moreover, high Ca scavenging by cyanobacteria biomineralizing intracellular ACC, a trait shared with endolithic cyanobacteria, suggests that these cyanobacteria should be considered as potentially significant geochemical reservoirs of Ca.

58 1. Introduction

59 Cyanobacteria have played an important role in mediating the formation of carbonate
60 sedimentary deposits such as stromatolites for billions of years (Golubic & Lee, 1999;
61 Altermann, Kazmierczak, Oren, & Wright, 2006). It has been usually suggested that this occurs
62 through extracellular carbonatogenesis (e.g., Lee, Apel, & Walton, 2004; Riding, 2006;
63 Kamennaya, Ajo-Franklin, Northen, & Jansson, 2012; Bundeleva et al., 2014). This process
64 may be associated with CO₂-concentrating mechanisms, which comprises a set of diverse
65 molecular mechanisms contributing to the concentration of inorganic carbon within cells
66 (Riding, 2006; Jansson & Northen, 2010; Jiang, Cheng, Gao, & Qiu, 2013). More specifically,
67 bicarbonates (HCO₃⁻) are incorporated actively and transformed into CO₂ within carboxysomes
68 for fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Price, Maeda, Omata, &
69 Badger, 2002). This transformation releases OH⁻ which are balanced by the import of H⁺ so that
70 the intracellular pH of the cells keeps regulated at a near neutral value (e.g., Belkin & Boussiba,
71 1991; Jiang, Cheng, Gao, & Qiu, 2013). This locally raises the extracellular pH, inducing
72 CaCO₃ precipitation (Merz, 1992). Moreover, the intracellular import of H⁺ is associated with
73 an export of Ca²⁺ in some cyanobacteria, which also favors extracellular CaCO₃ precipitation
74 (Waditee et al., 2004). Overall, biomineralization of CaCO₃ by cyanobacteria has been
75 traditionally considered as a non-controlled and extracellular process (Riding, 2006).

76 However, this dogma has been recently challenged by the discovery of several
77 cyanobacterial species forming intracellular amorphous calcium carbonates (ACC) (Couradeau
78 et al., 2012; Benzerara et al., 2014). These cyanobacteria were found in diverse environments
79 all around the world (Ragon, Benzerara, Moreira, Tavera, & Lopez-Garcia, 2014). This ACC
80 formation capability is a synapomorphy at least in some cyanobacterial groups and may have
81 appeared several hundred million years ago (Benzerara et al., 2014). Interestingly,
82 *Gloeomargarita lithophora*, which forms intracellular ACC, is the closest modern relative of

83 plastid and bears information on the evolution of photosynthesis in eukaryotes by
84 endosymbiosis of a cyanobacterium sometimes during the Proterozoic (Ponce-Toledo et al.,
85 2017). Yet, the formation of intracellular ACC in these cyanobacteria is surprising. Indeed,
86 considering the pH and the concentrations of HCO_3^- and Ca^{2+} in the cytoplasm of cyanobacterial
87 cells (Badger & Andrews, 1982; Belkin & Boussiba, 1991; Barrán-Berdón, Rodea-Palomares,
88 Leganes, & Fernandez-Pinas, 2011), precipitation is not thermodynamically possible (Cam,
89 Georgelin, Jaber, Lambert, & Benzerara, 2015). Based on the size distribution and spatial
90 location of intracellular Ca-carbonates in diverse strains, several nucleation sites have been
91 suggested for these precipitates, including 1) carboxysomes for cyanobacteria showing ACC
92 inclusions throughout their cells or 2) cytoskeletal proteins for cyanobacteria with ACC
93 inclusions located at their septum and their poles (Li et al., 2016). Blondeau et al. (2018a)
94 showed by using cryo-electron microscopy of vitreous sections, that intracellular ACC were
95 systematically enclosed within an envelope which could be a protein shell or a lipid monolayer,
96 suggesting that chemical conditions (e.g., Ca^{2+} concentration) within these vesicles might be
97 different from those in the cytosol and more suitable to ACC precipitation.

98 However, differences in ability to manage Ca between cyanobacterial strains forming
99 intracellular ACC and other strains of cyanobacteria are still unclear. Calcium is notoriously
100 essential for all eukaryotes or prokaryotes as it fills multiple biological roles, including cellular
101 signaling for cell structure maintenance, gene expression, cell cycle and cell differentiation
102 processes, such as the development of heterocysts in cyanobacteria (e.g., Dominguez, 2004). In
103 microalgae, Ca is also an essential co-factor of the oxygen-evolving complex of photosystem
104 II (Debus, 1992). In contrast, Ca may be toxic at high cytosolic concentrations (Clapham, 2007).
105 Overall, the current view is that the intracellular concentration of dissolved Ca^{2+} is regulated in
106 a tight and very low concentration range (~100 nM), involving buffers usually composed of
107 proteins with high Ca binding-affinities, which can release or trap Ca^{2+} (e.g., Gilabert, 2012;

108 Dominguez et al., 2015). Intracellular ACC may serve as an additional overlooked inorganic
109 Ca-buffer. It has been demonstrated that *Gloeomargarita lithophora* C7, *Cyanothece* sp. PCC
110 7425 and *Thermosynechococcus elongatus* BP-1, three cyanobacteria forming ACC inclusions,
111 strongly incorporate dissolved Ca²⁺, whereas *Gloeocapsa* sp. PCC 73106, which does not form
112 intracellular ACC, incorporates only limited amounts of Ca intracellularly (Cam et al., 2018).
113 Based on these results, Cam et al. (2018) hypothesized that the ability to strongly incorporate
114 Ca might be a specificity of cyanobacterial strains forming CaCO₃ inclusions and that
115 differences in Ca homeostasis may therefore explain why some cyanobacteria form intracellular
116 ACC while others do not. Here, we tested that hypothesis by surveying the uptake of dissolved
117 Ca by a much larger set of cyanobacterial strains, including some forming intracellular ACC
118 and other not forming intracellular ACC. Moreover, we searched whether cyanobacteria
119 forming intracellular ACC need higher amounts of Ca for growth. Last, we searched in the
120 genomes of these cyanobacteria potentially shared genes coding proteins involved in Ca
121 transport.

122 2. Material & Methods

123 2.1. Strains and culture conditions

124 Fifty-two cyanobacterial strains scattered throughout the phylogenetic tree of cyanobacteria
125 (Fig. S1) were tested for their capability to sequester Ca (Table S1). Here, we will refer to
126 strains forming intracellular ACC as ACC+ strains and strains not forming intracellular ACC
127 as ACC- strains. Six of the ACC+ strains were previously studied: *Synechococcus* sp. PCC
128 6312, *Synechococcus lividus* PCC 6716 and PCC 6717, *Cyanothece* sp. PCC 7425,
129 *Chroococciopsis thermalis* PCC 7203, and *G. lithophora* C7 (Benzerara et al., 2014). These
130 strains were isolated from very diverse environments including mesophilic alkaline lakes, hot
131 springs at a temperature up to 53 °C and soils (Benzerara et al., 2014). Ten additional strains

132 phylogenetically close to these ACC+ strains were tested for their capability to form
133 intracellular ACC: *Cyanothece* sp. PCC 8303 (isolated from a thermal resort in the Vosges,
134 France), PCC 8955 (isolated from JB Bokassa's swimming pool in Central African Republic)
135 and PCC 9308, *Synechococcus* sp. PCC 6603 (isolated from a freshwater pond in California)
136 and PCC 6715 (isolated from a hot spring in Yellowstone), *Chroococidiopsis* sp. PCC 7432
137 (isolated from a water spring in Pinar del Rio, Cuba), PCC 7433 (isolated from a dried pool in
138 Cuba), PCC 7434 (isolated from a pool in a botanical garden in Havana, Cuba), PCC 7439
139 (isolated from a sand beach in Romania) and PCC 9819 (Table S1). Finally, 36 strains were
140 previously shown to not form intracellular ACC (Benzerara et al., 2014). Fifty-one strains
141 named PCC were axenic and were available from the Pasteur culture collection of cyanobacteria
142 (PCC). *G. lithophora* was isolated from Lake Alchichica (Couradeau et al., 2012) and was not
143 axenic but co-cultured with a single alphaproteobacterium closely related to the genus
144 *Sandarakinorhabdus* (Moreira et al., 2017). All cultures were inoculated at 1/6th, starting from
145 liquid pre-cultures in the stationary phase. They were grown to the stationary growth phase in
146 40 mL of liquid BG-11 (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979), without
147 agitation and under continuous light (8 $\mu\text{mol photon. m}^{-2} \text{ s}^{-1}$). The composition of the BG-11
148 medium was (in g.L^{-1}): NaNO_3 : 1.5; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$: 0.04; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.075; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$:
149 0.036; citric acid: 0.006; ferric ammonium citrate: 0.006; EDTA (disodium magnesium salt):
150 0.001; Na_2CO_3 : 0.02; trace metal mix A5+Co: 1 ml.L^{-1} ; vitamin B12: 10 pg. L^{-1} . Trace metal
151 mix A5+Co contained (in g.L^{-1}): H_3BO_3 : 2.86; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$: 1.81; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.222;
152 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$: 0.390; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: 0.079; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$: 0.0494. Most cultures were
153 grown at 22°C except *Cyanobacterium aponinum* PCC 10605, grown at 25°C, and the
154 thermophilic strains such as *Synechococcus lividus* PCC 6716 and PCC 6717 as well as
155 *Fischerella* sp. PCC 9431, which were grown at 37°C.

156 In parallel to the survey of Ca uptake by these 52 cyanobacterial strains, the growth of 3
157 planktonic ACC+ strains (*G. lithophora* C7, *Cyanothece* sp. PCC 7425 and *Synechococcus* sp.
158 PCC 6312) and 2 planktonic ACC– strains (*Synechococcus elongatus* PCC 7942 and
159 *Synechocystis* sp. PCC 6803) were monitored in duplicates with a higher temporal precision at
160 two different Ca²⁺ initial concentrations: 50 μM and 250 μM. Both concentrations are
161 environmentally relevant and typical of Ca concentrations encountered in alkaline/soda lakes
162 such as Lake Alchichica, where *G. lithophora* was first found (e.g., Zeyen et al., 2019; Boros
163 and Kolpakova, 2018). The standard Ca concentration in BG-11 is 250 μM. The concentration
164 of 50 μM corresponds to the standard Ca concentration in BG-11 divided by 5 and allows to
165 keep a concentration high enough so that it can be simply monitored over time.

166 Cultures were grown at 30°C, under continuous light (5 - 10 μmol photon. m⁻². s⁻¹) and
167 continuous agitation (120 rpm) for all strains, except *G. lithophora* C7 which was grown under
168 lower light intensity (2.5 - 5 μmol photon. m⁻². s⁻¹). In one experiment, cultures of these 5 strains
169 grown at an initial Ca concentration of 50 μM were split in half after 22 days of cultures.
170 Calcium was subsequently added to half of these subcultures at a concentration of 200 μM to
171 complement the initial deficit of Ca compared to the 250 μM of Ca in standard BG-11. No Ca
172 was added to the other half of the cultures.

173 2.2. Growth measurements

174 For comparison of Ca uptake by the fifty-two strains, growth was assessed by dry weight
175 measurements since some of the strains tended to form aggregates and could not be simply
176 numbered by optical density (OD) measurements. For this purpose, 200 μL of cultures were
177 deposited on 0.22 μm GTTP filters (Millipore) weighed beforehand using an XP6 ultra
178 microbalance (Mettler-Toledo). The GTTP filters were weighed after one week of drying at
179 45°C. The difference between the weight before and after filtering of the cultures provided the

180 mass of dry matter in $\text{g}\cdot\text{L}^{-1}$. Measured cell masses in 200 μL of culture amounted between 18
181 and 848 μg depending on the strains. The precision on this measurement was 1 μg . Cell growth
182 of the five planktonic strains cultured with 50 and 250 μM of Ca was compared by measuring
183 optical density at 730 nm (OD_{730}) every 2 to 3 days. The instrumental precision on OD
184 measurements was 0.005.

185 *2.3. Chemical analyses*

186 The concentration of dissolved Ca and the pH of the cultures were systematically measured at
187 the stationary phase for all fifty-two strains. Solution pH was measured on 0.5 μL of non-
188 filtered culture samples using a combined pH microelectrode (Fisherbrand). The accuracy of
189 pH measurements was estimated at ~ 0.01 units. The dissolved Ca concentrations were
190 measured by inductively coupled plasma atomic emission spectrometry (ICP-AES), using a
191 Thermo Scientific iCAP 6200 ICP emission spectrometer. For this purpose, cultures were
192 filtered at 0.22 μm . Depending on samples, from 200 to 400 μL of the filtrate were diluted in
193 10 mL of 2% HNO_3 . Three measurements were performed for each sample. The 2% HNO_3
194 solution was analyzed as a control to assess the contamination of the HNO_3 reagent by Ca^{2+} .
195 The Ca contamination was always lower than 6 ppb.

196 *2.4. Transmission electron microscopy*

197 Transmission electron microscopy analyses were performed on the ten PCC strains not tested
198 before for their capability to form intracellular ACC. Moreover, six ACC- strains were
199 analyzed to determine the chemical composition of their polyphosphate granules. For these
200 analyses, 0.5 mL of the cultures were centrifuged at 5000 g for 10 min. The cell pellets were
201 washed three times with milli-Q water and resuspended in 0.5 mL of milliQ water for analysis
202 by scanning transmission electron microscopy (STEM). Washing was necessary to avoid the

203 precipitation of salts upon drying but did not alter intracellular ACC as shown by Blondeau et
204 al. (2018b). After washing, 3 μ L of the cell suspensions were deposited on 200 mesh Formvar™
205 carbon coated copper grids and dried at room temperature. The grids were made hydrophilic
206 beforehand by glow discharge, i.e. exposition for 30 s to an Ar⁺ plasma. STEM analyses were
207 performed using a JEOL 2100F microscope equipped with a field emission gun and operating
208 at 200 kV. STEM images were acquired in the high angle annular dark field (HAADF) mode
209 with a probe size of 0.7 to 1 nm. Elemental mapping was performed based on energy dispersive
210 x-ray spectrometry (EDXS) analyses using the JEOL Analysis Station software. Semi-
211 quantitative analyses of EDXS spectra were processed to assess the Ca/Mg ratios of the
212 polyphosphate in the cells following the procedure by Li et al. (2016) based on the use of K
213 factors which provide the relationship between peak intensity and the element quantity. The
214 hypothesis that Ca/Mg ratios of the polyphosphate granules were significantly different
215 between ten ACC+ strains (number of polyphosphate granules, n=136) and six ACC- strains
216 (n=133) was tested by a statistical non-parametric Wilcoxon-Mann-Whitney procedure, which
217 does not require the assumption of normal distributions. This analysis was performed using the
218 software R version 3.2.0 (Team, 2013).

219 *2.5. Search of Ca-related transport genes in the genomes of cyanobacteria forming* 220 *intracellular ACC*

221 Several families of Ca-related transport proteins have been described in the literature. Three
222 hundred and thirty-nine reference sequences of known proteins involved in Ca transport were
223 retrieved from the transporter classification database (TCDB, Saier et al., 2015) by using the
224 substrate search tool (with Ca as requested substrate). Their homologs were searched in all the
225 available genomes of cyanobacteria forming intracellular ACC (Table 1) by BLAST with an e-
226 value threshold of 1e-05, resulting in 4280 sequences. Each sequence was further searched for

227 similarity with known domain profiles using CD-search (Marchler-Bauer et al., 2016; CDD
 228 database, version 3.6). The best specific hit (according to the hit classification provided by CD-
 229 search) was kept to validate the functional annotation of the sequences and their specific
 230 implication in Ca transport. As the genomes of PCC 6716 and PCC 6717 have not been
 231 structurally annotated yet, their genomic sequences were explored using tBLASTn in order to
 232 search for homologs of the Ca-related proteins previously found in the five other genomes. The
 233 hits were further validated with CD-search. Transport proteins shared by ACC+ strains were
 234 also searched in the available genomes of ACC- strains.

235

Transport type	Function	specific hit (CDD profile)	<i>G. lithophora</i> C7 NZ_CP017675.1	<i>S. sp.</i> PCC 6312 NC_019680.1	<i>S. lividus</i> PCC 6715 NZ_CP01809 2.1	<i>S. lividus</i> PCC 6716 Unpublished	<i>S. lividus</i> PCC 6717 Unpublished	<i>C. thermalis</i> PCC 7203 NC_019695.1	<i>C. sp.</i> PCC 7425 NC_011884.1	Reference
Active transport	Ca ²⁺ ATPase	cd02089	0	0	0	0	0	AFY89136.1, AFY89473.1	ACL44608.1	Berkelman, Garret-Engel, & Hoffman, 1994
	apnhaP (K ⁺ /H ⁺ antiporter, Ca ²⁺ /H ⁺ antiporter at alkaline pH)	COG0025	APB33279.1	AFY60943.1, AFY61477.1, AFY62329.1	ATS18144.1	1	1	AFY86316.1, AFY86773.1, AFY88512.1, AFY89267.1, AFY89685.1	ACL43274.1, ACL45641.1, ACL45953.1, ACL47119.1	Waditee et al., 2001
	UPF0016 (Putative Ca ²⁺ /cation antiporter)	COG2119, pfam01169	APB33251.1, APB33252.1	AFY59528.1, AFY61632.1, AFY62270.1	ATS18363.1, ATS18364.1	1	1	AFY86178.1, AFY86627.1, AFY86628.1	ACL43789.1, ACL46990.1, ACL46991.1	Demaegd et al., 2014
	Ca ²⁺ /H ⁺ antiporter	COG0387	APB32733.1	AFY62574.1	ATS17843.1	1	1	AFY88666.1, AFY88667.1	ACL43313.1, ACL45336.1	
	Ca ²⁺ /Na ⁺ antiporter	pfam01699	APB34699.1	0	ATS18027.1	0	1	AFY89133.1	ACL44147.1, ACL45335.1	
Passive transport	Mechanosensitive channel	COG0668	APB35048.1	AFY61749.1	0	0	0	AFY88096.1, AFY88215.1, AFY89282.1, AFY89283.1	0	Domínguez, Guragain, & Patrauchan, 2015
		pfam00924	APB33494.1, APB33956.1, APB32821.1, APB32856.1	AFY60788.1, AFY59468.1, AFY62613.1	ATS17940.1, ATS18073.1	3	2	AFY88498.1	ACL43383.1, ACL43916.1, ACL44170.1, ACL46737.1, ACL47385.1	
	hBI-1 (pH sensitive channel)	pfam01027	0	AFY61003.1	ATS17502.1	1	1	AFY90919.1	ACL43777.1	Chang et al., 2014
	Pit (CaHPO ₄ /H ⁺ symport)	pfam01384, COG0306	0	0	ATS18407.1	1	1	AFY87346.1	ACL43457.1	Domínguez, Guragain, & Patrauchan, 2015

236

237 **Table 1.** Proteins involved in Ca²⁺ transport detected in seven cyanobacterial strains forming
238 intracellular ACC. Protein sequence identifiers (Genbank accessions) are reported.

239 2.6. Phylogenetic analyses

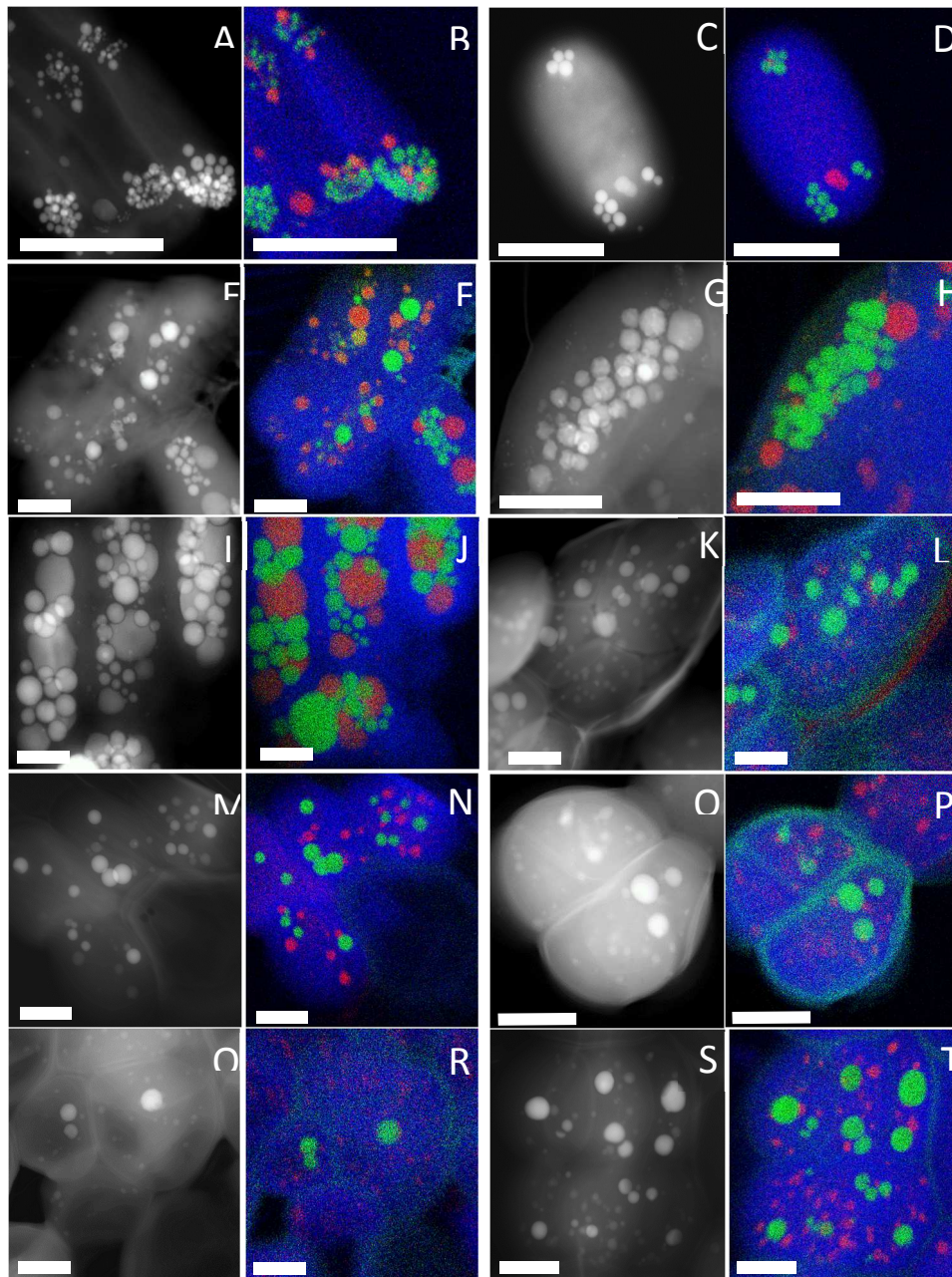
240 The 16S rRNA gene sequences were retrieved from genomes and aligned using CLUSTAL_W
241 (Thompson, Higgins, & Gibson, 1997). One thousand four hundred and fifteen conserved
242 positions in the sequences were considered in order to compute a phylogenetic tree using
243 PHYML (Guindon et al., 2003) and applying the Gamma Time Reversible model. One hundred
244 bootstrap replicates were performed to assess the statistical support of each node and the ones
245 with a value greater than 70% were kept. The sequences were retrieved from GenBank under
246 the accession numbers MK484706 to MK484714.

247 3. Results

248 3.1. Assessing the capability of cyanobacterial strains to form intracellular ACC

249 Among the fifty-two cyanobacterial strains studied here, six strains (*Synechococcus* sp. PCC
250 6312, PCC 6716 and PCC 6717, *Cyanothece* sp. PCC 7425, *Chroococciopsis* sp. PCC 7203,
251 and *G. lithophora* C7) were already known to form intracellular ACC (Benzerara *et al.*, 2014).
252 Additionally, ten strains phylogenetically close to some of these ACC+ strains were tested for
253 their capability to form intracellular ACC (Table S1) : *Cyanothece* sp. PCC 8303, PCC 8905
254 and PCC 9308 were close relatives of *Cyanothece* sp. PCC 7425; *Synechococcus lividus* PCC
255 6715 and *Synechococcus* sp. PCC 6603 were close relatives of *S. lividus* PCC 6716 and PCC
256 6717 and *Synechococcus* sp. PCC 6312, respectively; *Chroococciopsis* sp. PCC 7432, PCC
257 7433, PCC 7434, PCC 7439 and PCC 9819 were close relatives of *Chroococciopsis thermalis*
258 PCC 7203 (Fig. S1). STEM-EDXS analyses showed that these ten strains were also capable to
259 form intracellular ACC in BG-11 (Fig. 1). Cells of *Synechococcus* sp. PCC 6715 and PCC 6603
260 showed ACC inclusions mostly located at their poles as in *Synechococcus* sp. PCC 6312 and *S.*

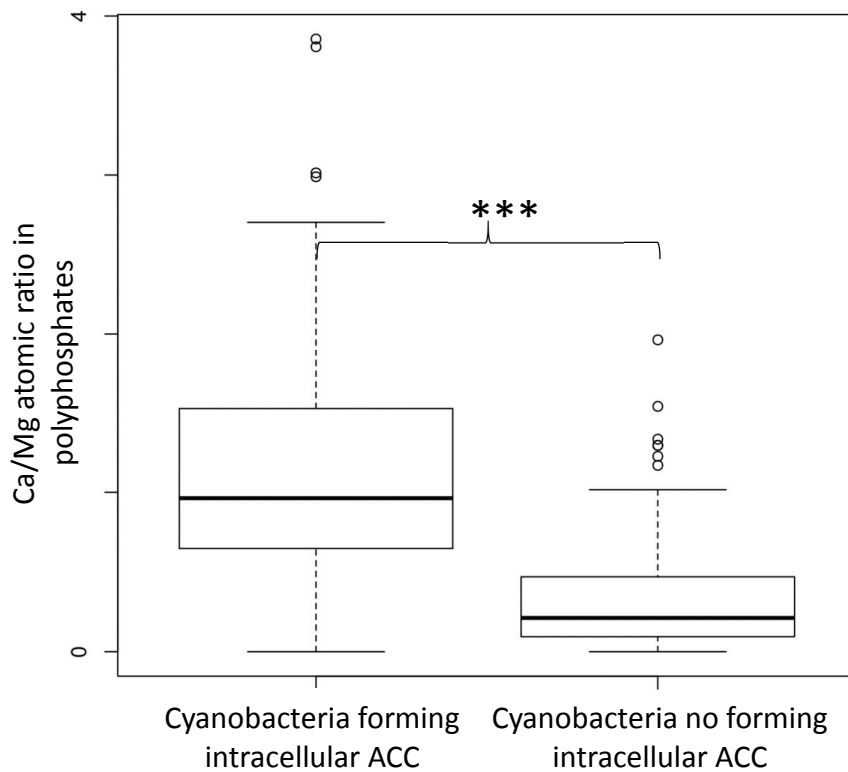
261 *lividus* PCC 6716 and 6717. In contrast, the cells of all other ACC+ strains showed inclusions
 262 scattered throughout the cells similarly to *Cyanothece thermalis* PCC 7425 and
 263 *Chroococciopsis* sp. PCC 7203. Overall, among the 52 analyzed strains, 16 strains formed
 264 intracellular ACC, while 36 did not form intracellular ACC.



265
 266 **Figure 1.** STEM HAADF-EDXS analyses of the 10 strains tested for their capability to form intracellular ACC.
 267 (A) and (B): STEM-HAADF image and EDXS map of *Synechococcus* sp. PCC 6715. (C) and (D): *Synechococcus*
 268 sp. PCC 6603. (E) and (F): *Cyanothece* sp. PCC 8303. (G) and (H) *Cyanothece* sp. PCC 8955. (I) and (J)
 269 *Cyanothece* sp. PCC 9308. (K) and (L) *Chroococciopsis* sp. PCC 7432. (M) and (N) *Chroococciopsis* sp. PCC

270 7433. (O) and (P) *Chroococidiopsis* sp. PCC 7434. (Q) and (R) *Chroococidiopsis* sp. PCC 7439. (S) and (T)
271 *Chroococidiopsis* sp. PCC 9819. For all EDXS maps, calcium is in green, phosphorus in red and carbon in blue.
272 As a result, Ca-carbonates appear in green and PolyP granules in red. All scale bars represent 2 μm .

273 As evidenced by EDXS maps, Ca was mostly contained in intracellular ACC inclusions for
274 these ten strains similarly to their ACC+ relatives. Some Ca was also detected by STEM-EDXS
275 in association with the polyphosphate granules in these strains, which mostly contained Mg as
276 a counter-cation (Fig. 2 and Fig. S2). Interestingly, some Ca was also detected in the
277 polyphosphate inclusions of some of the six strains not forming intracellular CaCO_3 that were
278 analyzed in this study (*Gloeocapsa* sp. PCC 7428, *Synechocystis* sp. PCC 6803, *Oscillatoria*
279 sp. PCC 6304, *Cyanobium gracile* PCC 6307, *Synechococcus* sp. PCC 6301, *Leptolyngbya* sp.
280 PCC 7104). Based on a Wilcoxon-Mann-Whitney statistical analysis, the Ca/Mg ratio of
281 polyphosphates was shown to be significantly higher in ACC+ strains than in ACC- strains
282 (Fig. 2). In one ACC- strain, *Gloeocapsa* sp. PCC 7428, STEM observations showed that a
283 significant amount of Ca was localized on/in the cell wall of the cells, in association with K,
284 Mg and S (Fig. S3).



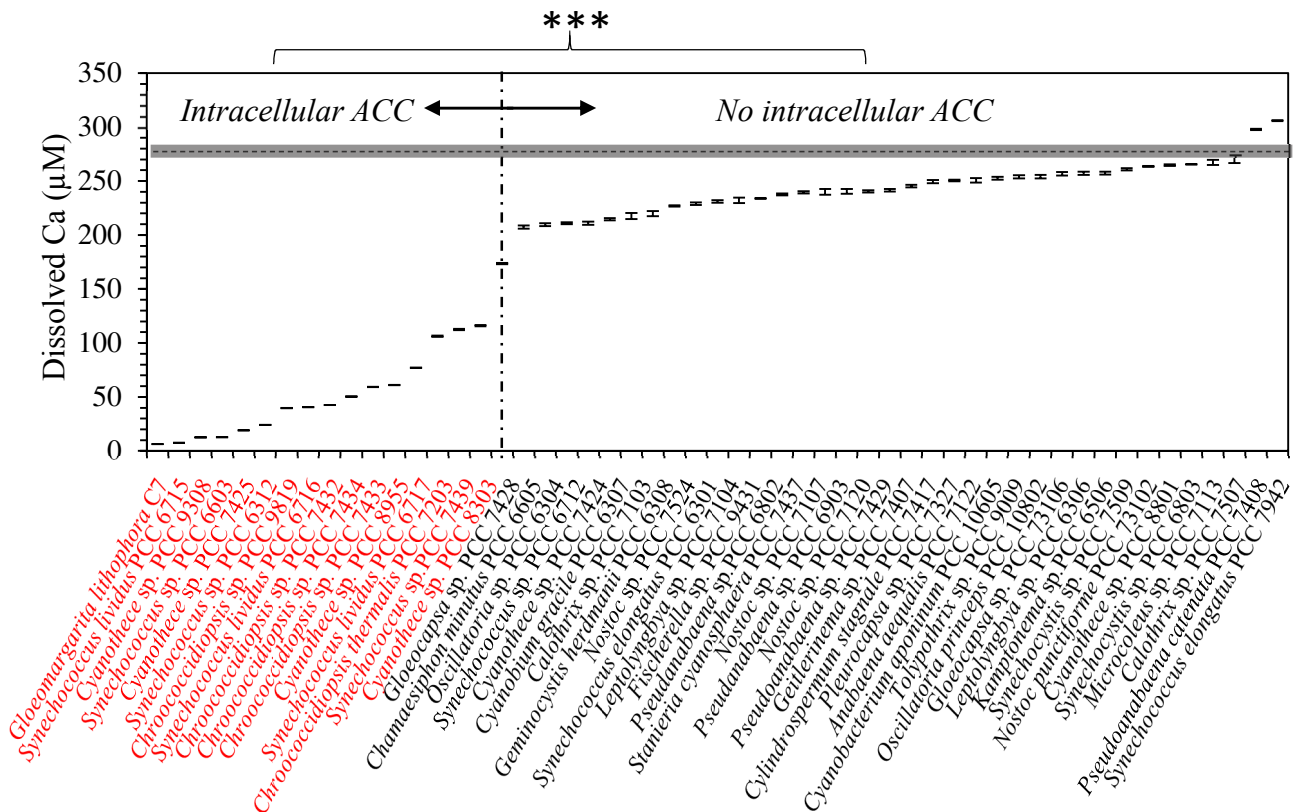
285

286 **Figure 2.** Ca/Mg atomic ratio of polyphosphates in strains forming intracellular ACC (left) vs. strains not forming
 287 intracellular ACC (right). The dataset includes the Ca/Mg ratios of 136 polyphosphates from ten strains forming
 288 intracellular ACC and 133 polyphosphates from six strains not forming intracellular ACC. The Ca/Mg values of
 289 the polyphosphates were calculated based on STEM-EDX analyses. The bold lines indicate the median values; the
 290 boxes span the second and third quartiles; the vertical dashed lines span 1.5 times the extent of the boxes. Open
 291 circles are outliers. The Wilcoxon-Mann-Whitney statistical analysis shows that the difference between the two
 292 groups is significant *** $p < 0.001$.

293 3.2. Assessment of the Ca uptake by the 52 cyanobacterial strains

294 The 52 cyanobacterial strains analyzed in this study were cultured in the same BG-11 medium
 295 (Table S1). The initial concentration of dissolved Ca^{2+} was measured at $281 \mu\text{M} (\pm 6)$. The final
 296 concentration of dissolved Ca^{2+} was measured after 40 to 61 days of incubation depending on
 297 the strains. This final concentration widely varied between strains (Fig. 3). Many strains (36
 298 out of 52) showed little to no Ca uptake, i.e. less than 22% of the initial Ca stock available in
 299 the solution. *Gloeocapsa* sp. PCC 7428 showed an intermediate uptake of ~38% of the initial

300 Ca²⁺ concentration. Sixteen strains showed a high Ca uptake, i.e. higher than 58% of the initial
 301 Ca stock and up to ~98% for *G. lithophora* C7. The very high Ca uptake correlated with the
 302 capability of the strain to form intracellular ACC: all 16 ACC+ strains showed a Ca uptake
 303 higher than ACC- strains (Fig. 3). Significance of this difference was supported by a Wilcoxon-
 304 Mann-Whitney statistical analysis with p <0.001.



305
 306 **Figure 3.** Plot of the concentrations of dissolved calcium remaining after 40 - 61 days of incubation for the 52
 307 tested strains. In red: strains forming intracellular calcium carbonates; in black: strains not forming intracellular
 308 carbonates. The dashed line represents the initial concentration of dissolved calcium in the culture medium (BG-
 309 11). The grey area corresponds to error bars around this value. Error bars were calculated based on the precision
 310 of ICP-AES.

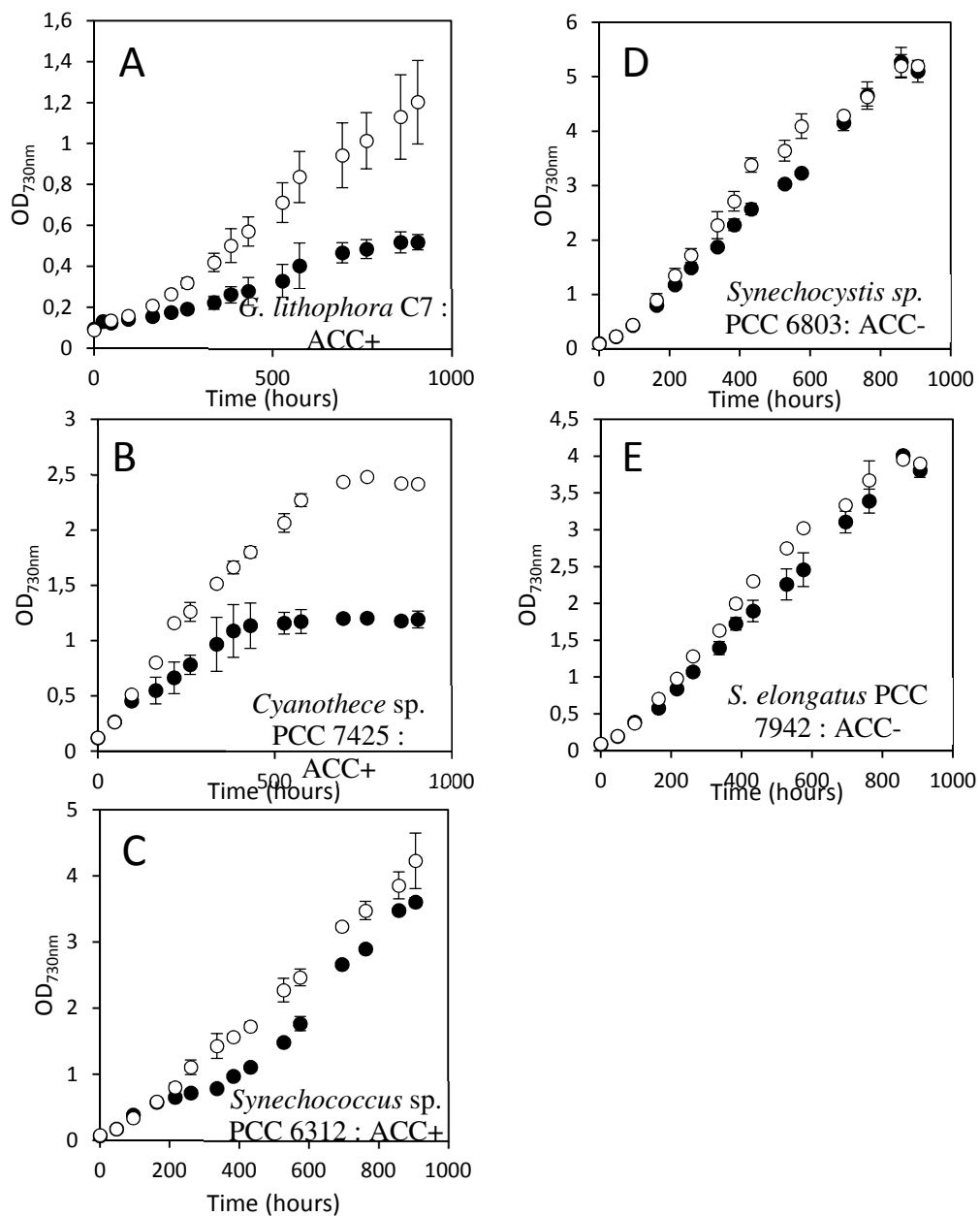
311
 312 Since parameters other than the capability of the cells to form intracellular ACC such as pH,
 313 the final dry mass and incubation duration may *a priori* impact the observed variability of Ca

314 uptake, we tested potential correlations between Ca uptake and these parameters. While the
315 initial pH of the BG-11 medium was 7.5, it systematically increased upon cell growth, reaching
316 a final value between 8 and 11 depending on the strains. No correlation was detected between
317 the final pH and Ca uptake (Fig. S4). Similarly, different strains showed different final dry
318 masses, but dry masses were not correlated with Ca uptake (Fig. S5). Last, we tested the
319 influence of an incubation time of 40 vs 60 days on Ca uptake for three ACC+ strains and two
320 ACC- strains (Fig. S6). Although we observed that uptake increased from 53% to 92% between
321 40 and 60 days for *Synechococcus* sp. PCC 6312, the ranking of the strains in terms of Ca
322 uptake did not vary over this time range. This is also consistent with the analyses by Cam et al
323 (2018), who showed that Ca uptake rate was higher in the first hours of cultures of several
324 ACC+ strains. Overall, these tests supported the conclusion that cultures of ACC+ strains
325 intrinsically showed a higher Ca uptake than ACC- strains regardless of their dry masses, the
326 duration of incubation (40 or 60 days) and/or extracellular pH.

327 *3.3. Impact of the initial concentration of dissolved Ca²⁺ on the growth of cyanobacteria*
328 *forming intracellular ACC*

329 We selected a subset of three strains representing the two types of intracellular ACC
330 distributions: scattered through the cell, i.e. *G. lithophora* C7 and *Cyanothece* sp. PCC 7425,
331 and at the cell poles: *Synechococcus* sp. PCC 6312. Their growth was compared with that of
332 two ACC- strains: *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803. The
333 growth of these three ACC+ strains and two ACC- strains was measured in BG-11 at two initial
334 Ca²⁺ concentrations: 50 μM and 250 μM. *G. lithophora* C7 and *Cyanothece* sp. PCC 7425
335 showed significantly higher growth rates when grown with an initial Ca concentration of 250
336 μM compared to 50 μM (Fig. 4). In contrast, growth was only slightly higher for *Synechococcus*
337 sp. PCC 6312 at an initial Ca concentration of 250 μM (compared to 50 μM) and not
338 significantly different between the two Ca concentrations for *Synechocystis* sp. PCC 6803 and

339 *S. elongatus* PCC 7942. The pH showed similar differences between cultures at 50 and 250 μM ,
 340 reaching significantly higher values at 250 μM for *G. lithophora* C7 and *Cyanothece* sp. PCC
 341 7425 but similar values at 50 and 250 μM for *Synechococcus* sp. PCC 6312, *S. elongatus* PCC
 342 7942 and *Synechocystis* sp. PCC 6803 (Fig. S7).



343
 344 **Figure 4.** Time evolution of OD_{730nm} for cultures in a BG-11 medium with an initial dissolved Ca
 345 concentration of 250 μM (open symbols) and 50 μM (closed symbols). (A) *G. lithophora* C7; (B)
 346 *Cyanothece* sp. PCC 7425; (C) *Synechococcus* sp. PCC 6312; (D) *Synechocystis* sp. PCC 6803; (E) and

347 *S. elongatus* PCC 7942. Error bars represent standard deviations calculated based on variations between
348 duplicates.

349 To further test the growth dependence on the Ca content of the growth medium, cultures with
350 50 μM of Ca were split in half after 527 hours. Calcium was subsequently added to half of these
351 subcultures at a concentration of 200 μM to complement the initial deficit of Ca compared to
352 the 250 μM of Ca in standard BG-11. No Ca was added to the other half of the cultures. For *G.*
353 *lithophora*, the culture was still growing at the time of Ca addition (Fig. 5). Yet, 300 h after Ca
354 addition, the Ca-supplemented subcultures reached an OD_{730} significantly higher than the
355 subcultures with no Ca addition. The difference was even larger for *Cyanothece* sp. PCC 7425.
356 These differences could also be observed on the time evolution of pH (Fig. S8). In contrast, the
357 OD_{730} of the Ca-supplemented subculture of *Synechococcus* sp. PCC 6312 was slightly lower
358 than that with no Ca addition (Fig. 5). The time evolution of the pH was similar for the two
359 subcultures (Fig. S8). For *Synechocystis* sp. PCC 6803 and *S. elongatus* PCC 7942, subcultures
360 with no Ca addition reached significantly higher OD_{730} than Ca-supplemented subcultures (Fig.
361 5).

362 3.4. Genome analyses

363 Genes coding for proteins possibly involved in passive and active transport of Ca were searched
364 and quantified in the annotated genomes of seven ACC+ strains (Table 1). Regarding active
365 transport, all genomes contained at least one copy (e.g., two for *Cyanothece* sp. PCC 7425 and
366 *Chroococciopsis thermalis* PCC 7203) of a gene coding for a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger and between
367 one and three copies of the UPF0016 gene coding for a putative calcium/cation exchanger
368 (Demaegd, Colinet, Deschamps, & Morsomme, 2014). All these genomes also encoded a
369 homolog of a $\text{Na}^{+}/\text{H}^{+}$ antiporter (apnhaP) that has been reported to have a high $\text{Ca}^{2+}/\text{H}^{+}$ antiport
370 activity at alkaline pH (Waditee et al, 2001). In contrast, homologs of other genes coding for

371 transporters possibly involved in Ca transport (Ca^{2+} ATPase, Pit, $\text{Ca}^{2+}/\text{Na}^{+}$ antiporter) were
372 found in some but not all these 7 genomes. Regarding passive transport, at least one copy of a
373 gene coding for a mechanosensitive channel was found in all genomes. A homolog of a pH-
374 sensitive Ca leak channel (Human Bax1 inhibitor) found in some intracellular membranes
375 (Chang et al., 2014) was detected in all genomes but the one of *G. lithophora*.

376 4. Discussion

377 4.1. Cyanobacteria forming intracellular CaCO_3 show a very strong Ca uptake

378 Several previous studies have assessed cell Ca uptake by measuring the temporal
379 changes of dissolved extracellular Ca^{2+} concentration (Singh & Mishra, 2014; Cam et al., 2016;
380 Blondeau et al., 2018b). Yet, changes in dissolved Ca concentrations can *a priori* be due to i)
381 extracellular precipitation of Ca-containing mineral phases, ii) adsorption at the cell surfaces
382 and/or by surface EPS and/or iii) Ca uptake within cells. Here, Ca uptake within cells is argued
383 to be the most important process responsible for the observed differences in the decrease of
384 extracellular dissolved Ca concentration between cultures of ACC+ and ACC- cyanobacterial
385 strains. This conclusion relies on several lines of evidence. First, cultures with higher solution
386 pH should experience higher rates of extracellular Ca-mineral precipitation and/or cell surface
387 adsorption (Bundeleva et al., 2014; Lee, Apel, & Walton, 2004). However, no correlation was
388 detected between pH and the decrease of dissolved Ca concentrations, i.e. the pH in the culture
389 media of ACC+ strains increased to diverse values with no systematic differences with ACC-
390 strains. In some cultures, where pH increased to high values (e.g., pH = 10.56 for
391 *Synechococcus* sp. PCC 6301), only a slight decrease of dissolved Ca concentration was
392 observed ($\Delta([\text{Ca}^{2+}]) = 49.56 \mu\text{M}$). Moreover, STEM observations clearly showed that Ca was
393 mostly contained within intracellular ACC inclusions in ACC+ strains and to a lesser extent
394 within polyphosphates. Overall, this supports the conclusion that the variations observed in the

395 decrease of dissolved Ca concentration was primarily dependent on the capability of the strains
396 to form intracellular ACC. Consistently, Cam et al. (2018) showed that BG-11 remained mostly
397 undersaturated with Ca-carbonate phases in the cultures of three ACC+ strains and that the
398 decrease of dissolved Ca concentrations in these cultures was primarily due to Ca uptake within
399 cells. Overall, the present study, based on the comparison between 52 strains, generalizes the
400 hypothesis formulated by Cam et al. (2018) based on only 4 strains, that cyanobacteria forming
401 intracellular ACC show a Ca uptake systematically higher than other cyanobacteria.

402 Although it is difficult to definitely demonstrate that ACC- strains never form ACC
403 (since this would require to culture them under an infinite number of diverse conditions), it can
404 be concluded that they do not form ACC at least under the specific conditions used here in the
405 present study. Moreover, it should be noted that some of them (e.g., PCC 6803, PCC7942) have
406 been scrutinized at different time points, therefore providing a much more extensive sampling
407 of diverse conditions. Last, we note that under the specific culture conditions we used, there is
408 a relationship between forming/not forming iACC and phylogeny (Fig. S1). This suggests that
409 this trait may be a synapomorphy for the *Chroococcidiopsis* and the *Cyanothece* sp PCC 7425
410 clusters as already mentioned for the *Synechococcus* sp. PCC 6312 cluster by Benzerara et al.
411 (2014) and that it is likely more controlled by genetics than environmental conditions.

412 4.2. Relatively high Ca adsorption by *Gloeocapsa* sp. PCC 7428

413 Although *Gloeocapsa* sp. PCC 7428 showed an affinity for Ca lower than ACC+
414 cyanobacteria, it was significantly higher than other ACC- cyanobacteria. STEM analyses
415 showed that Ca was clearly associated with the cell walls of *Gloeocapsa* sp. PCC 7428. Cell
416 walls of *Gloeocapsa* sp. PCC 7428 have been shown to be composed of a thick extracellular
417 sheath of mucopolysaccharides (Gonzalez-Esquer *et al.*, 2016), which may therefore be
418 responsible for this relatively high Ca sorption capability. Whether this is due to a high surface

419 Ca adsorption capacity of the strain and/or precipitation of extracellular Ca-mineral phases
420 could not be determined here but it is known that there is a continuum between surface
421 adsorption and extracellular mineral precipitation (Warren & Ferris, 1998). Consistently,
422 several studies have stressed on the significant calcification potential of the genus *Gloeocapsa*
423 (Pokrovsky, Martinez, Golubev, Kompantseva, & Shirokova, 2008; Bundeleva et al., 2014).
424 Moreover, cyanobacteria of the Chroococcales order, to which *Gloeocapsa* sp. PCC 7428
425 belong, have been proposed more generally to be particularly efficient at precipitating Ca-
426 carbonates (e.g., Saghai et al., 2015). Overall, the present survey of a large number of
427 cyanobacterial strains supports the idea that *Gloeocapsa* sp. PCC 7428 may be particularly
428 prone among cyanobacteria at inducing the precipitation of extracellular Ca-mineral phases.

429

430 *4.3. Comparison of the Ca content of ACC+ cyanobacterial strains with other known* 431 *bacteria*

432 Here, we normalized the Ca uptake by ACC+ strains to their dry weight and compared
433 them with similar data found in the literature for other bacteria (Table 2). As discussed by Cam
434 et al. (2018), these values did not represent the maximum values that may be achieved by these
435 strains as Ca input was limited to 250 μM in these batch cultures. Yet, the cell-normalized Ca
436 contents measured on cyanobacteria forming intracellular ACC are among the highest content
437 reported in the literature. As a comparison, more classically studied bacteria such as
438 *Escherichia coli*, *Vibrio cholera* or *Acetobacter aceti* contain two orders of magnitude less Ca.
439 In contrast, there are few other bacteria accumulating Ca to a high extent. For example,
440 *Achromatium* spp., a gammaproteobacterium forming intracellular calcite is to our knowledge
441 the strongest Ca-accumulating bacterium that has been reported so far, with a Ca content of
442 65400 fmol of Ca per cell (Gray, 2006). Assuming that the mass of C represents 50% of dry
443 weight, a carbon-to-volume conversion of 0.1 $\text{pgC}/\mu\text{m}^3$ for bacteria (Norland, Haldal, & Tumyr,

444 1987) and a cell volume of $3 \times 10^4 \mu\text{m}^3$ (Gray, 2006), this equals to 436 mg of Ca per gram of
445 dry matter, i.e. an order of magnitude higher than for cyanobacteria forming intracellular ACC.
446 *Bacillus cereus* spores also sequester high amounts of Ca on the same order of magnitude as
447 cyanobacteria forming intracellular ACC but with a different speciation, i.e. as Ca^{2+} -dipicolinic
448 acid chelates, incorporated in the spore cores (Steward, 1980).

Name	Type of organism	Ca uptake		Notes	Reference
		[HCl]mg/g of dry matter	fmol/cell		
Archaea					
<i>Haloferax volcanii</i>		0.36			Novoselov et al., 2017
<i>Natrialba magadii</i>		0.31			Novoselov et al., 2017
Bacteria					
Actinobacteria					
<i>Micrococcus roseus</i>		0.08			Rouf, 1964
<i>Nesterenkonia lacusekhoensis</i>		0.06			Novoselov et al., 2017
Firmicute					
<i>Alicyclobacillus acidoterrestris</i>		0.39			Novoselov et al., 2017
<i>Bacillus cereus</i> (vegetative cells)		0.3			Rouf, 1964
<i>Bacillus cereus</i> (spores)		28.00 to 30.00			Steward et al., 1980
Proteobacteria					
<i>Acetobacter aceti</i>		0.07			Novoselov et al., 2017
<i>Achromatium</i> spp.		436	65400	intracellular calcite	Gray, 2006
<i>Alcaligenes marinus</i>		0.09			Jones, Royle, & Murray, 1979
<i>Escherichia coli</i>		3.21			Novoselov et al., 2017
		1.10			Lawford & Rousseau, 1995 ; Demain & Solomon 1981
		0.11			Novoselov et al., 2017
		0.11			Novoselov et al., 2017
		0.07			Novoselov et al., 2017
		0.06			Novoselov et al., 2017
		0.06			BioMagnetech Corporation, 1990
		0.02			Rouf, 1964
<i>Sphaerotilus natans</i>		0.18			Rouf, 1964
<i>Vibrio cholerae</i>		0.09			Novoselov et al., 2017
Cyanobacteria					
<i>Chroococcidiopsis</i> sp. PCC 7432		8.88		intracellular ACC	This study
<i>Chroococcidiopsis</i> sp. PCC 7433		11.09		intracellular ACC	This study
<i>Chroococcidiopsis</i> sp. PCC 7434		12.55		intracellular ACC	This study
<i>Chroococcidiopsis</i> sp. PCC 7439		11.09		intracellular ACC	This study
<i>Chroococcidiopsis</i> sp. PCC 9819		8.36		intracellular ACC	This study
<i>Chroococcidiopsis thermalis</i> PCC 7203		6.58		intracellular ACC	This study
<i>Cyanothece</i> sp. PCC 7425		17.92	5.4	intracellular ACC	This study
		13.00	3.9	intracellular ACC	Cam et al., 2018
<i>Cyanothece</i> sp. PCC 8303		17.13		intracellular ACC	This study
<i>Cyanothece</i> sp. PCC 8955		22.57		intracellular ACC	This study
<i>Cyanothece</i> sp. PCC 9308		37.69		intracellular ACC	This study
<i>Gloeocapsa</i> sp. PCC 7428		8.34		adsorption/ precipitation	This study
<i>Gloeomargarita lithophora</i> C7		31.41	2.7	intracellular ACC	This study
		26.00	1.7	intracellular ACC	Cam et al., 2018
<i>Mastigocoleus testarum</i>		20.00	100	calcicyte	Guida & Garcia-Pichel, 2016
<i>Microcystis aeruginosa</i> Kützing		0.44			Krivtsov, Bellinger, & Sigeo, 2005
<i>Synechococcus</i> sp. PCC 6312		29.13		intracellular ACC	This study
<i>Synechococcus</i> sp. PCC 6603		42.82		intracellular ACC	This study
<i>Synechococcus lividus</i> sp. PCC 6715		25.46		intracellular ACC	This study
<i>Synechococcus lividus</i> sp. PCC 6716		15.15		intracellular ACC	This study
<i>Synechococcus lividus</i> sp. PCC 6717		33.99		intracellular ACC	This study
<i>Thermosynechococcus elongatus</i> BP-1		15.00	4.8	intracellular ACC	Cam et al., 2018

450 **Table 2.** Ca uptake by diverse prokaryotes compiled from the literature and our data.

451 Among cyanobacteria, the differentiated cells called calcicytes, observed in the
452 filamentous euendolithic cyanobacterium *Mastigocoleus testarum* have been measured to
453 contain 100 fmol of Ca per cell, which measured $\sim 1 \times 10^3 \mu\text{m}^3$ (Guida & Garcia-Pichel, 2016).
454 This represents 20 mg of Ca per g of dry weight, a value similar to that estimated for
455 cyanobacteria forming intracellular ACC (**Table 2**). The speciation of Ca in calcicytes has not
456 been determined so far and the possibility that it is mostly contained in intracellular ACC has
457 not been yet explored to our knowledge. Moreover, Cam et al. (2018) noted that since some
458 cyanobacteria forming intracellular ACC tend to decrease the extracellular concentration of
459 dissolved Ca^{2+} , they may favor the dissolution of extracellular Ca-carbonates. This conclusion
460 can be generalized to all cyanobacteria forming intracellular ACC and future studies should
461 investigate their capabilities to bore into calcium carbonate crystals.

462 4.4. Molecular mechanisms of high Ca sequestration

463
464 Whether cyanobacteria forming intracellular ACC may share Ca-sequestering
465 molecular pathways that are similar and possibly homologous to those of *Mastigocoleus*
466 *testarum*, would be interesting to test in future studies. Since Ca uptake involves Ca transport
467 proteins in any case, we searched genes that might be shared by the seven genomes available
468 for the divergent strains forming intracellular ACC.

469 The influx (from the extracellular solution to the cytosol) of Ca is usually assumed to
470 occur passively through channels, which show little ionic specificity for most of them
471 (Domínguez, Guragain, & Patrauchan, 2015). Here, a mechanosensitive channel was shown to
472 be present in all the ACC+ cyanobacteria. However, it should be noted that mechanosensitive
473 channels can also be found in ACC- cyanobacteria, such as *Synechocystis* sp. PCC 6803
474 (Nazarenko, Andreev, Lyukevich, Pisareva, & Los, 2003). Interestingly, inorganic phosphate

475 transport systems (PitB in *E. coli*) also seem able to transfer divalent cation-HPO₄ neutral
476 complexes intracellularly under some conditions (van Veen, Abee, Kortstee, Konings, &
477 Zehnder, 1994). This could connect Ca uptake with P sequestration which results in the
478 formation of polyphosphate granules in some of these cyanobacteria (e.g., Cam et al., 2018;
479 Blondeau et al., 2018a). However, these transport systems were detected in only some of the
480 ACC+ cyanobacteria.

481 The out-flux of Ca is usually assumed to occur actively through transporters. Out-flux
482 may proceed from the cytosol towards the extracellular solution or from the cytosol towards an
483 intracellular compartment as the one delimitating intracellular ACC (Blondeau et al., 2018a).
484 Here, we identified in all ACC+ cyanobacteria at least one gene coding for a Ca²⁺/H⁺
485 transporter, and at least one gene coding for a putative calcium exchanger of the UPF0016
486 family. Again, it should be noted that Ca²⁺/H⁺ antiports and membrane proteins of the UPF0016
487 family have been identified in numerous other bacteria (Domínguez, Guragain, & Patrauchan,
488 2015). Identifying their localization, in the plasma membrane or in intracellular compartments
489 would help deciphering if they are involved in Ca export extracellularly or within an
490 intracellular compartment. Mansor, Hamilton, Fantle, and Macalady (2015) identified in
491 *Achromatium* a Ca²⁺-ATPase and noted that they could also be found in *C. thermalis* PCC 7203,
492 *Cyanothece* sp. PCC 7425 and *Thermosynechococcus elongatus* BP-1. Here, we confirm that a
493 Ca²⁺-ATPase is indeed present in the genomes of *C. thermalis* and *Cyanothece* sp. but it is
494 absent from the genomes of the five other ACC+ strains. Garcia-Pichel, Ramírez-Reinat, and
495 Gao (2010) also detected a Ca²⁺-ATPase in *Mastigocoleus testarum*, an endolithic
496 cyanobacterium but stressed that this pump extrudes Ca²⁺ outside of the cell, confirming that it
497 is not likely involved in Ca sequestration within cells.

498 Overall, this analysis provides some indications about possible actors involved in Ca
499 homeostasis but it should be noted that this clearly does not provide definitive clues about the

500 mechanisms involved in ACC formation since: 1) similar genes might be found in ACC-
501 cyanobacteria; 2) the diverse cyanobacteria forming intracellular ACC may use different
502 biomineralization mechanisms involving different sets of genes; 3) formation of intracellular
503 ACC likely involves other processes than in- and out-fluxes of Ca. Only future genetic studies
504 targeting and deleting such candidate genes will provide definitive answers about their possible
505 role in the future.

506

507 *4.5. Cause for a high Ca demand by cyanobacteria forming intracellular ACC*

508 Calcium at a concentration of 50 μ M in BG-11 was shown to be limiting for the growth
509 of *Cyanothece* sp. PCC 7425 and *G. lithophora* C7. In contrast, Ca was not limiting at this
510 concentration for the growth of *Synechococcus* sp. PCC 6312. Ca accumulation seems therefore
511 to be more essential for *Cyanothece* sp. PCC 7425 and *G. lithophora* C7 than for *Synechococcus*
512 sp. PCC 6312. Additional differences can be noted between *Synechococcus* sp. PCC 6312 and
513 the two other ACC+ strains: while *Synechococcus* sp. PCC 6312 forms intracellular ACC
514 granules in connection with cell division and between the cytoplasmic membrane and the
515 outermost thylakoids, the two other strains form ACC granules in the center of the cells with
516 no apparent connection with cell division (Benzerara et al., 2014; Blondeau et al., 2018a).
517 Overall, intracellular carbonatogenesis likely follows different pathways in the clade of
518 *Synechococcus* sp. PCC 6312 compared to other cyanobacteria and may have different
519 functions. Li et al. (2016) suggested that in the clade of *Synechococcus* sp. PCC 6312, ACC
520 may form by nucleating on cell division proteins such as FtsZ, which require relatively high
521 Ca^{2+} for polymerization (e.g., Yu & Margolin 1997). If true, this needed amount of Ca may still
522 remain relatively modest compared with the one required by other ACC+ cyanobacteria.
523 Thereafter, we tentatively discussed potential causes for a higher demand in Ca observed for

524 *Cyanothece* sp. PCC 7425 and *G. lithophora* C7. Calcium may either be directly needed for
525 some biochemical processes and/or it may have a more indirect role through its involvement in
526 the formation of intracellular ACC which themselves fill a biological function. Calcium is
527 notoriously essential for bacteria (e.g., Dominguez, 2004). In particular, Ca is a co-factor for
528 cyanobacteria in the water-splitting in photosystem II complex (Debus, 1992). However, since
529 this need is shared by all cyanobacteria, it does not explain the observed higher requirement of
530 some of the ACC+ strains . Few studies have shown a similar need by other bacteria for
531 relatively high Ca concentrations. For example, Webb (1988) reported that the filamentous
532 bacterium, *Haliscomenobacter hydrossis*, grew better at Ca concentrations ≥ 0.69 mM and
533 suggested that relatively high concentrations of Ca may have been needed for the formation of
534 sheath by these bacteria. Shuttleworth and Unz (1991) showed that four strains of the
535 gammaproteobacterium *Thiothrix* sp. and one strain of the betaproteobacterium *Zoogloea*
536 *ramigera* need relatively high Ca concentrations for the formation of their sheath and mitigation
537 of the toxicity of heavy metals present in the culture media. However, since heavy metals were
538 only at trace concentrations in BG-11 and *Cyanothece* sp. PCC 7425, and *G. lithophora* C7 are
539 not filamentous, none of these functions apply to explain the differences in Ca requirements
540 between cyanobacterial strains forming intracellular ACC.

541 Similarly, high amounts of Ca are needed by sporulating bacteria (Stewart 1980) for
542 resistance of spores to wet heat (e.g., Kochan et al., 2018). Again, there is no obvious
543 connection with the cyanobacteria studied here, since they do not sporulate. Last, calcicytes in
544 the filamentous cyanobacterium *Mastigocoleus testarum* also accumulate large amounts of Ca
545 but this accumulation in a few cells has been proposed as a way to keep Ca concentration low
546 in the other cells, which does not apply to unicellular cyanobacteria such as the ones considered
547 here. Moreover, it has been suggested that the significant alkalization of the cytoplasm involved
548 by the presence of Ca^{2+} might be detrimental to the photosynthetic capacity of these cells.

549 Overall, unless a presently unknown biochemical process requiring high amounts of Ca
550 exists in cyanobacteria forming intracellular ACC only, it is possible that ACC+ strains require
551 high amounts of Ca so that they can form significant amounts of intracellular ACC, which are
552 beneficial to their growth. Several biological functions have been suggested for these ACC
553 granules in cyanobacteria by Couradeau et al. (2012) or for intracellular calcite in *A. oxaliferum*
554 (Gray & Head, 2014): 1) they may serve as ballasts for the cells as an adaptation to a benthic
555 form of life. However, this sounds unlikely since *Cyanothece* sp. PCC 7425, and *G. lithophora*
556 C7 were grown in the present study as planktonic cells. 2) Intracellular Ca-carbonates may
557 buffer intracellular pH and balance the formation of hydroxide by conversion of HCO_3^- to CO_2
558 upon carbon fixation. 3) Alternatively, they may serve as a storage form of inorganic carbon
559 available to the cells upon C-limited periods. Only future genetics studies providing mutants
560 impeded in their capability to form Ca-carbonates may help answering this question. Culturing
561 under C-limited conditions coupled with measurements of calcium carbonate dissolution may
562 be helpful too.

563 Whatever their cause, the observed differences in Ca requirement for growth between
564 cyanobacterial strains call for special care when attempting to culture and/or enrich these strains
565 from the environment. The BG-11 medium has proved to be a particularly useful generic growth
566 medium to culture a broad diversity of cyanobacterial strains (Rippka, Deruelles, Waterbury,
567 Herdman, & Stanier, 1979). Here, it is confirmed that 250 μM as a standard Ca concentration
568 provides a good compromise, allowing significant growth of strains forming intracellular
569 CaCO_3 and not being detrimental to strains not forming intracellular CaCO_3 . However, for
570 enrichment cultures, which are performed over extended durations, strains not limited by Ca^{2+}
571 may be favored over time if Ca^{2+} has been consumed by those forming intracellular CaCO_3 .
572 The use of BG-11 alone may therefore hinder the enrichment of the latter. Interestingly, it can
573 be noted that *G. lithophora* was successfully enriched by adding fragments of rocks containing

574 Ca-carbonates, which likely buffered the dissolved $[Ca^{2+}]$ by constant Ca^{2+} input over
575 prolonged periods (Couradeau et al., 2012). Therefore, this strategy should be useful for future
576 studies aiming at enriching ACC+ cyanobacteria.

577

578

579 **Acknowledgments**

580 Funding for this work was provided by the European Research Council under European
581 Community's Seventh Framework Programme FP7/2007-2013 Grant 307110, ERC
582 CALCYAN. ADW was funded by French state funds managed by the ANR within the
583 Investissements d'Avenir programme under reference ANR-11-IDEX-0004-02, within the
584 framework of the Cluster of Excellence MATISSE. The Pasteur Culture Collection of
585 cyanobacteria was supported by the Institut Pasteur (M.G. and T.L.). The SEM facility at
586 IMPMC was purchased owing to funding by Région Ile de France Grant SESAME 2006 I-07-
587 593/R; the transmission electron microscopy facility at IMPMC was purchased owing to
588 funding by Region Ile de France Grant SESAME 2000 E 1435.

589 **References**

- 590 Altermann, W., Kazmierczak, J., Oren, A., & Wright, D. T. (2006). Cyanobacterial calcification
591 and its rock-building potential during 3.5 billion years of Earth history. *Geobiology*, *4*,
592 147-166.
- 593 Badger, M., & Andrews, T. (1982). Photosynthesis and inorganic carbon usage by the marine
594 cyanobacterium, *Synechococcus* sp. *Plant Physiology*, *70*, 517-523.
- 595 Barrán-Berdón, A., Rodea-Palomares, I., Leganes, F., & Fernandez-Pinas, F. (2011). Free Ca^{2+}
596 as an early intracellular biomarker of exposure of cyanobacteria to environmental
597 pollution. *Analytical and Bioanalytical Chemistry*, *400*, 1015-1029.
- 598 Belkin, S., & Boussiba, S. (1991). High internal pH conveys ammonia resistance in spirulina
599 platensis. *Bioresource Technology*, *38*, 167-169.
- 600 Benzerara, K., Skouri-Panet, F., Li, J., Ferard, C., Gugger, M., Laurent, T., ... Moreira, D.
601 (2014). Intracellular Ca-carbonate biomineralization is widespread in cyanobacteria.
602 *Proceedings of the National Academy of Sciences of the United States of America*, *111*,
603 10933-10938.

604 Berkelman, T., Garret-Englele, P., & Hoffman, N. E. (1994). The *pacL* gene of *Synechococcus*
605 sp. strain PCC 7942 encodes a Ca²⁺-transporting ATPase. *Journal of Bacteriology*, *176*,
606 4430-4436.

607 BioMagnetech Corporation, (1990). Physical characteristics of magnetic bacteria and their
608 electromagnetic properties in the frequency range of 1–400 GHz, *BioMagnetech*
609 *Corporation*, New York.

610 Blondeau, M., Sachse, M., Boulogne, C., Gillet, C., Guigner, J.M., Skouri-Panet, F., ...
611 Benzerara, K. (2018a) Amorphous calcium carbonate granules form within an intracellular
612 compartment in calcifying cyanobacteria. *Frontiers in Microbiology*, *9*, 1768.

613 Blondeau, M., Benzerara, K., Ferard, C., Guigner, J.M., Poinot, M., Coutaud, M., ... Skouri-
614 Panet, F. (2018b). Impact of the cyanobacterium *Gloeomargarita lithophora* on the
615 geochemical cycles of Sr and Ba. *Chemical Geology*, *483*, 88-97.

616 Boros, E., Kolpakova, M. (2018). A review of the defining chemical properties of soda lakes
617 and pans: An assessment on a large geographic scale of Eurasian inland saline surface
618 waters. *PLoS One*, *13*, e0202205.

619 Bundeleva, I. A., Shirokova, L. S., Pokrovsky, O. S., Bénézeth, P., Ménez, B., Gérard, E., &
620 Balor, S. (2014). Experimental modeling of calcium carbonate precipitation by
621 cyanobacterium *Gloeocapsa* sp. *Chemical Geology*, *374-375*, 44-60.

622 Cam, N., Georgelin, T., Jaber, M., Lambert, J.-F., & Benzerara, K. (2015). In vitro synthesis of
623 amorphous Mg-, Ca-, Sr- and Ba-carbonates: What do we learn about intracellular
624 calcification by cyanobacteria? *Geochimica Et Cosmochimica Acta*, *161*, 36-49.

625 Cam, N., Benzerara, K., Georgelin, T., Jaber, M., Lambert, J.-F., Poinot, M., ... Cordier, L.
626 (2016). Selective uptake of alkaline earth metals by cyanobacteria forming intracellular
627 carbonates. *Environmental Science & Technology*, *50*, 11654–11662.

628 Cam, N., Benzerara, K., Georgelin, T., Jaber, M., Lambert, J.-F., Poinot, M., ... Jézéquel, D.
629 (2018). Cyanobacterial formation of intracellular Ca-carbonates in undersaturated
630 solutions. *Geobiology*, *16*, 49-61.

631 Chang, Y., Bruni, R., Kloss, B., Assur, Z., Kloppmann, E., Rost, B., ... Liu, Q. (2014).
632 Structural basis for a pH-sensitive calcium leak across membranes. *Science*, *344*,
633 1131-1135.

634 Clapham, D.E. (2007) Calcium signaling. *Cell*, *131*, 1047-1058

635 Couradeau, E., Benzerara, K., Gerard, E., Moreira, D., Bernard, S., Brown, G. E., & Lopez-
636 Garcia, P. (2012). An early-branching microbialite cyanobacterium forms intracellular
637 carbonates. *Science*, *336*, 459-462.

638 Debus, R. J. (1992). The manganese and calcium ions of photosynthetic oxygen evolution.
639 *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1102*, 269-352.

640 Demaegd, D., Colinet, A.-S., Deschamps, A., & Morsomme, P. (2014). Molecular evolution of
641 a novel family of putative calcium transporters. *PLoS ONE*, *9*, e100851.

642 Demain, A.L. & Solomon, N.A., (1981). Manual of industrial microbiology and biotechnology,
643 *American Society for Microbiology*, Washington, DC.

644 Domínguez, D. C. (2004). Calcium signaling in bacteria. *Molecular Microbiology*, *54*, 291-297.

645 Domínguez, D. C., Guragain, M., & Patrauchan, M. (2015). Calcium binding proteins and
646 calcium signaling in prokaryotes. *Cell Calcium*, *57*, 151-165.

- 647 Garcia-Pichel, F., Ramírez-Reinat, E., & Gao, Q. (2010). Microbial excavation of solid
648 carbonates powered by P-type ATPase-mediated transcellular Ca^{2+} transport. *Proceedings*
649 *of the National Academy of Sciences*, *107*, 21749-21754.
- 650 Gilabert, J. A. (2012). Cytoplasmic calcium buffering. In M. S. Islam (Ed.), *Calcium signaling*
651 (pp. 483–498). Dordrecht: Springer Science & Business Media.
- 652 Golubic, S., & Lee S.-J., (1999). Early cyanobacterial fossil record: Preservation,
653 palaeoenvironments and identification. *European Journal of Phycology*, *34*, 339-348.
- 654 Gonzalez-Esquer, C. R., Smarda, J., Rippka, R., Axen, S. D., Guglielmi, G., Gugger, M., &
655 Kerfeld, C. A. (2016). Cyanobacterial ultrastructure in light of genomic sequence data.
656 *Photosynthesis Research*, *129*, 147-157.
- 657 Gray, N. D. (2006). The unique role of intracellular calcification in the Genus *Achromatium*. In
658 *Inclusions in Prokaryotes* (p. 299-309). Springer, Berlin, Heidelberg.
- 659 Gray, N., & Head, I. (2014). The Family Achromatiaceae. In E. Rosenberg, E. F. De Long, S.
660 Lory, E. Stackebrandt, & F. Thompson (Éd.), *The Prokaryotes: Gammaproteobacteria* (p.
661 1-14). Berlin, Heidelberg: Springer Berlin Heidelberg.
- 662 Guida, B. S., & Garcia-Pichel, F. (2016). Extreme cellular adaptations and cell differentiation
663 required by a cyanobacterium for carbonate excavation. *Proceedings of the National*
664 *Academy of Sciences*, *113*, 5712-5717.
- 665 Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010).
666 New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the
667 performance of PhyML 3.0. *Systematic Biology*, *59*, 307-321.
- 668 Jansson, C., & Northen, T. (2010). Calcifying cyanobacteria—the potential of
669 biomineralization for carbon capture and storage. *Current Opinion in Biotechnology*, *21*,
670 365-371.
- 671 Jiang, H.-B., Cheng, H.-M., Gao, K.-S., & Qiu, B.-S. (2013). Inactivation of $\text{Ca}^{2+}/\text{H}^{+}$ exchanger
672 in *Synechocystis* sp. strain PCC 6803 promotes cyanobacterial calcification by
673 upregulating CO_2 -concentrating mechanisms. *Applied and Environmental Microbiology*,
674 *79*, 4048-4055.
- 675 Jones, G. E., Royle, L. G., & Murray, L. (1979). Cationic composition of 22 species of bacteria
676 grown in seawater medium. *Applied and Environmental Microbiology*, *38*, 800-805.
- 677 Kamennaya, N. A., Ajo-Franklin, C. M., Northen, T., & Jansson, C. (2012). Cyanobacteria as
678 biocatalysts for carbonate mineralization. *Minerals*, *2*, 338-364.
- 679 Kochan, T. J., Foley, M. H., Shoshiev, M. S., Somers, M. J., Carlson, P. E., & Hanna, P. C.
680 (2018). Updates to *Clostridium difficile* Spore Germination. *Journal of Bacteriology*, *200*,
681 e00218-18.
- 682 Krivtsov, V., Bellinger, E. G., & Sigeo, D. C. (2005). Elemental composition of *Microcystis*
683 *aeruginosa* under conditions of lake nutrient depletion. *Aquatic Ecology*, *39*, 123-134.
- 684 Lawford, H.G., & Rousseau, J.D., (1995). Establish medium requirements for high yield
685 ethanol production from xylose by existing xylose-fermenting microorganisms. Technical
686 Report NREL AAP-4-11195-03.
- 687 Lee, B. D., Apel, W. A., & Walton, M. R. (2004). Screening of cyanobacterial species for
688 calcification. *Biotechnology Progress*, *20*, 1345-1351.

689 Li, J., Margaret Oliver, I., Cam, N., Boudier, T., Blondeau, M., Leroy, E., ... Benzerara, K.
690 (2016). Biomineralization patterns of intracellular carbonatogenesis in cyanobacteria:
691 Molecular hypotheses. *Minerals*, 6, 10.

692 Mansor, M., Hamilton, T. L., Fantle, M. S., & Macalady, J. (2015). Metabolic diversity and
693 ecological niches of *Achromatium* populations revealed with single-cell genomic
694 sequencing. *Frontiers in Microbiology*, 6, 822.

695 Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., ... & Gwadz, M. (2016).
696 CDD/SPARCLE: functional classification of proteins via subfamily domain architectures.
697 *Nucleic acids research*, 45, D200-D203.

698 Merz, M. U. E. (1992). The biology of carbonate precipitation by cyanobacteria. *Facies*, 26,
699 81-101.

700 Moreira, D., Tavera, R., Benzerara, K., Skouri-Panet, F., Couradeau, E., Gérard, E., ... López-
701 García, P. (2017). Description of *Gloeomargarita lithophora* gen. nov., sp. nov., a
702 thylakoid-bearing, basal-branching cyanobacterium with intracellular carbonates, and
703 proposal for Gloeomargaritales ord. nov. *International Journal of Systematic and*
704 *Evolutionary Microbiology*, 67, 653-658.

705 Nazarenko, L. V., Andreev, I. M., Lyukevich, A. A., Pisareva, T. V., & Los, D. A. (2003).
706 Calcium release from *Synechocystis* cells induced by depolarization of the plasma
707 membrane: MscL as an outward Ca²⁺ channel. *Microbiology*, 149, 1147-1153.

708 Norland, S., Heldal, M., & Tumor, O. (1987). On the relation between dry matter and volume
709 of bacteria. *Microbial Ecology*, 13, 95-101.

710 Novoselov, A. A., Silva, D., Schneider, J., Abrevaya, X. C., Chaffin, M. S., Serrano, P., ...
711 Filho, C. R. de S. (2017). Geochemical constraints on the Hadean environment from
712 mineral fingerprints of prokaryotes. *Scientific Reports*, 7, 4008.

713 Pokrovsky, O. S., Martinez, R. E., Golubev, S. V., Kompantseva, E. I., & Shirokova, L. S.
714 (2008). Adsorption of metals and protons on *Gloeocapsa* sp. cyanobacteria: A surface
715 speciation approach. *Applied Geochemistry*, 23, 2574-2588.

716 Ponce-Toledo, R. I., Deschamps, P., López-García, P., Zivanovic, Y., Benzerara, K., Moreira,
717 D. (2017) An early-branching freshwater cyanobacterium at the origin of chloroplasts.
718 *Current Biology*, 27, 1-6.

719 Price, G. D., Maeda, S., Omata, T., & Badger, M. R. (2002). Modes of active inorganic carbon
720 uptake in the cyanobacterium, *Synechococcus* sp. PCC 7942. *Functional Plant Biology*,
721 29, 131-149.

722 Ragon, M., Benzerara, K., Moreira, D., Tavera, R., & Lopez-Garcia, P. (2014). 16S rDNA-
723 based analysis reveals cosmopolitan occurrence but limited diversity of two cyanobacterial
724 lineages with contrasted patterns of intracellular carbonate mineralization. *Frontiers in*
725 *Microbiology*, 5, 331.

726 Riding, R. (2006). Cyanobacterial calcification, carbon dioxide concentrating mechanisms, and
727 Proterozoic-Cambrian changes in atmospheric composition. *Geobiology*, 4, 299-316.

728 Rippka, R., Deruelles, J., Waterbury, J., Herdman, M., & Stanier, R. (1979). Generic
729 assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of*
730 *General Microbiology*, 111, 1-61.

731 Rouf, M. A. (1964). Spectrochemical Analysis of inorganic elements in bacteria. *Journal of*
732 *Bacteriology*, 88, 1545-1549.

733 Saghai, A., Zivanovic, Y., Zeyen, N., Moreira, D., Benzerara, K., Deschamps, P., ... López-
734 García, P. (2015). Metagenome-based diversity analyses suggest a significant contribution
735 of non-cyanobacterial lineages to carbonate precipitation in modern microbialites.
736 *Frontiers in Microbiology*, 6.

737 Saier, M. H., Reddy, V. S., Tsu, B. V., Ahmed, M. S., Li, C., & Moreno-Hagelsieb, G. (2016).
738 The transporter classification database (TCDB): Recent advances. *Nucleic Acids*
739 *Research*, 44, 372-379.

740 Shuttleworth, K. L., & Unz, R. F. (1991). Influence of metals and metal speciation on the
741 growth of filamentous bacteria. *Water Research*, 25, 1177-1186.

742 Singh, S., & Mishra, A. K. (2014). Regulation of calcium ion and its effect on growth and
743 developmental behavior in wild type and ntcA mutant of *Anabaena* sp. PCC 7120 under
744 varied levels of CaCl₂. *Microbiology*, 83, 235-246.

745 Stewart, M., Somlyo, A. P., Somlyo, A. V., Shuman, H., Lindsay, J. A., & Murrell, W. G.
746 (1980). Distribution of calcium and other elements in cryosectioned *Bacillus cereus* T
747 spores, determined by high-resolution scanning electron probe x-ray microanalysis.
748 *Journal of Bacteriology*, 143, 481-491.

749 Team R. C. (2013) R: A language and environment for statistical computing.

750 Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the
751 sensitivity of progressive multiple sequence alignment through sequence weighting,
752 position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 22, 4673-
753 4680.

754 van Veen, H. W., Abee, T., Kortstee, G. J. J., Konings, W. N., & Zehnder, A. J. B. (1994).
755 Translocation of metal phosphate via the phosphate inorganic transport system of
756 *Escherichia coli*. *Biochemistry*, 33, 1766-1770.

757 Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., & Takabe, T. (2001).
758 Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na⁺/H⁺ antiporter,
759 homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail.
760 *Journal of Biological Chemistry*, 276, 36931-36938.

761 Waditee, R., Hossain, G. S., Tanaka, Y., Nakamura, T., Shikata, M., Takano, J., ... Takabe, T.
762 (2004). Isolation and functional characterization of Ca²⁺/H⁺ antiporters from
763 cyanobacteria. *Journal of Biological Chemistry*, 279, 4330-4338.

764 Webb, L. E. (1988). Calcium dependence of the filamentous bacterium *Haliscomenobacter*
765 *hydrossis*. *Water Research*, 22, 1317-1320.

766 Warren, L. A., & Ferris, F. G. (1998). Continuum between sorption and precipitation of Fe(III)
767 on Microbial Surfaces. *Environmental Science & Technology*, 32, 2331-2337.

768 Yu, X. C., & Margolin, W. (1997). Ca²⁺-mediated GTP-dependent dynamic assembly of
769 bacterial cell division protein FtsZ into asters and polymer networks in vitro. *Embo*
770 *Journal*, 16, 5455-5463.

771
772
773
774
775

776 **Supplementary information of “Evidence of high Ca uptake by cyanobacteria forming**
777 **intracellular CaCO₃ and impact on their growth”**

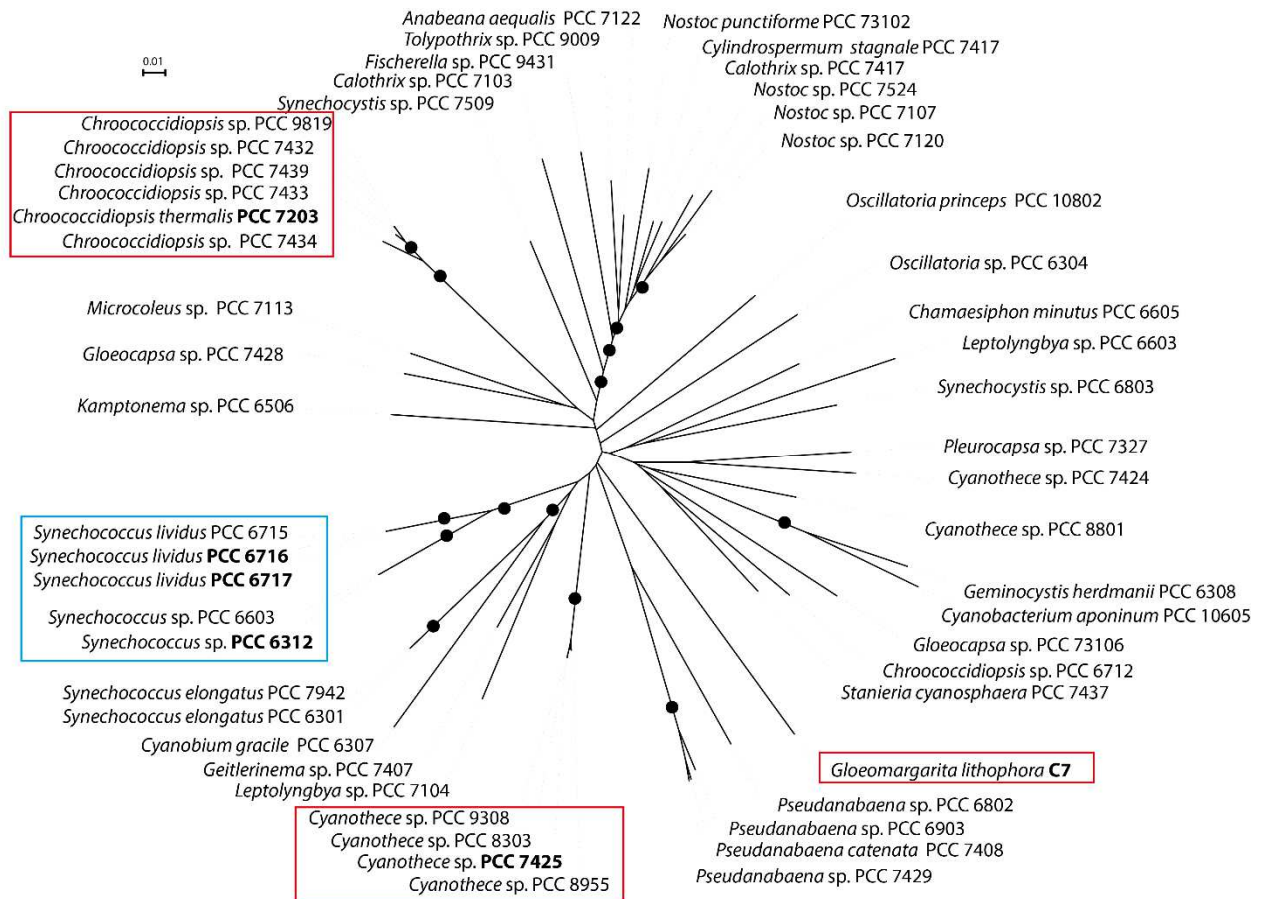
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829

Supplementary information file contains 1 table and 8 figures

830 **Table S1.** List of the studied cyanobacterial strains together with the main chemical data measured
 831 after their incubation in BG11.

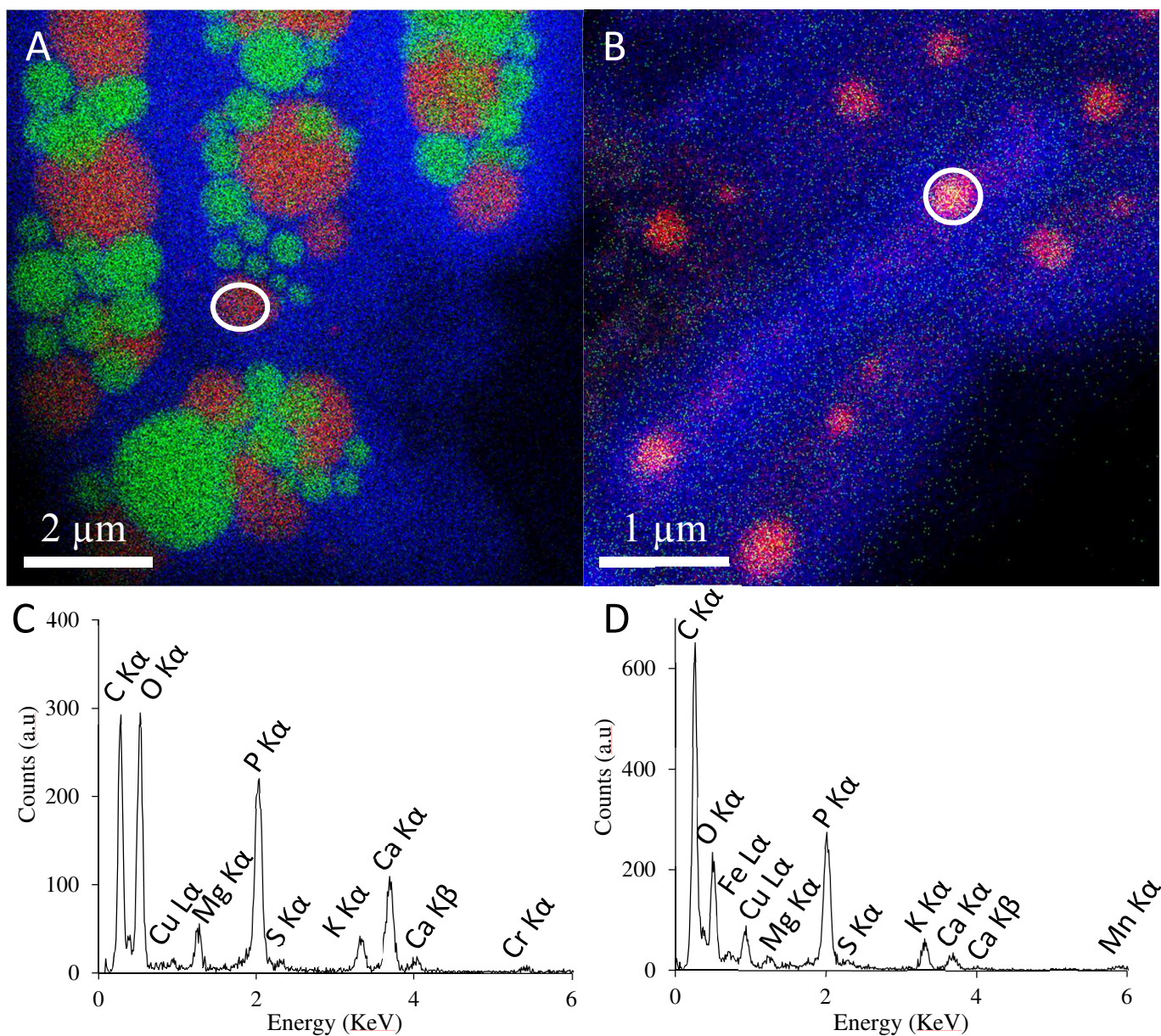
Strains	Species	Final dissolved [Ca] (μM)	Uptake [Ca] (μM)	Dry weight (g/L)($\pm 1\mu\text{g}$)	Final pH (± 0.01)	Incubation time (days)	Presence of intracellular ACC (yes/no)
C7	<i>Gloeomargarita lithophora</i>	6.3 \pm 0.1	274.3	0.35	9.50	60	Yes
PCC 6301	<i>Synechococcus elongatus</i>	231.1 \pm 1.6	49.6	0.49	10.56	40	No
PCC 6304	<i>Oscillatoria</i> sp.	209.6 \pm 1.6	71.0	0.08	9.73	40	No
PCC 6306	<i>Leptolyngbya</i> sp.	257.2 \pm 2.0	23.4	0.44	10.35	40	No
PCC 6307	<i>Cyanobium gracile</i>	217.5 \pm 3.7	63.2	0.20	9.83	40	No
PCC 6308	<i>Geminocystis herdmanii</i>	229.0 \pm 1.6	51.7	0.50	11.07	40	No
PCC 6312	<i>Synechococcus</i> sp.	29.9 \pm 0.1	250.8	0.35	9.92	60	Yes
PCC 6506	<i>Kamptonema</i> sp.	257.3 \pm 2.0	23.3	0.63	10.57	40	No
PCC 6603	<i>Synechococcus</i> sp.	12.7 \pm 0.1	268.0	0.26	10.52	61	Yes
PCC 6605	<i>Chamaesiphon minutus</i>	207.3 \pm 2.0	73.4	0.54	9.40	40	No
PCC 6712	<i>Chroococciopsis</i> sp.	210.9 \pm 1.9	69.8	0.70	10.03	40	No
PCC 6715	<i>Synechococcus lividus</i>	7.4 \pm 0.1	273.2	0.43	9.00	61	Yes
PCC 6716	<i>Synechococcus lividus</i>	40.6 \pm 0.1	240.0	0.64	9.22	61	Yes
PCC 6717	<i>Synechococcus lividus</i>	77.1 \pm 0.1	203.6	0.24	8.90	61	Yes
PCC 6802	<i>Pseudanabaena</i> sp.	237.6 \pm 1.1	43.1	0.50	7.96	40	No
PCC 6803	<i>Synechocystis</i> sp.	203.0 \pm 1.8	77.7	0.52	10.05	60	No
PCC 6903	<i>Pseudanabaena</i> sp.	240.4 \pm 1.4	40.2	0.33	10.47	54	No
PCC 7103	<i>Calothrix</i> sp.	219.9 \pm 3.3	60.8	0.88	10.42	47	No
PCC 7104	<i>Leptolyngbya</i> sp.	232.1 \pm 3.4	48.6	1.03	10.70	40	No
PCC 7107	<i>Nostoc</i> sp.	240.0 \pm 3.6	40.6	0.42	10.17	47	No
PCC 7113	<i>Microcoleus</i> sp.	267.2 \pm 3.2	13.5	0.68	9.36	47	No
PCC 7120	<i>Nostoc</i> sp.	240.4 \pm 2.9	40.3	0.41	9.71	47	No
PCC 7122	<i>Anabaena aequalis</i>	250.7 \pm 2.6	29.9	0.40	9.14	54	No
PCC 7203	<i>Chroococciopsis thermalis</i>	112.5 \pm 0.7	168.2	1.03	9.99	61	Yes
PCC 7327	<i>Pleurocapsa</i> sp.	249.5 \pm 1.9	31.2	0.39	9.21	54	No
PCC 7407	<i>Geitlerinema</i> sp.	245.3 \pm 1.7	35.3	0.09	8.95	54	No

PCC 7408	<i>Pseudanabaena catenata</i>	297.8 ± 0.7	0.0	1.19	10.04	54	No
PCC 7417	<i>Cylindrospermum stagnale</i>	250.5 ± 1.0	30.1	1.26	9.71	40	No
PCC 7424	<i>Cyanothece</i> sp.	214.6 ± 1.3	66.0	0.56	9.77	54	No
PCC 7425	<i>Cyanothece</i> sp.	28.1 ± 0.1	252.6	0.57	9.37	60	Yes
PCC 7428	<i>Gloeocapsa</i> sp.	173.4 ± 0.8	107.2	0.52	9.48	54	No
PCC 7429	<i>Pseudanabaena</i> sp.	241.4 ± 1.7	39.2	0.30	10.07	54	No
PCC 7432	<i>Chroococciopsis</i> sp.	42.6 ± 0.1	238.1	1.08	10.25	61	Yes
PCC 7433	<i>Chroococciopsis</i> sp.	59.3 ± 0.3	221.4	0.80	10.25	61	Yes
PCC 7434	<i>Chroococciopsis</i> sp.	50.4 ± 0.2	230.2	0.74	10.2	61	Yes
PCC 7437	<i>Stanieria cyanosphaera</i>	239.5 ± 1.3	41.2	0.82	9.51	54	No
PCC 7439	<i>Chroococciopsis</i> sp.	106.3 ± 0.8	174.3	0.63	9.95	61	Yes
PCC 7507	<i>Calothrix</i>	270.5 ± 5.0	10.1	1.17	9.99	54	No
PCC 7509	<i>Synechocystis</i> sp.	261.0 ± 1.4	19.6	0.36	9.92	54	No
PCC 7524	<i>Nostoc</i> sp.	227.0 ± 0.7	53.6	0.72	9.41	40	No
PCC 7942	<i>Synechococcus elongatus</i>	305.9 ± 1.0	0.0	0.67	9.83	40	No
PCC 8303	<i>Cyanothece</i> sp.	116.1 ± 0.8	164.5	0.39	10.7	61	Yes
PCC 8801	<i>Cyanothece</i> sp.	264.7 ± 0.9	15.9	0.40	9.79	47	No
PCC 8955	<i>Cyanothece</i> sp.	61.0 ± 0.2	219.7	0.39	8.73	61	Yes
PCC 9009	<i>Tolypothrix</i> sp.	253.9 ± 2.0	26.7	0.26	10.14	47	No
PCC 9308	<i>Cyanothece</i> sp.	12.6 ± 0.1	268.0	0.29	8.74	61	Yes
PCC 9431	<i>Fischerella</i> sp.	233.9 ± 0.6	46.8	1.19	9.01	47	No
PCC 9819	<i>Chroococciopsis</i> sp.	39.7 ± 0.1	240.9	1.16	9.35	61	Yes
PCC 10605	<i>Cyanobacterium aponinum</i>	252.5 ± 1.8	28.2	0.43	9.48	47	No
PCC 10802	<i>Oscillatoria princeps</i>	254.2 ± 2.2	26.5	4.24	10.08	47	No
PCC 73102	<i>Nostoc punctiforme</i>	263.4 ± 0.6	17.2	0.34	9.59	47	No
PCC 73106	<i>Gloeocapsa</i> sp.	256.7 ± 2.1	24.0	0.20	8.88	47	No



832
 833
 834
 835
 836
 837
 838
 839
 840
 841
 842
 843
 844
 845
 846
 847
 848
 849

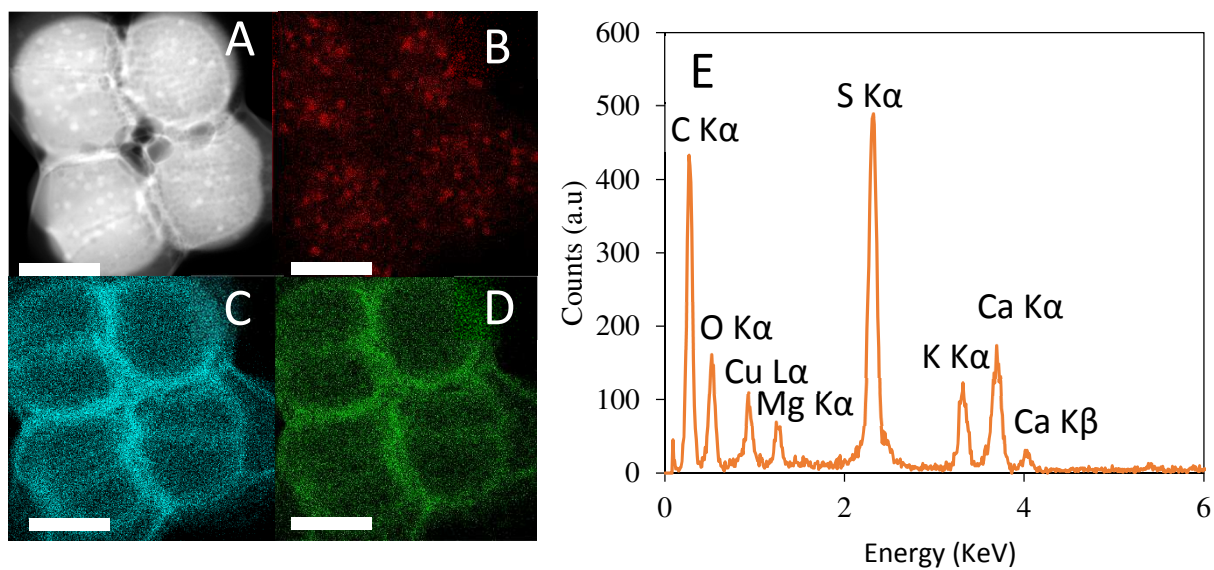
Figure S1. PhyML phylogenetic tree of the 52 tested strains based on the 16S rRNA gene. Nodes supported at 70% or more are indicated by a black dot. The ACC+ strains are outlined by a red square for granules scattered throughout the cells and a blue square for cells with granules located at cell poles and septa. The ACC+ strains described by Benzerara et al. (2014) are highlighted in bold.



851

852 **Figure S2.** STEM-EDXS analyses of the ACC+ *Cyanothecce* sp. PCC 9308 and the ACC-
 853 *Synechococcus elongatus* PCC 6301 strains grown in BG-11. (A) STEM-EDXS map of a cell of
 854 *Cyanothecce* sp. PCC 9308. (B) STEM-EDXS map of a cell of *Synechococcus elongatus* PCC 6301. (C)
 855 EDXS spectrum of the PolyP inclusion of *Cyanothecce* sp. PCC 9308 outlined by a white circle in (A).
 856 (D) EDXS spectrum of the PolyP inclusion of *Synechococcus elongatus* PCC 6301 outlined by a white
 857 circle in (B). For all EDXS maps, calcium is in green, phosphorus in red and carbon in blue. As a result,
 858 Ca-carbonates appear in green and PolyP granules in red.

859



860

861 **Figure S3.** STEM analyses of *Gloeocapsa* sp. PCC 7428 cells grown in BG-11. (A) STEM-HAADF
 862 image. (B) STEM-EDXS maps of phosphorus. (C) STEM-EDXS map of sulfur. (D) STEM-EDXS map
 863 of calcium. (E) EDXS spectrum of the cell wall of the cells. The scale bar represents 2 μm .

864

865

866

867

868

869

870

871

872

873

874

875

876

877

878

879

880

881

882

883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915

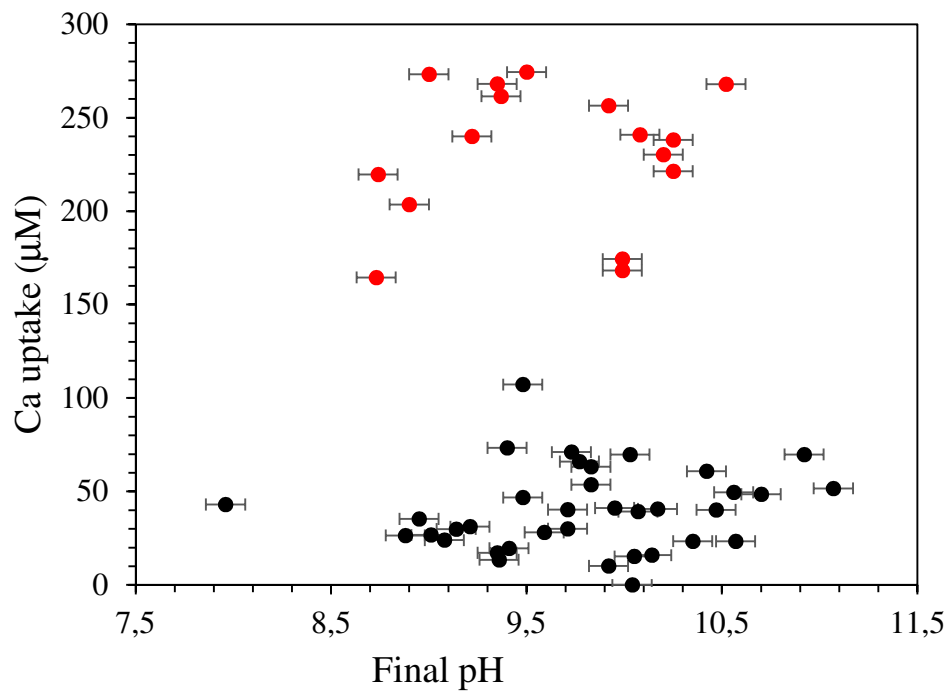


Figure S4. Calcium uptake (obtained by subtracting the dissolved Ca concentration left after cell incubation from the initial dissolved Ca concentration in the culture medium) vs pH for the 52 cyanobacterial strains. In red: strains producing intracellular ACC; in black: strains not forming intracellular ACC.

916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947

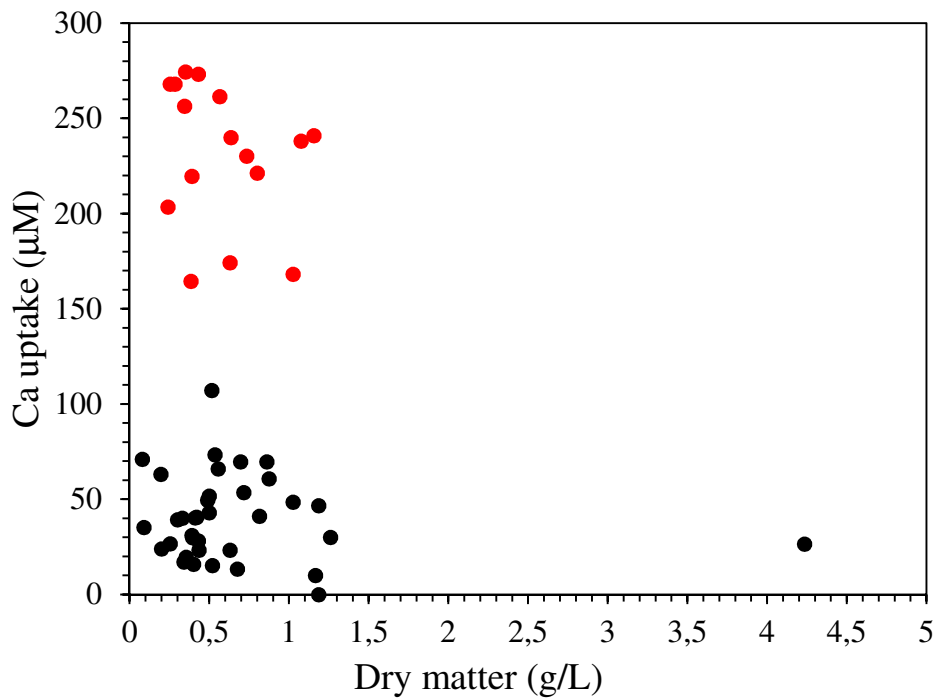
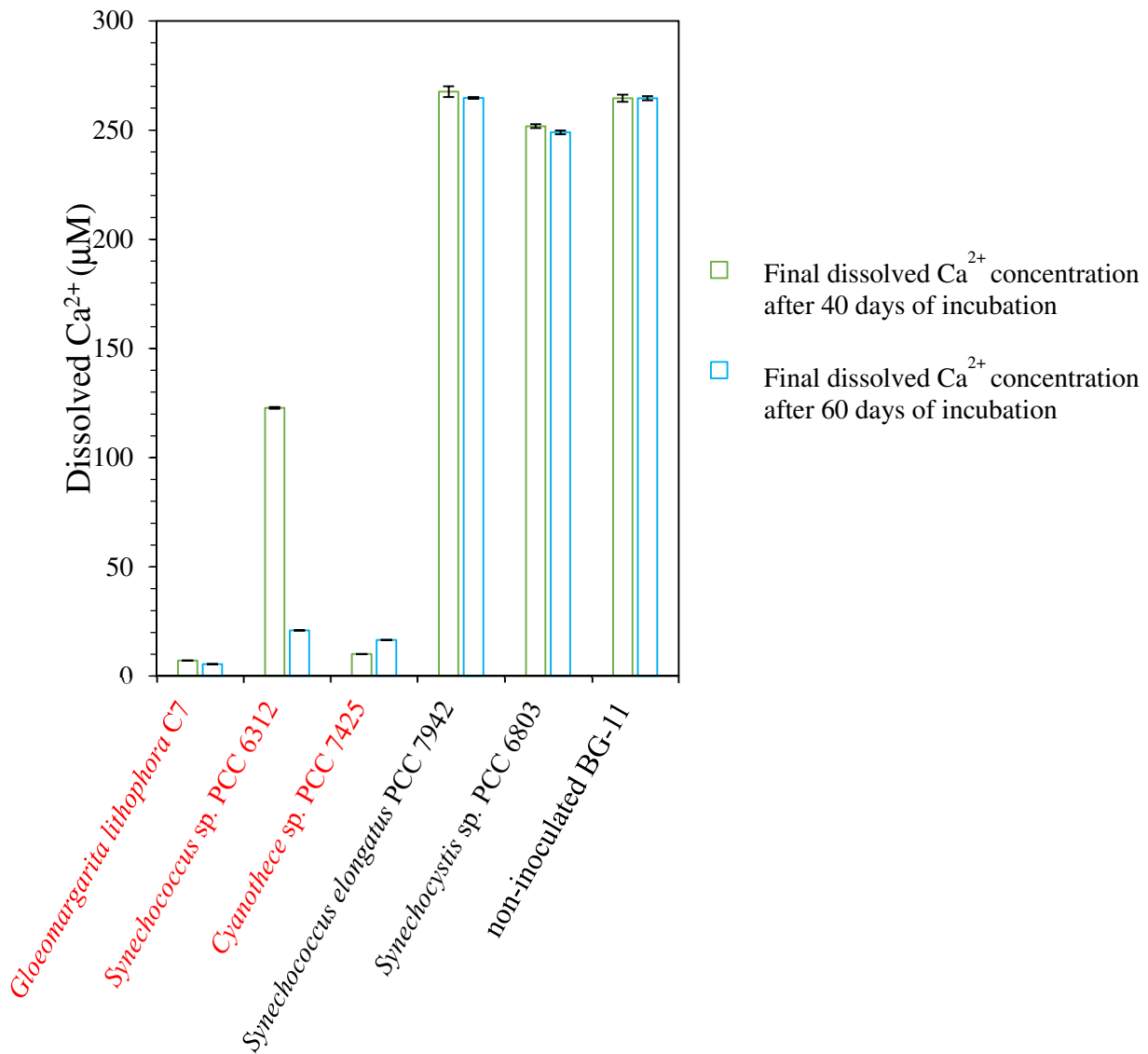


Figure S5. Calcium uptake (obtained by subtracting the dissolved Ca concentration left after cell incubation from the initial dissolved Ca concentration in the culture medium) vs dry mass for the 52 cyanobacterial strains of cyanobacterial. In red: strains producing intracellular ACC; in black: strains not forming intracellular ACC.



948

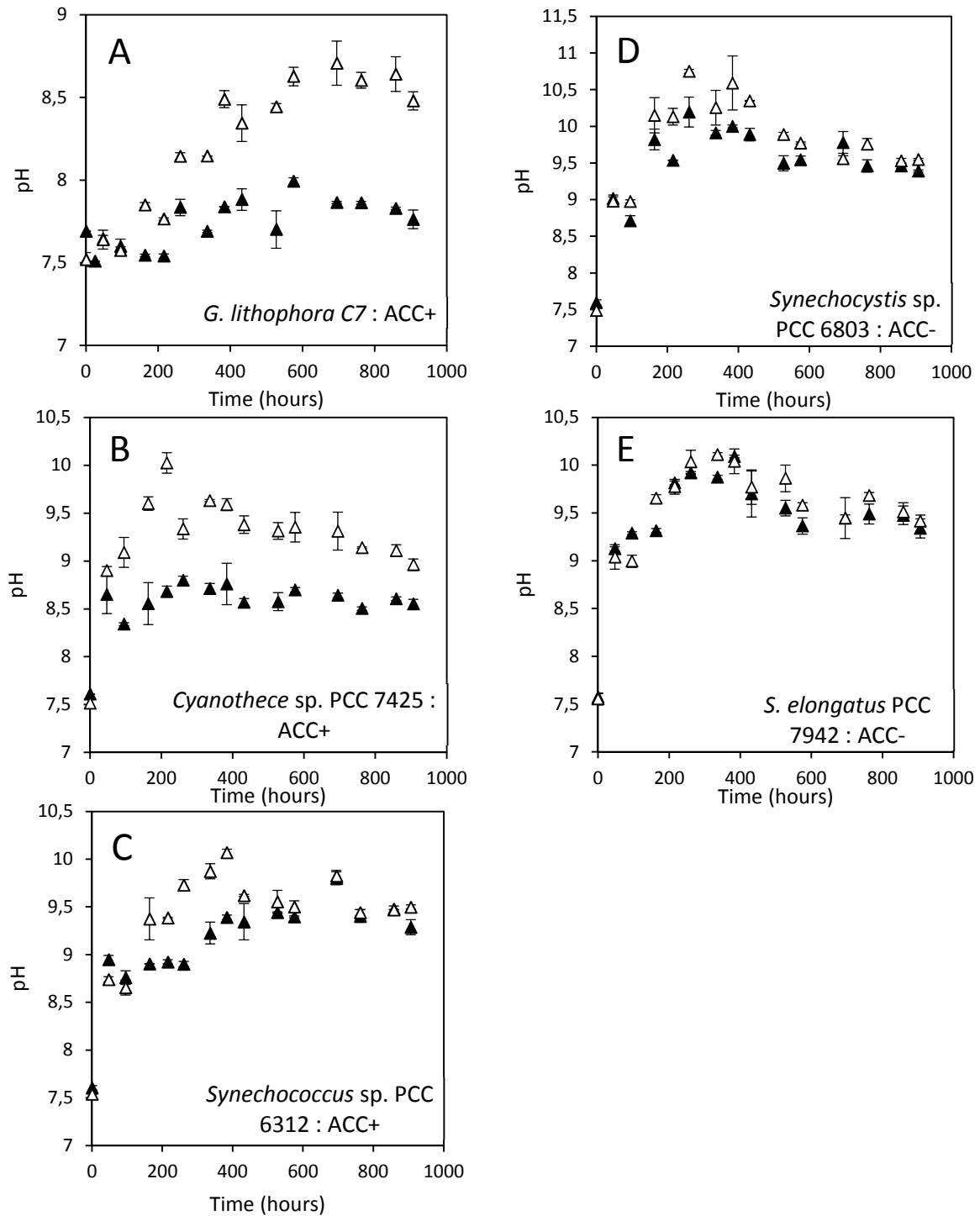
949 **Figure S6.** Final dissolved calcium concentration for cultures grown in sterile BG-11 after 40 (green)
 950 and 60 (blue) days of incubation. Strains forming intracellular ACC (*G. lithophora*, *Synechococcus* sp.
 951 PCC 6312, *Cyanothece* sp. PCC 7425) are indicated in red, and strains without ACC (*Synechococcus*
 952 *elongatus* PCC 7942, *Synechocystis* sp. PCC 6803) are indicated in black. Error bars were calculated
 953 based on the precision of ICP-AES measurements.

954

955

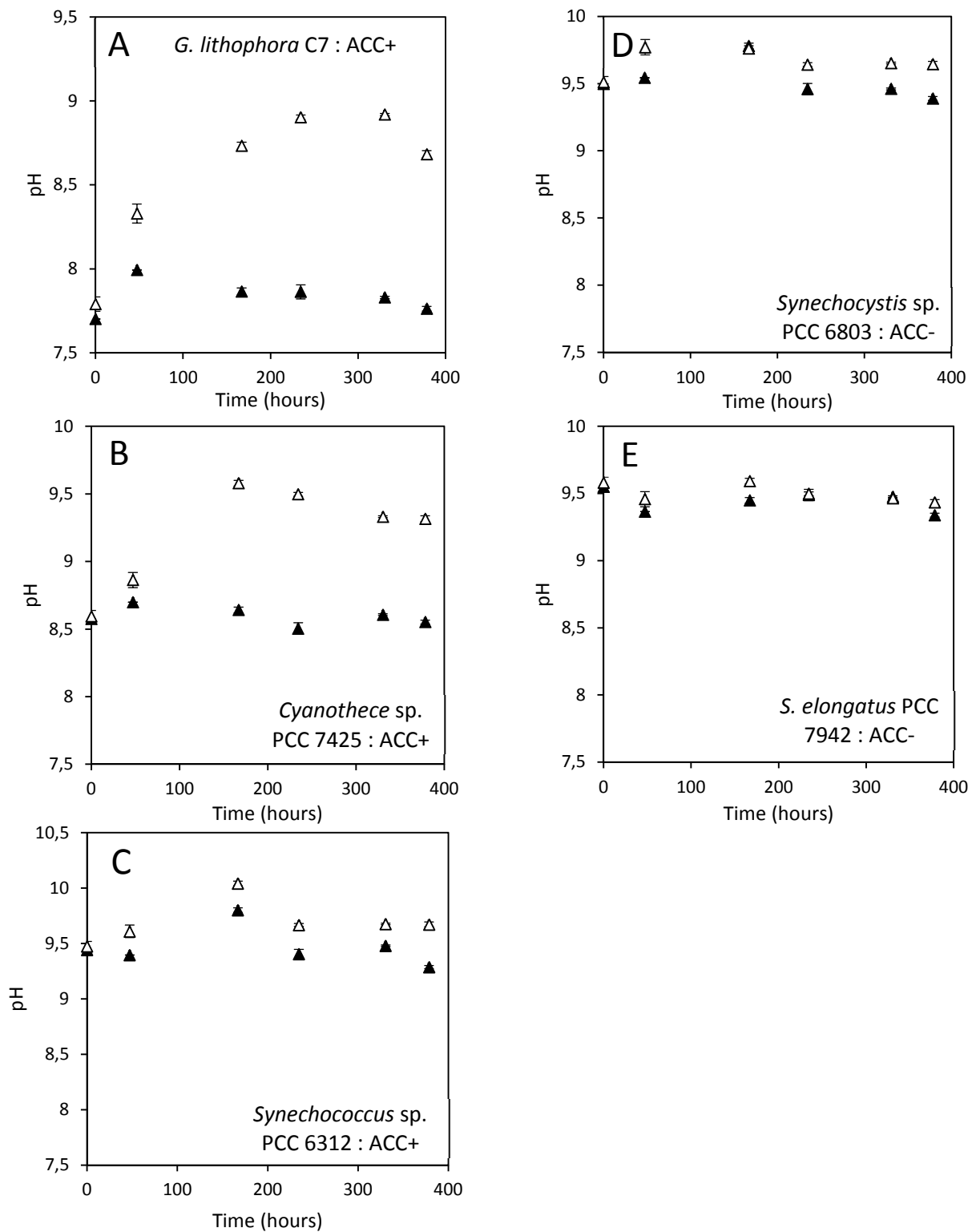
956

957



958

959 **Figure S7.** Time variations of pH in cultures with an initial Ca concentration of 50 μM (closed symbols)
 960 and 250 μM (open symbols). (A) *G. lithophora* C7; (B): *Cyanothece* sp. PCC 7425; (C): *Synechococcus*
 961 sp. PCC 6312; (D): *Synechocystis* sp. PCC 6803; (E): *Synechococcus elongatus* PCC 7942. Error bars
 962 represent standard deviations calculated based on variations between duplicates.



963
 964 **Figure S8.** Time evolution of pH measured in subcultures first grown in BG-11 with 50 μM of Ca, then
 965 supplemented (open symbols) or not (closed symbols) with 200 μM of Ca. Addition of 200 μM of Ca
 966 was done at $t=0$. (A) *G. lithophora* C7; (B) *Cyanothece* sp. PCC 7425; (C) *Synechococcus* sp. PCC
 967 6312; (D) *Synechocystis* sp. PCC 6803; (E) *Synechococcus elongatus* PCC 7942. Error bars represent
 968 standard deviations calculated based on variations between duplicates.