

1 **A toxin-mediated policing system in *Bacillus* improves population fitness via penalizing**
2 **non-cooperating phenotypic cheaters**

3 **Authors**

4 Rong Huang¹, Jiahui Shao¹, Zhihui Xu¹, Yuqi Chen¹, Yunpeng Liu², Dandan Wang³, Haichao Feng¹,
5 Weibing Xun¹, Qirong Shen¹, Nan Zhang^{1*}, Ruifu Zhang^{1,2*}

6 **Affiliations**

7 ¹Jiangsu Provincial Key Lab of Solid Organic Waste Utilization, Jiangsu Collaborative Innovation
8 Center of Solid Organic Wastes, Educational Ministry Engineering Center of Resource-saving
9 fertilizers, Nanjing Agricultural University, Nanjing 210095, Jiangsu, P. R. China

10 ²Key Laboratory of Microbial Resources Collection and Preservation, Ministry of Agriculture,
11 Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural
12 Sciences, Beijing 100081, P. R. China

13 ³National Engineering Research Center for Efficient Utilization of Soil and Fertilizer Resources,
14 College of Resources and Environment, Shandong Agricultural University, Daizong Road, Tai'an
15 271018, Shandong, P. R. China

16 ***Corresponding authors**

17 **Ruifu Zhang** (rfzhang@njau.edu.cn), **Nan Zhang** (nanzhang@njau.edu.cn), College of Resources
18 & Environmental Sciences, Nanjing Agricultural University, 210095, Nanjing, P. R. China.

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20 **Abstract**

21 Microbial cooperation is vulnerable to exploitation by social cheaters. Although the strategies for
22 controlling genotypic cheaters have been well investigated, the mechanism and significance of
23 preventing phenotypic cheating remain largely unknown. Here, we revealed the molecular
24 mechanism and ecological significance of a policing system for punishing phenotypic cheaters in
25 the community of a plant beneficial strain *Bacillus velezensis* SQR9. Coordinated activation of
26 extracellular matrix (ECM) production and autotoxin bacillunoic acids (BAs)
27 biosynthesis/self-immunity, punished public goods-nonproducing cheaters in strain SQR9's
28 community. Spo0A was identified to be the co-regulator for triggering both ECM production and
29 BAs synthesis/immunity, which activates acetyl-CoA carboxylase (ACC) to produce malonyl-CoA,
30 an essential precursor for BAs biosynthesis, thereby stimulating BAs production and self-immunity.
31 Elimination of phenotypic cheaters by this policing system, significantly enhanced population
32 fitness under different stress conditions and in plant rhizosphere. This study provides insights into
33 our understanding of maintenance and evolution of microbial cooperation.

34

35 **Introduction**

36 Cooperative interactions are not restricted to complex, higher organism, but also prevalent among
37 microbial communities in many contexts^{1,2}. Production of costly public goods that can be used by
38 any cells in a population, is a common cooperative behavior consistently found in diverse
39 microorganisms³. Typical public goods include extracellular enzymes for substances digesting⁴,
40 siderophore for iron-scavenging⁵, matrix components for biofilm formation^{6,7}, biosurfactants for

41 cooperative swarming^{8,9}, and so on. Intriguingly, the considerable cost for producing public goods
42 usually raises cheating individuals in the evolution of cooperation, who contributes no or just a little
43 of their share of the common good^{3,4,10}. Therefore, cheaters will have a fitness advantage over fully
44 participating cooperators, and their frequency will increase rapidly, eventually leading to the
45 collapse of cooperative behavior¹¹. This “tragedy of the commons” is predicated by natural selection
46 and game theory^{12,13}, and has been widely illustrated in various cooperation systems^{14,15}.

47 Despite the exploitation of public goods by cheating individuals, cooperation principally
48 survives cheating during the evolutionary history¹⁶. Several mechanisms have been proposed to play
49 significant roles in maintaining cooperation by preventing cheater invasion^{3,16,17}, mainly including
50 kin selection/discrimination that selectively direct cooperation to genetic relatives^{18,19}, facultative
51 cooperation regulated by quorum-sensing (QS) system²⁰ or nutrient fitness cost²¹, coupling
52 production of public and private goods²², punishment of cheating individuals by
53 cooperator-produced antibiotics^{10,23}, partial privatization of public goods under certain
54 conditions^{24,25}, and spatial structuring to surround the producers more likely by other cooperators²⁶.
55 In general, the emergency of multiple sanction strategies is a consequence of natural selection,
56 which suppress social cheaters and promote public goods production, thereby maintaining microbial
57 community stability and improving their adaptation in different niches³.

58 Microbial social cheating can occur either at the genotypic or phenotypic level. The genotypic
59 cheaters indicate the lost or mutation in specific gene(s) thus deficiency in the related biological
60 function^{14,27}; while the phenotypic cheaters are individuals with identical genetic background but
61 silencing or down-regulation in public goods production (heterogeneous expression or division of

62 labor)^{25,28}. Despite the well-studied mechanisms of cheater control on the genotypic level³, those
63 regarding to the phenotypic level remain largely unknown^{3,25}; also unlike the definite significance of
64 suppressing obligate genotypic cheaters¹⁷, the ecological roles of controlling phenotypic cheaters in
65 mediating microbial population fitness have been rarely concerned²⁹. Accordingly, lacking of the
66 knowledge about phenotypic cheating limits our understanding of the cooperation behavior within
67 microbial social communities.

68 Biofilms are extracellular matrix (ECM)-enclosed multicellular communities that sustain
69 bacterial survival in diverse natural environments³⁰⁻³², where the tightly associated cells are
70 heterogeneously expressed with only a subpopulation of matrix producers³³⁻³⁵. As the ECM
71 components (mainly include extracellular polysaccharides (EPS) and TasA fibers) are costly public
72 goods shared by all cells within the biofilm, the nonproducing phenotypic cheaters can emerge, and
73 thus disrupt the biofilm and community fitness^{25,36}. Although a few studies have investigated the
74 matrix production-cannibalism overlap and ECM privatization within biofilm individuals^{25,29}, the
75 molecular mechanism involved in punishment of nonproducing cheaters, as well as the ecological
76 significance of the policing system in regulating population stability and fitness, remain unclear.
77 *Bacillus velezensis* SQR9 (formerly *B. amyloliquefaciens* SQR9) is a well-studied beneficial
78 rhizobacterium that form robust and highly structured biofilms on air-liquid interface and plant
79 roots³⁷⁻⁴⁰. Production of toxic bacillunoic acids (BAs), encoded by a unique genomic island in strain
80 SQR9, was proved to occur in subfraction of cells with the self-immunity ability induced by BAs
81 during biofilm formation, where the nonproducing siblings will be lysed by BAs^{41,42}. Based on the
82 manifestation that the BA-mediated cannibalism enhanced biofilm formation of strain SQR9, we

83 hypothesized the ECM and BAs synthesis can be co-regulated to restrain cheaters and sustain
84 population stability. Using a combination of single-cell tracking technique, molecular approaches,
85 and ecological evaluation, we demonstrated the ECM and BAs production are coordinated in the
86 same subpopulation by the same regulator during biofilm formation, which enforces punishment of
87 the nonproducing phenotypic cheaters to maintain community stabilization; also this genomic
88 island-governed policing system is significant to promote community fitness in various conditions.

89

90 **Results**

91 **Coordinated production of extracellular matrix (ECM) and autotoxin bacillunoic acids (BAs)** 92 **punishes public goods-nonproducing cheaters in *B. velezensis* SQR9 community**

93 To test the hypothesis that secretion of cannibal toxin eliminates the public goods-nonproducing
94 cheaters in *B. velezensis* SQR9 community, we firstly tried to determine whether ECM (public
95 goods) production and BAs (autotoxin) biosynthesis/BAs-induced self-immunity occur in the same
96 subpopulation. We fused promoters for genes related to extracellular polysaccharides (EPS) and
97 TasA fibers biosynthesis with *mCherry*, while the promoters for genes related to the autotoxin BAs
98 biosynthesis and the self-immunity with *gfp*, obtained the *P_{eps}-mCherry*, *P_{tapA}-mCherry*, *P_{bnaf}-gfp*,
99 and *P_{bnab}-gfp*, respectively. Their expression patterns were monitored using confocal laser scanning
100 microscopy (CLSM) during the biofilm community formation. Photographs show that expression of
101 the *P_{eps}-mCherry*, *P_{tapA}-mCherry*, *P_{bnaf}-gfp*, and *P_{bnab}-gfp* were all observed in a subpopulation
102 cells of the whole community (Fig. 1), which suggests a differential expression pattern of each
103 function among subpopulations during biofilm formation, where the ECM-nonproducers can be

104 recognized as phenotypic cheaters²⁵. Importantly, the overlay of the double fluorescent reporters
105 indicates that ECM and BAs production is generally raised in the same subpopulation (Fig. 1; the
106 yellow cells represent co-expression of *mCherry* and *gfp*); as expected, since the self-immunity
107 gene *bnAAB* was reported to be specifically activated by endogenous BAs⁴², it was also
108 preferentially expressed in the same subpopulation with ECM-producers (Fig. 1). These
109 observations demonstrate a general coordination of ECM production and BAs synthesis/immunity
110 in the same subpopulation of *B. velezensis* SQR9 biofilm community.

111 Based on the co-expression pattern, we postulated that the ECM-nonproducing cheaters,
112 synchronously being sensitive to the BAs, will be killed by their siblings that produce both public
113 goods ECM and the autotoxin BAs. Combining propidium iodide (a red-fluorescent dye for labeling
114 dead cell) staining with reporter labelling, we monitored the cell death dynamics during the biofilm
115 formation process in real time. It was observed that a portion of the cells that didn't produce public
116 ECM (Fig. 2A & 2B) or toxic BAs (Fig. 2C), or silenced in expression of the self-immunity gene
117 *bnAAB* (Fig. 2D), were killed during the biofilm development process, while the corresponding
118 producers remained alive throughout the incubation (red arrows indicate the dead cells in Fig. 2;
119 Movies S1~S4). This lysis can be attributed to the BAs produced by the *gfp*-activated cells, as
120 cannibalism of *B. velezensis* SQR9 was largely dependent on the production of this secondary
121 metabolism⁴². Taken together, the double-labelling observation and cell death dynamics detection
122 indicate that the subpopulation of ECM and BAs producers selectively punish the nonproducing
123 siblings depend on a coordinately activated cell-differentiation pathway.

124

125 **Spo0A is the co-regulator for triggering ECM production and BAs synthesis/immunity**

126 To identify the potential co-regulator(s) of ECM production and BAs synthesis/immunity in *B.*
127 *velezensis* SQR9, we evaluated the BAs production in an array of mutants that known to be altered
128 in ECM synthesis ($\Delta degU$, $\Delta comPA$, $\Delta abrB$, $\Delta sinI$, $\Delta sinR$, and $\Delta spo0A$), by measuring their
129 antagonism towards *B. velezensis* FZB42, a target strain specifically inhibited by BAs but no other
130 antibiotics secreted by SQR9⁴¹. The crude extract of BAs of wild-type SQR9 showed remarkable
131 antagonism to the of lawn of strain FZB42 (Fig. 3A & 3B); only $\Delta spo0A$ but no other mutants (all
132 with the equal cell density of the wild-type), revealed significantly reduced inhibition zone towards
133 FZB42, and the complementary strain generally restored the antagonistic ability (Fig. 3A & 3B).
134 Spo0A is a well-investigated master regulator that governs multiple physiological behaviors in *B.*
135 *subtilis* and closely-related species^{43,44}; as expected, the EPS production and biofilm formation was
136 seriously impaired in $\Delta spo0A$ (Fig. S1). Intriguingly, $\Delta spo0A$ but neither its complementary strain
137 nor the wild-type, can be substantially inhibited by the crude extracted BAs of strain SQR9, while
138 $\Delta spo0A$ was not inhibited by $\Delta GI3$ that disabled in BAs production (Fig. 3C), suggesting Spo0A
139 does participate in the immunity to BAs. In addition, we constructed *gfp* transcriptional fusions to
140 the promoter of genes involved in ECM production (*eps* & *tapA*) and BAs biosynthesis/immunity
141 (*bnaF/bnaAB*), and discovered that under both liquid culture (Fig. 3D) and plate colony conditions
142 (Fig. S2), their expression level was significantly decreased in $\Delta spo0A$ as compared with the
143 wild-type, which was restored in the complementary strain $\Delta spo0A/spo0A$. These results suggest
144 that the global regulator Spo0A is the co-regulator for controlling ECM production and BAs
145 biosynthesis/immunity in *B. velezensis*, which is probably dependent on the transcriptional

146 regulation of certain relevant genes.

147

148 **Spo0A activates acetyl-CoA carboxylase (ACC) to support BAs synthesis and self-immunity**

149 Despite the well-known Spo0A pathway in governing ECM production and biofilm formation in
150 *Bacillus*⁶, how does Spo0A regulate BAs synthesis and self-immunity remains unknown. By using
151 the biolayer interferometry analysis (BLI) for detecting molecular interaction, we revealed that the
152 purified protein Spo0A cannot directly bind to the promoter of *bnaf*, suggesting it doesn't induce
153 BAs production through direct transcriptional activation (Fig. S3). Alternatively, Spo0A has been
154 reported to stimulate the expression of *accDA* that encodes acetyl-CoA carboxylase^{45,46}, which
155 catalyzes acetyl-CoA to generate malonyl-CoA, an essential precursor for BAs biosynthesis (Fig.
156 4A)⁴¹; therefore we postulated *accDA* may be involved in the regulation of BAs
157 production/immunity by Spo0A. We firstly verified the positive regulation of Spo0A on *accDA*
158 expression in *B. velezensis* SQR9 by *gfp* fusion (Fig. 4B & Fig. S4). Since knockout of *accDA*, the
159 essential gene for fatty acids biosynthesis, significantly impact bacterial growth, we alternatively
160 constructed a strain in which the original promoter of *accDA* was replaced by a xylose-inducible
161 promoter (*P_{xyt}*), and monitored its BAs synthesis/immunity under different xylose induction
162 conditions. The SQR9-*P_{xyt}-accDA* lost the antagonism ability towards target strain FZB42 in the
163 absence of xylose, while the inhibition was significantly enhanced with the induction of xylose in a
164 dose-dependent manner (Fig. 4c & 4d). Since exogenous xylose didn't influence the suppression of
165 wild-type SQR9 on FZB42 (Fig. 4C & 4D), these results suggest that *accDA* expression positively
166 contribute to BAs production. Importantly, the SQR9-*P_{xyt}-accDA* was proved to be sensitive to

167 SQR9-produced BAs without xylose addition, and the immunity was gradually restored with xylose
168 supplement (Fig. 4E). The xylose-induced transcription of *accDA*, also resulted in enhanced
169 expression of genes involved in self-immunity (*bnAAB*; Fig. 4F & Fig. S5A) but not BAs synthesis
170 (*bnAF*; Fig. 4F & Fig. S5B), as the *AccDA*-derived malonyl-CoA accumulation affects BAs
171 production in a post-transcriptional manner. The CLSM photographs also reveal that the activation
172 of *accDA* (*mCherry* fusion) and *bnAAB* (*gfp* fusion) was located in the same subpopulation cells
173 (Fig. S6). Accordingly, these results indicate the positive regulation of Spo0A on BAs
174 production/immunity in *B. velezensis* SQR9, is strongly dependent on *accDA* that encodes
175 acetyl-CoA carboxylase.

176

177 **The co-regulation policing system enhances population stability and fitness**

178 Having illustrated the molecular mechanism of the co-regulation pathway for punishing
179 nonproducing cheaters in *B. velezensis* SQR9, we wondered the broad-spectrum ecological
180 significance of this policing system for *B. velezensis* SQR9 in a community level. We constructed
181 two mutants with disabled sanction mechanism, the $\Delta bnAV$ deficient in BAs synthesis (loss of the
182 punishing weapon) and the SQR9-*P₄₃-bnAAB* that continually expresses the self-immunity genes
183 (cheaters cannot be punished by the weapon BAs), both mutants showed similar growth
184 characteristics with the wild-type (Fig. S7). We firstly applied flow cytometry analysis to test
185 whether lack of the policing system ($\Delta bnAV$ and SQR9-*P₄₃-bnAAB*) impair the punishment of public
186 goods nonproducing cheaters during biofilm formation. The proportion of matrix-producing
187 cooperators (*eps* & *tapA* active cells) in the wild-type community, as well as the average expression

188 level of corresponding genes, were significantly higher than that in the $\Delta bnaV$ or SQR9- P_{43} -*bnaAB*
189 community (Fig. 5A & 5B), suggesting the cheating individuals were not effectively controlled in
190 the two mutants population. Consequently, the wild-type established a more vigorous biofilm as
191 compared with the two mutants, as shown by the earlier initial progress, larger maximum biomass,
192 and delayed dispersal process (prolonged stationary phase) (Fig. 5C & 5D). Additionally, the robust
193 biofilm formed by the wild-type also endowed them stronger resistance against different stresses,
194 including antibiotics, salinity, acid-base, and oxidation (Fig. 5D, Figs. S8 & S9).

195 Besides the well-known regulation on biofilm matrix production, Spo0A also controls the
196 production of other public goods, such as proteases and siderophore^{44,47}; it can be recognized as a
197 critical switch that governs the cell transition from a free-living and fast-growing status
198 (Spo0A-OFF), to a multicellular and cooperative style (Spo0A-ON)^{34,48}. Intrinsically, the punishing
199 targets of this policing system are supposed not limited to the matrix-nonproducing cheaters, but all
200 of the Spo0A-OFF individuals (cells that don't express the immune genes *bnaAB*). Therefore we
201 determined the production of extracellular proteases and siderophore among the three strains,
202 revealing that these public goods were also accumulated more in the wild-type than in these two
203 mutants community (Fig. 5E & Fig. S10). Importantly, the wild-type SQR9 demonstrated a
204 significantly stronger root colonization comparing with the two mutant strains losing the cheater
205 punishing system (Fig. 5F). In summary, the Spo0A governed co-regulation punishment system
206 effectively excludes the nonproducing cheaters of public goods in *B. velezensis* population, thereby
207 improving the population stability and ecological fitness under different conditions.

208

209 **Discussion**

210 Microbes have evolved diverse strategies for preventing cheaters in their communities. Despite the
211 well-established mechanisms for controlling genotypic cheaters^{3,16,17,49}, those regarding the
212 phenotypic cheaters remain largely unknown^{25,29}. Phenotypic cheaters are ordinarily derived from
213 heterogeneous expression of different biological functions within a cell population³⁵, this division of
214 labor is postulated to afford bacterial community a better adaptation to unexpected environmental
215 fluctuations³⁴; however, when cells are supposed to be developed into a certain type in response to
216 the surroundings, such as producing ECM to form surface-attached biofilm, or secreting
217 extracellular enzymes to excavate resources, the individuals that don't perform these assignments
218 (but still share the community's public goods) become actual cheaters and may disturb the
219 community stability and fitness^{15,29}. In the present study, we demonstrated that during biofilm
220 formation, the beneficial rhizobacterium *B. velezensis* SQR9 engages a policing system that
221 coordinately activates ECM production and autotoxin synthesis/immunity, to punish the phenotypic
222 cheaters silencing in public goods secretion and reduce their proportion in the community (Fig. 6).
223 This finding coincides with the coordinated cannibalism phenomenon reported in biofilm formation
224 by *B. subtilis*²⁹. Specifically, the toxic BAs for punishment is synthesized by a horizontal gene
225 transfer (HGT)-acquired genomic island⁴¹, where its production is regulated by a
226 precursor-dependent post-transcriptional manner (Fig. 4), and self-immunity is induced by the BAs
227 through a two-component system⁴²; importantly, this sanction mechanism not only facilitates ECM
228 accumulation, but also contributes to enhanced production of other public goods including proteases
229 and siderophore, thereby effectively improving the community fitness under different stressful

230 conditions and in plant rhizosphere (Fig. 5). Actually, the coordination policing system eliminates
231 phenotypic cheaters that stay in a fast-growing, motility phase (Spo0A~OFF state), to promote the
232 population to a stationary, resource-mining phase (Spo0A-ON state) when environment required
233 (Fig. 6). It should be noted that the phenotypic cheaters are not so obligate or detrimental, and this
234 punishment is relatively temperate than those for genotypic cheaters^{10,50} as only a subpopulation of
235 the cheaters were killed (Fig. 2); we think this scene is a balance between restraining the temporary
236 cheaters and retaining the advantages of heterogeneous population^{34,51}.

237 The diversified cheater-controlling mechanisms used by microorganisms, reveal different
238 applicability features and can occur in various types of microbial cooperation¹⁷. For instance, kin
239 discrimination is effective for controlling non-kin cheaters with different genetic backgrounds^{52,53},
240 but appears incapable of preventing spontaneous genotypic cheaters in the same population, quite
241 apart from the phenotypic cheaters³; facultative cooperation enables microbial population to
242 optimize the occasion for producing public goods, which is an economic-style strategy for
243 minimizing resource exploitation by cheaters while is unlikely to suppress them directly^{20,54,55};
244 partial privatization and spatial structuring can immediately restrain the cheaters by physical
245 separation^{25,26}. Comparatively, the targeted benefit (private benefit) and punishment mechanisms
246 afford cooperators direct fitness advantage over cheaters^{10,22}, especially the latter precisely
247 antagonizes the cheating individuals to eliminate them from the community^{4,10}. The punishment
248 strategy is usually elaborately regulated by QS or QS-like system for coupling the public goods
249 production and autotoxins synthesis/immunity⁵⁶, therefore it is both complicated for cheaters to
250 overcome and costly for cooperators to implement¹⁶. Here we prove that the policing system in *B.*

251 *velezensis* SQR9 contributes to optimized cell differentiation and population fitness, suggesting its
252 ecological benefits does overcome the costs for expressing antibiotic production and immunity (Fig.
253 5). Alternatively, this sanction system can work in concert with privatization strategy to collectively
254 prevent cheater invasion during biofilm formation²⁵.

255 Interestingly, the secondary metabolites applied by *B. velezensis* SQR9 to punish the cheaters,
256 are governed by a unique genomic island acquired through HGT⁴¹. Since *accDA* that is important
257 for both BAs biosynthesis and the corresponding self-immunity, and *eps* and *tapA* operon required
258 for ECM production, are all activated by Spo0A with moderate phosphorylation level (Fig. 4)^{6,42,45},
259 these genes constitute an ingenious co-regulatory network to appoint the cooperators to be BAs
260 producers and defenders, while the cheaters to be sensitive individuals that can be eliminated (Figs.
261 1 & 2). It was known that clusters carrying antibiotic biosynthesis and resistance genes (ARGs) are
262 usually transformed among microbes through HGT in natural environment^{57,58}, but since these
263 elements also brought certain costs such as DNA replication and metabolic burden, they must
264 produce considerable benefits to be reserved in the new host. Here the SQR9-acquired GI3 not only
265 act as a weapon for antagonizing closely related competitors⁴¹, but also establishes a policing
266 system for punishing cheaters within the internal community. We consider this dual function of the
267 antibacterial fatty acids could explain why this large cluster was integrated in the genome of *B.*
268 *velezensis* SQR9, and this case can provide inspirations for discovering novel molecular regulatory
269 mechanisms and understanding microbial evolution events.

270 In conclusion, the present study highlights the beneficial rhizobacterium *B. velezensis* SQR9
271 engages a policing system that coordinately activates ECM production and autotoxin

272 synthesis/immunity, to eliminate the phenotypic cheaters silencing in public goods secretion thereby
273 enhancing the community fitness. This study provides insights of the molecular mechanism
274 involved in controlling phenotypic cheaters, as well as the ecological roles of the policing system,
275 which deepens our understanding of maintenance and evolution of microbial cooperation.

276

277 **Materials and Methods**

278 **Bacterial strains and growth conditions**

279 The strains and plasmids used in this study are listed in Table S1. *Bacillus velezensis* SQR9
280 (formerly *B. amyloliquefaciens* SQR9, China General Microbiology Culture Collection Center
281 (CGMCC) accession no. 5808) was used throughout this study. *B. velezensis* FZB42 (Bacillus
282 Genetic Stock Center (BGSC) accession no. 10A6) was used to test the bacillunoic acids (BAs)
283 production by wild-type SQR9 and its mutants. *Escherichia coli* TOP 10 (Invitrogen, Shanghai,
284 China) was used as the host for all plasmids. *E. coli* BL21 (DE3) (Invitrogen, Shanghai, China) was
285 used as the host for recombinant protein expression. All strains were routinely grown at 37°C in
286 low-salt Luria-Bertani (LLB) medium (10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, 3 g L⁻¹ NaCl). For
287 biofilm formation, *B. velezensis* SQR9 and its mutants were cultivated in MSgg medium (5 mM
288 potassium phosphate, 100 mM morpholine propanesulfonic acid, 2 mM MgCl₂, 700 μM CaCl₂, 50
289 μM MnCl₂, 50 μM FeCl₃, 1 μM ZnCl₂, 2 mM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg of
290 tryptophan per milliliter, 50 μg of phenylalanine per milliliter, and 50 μg of threonine per milliliter)
291 at 37°C⁵⁹. To collect the fermentation supernatant for antagonism assessment, *B. velezensis* SQR9
292 and its mutants were cultured in Landy medium⁶⁰ containing 20 g L⁻¹ glucose and 1 g L⁻¹ yeast

293 extract. When necessary, antibiotics were added to the medium at the following final concentrations:
294 zeocin, 20 $\mu\text{g mL}^{-1}$; spectinomycin, 100 $\mu\text{g mL}^{-1}$; kanamycin, 30 $\mu\text{g mL}^{-1}$; ampicillin, 100 $\mu\text{g mL}^{-1}$;
295 chloramphenicol, 5 $\mu\text{g mL}^{-1}$ for *B. velezensis* strains and 12.5 $\mu\text{g mL}^{-1}$ for *E. coli* strains;
296 erythromycin, 1 $\mu\text{g mL}^{-1}$ for *B. velezensis* strains and 200 $\mu\text{g mL}^{-1}$ for *E. coli* strains. The medium
297 was solidified with 2% agar.

298

299 **Reporter construction**

300 For single-labelled strain, the promoter region of the testing gene and *gfp* fragment were fused
301 through overlap PCR, and this transcriptional fusion was cloned into vector pNW33n using primers
302 listed in Table S2. For double-labelled strains, one promoter region was fused with *gfp* fragment and
303 the other promoter region was fused with *mCherry* fragment. The two fusions were then fused in
304 opposite transcription directions and cloned into vector pNW33n using primers listed in Table S2.
305 All constructions were transferred into competent cells of *B. velezensis* SQR9 and mutants when
306 required.

307

308 **Promoter replacement**

309 Strain SQR9-*P_{xyI}-accDA* was constructed by replacing the original promoter of *accDA* (*P_{accDA}*) by a
310 xylose-inducible promoter *P_{xyI}*. The approximately 800 bp fragments of upstream and downstream
311 of the *P_{accDA}* region were amplified from the genomic DNA of strain SQR9; the *Sp^c* fragment was
312 amplified from plasmid P7S6⁶¹, and the *P_{xyI}* promoter was amplified from the plasmid PWH1510⁶².
313 The four fragments were fused using overlap PCR in the order of the upstream fragment, *Sp^c*, *P_{xyI}*,

314 and the downstream fragment. The fusion was transferred into competent cells of *B. velezensis*
315 SQR9 for generating transformants. Strain SQR9-*P*₄₃-*bn**aAB* was obtained by replacing the original
316 promoter (*P*_{*bn**aAB*}) by a constitutive promoter *P*₄₃. The primers used for constructing the
317 four-fragment fusion are listed in Table S2.

318

319 **Fluorescence microscopy**

320 Cells were inoculated from a fresh pre-culture and grown to mid-exponential growth at 37°C in
321 LLB medium. Bacterial cultures were centrifuged at 4000 × g for 5 min, the pellets were washed
322 and suspended in liquid MSgg to reach an OD₆₀₀ of 1.0. One μL suspension was placed on solid
323 MSgg medium and were cultured at 37°C for 12 h. Agarose MSgg pads were then inverted on a
324 glass bottom dish (Nest). Cells were imaged using the Leica TCS SP8 microscope with the 63 ×
325 oil-immersion objective lens. For GFP observation, the excitation wavelength was 488 nm and the
326 emission wavelength was 500~560 nm; for mCherry observation, the excitation wavelength was
327 587 nm and the emission wavelength was 590~630 nm. Wild-type biofilms containing no
328 fluorescent fusions were analyzed to determine the background fluorescence.

329 For time-lapse experiment, after staining with propidium iodide (PI) for 15 min, images of
330 colonies on the agarose pad were recorded for 20 min, with interval of 5 min. Image acquisitions
331 were also performed with the Leica TCS SP8 microscope with the 63 × oil-immersion objective
332 lens. Detectors and filter set for monitoring of GFP and PI (excitation wavelength of 536 nm and
333 emission wavelength of 608~652 nm) were used.

334

335 **Preparation of the crude extract of BAs**

336 The crude extract of BAs was prepared by thin layer chromatography (TLC). According to a
337 previous study⁴¹, the fermentation supernatant of strain SQR9 were separated on a TLC plate, and
338 the inhibition zone on the lawn of strain FZB42 indicated the position of BAs. Then, silica gel
339 powder with BAs was scraped and extracted by MeOH, which was used as the crude extract of
340 BAs.

341

342 **Oxford cup assay**

343 Inhibition of different SQR9-derived mutants on *B. velezensis* FZB42 was evaluated by Oxford cup
344 method. The suspension of strain FZB42 ($\sim 10^6$ CFU mL⁻¹) was spread onto LLB plates (10 × 10 cm)
345 to grow as a bacterial lawn. A volume of 100 μL crude extract of BAs produced by different mutants
346 was injected into an Oxford cup on the lawn of strain FZB42. The plates were placed at 22°C until a
347 clear zone formed around the cup, and the inhibition diameter was scored. Each treatment includes
348 three biological replicates.

349

350 **BAs-sensitivity assessment**

351 Cells were inoculated from a fresh pre-culture and grown to mid-exponential growth at 37°C in
352 LLB medium. Afterwards, diluted cell suspension ($\sim 10^6$ CFU mL⁻¹) was spread onto LLB plates to
353 grow as a bacterial lawn. A volume of 100 μL crude extract of BAs from the wild-type SQR9 was
354 injected into an Oxford cup on the lawn. The plates were placed at 22°C for observation and
355 determination of the inhibition zone. Each treatment includes three biological replicates.

356

357 **Biolayer interferometry (BLI) measurements**

358 To confirm whether Spo0A can bind P_{bnaF} directly, determination of binding kinetics was performed
359 on an Octet® RED96 device (ForteBio, Inc., Menlo Park, US) at 25°C with orbital sensor agitation
360 at 1000 rpm. Streptavidin (SA) sensor tips (ForteBio) were used to immobilize 100 nM
361 biotin-labeled P_{bnaF} . Then, a baseline measurement was performed in the buffer PBST (PBS, 0.1%
362 BSA, 0.02% Tween-20) for 300 s. The binding of Spo0A at different concentrations (100 nM, 250
363 nM, 500 nM, and 1000 nM) to P_{bnaF} was recorded for 600 s followed by monitoring protein
364 dissociation using PBST for another 600 s. The BLI data for each binding event were summarized
365 as a “nm shift” (the wavelength/spectral shift in nanometers) and KD values determined by fitting to
366 a 1:1 binding model.

367

368 **Promoter activity testing via fluorescence intensity**

369 For colony fluorescence, cells were inoculated from a pre-culture into fresh LLB medium and
370 grown at 37°C with 170 rpm shaking until OD₆₀₀ reached 0.5. One µL of the suspension were
371 inoculated on solid LLB medium and were cultured at 37°C. Colony morphology and fluorescence
372 were recorded by the stereoscope. ImageJ software was used to measure GFP intensity. For liquid
373 culture fluorescence, overnight cultures were transferred to fresh LLB medium. Fluorescence
374 intensity was determined by a microtiter plate reader. Each treatment includes three biological
375 replicates.

376

377 **Xylose induction assay**

378 For the xylose-induced BAs production assay, 30 μ L overnight culture of SQR9-*P_{xyI}-accDA* or
379 wild-type SQR9 was transferred respectively into 3 mL fresh LLB liquid with different
380 concentrations of xylose (0%, 0.1%, 0.2%) and incubated at 37°C, 170 rpm for 24 h. Cell
381 suspensions were adjusted to the same OD₆₀₀ and were centrifuged at 12000 \times g for 1 min. The
382 cell-free supernatant was mixed with MeOH (volume ratio 2:1) to extract BAs. A volume of 100 μ L
383 extract was injected into an Oxford cup on the lawn of strain FZB42 (as described above). The
384 plates were placed at 22°C.

385 For the xylose-induced self-immunity assay, strain SQR9-*P_{xyI}-accDA* was grown in LLB
386 without xylose for 24 h. Cell suspension was spread onto LLB plates containing different
387 concentrations of xylose (0%, 0.1% and 0.2%) to grow as the lawn. A volume of 100 μ L (1 \times) or 200
388 μ L (2 \times) crude extract of BAs from the wild-type SQR9 was injected into an Oxford cup on the lawn.
389 The plates were placed at 22°C.

390 For xylose-induced gene expression assay, cells were inoculated from a pre-culture into fresh
391 LLB medium with different concentrations of xylose (0%, 0.1%, 0.2%), and were grown at 37°C
392 with 170 rpm shaking until OD₆₀₀ reached 0.5. One μ L of suspension was inoculated on solid LLB
393 medium and was cultured at 37°C, colony morphology and fluorescence were recorded by the
394 stereoscope.

395 Each treatment in these assays includes three biological replicates.

396

397 **Biofilm formation**

398 Cells were inoculated from a fresh pre-culture and grown to mid-exponential growth at 37°C in
399 LLB medium. Bacterial cultures were centrifuged at $4000 \times g$ for 5 min, the pellets were washed
400 and suspended in MSgg medium to an OD_{600} of 1.0. For colony observation, 1 μL of suspension
401 were inoculated on solid MSgg medium and were cultured at 37°C, then the colony morphology
402 was recorded by the stereoscope. For pellicle observation, suspension was inoculated into MSgg
403 medium with a final concentration of 1% in a microtiter plate well, and the cultures were incubated
404 at 37°C without shaking.

405 Besides, the ability of strain to form biofilm under stress was measured in the 48-well
406 microtiter plate according to the method described above. When required, reagents that simulate
407 stress were supplemented in the MSgg medium before inoculating, including oxidative stress
408 (0.0025% H_2O_2), salt stress (7% NaCl), acid stress (pH 5), alkaline stress (pH 8), and antibiotic
409 stress (4 $\mu\text{g mL}^{-1}$ tetracycline or 20 $\mu\text{g mL}^{-1}$ streptomycin). The amount of reagent added was
410 determined according to a concentration gradient in pre-experiment, and a concentration was chosen
411 to inhibit wild-type growth without killing it. At different stages of biofilm development (initiation,
412 progress, maturity, and dispersal), the MSgg medium underneath the biofilm was carefully removed
413 by pipetting and then the biofilm was taken and weighed.

414 Each treatment includes three biological replicates.

415

416 **Flow cytometry**

417 Biofilms were collected and re-suspended in 1 mL PBS buffer, and single cells were obtained after
418 mild sonication. Cells were centrifuged at $4000 \times g$ for 5 min and washed briefly with PBS. For

419 flow cytometry, cells were diluted to 1:100 in PBS and measured on BD FACSCanto II. For GFP
420 fluorescence, the laser excitation was 488 nm and coupled with 500-560 nm. Every sample was
421 analyzed for 20000 events. FlowJo V10 software was used for data analysis and graphs creating.
422 Three replicates for each treatment were analyzed.

423

424 **Root colonization assay in hydroponic culture**

425 Bacterial suspension was inoculated into 1/4 Murashige-Skoog medium to make the final OD₆₀₀
426 value to be 0.1, into which sterile cucumber seedlings with three true leaves were immersed. After
427 cultured with slowly shaking for two days, cells colonized on cucumber roots were determined by
428 plate colony counting. In detail, roots were washed eight times in PBS to remove free and weakly
429 attached bacterial cells. After vortexing for 5 min until colonized bacteria were detached from roots,
430 100 μ L of the bacterial suspension was plated onto LLB agar plates for quantification. Each
431 treatment includes three biological replicates.

432

433 **Measurement of public goods production**

434 Qualitative measurement of proteases production was done by inoculating 1 μ L of bacterial
435 suspension on solid 2% skim milk medium and cultured at 30°C until transparent zone formed
436 around colonies; quantitative measurements of alkaline protease and neutral protease activity were
437 conducted according to a previous study⁶³. Qualitative and quantitative measurement of siderophore
438 production were based on the universal chemical assay described by Schwyn and Neilands⁶⁴. Each
439 treatment includes three biological replicates.

440

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446

447 **Competing Interests Statement**

448 The authors declare no conflict of interest.

449

450 **References**

- 451 1. Rakoff-Nahoum, S., Foster, K.R. & Comstock, L.E. The evolution of cooperation within the gut
452 microbiota. *Nature* **533**, 255-259 (2016).
- 453 2. Kehe, J. *et al.* Positive interactions are common among culturable bacteria. *Sci. Adv.* **7**, eabi7159
454 (2021).
- 455 3. Smith, P. & Schuster, M. Public goods and cheating in microbes. *Curr. Biol.* **29**, R442-R447 (2019).
- 456 4. Chen, R. *et al.* Social cheating in a *Pseudomonas aeruginosa* quorum-sensing variant. *Proc. Natl.*
457 *Acad. Sci. USA* **116**, 7021-7026 (2019).
- 458 5. Griffin, A.S., West, S.A. & Buckling, A. Cooperation and competition in pathogenic bacteria. *Nature*
459 **430**, 1024-1027 (2004).
- 460 6. Vlamakis, H. *et al.* Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev.*
461 *Microbiol.* **11**, 157-168 (2013).
- 462 7. Dragoš, A. & Kovács, Á.T. The peculiar functions of the bacterial extracellular matrix. *Trends*
463 *Microbiol.* **25**, 257-266 (2017).
- 464 8. Lyons, N.A. & Kolter, R. *Bacillus subtilis* protects public goods by extending kin discrimination to
465 closely related species. *mBio* **8**, e00723-17 (2017).
- 466 9. Kraigher, B. *et al.* Kin discrimination drives territorial exclusion during *Bacillus subtilis* swarming
467 and restrains exploitation of surfactin. *ISME J.* **16**, 833-841 (2022).
- 468 10. Wang, M. *et al.* Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. *Proc.*
469 *Natl. Acad. Sci. USA* **112**, 2187-2191 (2015).
- 470 11. Stewart, A.J. & Plotkin, J.B. Collapse of cooperation in evolving games. *Proc. Natl. Acad. Sci. USA*
471 **111**, 17558-17563 (2014).

- 472 12. Hardin, G. The tragedy of the commons. *Science* **162**, 1243-1248 (1968).
- 473 13. West, S.A. *et al.* Social evolution theory for microorganisms. *Nat. Rev. Microbiol.* **4**, 597-607 (2006).
- 474 14. Sandoz, K.M., Mitzimberg, S.M. & Schuster, M. Social cheating in *Pseudomonas aeruginosa*
475 quorum sensing. *Proc. Natl. Acad. Sci. USA* **104**, 15876-15881 (2007).
- 476 15. Martin, M. *et al.* Cheaters shape the evolution of phenotypic heterogeneity in *Bacillus subtilis*
477 biofilms. *ISME J.* **14**, 2302-2312 (2020).
- 478 16. Travisano, M. & Velicer, G.J. Strategies of microbial cheater control. *Trends Microbiol.* **12**, 72-78
479 (2004).
- 480 17. Özkaya, Ö. *et al.* Maintenance of microbial cooperation mediated by public goods in single- and
481 multiple-trait scenarios. *J. Bacteriol.* **199**, e00297-17 (2017).
- 482 18. Diggle, S.P. *et al.* Cooperation and conflict in quorum-sensing bacterial populations. *Nature* **450**,
483 411-414 (2007).
- 484 19. McNally, L. *et al.* Killing by Type VI secretion drives genetic phase separation and correlates with
485 increased cooperation. *Nat. Commun.* **8**, 14371 (2017).
- 486 20. Allen, R.C. *et al.* Quorum sensing protects bacterial co-operation from exploitation by cheats. *ISME*
487 *J.* **10**, 1706-16 (2016).
- 488 21. Sexton, D.J. & Schuster, M. Nutrient limitation determines the fitness of cheaters in bacterial
489 siderophore cooperation. *Nat. Commun.* **8**, 230 (2017).
- 490 22. Dandekar, A.A., Chugani, S. & Greenberg, E.P. Bacterial quorum sensing and metabolic incentives
491 to cooperate. *Science* **338**, 264-266 (2012).
- 492 23. García-Contreras, R. *et al.* Quorum sensing enhancement of the stress response promotes resistance
493 to quorum quenching and prevents social cheating. *ISME J.* **9**, 115-125 (2015).
- 494 24. Jin, Z. *et al.* Conditional privatization of a public siderophore enables *Pseudomonas aeruginosa* to
495 resist cheater invasion. *Nat. Commun.* **9**, 1383 (2018).
- 496 25. Otto, S.B. *et al.* Privatization of biofilm matrix in structurally heterogeneous biofilms. *mSystems* **5**,
497 e00425-20 (2020).
- 498 26. van Gestel, J. *et al.* Density of founder cells affects spatial pattern formation and cooperation in
499 *Bacillus subtilis* biofilms. *ISME J.* **8**, 2069-2079 (2014).
- 500 27. Andersen, S.B. *et al.* Long-term social dynamics drive loss of function in pathogenic bacteria. *Proc.*
501 *Natl. Acad. Sci. USA* **112**, 10756-10761 (2015).
- 502 28. Dragoš, A. *et al.* Division of labor during biofilm matrix production. *Curr. Biol.* **28**, 1903-1913
503 (2018).
- 504 29. López, D. *et al.* Cannibalism enhances biofilm development in *Bacillus subtilis*. *Mol. Microbiol.* **74**,
505 609-618 (2009).
- 506 30. Branda, S.S. *et al.* Biofilms: the matrix revisited. *Trends Microbiol.* **13**, 20-26 (2005).
- 507 31. Vlamakis, H. *et al.* Control of cell fate by the formation of an architecturally complex bacterial
508 community. *Genes Dev.* **22**, 945-953 (2008).
- 509 32. Cairns, L.S., Hobley, L. & Stanley-Wall, N.R. Biofilm formation by *Bacillus subtilis*: new insights
510 into regulatory strategies and assembly mechanisms. *Mol. Microbiol.* **93**, 587-598 (2014).
- 511 33. López, D. *et al.* Paracrine signaling in a bacterium. *Genes. Dev.* **23**, 1631-1638 (2009).
- 512 34. López, D., Vlamakis, H. & Kolter, R. Generation of multiple cell types in *Bacillus subtilis*. *FEMS*
513 *Microbiol. Rev.* **33**, 152-163 (2009).

- 514 35. López, D. & Kolter, R. Extracellular signals that define distinct and coexisting cell fates in *Bacillus*
515 *subtilis*. *FEMS Microbiol. Rev.* **34**, 134-149 (2010).
- 516 36. West, S.A. *et al.* The social lives of microbes. *Annu. Rev. Ecol. Evol. S.* **38**, 53-77 (2007).
- 517 37. Cao, Y. *et al.* *Bacillus subtilis* SQR 9 can control Fusarium wilt in cucumber by colonizing plant
518 roots. *Biol. Fert. Soils* **47**, 495-506 (2011).
- 519 38. Xu, Z. *et al.* Contribution of bacillomycin D in *Bacillus amyloliquefaciens* SQR9 to antifungal
520 activity and biofilm formation. *Appl. Environ. Microbiol.* **79**, 808-815 (2013).
- 521 39. Qiu, M. *et al.* Comparative proteomics analysis of *Bacillus amyloliquefaciens* SQR9 revealed the key
522 proteins involved in in situ root colonization. *J. Proteome Res.* **13**, 5581-5591 (2014).
- 523 40. Xu, Z. *et al.* Antibiotic bacillomycin D affects iron acquisition and biofilm formation in *Bacillus*
524 *velezensis* through a Btr-mediated FeuABC-dependent pathway. *Cell Rep.* **29**, 1192-1202 (2019).
- 525 41. Wang, D. *et al.* A genomic island in a plant beneficial rhizobacterium encodes novel antimicrobial
526 fatty acids and a self-protection shield to enhance its competition. *Environ. Microbiol.* **21**, 3455-3471
527 (2019).
- 528 42. Huang, R. *et al.* A unique genomic island-governed cannibalism in *Bacillus* enhanced biofilm
529 formation through a novel regulation mechanism. *bioRxiv*, doi: 10.1101/2021.10.04.462985. (2021).
- 530 43. Hamon, M.A. & Lazazzera, B.A. The sporulation transcription factor Spo0A is required for biofilm
531 development in *Bacillus subtilis*. *Mol. Microbiol.* **42**, 1199-1209 (2001).
- 532 44. Molle, V. *et al.* The Spo0A regulon of *Bacillus subtilis*. *Mol. Microbiol.* **50**, 1683-1701 (2003).
- 533 45. Pedrido, M.E. *et al.* Spo0A links de novo fatty acid synthesis to sporulation and biofilm development
534 in *Bacillus subtilis*. *Mol. Microbiol.* **87**, 348-367 (2013).
- 535 46. Diomandé, S.E. *et al.* Role of fatty acids in *Bacillus* environmental adaptation. *Front. Microbiol.* **6**,
536 813 (2015).
- 537 47. Fujita, M., Gonzalez-Pastor, J.E. & Losick, R. High- and low-threshold genes in the Spo0A regulon
538 of *Bacillus subtilis*. *J. Bacteriol.* **187**, 1357-1368 (2005).
- 539 48. Shank, E.A. & Kolter, R. Extracellular signaling and multicellularity in *Bacillus subtilis*. *Curr. Opin.*
540 *Microbiol.* **14**, 741-747 (2011).
- 541 49. Bruger, E. & Waters, C. Sharing the sandbox: Evolutionary mechanisms that maintain bacterial
542 cooperation. *F1000Res.* **4**, 1504 (2015).
- 543 50. Özkaya, Ö. *et al.* Cheating on cheaters stabilizes cooperation in *Pseudomonas aeruginosa*. *Curr. Biol.*
544 **28**, 2070-2080 (2018).
- 545 51. Momeni, B. Division of labor: how microbes split their responsibility. *Curr. Biol.* **28**, R697-R699
546 (2018).
- 547 52. Strassmann, J.E., Gilbert, O.M. & Queller, D.C. Kin discrimination and cooperation in microbes.
548 *Annu. Rev. Microbiol.* **65**, 349-367 (2011).
- 549 53. Rendueles, O. *et al.* Rapid and widespread de novo evolution of kin discrimination. *Proc. Natl. Acad.*
550 *Sci. USA* **112**, 9076-9081 (2015).
- 551 54. Lyons, N.A. & Kolter, R. A single mutation in *rapP* induces cheating to prevent cheating in *Bacillus*
552 *subtilis* by minimizing public good production. *Commun. Biol.* **1**, 133 (2018).
- 553 55. Xavier, J.B., Kim, W. & Foster, K.R. A molecular mechanism that stabilizes cooperative secretions in
554 *Pseudomonas aeruginosa*. *Mol. Microbiol.* **79**, 166-179 (2011).
- 555 56. Whiteley, M., Diggle, S.P. & Greenberg, E.P. Progress in and promise of bacterial quorum sensing

- 556 research. *Nature* **551**, 313-320 (2017).
- 557 57. Steinke, K. *et al.* Phylogenetic distribution of secondary metabolites in the *Bacillus subtilis* species
558 complex. *mSystems* **6**, e00057-21 (2021).
- 559 58. Xia, L. *et al.* Biosynthetic gene cluster profiling predicts the positive association between antagonism
560 and phylogeny in *Bacillus*. *Nat. Commun.* **13**, 1023 (2022).
- 561 59. Branda, S.S. *et al.* Fruiting body formation by *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **98**,
562 11621-11626 (2001).
- 563 60. Landy, M., Rosenman, S.B. & Warren, G.H. An antibiotic from *Bacillus subtilis* active against
564 pathogenic fungi. *J. Bacteriol.* **54**, 24 (1947).
- 565 61. Feng, H. *et al.* Identification of chemotaxis compounds in root exudates and their sensing
566 chemoreceptors in plant-growth-promoting rhizobacteria *Bacillus amyloliquefaciens* SQR9. *Mol.*
567 *Plant Microbe Interact.* **31**, 995-1005 (2018).
- 568 62. Xu, Z. *et al.* Enhanced control of plant wilt disease by a xylose-inducible *degQ* gene engineered into
569 *Bacillus velezensis* strain SQR9XYQ. *Phytopathology* **109**, 36-43 (2019).
- 570 63. Kunitz, M. Crystalline soybean trypsin inhibition: II. General properties. *J. Gen. Physiol.* **30**,
571 291-310 (1947).
- 572 64. Schwyn, B. & Neilands, J. Universal chemical assay for the detection and determination of
573 siderophores. *Anal. Biochem.* **160**, 47-56 (1987).
- 574
- 575

576 **Figure captions**

577 **Fig. 1 Expression of extracellular matrix (ECM) production and bacillunoic acids (BAs)**

578 **biosynthesis/immunity were located in the same subpopulation.** Colony cells of different

579 double-labeled strains were visualized using a confocal laser scanning microscopy (CLSM) to

580 monitor the distribution of fluorescence signal from different reporters. *P_{eps}-mCherry* and

581 *P_{tapA}-mCherry* were used to indicate cells expressing extracellular polysaccharides (EPS) and TasA

582 fibers production, respectively; *P_{bnaf}-gfp* and *P_{bnab}-gfp* were used to indicate cells expressing BAs

583 synthesis and self-immunity, respectively. The bar represents 5 μ m.

584 **Fig. 2 ECM and BAs producing subpopulation eliminated the nonproducing cheaters.** Colony

585 cells of different *gfp*-labeled strains were stained with propidium iodide (PI, a red-fluorescent dye

586 for labeling dead cell) for 15 min, and then visualized by a CLSM to monitor the distribution of

587 fluorescence signal from reporters and the PI dye, at 0, 10, and 20 min after treatment. *P_{eps}-gfp* and

588 *P_{tapA}-gfp* were used to indicate cells expressing EPS and TasA fibers production, respectively;

589 *P_{bnaf}-gfp* and *P_{bnab}-gfp* were used to indicate cells expressing BAs synthesis and self-immunity,

590 respectively.

591 **Fig. 3 Spo0A is the co-regulator for triggering ECM production and BAs synthesis/immunity.**

592 **(A)** Inhibition of the lawn of *B. velezensis* FZB42 by the crude extracted BAs of wild-type SQR9,

593 its different mutants altered in ECM production, and complementary strain Δ *spo0A/spo0A*. **(B)**

594 Diameter of the inhibition zones observed in **(A)**. **(C)** Sensitivity of wild-type SQR9, Δ *spo0A*, and

595 Δ *spo0A/spo0A* (as the lawn) to the extracellular extract of SQR9 and its mutant Δ GI3 that disable in

596 BAs synthesis. **(D)** Expression level of *eps*, *tapA*, *bnaf*, and *bnab* in wild-type SQR9, Δ *spo0A*,

597 and $\Delta spo0A/spo0A$, as monitored by using *gfp* reporters fused to the corresponding promoters. Data
598 are means and standard deviations from three biological replicates. * indicates significant difference
599 with the Control (SQR9) column as analyzed by Duncan's multiple range test ($P < 0.05$).

600 **Fig. 4 Spo0A activates acetyl-CoA carboxylase (ACC) for BAs synthesis and self-immunity. (A)**

601 Involvement of ACC in biosynthesis of BAs in *B. velezensis* SQR9. ACC catalyzes acetyl-CoA to
602 generate malonyl-CoA, which is transformed to malonyl-ACP under the catalyzation of ACP
603 transacylase; then malonyl-ACP and acetyl-CoA are aggregated into a C₅ primer, the precursor for
604 BAs synthesis. **(B)** Expression level of *accDA* in wild-type SQR9, $\Delta spo0A$, and $\Delta spo0A/spo0A$, as
605 monitored by using the *P_{accDA}-gfp* reporter. **(C)** Inhibition of the lawn of *B. velezensis* FZB42 by the
606 crude extracted BAs of wild-type SQR9 and SQR9-*P_{xyI}-accDA*, with addition of different
607 concentrations of xylose (0%, 0.1% and 0.2%). **(D)** Diameter of the inhibition zones observed in
608 **(C)**. **(E)** Sensitivity of wild-type SQR9 and SQR9-*P_{xyI}-accDA* (as the lawn) to the crude extracted
609 BAs of SQR9 (100 μ L (1 \times) or 200 μ L (2 \times)), with addition of different concentrations of xylose (0%,
610 0.1%, and 0.2%). **(F)** Expression of *bnaf* and *bnab* in the colony cells of wild-type SQR9 and
611 SQR9-*P_{xyI}-accDA*, with addition of different concentrations of xylose (0%, 0.1% and 0.2%).
612 Colonies were observed under both bright field (BF in the figure) and GFP channel, to monitor the
613 florescence of *P_{bnaf}-gfp* and *P_{bnab}-gfp* reporters in different strains. Data are means and standard
614 deviations from three biological replicates. * in **(B)** indicates significant difference ($P < 0.05$) with
615 the Control (SQR9) column as analyzed by Duncan's multiple range tests; columns with different
616 letters in **(D)** are statistically different according to the Duncan's multiple range test ("a" for
617 wild-type SQR9 under different concentrations of xylose and "a" for SQR9-*P_{xyI}-accDA*; $P < 0.05$).

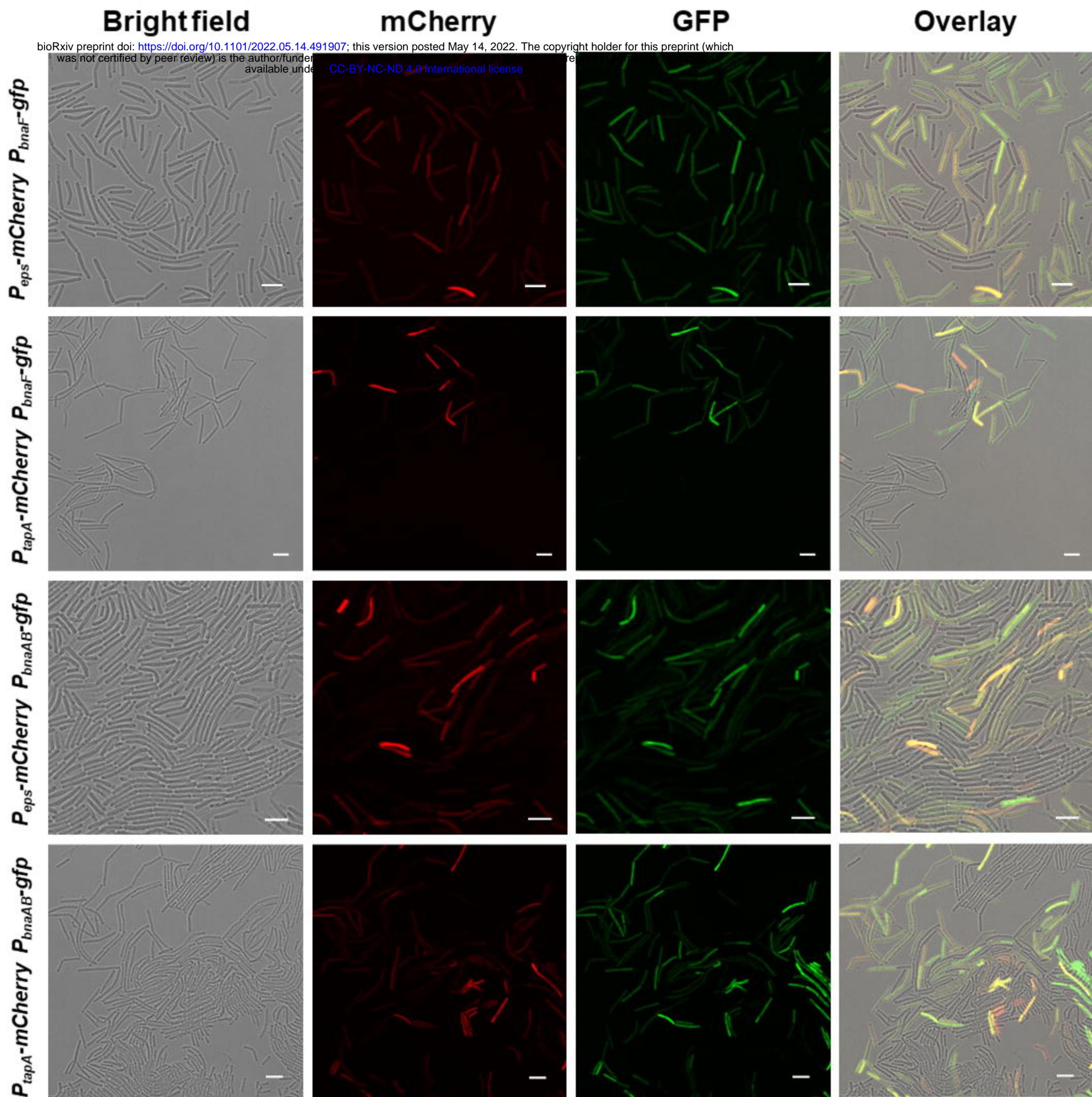
618 **Fig. 5 The co-regulation policing system eliminates cheaters and enhances population fitness.**

619 (A) Flow cytometry monitoring the expression of P_{eps} -*gfp* and P_{tapA} -*gfp* reporters in wild-type SQR9,
620 SQR9 Δ *bnaV* and SQR9- P_{43} -*bnaAB*. (B) Proportion of the active cells (%) and average FITC in
621 wild-type SQR9, SQR9 Δ *bnaV* and SQR9- P_{43} -*bnaAB*, as monitored by P_{eps} -*gfp* and P_{tapA} -*gfp*
622 reporters using flow cytometry. (C) Pellicle formation dynamics of wild-type SQR9, SQR9 Δ *bnaV*
623 and SQR9- P_{43} -*bnaAB* in MSgg medium. (D) Pellicle weight dynamics of wild-type SQR9,
624 SQR9 Δ *bnaV* and SQR9- P_{43} -*bnaAB* in MSgg medium under normal (corresponds to (C)) or stressed
625 conditions (H_2O_2 , tetracycline, or 7% NaCl). (E) Production of proteases and siderophore by
626 wild-type SQR9, SQR9 Δ *bnaV* and SQR9- P_{43} -*bnaAB* colonies. (F) Comparison of root colonization
627 of wild-type SQR9, SQR9 Δ *bnaV* and SQR9- P_{43} -*bnaAB*. Data are means and standard deviations
628 from three biological replicates; columns with different letters are significantly different according
629 to Duncan's multiple range tests, $P < 0.05$.

630 **Fig. 6 Working model and ecological significance of the co-regulation policing system in *B.***

631 *velezensis*. In certain conditions (e.g., environmental or self-produced clues, surface attachments,
632 etc.), *Bacillus* cells can differentiate into Spo0A-ON (~moderate phosphorylated) and Spo0A-OFF
633 (unphosphorylated) subpopulation. The Spo0A-ON subpopulation are cooperators that produce
634 public goods for the community, such as extracellular matrix (ECM) or proteases; simultaneously
635 they express AccDA to produce malonyl-CoA as the precursor for bacillunoic acids (BAs)
636 biosynthesis, and the endogenous autotoxin activates immunity-required transporter BnaAB to
637 pump them out. Comparatively, the Spo0A-OFF subpopulation are phenotypic cheaters that
638 silenced in public goods secretion, which are also disable in malonyl-CoA production and BAs

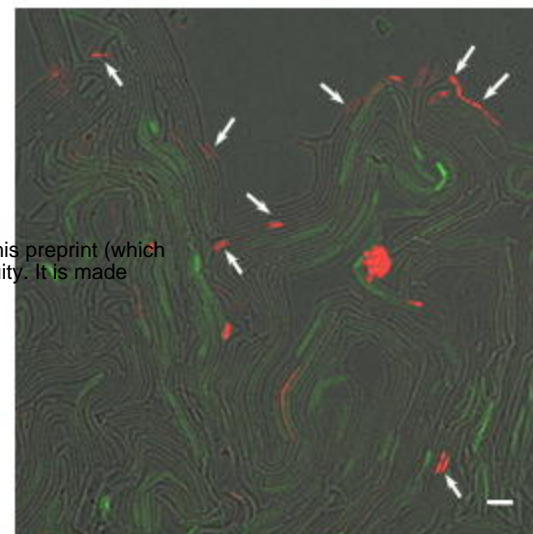
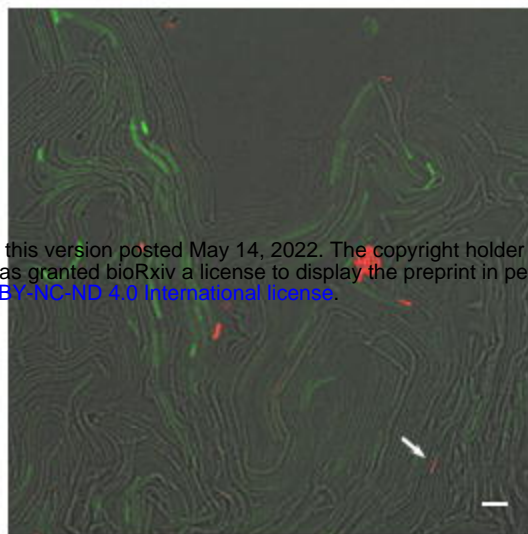
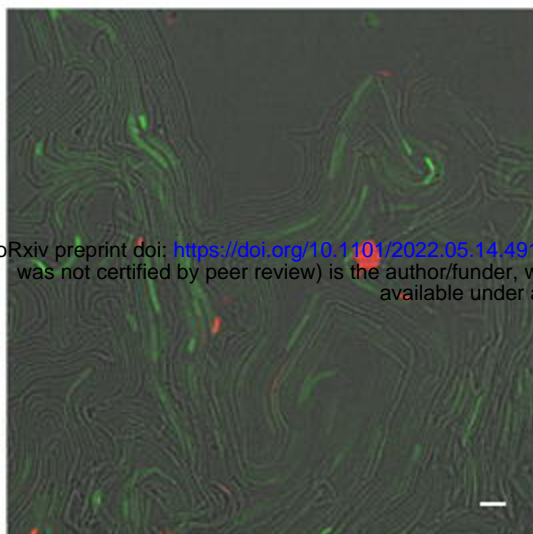
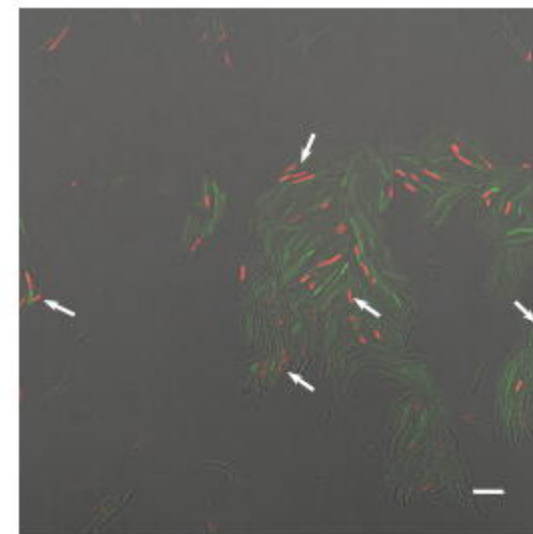
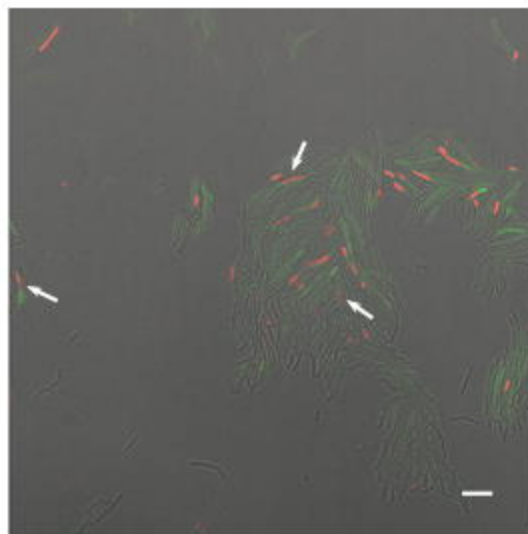
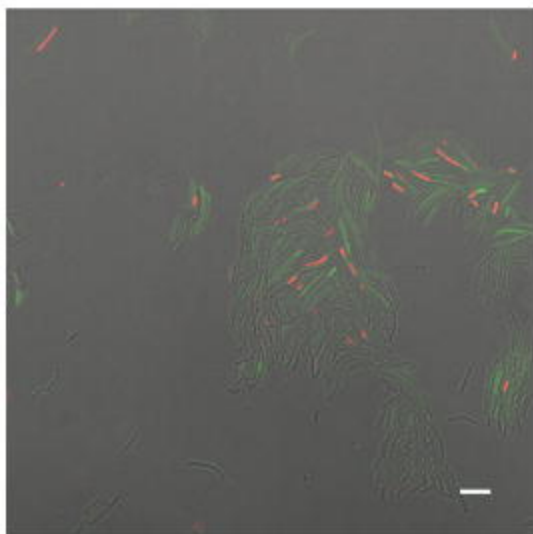
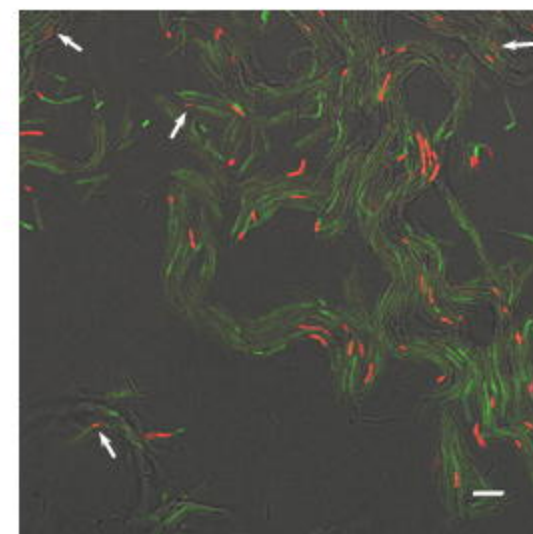
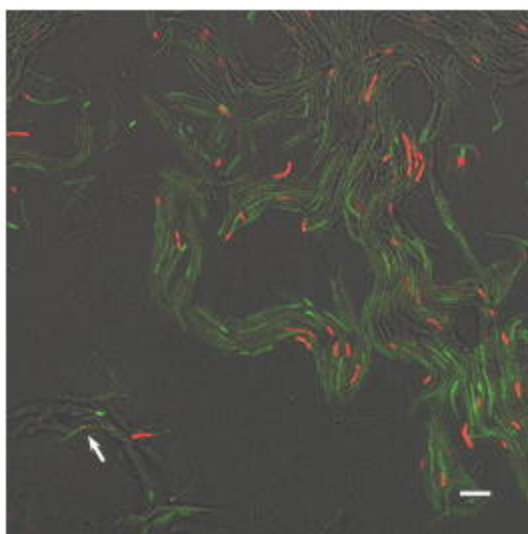
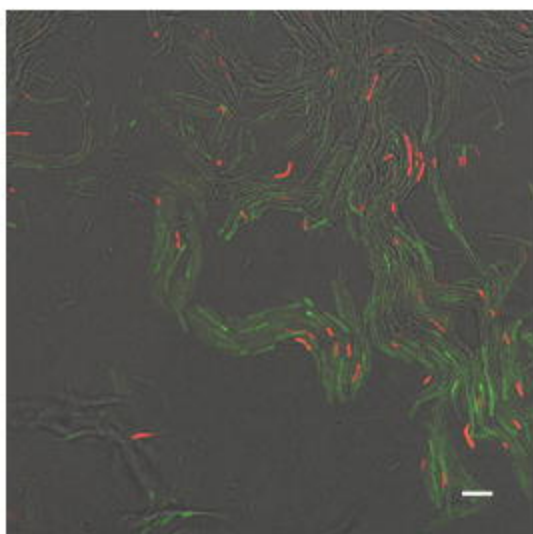
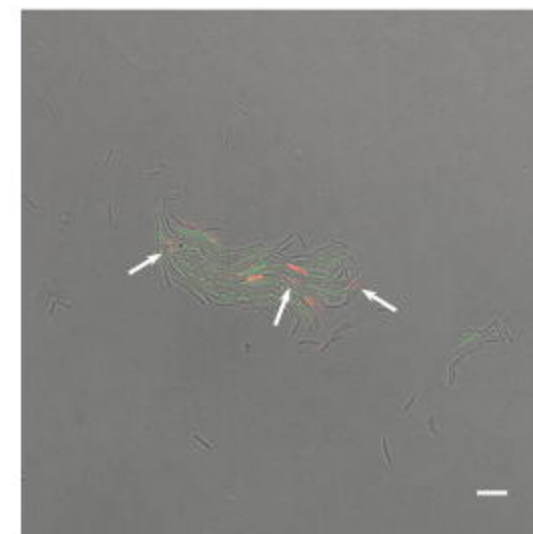
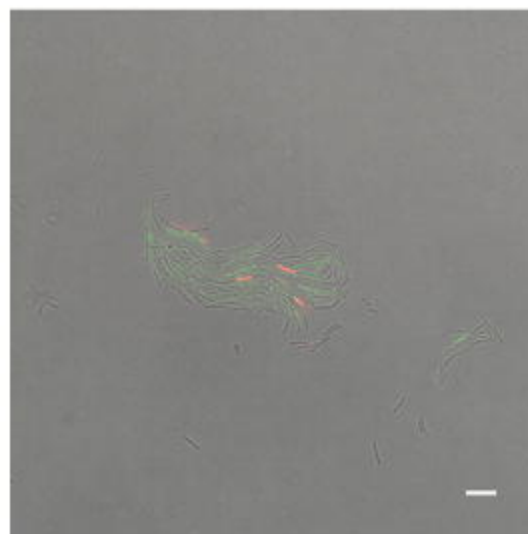
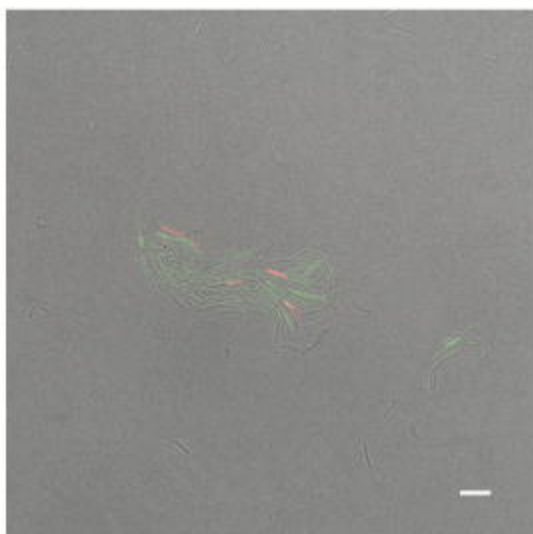
639 biosynthesis/self-immunity. Consequently, the cooperators-produced BAs can effectively eliminate
640 the cheating individuals, thereby enhancing the population stability and fitness.



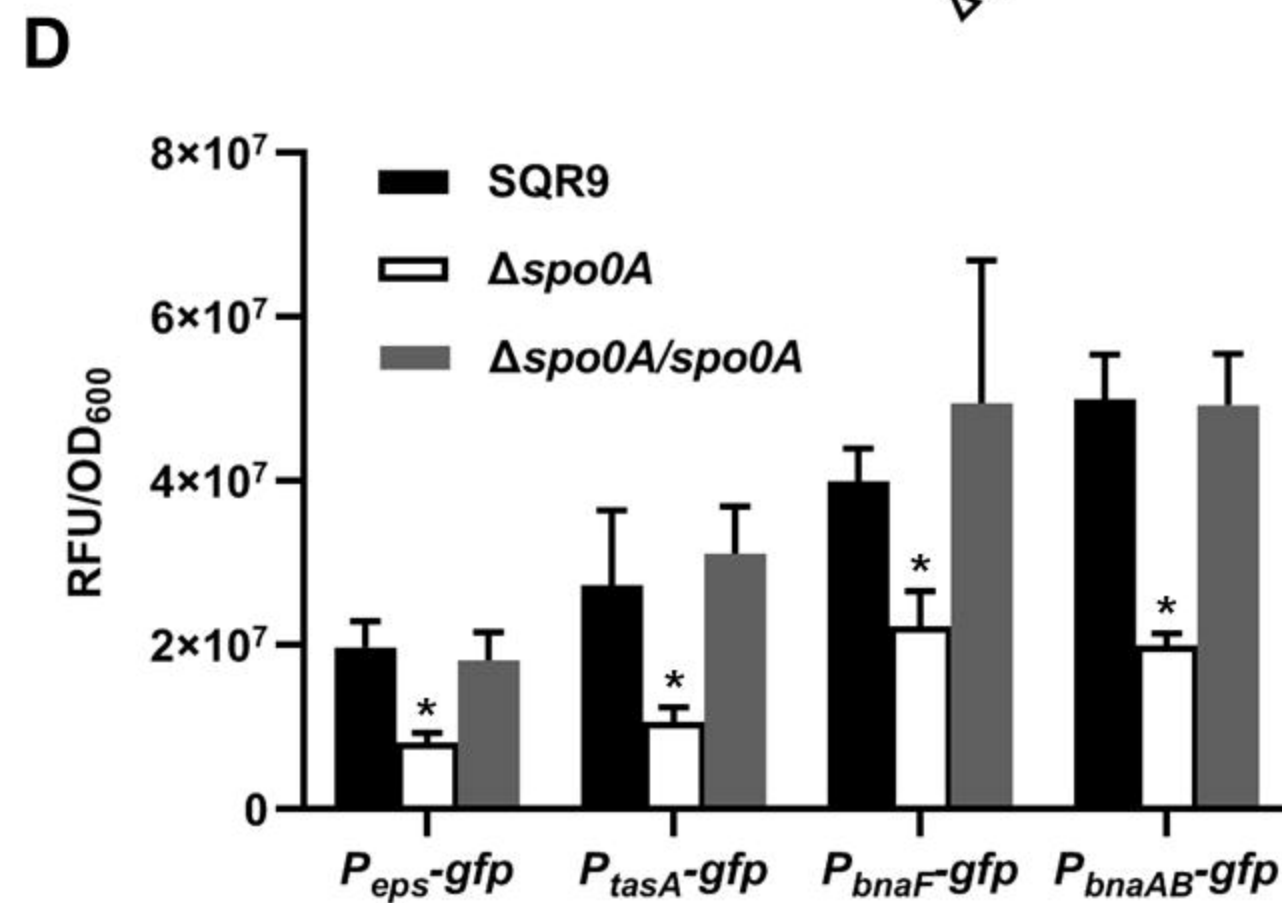
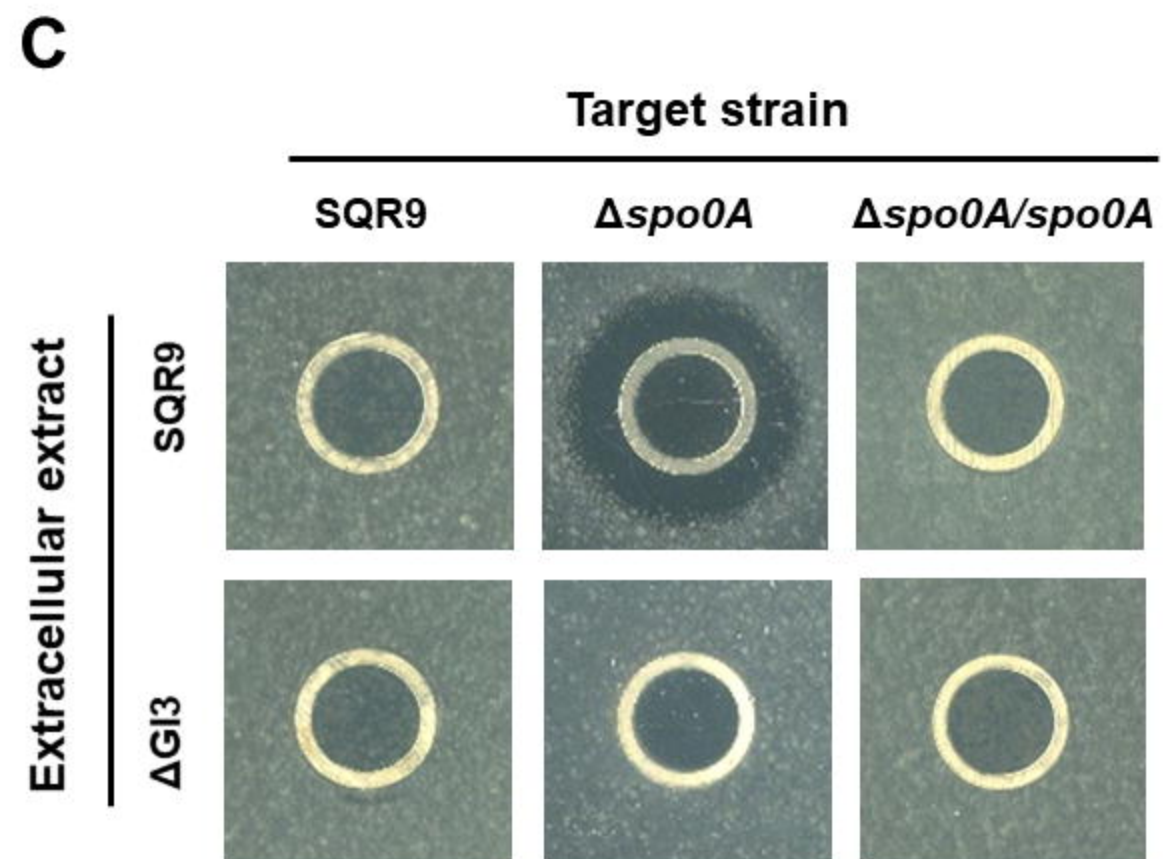
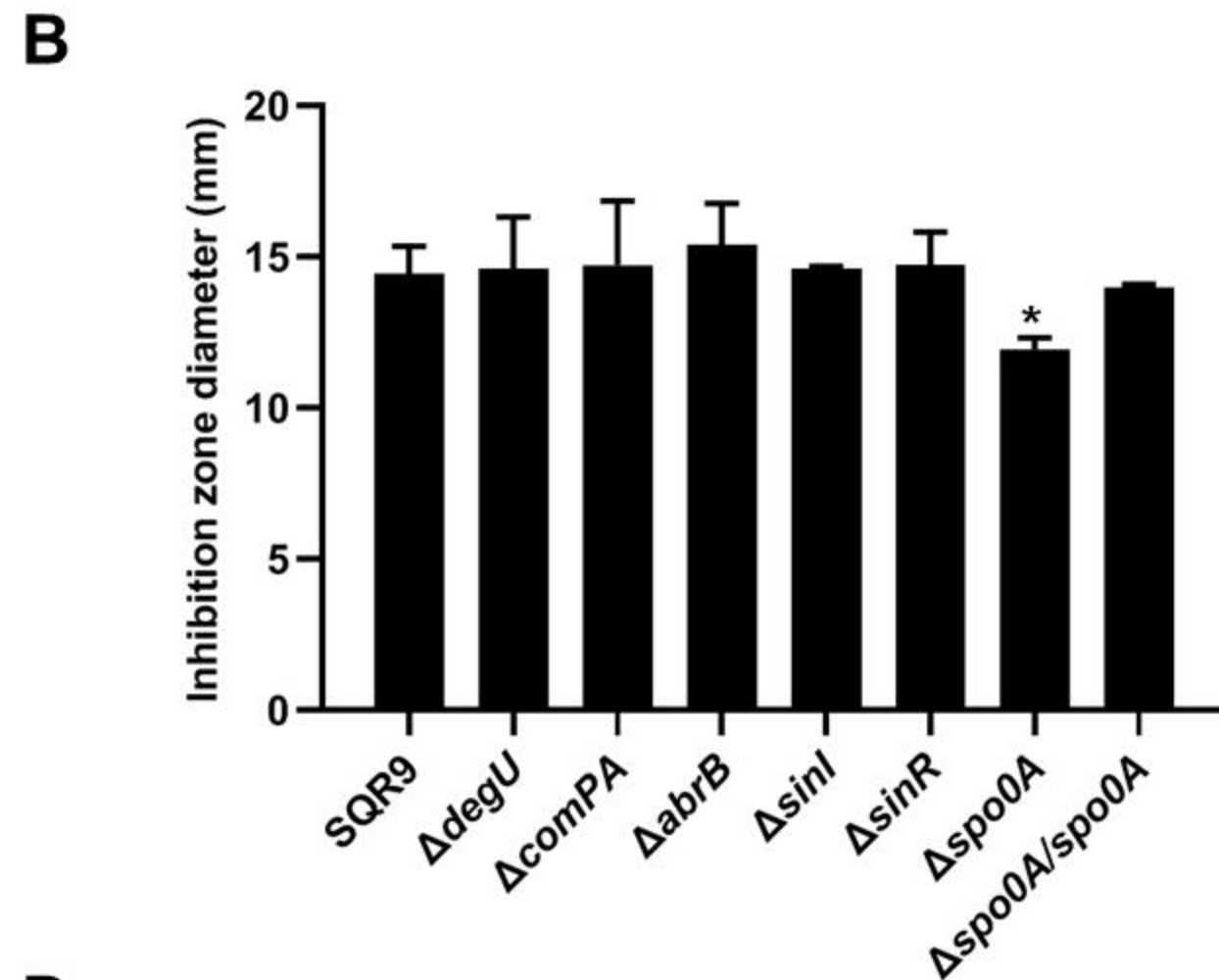
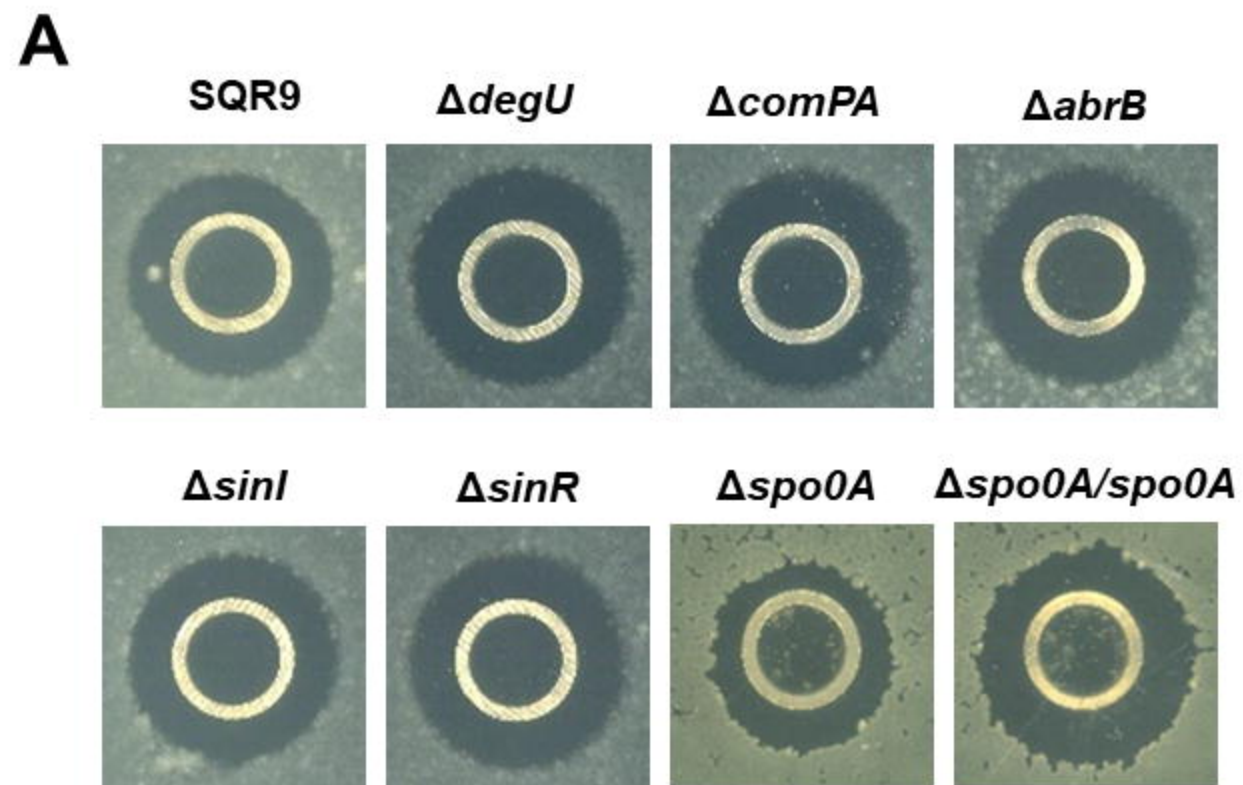
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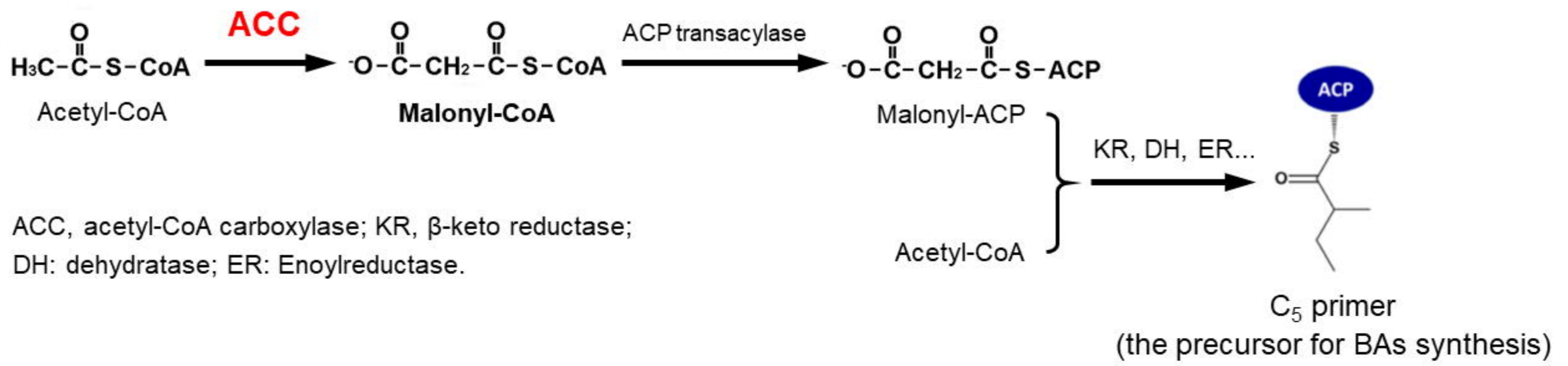
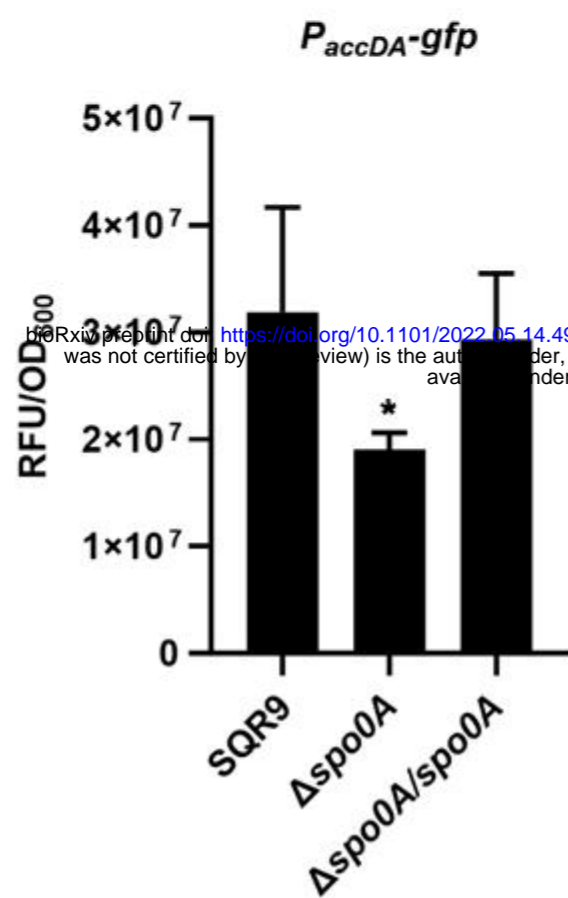
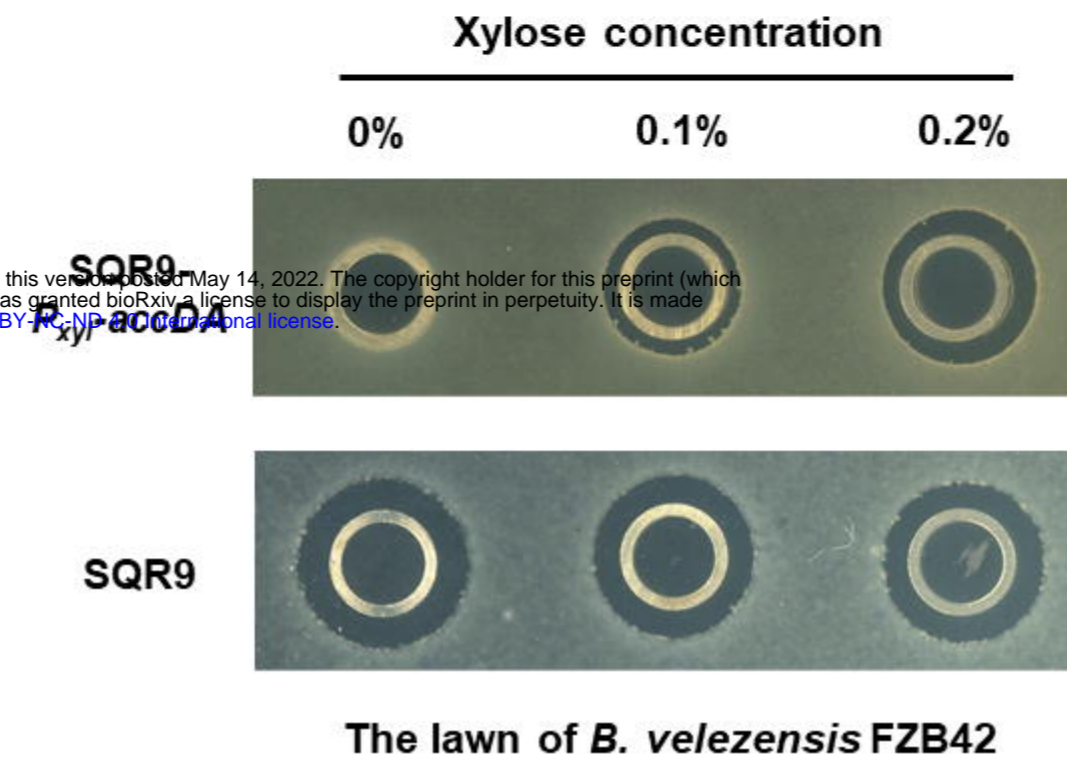
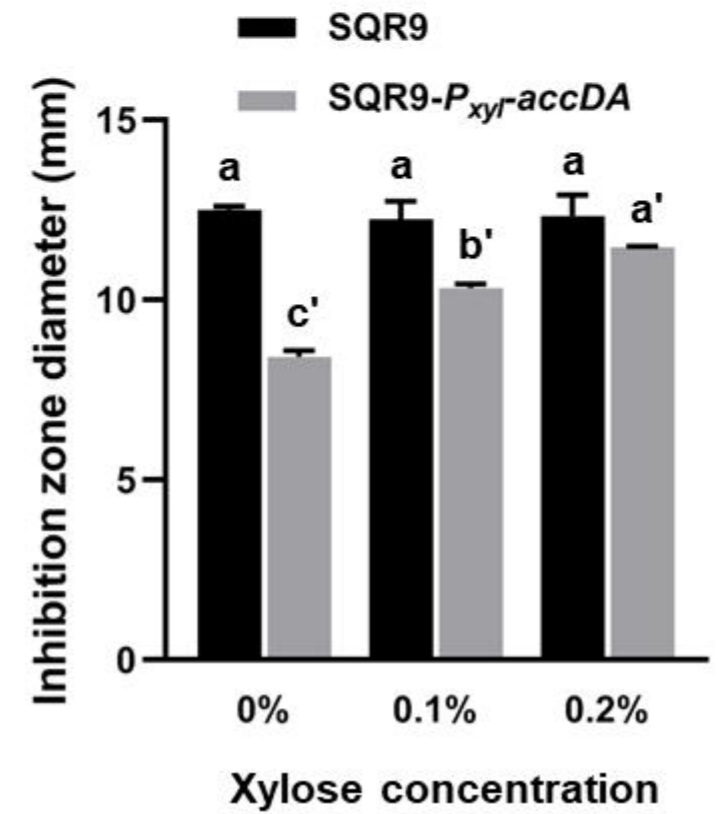
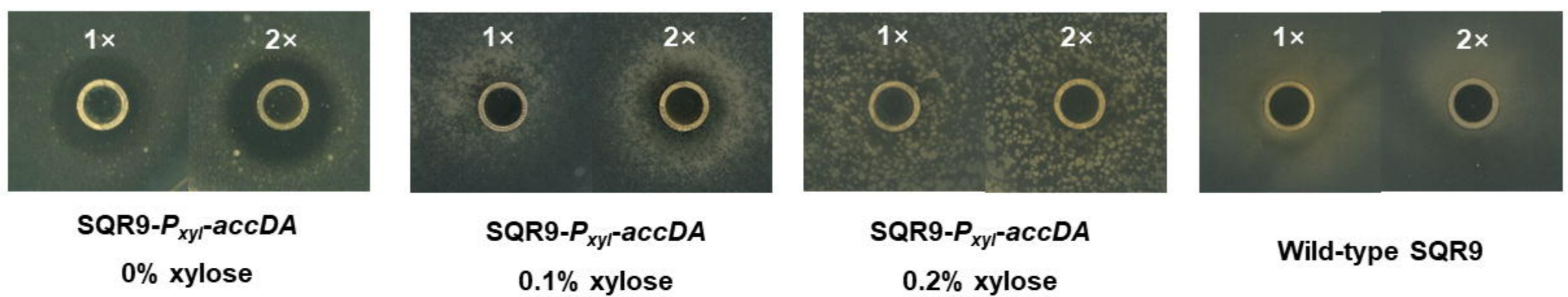
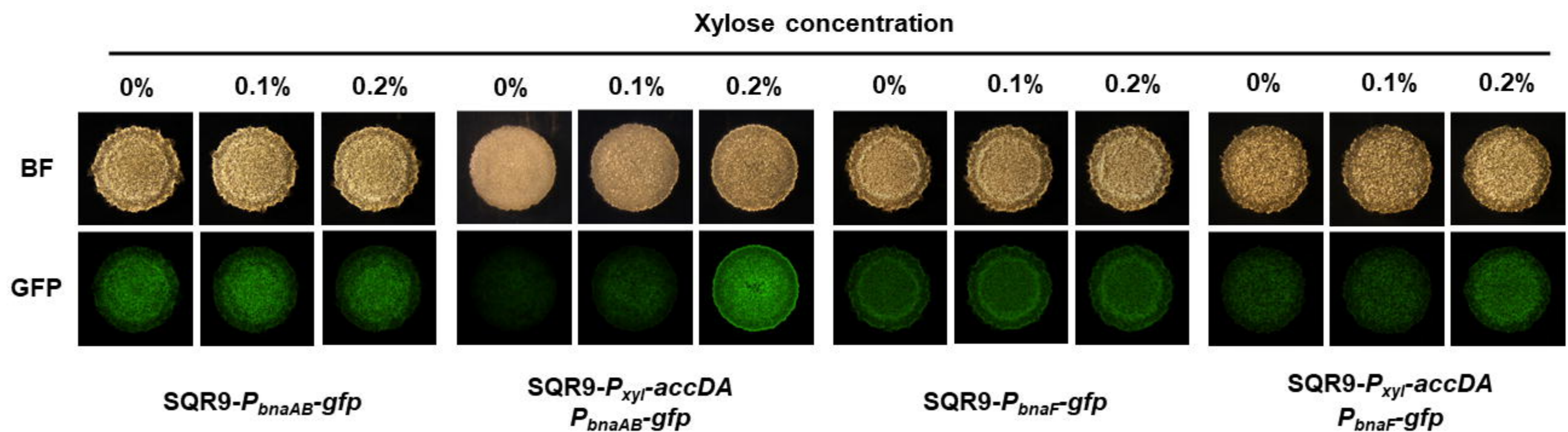
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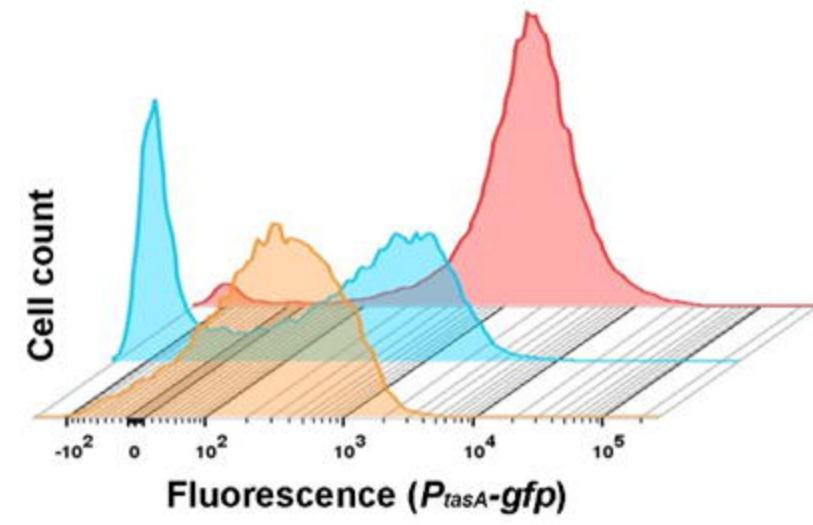
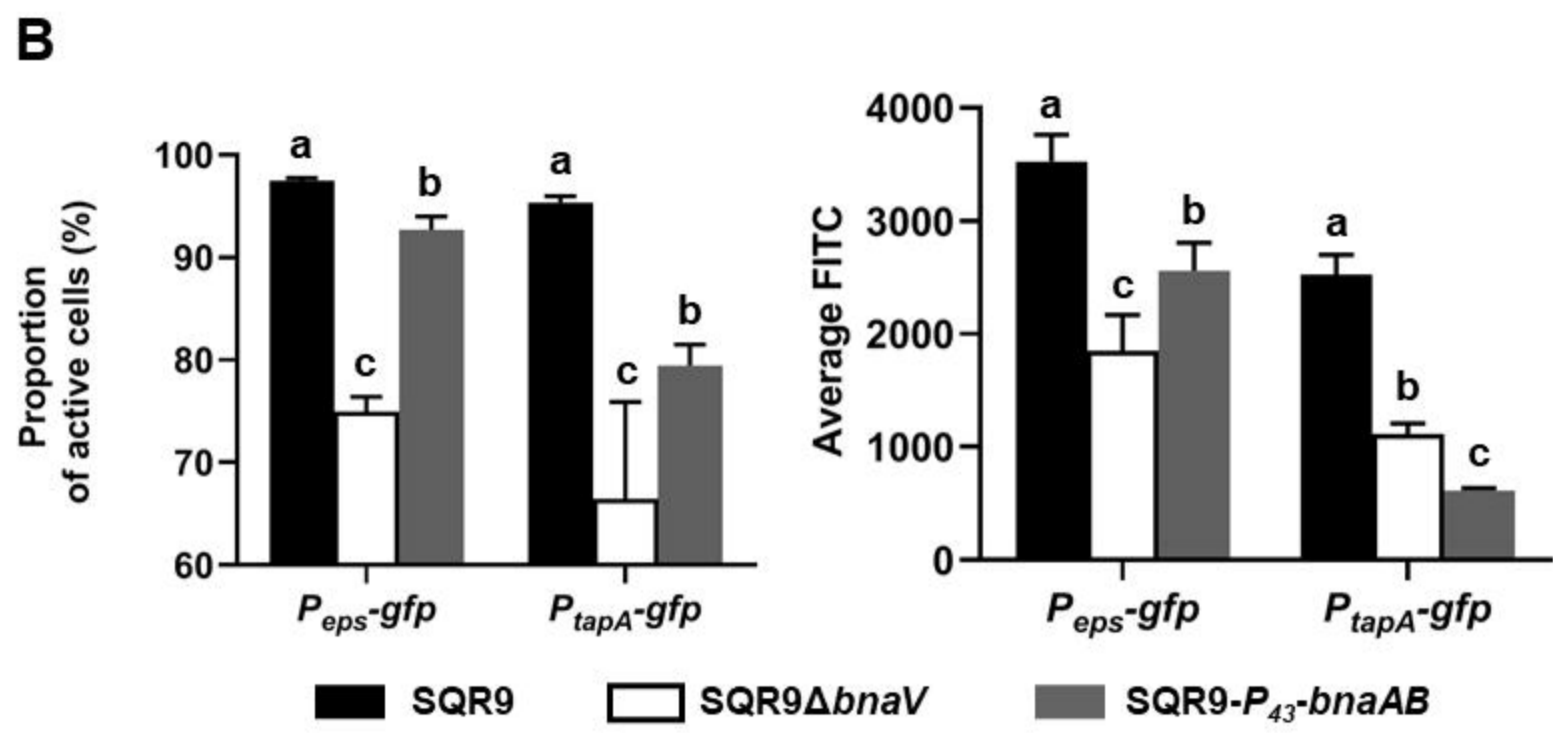
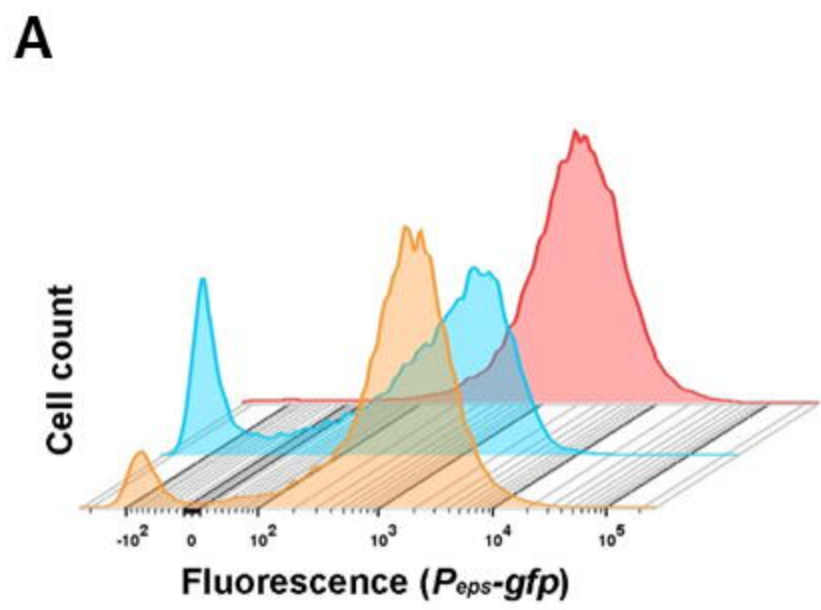
20 min

 $P_{eps-gfp} + PI$  $P_{tapA-gfp} + PI$  $P_{bnaF-gfp} + PI$  $P_{bnaAB-gfp} + PI$ 

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A**B****C****D****E****F**



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■ SQR9 □ SQR9 $\Delta bnaV$ ■ SQR9- $P_{43-bnaAB}$

