The stress-responsive alternative sigma factor SigB plays a positive role in the antifungal proficiency of *Bacillus subtilis*

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Abstract

Different *Bacillus* species with PGPR (plant growth-promoting rhizobacterium) activity produce potent biofungicides and stimulate plant defense responses against phytopathogenic fungi. However, very little is known about how these PGPRs recognize phytopathogens and exhibit the antifungal response. Here, we report the antagonistic interaction between *B. subtilis* and the phytopathogenic fungus *Fusarium verticillioides*. We demonstrate that this bacterial-fungal interaction triggers the induction of the SigB transcription factor, the master regulator of *B. subtilis* stress adaptation. Dual-growth experiments performed with live or dead mycelia or culture supernatants of *F. verticillioides* showed that SigB was activated and required for the biocontrol of fungal growth. Mutations in the different regulatory pathways of SigB activation in the isogenic background revealed that only the energy-related RsbP-dependent arm of SigB activation was responsible for the specific fungal detection and triggering the antagonistic response. The activation of SigB increased the expression of the operon responsible for the production of the antimicrobial cyclic lipopeptide surfactin (the *srfA* operon). SigB-deficient *B. subtilis* cultures produced decreased amounts of surfactin, and *B. subtilis* cultures defective in surfactin production (Δ*srfA*) were unable to control the growth of *F. verticillioides*. *In vivo* experiments of seed germination efficiency and early plant-growth inhibition in the presence of *F. verticillioides* confirmed the physiological importance of SigB activity for plant bioprotection.

Importance

Biological control using beneficial bacteria (PGPRs) represents an attractive and environment-friendly alternative approach to pesticides for controlling plant diseases.

Different PGPR *Bacillus* species produce potent biofungicides and stimulate plant defense responses against phytopathogenic fungi. However, very little is known about how PGPRs recognize phytopathogens and process the antifungal response. Here, we report how *B. subtilis* triggers the induction of the stress-responsive sigma B transcription factor and the synthesis of the lipopeptide surfactin to fight the phytopathogen. Our findings show the participation of the stress-responsive regulon of a PGPR *Bacillus* in the detection and biocontrol of a phytopathogenic fungus of agronomic impact.

Introduction

In nature, microbes continuously interact with each other, while axenic or pure culture conditions occur only under laboratory conditions (1, 2). These microbial interactions can be synergistic, neutral or antagonistic and might positively or negatively affect the colonization of a given niche or host by a specific microbe (2-4). In particular, an antagonistic interaction between bacteria and fungi would be beneficial to protect plants of agronomical importance against phytopathogenic fungi that reduces the yield and quality of crops (5-7). One strategy to achieve this goal is to control soil-borne plant diseases via the utilization of plant growth-promoting rhizobacteria (PGPRs), which have the ability to maintain the population of plant pathogenic microbes below the disease threshold level in soil and plant tissues (8-12). A large number of biocontrol agents have been identified, but to date, *Bacillus* species, in particular those that belong to the *B. amyloliquefaciens*/*B. subtilis* group, are considered the most efficient biocontrol bacteria because these bacilli have the ability to produce long-lasting and robust biofilms that colonize and protect plant surfaces (i.e., the rhizosphere) and to produce spores that can survive in adverse environments (13). In addition to biofilm

formation and sporulation proficiency, most PGPR *Bacillus* species produce cyclic lipopeptide molecules, mainly those of the iturin, surfactin and fengycin families, which possess strong antifungal activity and the ability to induce systemic plant resistance against pathogens (8, 11, 12, 14).

B. subtilis is recognized as PGPR for its ability to promote plant growth and provide protection against pests but also as an important model organism to investigate complex regulatory pathways and bacterial behaviors (15-20). We hypothesize that the antagonistic interaction with fungi might represent a stressful situation for *B. subtilis* (because, for example, of the mutual fungus-bacterium competition for nutrient availability and sites to settle and due to the microbicidal metabolites produced by each microbe against the other) that might be genetically controlled. In *B. subtilis* and other bacilli, the genetic regulatory network that responds to danger (i.e., stress) is under the control of the alternative sigma factor of the RNA polymerase SigB (21). This transcription factor controls the general stress regulon, which involves more than 200 genes (~5% of the genome) whose products confer resistance to multiple forms of stress in the bacterium (22, 23). The activation of SigB is under the control of the partner-switching RsbV-RsbW-SigB module (22, 23). Under nonstress conditions, SigB is held inactive in a complex with the anti-sigma factor/kinase RsbW, and the third partner, the anti-anti-sigma factor RsbV, is inactive because of phosphorylation by RsbW (24-29). Under stress conditions, the release of SigB from the inactive SigB::RsbW complex is achieved by the dephosphorylated form of the anti-anti-sigma factor RsbV. In *B. subtilis*, activation (dephosphorylation) of RsbV, and therefore SigB activation, is achieved by alternative phosphatases that sense energy-related or environmental stress (the RsbP or RsbU phosphatase, respectively) (24-29). In addition, SigB is also activated by low temperature (30, 31) independently of RsbP, RsbU and RsbV activities

(30). However, to the best of our knowledge, the participation of SigB in the adaptive response of *B. subtilis* and its closest relatives (i.e., *B. thuringiensis*, *B. amyloliquefaciens*) to the presence of harmful organisms (for example, fungi) has not been previously explored (22, 23). In particular, *Fusarium verticillioides* is a phytopathogenic fungus of economic importance because it is the most commonly reported fungal species that infects maize (32), in addition to causing stalk rot disease in sorghum (33) and Pokkah Boeng disease in sugarcane (34). The diseases caused by *Fusarium* spp. are difficult to control with existing fungicides, and many transgenic plants lack resistance to these diseases (35). In this work, we report that *B. subtilis* recognizes and responds to the presence of the phytopathogenic fungus *F. verticillioides* via the induction of SigB and increases production of the lipopeptide surfactin. We discuss the significance of these findings for the fitness of the bacterium and the enhancement of *Bacillus*-mediated plant protection.

Results and discussion

SigB is induced during the antagonistic interaction of *B. subtilis* with *F. verticillioides*. As shown in Fig. 1A, *B. subtilis* was able to repress the growth of *F. verticillioides*, and SigB was activated as suggested by the development of the blue color (because of the SigB-dependent *ctc-lacZ* activity) inside the *B. subtilis* colony (13, 16, 18) after 96 h of co-incubation with the fungus. Interestingly, as observed in the figure, the activation of SigB was not uniform, increasing with the proximity of *B. subtilis* to the fungus. The *B. subtilis* cells situated in the part of the bacterial colony closest to the fungus and those furthest from the fungus (left and right rectangles in the figure, respectively) were scraped, and the SigB-dependent specific β-galactosidase activity was determined (see Experimental Procedures for details). The specific β-

galactosidase activities measured were 1,255 ± 25 and 234 ± 12 Miller units (M.U.) for the *B. subtilis* cells located closest or furthest from the fungus, respectively. Accordingly, when the *B. subtilis* colony was completely surrounded by the fungus, the induction of sigma B occurred uniformly throughout the boundary of the colony, and average values of 1,050 ± 18 M.U. were measured in the *B. subtilis* cells scraped after 96 h of co-incubation from any of the two area indicated in Fig. 1B. However, when *B. subtilis* developed in the absence of the fungus (Fig. 1C), sigma B induction occurred later (after 144 h of incubation) and only in the center of the bacterial colony because of the metabolic stress generated by the insufficient availability of nutrients in that inner part of the biofilm (36, 37).

The results presented in Fig. 1A-C suggested that the antagonistic interaction of *B. subtilis* with *F. verticillioides* would represent a stressful (or threatening) condition that could be sensed by the bacterium which induced SigB activity as a response. In order to test this hypothesis, we separately cultured the wild-type strain NCIB3610 and its isogenic Δ*sigB* derivative, deficient in SigB activity (strain DG599, Table 1), in LB broth (see Experimental Procedures) with shaking at 28 ºC until the middle logarithmic phase of growth. At that time, we divided each bacterial culture into two flasks and added to one of them live mycelia from a *F. verticillioides* culture grown for 24 h (see Experimental Procedures for details). As shown in Fig. 1D, there were no significant differences between the kinetic of growth and the final cellular yields (UFC ml^{-1}) of the SigB-proficient and SigB-deficient *B. subtilis* cultures grown in the absence of the fungus. However, confirming the hypothesized antagonistic interaction between the bacterium and the fungus, there was an appreciable decrease of the rate of growth and final cellular yield of the SigB-deficient *B. subtilis* strain co-cultured with the fungus compared to the bacterial yield reached in co-culture of the SigB-proficient *B. subtilis*

141 strain with the fungus (Fig. 1D). The overall results (Fig. 1A-D) suggest that *B. subtilis*
142 senses the presence of *F. verticillioides* as a hostile situation and induces SigB as an
143 adaptive response. The other adaptive pathway that *B. subtilis* might employ to protect
144 itself from the fungal presence is the onset of sporulation (38). However, sporulation is
145 largely prevented in nutrient-enriched media because it is subject to nitrogen and carbon
146 catabolite control (38). Under our experimental growth conditions in rich media (i.e.,
147 LB), the sporulation frequency of the wild-type and Δ*sigB B. subtilis* strains after 96 h
148 of growth was approximately 0.15 % (9.0×10^5 spores in 6.0×10^8 viable cells per ml),
149 a sporulation value that was not significantly affected by the presence of *F.*
150 *verticillioides* (Fig. 1E). Therefore, the sporulation pathway does not significantly
151 influence the *B. subtilis* fungal adaptation under our experimental conditions.
152 To obtain new insights into the kinetics of the induction of SigB in response to the
153 presence of the fungus, we exposed sub-cultures of the DG555 strain to different
154 amounts of live mycelia from a *F. verticillioides* culture grown for 24 h (see
155 Experimental procedures for details). As shown in Fig. 2A, 30 minutes after the fungal
156 addition, there was a rapid and dose-dependent induction of SigB in response to the
157 presence of different amounts of fungal mycelia. One hour after the peak of fungal-
158 induced β-galactosidase activity, the SigB activity decreased and became stable but
159 significantly higher than the SigB-directed β-galactosidase activity of the untreated
160 culture even after prolonged incubation (two hours and beyond in Fig. 2A).
161 To obtain more information regarding the novel fungus-directed SigB induction, we
162 performed two modified versions of the experiment described in Fig. 2A. In one case,
163 the mycelia of the *F. verticillioides* culture grown for 24 h were heat-killed (i.e.,
164 autoclaved) before being added to the SigB reporter strain DG555 (Fig. 2B). In the
165 other type of experiment, we used the culture supernatant of a 24-h culture of the fungus

(Fig. 2C). In both cases (Fig. 2B-C), SigB was activated, and the induction of SigB was once again rapid and dose-dependent. The values for β-galactosidase activity obtained in these experiments (Fig. 2B-C) were slightly lower than those obtained after the addition of live mycelia (Fig. 2A). This observation suggested that the fungal metabolite(s) responsible for the activation of SigB was not completely heat resistant and/or was intrinsically unstable, and therefore, the metabolite must be continuously replenished by the fungus to obtain maximal SigB induction. Regardless, the overall results (Fig. 2) were consistent with the results shown in Fig. 1 and indicated that physical contact of the fungus with the bacterium was not necessary to induce SigB.

***F. verticillioides* activates the RsbP-dependent pathway of the SigB-controlling cascade.** What is the SigB activation pathway (Fig. 3A) that controls the activation of the alternative sigma factor when the bacterium is confronted with the fungus? To answer this question, we monitored the expression of the SigB-dependent *ctc-lacZ* reporter fusion in isogenic *B. subtilis* strains whose SigB activation pathways had been altered (i.e., the DG556-Δ*rsbU*, DG557-Δ*rsbP* and DG558-Δ*rsbUP* strains; see Table 1 and Introduction for details). For the Δ*rsbU* strain, the SigB reporter fusion (*ctc-lacZ*) was induced in the presence of mycelia from *F. verticillioides* (Fig. 3B), and the level and kinetics of β-galactosidase expression were very similar to those exhibited by wild-type DG555 cells exposed to the fungus (Fig. 2A). Interestingly, the *B. subtilis* mutant strains Δ*rsbP* (DG557) and Δ*rsbUP* (DG558) were unable to induce SigB in response to the presence of the fungus (Fig. 3C-D). These results strongly suggested that during the antagonistic interaction between *B. subtilis* and *F. verticillioides*, stress sensed by the RsbP pathway represented a unique or primary input responsible for the activation of SigB when *B. subtilis* is confronted with the fungus. The involvement of the RsbP-dependent pathway (Fig. 3A), and the previously described results (Fig. 2), suggested

that the fungus might produce one or more metabolites that interfere with the ability of *B. subtilis* to produce and/or utilize energy. Notably, many *Fusarium* species, including *F. verticillioides*, produce the mycotoxin fusaric acid, which in addition to its ability to modulate the production of antifungal bacterial metabolites (39-41), it has also been reported that, in treated plants, this mycotoxin blocked mitochondrial respiration, decreased the ATP concentration and destroyed membrane integrity (42). However, our preliminary results indicated that the addition of fusaric acid to *B. subtilis* does not induce SigB (data not shown).

Up to this point, all the experiments have been performed at 28 ºC, a growth temperature that not only favors fungal growth but also activates the RsbUP-independent low-temperature pathway of SigB activation (Fig. 3A) (30, 31). Therefore, the incubation temperature at which the former experiments were performed (28 ºC) indicated the possibility that the low-temperature pathway of SigB activation would also be involved in the specific fungal detection (30, 31). However, as shown in Fig. 3E, experiments similar to those described in Fig. 3B but performed at 37 ºC (a growth temperature that inactivated the low-temperature-dependent pathway of SigB activation), and other incubation temperatures higher than 37 ºC (Fig. 3F), in wild-type and *rsbP* minus backgrounds confirmed that SigB was specifically induced by the presence of the fungus independently of the incubation temperature.

SigB is required for the proficiency of *B. subtilis* to control *F. verticillioides* growth.

B. subtilis is known for its ability to inhibit fungal growth (8, 14), and SigB is induced when the fungus *F. verticillioides* or its metabolites are present (Figs. 1-3). Therefore, we wondered whether SigB plays a role in fungal biocontrol. To answer this question, we monitored the growth of *F. verticillioides* in dual-growth experiments where the fungus was grown in the presence of the wild-type strain NCIB3610 or in the presence

of the isogenic strain DG559, which lacked SigB activity (Δ*sigB* strain, Table 1). As shown in Fig. 4A, the wild-type *B. subtilis* strain NCIB3610 was able to control the growth of the fungus even after more than one week of co-incubation, and showed a fungal growth inhibition index of 62.5% (see experimental Procedures) (40). In the case of the DG559 strain, which lacked SigB activity (Δ*sigB*), the inhibition of the mycelial growth of *F. verticillioides* was weaker (the fungal growth inhibition index was 17.5%) than that observed with NCIB3610 (Fig. 4B). Furthermore, the culture supernatants of *B. subtilis* cultures deficient in SigB activity were less efficient at controlling the growth of the fungus than the culture supernatants of *B. subtilis* cultures proficient in SigB activity (35 % and 75 % of fungal growth inhibition, respectively) (Fig. 4C). These results showed that SigB plays a previously unidentified and important, although not essential, role in the biocontrol of *F. verticillioides* growth. To confirm this conclusion, we quantified the fungal growth ($CFUml^{-1}$) in dual-cultures of *F. verticillioides* developed in LB broth in the presence of the wild-type strain NCIB3610 or the isogenic Δ*sigB* derivative, with shaking at 28 ºC during 5 days. As shown in Fig. 4D, the fungal yield was significantly decreased in the co-culture of *F. verticillioides* with the wild-type (SigB-proficient) *B. subtilis* strain (4×10^5 $CFUml^{-1}$) compared to the final cellular yield reached by the fungus developed in absence of bacteria (3×10^8 $CFUml^{-1}$). As expected from the results showed in Fig. 4B-C, the fungal yield obtained in co-culture with the SigB-deficient *B. subtilis* strain was intermediate (5×10^7 $CFUml^{-1}$) between the cellular yield reached by the fungus grown in the absence or presence of the wild-type *B. subtilis* strain (Fig. 4D).

***F. verticillioides* induces surfactin production via activation of SigB.** *B. subtilis* and its closest relatives (i.e., *B. amyloliquefaciens*) are recognized to constitute powerful biological weapons against phytopathogenic fungi because of their proficiency in

241 synthesizing natural compounds with strong antifungal activity (i.e., the cyclic
242 lipopeptides iturin, fengycin and surfactin) (8, 43-45). The NCIB3610 strain does not
243 produce iturins but harbors the operons for surfactin and plipastatin (a member of the
244 fengycin family) production, i.e., the *srfA* and *ppsB* operons, respectively (46).
245 However, it has been reported that the promoter of the plipastatin operon in the
246 NCIB3610 isolate and its isogenic derivatives (i.e., strain 168) is weak; therefore,
247 surfactin would be the only lipopeptide produced in significant amounts by this wild
248 isolate under laboratory conditions (47, 48). Therefore, we investigated whether the
249 ability of *B. subtilis* NCIB3610 to control the growth of *F. verticillioides* depended on
250 the proficiency of this strain in producing surfactin. To this end, we constructed an
251 NCIB3610 isogenic strain deficient in surfactin production (Δ*srfA*, DG560 strain, Table
252 1). Interestingly, the surfactin-deficient strain DG560 completely lost the ability to
253 control the mycelial growth of *F. verticillioides* (Fig. 5A-B, 0 % of fungal growth
254 inhibition index). Accordingly, the fungal yield in co-culture with the surfactin-deficient
255 (Δ*srfA*) *B. subtilis* strain was unaffected and indistinguishable of the final cellular yield
256 reached by the fungus developed in the absence of bacteria (Fig. 5C).
257 Because the biocontrol ability of SigB-deficient *B. subtilis* DG559 was partial (Fig. 4)
258 and surfactin synthesis was essential for the control of fungal growth (Fig. 5), we
259 analyzed whether this defective biocontrol phenotype of the SigB-deficient strain was
260 due to decreased surfactin production. As shown in Fig. 6A, the expression of a reporter
261 *lacZ* fused to the promoter of the surfactin operon (*srf-lacZ*) was lower in the strain
262 deficient in SigB activity than in the wild-type strain proficient in SigB activity (strains
263 DG562 and DG561, respectively). Therefore, SigB plays a previously unknown, direct
264 or indirect, positive, although nonessential, role in surfactin production. Interestingly,
265 the β-galactosidase activity driven by *srf-lacZ* in wild-type and Δ*sigB* cultures

confronted with *F. verticillioides* (Fig. 6B and 6C, respectively) indicated that SigB was essential for the augmentation of surfactin production in the presence of the fungus. Accordingly, the RsbP-dependent pathway of SigB activation, but not the RsbU-dependent pathway, was required for the increase in surfactin production triggered by the presence of the fungus (Fig. 6D and 6E, respectively). The overall results indicate that *F. verticillioides* induces the energy-related RsbP-dependent activation pathway of SigB (see Conclusions below), which in turn increases the expression of the operon that controls surfactin synthesis.

To evaluate the *in vivo* importance of SigB to protect plants against infections by *F. verticillioides*, we used Zea mays (i.e., maize) as a model plant for the infective assays. Untreated and bacilli-treated maize seeds were sowed in vermiculite infected with *F. verticillioides*, and the germination efficiency and early plant development was monitored (see Experimental Procedures for details). The average inhibition of germination rate produced by *F. verticillioides* was 70 % and 15 % for untreated and treated seeds with the wild-type strain NCIB3610 (Fig. 7A). The proficiency of *B. subtilis* to protect the seeds from the fungal attack significantly decreased in seeds treated with the SigB-deficient (Δ*sigB*) *B. subtilis* strain (58 % of germination inhibition compared to germination of uninfected Δ*sigB*-treated seeds) and was null in the case of seeds treated with the surfactin defective (Δ*srfA*) strain (90 % of germination inhibition compared with the germination of uninfected Δ*srfA*-treated seeds) (Fig. 7A). Consequently, the in vivo PGPR activity of wild-type, Δ*sigB,* and Δ*srfA B. subtilis* cells, evaluated by the average root length of emerged plants infected by *F. verticillioides*, confirmed the important and essential roles of SigB and surfactin, respectively, for the biocontrol proficiency of the bacterium against phytopathogens of agronomical importance (Fig. 7B).

291

292 **Conclusions**

293 From the present results, we demonstrated that the interaction of *B. subtilis* with fungi
294 induced SigB and its stress-responsive regulon (Figs. 1-2). The activation of SigB was
295 required for biocontrol when *B. subtilis* is co-cultured with *F. verticillioides* (Fig. 4).
296 The activation of SigB depended on the functionality of the RsbP route (Fig. 3) that
297 senses energy depletion in planktonic pure-cultures of *B. subtilis* (22, 23). Therefore, it
298 could be expected that *F. verticillioides* is able to interfere with energy production in *B.*
299 *subtilis*. Another possibility for the nature of the fungus-mediated signal working on
300 SigB activation, that cannot be excluded, is that RsbP senses a novel and so far
301 unknown signal, nonrelated to energy depletion, that might be exclusively present when
302 *B. subtilis* is co-cultivated with other organisms (i.e., fungi) and absent (or weaker) in
303 axenic *B. subtilis* cultures. Unfortunately, all studies on regulation of SigB activity have
304 so far been uniquely confined to the planktonic and axenic growth style of *B. subtilis*
305 but not related to its life style with other organisms (22, 23, 49).

306 The biocontrol of the growth of *F. verticillioides* by *B. subtilis* depended in the
307 proficiency of surfactin production (Fig. 5), and SigB upregulated this synthesis (Fig.
308 6). Accordingly, with the *in vitro* results, wild-type (surfactin- and SigB-proficient) *B.*
309 *subtilis* completely protected a model plant (maize) from the fungal attack (Fig. 7A-B).
310 How SigB regulates *srfA* expression is an unsolved question. The simplest scenario for
311 the influence of SigB on *srfA* expression would be direct binding of SigB to the *srfA*
312 promoter. The expression of the *srfA* operon is known to be regulated by ComPA two
313 component regulatory system and the RapC phosphatase (50), but to the best of our
314 knowledge, SigB does not regulate ComPA or RapC (22, 23). Moreover, even the most
315 comprehensive characterization of the transcriptional landscape of *B. subtilis* performed

to date (49) did not reveal a SigB-dependent promoter directly upstream of *srfA*. Therefore, it is likely that SigB activates *srfA* expression indirectly via an unidentified pathway.

Pesticides have been extensively used to combat plant diseases (51). However, the increased use of pesticides has had negative impacts on human health and the environment (52-53). Therefore, many efforts have been directed at improving biological control, and biocontrol using PGPRs represents an attractive and environment-friendly alternative approach for controlling plant diseases (54). The main strategies that PGPRs use to control phytopathogenic fungi are the production of natural antifungal compounds (45) and/or the induction of plant systemic resistance (55). *Bacillus* species are able to use both strategies (8, 14, 45, 56). *Bacillus* species can produce three types of antifungal lipopeptides: iturins, fengycins and surfactins (8). In addition, surfactins are very important molecules for the proficiency of bacilli at establishing robust and persistent beneficial biofilms in the plant rhizosphere (Fig. 7C), and to work synergistically with fengycins and bacillomycins (a member of the iturin family) against fungi (57-59). Additionally, surfactins, but not iturins or fengycins, are able to induce the plant systemic resistance (ISR) against pathogens (Fig. 7C) (55). As a specific mode of positive feedback from the plant to the bacterium, it has been reported that plant polysaccharides stimulate *B. subtilis* biofilm formation (60) and induce the synthesis of surfactins by *Bacillus* spp. (61). Therefore, the selection and use of a *Bacillus* strain or a cocktail of bacilli that overexpress SigB and produce important amounts of surfactins would be of interest for environmental applications. (Fig. 7C).

Experimental Procedures

Bacterial strains, media and general conditions. The different NCIB3610 isogenic *B. subtilis* strains used in this work (Table 1) were streaked on Luria Bertani (LB) agar plates and cultured at 37 °C for 14 h. A single colony was transferred to 30 ml of SM broth (Difco, USA) and grown on a reciprocating shaker (150 rpm) at 37 °C for 36 h to obtain a high titer of mature spores. This 36-h-old culture was heat-treated at 80 °C for 15 minutes to remove nonsporulated cells, diluted to a concentration of 1×10^7 colony-forming units (CFU) ml^{-1} and stored at -20 °C until use. The fungus *F. verticillioides* (strain HFV1904ccc143-2005) was obtained from CEREMIC (Centro de Referencia en Micología, Universidad Nacional de Rosario) and maintained on potato dextrose agar (PDA) plates. Bacterial and fungal quantification ($CFUml^{-1}$) was performed on LB or PDA plates incubated at 37 °C or 28 °C, respectively, for 48 h. For sporulation efficiency, cells were grown in LB or SM broth with shaking (150 rpm) during 96 h at 28 ºC or 36 h at 37 ºC, and then diluted and plated on LB agar plates before and after heat treatment at 80 ºC for 15 min to quantify the number of viable cells and spores expressed as $CFUml^{-1}$, respectively (18, 31).

When appropriate, antibiotics were included at the following final concentrations: 1 μg/ml erythromycin (Ery), 5 μg/ml kanamycin (Kan), 5 μg/ml chloramphenicol (Cm), and 50 μg/ml spectinomycin (Spc). Transformation of *B. subtilis* to obtain isogenic derivatives of the parental strains was carried out as previously described (18, 31). The specific β-galactosidase activity is expressed in Miller Units (M.U.) and was calculated as previously reported (18, 31). The cultures used to measure β-galactosidase activity were grown in LB or PDA media at the indicated temperatures.

***In vitro* antifungal activity.** The *B. subtilis* strain NCIB3610 and its isogenic derivatives were subjected to an *in vitro* antifungal activity assay against mycelia of *F. verticillioides*. PDA medium was used as the basal medium and PDA plate

supplemented with 60 µg/ml of X-gal (as an indicator of β-galactosidase) was used as indicated. A plug (0.3 cm in diameter) containing mycelia of the phytopathogen *F. verticillioides* was taken from an 8-day-old fungus developed on PDA and placed at the center of a fresh 100-mm PDA Petri dish. A single 3-µl inoculum containing 3×10^7 spores of NCIB3610 (or its isogenic strains) was placed 1 cm away from the edge of the mycelium-inoculated PDA or PDA-Xgal plate. The PDA plates were incubated at 28 °C, and the evolution of fungal growth was monitored daily for a 2-week period. The fungal growth inhibition index was calculated from measurements of fungal radial growth toward (X_1) versus perpendicular to (X_2) the bacterial colony according to the formula [1 - (X_1/X_2)] x100 (40). The co-inoculated PDA-Xgal was incubated at 28 °C for 96 h. The bacteria to obtain culture supernatants were grown at 28 °C for 18 h before filtering with 0.22 µm sterile filters.

Co-culture of bacteria and fungi. The co-culture of *B. subtilis* and *F. verticillioides* was initiated by mixing 1×10^5 CFU of the corresponding *B. subtilis* strain and 5×10^5 CFU of *F. verticillioides* per ml of LB broth. The co-cultivation was prolonged during the indicated times with shaking (150 rpm) at 28 ºC. Aliquots of the co-culture were taken at the indicated times, diluted, plated on LB or PDA, and incubated for 36 h at 37 ºC or 28 ºC for bacterial and fungal cellular yield determination ($CFUml^{-1}$), respectively. For the determination of the β–galactosidase activity in co-cultures of bacteria and fungi, five hundred milliliters of the indicated *B. subtilis* strain were cultured in LB broth in 2-L Erlenmeyer flasks at 28 °C with shaking (125 rpm) until the midlogarithmic phase of growth ($O.D._{600}$ = 0.5). At this time, the bacterial culture was divided in five 0.5-L Erlenmeyers. Four of them, containing 98.0 ml, 99.0 ml, 99.5 ml, and 99.9 ml of the *B. subtilis* culture, were supplemented with 2 ml, 1 ml, 0.5 ml or 0.1 ml, respectively, of a live or dead (autoclaved) or cell-free supernatant culture of *F.*

verticillioides grown for 24 h at 28 °C in LB broth. The final fungal concentration in each 0.5-L Erlenmeyer was of 2%, 1%, 0.5 %, and 0.1 %, respectively. The fifth 0.5-L Erlenmeyer only contained 100.0 ml of the *B. subtilis* culture (positive control culture). The fungal-inoculated and non-inoculated bacterial cultures were incubated at 28 °C with shaking as shown in the corresponding experiments, and aliquots for the determination of β-galactosidase activity, were taken at the indicated times and processed.

***In vivo* antifungal activity.** Maize (Zea mays) seeds were surface disinfected by dipping in 70 % ethanol (v/v) for 2 minutes followed by 10 mM sodium hypochlorite for 5 minutes, rinse three times with sterile water, and soaked for 10 min in sterile water containing a *B. subtilis* cell suspension at a concentration of 5 x 10^7 CFU ml^{-1}, or sterile water alone. Finally, the treated seeds were dried under a filter-sterilized air flow at room temperature. To quantify the average number of *B. subtilis* cells adhered per seed, fifteen *B.subtilis*-treated seeds were dipped in a Falcon tube of 50-ml of capacity containing 15 ml of sterile water and shaken during 60 min at 75 rpm at room temperature. At this time, without shaking, and after the seeds completely decanted to the bottom of the Falcon tube, one ml of the suspension, containing the eluted bacteria, was used to make serial dilutions before plated on LB agar plates and incubated at 37 ºC during 36 h. The average number of *B. subtilis* cells per seed was of 1 x 10^6 CFU. The bacterial-treated seeds were sown in plastic pots (30-cm and 50-cm of diameter and depth, respectively) containing sterilized vermiculite previously infected with *F. verticillioides* by adding a suspension of mycelial fragments to obtain a final fungal concentration of 5 x 10^4 CFU g^{-1} of vermiculite. For each treatment with the different *B. subtilis* strains, ten seeds were placed in each pot and seven pods were used. The pots were incubated in a room set at 28 °C, 95 % relative humidity and a photoperiod of 16

h. Seedling emergence and root lengths were recorded after 7 days and 14 days of soaring, respectively.

The *in vitro* and *in vivo* antifungal experiments of each treatment were repeated five times, and the statistical analysis to evaluate the effect of the organisms in vitro and on plants was carried out using one-way analysis of variance (ANOVA) ($P < 0.01$).

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Author contributions

B.M., C.S., V.D., C.B., and R.W. carried out the experiments. All authors contributed to the experimental design and concepts, and all authors contributed to the text. G.R. designed the experiments and wrote the main text with contributions from all other authors.

The authors declare no conflict of interest.

Figure legends

Figure 1. Antagonistic response of *B. subtilis* confronted with *F. verticillioides*. (A-B) The co-culture of a *B. subtilis* NCIB3610 isogenic strain harboring a *ctc-lacZ* fusion as a reporter of SigB activity (strain DG555, Table 1) with *F. verticillioides* allowed

observation of the antagonistic fungal-bacterium interaction. The pattern of induction of SigB is evidenced by the development of blue color (derived from expression of the *ctc-lacZ* fusion) inside the bacterial colony. The areas represented by the squares correspond to the colony areas that were used to quantify the level of SigB-directed β-galactosidase activity (see text for details). (C) Pattern of SigB expression when the DG555 strain was developed in the absence of *F. verticillioides*. For A-C, cells were grown on PDA plates supplemented with X-gal (60 µg/ml) for 96 h at 28 ºC. (D-E) Planktonic growth and sporulation proficiency of the wild-type strain NCIB3610 and its isogenic SigB-deficient derivative (Δ*sigB*, strain DG559, Table 1) in the absence and presence of live *F. verticillioides* (see Experimental Procedures for details). Typical results from five independent experiments performed in duplicate are shown for A to E.

Figure 2. *F. verticillioides* induces the stress-responsive SigB regulon. β-Galactosidase activity of LB cultures of the wild-type strain DG555 in response to different amounts of live (A), dead (B) or supernatant (C) of *F. verticillioides*. β-galactosidase values are expressed in M.U. ± SEM and time zero corresponds to the moment that the bacterial cultures reached the middle logarithmic phase of growth ($O.D._{600}$ = 0.5) and fungal addition (see Experimental Procedures for details). A typical output of three independent experiments performed in parallel is shown.

Figure 3. *B. subtilis* recognizes *F. verticillioides* via the energy-dependent pathway of the SigB regulatory cascade. (A) A cartoon summarizing the three known pathways of SigB activation, one of which is likely responsible for sensing the presence of the fungus (see text for details). (B-F) β-Galactosidase activity of NCIB3610 isogenic strains harboring the *ctc-lacZ* fusion in wild-type background (strain DG555) (E, F) or affected in the different pathways of SigB activation: Δ*rsbU* (strain DG556) (B), Δ*rsbP* (strain DG557) (C), and Δ*rsbUP* (strain DG558) (D, F). Each bacterial culture was

grown in LB broth with shaking at 28 °C (B-D), 37 °C (E) or the indicated temperatures (F) until the middle logarithmic phase, at which time (time zero), the culture was divided into two sub-cultures and *F. verticillioides* was added to one of them (final fungal concentration 1 %). The incubation was continued as shown in the figure, and aliquots for the determination of β-galactosidase activity, were taken at the indicated times and processed. For the experiment shown in panel (F), β-galactosidase activity was determined 40 min after time zero. For B-F, a typical output of three independent experiments performed in parallel is shown.

Figure 4. Role of Sig B in the *in vitro* antifungal activity of *B. subtilis*. (A-B) *F. verticillioides* and the wild-type *B. subtilis* strain NCIB3610 and its isogenic Δ*sigB* strain (DG559) (A and B, respectively) were grown on PDA plates at 28 °C as indicated in Experimental Procedures. (C) Four-day growth of *F. verticillioides* inoculated in the middle of a PDA plate without supplementation (left plate) or supplemented with 10% culture supernatant from NCIB3610 (wt) or DG599 (Δ*sigB*) *B. subtilis* cultures. (D) Growth of *F. verticillioides* under axenic conditions or co-cultured with the wild-type strain NCIB3610 or the isogenic SigB-deficient derivative (Δ*sigB*). Cultures were developed in LB broth with shaking at 28 ºC, and fungal quantification ($CFUml^{-1}$ ± SEM) at different times of growth was carried out as described in Experimental Procedures. The results of five independent experiments performed in duplicate are shown for A to D.

Figure 5. Surfactin has an essential role in the *in vitro* antifungal activity of *B. subtilis*. (A-B) Absence of antifungal activity of an NCIB3610 isogenic strain deficient in surfactin production (Δ*srfA*, strain DG560, Table 1). (C) Growth of *F. verticillioides* in the absence or presence of the surfactin-deficient derivative (Δ*srfA*) DG560 in LB broth

with shaking at 28 ºC as described in Experimental Procedures. The results of five representative experiments are shown.

Figure 6. SigB-dependent surfactin production. β-Galactosidase activity of NCIB3610 isogenic strains, proficient and deficient in SigB activity, and harboring a *srf-lacZ::amyE* as a reporter of surfactin production (strains DG561-wt, DG562-Δ*sigB*, DG563-Δ*rsbP*, and DG564-Δ*rsbU*). Each bacterial culture (with or without 1 % fungal addition) was grown in LB broth with shaking at 28 °C and processed as indicated in Experimental Procedures.

Figure 7. Biocontrol proficiency of PGPR *B. subtilis*: *in vivo* roles of SigB and surfactin (A-B) Germination efficiency and plant-growth (root length) (A and B, respectively) of Zea mays infected with *F. verticillioides* in the absence or presence of *B. subtilis* (see Experimental Procedures for details). A typical output of three independent experiments is shown. (C) A cartoon summarizing the beneficial interactions between surfactin-producing PGPR *B. subtilis* cells and plants to resist phytopathogenic fungi. The stimulatory effect of plant polysaccharides on biofilm formation and surfactin synthesis is not indicated for simplicity (see text for details).

510 **Table 1.** Strains used in this work

Strains	Relevant phenotype and/or genotype	Comments and/or source (reference)
F. verticillioides	Wild-type isolate	CEREMIC*
NCIB3610	*B. subtilis* Marburg strain Wild-type isolate, Surfactin (Srf)-proficient and biofilm formation (Bio)-proficient	Laboratory collection (18)
MR101	JH642 *amyE*::*Pctc-lacZ*::*cat*	Laboratory collection (31)
DG555	*amyE*::*Pctc-lacZ*::*cat* Idem to NCIB3610, but also reporter of SigB activity	This work, MR101→NCIB3610**
DG5572	JH642 Δ*rsbU*::*kan*	Laboratory collection (30)
DG5573	JH642 Δ*rsbP*::*spc*	Laboratory collection (30)
DG5574	JH642 Δ*rsbUP*::*kan-spc*	Laboratory collection (30)
DG556	Δ*rsbU*::*kan* Idem to NCIB3610, but deficient in the environmental pathway of SigB activation	This work, DG5572→NCIB3610
DG557	Δ*rsbP*::*spc* Idem to NCIB3610, but deficient in the energy-related pathway of SigB activation	This work, DG5573→NCIB3610
DG558	Δ*rsbUP*::*kan-spc* Idem to NCIB3610, but deficient in the energy-related and environmental pathways of SigB activation	This work, DG556→DG558
MR644	JH642 Δ*sigB*::*neo*	Laboratory collection (31)
DG559	Δ*sigB*::*neo* Idem to NCIB3610, but deficient in SigB activity (strain sensitive to stress)	This work, MR644→NCIB3610

DG560	NCIB3610 Δ*srfAA::ery* Idem to NCIB3610, but deficient in surfactin synthesis (also deficient in biofilm formation)	Laboratory collection (18)
MR760	JH642 *amyE*::P*srf-lacZ*::*cat*	Laboratory collection (18)
DG561	*amyE*::P*srf-lacZ*::*cat* Idem to NCIB3610, but also reporter of surfactin production	This work, MR760→NCIB3610
DG562	Δ*sigB::neo amyE*::P*srf-lacZ*::*cat*	This work, DG559→DG561
DG563	Δr*sbU::kan amyE*::P*srf-lacZ*::*cat*	This work, DG556→DG561
DG564	Δr*sbP::spc amyE*::P*srf-lacZ*::*cat*	This work, DG557→DG561

511 * Centro de Referencia en Micología, Universidad Nacional de Rosario

512 **Donor DNA → receptor strain

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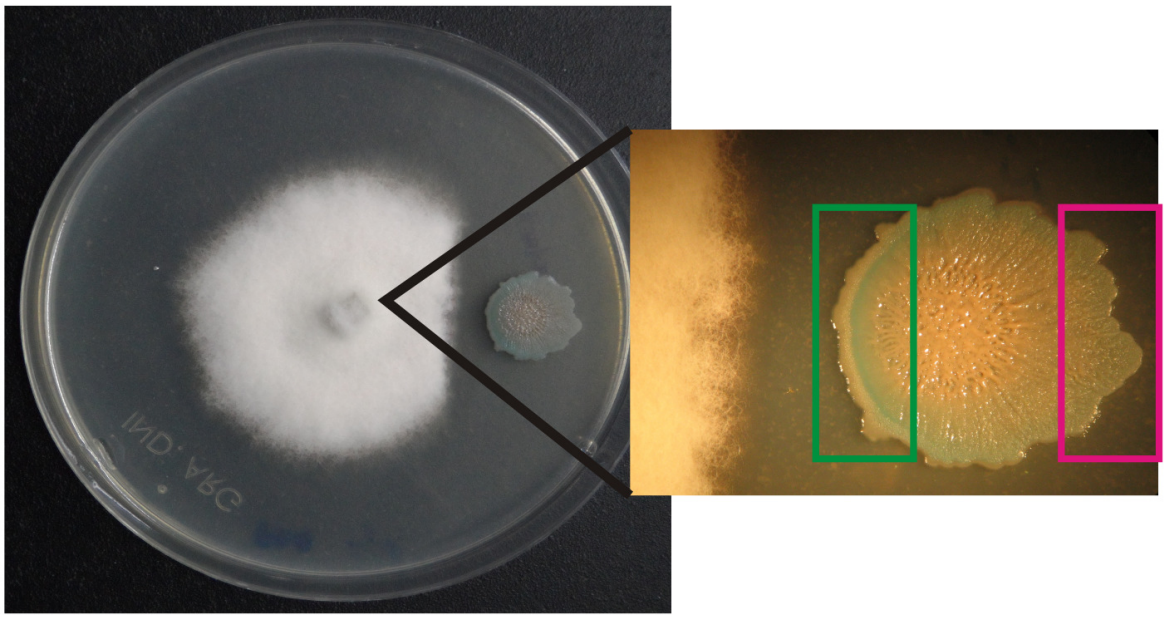

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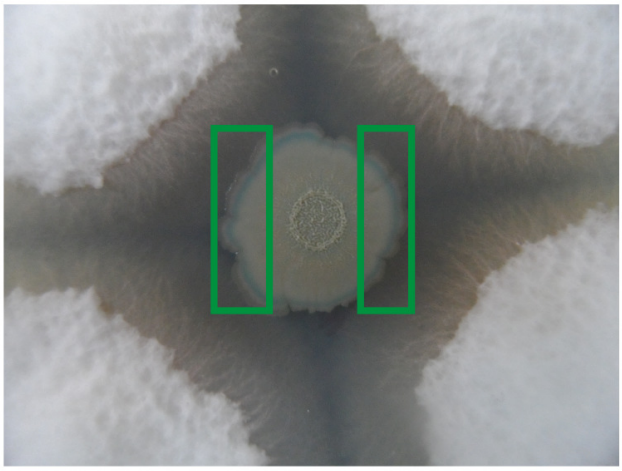

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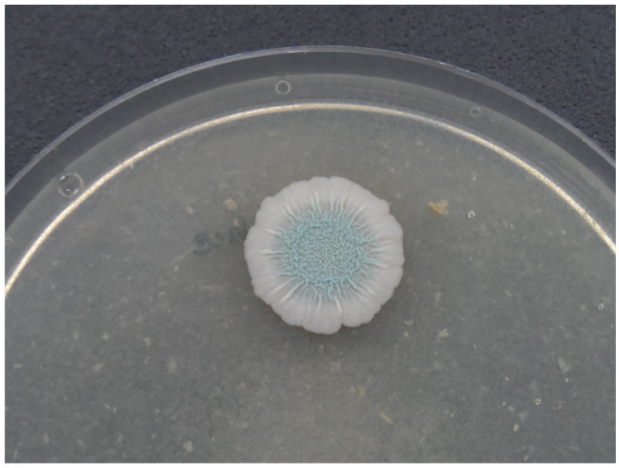
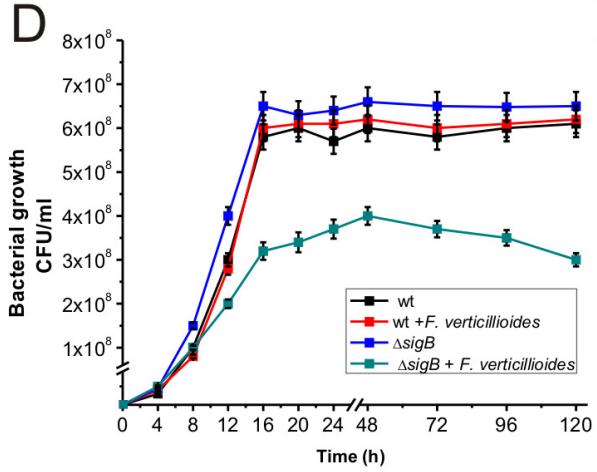
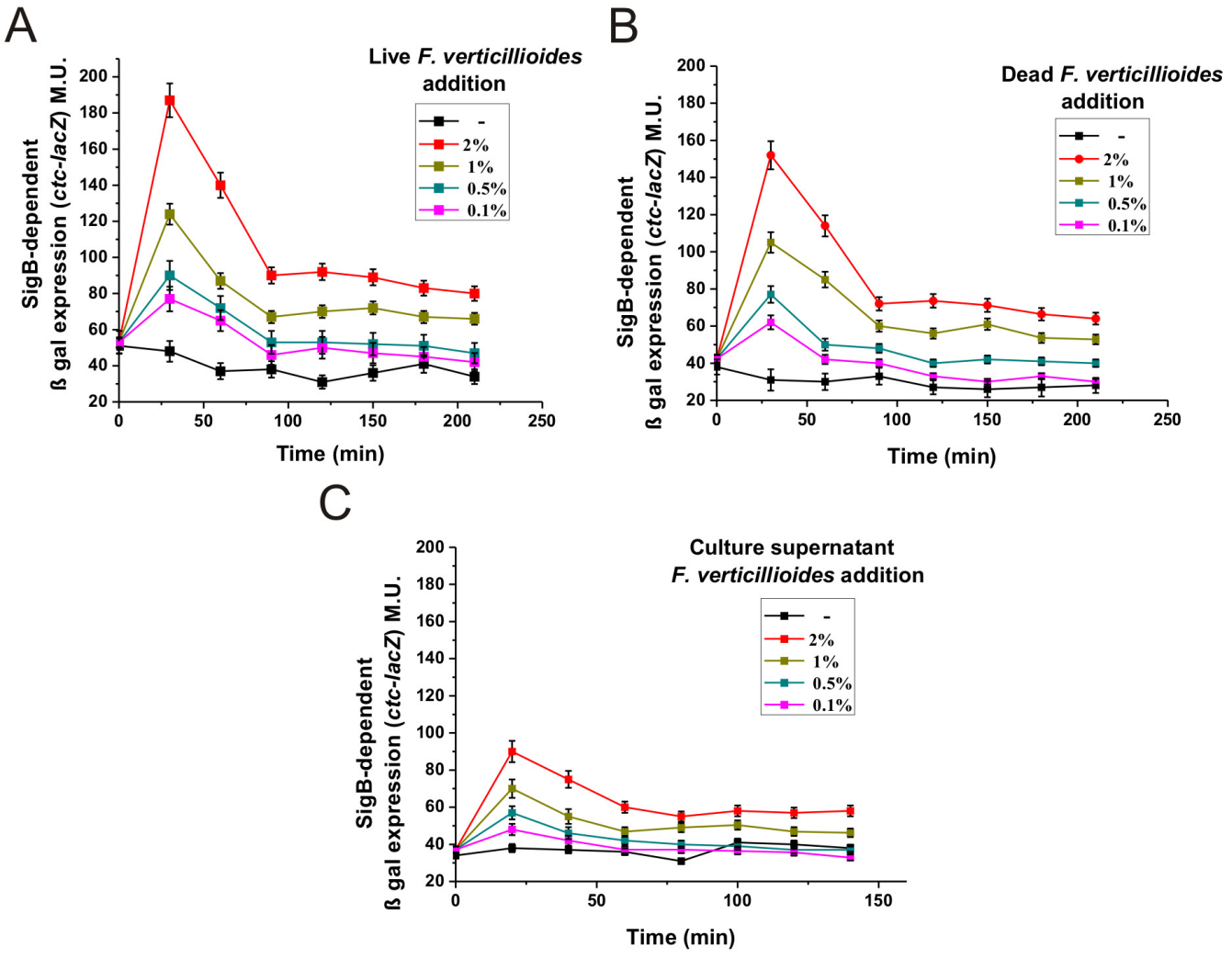


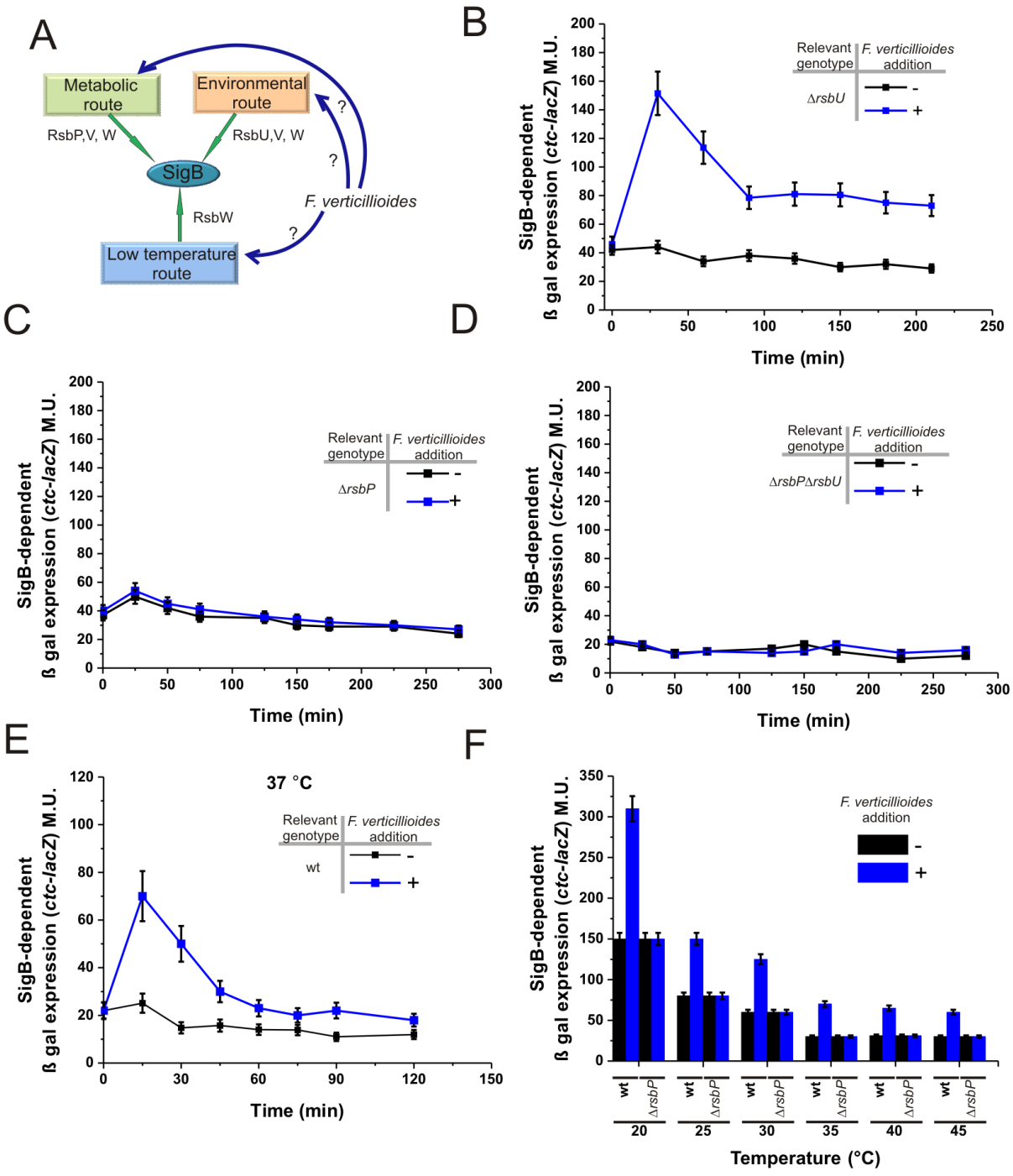
D

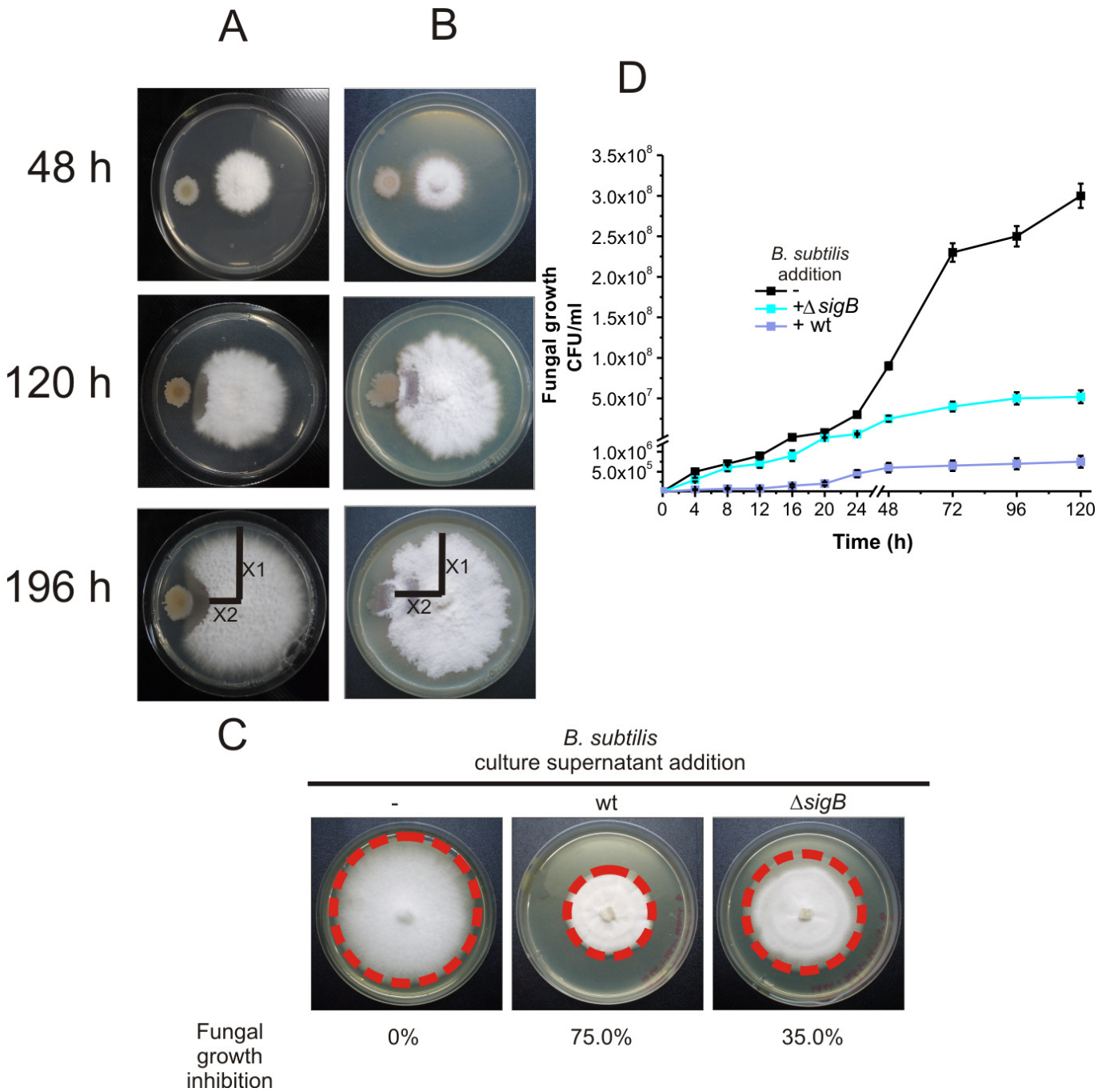


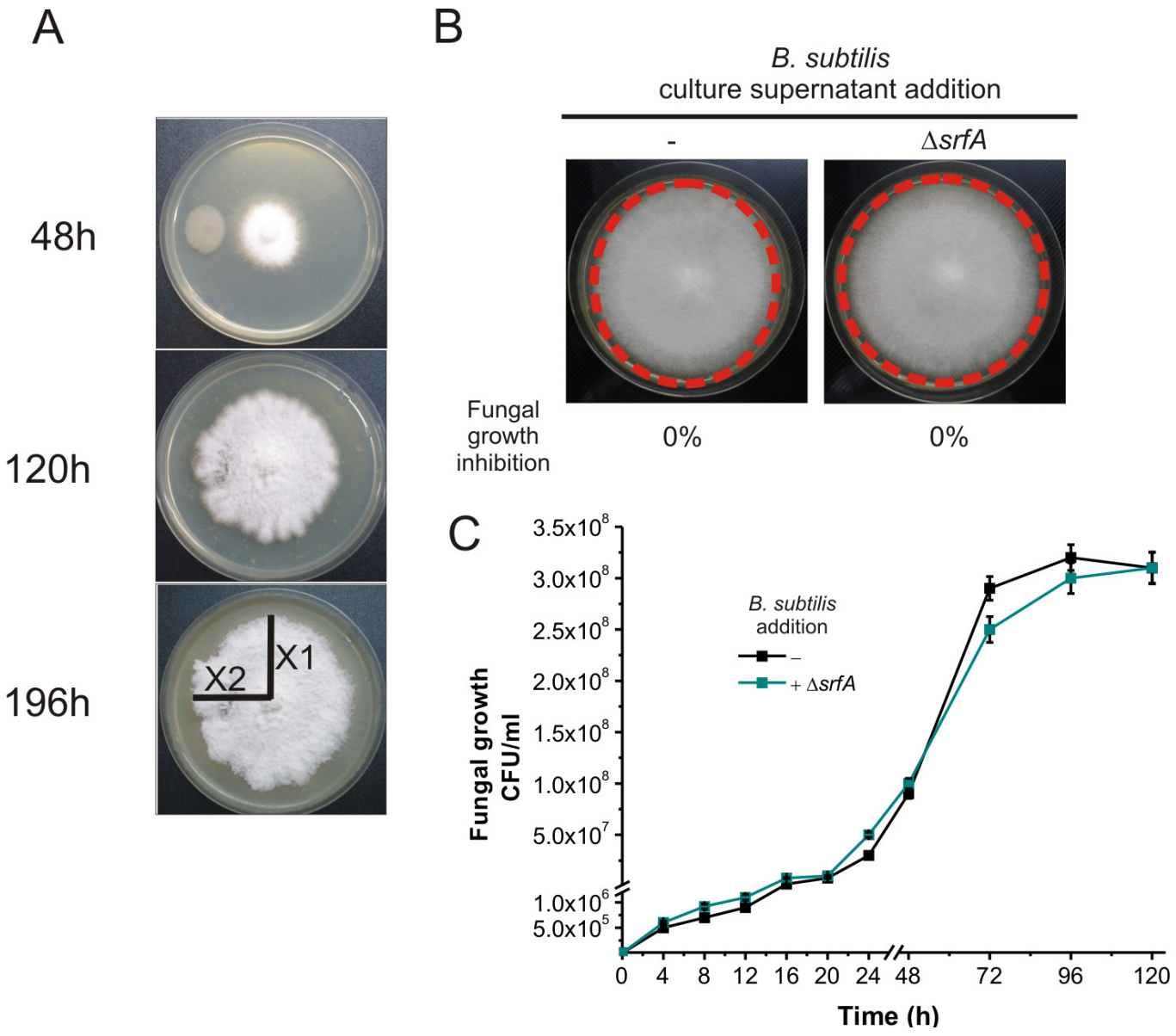
E

B. subtilis strain	*F.verticillioides* presence	*B. subtilis* (UFC/ml)		% of sporulation
		Viable	Spore	
Wild-type	No	$6.0x10^8$	$9.0x10^5$	0.15
	Yes	$6.2x10^8$	$8.1x10^5$	0.13
Δ*sigB*	No	$6.5x10^8$	$7.1x10^5$	0.11
	Yes	$3.6x10^8$	$4.3x10^5$	0.12

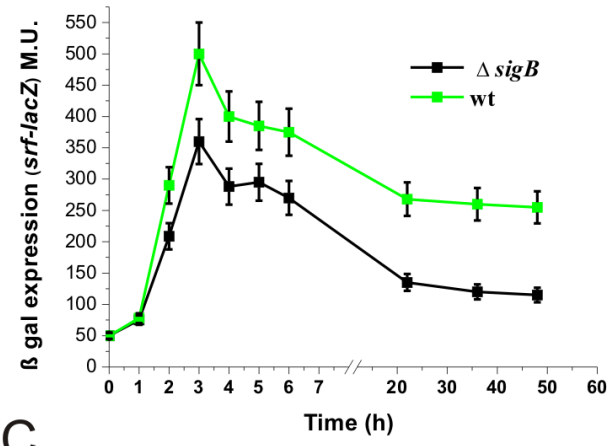




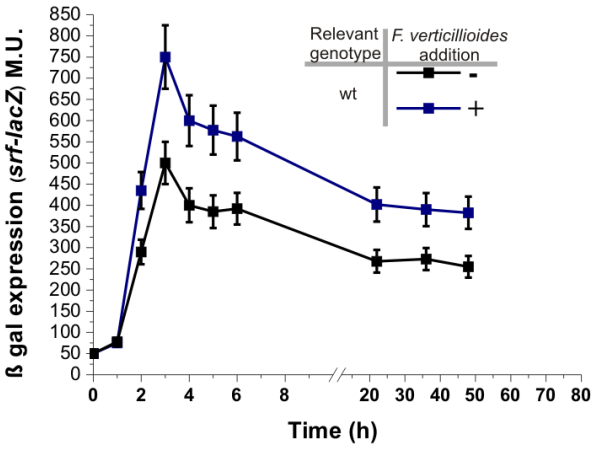




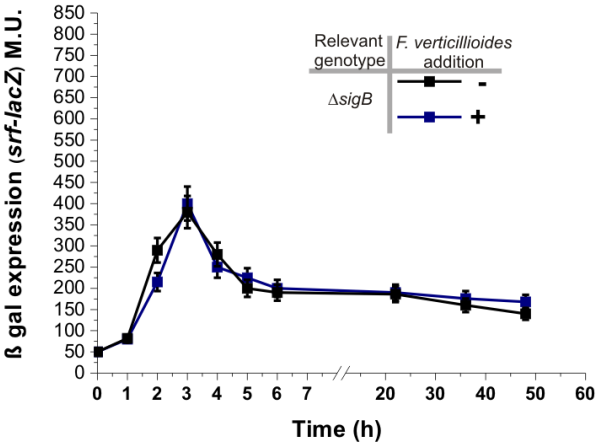
A



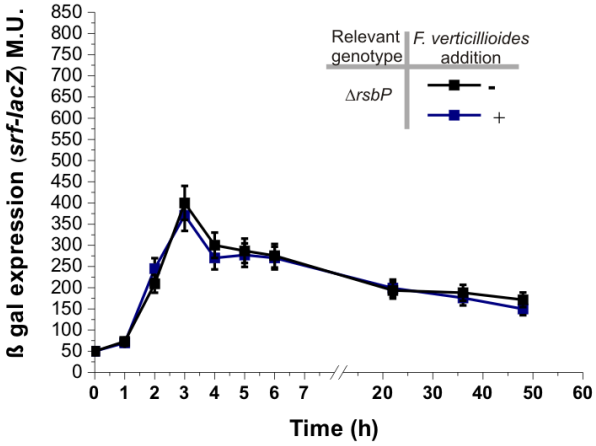
B



C



D



E