

1 Factors affecting formation of large calcite crystals (≥ 1 mm) in *Bacillus subtilis* 168 biofilm

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15 Abstract

16 B4 is the most common medium used in general organomineralization studies and has been used to
17 assay or to characterize mineral precipitation potential. In an exercise for the optimization of the laboratory
18 conditions of crystal precipitation *in vitro*, we used *Bacillus subtilis* 168 as a type strain and its isogenic
19 mutants. While literature is mainly focused on observing generic precipitation, we investigated the
20 requirement to obtain large crystals (≥ 1 mm), which could be advantageous in wide-ranging implications
21 for bioconsolidation of soil, sand, stone, and cementitious materials. Calcite crystals are visible on B4 agar
22 plates within 7 days at 37°C after inoculum of *B. subtilis* 168 strain. In this study we show that to form
23 large crystals with a diameter ≥ 1 mm several conditions must be met: i) Reduced amount of B4 medium
24 into the Petri plate improve crystal formation. 55mm Petri plates contained only 4mL of B4 agar medium
25 reached a plateau in 6 days at 37°C. High moisture and presence of water condense would decrease
26 crystal formation. ii) Inoculation of cells using a rod instead of a circular shaped spot. When the same
27 number of *B. subtilis* cells was streaked, rod-shape biofilm significantly fostered crystal precipitation, while
28 spot-shape prevented precipitation. iii) When more than one biofilm is present within the same plate,

29 mutual interactions can affect precipitation in each biofilm. iv) Spherical nucleation sites are identified as
30 initial step during the formation of large calcite crystal.

31

32 Keywords: Biofilm formation, *Bacillus subtilis*, calcite crystals, CaCO₃, organomineralization, B4

33

34 Introduction

35 B4 is extensively used to assess potential mineral precipitation on biofilm *in vitro*. We used *Bacillus*
36 *subtilis* 168 as a type strain and its isogenic mutants as model organism to study calcite
37 organomineralization on bacterial biofilms while growing on it. No work is currently focusing to the size of
38 the crystal *in vitro* conditions. The investigation of the requirement to obtain large crystals (≥1mm) could
39 be advantageous in wide-ranging implications for the bioconsolidation of soil, sand, stone, and
40 cementitious materials. Recently, bacterial biomineralization has drawn the attention for its importance in
41 many fields. It is relevant in ecology: CaCO₃ precipitation in hot springs (Jones and Peng 2014), fresh
42 water (Saunders et al. 2014) and marine cyanobacterial mats (Kazmierczak et al. 2015) to cite some
43 examples. From a more practical stand point, CaCO₃ mineral precipitation is involved in innovative
44 technologies for stone consolidation, for example, the generation of inorganic material on bed biofilm
45 reactor-membrane bioreactors (Gonzalez-Martinez et al. 2015), and as a new tool in the conservation of
46 monumental calcareous stones (Perito et al. 2014; Zamarreño et al. 2009).

47 For these applied subjects, the need for a laboratory *in vitro* model system is required and also the size of
48 the crystal is important. Previous studies have already used *Bacillus subtilis* 168 as a type strain to study
49 calcite precipitation on biofilms by using the maximum medium B4 rich in calcium (Barabesi et al. 2007;
50 Marvasi et al. 2010; Marvasi et al. 2012; Boquet et al. 1973). Calcite crystals are obtained by streaking *B.*
51 *subtilis* 168 strain on B4 medium and calcite crystals are visible on B4 within 7 days at 37°C.

52 Furthermore, we have previously isolated isogenic *B. subtilis* 168 mutants unable to precipitate calcite
53 crystals on B4 (Barabesi et al. 2007). The mutated genes belong to the *lcfA* operon, with functions
54 involved in fatty acid metabolism. Our data pointed to an involvement of the whole cluster in the
55 precipitation phenotype and suggested a link between calcite precipitation and fatty acid metabolism. We
56 hypothesized possible pleiotropic effects of mutations in fatty acid pathways on cellular metabolism or *lcfA*

57 operon could be involved in the synthesis of a lipid intermediate (e.g. an acyl CoA intermediate) directly
58 involved in biomineralization (Barabesi et al. 2007, Tojo et al. 2011; Surorova et al. 2015). In a following
59 study we demonstrated that the decrease in pH of the FBC5 biofilm, the strain carrying a mutation in last
60 gene of the *lcfA* operon, *etfA*, was the main process responsible for the impairment in precipitation. Since
61 *EtfA* is known to be involved in redox reactions of NAD⁺/NADH, we proposed that a misregulation in some
62 part of the *EtfA* pathway could be responsible for NADH accumulation in FBC5 and consequently for
63 exceeding extrusion of H⁺ (Marvasi et al. 2010).

64 Calcium carbonate deposition by bacteria is largely dependent on environmental conditions (Wei et al.
65 2015; Pedley 2014; Beveridge 1989; Perito and Mastromei 2011). On B4, precipitation is controlled by
66 factors such as the pH of the medium and the acidification released by the biofilm during metabolic
67 processes including carbonic acids and extrusion of protons via respiration chain (Marvasi et al. 2010). In
68 this sense *in vitro* studies may help to compare carbonate structures typical isolated from the
69 environment. For example spherical shapes (typical of spherulites or leiolites at macroscale), which
70 originate from a combination of extracellular polymeric substances (EPS) and chemical crystallization,
71 have been observed in the environment (Dupraz et al 2009a; Wei et al. 2015; Shirakawa et al. 2011;
72 Banerjee and Joshi 2014). For example in freshwater microbial deposits often show carbonate
73 precipitation on impregnation of cyanobacterial sheaths or cells. Likewise, spherulites (calcitic fibro-radial
74 spherulitic poly- crystals) in subaerial calcrete laminar crusts are also a product of photosynthetically
75 induced calcium carbonate precipitation (Verrecchia et al, 1995; Dupraz et al 2009a). We were intrigued
76 whether the formation of large crystals would have let the observation of such shapes in the *B. subtilis*
77 biofilm – in a not photosynthetic environment.

78
79 In this study we show that to form large crystals with a diameter ≥1mm several conditions must be met: i)
80 Low water content; ii) Inoculation methods, iii) Mutual interactions of biofilms co-inoculated in the same
81 plate. Finally, spherical nucleation sites have been observed during the formation of large calcite crystal.

82
83 Materials and Methods

84 *Bacterial strains and B4 precipitation medium.* Strains used in this study were *Bacillus subtilis* 168
85 (Anagnostopoulos and Spizizen 1961) and *B. subtilis* mutants described in the Supplementary Material
86 Table S1 (Barabesi et al. 2007). To test calcite crystal formation the cultures were routinely grown on B4
87 solid (0.4% yeast extract, 0.5% dextrose, 0.25% calcium acetate, 1.4% agar) (Boquet et al. 1973). Calcite
88 was identified via energy-dispersive X-ray spectroscopy (Barabesi et al. 2007). Mutant strains were
89 supplemented with chloramphenicol 5 mg/ml (SIGMA) to maintain the selective pressure for maintaining
90 the inserted plasmid. Unless otherwise specified, biofilms were grown on plates incubated at 37°C inside a
91 plastic bag to prevent dehydration. Bromothymol blue (SIGMA) which transition of pH ranges from yellow
92 pH 6.0, green pH 6.7 and blue pH 7.6 was used as a pH indicator. Bromothymol blue stock solutions (20
93 mg/ml in NaOH 0.1N) were added to B4 medium before autoclaving at a final concentration of 0.0025%
94 (v/v).

95
96 *Test for calcium carbonate precipitation (in-vitro).* Petri dishes 55mm (Fisher) were filled with 4 mL or 10
97 mL B4 agar medium according with the experiment. All strains were streaked as a single 4-cm streak at
98 the center of the B4 agar plate by using a volume of 50 μ L of 10⁸ CFU/mL from an overnight culture of *B.*
99 *subtilis* in LB (Oxoid). Standard incubation occurred by putting plates into a plastic bag to prevent
100 dehydration during the 14 days of incubation at 37°C. When additional moisture was requested, a 2x2cm
101 filter paper (Whatman) soaked with 2 mL of sterile water was placed on the lid during the incubation of the
102 B4 agar plates. B4 plates inoculated with *B. subtilis* 168 were daily observed to measure crystal
103 precipitation. Five replicas for each experiment were performed. We were interested in the formation of
104 large and visible calcite crystals on the biofilm. To that end only crystals with a diameter larger than ≥ 1 mm
105 were counted. According with the formation of single, isolated crystals on the biofilm, the following
106 numerical score was associated: (0) no crystal formation; (1) First single crystal (≥ 1 mm) was observed; (2)
107 Between 2 and 10 crystals (≥ 1 mm) were counted; (3) More than 10 crystals (≥ 1 mm) were counted.
108 Observations occurred visually by using a stereomicroscope (Olympus SZ51). Pictures of the streaks
109 were taken by using an Olympus camera mounted on the stereomicroscope (Olympus SZ51).

110
111 *Diffusible extracellular factor assays.* Two streaks of different *B. subtilis* 168 isogenic pairs (Supplemental

112 Material Table SI) were inoculated with an L shape with one arm of the L parallel and adjacent to the other
113 strain (but not touching the other strain). The other arm of the L shape was distal. Distal arms were used
114 as controls (Barber et al. 1997). In control plates a portion of agar medium was removed with a sterile
115 scalpel to generate a void between the two parallel strains, this prevented passage of diffusible factors
116 (and possible acidification or alkalization effects) between biofilms (Supplementary Material Figure S1).
117 Plates were incubated at 37°C for 15 days.

118
119 *Crystals desiccation.* Biofilms were grown until well-developed calcite crystals were formed, crystals were
120 desiccated at 40° C for 6 days. Pictures of crystal development in the biofilms according to the inoculation
121 mode used were taken by using an Olympus camera mounted on the stereomicroscope (Olympus SZ51).

122
123 Scanning electron microscope (SEM) images. Crystals morphology was observed in fresh samples by
124 electron microscopy (ESEM Quanta-200 FEI) without fixation.

125
126 Statistical analysis: JMP (SAS) statistical software was used to infer the *t*-test.

127
128 Results

129 In the course of the study, a number of conditions were considered as essential for large ($\geq 1\text{mm}$) *in vitro*
130 crystal formation on *Bacillus subtilis* biofilms. The conditions are reported in the following paragraphs.

131
132 *1. Moisture and total volume of B4 affect calcite crystal formation on B4 agar medium.*

133 In order to investigate to what extent moisture during biofilm development impaired calcite crystals
134 formation, 55mm Petri plates with different B4 agar content were prepared. Biofilms (4 cm long) of *B.*
135 *subtilis* 168 were streaked on B4 plates filled with 10mL of B4 agar and crystals formation was recorded
136 for 14 days as described in material and methods. Crystal formation was completed at day 12 (Figure 1,
137 panel A). To measure whether the volume of B4 agar inside the plates contributed to calcite precipitation,
138 plates with only 4 mL of B4 agar medium were also inoculated. Interestingly, plates containing 4 mL
139 instead of 10 mL of precipitation medium reached a plateau in 6 days (Figure 1, Panel A).

140 To determine if an excess of humidity during incubation affected calcite precipitation, 10 mL plates with a
141 filter paper soaked with water was placed inside the lid and plates were incubated face-down as usual.
142 Under these conditions no crystal formation was observed (Fig. 1, Panel B). However, when the amount
143 of B4 agar medium in the plates was reduced to 4 mL, precipitation was completed after 9 days of
144 incubation (Fig 1, Panel B).

145
146 *2. Rod-streaking shape and initial number of cell seem to favor precipitation of large crystals onto*
147 *precipitation media.*

148 We tested to what extent the initial number of cells and inoculation method (spotting vs streaking) affected
149 calcite precipitation on B4 media. 50 μ L of a 10^9 CFU/mL *B. subtilis* 168 suspension and relative dilutions
150 (10^7 and 10^5 CFU/mL) were streaked as a rod-shape (4 cm long rod streak) or spotted (1.5 cm of
151 diameter) (Figure 2, A and B) onto B4 agar plates. No significant differences were reported for any
152 dilutions on the streak, however the type of plating – streak versus spot – significantly affected calcite
153 precipitation (Figure 2). Interestingly, when cells were plated as spot, complete precipitation (≥ 10 crystals
154 on the biofilm) was never observed (Figure 2, B). An overall reduction of calcite precipitation was
155 measured when spots were compared with streaks. Significant increase in precipitation was observed on
156 the spot where 10^5 CFU/mL were used when compared with suspensions with higher cellular
157 concentrations (Figure 2, panel B, days 12 and 13). At the end of the 14-day test, cellular concentrations
158 were not significantly different. Visual differences in precipitations may be appreciated in Figure 3, where
159 a representative picture is shown (Figure 3, streak panel A and spot panel B).

160
161 *3. Mutual interaction of biofilms contributes to calcite precipitation*

162 To test to what extent *B. subtilis* biofilms are able to mutually affect each other for calcite precipitation, a
163 diffusion test was performed (Barber et al. 1997). In this study we used strains of *B. subtilis* mutated in the
164 genes of *lcfA* operon, a gene cluster with functions in fatty acid metabolism that we identified as involved
165 in the precipitation phenotype in a previous study (Barabesi et al. 2007). Mutants used in this study are
166 reported in Table I, many of them are not able to precipitate calcite crystals in their biofilms (Table I).
167 Diffusion test was performed by streaking *B. subtilis* wild type and its isogenic form in an L-shape. Two

168 arms of the L are adjacent, while the distal arms are used as control and to test how far the diffusion
169 proceeds. After 15 days of incubation, strains FBC2 and FBC3 coupled with *B. subtilis* 168 were able to
170 restore the precipitation on parallel arm (Table I). When pairs of the same *B. subtilis* mutant impaired in
171 calcite formation were tested, precipitation did not occur (Table I).

172 Strains FBC4 and FBC5 coupled with *B. subtilis* 168 were unable to precipitate calcite in standard B4
173 medium (Table I). In FBC3, diffusion test showed that the adjacent streak section was able to partially
174 restore the precipitation (Figure 4). As control, a portion of agar in between the two parallel strains (168
175 and FBC3) was removed. Mutual interactions were not observed due to the lack of diffusion (Table II).

176 We previously demonstrated that pH was indeed a main determinant in mineral precipitation since
177 buffering B4 medium re-established calcite formation in *B. subtilis* mutants (Marvasi et al. 2010). To
178 determine if pH changes could affect calcite precipitation behavior of FBC3 when streaked together with
179 168, a bromothymol blue pH indicator was added to the B4 precipitation medium. The arm of FBC3 streak
180 adjacent to that of the wild type 168 was less acidic than the distal arm, indicating that re-establishment of
181 calcite formation in the mutant can be due to alkalization released by the wild type (Supplementary
182 material S2).

183

184 4. Spherical crystals serve as nucleation sites on *B. subtilis* biofilms.

185 Even though crystal formation on *B. subtilis* biofilms has been extensively studied, only a few studies
186 focused on the initial nucleation step(s) of crystal nucleation during growth on B4 precipitation medium.
187 Nanospheres of calcite were previously identified at early stages in calcite precipitation microbial mats
188 (Dupraz et al. 2004). We therefore hypothesized that if early nucleation occurred within the biofilm,
189 spherical calcite structures would be identified within the biofilm depth. Spherical structures were indeed
190 observed after biofilm removal (Figure 5, A). Interestingly, spherical structures were connected with the
191 external part of the crystal, revealing a putative early stage of crystal formation (Figure 5, B). SEM images
192 also revealed spherical structure beneath the flat crystal structure (Figure 5, C and D) indicating that as
193 the potential origin of nucleation sites on *B. subtilis* biofilms.

194

195 Discussion

196 Due to the relevance of biologically induced mineralization we decided to study several physical
197 conditions required for calcite formation of large crystals (≥ 1 mm in diameter) in *B. subtilis* by using B4 as
198 precipitating medium. Several factors were considered as essential for large crystal formation in *B. subtilis*
199 biofilm. First, water content (thickness of the medium, presence of moisture). Active carbonate nucleation
200 occurs when bacterial cell surfaces are utilized as nucleation sites, owing to the chemically heterogeneous
201 macromolecules that impart a net electronegative charge, which favors the adsorption of cations with
202 CO_3^{2-} or HCO_3^- (Schultze-Lam and Beveridge 1994). In this context water plays a pivotal role. The
203 equilibrium constant for the dissolution of CaCO_3 is $K_{ps} 4.5 \times 10^{-9}$ and when the ionic product $Q_{sp} > K_{sp}$ a
204 precipitate is expected to form (Blackman A et al. 2016). A reduced amount of water would push the
205 equilibrium of the reaction toward the formation of salt. High concentration of calcium will favor the
206 formation of calcite on biofilm, and indeed precipitation occurred faster and at higher rate where on the
207 plates that don't contain the paper soaked with water. It is reasonable to assume that a minor volume of
208 B4 (4 ml), and therefore water, would foster precipitation.

209 Water is not the only factor limiting crystal formation. Streak's shape curiously also affects crystal
210 formation. We found that spotting *B. subtilis* 168 cells on B4 resulted in a decreased and delayed
211 production of crystals when compared with the a 4-cm long streak. On the other hand, the number of cells
212 seems to affect precipitation in the spot, with diluted inoculum (10^5 CFU/mL) inducing a better precipitation
213 on day 12th and 13th (Figure 2, panel B). We can speculate on causes that can be responsible for the
214 reduced precipitation in spot: i) Higher number of cells per cm^2 in the spot compared with the streak due to
215 the dilution of cells along the 4 cm of the streak itself; ii) unequal total area covered by the spot and the
216 streak leading to difference given the resources available to the cells; iii) the differences seen in spotting
217 and longitudinal streaking might be a function of chemical concentration. A lateral (longitudinal) streak
218 would allow the agar near the middle of the streak to be exposed to a higher concentration of crystal-
219 inducing chemical environment, which would not be evident in a spot inoculation. iv) The physiology of the
220 biofilm at the edge. Spatiotemporal analysis showed differentiation within a biofilm with zones (Vlamakis et
221 al. 2008) where motile cells, and therefore more active in respiration, NAHD production are at the edges
222 ultimately resulting in an increase of acidity (Vlamakis et al. 2008).

223 This observation is not surprising since microorganisms can change the microenvironmental conditions
224 with their metabolism (Marvasi et al. 2010; Marvasi et al. 2012; Dupraz et al. 2009b; Dupraz et al. 2008).
225 Biologically induced biomineralization is characterized through a delicate equilibrium where the
226 environment and the microbial metabolism play together to foster and shape crystals. In this study we
227 have shown that biofilms can affect each other by mutual pH interactions by using a diffusion test. Results
228 from the diffusion tests indicate that the alkalization of the medium induced by stronger alkalizing strains
229 (such as *B. subtilis* 168), do foster crystal formation on the parallel section of the acidifying biofilm.
230 However, strains previously reported to decrease the pH of their biofilms during growth (ie. strains FBC5
231 or FBC4 (Marvasi et al. 2010) prevented crystal formation and reduced precipitation in 168 biofilm (Table
232 I). We cannot exclude that other molecules may be responsible for the restoration of the precipitation from
233 strains capable of calcite precipitation to impaired mutant, however pH is currently the main responsible of
234 the restored precipitation, since using media buffered at different pH values calcite formation was
235 controlled (Marvasi et al. 2010). These data show it is necessary to streak one single bacterial strain per
236 plate to observe real precipitation and prevent influences from other strains. Other factor that affects
237 precipitation when two or more bacteria are closely grown is the depletion of calcium. Distinct strains can
238 deplete different amount of calcium (Shirakawa et al. 2011). *Pseudomonas putida*, for example,
239 consumed on average 96% of all Ca^{2+} available, *Lysinibacillus sphaericus* 74% and *Bacillus subtilis* only
240 28% calcium ion compared to the control of B4 without bacteria within 12 days of incubation. In addition,
241 the ratio of different competing ions such as magnesium and calcium has been proved to affect the
242 complexation with CO_3^{2-} (Mg/Ca) (Saunders et al. 2014). Experiments conducted from lithifying biofilm
243 isolated from River Lathkill (UK) showed that the $(\text{Mg}/\text{Ca})_{\text{calcite}}$ precipitation rate, rapidly formed
244 precipitates with very low magnesium content indicating kinetic control on fractionation (Saunders et al.
245 2014). These data show as the type of biofilm and biofilm growth rate control calcite precipitation
246 (Saunders et al. 2014). These results support, once more, the necessity to streak one strain per Petri
247 plate when testing its calcinogenic potential.

248
249 Another interesting finding of this *in vitro* system was the presence of spherical crystals as nucleation sites
250 on *B. subtilis* biofilms as reported in the SEM images. The initial nucleation may occur as a sphere and

251 when the crystal reaches the upper surfaces the shape changes by growing as a flat crystal. We
252 recognize that this spherical shape may occur on *B. subtilis* 168 due to the presence of a defined
253 extracellular polymeric substances (EPS) composition which affects crystal shape (Braissant et al. 2007).
254 Such spherical shapes have been observed in different bacterial genera and species and the model may
255 play a role in other biofilms and environments (Wei et al. 2015; Shirakawa et al. 2011; Banerjee and Joshi
256 2014).

257 In conclusion, we show that to form large crystals with a diameter ≥ 1 mm several conditions must be met.
258 Reduced amount of B4 medium in the Petri plate we recommend 55mm Petri plates contained only 4mL
259 of B4 agar showed the best performance. Presence of water condense would decrease crystal formation.
260 Inoculation of cells using a rod instead of inoculating a circular shaped spot. Mutual interactions can affect
261 precipitation in each biofilm, so we recommend streak one biofilm per plate. This inoculation strategy will
262 lead to the formation of spherical nucleation.

263 Further experiments of such experimental conditions could be used to make mathematical predictions
264 regarding the geochemical conditions required to yield mineral precipitation, including crystal size.

265

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268

269 Captions

270

271 Table I. Results of diffusible factor assay.

272 Table II. Diffusion test on plates with agar removed.

273

274 Figure 1. Effect of moisture and volume of B4 medium on crystal formation. Panel A shows crystals
275 growth on standard B4 medium. Panel B are plates in which contains a 2x2 cm filter paper soaked with
276 water and incubated on the lid into face-down incubated 55mm Petri plates. Error bars represent the
277 standard errors. Five replicas for each measurement were performed. The lines show a smooth curve
278 through the data. It is an approximating function that attempts to capture the pattern of the data.

279
280 Figure 2. Crystal formation by spotting or streaking serial dilutions on *B. subtilis* 168 on B4 medium. Panel
281 A, crystal formation of different dilutions of rod-shape biofilms. Panel B, crystal formation of serial dilution
282 with a circular-shaped biofilm. Asterisk (*) shows significant difference ($p < 0.05$). Error bars represent the
283 standard errors. The lines show a smooth curve through the data. It is an approximating function that
284 attempts to capture the pattern of the data.

285
286 Figure 3. Crystal formation on rod and spot shaped biofilm. *B. subtilis* cells were spotted with different
287 shape resulting in different crystal precipitation. Panel A, B shows 50 μ L of 10^9 CFU mL⁻¹. Panels C, D are
288 50 μ L of 10^9 CFU mL⁻¹. Arrows show representative crystals with a diameter ≥ 1 mm. In panel B only one
289 crystal >1 mm is identified. Each panel is made by recomposing a number of individual pictures taken with
290 the stereomicroscope. Photos taken from biofilms grown on 90mm Petri plates.

291
292 Figure 4. Diffusion test of the pair *B. subtilis* 168/FBC3 after 7 days of incubation at 37°C. Representative
293 crystals with a diameter ≥ 0.8 mm are shown with an arrow. Dotted bracket highlights an area with small
294 calcite crystals.

295
296 Figure 5. Spherical nucleation sites of calcite crystals produced by *B. subtilis* 168 biofilm. Panel A,
297 stereomicroscope image of calcite crystals. The black arrow shows a spherical nucleation site. Panel B,
298 stereomicroscope image at higher magnification of a crystal with the characteristic “umbrella” shape.
299 Panel C, ESEM micrograph of an agglomerate of calcite crystals produced by *B. subtilis* 168 biofilm. The
300 micrograph shows the bottom faces of the agglomerate. White arrows show calcite spheroid nucleation
301 sites. Panel D, Upper face. White arrows show flat circular area where crystals emerge from biofilm.

302
303 Supplemental Material Figure S1. The two strains are depicted by a dotted and continuous line. Gray
304 rectangle represent agar removed for the control.

305

306 Supplemental Material Figure S2. Qualitatively evidence of pH gradient (diffusion). pH of B4 medium
307 where strains 168 and FBC3 were streaked highlighted with the pH indicator bromothymol blue. In the
308 picture the indicator is lightly gray (pH 6.0), and dark gray where the pH is 7.6 where the precipitation
309 occurs. The distal arm of the mutant strain *B. subtilis* BCF3 is more acid (light gray in the picture) when
310 compared with the arm adjacent to the wild type *B. subtilis* 168. Arrows indicate the differences in color.

311

312 Supplemental Material Table S I. Strains used in this study.

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