



Biofilm Formation and Synthesis of Antimicrobial Compounds by the Biocontrol Agent *Bacillus velezensis* QST713 in an *Agaricus bisporus* Compost Micromodel

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1 **Biofilm formation and synthesis of antimicrobial compounds by the biocontrol agent**
2 ***Bacillus velezensis* QST713 in *Agaricus bisporus* compost micromodel**

3
4 Running title: Bioprotection by *Bacillus velezensis* QST713

5
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14
15 **Abstract**

16 *Bacillus velezensis* QST713 is widely used as a biological control agent for crop protection
17 and disease suppression. This strain is used industrially in France for the protection of *Agaricus*
18 *bisporus* against *Trichoderma aggressivum* f. *europaeum*, the green mould disease. The efficacy
19 of this biocontrol process had been evaluated in a previous report, yet the mode of its action has
20 not been explored under production conditions. In order to decipher the underlying biocontrol
21 mechanisms for effective biofilm formation of strain QST713 in the compost and for the
22 involvement of antimicrobial compounds, we developed a simplified micromodel for the culture
23 of *A. bisporus* during its early culture cycle. By using this micromodel system, we studied the
24 transcriptional response of strain QST713 in the presence or absence of *A. bisporus* and/or *T.*

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aggressivum in axenic industrial compost. We report an over-expression of several genes of the biocontrol agent involved in biofilm formation in the compost compared to expression during growth in broth compost extract, either in the exponential growth phase (*epsC*, *blsA* and *tapA* genes) or in the stationary growth phase (*tapA* gene), while a gene encoding a flagellar protein (*hag*) was under-expressed. We also report an over-expression of *Bacillus velezensis* QST713 genes related to surfactin (*urfAA*) and fengycin (*fenA*) production in the presence of the fungal pathogen in the compost.

Importance

Biocontrol agents are increasingly used to replace chemical pesticides to prevent crop diseases. In the button mushroom field in France, the use of *Bacillus velezensis* QST713 as a biocontrol agent against the green mold *Trichoderma aggressivum*, has been shown to be efficient. However, the biocontrol mechanisms effective in the pathosystem *Agaricus bisporus*/*Trichoderma aggressivum*/*Bacillus velezensis* QST713 are still unknown. Our paper focuses on the exploration of the bioprotection mechanisms of the biocontrol agent *Bacillus velezensis* QST713 during the culture of the button mushroom (*Agaricus bisporus*) in a micromodel culture system to study the specific response of strain QST713 in the presence of *T. aggressivum* and/or *A. bisporus*.

Keywords : *Bacillus velezensis*, *Agaricus bisporus*, Biofilm, Compost, Biocontrol, Secondary metabolites, qPCR, GFP, CLSM.

1. Introduction

The strain *Bacillus velezensis* QST713 is a biocontrol agent used in France to protect the white button mushroom “*Agaricus bisporus*” crop against the fungal pathogen *Trichoderma aggressivum* responsible for the green mould disease. This fungal pathogen impedes the growth of *A. bisporus* mycelium and is characterized by the presence of a green color in the infected areas, due to the sporulation of *T. aggressivum* (1–3). Historically, the strain QST713 was initially commercialized as a *Bacillus subtilis*, yet sequencing of its genome allowed us to recently reclassify it as a *Bacillus velezensis* (4). Hence, we had assessed the impact of this strain on the microbial communities in the culture substrate of *A. bisporus* and its biocontrol effect on *T. aggressivum* (3). Many studies had shed light on the involvement of biofilm formation and production of antimicrobial compounds by *Bacillus* species in the crop bioprotective mechanisms (5–9). In this pathosystem, mechanisms developed by *B. velezensis* QST713 during its bioprotective process have not been investigated yet. To determine the involvement of biofilm formation and synthesis of antimicrobial compounds by *B. velezensis* QST713 in the bioprotective mechanisms during the cultivation of *A. bisporus*, a well-documented regulation network knowledge of *B. subtilis* biofilm formation is first needed. The transition from the “planktonic” to the “biofilm” state requires regulatory pathways to set up the expression of genes involved in matrix production or motility at the origin of cellular heterogeneity (10, 11). This cellular heterogeneity is illustrated by the coexistence of different cell phenotypes such as matrix-producing cells, surfactin-producing cells, flagellated cells or spore-forming cells. Each cell type is characterized by differential expression profile with specific genes regulated by genetic determinants involved in various regulatory pathways for biofilm formation (10, 12, 13). These different regulatory pathways are activated by extracellular signals (presence of biocides, surfactants, plant exudates, etc.) setting up a phosphorylation cascade relayed by the intracellular

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kinase phosphatases KinA, KinB, KinC and KinD. The resulting phosphorylation of the Spo0A transcription factor leads to the activation of *B. subtilis* biofilm formation (11).

The transcriptional regulator Spo0A controls the expression of about hundred genes, including the genes encoding the transcriptional regulators AbrB, SinI/SinR and SlrA/SlrR triggering the direct or indirect regulation of genes involved in the synthesis of extracellular matrix components (*tasA* operon, *eps* operon) or motility (*hag*) (11). The *tasA* operon (*tapA-sipW-tasA*) is responsible for the synthesis of the TasA structural protein forming amyloid fibres in the matrix. The *eps* operon (*epsA-O*) is responsible for the production of exopolysaccharides present in the matrix (11, 14, 15). The *hag* gene encodes a flagellar protein expressed in motile cells (11). Spo0A is involved in the regulation of *srf* genes allowing the synthesis of surfactin, a surface-active lipopeptide involved in biofilm formation by induction of polymer synthesis and involved in the regulation of *swr* genes for the swarming motility system (16). Surfactin can act as a signalling molecule that promotes other bacilli biofilm formation and also as a wide spectrum antimicrobial (8, 17). In addition, Spo0A allows the regulation of the *sigF* gene involved in sporulation (11–13). DegU, a pleiotropic transcriptional factor, is also involved in the regulation of genes encoding matrix components including (i) *blsA* encoding an amphiphilic hydrophobin (BlsA) forming a hydrophobic protective layer on the interface of the biofilm in contact with air and (ii) the *pgsBCD* operon that allows the synthesis of a polymer giving a mucosal appearance to the colonies. DegU is also involved in antibiotic production, and it controls the expression of *swr* genes involved in swarming motility (6, 11, 12, 16, 18). Moreover, the ComA regulator controls the production of surfactin, as well as the ability to incorporate exogenous DNA from the environment (12, 13, 19).

It has been shown that certain secondary metabolite antimicrobials can be overproduced by the biofilm form of the biocontrol agents (20). We hypothesized that both, the biofilm-associated

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spatial competition and the production of antifungal by *B. velezensis* QST713 could be involved in the bioprotective mechanisms against *T. aggressivum* during the cultivation of *A. bisporus*. In this work a reverse-transcription quantitative PCR was used to follow the expression of genes related to biofilm formation (*epsC*, *tapA*, *blsA*), motility (*hag* and *swrA*) and secondary metabolite synthesis (e.g. the antifungal surfactine (*srfAA*), fengycin (*fenA*), bacillomycin D (*bmyA*), the siderophore bacillibactin (*dhbA*), and two potential antimicrobials (*nrpsB*, *tnrpsC*)) of *B. velezensis* QST713 in broth compost extract for exponential and stationary growth phase, and in a compost culture with or without the presence of *T. aggressivum* f. *europaeum* and *A. bisporus*. Putative open-reading frames of these genes have been identified *in silico* according to the previously published genomic sequence of *B. velezensis* QST713 (4). The culture compost of *A. bisporus* is naturally extremely rich in *Bacillus* species (3), we have therefore chosen to carry out this study with autoclaved axenic compost in order to specifically study the cell reprogramming of strain QST713. The spatial organization of the biocontrol agent in the compost, with a recombinant *B. velezensis* strain constitutively producing the green fluorescent protein (Gfp), has been studied with non-invasive confocal laser scanning microscopy (CLSM).

110

2. Materials and Methods

2.1. Strains and culture conditions.

The strains and plasmid used in this study are described in Table 1. The *Bacillus* strains used were *Bacillus velezensis* QST713 (4), *Bacillus velezensis* SQR9-gfp (21) and *Bacillus velezensis* FZB42-FB01. *Escherichia coli* GM48 was used as the host strain for the plasmid pHAPII. *Bacillus* strains were cultured in 10 mL of Trypticase Soy Broth (TSB, bioMérieux, France) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) under aerobic conditions (200 rpm) 24h at 25°C in an incubator shaker (Infors HT Minitron, Basel, Switzerland). Antibiotics were added as

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required at the following concentrations: 25 $\mu\text{g mL}^{-1}$ kanamycin (Kan), 1 $\mu\text{g mL}^{-1}$ erythromycin (Em). *E. coli* GM48 cells were grown in 10 mL of LB medium for 24h at 37°C under agitation at 200 rpm. When necessary, 25 $\mu\text{g mL}^{-1}$ of kanamycin was added. *Trichoderma aggressivum* f. *europaeum* Ta2 strain Z (1, 2) and *Agaricus bisporus* (Amycel Delta, white hybrid variety, Amycel, Vendôme) were cultured on Yeast Malt Extract Agar medium (YMEA, 2 g L⁻¹ yeast extract, 20 g L⁻¹ malt extract, 15 g L⁻¹ Agar) supplemented with a mixture of citric acid (250 $\mu\text{g mL}^{-1}$), streptomycin (100 $\mu\text{g mL}^{-1}$), and tetracycline (50 $\mu\text{g mL}^{-1}$) for 15 days at 25°C. All media components derived from Sigma-Aldrich, Saint-Quentin Fallavier, France. Cubes of *A. bisporus* YMEA cultures were used to produce an initial culture of *A. bisporus* in autoclaved compost and incubated in a climatic chamber for 15 days (25°C, air relative humidity of 90%).

2.2. Preparation of axenic compost and broth compost extract.

The industrial compost used in this experiment is composed of straw and horse manure, supplemented with nitrogen (urea and ammonium sulphate), minerals (gypsum and calcium carbonate), and water (France Champignon, La Tourte, Longué-Jumelles, France). For the preparation of autoclaved axenic compost, 50 g of fresh compost were weighted into individual pots (Microbox Full Gas 560 mL 97x80 mm XXL filter lid Combiness, Dutscher, Brumath, France) and autoclaved three times (30 min; 121°C) spaced by 24 hours at 25°C and finally stored at 4°C. For the preparation of broth compost extract, 300 g of fresh compost was boiled in 1 L of water for 20 min, then centrifuged. The supernatant was collected and supplemented with 5 g of anhydrous glucose (Sigma-Aldrich, Saint-Quentin Fallavier, France) and autoclaved for 30 min at 121°C then filtered with Stericup-GP-0.22 μm (Merck-Millipore, Guyancourt, France).

2.3. Broth compost extract experiment.

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143 *B. velezensis* QST713 was cultivated in broth compost extract for 24h at 25°C, 200 rpm.
144 Optical density at 600 nm (OD₆₀₀) was measured, serial dilution and enumeration were made to
145 determine cell concentration (CFU mL⁻¹) according to culture age. Samples were collected during
146 the exponential and stationary growth phases to perform RNA extraction. For the broth compost
147 extract samples, 4 mL of the culture was collected in mid-exponential growth phase and 2 mL in
148 late stationary growth phase. Cells were harvested by centrifugation and 1 mL of Lifeguard
149 solution (Qiagen, Courtaboeuf, France) was added to the pellet. Samples were stored at -80°C
150 (performed in triplicate) until RNA extraction.

151

152 **2.4. Culture compost axenic micromodel experiments.**

153 Cultures in compost were performed and treated with *B. velezensis* QST713 with (A. b +)
154 or without (A. b -) seeding of *A. bisporus*, and with (T. a +) or without (T. a -) inoculation of *T.*
155 *aggressivum*. Hence, four conditions were studied: strain *B. velezensis* QST713 alone in the
156 compost (QST713 +; A. b -; T. a -), strain QST713 with *T. aggressivum* in the compost (QST713
157 +; A. b -; T. a +), strain QST713 with *A. bisporus* in the compost (QST713 +; A. b +; T. a -), and
158 strain QST713 with both fungi in the compost (QST713 +; A. b +; T. a +). For all conditions,
159 individual pots containing 50 g of autoclaved compost were used. At day 0, 5 mL of *B. velezensis*
160 QST713 suspension in physiological saline (0.85% NaCl) at 50 CFU mL⁻¹ was added into each
161 pot to reach the final concentration of 2.5 x 10² CFU per pot of strain QST713. When required,
162 the compost was then seeded at day 0 with 1.6 g of a 15 day-old culture of *A. bisporus* in
163 autoclaved compost, and/or inoculated with 5 mL of *T. aggressivum* conidiospores solution in
164 physiological saline at 200 spores mL⁻¹ made with conidia collected from a 15 day-old YMEA
165 culture to get the final concentration of 1,000 conidiospores per pot. When required, 5 mL of
166 physiological saline was added into the pots to equilibrate the water supply. The compost was

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167 then homogenized and compacted at the bottom of the pot. Three replicates per condition were
168 performed representing twelve pots in total. The pots were then incubated under controlled
169 conditions during 10 days of cultivation in climatic chamber (Votsh VC 2020; 25°C, air relative
170 humidity 90%). Samples were taken on the 6th day of cultivation. For each compost sample
171 (twelve in total), 5 g of compost was collected and crushed with mortar and pestle in liquid
172 nitrogen for homogenization. Samples were finally stored at -80°C until RNA extraction.

173

174 **2.5. *Bacillus* enumeration from the compost.**

175 For each pot, compost was collected at days 0, 3, 6 and 10. Enumeration of strain QST713
176 was performed from 5 g compost in 45 mL sterile physiological saline water (0.85% NaCl) with a
177 sterile filter bag of Interscience Bag System ® (Interscience, Saint-Nom, France). The mixture
178 was homogenized for 30 min at room temperature and then blended in a commercial laboratory
179 blender according to the manufacturer's instructions (Seward Laboratory Lab-Blender 400,
180 Worthing, UK). The filtered liquid was collected and readjusted to 50 mL with sterile
181 physiological saline. Serial dilutions were made from the extracted suspensions in sterile
182 physiological saline (0.85% NaCl) and 0.1 mL of each serial dilution was used to inoculate
183 Trypticase Soy Agar (TSA; Biomerieux, France) in a 90 mm Petri dish. All Petri dishes were
184 incubated for 48h at 25°C.

185

186 **2.6. *RNA extraction.***

187 RNA extraction from strain QST713 in the compost was performed with the previously
188 published Lysozyme / CTAB (hexadecyltrimethylammonium bromide) / TriReagent /
189 Chloroform method with some modifications (22). Briefly, the CTAB extraction buffer was
190 prepared according to Wang and Stegemann (2010) with some modifications and was composed

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191 of 2% CTAB, 2.5% polyvinylpyrrolidone (PVP 40), 2 M sodium chloride (NaCl), 100 mM Tris-
192 HCl pH=8, 20 mM ethylene-diamine-tetra-acetic acid (EDTA) and 2% beta-mercaptoethanol
193 (added just before use) in RNase free water (Merck-Millipore, Guyancourt, France) to a final
194 volume of 100 mL. All media components except RNase free water derived from Sigma-Aldrich,
195 Saint-Quentin Fallavier, France. Before the RNA extraction, the broth compost extract culture
196 samples were centrifuged to remove the LifeGuard solution and 100 mg of each homogenized
197 compost sample was collected. A lysozyme treatment for 30 min at 37°C in 500 µL of lysozyme
198 buffer (30 mM Tris, 10 mM EDTA, 10 mg mL⁻¹ lysozyme (Sigma-Aldrich, Saint-Quentin
199 Fallavier, France), pH 6.2 in RNase free water) was performed on the eighteen samples. After the
200 lysozyme treatment, 500 µL of CTAB buffer supplemented with proteinase K at 20 mg mL⁻¹ final
201 concentration was added, vortexed for 2 min and incubated in a water bath at 55°C for 10 min.
202 The next steps of the RNA extraction were carried out according to appendix 5, step 30 to 56 of
203 [Jordon-Thaden et al. \(2015\)](#), followed by DNase treatment in solution with the RNase-Free
204 DNase I Kit (Norgen Bioteck, Proteigene distributor, Saint-Marcel, France) and RNA Clean-Up
205 Kit (Norgen bioteck, Proteigene distributor, Saint-Marcel, France). The RNA concentration was
206 measured with NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Illkirch, France)
207 and Qubit 3.0 Fluorometer (ThermoFisher Scientific, Illkirch, France). Quality and concentration
208 of RNA was assessed by Bioanalyzer Agilent 2100 (Agilent Technologies, Les Ulis, France) at
209 the @BRIDge platform (<http://abridge.inra.fr/>). When required, RNA extracts were precipitated
210 with glycogen (molecular biology grade; ThermoFisher Scientific, Illkirch, France) according to
211 the manufacturer's protocol to obtain 1 µg of total RNA required for reverse transcription.

212

213 **2.7. Gene transcription analyses by quantitative real-time PCR.**

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214 The reverse transcription of RNA was conducted with the Omniscript Reverse
215 Transcription kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol with
216 random hexamers (Qiagen, Courtaboeuf, France). Reverse transcripts of the genes involved in
217 biofilm formation (*epsC*, *tapA*, *blsA*), secondary metabolite synthesis (*srfAA*, *fenA*, *bmyA*, *dhbA*,
218 *nrpsB*, *tnrpsC*) and motility (*hag* and *swrA*) were quantified by quantitative real-time polymerase
219 chain reaction (qPCR) with the Takyon™ Rox SYBR® MasterMix dTTP Blue (Eurogentec,
220 Angers, France) according to the manufacturer's protocol (10 µL of Takyon™ MasterMix, 0.4
221 µL of each primer (final concentration 200 nM), 5 µL of cDNA template diluted 20X, 4.2 µL of
222 water for molecular biology). The qPCR protocol for maximal efficiency was performed: 5 min
223 at 95°C followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 1 min at 60°C,
224 elongation for 30 s at 72°C. A final elongation was performed: 5min at 72°C followed by a
225 melting curve step. qPCR efficiency varied from 89% to 100% depending on the genes to be
226 tested. The *gyrA* gene was used as housekeeping internal control (Table 1). The $2^{-\Delta\Delta CT}$ method
227 was used to analyse the qPCR data and results were presented as Log2(fold change) (24).

228

229 2.8. *Bacillus velezensis* QST713 transformation.

230 In order to fluorescently contrast the strain QST713 in the compost by CLSM, it was
231 transformed by the pHAPII plasmid carrying a *gfp* gene under the control of a constitutive
232 promoter (21). However, transformation of strain QST713 is hard to carry out and has not been
233 published yet. We tested several methods (natural transformation, conjugation,
234 electrotransformation, tribo transformation) and electrotransformation was the only successful
235 one, however, with a very low efficacy. The electrotransformation method used here combines
236 methods described in Zhang et al. (2011) and Yi and Kuipers (2017), with some modifications.
237 Cells were grown in a hyperosmotic growth medium with glycine to weaken the peptidoglycan.

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238 Plasmid pHAPII (carrying the *gfp* gene) was first extracted from *Bacillus velezensis* strain SQR9-
239 *gfp* with the GeneJET Plasmid Miniprep Kit (ThermoFisher, Illkirch, France) and then
240 transferred to *Escherichia coli* GM48 (*dam*⁻/*dcm*⁻) strain to obtain an unmethylated pHAPII
241 plasmid. For the transformation of strain QST713, competent cells were prepared according to
242 the following protocol: cells were grown in 50 mL BHIS medium (34 g BHI (Difco), and 91.1 g
243 sorbitol in 1 L deionized water, pH 7.2) at 30°C, 200 rpm to OD₆₀₀ = 0.85. After 1 h of additional
244 incubation with 1% glycine, the cell culture was transferred into a 50 mL centrifuge tube and
245 cooled on ice for 20 min, and cells were collected (OD₆₀₀ = 0.95) by centrifugation at 4 °C, 8000
246 rpm for 5 min with an Eppendorf centrifuge (Eppendorf, Montesson, France). Cells were washed
247 four times in electroporation buffer (pre-chilled) (10% glycerol, 0.25 M sorbitol, 0.25 M
248 mannitol) and electroporation buffer was added to the pellet at 1/100 vol of the original culture to
249 obtain electro-competent cells. Electroporation was performed in 100 µL of electro-competent
250 cells with 250 ng of pHAPII plasmid in 2-mm gap electroporation cuvettes (pre-chilled) with the
251 Eppendorf Eporator ® (strength 2,1 kV cm⁻¹) (Eppendorf, Montesson, France). After pulse, 1
252 mL of pre-warmed growth medium was added to the electroporation cuvettes and transferred to a
253 2 mL Eppendorf tube that was then incubated for a recovery time of 5h at 30°C, 180 rpm.
254 Electroporation was then centrifuged for 5 min at 8000 rpm with a Sigma 1k15 centrifuge
255 (Sigma-Aldrich, Saint-Quentin Fallavier, France) and supernatant was removed. A volume of 100
256 µL of sterile growth medium was added to resuspend the pellet and the total resulting volume
257 was plated on LB agar plates (90 mm) containing 25 µg mL⁻¹ of kanamycin. Positive
258 transformants were screened after 48h of incubation according to their colony fluorescence on a
259 Safe Imager™ 2.0 Blue Light Transilluminator (ThermoFisher, Illkirch, France).

260

261 2.9. Confocal Laser Scanning Microscopy of *Gfp-bacilli* in axenic compost.

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262 The condition *Bacillus/Trichoderma/Agaricus* in compost described above was used to
263 visualize the spatial organization of the biocontrol agent at day 6 in the compost by confocal laser
264 scanning microscopy (CLSM), using a Leica SP8 AOBS inverter microscope (Leica,
265 Microsystems, Germany). The fluorescent bacteria used were *Bacillus velezensis* QST713
266 carrying a plasmid encoding a Gfp (this study), and two relative biocontrol strains: *Bacillus*
267 *velezensis* SQR9 carrying a plasmid encoding a Gfp (21) or *Bacillus velezensis* FZB42-FB01 Gfp
268 tagged (27). Samples were collected from the compost pots and directly scanned using 10x and
269 20x HC PL FLUOTAR dry objective lens. Samples were scanned at 600 Hz (1024 x 1024 pixels
270 images, z step of 4 μm , z range from 480 to 900 μm), with dual excitation wavelengths of 488
271 nm (argon laser: intensity 44%) and 561 nm (DPSS 561 laser; intensity 1.88%). Using ultra-
272 sensitive hybrid detectors (HyD Leica Microsystems, Germany), the emitted fluorescences were
273 collected in the range 497 to 533 nm for the green Gfp (bacilli) and in the range of 580 to 740 nm
274 for the red autofluorescence of the compost. Laser reflexion was used simultaneously to contrast
275 the mycelium of *A. bisporus*. Three-dimensional projections from the xyz image series were
276 constructed using the Easy 3D function of the IMARIS software (Bitplane, Switzerland).

277

278 **2.10. Statistical analysis**

279 The Shapiro-Wilk and Levene tests were applied to determine the normality of the data
280 distribution and homogeneity of variances. The data were not normally distributed and the
281 variances were not homogeneous. Thus, a non-parametric Kruskal-Wallis ANOVA test and the
282 Conover-Iman post-hoc test were performed to determine whether the observed differences were
283 significant using XLSTAT-Premium trial software (Microsoft Corporation, USA).

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285 3. Results

286 3.1. Growth and biocontrol effect of *B. velezensis* QST713 in compost micromodels.

287 The validation of *A. bisporus* culture micromodels in axenic autoclaved compost was based
288 on the invasion of compost by the mycelium of *A. bisporus* at the end of the vegetative phase (15
289 days of cultivation), and on the observation of the biocontrol effect on *T. aggressivum* by *B.*
290 *velezensis* QST713. Three controls were carried out: (1) the normal development of *A. bisporus*
291 alone in the compost resulting in the progressive invasion of the compost in pots by a white
292 mycelium (line 1, Fig. 1); (2) The development of the competitor *T. aggressivum* alone in the
293 compost with a massive sporulation at day 6 (line 2, Fig. 1); (3) The pathogenic effect of *T.*
294 *aggressivum* on *A. bisporus*, resulting in the absence of *A. bisporus* mycelium development
295 during the culture and the colonisation of *T. aggressivum* that was the most observable at day 6
296 (line 3; Fig. 1). Moreover, presence of strain QST713 alone did not affect the natural appearance
297 of the compost (line 4; Fig. 1). Furthermore, when strain QST713 and *T. aggressivum* were
298 cocultured, we observed the absence of *T. aggressivum* sporulation in a healthy compost
299 highlighting the inhibition effect of *B. velezensis* QST713 on *T. aggressivum* (line 5; Fig. 1). To
300 determine whether the development of *A. bisporus* was affected by strain QST713, cocultures of
301 *A. bisporus* and strain QST713 were assessed. We observed a normal growth of *A. bisporus* in the
302 presence of strain QST713 (line 6; Fig.1). The last condition studied was the coculture of the tri-
303 partite *A. bisporus*/*T. aggressivum*/*B. velezensis* QST713, where we observed a normal growth of
304 the mycelium of *A. bisporus* and an inhibition of the development of *T. aggressivum* which thus
305 validated the biocontrol efficiency of strain QST713 (line 7; Fig.1). *B. velezensis* QST713 was
306 enumerated to determine its effective development in the compost and results are represented in
307 Fig. S1A. Strain QST713 alone in the compost reached 10^9 CFU g⁻¹ of compost at day 6 of the
308 cultivation and its development was not affected by the presence of the fungi ($P > 0.05$). We

309 demonstrated the biocontrol effect of *B. velezensis* QST713 on *T. aggressivum* using *A. bisporus*
310 culture micromodels in axenic autoclaved compost in early vegetative phase.

311

312 **3.2. Biofilm is the preferential mode of life of *B. velezensis* QST713 in compost micromodels.**

313 To determine the preferential lifestyle of *B. velezensis* QST713 in the compost, we
314 compared the expression of genes related to biofilm formation of strain QST713 cultivated in
315 broth compost extract (planktonic state) at two different times (Exponential Growth Phase =
316 EGP; Stationary Growth Phase = SGP), and in the compost after six days of cultivation. The
317 growth curve of *B. velezensis* QST713 in broth compost extract was determined (Fig. S1B).
318 Strain QST713 reaches the stationary growth phase in the broth compost extract after eight hours
319 of cultivation at 25°C (200 rpm) with a maximum OD₆₀₀ = 0.74, corresponding to 4 x 10⁷ CFU
320 mL⁻¹. Exponential growth phase occurred from 3 to 6 hours. Cells were collected in the mid
321 exponential growth phase after 5 h and in the stationary growth phase after 14 h of cultivation.

322 Gene expressions of strain QST713 in the compost relative to its growth in the broth
323 compost extract at EGP and SGP were represented as a heatmap (Fig. 2(Aa); (Ba)). Genes related
324 to biofilm formation (*epsC* involved in the synthesis of extracellular polysaccharide, *blsA*
325 involved in the production of the hydrophobin BlsA, and *tapA* involved in the production of
326 amyloid fibres) were up-regulated in the compost compared to EGP (Fig. 3A). On the opposite,
327 *hag*, a gene involved in flagellin biosynthesis, was strongly down-regulated in the compost as
328 compared to both EGP and SGP (Fig. 3A). The gene *swrA* involved in swarming motility was up-
329 regulated in the compost compared to EGP. Altogether, these results strongly suggest that biofilm
330 is the dominant mode of life of the strain QST713 in the compost.

331

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332 **3.3. Secondary metabolites synthesis of *B. velezensis* QST713 in axenic compost**
333 ***micromodels.***

334 Differential expression of genes involved in the production of secondary metabolites by
335 strain QST713 was studied in planktonic lifestyle (broth compost extract) and compared to
336 compost-associated mode of life (compost) (Fig. 2 (A,a); (B,a)). Expression of the gene encoding
337 surfactin, a molecule involved in biofilm formation and associated with antimicrobial activity,
338 was not differentially expressed between the biofilm (compost) and the planktonic lifestyle (EGP
339 and SGP) (Fig. 3B). Three genes encoding secondary metabolites were up-regulated in the
340 compost when compared to EGP: *fenA* involved in the synthesis of the antifungal fengycin, *bmyA*
341 involved in the synthesis of the antifungal bacillomycin D, and *dhbA* involved in the synthesis of
342 the siderophore bacillibactin. Only the gene *fenA* was significantly up-regulated in the compost
343 when compared to both EGP and SGP (Fig. 3B). The genes *nrpsB* and *tnrpsC* belonging to two
344 clusters of secondary metabolites with potential antimicrobial activities (4) were both up-
345 regulated in the compost compared to SGP and EGP. These results suggest that *fenA*, *nrpsB* and
346 *tnrpsC* were over-expressed in the biofilm form in the compost.

348 **3.4. Antifungal compounds surfactin and fengycin are over-expressed in the presence of *T.***
349 ***aggressivum.***

350 To determine the genes potentially involved in the bioprotection of *A. bisporus* culture by
351 *B. velezensis* QST713 against *T. aggressivum*, the expression of genes involved in biofilm
352 formation and secondary metabolite production was studied in compost micromodels. The four
353 conditions studied were: (a) *B. velezensis* QST713 alone in the compost (QST713 +; A. b -; T. a -
354), (b) the co-culture of *B. velezensis* QST713 and *T. aggressivum* (QST713 +; A. b -; T. a +), (c)
355 the co-culture of *B. velezensis* QST713 and *A. bisporus* (QST713 +; A. b +; T. a -), and (d) the

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co-culture of *B. velezensis* QST713 with *T. aggressivum* and *A. bisporus* (QST713 +; A. b +; T. a +). Gene expression profiles of strain QST713 in the presence of *T. aggressivum*, *A. bisporus* or both in the compost were compared to those of strain QST713 alone in the compost and represented in Fig. 2 (C. a, b, c, d). Genes related to biofilm formation did not appear to be significantly up- or down-regulated in the presence of *T. aggressivum*, *A. bisporus* or both compared to strain QST713 alone in the compost (Fig. 3C). The gene *srfAA* involved in the synthesis of surfactin, a molecule involved in the initiation of biofilm formation and with an antifungal activity, was up-regulated in the presence of *T. aggressivum* or *A. bisporus* compared to strain QST713 alone in the compost. The *fenA* gene encoding an antifungal compound was up-regulated in the presence of *T. aggressivum* compared to strain QST713 alone in the compost (Fig. 3D). The *tnrpsC* gene encoding a potential secondary metabolite compound was down-regulated in the presence of *T. aggressivum*. The *dhbA* gene encoding the siderophore bacillibactin was up-regulated in the presence of *A. bisporus* compared to strain QST713 alone in the compost (Fig. 3D). A competition for iron uptake might occur in the compost between strain QST713 and *A. bisporus*, an effect that we did not observe in the presence of the three partners. These results suggest that fengycin and surfactin may be involved in the inhibition of *T. aggressivum*.

373

374

3.5. Direct visualisation of biofilm formation in the compost by fluorescent *B. velezensis* strains.

Non-invasive confocal laser scanning microscopy experiment was performed for the condition where the three partners were present to confirm the effective biofilm formation by strain QST713 in compost micromodels. The plasmid pHAPII (carrying the Gfp-encoding gene)

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380 was electroporated into strain QST713. The transformation of the recalcitrant strain QST713 was
381 complicated and hardly reproducible. The constitutively fluorescent strain QST713/pHAPII
382 allowed to visualize the spatial organization of these bacteria cocultured with *A. bisporus* and *T.*
383 *aggressivum* in the compost at the 6th day of cultivation (Fig. 4A). In the compost, strain
384 QST713 mainly formed micro-aggregates of cells (Fig. 4A, left picture) and sometimes massive
385 spatially organized biofilms (Fig. 4A, right picture). We did not observe systematic colocalization
386 of strain QST713 with *A. bisporus* mycelium during this experiment.

387 To determine if other biocontrol strains of *B. velezensis* form similar biofilm structures in the
388 compost cocultured with *T. aggressivum* and *A. bisporus*, two other biocontrol *B. velezensis*
389 isolates, FZB42-FB01 and SQR9-*gfp* were observed in the compost micromodels by CLSM.
390 Similarly to strain QST713, both strains showed heterogeneous colonization of the compost with
391 sparse biofilm structures (Fig. 4B). A specific spatial interaction of strain SQR9 (in green) and
392 the mycelium of *A. bisporus* (contrasted in grey with reflected light) was observed after 6 days of
393 cocultivation, suggesting an interaction between the two species that could be a metabolic
394 cooperation (28).

395 Antagonism experiments in Petri dishes confirmed that the three *B. velezensis* strains were
396 able to inhibit *T. aggressivum* (Fig. S2). However, further investigations are needed to determine
397 if the mechanisms of inhibition of *T. aggressivum* are similar among these three *B. velezensis*
398 isolates.

399

400 4. Discussion

401 We previously reported an effective biocontrol effect of *B. velezensis* QST713 against *T.*
402 *aggressivum* during the cultivation of *A. bisporus*. We showed that this protective effect is
403 significant before the seventh day of cultivation, and that the industrial compost is rich in strains

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404 assigned to the *Bacillus velezensis* species (3). We also observed that strain QST713 was capable
405 to form dense and robust biofilms on inert surfaces and to inhibit the growth of *T. aggressivum* in
406 *vitro* (4). In this study, we attempted to explore the mechanisms of bioprotection in a compost
407 micromodel, including both the effective biofilm formation of strain QST713 in the compost and
408 the involvement of antimicrobial compounds. The relative quantification of gene expression
409 related to biofilm formation and antimicrobial production in strain QST713 was assessed by
410 quantitative real-time PCR, through the development of a simplified micromodel of *A. bisporus*
411 cultivation with the addition of the pathogen *T. aggressivum*. An axenic autoclaved industrial
412 compost was used to remove the native microbiota rich in *Bacillus* species and to focus
413 specifically on the response of strain QST713 to the presence of *T. aggressivum* and/or *A.*
414 *bisporus* in the compost.

415 The results showed that genes related to biofilm formation (*epsC*, *tapA* and *blsA*) were up-
416 regulated and the motility gene *hag* strongly down-regulated in the compost compared to the
417 exponential growth phase in broth compost extract. The swarming motility gene (*swrA*) was also
418 active in the compost. The expression profile of strain QST713 in the compost was close to the
419 stationary growth phase profile in the broth compost extract with the exception of the *hag* gene
420 which was under-expressed and the *tapA* gene which was over-expressed, suggesting an effective
421 biofilm formation in the compost. The large variability in gene expression in the compost
422 samples would be related to the heterogeneity of the compost colonisation observed by CLSM.
423 Moreover, the expression profile of genes encoding secondary metabolites was close to the
424 stationary growth phase profile in the broth compost extract with the exception of *fenA*, which
425 was upregulated and *dhbA*, which was downregulated in the compost, suggesting a higher
426 expression of *fenA* in the compost biofilm mode of life.

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We also showed that *srfAA* and *fenA* were up-regulated in the presence of *T. aggressivum*, suggesting that surfactin and fengycin would be overproduced in the presence of the pathogen. Previous studies have demonstrated in other *Bacillus* strains that fengycin synthesis could be regulated by the presence of pathogens (29, 30) and that its toxicity varied depending on the pathogen (30, 31). In addition, surfactin has been shown to be involved in biofilm formation (8), swarming motility and environmental colonisation (32, 33), microbial communication as a signalling molecule that triggers matrix production *via* the sensor histidine kinase KinC (17, 19), an elicitor of plant defence (34) and also as an antifungal compound (35).

In order to evaluate the specificity of the QST713 strain's behaviour, we also studied two biocontrol strains for their ability to colonize the compost and to antagonize *T. aggressivum*. The strain FZB42 has recently been authorized as a market biocontrol strain for mushroom cultivation in France (personal communication, French Technical Mushroom Centre). However, no public data are currently available either on its effect on the yield of *A. bisporus* or on its biocontrol effect against *T. aggressivum*. The strain SQR9 is not currently used in France as a biocontrol agent. Both strains exhibit genes related to biofilm formation, fengycin and surfactin production (4), and can inhibit the growth of *T. aggressivum* both *in vitro* and in the compost. Spatial interaction of strain SQR9 and the mycelium of *A. bisporus* suggests a potential specific interaction between these organisms. Similar observations have been reported for strain SQR9 used to protect maize crops, where the genes implicated in biofilm formation were up-regulated in the presence of root exudates (28). This proximity with the mycelium of *A. bisporus* could also form a protective barrier against the different molecules emitted by *T. aggressivum* as the antifungal compound 3,4-dihydro-8-hydroxy-3-methylisocoumarin (36, 37), as well as proteinase, endochitinase and β -glucanase which are cell wall degrading enzymes that could be involved in *T. aggressivum* mycoparasitism (38, 39).

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451 Finally, the use of biocontrol products formulated in the biofilm form with
452 microencapsulated biocontrol agents would permit their introduction in agrosystems.
453 Furthermore, a recent study demonstrated a better persistence of the biocontrol agents at the
454 entrance to the system, as well as a better biocontrol effect on pathogens (40). Hence, additional
455 investigations are needed to formally assign the role of biofilm formation, surfactin and fengycin
456 production in the biocontrol mechanisms that occur against *T. aggressivum* during the cultivation
457 of *A. bisporus*.

458

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468

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580

581 **Table and Figures**582 **Table 1:** Strains, plasmid and primers used in this study.

583

584 **Fig. 1:** Growth of *A. bisporus* and *T. aggressivum* in culture micromodels and effect of *B.*
585 *velezensis* QST713 in co-culture with one or both partners during 15 days of cultivation. Pictures
586 of culture pots were taken at days six (6d), ten (10d) and fifteen (15d) of cultivation. From the top
587 line to the bottom line: (line 1: QST713 -; A. b +; T. a -) *A. bisporus* alone in the compost
588 resulting in the progressive invasion of the compost by a white mycelium; (line 2: QST713 -; A.
589 b -; T. a +) The development of *T. aggressivum* alone in the compost with a massive sporulation
590 (mostly at day six); (line 3: QST713 -; A. b +; T. a +) Pathogenic effect of *T. aggressivum* on *A.*
591 *bisporus*, absence of *A. bisporus* mycelium development and massive sporulation of *T.*
592 *aggressivum* (mostly at 6 days); (line 4: QST713 +; A. b -; T. a -) *B. velezensis* QST713 alone in
593 the compost, compost has a natural aspect; (line 5: QST713 +; A. b -; T. a +) the co-culture of *B.*
594 *velezensis* QST713 and *T. aggressivum*, absence of development of *T. aggressivum* that showed

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the inhibitory effect on *T. aggressivum* by strain QST713, and natural aspect of the compost; (line 6: QST713 +; A. b +; T. a -) the co-culture of *B. velezensis* QST713 and *A. bisporus*, normal development of *A. bisporus* mycelium; (line 7: QST713 +; A. b +; T. a +) the co-culture of *B. velezensis* QST713 with *T. aggressivum* and *A. bisporus*, normal development of *A. bisporus* mycelium that showed the biocontrol effect of *B. velezensis* QST713 on *T. aggressivum*.

Fig. 2: Heatmap of the transcriptional levels of biofilm-related, secondary metabolite production and motility genes of *B. velezensis* QST713 in the compost and broth compost extract. Transcriptional levels of motility related genes (*hag* and *swrA*), biofilm-related genes (*epsC*, *blsA*, *tapA*, *srfAA*) and secondary metabolite synthesis genes (*fenA*, *bmyA*, *dhbA*, *nrpsB*, *tnrpsC*) for six different conditions: (a) *B. velezensis* QST713 alone in the compost, (b) the co-culture of *B. velezensis* QST713 and *T. aggressivum*, (c) the co-culture of *B. velezensis* QST713 and *A. bisporus*, and (d) the co-culture of *B. velezensis* QST713 with *T. aggressivum* and *A. bisporus*, (e) Exponential growth phase (EGP) of *B. velezensis* QST713 alone in the broth compost extract and (f) Stationary growth phase (SGP) of *B. velezensis* QST713 alone in the broth compost extract. Letters A, B and C represent the transcription levels of QST713 genes relative to EGP, SGP or QST713 alone in the compost, respectively. The green color represents the values of up-regulated genes (max value: +7.19), the red color represents the values of down-regulated genes (min value: -7.49), both compared to a control value of 0 (in black). Data are represented in Log2Fold change.

Fig. 3: Compost and interaction effects on the expression of genes related to motility, biofilm formation and secondary metabolite production in *B. velezensis* QST713. Transcriptional levels of motility-related genes (*hag* and *swrA*), biofilm-related genes (*epsC*, *blsA*, *tapA*, *srfAA*) and

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619 secondary metabolite synthesis genes (*fenA*, *bmyA*, *dhbA*, *nrpsB*, *tnrpsC*) relative to that of (A
620 and B) the exponential growth phase of strain QST713 alone in the broth compost extract, and of
621 (C and D) strain QST713 alone in the compost. Different culture conditions have been studied:
622 strain QST713 alone in the compost (QST713 +; A. b -; T. a -; compost), co-cultured with *T.*
623 *aggressivum* in the compost (QST713 +; A. b -; T. a +; compost), co-cultured with *A. bisporus* in
624 the compost (QST713 +; A. b +; T. a -; compost), co-cultured with *T. aggressivum* and *A.*
625 *bisporus* (QST713 +; A. b +; T. a +; compost), and its stationary growth phase alone in the broth
626 compost extract (QST713 +; A. b -; T. a -; broth compost extract SGP). The gene *gyrA* was used
627 as an internal reference gene. Bars represent standard deviations of data from three biological
628 replicates. Results are presented as Log2Fold Change with Fold Change = $2^{-\Delta\Delta CT}$. (*) represents
629 the significant difference compared with the exponential growth phase for A and B, and to strain
630 QST713 alone in the compost for C and D ($P < 0.05$). (**) represents the significant difference
631 between conditions ($P < 0.05$).

632

633 **Fig. 4:** *Bacillus* microcolonies and biofilm on the culture compost of *A. bisporus*. Confocal laser
634 scanning microscopy observations of (A) *B. velezensis* QST713 (in green) cultivated with *A.*
635 *bisporus* and *T. aggressivum* in the compost (in red). (B) *B. velezensis* FZB42 (in green) or *B.*
636 *velezensis* SQR9 (in green) cultivated with *A. bisporus* (in grey) and *T. aggressivum* in the
637 compost. The positive and colocalized spatial interaction between the strain SQR9 and the
638 mycelium of *A. bisporus* is indicated with white arrow.

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Table 1: Strains, plasmid and primers used in this study.

Strains	Description	Source
<i>Escherichia coli</i> GM48	F ⁻ , hr-1, araC14, leuB6(Am), fhuA31, lacY1, tsx-78, glnX44(AS), galK2(Oc), galT22, λ ⁻ , dcm-6, dam-3, thiE1	Lab strain
<i>Bacillus velezensis</i> QST713	Wild type	Serenade (Bayer)
QST713- <i>gfp</i>	carrying pHAPII plasmid, <i>gfp</i> +, Km ^r	This study
SQR9- <i>gfp</i>	carrying pHAPII plasmid, <i>gfp</i> +, Km ^r	Cao et al. 2011 (21)
FZB42-FB01	<i>amyE::</i> (Ery ^r - <i>gfp</i> +))	Fan et al. 2011 (27)
<i>Trichoderma aggressivum</i> f. <i>europaeum</i> (Ta2) strain Z	Wild type	Mamoun et al. 2000 (2)
<i>Agaricus bisporus</i> IE-751 (Delta)	Wild type	Amycel
Plasmid		
pHAPII	pUBC19, <i>apha3</i> containing <i>gfp</i> transcriptionally fused to HAPII promoter Kan ^r	Cao et al. 2011 (21)
Primers for <i>B. velezensis</i> QST713	Oligonucleotide sequence 5' – 3'	
ForblsA	AATTTTCTCAACTGTCATGGCAAG	This study
RevblsA	CGTTGACTGTATCTTTTGTGTAGC	This study
ForbmyA	GAGCTCTGTGACGAATTTTGA	This study
RevbmyA	CTACTCTTTCATTGTTTCATCGGA	This study
FordhbA	CGTAAATGTGGCAGCTATAGATA	This study
RevdhbA	GCAACGTTGCGCAATATTTCAAT	This study
ForepsC	TCAGGCTATACGACAGAACAGAT	This study
RevepsC	AATCTGCTCCGTATGTAATTCA	This study
ForfenA	CAGAAACGGATGTATGTGCTC	This study
RevfenA	GCTTCCTCCAGACCAACG	This study
Forhag	ATATCGCGGCTCTTAACACTAG	This study
Revhag	ATACGCTGAAGAATGCTGTGA	This study
FornrpsB	TTTGGTGGATGTTAGCGGGTGGTA	This study
RevnrpsB	TGTAGTTTCTGCTGGACCATAG	This study
ForsrfAA	CCTGTCCCGTGTGATCAAAC	This study
RevsrfAA	GACTTCGGTATTGCTGACCG	This study
ForswrA	GTGGAACAACTAAAAGACAGAACA	This study
RevswrA	TCAGATAGTGGTCAACCTCC	This study
FortapA	CAAGGTGAGATGGCGGATGA	This study
RevtapA	GCTCTTTCCCTTATTCTCCAG	This study
FortnrpsC	ACTGGAGAATACGGTGGGGA	This study
RevtnrpsC	CAAGTACAACATGACATCAAACA	This study
ForgyrA	CAGGTGAACAAAGCGAGATTAATT	This study
RevgyrA	GTGATCAAGATAATGCTCCAGACA	This study

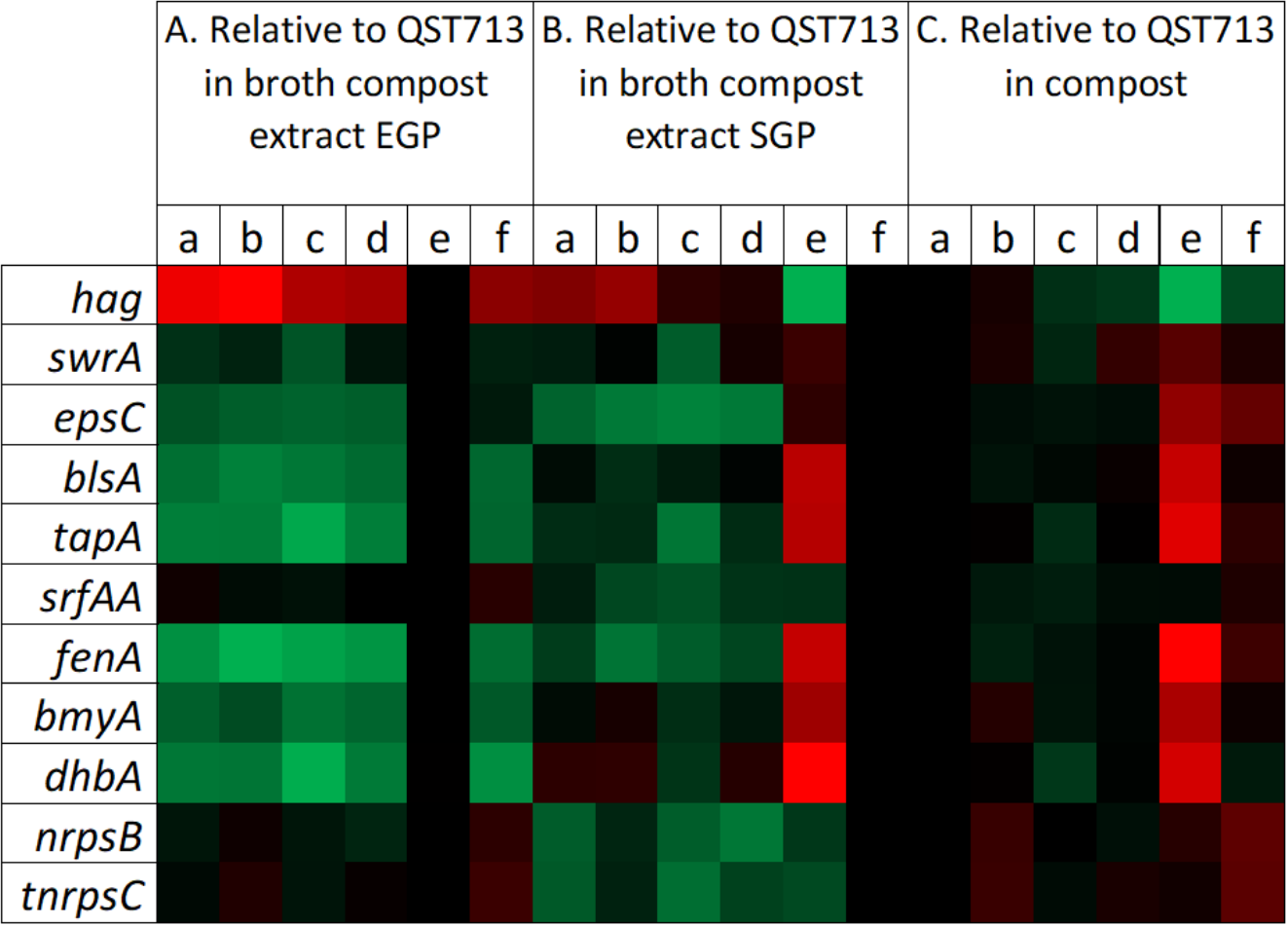
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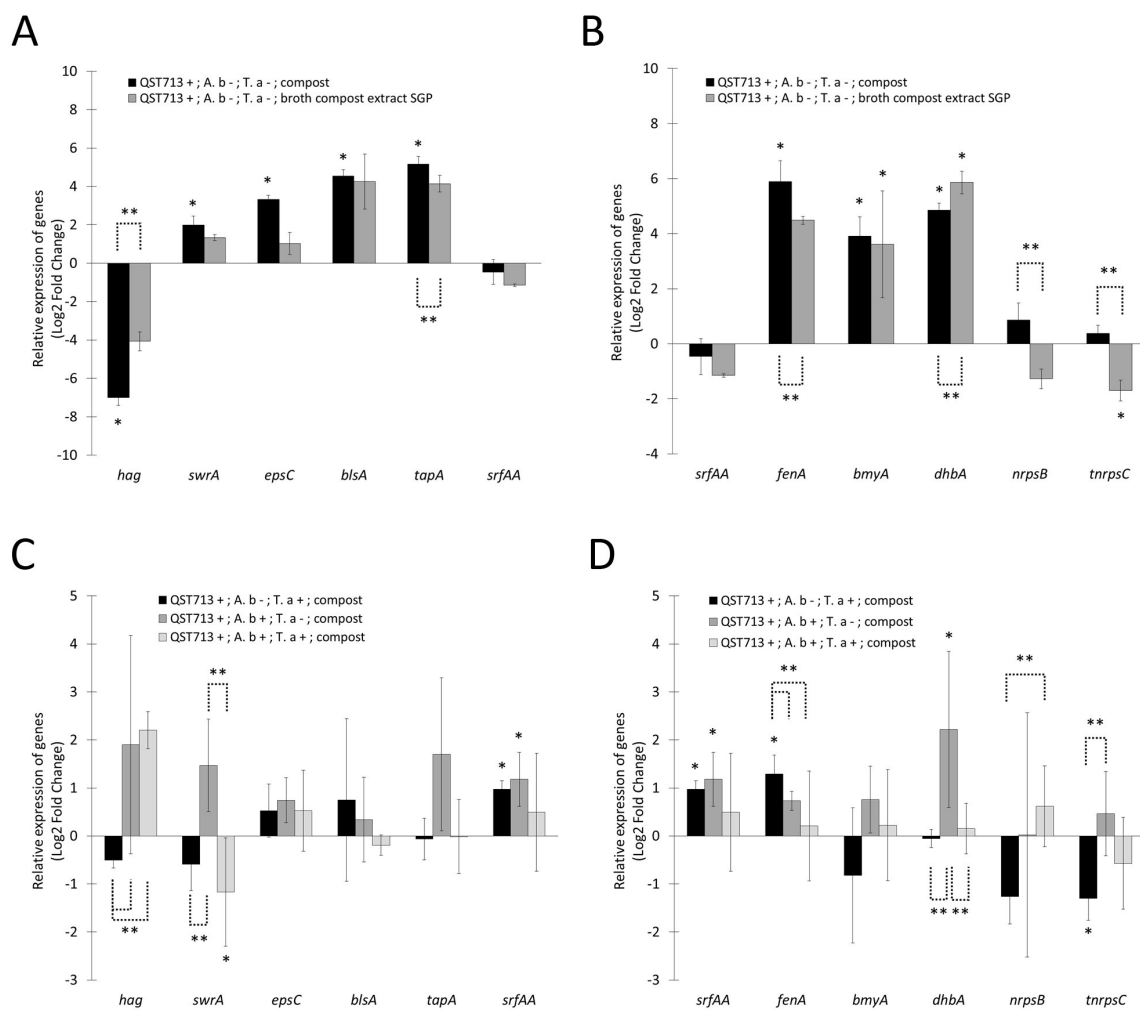
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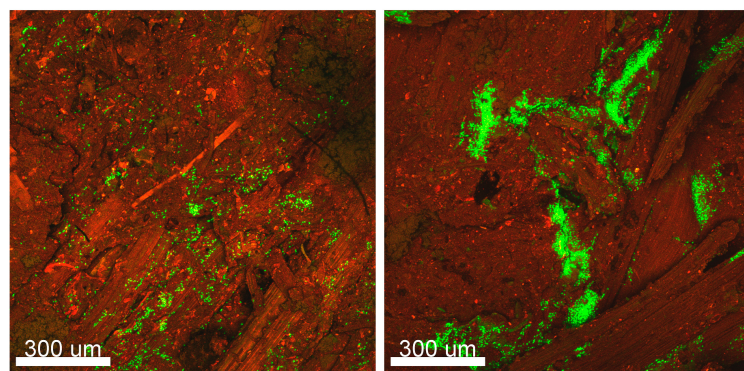


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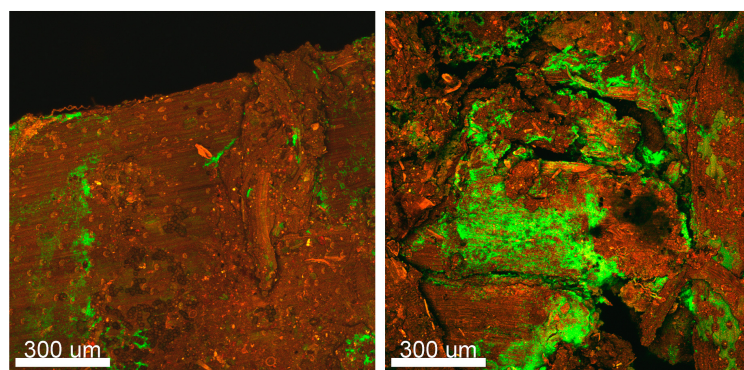
A

QST713



B

FZB42



SQR9

