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THE MATRIX IS EVERYWHERE:

CACO₃ BIOMINERALIZATION BY THE *BACILLUS LICHENIFORMIS*

PLANKTONIC CELLS

Running title: The *Bacillus* planktonic cells in CaCO₃ mineralization

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26

ABSTRACT

27 To date, the mechanisms of CaCO₃ nucleus formation and crystal growth induced by bac-
28 terial cells still remain debatable. Here, an insight on the role of planktonic cells of *Bacillus li-*
29 *cheniformis* DSMZ 8782 in the biomineralization is presented. We showed that during 14-days
30 bacterial growth in a liquid urea/Ca²⁺-containing medium the transformation of CaCO₃ poly-
31 morphs followed the classical pathway "ACC-vaterite-calcite/aragonite". By microscopic tech-
32 niques, we detected the formation of extracellular matrix (ECM) around the cells at the stage of
33 exponential growth and appearance of electron-dense inclusions at 24 h after the inoculation. The
34 cells formed filaments and created a network, the nodes of which served as sites for further crystal
35 growth. The ECM formation accompanied with the expression of proteins required for biofilm
36 formation, the aldehyde/alcohol dehydrogenase, stress-associated Clp family proteins, and a porin
37 family protein (ompA ortholog) associated with bacterial extracellular vesicles. We demonstrated
38 that urea and CaCl₂ acted as denaturing agents causing matrix formation in addition to their tradi-
39 tional role as a source of carbonate and Ca²⁺ ions. We showed that CaCO₃ nucleation occurred
40 inside *B. licheniformis* cells and further crystal growth and polymorphic transformations took
41 place in the extracellular matrix without attaching to the cell surface. The spatial arrangement of
42 the cells was important for the active crystal growth and dependent on environmental factors. The
43 extracellular matrix played a double role being formed as a stress response and providing a favor-
44 able microenvironment for biomineralization (a high concentration of ions necessary for CaCO₃
45 crystal aggregation, fixation and stabilization).

46

1.INTRODUCTION

47 Biom mineralization is the phenomenon of mineral formation by living organisms within
48 their metabolic reactions with the environment. Calcium carbonate is one of the most widespread
49 material on the Earth, being the main constituent of limestone, marble, chalk, travertine, dolomite.
50 In the world's oceans its content reaches 10%. It is an essential component in biological systems
51 such as shells of marine organisms, pearls, and eggshells. Besides that, numerous microorganisms
52 are involved in the calcium carbonate mineralization. The process of microbially induced calcium
53 carbonate precipitation (MICP) has received a lot of attention from researchers working in various
54 fields. Thus, one of the most intensively studied subjects is the development of technologies that
55 use microorganisms able to induce the precipitation of calcium carbonate to restore concrete
56 structures. In the most cases, the microbiological method has been developing for the restoration
57 of historical stone buildings (1, 2). The main advantage is the imitation of the natural mineral
58 formation that guarantees good compatibility of the newly formed CaCO_3 mineral and the building
59 material (2, 3).

60 Three anhydrous polymorphs of crystalline calcium carbonate (aragonite (4), calcite (5)
61 and vaterite (6)) and two hydrated phases (monohydrocalcite and ikaite) (7) are well-described. In
62 biogenic and non-biogenic calcium carbonate minerals, the amorphous calcium carbonate (ACC)
63 phase is also found and has been received increasing attention by researchers (8). The most
64 thermodynamically stable CaCO_3 polymorph is calcite, the least stable non-crystalline polymorph
65 is ACC (9, 10). The latter often serves as a precursor in the formation of more stable crystalline
66 polymorphs (11). Recent works report various applications of CaCO_3 polymorphs including ACC
67 to create new biomaterials (12). As a consequence, morphological control of crystal growth is
68 necessary during the development of new products with unique characteristics (13).

69 To date, the mechanism of crystal formation by bacterial cells still remains debatable. In a
70 recent study (14), Keren-Paz and Kolodkin-Gal suggested a possible scheme of biogenic CaCO_3
71 crystallization pathway. The authors offered to divide the process into 4 stages: a) formation of a

72 saturated CaCO₃ micro-environments; b) nucleus formation; c) crystal nucleus transfer out of the
73 cell; and d) crystal growth on or near the cell surface. The authors supposed that at the first stage,
74 Ca²⁺ is accumulated in a cell as a result of ATP-dependent calcium transporters action or as a
75 product of carbonic anhydrases function within caboxysomes. During the second stage the initial
76 nuclei are forming and accumulating to be further transferred and to evolve at the third and fourth
77 stages. Very often, nucleation is thought to occur on microbial membranes. It is expected that
78 viable cells cannot produce crystals larger than their size; accordingly, at the third phase the formed
79 nuclei are transferred to the surface of the bacterial cell using active transport or lysis. At the last
80 stage, crystal growth occurs through the aggregation of nuclei on charged cell walls. The rate, at
81 which a crystal grows, can be affected by the shape of the crystal and its morphology, the
82 environment, and external conditions. Despite the rather logical proposed scheme of biogenic
83 crystal formation, a large number of questions arise at all stages. For example, nucleus formation
84 and initial crystal growth are the most questionable issues. To date, there are a lot of contradictory
85 results obtained regarding the influence of the microbial cell and its closest environment on
86 mineral formation.

87 Several research groups reported the intracellular carbonate ion formation within metabolic
88 reactions of the cell together with free calcium ions attraction out of the cell followed by the
89 formation of insoluble CaCO₃ crystals at the negatively charged cell wall surface (15). In this case,
90 the crystal morphology depends on many factors: the bacterial cell morphology, environmental
91 components, as well as physical factors (temperature, pH, and aeration) (16). This was confirmed
92 by Ghosh et al. who demonstrated appearance of nanoscale calcium carbonate crystals at the
93 surface of *Sporosarcina pasteurii* (17) supporting the hypothesis of the critical role of a negatively
94 charged surface in the formation of crystals. In contrast, W. Zhang et al. claimed that the
95 mechanism of crystal formation proceeds according to a different scheme (18). In addition,
96 Bundeleva et al. (19) reported studies on anoxygenic *Rhodovulum* sp., in which no crystals were
97 observed on the surface and near the cell. The authors hypothesized about the mechanisms of cell

98 protection against mineral deposition, and they proposed the idea of crystal deposition at some
99 distance from the cell surface. Zhang C. et al. (20) assumed that it is a spherical core formed at the
100 initial stage followed by the formation of a crystal structure with various shapes depending on the
101 selected conditions. The amorphous phase (ACC) also plays an important role, being one of the
102 main components of the spherical core (12). Thus, the question of the bacterial cells role remains
103 unexplicit and still requires a more detailed study of the exact mechanism of calcium carbonate
104 bioprecipitation.

105 In previously performed screening for the bacterial strains able to MCIP we found that one
106 of the most potential cultures was the strain *Bacillus licheniformis* DSMZ 8782 (results will be
107 published elsewhere). In the present study we used several microscopic and biochemical
108 techniques to demonstrate the behavior of planktonic bacterial cells during the growth in urea-
109 containing medium and their effect on initiation of CaCO₃ crystallization and crystal evolution.

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111

2.RESULTS

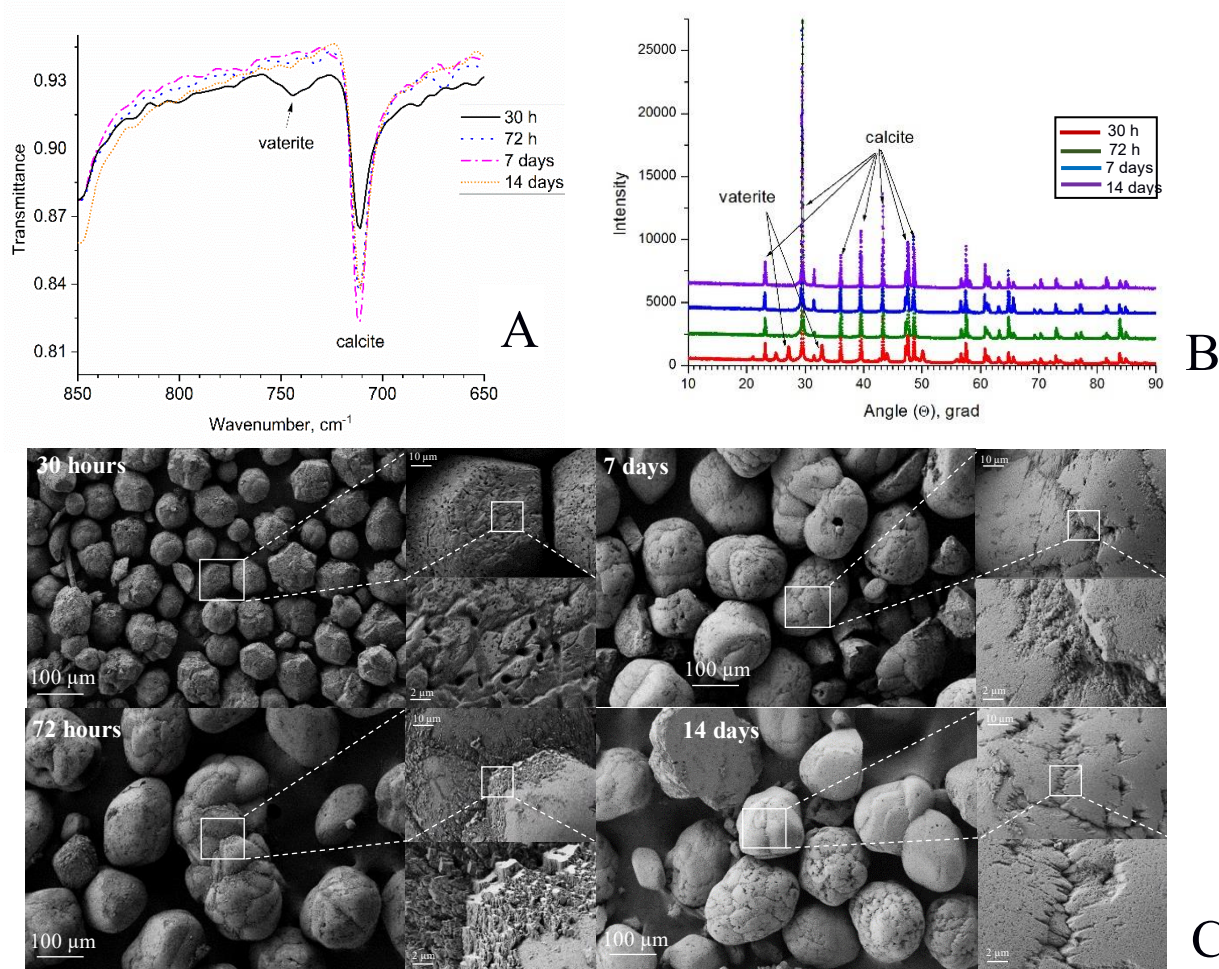
112 2.1. Particle analysis produced by *B. licheniformis* DSMZ 8782 during 14 days

113 Samples with CaCO₃ precipitates obtained during the growth of the bacterium in the liquid
114 medium B4-UCa for 14 days were analyzed by FTIR, XRD and SEM methods (Fig. 1). Intense
115 absorption bands of the carbonate ion (ν_{1-4} CO₃²⁻), OH-groups and water (21) can be easily
116 distinguished on the FTIR spectra of the obtained samples (Fig. 1A). Low-intensity bands with
117 maxima at 1800 and 2500 cm⁻¹ can be attributed to the composite vibrational frequencies of the
118 carbonate ion or organic impurities (ketones, thiols, or amines) (22). Intense bands ν_{2-4} CO₃²⁻
119 (~710, ~870 and ~1400 cm⁻¹) correspond to the most abundant calcite phase in the obtained
120 samples. However, the presence of the ν_1 CO₃²⁻ band (~ 1080 cm⁻¹) indicates the presence of small
121 impurities of aragonite, vaterite, or amorphous calcium carbonate (ACC) in the studied samples
122 (23, 24). The band with a maximum of ~ 750 cm⁻¹ has been found only in the spectra of samples
123 collected at points 30 and 48 hours after the inoculation indicating the presence of vaterite (24, 25)
124 in their composition. The amorphous phase is usually characterized by the ratio of the ν_2 to ν_4 band
125 intensities for the carbonate ion (20). Accordingly, this ratio increased in the series of samples: at
126 14 days, 7 days, 48 h, and 30 h after inoculation and reached a maximum for samples containing
127 traces of vaterite phase. Thus, the data of FTIR spectroscopy are in a good agreement with the
128 known model of calcium carbonate polymorphs transformation: ACC → vaterite → calcite,
129 aragonite (25).

130 The X-ray diffraction analysis (Fig. 1B) revealed changes in the distribution of calcium
131 carbonate polymorphs during the microorganism growth. Therefore, for precipitated samples
132 washed out from the culture liquid at the point 30 hours after the *B. licheniformis* inoculation, a
133 noticeable amount of vaterite, consisting about 10% of the total content of calcium carbonate
134 crystals, was detected. Further, the vaterite phase gradually transformed into calcite so that in the
135 samples at 72 hours after the inoculation the vaterite peaks were no longer detected. Accumulation
136 of calcite phase was accompanied with ACC disappearance detected by scanning electron

137 microscopy (Fig. 1C). At the point of 30 h after the inoculation small spherulites (50-70 μm in
138 size) with bacterial cell traces on their surfaces were located among the amorphous phase as seen
139 in the sample microphotographs (Fig. 1C: left top image). After 72 hours of cultivation, the
140 inorganic precipitates increased in size reaching a structure with an average diameter from 300 to
141 500 μm (Fig. 1C: left bottom image). Noteworthy, the smaller precipitates had better crystal
142 structure than their 30-hour precursors. During the following period, the precipitates hardly
143 increased in size, their structures looked crystalline, their amorphous environment disappeared
144 completely (Fig. 1C: right).

145



146

147 **Fig. 1. Mineral analysis of CaCO_3 precipitates induced by the *B. licheniformis* DSMZ 8287**
148 **in the liquid medium B4-UCa for 14 days.** FTIR spectra of precipitates at different times of
149 growth shows vaterite peak loss after 30 hours: A, X-ray diffraction patterns of the samples shows

150 polymorph transition from vaterite to calcite: **B**, variation of SEM topologies, particles growth and
151 loss of amorphous environment by 14th day: **C**.

152

153 **2.2. Analysis of the *B. licheniformis* DSMZ 8782 growth and biomineralization during**
154 **the first 48 hours**

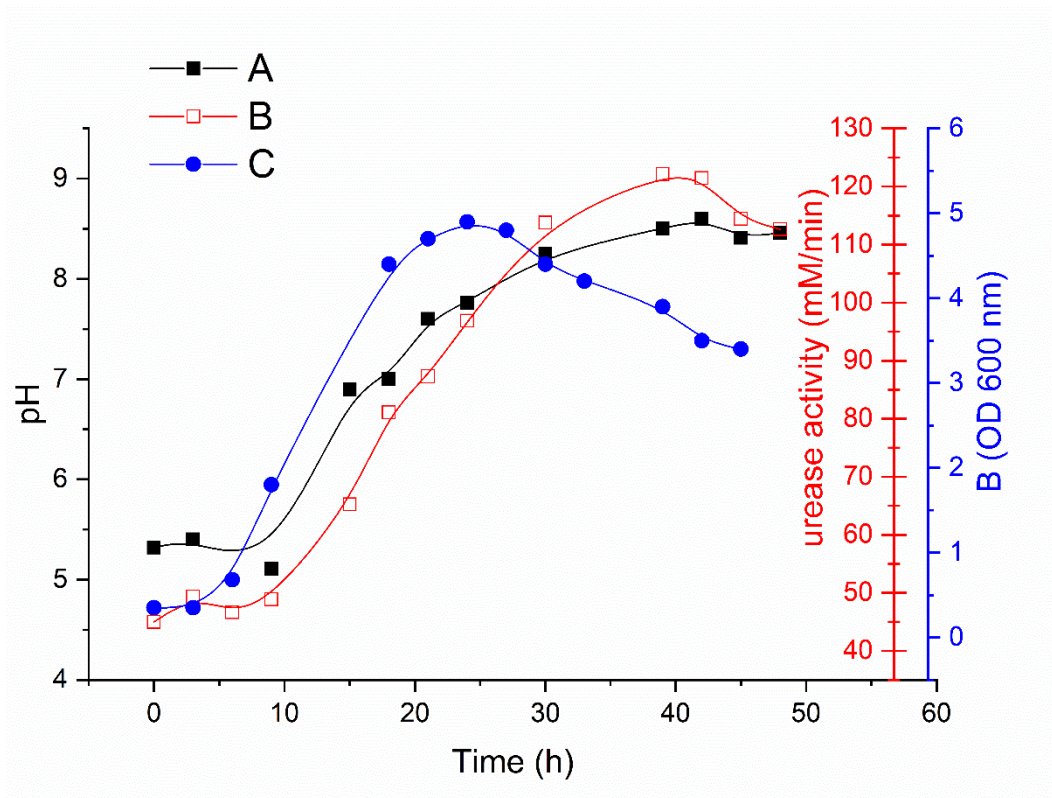
155 The most intriguing questions in understanding the processes of the calcium carbonate
156 biomineralization is the role of bacteria in the mineral formation. To address this question, we
157 conducted a series of experiments using several different microscopic and biochemical techniques
158 observing the cell behavior during the first 48 hours of the bacterium growth. Another reason to
159 choose this period for further studies was the absence of significant changes in cell behavior and
160 noticeable crystal transformation according to the results described above.

161

162 **2.2.1. Growth parameters.**

163 To study the process of mineral formation induced by *B. licheniformis* DSMZ 8782 in
164 details, pH parameters, biomass accumulation and the specific urease activity were monitored
165 during the bacterial growth for the first two days in the B4-UCa medium (Fig. 2).

166



167

168 **Fig 2. Time-dependences of *B. licheniformis* DSMZ 8782 growth parameters in the**
169 **B4-UCa medium for the first 48 hours. A: pH; B: specific urease activity; C: biomass**
170 **accumulation.**

171

172 After the first 24 hours of the bacterium growth, we observed an increase of the urease
173 activity along with cell biomass accumulation and a subsequent increase in the medium pH. Then,
174 changes in the parameters were stabilized and after about 40 hours the level of the urease activity
175 and cell biomass slightly decreased. We supposed that this was due to the formation of the calcium
176 carbonate precipitates (8).

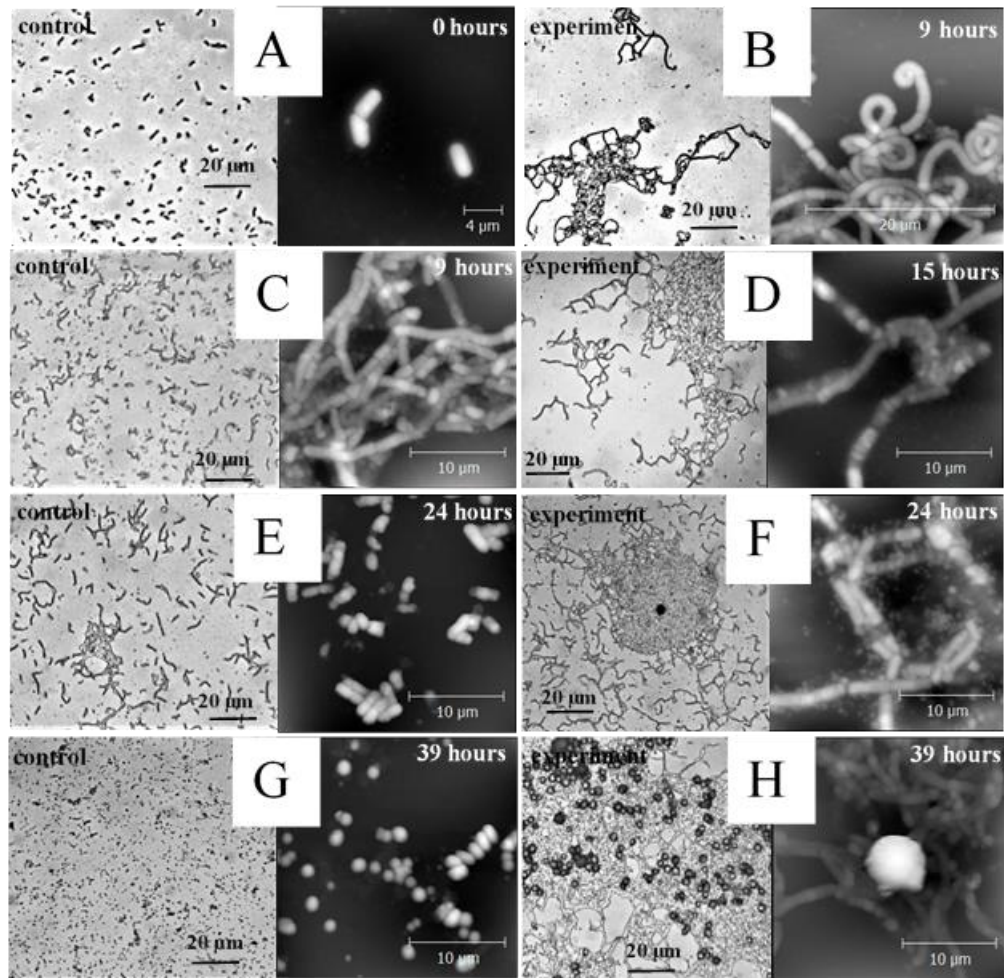
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178 **2.2.3. Light microscopy and AFM**

179 The initiation of CaCO_3 crystal formation was monitored by the light and atomic-force
180 microscopies (Fig. 3) using samples taken each 3 hours during the bacterial growth in the control
181 (B4-C, urea- and Ca^{2+} -free) and the experimental (B4-UCa, with the excess of urea and Ca^{2+})
182 media. At the point 0 h separated cells were seen in the images of the control samples (Fig. 3A),

183 followed by their apical collection in 2-4 cell straight chains. By 9 hours of inoculation (Fig. 3C),
184 further chain elongation tended to create an untight network. By the same time (9 hours after
185 inoculation), cells in the B4-UCa medium have formed long twisted chains and loops followed by
186 disappearance of clear contacts between pairs of cells and the appearance of amorphous
187 surrounding around bacterium by 15 hours of the growth (Fig. 3B and D). We supposed this
188 amorphous surrounding to be an extracellular matrix (ECM). At the 24-h point, the images of the
189 samples grown in two media were completely different (Fig. 3E and 3F): in the B4-C medium
190 separate cells and their stacks were still observed with hardly detectable extracellular matrix in the
191 places of cell accumulation (Fig. 3E). The light microscopy and AFM images of the cells in B4-
192 UCa medium demonstrated clearly visible ECM around a cluster of cells with “early” CaCO₃
193 crystal inclusions in the network nodes (Fig. 3F). By 39 hours of observation, in the images of the
194 control samples separate spores and their accumulations were seen exclusively (Fig. 3G), while in
195 the experimental sample the cells have continued to form dense structures around themselves until
196 the height of the "early crystals" reached the maximum to be hardly detected by atomic-force
197 microscopy (Fig. 3H). However, the bacterial cell network with numerous crystal precursors were
198 still good visible by light microscopy showing a sharp increase of their number (Fig. 3H).

199



200

201 **Fig. 3. *B. licheniformis* cell morphology evolution during the first 48 hours of the growth.**

202 Images of bacterial cells grown in the B4-C (control: **A, C, E, G** pictures) and B4-UCa
203 (experiment: **B, D, F, H**) media were obtained by the light (left) and atomic force (right)
204 microscopies.

205

206 The main result of the light microscopy and AFM observations was a clear increase in the
207 amount of extracellular matrix around the cells during the cultivation in the B4-UCa medium,
208 while this phenomenon was not observed for the samples in the urea- and Ca²⁺-free media during
209 the same period. Observations of the cell behavior in both media revealed two trends. First, in an
210 environment with urea and calcium, the life cycle of cells was longer, and the cells formed a strong
211 network, in the nodes of which crystal nucleation occurred followed by the subsequent active
212 crystal growth. In the B4-UCa medium, cells combined into longer chains as compared to the

213 control B4-C medium; their terminal parts were twisted into loops not observed in the control
214 samples. In the control B4-C medium, spores have started to appear 18 hours after the inoculation
215 and their number increased significantly during the studied period. No precipitates were detected
216 in this case. Second, we found the appearance of an extracellular matrix around planktonic cells
217 after 9 hours of the growth in B4-UCa medium. This is typical for the representatives of the
218 *Bacillus* genus including *B. licheniformis*, though it was shown during the growth of
219 microorganisms on a solid medium and was associated with biofilm formation (see for example,
220 (26) or (14)). These observations tended us to find out, which components of the B4-UCa medium
221 make the cells to behave unusually in a liquid medium forcing them to form a specific network, at
222 the nodes of which CaCO₃ crystal nucleation and growth occurs. To understand what may affect
223 mineral formation in planktonic cells, analysis of the protein content of the extracellular matrix of
224 *B. licheniformis* was required.

225

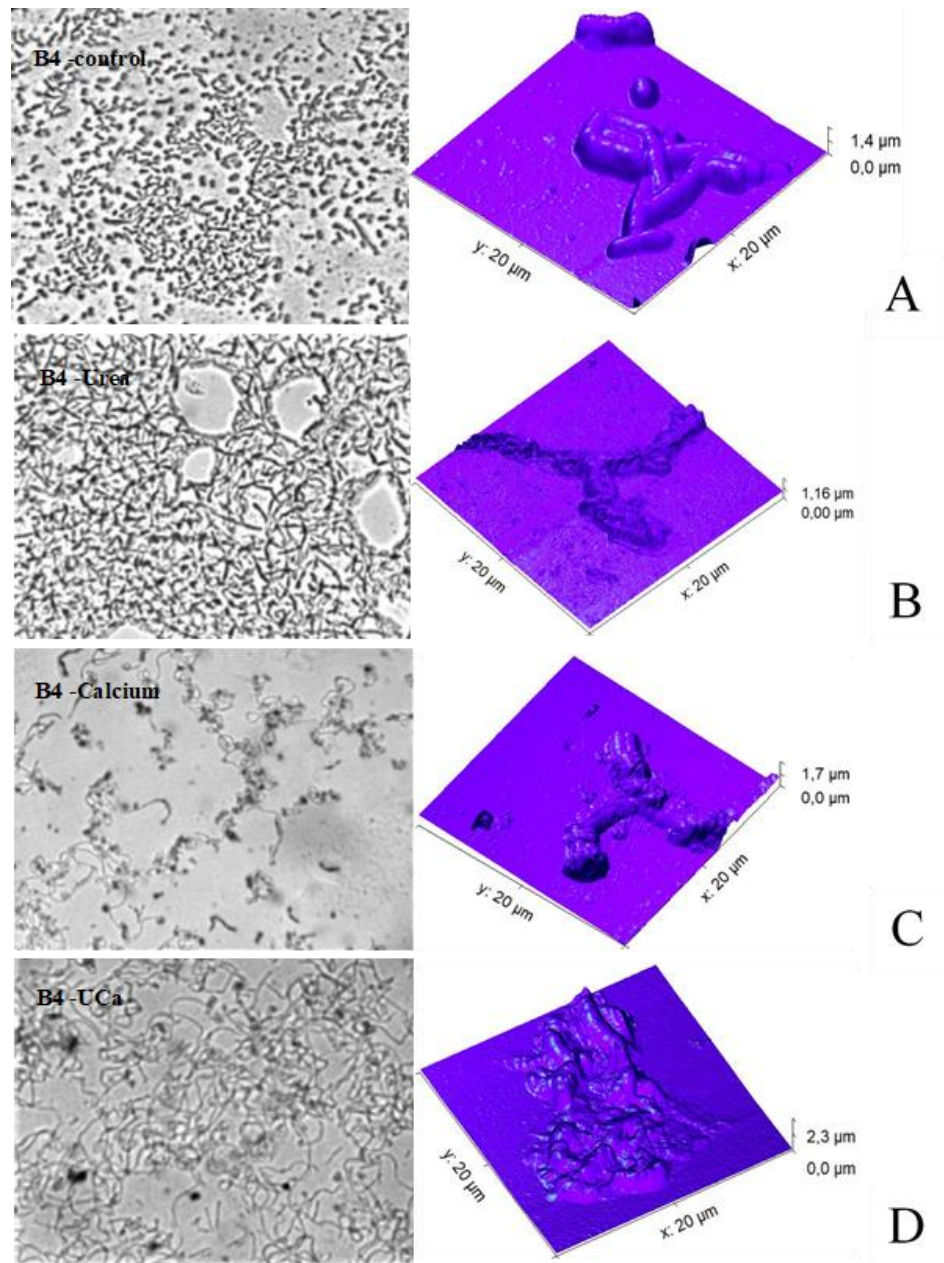
226 **2.3. Effect of medium components on CaCO₃ crystallization**

227 To study the influence of the medium composition on the cell behavior and the formation
228 of the extracellular matrix, the following media were used: B4-C, control medium without urea
229 and calcium ions; B4-U contained only urea; B4-Ca contained calcium ions and the experimental
230 medium B4-UCa contained urea and Ca²⁺ (CaCl₂). Changes in biomass were monitored during the
231 growth of bacteria in all media over a 72-hour period. In B4-U and B4-C media the strain
232 *B. licheniformis* DSMZ 8782 showed similar growth rate reaching their peak after 10 hours of
233 cultivation, after which some decrease was seen (Fig. S1, Supplementary material). One can
234 assume that urea served as an additional nutritional source for the bacterium. Slower growth with
235 a peak at 20 hours was observed in the experimental medium B4-UCa. After 24-hour growth in
236 this medium, intensive crystal formation started to be visible that interfered with density
237 measurement and made it difficult to evaluate biomass accumulation. By the second day of

238 cultivation, amount of *B. licheniformis* cells reached approximately the same values in all media
239 and then their growth was constant.

240 We selected a point of 24 hours of the bacteria cultivation to make light and atomic force
241 microscopy images for the sample withdrawn from studied media (Fig. 4). Cells in control B4-C
242 medium behaved disassociately gathering into clusters of separable cells (Fig. 4A, left). At the 3D-
243 AFM image (Fig. 4A, right) the absence of extracellular matrix around bacterial cells were clearly
244 seen. In the presence of urea in B4-U medium (Fig. 4B), dense clusters of poorly distinguishable
245 cells were observed which apparently formed a dense extracellular matrix. The excess of calcium
246 ions (B4-Ca medium, Fig. 4C) forced the bacterial cells to apically coalesce into twisted chains
247 with coiled strands. Most likely, this cell behavior is associated with the redistribution of ions on
248 the membrane and was also observed in the complete B4-UCa medium (Fig. 4D). The AFM image
249 of cells in B4-Ca medium (Fig. 4C, right) also demonstrated the accumulation of ECM around
250 bacterial cells, however, in a smaller amount than in the presence of urea (Fig. 4B). The light-
251 microscopic image of the sample from the medium B4-UCa (Fig. 4D, left) demonstrated a
252 combination of the patterns observed for B4-U and B4-Ca media: the twisted chains of bacterial
253 cells were obviously surrounded by dense extracellular matrix. On the AFM 3D-image of this
254 sample (Fig. 4D, right), individual cells were indistinguishable from each other in the extracellular
255 matrix and inclusions of calcium carbonate were clearly observed.

256



257

258 **Fig. 4. *B. licheniformis* cell morphology at the 24 hours after inoculation.** Bacterial
259 cells were grown in four media: the B4-C (control, **A**), B4-U (with urea, **B**), B4-Ca (with Ca²⁺, **C**)
260 and B4-UCa (with urea and calcium, **D**). Samples were observed by the light (left) and atomic
261 force (right) microscopies.

262

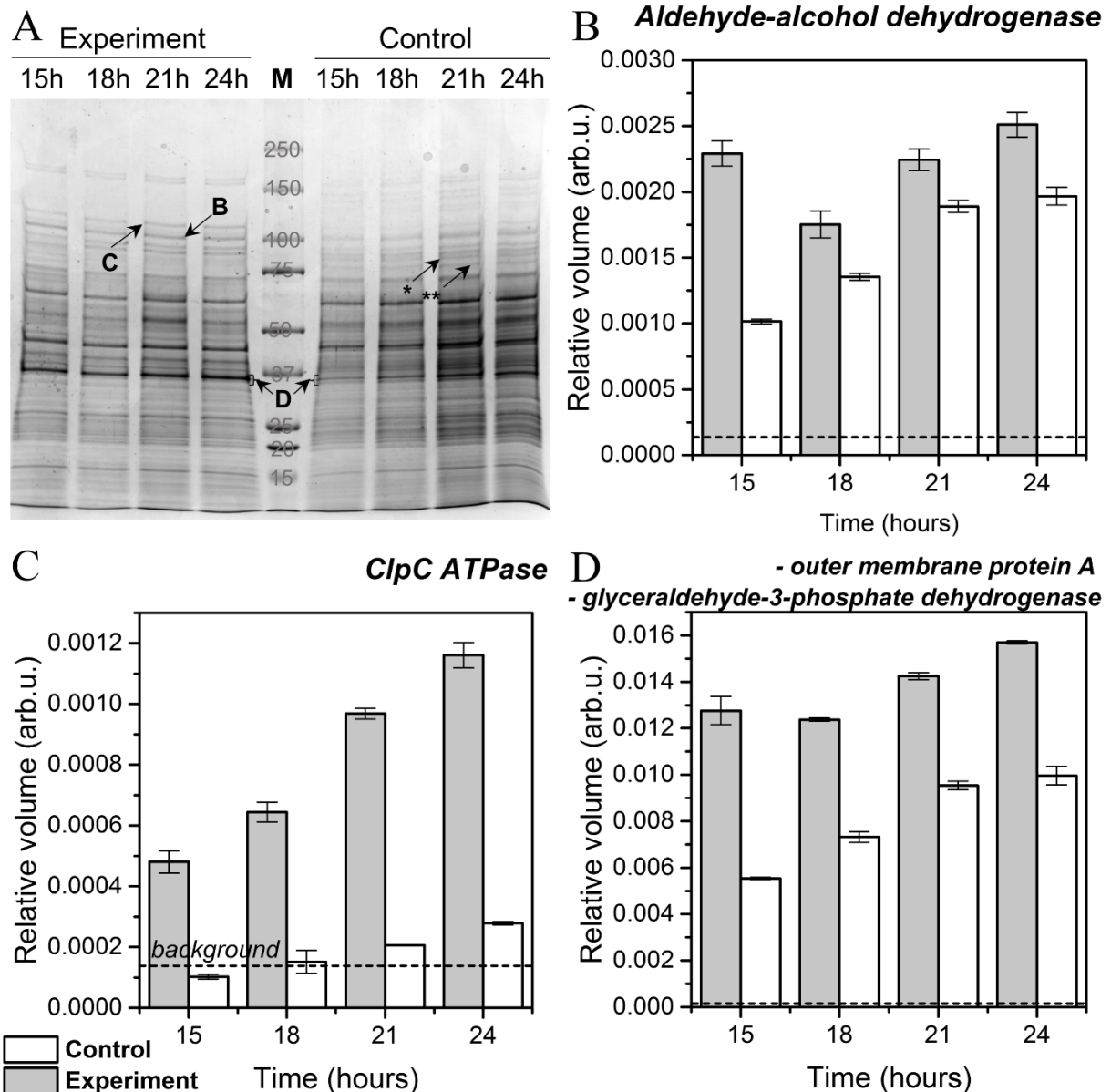
263 **2.4. ECM proteome analysis.**

264 To analyze differences in proteome composition in ECM of cells grown in the control B4-
265 C (urea- and Ca²⁺-free) and full B4-UCa media, the protein fractions obtained from PBS-washed

266 cells containing extracellular matrix (fractions C, see Materials and Methods, sections 4.4-4.5)
267 were analyzed by PAGE separation (Fig. 5A) followed by mass-spectrometric identification
268 (Figures S2, S3, S4, supplemental materials) and densitometric analysis (Fig. 5B-D). Analysis of
269 the experimental cell washes revealed a protein zone practically absent in the control (Fig. 5A)
270 and accumulated during the bacterium growth. The protein content of another two zones for the
271 experimental cells was more than twice higher than in the control cells, which was most
272 pronounced for the samples corresponding to 15 hours after the inoculation (Fig. 5A).

273 Homology search with the BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)
274 revealed about 80% homology between ATPase ClpC, aldehyde-alcohol dehydrogenase from
275 *Enterobacter cloacae* and *B. licheniformis* proteins, and outer membrane protein A from *E. cloacae*
276 mixed with glyceraldehyde-3-phosphate dehydrogenase identified using mass-spectrometric
277 databases. The discrepancy between the amino acid sequences of some of the peptides, for which
278 the identification of proteins was carried out, can be explained by the poor presentation of the
279 amino acid sequences of Gram-positive bacteria, in particular, *B. licheniformis*, in the NCBI and
280 SwissProt databases.

281



282

283 **Fig. 5. Proteome analysis of the ECM washed with cells during the growth for 24 h.**

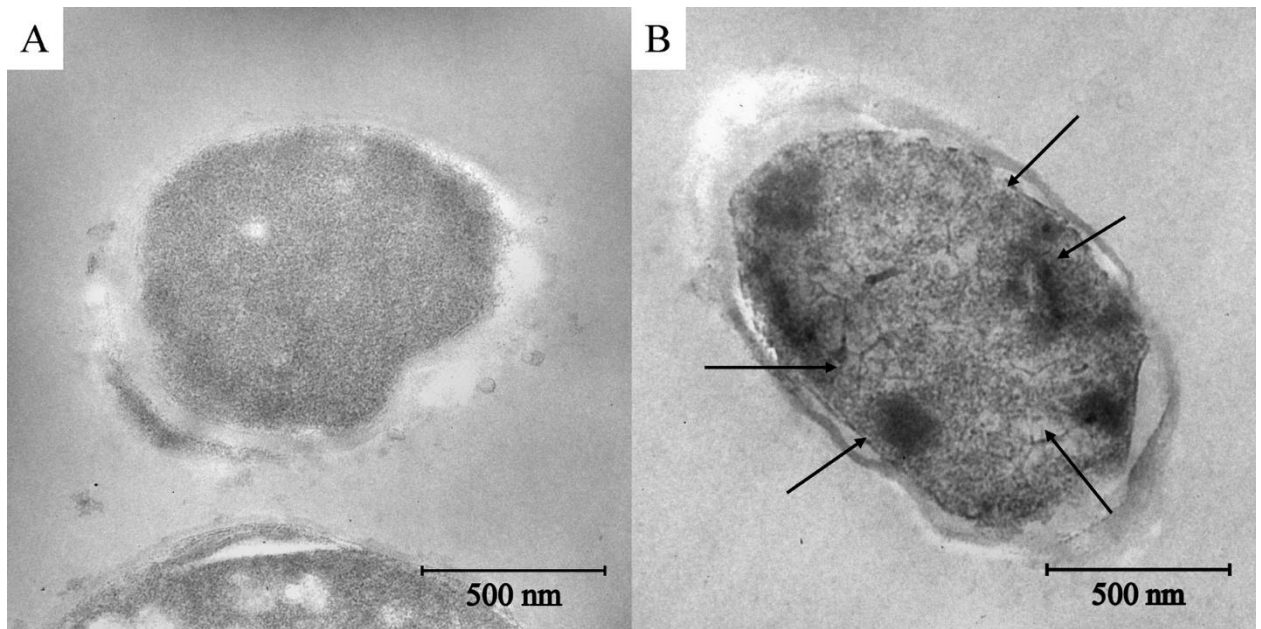
284 **A:** image of a protein PAGE separation of the fraction C (PBS-washed cells and ECM) produced
 285 by the cells in the control and experimental media; **B – D:** densitometric analysis of the zones
 286 differed in color intensity (marked with arrows on the panel **A**). The relative volume values were
 287 obtained by normalizing the color intensity of a zone to the total color intensity of the
 288 corresponding lane. The background value of the relative intensity (indicated by the dashed line)
 289 corresponds to the normalized random area of the corresponding track of a similar size.

290

291

292 **2.5. TEM analysis**

293 Electron microscopy of ultrathin slices of *B. licheniformis* cells showed homogeneous,
294 fine-grained with a moderate electron density cytoplasm of the control cells. Rounded vacuoles
295 corresponding to the invaginations of the bacterial membrane were regularly detected (Fig. 6A).
296 In experimental cells a higher electron density of the cytoplasm was observed indicating the
297 differences in the chemical composition of the intracellular contents. In the almost all studied
298 experimental samples, rod-shaped, branching dense formations were clearly seen in the whole cell
299 volume and especially along the edge of the inner membrane (Fig. 6B, marked by black arrows).
300



302 **Fig. 6. Transmission electron micrographs of unstained cells of *Bacillus licheniformis***
303 **at 24 h after the inoculation. A:** a sample withdrawn from the control medium B4-C; **B:** a sample
304 from the B4-UCa medium. Electron dense inclusions within the cell are indicated by black arrows.
305

306

3. DISCUSSION

307 At present a few research groups study intensively the biogenic process of CaCO₃
308 mineralization induced by microorganisms, in particular, by representatives of the genus *Bacillus*,
309 as well as the relationship between mineral and biofilm formation (14, 27, 28). However, there are
310 practically no works devoted to the behavior of planktonic cells during the biomineralization
311 induction. Our previous screening for bacteria capable of inducing calcium carbonate precipitation
312 resulted in a choice of the *B. licheniformis* strain DSMZ 8287 as the most promising
313 microorganisms to induce mineral formation (results will be published elsewhere). Our purpose in
314 the present work was to deepen the understanding of the role of planktonic bacterial cells in the
315 biomineralization process. We have carried out detailed studies of the induction of CaCO₃
316 precipitation by the studied strain during its 14-days growth in a liquid medium with urea and
317 calcium salts. Under the conditions of our experiments with this strain, the calcium carbonate
318 crystal nucleation, most likely, occurred in the first 24 hours, then the action was transferred to the
319 extracellular space, where the growth of crystals became chemically controlled depending on the
320 physico-chemical parameters of the microenvironment. Our observations revealed that the most
321 important events took place during the first two days, then the changes in the quantity and quality
322 of the formed minerals occurred at a noticeably lower rate and were not so significant. Like it has
323 been reported previously for other bacteria (28, 29) the transformation of CaCO₃ polymorphs
324 induced by *B. licheniformis* DSMZ8782 followed the classical pathway "ACC-vaterite-
325 calcite/aragonite". Interestingly, the polymorph transformations resembled a stepwise mechanism
326 of the formation of a substance characterized by several polymorphic modifications, according to
327 the rule of Ostwald steps (30). Although we have not had satisfied data to state this with certainty.

328 Our observations of the events occurring in the reaction medium with cells in course of
329 crystallization induction for the first 48 hours prompted us to investigate this period in more detail.
330 Analysis of cell images made every 3 hours for the first 48 hours of the bacterial growth in the
331 experimental (with an excess of urea and calcium salts) and in the control (urea/Ca²⁺-free) media

332 led us to the following conclusions. At the stage of exponential growth, ECM started to be formed
333 around the cells. By the end of this stage (at the point of 24 h after inoculation) dense clots were
334 clearly visible in the ECM (Fig. 3F), which, in our opinion, should be considered as crystal nuclei
335 that subsequently transform in the most thermodynamically stable form, calcite. It is interesting
336 that the cells in this environment formed filaments joined with each other in long twisted chains
337 and created a network, the nodes of which served as sites for the further active crystal growth. In
338 2015, van Gestel et al (31) carried out an elegant study, where they demonstrated intercellular
339 interactions and division of labor during the migration of *B. subtilis* cells throughout biofilm
340 formation. The researchers showed the enhancement of the expression of matrix-forming genes in
341 cells at the edges of the colonies and organization of the cells into so-called van Gogh bundles
342 consisting of filamentous loops. We have observed a similar picture for planktonic cells (Fig. 3):
343 the cells united into long twisted chains, folded in stacks and, what was the most remarkable, an
344 extracellular matrix has appeared around them. In their work, van Gestel et al suggested that the
345 appearance of bending filaments was a response to external conditions. In our work, this was
346 evidenced by the proteins required for matrix formation expression increase, in particular the
347 aldehyde/alcohol dehydrogenase accumulated in experimental cells (Fig. 5A and B). Expression
348 of the aldehyde/alcohol dehydrogenase *B. licheniformis* gene ortholog, which protein product
349 catalyzes the reaction of acetyl CoA into ethanol necessary for matrix formation, has been reported
350 to maximize during the induction of matrix formation in bacteria of other species (32).

351 The next conclusion we made was that the matrix not only served as a glue for binding
352 cells to each other and as a site of the crystal growth, but also as a response to stress caused by
353 conditions in the reaction medium (high pH, elevated concentrations of urea and calcium ions). It
354 was evidenced by the accumulation of protease of the Clp family during the growth of *B.*
355 *licheniformis* in the presence of urea and CaCl₂ (Fig. 5A and C). We showed that urea acted not
356 only as a source of carbonate ions generated by urease as described earlier for urease-driven
357 bacterial induction of mineralization (33–35), but also as a denaturing agent causing matrix

358 formation (Fig. 4B). On the one hand, for some bacteria, for example, *Staphylococcus aureus* (36)
359 and *B. amyloliquefaciens* (37), *B. cereus* (32) and *B. subtilis* (38), representatives of Clp family
360 ATPases were reported to be required for biofilm (and ECM) formation. On the other hand, the
361 expression of Clp genes, which are highly conserved among bacteria, is a typical response to stress
362 (for example, heat, chemical or osmotic shock) leading to the presence of proteins with a
363 misfolding of the polypeptide chain (39). Besides urea, an increase in the production of the stress-
364 associated Clp family proteins can be explained by the effect of used elevated concentration of
365 calcium ions (22 mM), which was close to or exceeded the value typical for the induction of stress
366 reactions (40). This idea was supported by atomic force and light microscopy images of *B.*
367 *licheniformis* cells incubated in the urea-free Ca²⁺-containing medium resulted in induction of the
368 ECM formation (Fig. 4C). Noteworthy, at the concentrations used of the present work, both CaCl₂
369 and urea, as well as their mixture induced the formation of extracellular matrix during the first 24
370 hours of incubation (Fig. 4B-D).

371 We also demonstrated the presence of electron-dense inclusions inside bacteria under our
372 experimental conditions (Fig. 6B). This observation remarkably agrees with similar data of other
373 authors (29). In particular, by TEM analysis of *B. licheniformis* DB1-9 the group of Han with
374 coauthors revealed the dark and denser spots inside the cells and within the cell EPS (29). This
375 may indicate the calcium carbonate nucleation inside the bacterial cell. Moreover, the fact that the
376 protein belonging to the porin family (ompA ortholog) was identified (in a mixture with
377 glyceraldehyde-3-phosphate dehydrogenase), led us to another conclusion. Accumulation of this
378 protein we reliably observed in experimental cells between 15 and 24 h after inoculation (Fig. 5A
379 and D). Taken together data on the experimentally observed increase in the concentration of this
380 protein and its potential in the formation of bacterial extracellular vesicles (41) could lead to a
381 hypothesis about a possible transport of crystal precursors outside of the cell by vesicles that were
382 described earlier for magnetotactic bacteria (42).

383 In conclusion, our results support the hypothesis (29) that CaCO₃ nucleation occurs inside
384 *B. licheniformis* cells and further crystal growth and polymorphic transformations occur in the
385 extracellular matrix. We demonstrated that the spatial arrangement of the cells is important for the
386 active crystal growth and is dependent on stress-induced environmental factors. The extracellular
387 matrix forms a favorable microenvironment for crystal growth providing a high concentration of
388 ions necessary for CaCO₃ crystal aggregation, fixation, and stabilization.
389

390

4. MATERIAL AND METHODS

391

4.1 Bacterium growth conditions and sample preparations

392

The strain *Bacillus licheniformis* DSMZ 8782 used in the study was kept at -20°C in LB

393

medium (peptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) containing 20% (v/v) glycerol.

394

Prior to each experiment, an overnight culture of *B. licheniformis* was prepared from frozen

395

glycerol stock by culturing in LB at 37°C with an orbital shaker operated at 110 rpm. After the

396

growth, cells were separated aseptically by centrifugation at 2000 g for 10 minutes and re-

397

suspended in 2 mL of 0.9% NaCl to prepare the inoculum.

398

Routinely, to evaluate mineralizing ability of the strain *B. licheniformis* DSMZ 8782 during

399

the growth in a liquid medium, the inoculum of the microorganism was transferred into the flasks

400

with 100 mL of the standard B4 liquid medium containing: yeast extract, 2 g/L; glucose, 10 g/L;

401

urea, 2.5 g/L; CaCl₂, 2.5 g/L. Pre-sterilized solutions of urea and CaCl₂ were added separately to

402

the medium after autoclaving. In each case, bacterium was cultivated at 37°C with shaking at 110

403

rpm with periodic removing of aliquots. Withdrawn aliquots were cooled to the room temperature

404

25°C followed by measurements of pH, optical density at 600 nm and urease activity.

405

To analyze CaCO₃ particles, precipitates were filtered on a vacuum filter with a pore size

406

of 0.2 µm, dried at 37°C in a thermostat and analyzed by powder X-ray diffraction, optical

407

stereoscopic and scanning electron microscopes and IR Fourier spectrometer (see below).

408

To assess the influence of the medium components on cell behavior during CaCO₃

409

mineralization, 4 variants of standard B4 medium were used:

410

1. B4 without urea and CaCl₂ (B4-C, control medium);

411

2. B4 with the addition of urea (2.5 g/L) and without CaCl₂ (B4-U);

412

3. B4 with the addition of CaCl₂ (2.5 g/L) and without urea (B4-Ca);

413

4. B4 with urea and CaCl₂ (B4-UCa).

414 Preliminary prepared inoculum was transferred into the flasks with different media and
415 cultivated for 48 hours at 37°C with shaking. Two-mL aliquots were withdrawn each 3 hours, cells
416 were centrifuged and re-suspended in 1 mL of sterilized distilled water. Bacterial surface was
417 studied using an NT-MDT Solver Bio scanning probe microscope in a semi-contact mode, using
418 an NSG01 probe and by MSP-1 optical stereoscopic microscope.

419

420 **4.2. Urease assay**

421 Urease activity was determined according to the method described in (43) by evaluation of
422 conductivity of a culture medium. To assess the urease activity, the aliquot was cooled to 25°C and
423 the conductivity was measured using a conductometer Gravity: Analog Electrical Conductivity
424 Sensor/Meter (K=10). The formation of ionic particles of non-ionic substrates led to an increase
425 in the total conductivity of the solution and the rate, at which the conductivity increased, was
426 proportional to the concentration of the active urease presented in the reaction mixture. The
427 conductivity of the medium with growing *E. coli* DH5 α strain was taken as a negative control.
428 Molar concentration of degraded urea was calculated using the equation (1) with the coefficient
429 taken from (43):

$$430 \quad \text{Ureahydrolysed}(mM) = \text{Conductivity}(mS) \times 11,11 \quad (1)$$

431

432 One unit of the urease activity corresponded to the enzyme amount capable of catalyzing
433 the conversion of 1 μ M urea per minute under the standard assay conditions (pH 5.5, 37° C, 20
434 min). The data points are presented as the means of at least three independent experiments, and
435 the errors were calculated for each data point using Excel Solver add-in (Microsoft, Redmond
436 WA). Reported below time-dependent curves for pH- and urease activity assays during the
437 bacterial growth were generated with ORIGIN 8.0 software (OriginLab, Northampton, MA, USA).

438

439 **4.3. *CaCO₃ particle analysis***

440 Powder X-ray diffraction (XRD) analysis of the samples was performed on a Rigaku
441 Miniflex 600 diffractometer (Bragg–Brentano geometry) with Ni-filtered CuK α ($\lambda=1.5418\text{\AA}$)
442 radiation and a LYNXEYE detector. Diffraction patterns were recorded in the 10–70° 2 θ range,
443 with a step of 0.02° and collection time of 0.3 s/step. Rietveld analysis of the patterns was carried
444 out with a help of FullProf Suite (44). Structure models were obtained from Crystallography Open
445 Database (45).

446 The microstructure of the samples was investigated using a Carl Zeiss NVision 40 high
447 resolution scanning electron microscope at 1 kV acceleration voltage.

448 The IR spectra of samples were recorded on an ALPHA IR Fourier spectrometer (Brucker)
449 in the disturbed total internal reflection mode in the range of 400–4000 cm⁻¹ with a resolution of
450 1.5 cm⁻¹.

451

452 **4.4. *Extracellular matrix isolation***

453 To isolate fractions containing extracellular matrix (ECM), 2-mL aliquots were withdrawn
454 from the 100-mL flasks containing medium B4-UCa or control B4-C (without urea and CaCl₂) at
455 15 hours, 18 hours, 21 hours, and 24 hours during the growth of *B. licheniformis* at 37°C with
456 shaking. Each sample was centrifuged at 8000 g for 10 minutes. Fractions containing supernatant
457 (S1) were frozen at -20°C for further analysis; the pellets were re-dissolved in 2 mL of PBS and
458 centrifuged for 10 minutes at 8000 g. The cell fraction containing extracellular matrix was frozen
459 at -20°C for further analysis (fraction C). The number of cells was estimated by the suspension
460 absorption at 600 nm using spectrophotometer Hitachi U-3310 and normalized. Cells were
461 destroyed by ultrasound treatment followed by electrophoretic separation of proteins.

462

463

464 **4.5. Extracellular matrix analysis**

465 Samples of PBS-washed bacteria pellet (fractions C) were mixed with Laemmli's buffer
466 containing β -mercaptoethanol (46), denatured at 99°C for 5 minutes, and loaded into the wells of
467 a polyacrylic gradient gel (8-16%) using a Kaleidoscope kit (BioRad) as a molecular weight
468 marker. Fragments of the Coomassie-stained gel were excised and prepared for mass-spectrometric
469 analysis as described in (47). Briefly, the gel fragments were washed from the dye twice with
470 30 mM NH_4HCO_3 , 40% acetonitrile, dehydrated with 100% acetonitrile, and treated with trypsin
471 (Promega) (20 $\mu\text{g}/\text{mL}$ in 50 mM NH_4HCO_3) at 37°C for 5 hours. Tryptic peptides were mixed with
472 a 2,5-DHB matrix (Bruker), applied to a target, and mass spectra were obtained on a MALDI-
473 TOF/TOF mass spectrometer UltrafleXtreme (Bruker) in the positive ion mode. For each
474 spectrum, 5000 laser pulses were summed up. Protein identification was performed using
475 MASCOT (www.matrixscience.com) using the NCBI database (www.ncbi.nlm.nih.gov). The error
476 was limited to 20 ppm. Methionine oxidation and deamidation were indicated as variable
477 modifications. Identification was considered reliable ($p < 0.05$) if the score value exceeded the
478 threshold value.

479

480 **4.6. TEM analysis**

481 Bacteria-containing suspensions were centrifuged at 5000 g for 10 min and fixed with 2.5%
482 PBS-buffered glutaraldehyde solution (Sigma Inc.) for 1 h. The glutaraldehyde-fixed bacterial
483 cells were twice washed by PBS and post-fixed in 1% OsO_4 solution for 1 h. Then, the cells were
484 dehydrated in series of ethanol solutions of gradually increasing concentration, infiltrated with
485 acetone and embedded in the Epon epoxy resin. The ultrathin sections (50-70 nm) of the bacteria
486 under study were obtained using ultramicrotome Leica UC7 (Germany). The sections were
487 collected on the 200 mesh copper grids and contrasted with uranyl-acetate and lead citrate. TEM
488 analysis of the sections was performed using the electron microscope JEOL JEM 1011 (Japan),
489 equipped with a high-resolution digital camera Morada (Olympus, Japan).

490

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497

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- 616
- 617

618

FIGURE LEGENDS

619

620 **Fig. 1. Mineral analysis of CaCO₃ precipitates induced by the *B. licheniformis* DSMZ 8287**
621 **in the liquid medium B4-UCa for 14 days.** FTIR spectra of precipitates at different times of
622 growth shows vaterite peak loss after 30 hours: **A**, X-ray diffraction patterns of the samples shows
623 polymorph transition from vaterite to calcite: **B**, variation of SEM topologies, particles growth and
624 loss of amorphous environment by 14th day: **C**.

625 **Fig 2. Time-dependences of *B. licheniformis* DSMZ 8782 growth parameters in the**
626 **B4-UCa medium for the first 48 hours.** **A:** pH; **B:** specific urease activity; **C:** biomass
627 accumulation.

628 **Fig. 3. *B. licheniformis* cell morphology evolution during the first 48 hours of the growth.**
629 Images of bacterial cells grown in the B4-C (control: **A**, **C**, **E**, **G** pictures) and B4-UCa
630 (experiment: **B**, **D**, **F**, **H**) media were obtained by the light (left) and atomic force (right)
631 microscopies.

632 **Fig. 4. *B. licheniformis* cell morphology at the 24 hours after inoculation.** Bacterial cells
633 were grown in four media: the B4-C (control, **A**), B4-U (with urea, **B**), B4-Ca (with Ca²⁺, **C**) and
634 B4-UCa (with urea and calcium, **D**). Samples were observed by the light (left) and atomic force
635 (right) microscopies.

636 **Fig. 5. Proteome analysis of the ECM washed with cells during the growth for 24 h.** **A:**
637 image of a protein PAGE separation of the fraction C (PBS-washed cells and ECM) produced by
638 the cells in the control and experimental media; **B – D:** densitometric analysis of the zones differed
639 in color intensity (marked with arrows on the panel **A**). The relative volume values were obtained
640 by normalizing the color intensity of a zone to the total color intensity of the corresponding lane.
641 The background value of the relative intensity (indicated by the dashed line) corresponds to the
642 normalized random area of the corresponding track of a similar size.

643

644 **Fig. 6. Transmission electron micrographs of unstained cells of *Bacillus licheniformis* at**
645 **24 h after the inoculation. A:** a sample withdrawn from the control medium B4-C; **B:** a sample
646 from the B4-UCa medium. Electron dense inclusions within the cell are indicated by black arrows.
647

