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The Alternative Sigma Factor SigB Is Required for the Pathogenicity of *Bacillus thuringiensis*

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ABSTRACT To adapt to changing and potentially hostile environments, bacteria can activate the transcription of genes under the control of alternative sigma factors, such as SigB, a master regulator of the general stress response in several Gram-positive species. *Bacillus thuringiensis* is a Gram-positive spore-forming invertebrate pathogen whose life cycle includes a variety of environments, including plants and the insect hemocoel or gut. Here, we assessed the role of SigB during the infectious cycle of *B. thuringiensis* in a *Galleria mellonella* insect model. We used a fluorescent reporter coupled to flow cytometry and showed that SigB was activated *in vivo*. We also showed that the pathogenicity of the $\Delta sigB$ mutant was severely affected when inoculated via the oral route, suggesting that SigB is critical for *B. thuringiensis* adaptation to the gut environment of the insect. We could not detect an effect of the *sigB* deletion on the survival of the bacteria or on their sporulation efficiency in the cadavers. However, the gene encoding the pleiotropic regulator Spo0A was upregulated in the $\Delta sigB$ mutant cells during the infectious process.

IMPORTANCE Pathogenic bacteria often need to transition between different ecosystems, and their ability to cope with such variations is critical for their survival. Several Gram-positive species have developed an adaptive response mediated by the general stress response alternative sigma factor SigB. In order to understand the ecophysiological role of this regulator in *Bacillus thuringiensis*, an entomopathogenic bacterium widely used as a biopesticide, we sought to examine the fate of a $\Delta sigB$ mutant during its life cycle in the natural setting of an insect larva. This allowed us, in particular, to show that SigB was activated during infection and that it was required for the pathogenicity of *B. thuringiensis* via the oral route of infection.

KEYWORDS *Bacillus thuringiensis*, infectious cycle, larva insect model, stress response

During their life cycle, bacteria can be confronted with environmental changes that are often considered stresses. These microorganisms are able to cope with rapid environmental changes in various ways, but alterations in the transcriptional patterns of the cells—mediated by alternative sigma factors and other transcriptional regulators—constitute one of the main responses to these stresses. Sigma factors are components of the RNA polymerase machinery that allow the recognition of specific promoter sequences to initiate gene transcription. In Gram-positive bacteria, the SigA sigma factor is required for the transcription of most genes in exponentially growing cells (1). In *Firmicutes*, one of the main regulators for the adaptation to changing environmental conditions is SigB. This alternative sigma factor was first described in the model organism *Bacillus subtilis* (2, 3). *sigB* regulation is complex and tightly controlled at the transcriptional and posttranslational levels. Briefly, in the absence of stress, SigB is sequestered by the anti-sigma factor RsbW. When a stress is sensed, the anti-anti-sigma factor RsbV is activated and sequesters RsbW, liberating SigB, which is then free to associate with the core RNA polymerase (4). The genes encoding these proteins are in an operon, and their transcription is under the control of SigB. Depending on the

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bacteria, the sensing portion of this signaling pathway is different and the suite of genes controlled by this alternative sigma factor is variable (4–7). SigB has been characterized and shown to be a key protein in *B. subtilis* and several other Gram-positive species by stimulating resistance to various stresses, such as heat or ethanolic shocks, as well as being involved in the infectious lifestyle of pathogens such as *Listeria monocytogenes* or *Staphylococcus aureus* (8–10). A recent study of *Clostridium difficile* has demonstrated the importance of SigB for the resistance of this bacterium to various stresses, including resistance to cationic antimicrobial peptides, as well as for its ability to colonize axenic mice (11). Since many of the SigB-harboring species are spore formers, the involvement of this factor in this process was also investigated. It was reported that sporulation was inhibited by SigB in *B. subtilis* and *C. difficile* (11, 12). Besides its importance in the resistance to acute stresses, it was proposed that a major function of SigB, as the master regulator of the general stress response, is to allow bacteria, such as *B. subtilis*, to cope with long-term survival by providing them with a preventive arsenal of proteins involved in the response to nonspecific stresses and that it might also offer an alternative to sporulation as a means to deal with adverse conditions (8, 13–15).

The role of SigB has also been investigated in bacteria of the *Bacillus cereus* group. This group of Gram-positive spore formers includes *Bacillus anthracis*, the agent of anthrax, *B. cereus sensu stricto*, responsible for foodborne toxi-infections, and *Bacillus thuringiensis*, an entomopathogenic bacterium used as a biopesticide (16). As in other bacteria, SigB also responds to acute stresses in *B. cereus* and *B. anthracis* (17, 18). In contrast to findings for *B. subtilis* and *C. difficile*, it appeared that sporulation efficiency was not affected in a *B. cereus* $\Delta sigB$ mutant (19). However, this study showed that germination was less efficient when SigB was absent than in the parental strain. Virulence of a *B. anthracis* $\Delta sigB$ mutant was assayed, and it was shown to be impaired in a mouse model of infection (18). Although it was reported that SigB did not control the production of virulence factors in *B. cereus* (17), its involvement in the pathogenic properties or life cycle of *B. cereus* and *B. thuringiensis* was not investigated. These pathogens are confronted with diverse ecosystems during their life cycle (such as the phylloplane, the rhizosphere, and the host digestive tract or circulating fluid, for instance) and must consequently cope with many environmental challenges. The life cycle of *B. thuringiensis* was investigated in several studies using an insect model of infection (20). It was shown that this bacterium activated various virulence genes, including those under the control of the quorum-sensing regulator PlcR, to kill its host (21, 22) and then entered a necrotrophic phase, controlled by the quorum sensor NprR, that allowed the bacteria to survive in the cadaver of the insect larva (23). A subpopulation of the necrotrophic cells then entered the sporulation pathway (22). These studies reported that spores constituted between 30 and 60% of the population, and it was also reported that a portion of the population was in an unknown physiological state (24).

Here, we investigated the involvement of SigB in the successive stages of the *B. thuringiensis* infectious cycle using the insect model *Galleria mellonella* and two modes of infection exposing the bacteria to different stresses. Using a fluorescent reporter coupled to flow cytometry, we have shown that SigB was activated *in vivo*. We have also determined that it was required for the virulence of *B. thuringiensis* when the bacteria were ingested by the larvae. No effect on the survival of the bacteria or on the sporulation efficiency in the cadavers was detected. However, there was a higher expression of the pleiotropic regulator gene *spo0A* in the $\Delta sigB$ mutant than in the parental strain.

RESULTS

Comparison of the *B. thuringiensis* and *B. cereus sigB* loci and generation of a *sigB* deletion mutant. SigB was previously studied in *B. cereus* strain ATCC 14579, a close relative to the *B. thuringiensis* 407 Cry⁻ strain (*B. thuringiensis* 407⁻) used in this study (25). Comparison of the genetic organizations of the *sigB* locus in these two

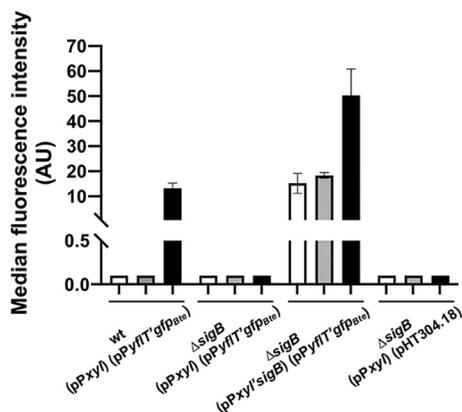


FIG 1 Transcriptional analysis of the SigB-dependent gene *yflT* following a heat shock stress *in vitro*. wt and $\Delta sigB$ mutant cells (carrying the empty vector pPxyl) as well as $\Delta sigB$ mutant cells complemented for the *sigB* deletion (carrying pPxyl'-*sigB*) were grown at 30°C in HCT. These bacteria harbor a vector carrying a transcriptional fusion between the PyflT promoter and the reporter gene *gfp_{Bte}* or the empty vector pHT304.18. At an OD₆₀₀ of 0.4 (T0), xylose was added to induce transcription of *sigB*, and the cultures were split in 2. One half was subjected to a heat shock at 42°C for 30 min, whereas the rest of the cells remained at 30°C. The bacteria were then harvested and subjected to flow cytometry analysis. The data are the result of two independent experiments. The error bars represent the standard error of the mean (SEM). White bars, cells harvested at T0 before heat shock; gray bars, cells kept at 30°C for 30 min; black bars, cells subjected to heat shock for 30 min.

bacteria showed that they present the same synteny (see Fig. S1 in the supplemental material). Moreover, the proteins encoded by the genes in the locus share between 95.5 and 99.3% identity at the peptide sequence level. This strongly suggests that *sigB* regulation in *B. thuringiensis* is similar to that reported for *B. cereus* (26).

We generated a $\Delta sigB$ clean deletion mutant by removing most of the *sigB* open reading frame (ORF) in the chromosome of *B. thuringiensis* strain 407⁻. We then complemented this strain by introducing the pPxyl'-*sigB* plasmid harboring the *sigB* gene under the control of a xylose-inducible promoter. To explore the activity of SigB, we used a transcriptional fusion between the reporter gene *gfp* optimized for *B. thuringiensis* (*gfp_{Bte}*) (24) and the promoter of *yflT*. This gene encodes a general stress protein and has been reported as one of the most induced SigB-dependent genes in *B. cereus* strain ATCC 14579 during heat shock (5, 26). Before using our constructs *in vivo*, we verified their functionality *in vitro*. We grew the bacteria at 30°C until mid-exponential phase, added xylose to induce *sigB* transcription from the Pxyl promoter, and transferred half of the culture from 30°C to 42°C for 30 min. The rest of the cells were kept at 30°C for the duration of the experiment. The $\Delta sigB$ strain harboring the empty vectors pPxyl and pHT304.18 was used as a negative control for fluorescence (Fig. 1; see also Fig. S2, right panel, in the supplemental material). The results show that the PyflT'-*gfp_{Bte}* fusion was expressed in the wild-type (wt) cells after being subjected to heat shock (mean of 13 arbitrary units [AU]), which contrasted with the $\Delta sigB$ mutant in which the level of fluorescence intensity was similar to that of the bacteria incubated at 30°C (mean of 0.1 AU) (Fig. 1; see also Fig. S2, two left panels). We also examined the activation of PyflT in the complemented $\Delta sigB$ mutant. The bacterial cells already presented a high level of fluorescence (mean of 15 AU) before being submitted to the heat shock (Fig. 1; see also Fig. S2, 3rd panel from the left), suggesting that the *sigB* gene was expressed and that SigB was active. This could reflect a leakage from the Pxyl promoter in these conditions. Nevertheless, this showed that the cells expressed the PyflT'-*gfp_{Bte}* fusion and we could observe an increase in fluorescence after heat shock (mean of 50 AU), indicating that the bacteria responded to the stress. Altogether, these results show that the PyflT'-*gfp_{Bte}* reporter fusion is functional and reflects the activity of SigB.

The alternative sigma factor SigB is active *in vivo*. We then assayed the activity of the SigB factor *in vivo* in a *G. mellonella* model of infection (Fig. 2; see also Fig. S3 in

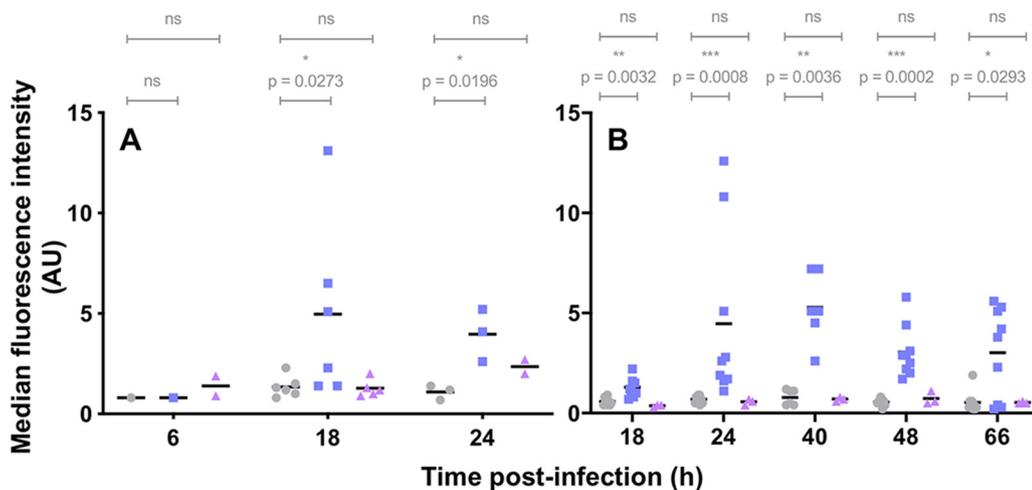


FIG 2 Transcriptional analysis of the SigB-dependent gene *yfIT* *in vivo*. wt and $\Delta sigB$ mutant bacteria harboring a transcriptional fusion between the *PyfIT* promoter and the reporter gene *gfp_{Bte}* and wt bacteria harboring the empty vector pHT304.18 were inoculated to *G. mellonella* larvae via the oral route by force feeding (A) or via intrahemocoelic injection (B). The larvae were then incubated at 30°C. Bacteria were isolated from the cadavers of the larvae at each time point and subjected to flow cytometry analysis. The data are the results of 2 independent experiments, with each symbol representing bacteria isolated from one larva. The data were analyzed using a Kruskal-Wallis test followed by an uncorrected Dunn's test; ns, not significantly different. Gray dots, wt cells harboring the empty vector pHT304.18 as a control for autofluorescence; violet squares, wt cells harboring pPyfIT-*gfp_{Bte}*; pink triangles, $\Delta sigB$ mutant cells harboring pPyfIT-*gfp_{Bte}*.

the supplemental material). The *G. mellonella* larvae can be infected by the following methods: intrahemocoelic injection and oral route via force-feeding. The former allows for the injection of the bacterial cells directly into the hemocoel of the larva. For the latter, the bacteria are fed to the larva along with Cry1C toxins. It was previously demonstrated that the toxins or the bacterial cells alone generate little mortality (less than 10%), whereas the association of the two creates a synergistic effect that reveals the pathogenic properties of the bacteria (21). This method is closer to the natural infection route of larvae, which mostly ingest *B. thuringiensis* while eating (27). The injection method is a means to assay the capacity of the bacteria to multiply in the host. The force-feeding method allows us to examine if the bacteria are able to multiply in the gut and cross the epithelial barrier before reaching the hemocoel. The results show that the *PyfIT*-*gfp_{Bte}* fusion is activated following both inoculation methods in the wild-type background (Fig. 2). At the earlier time points postinfection, the histograms present only one narrow peak, indicating that most of the bacteria have activated the *PyfIT*-*gfp_{Bte}* fusion (Fig. S3A and C). A higher level of activation was reached earlier postinoculation following force-feeding than following injection (mean of 5 AU 18 h post-force-feeding compared to a mean of 1 AU at the same time postinjection). However, the peak level of activation was similar in both cases (mean of 5 AU 18 h post-force-feeding and 5 AU at 40 h postinjection). The *PyfIT*-*gfp_{Bte}* fusion was not activated in the $\Delta sigB$ mutant cells (Fig. 2; see also Fig. S3B and D), indicating that the activation of the *PyfIT* promoter is SigB dependent. No effect of the Cry1C toxin was detected on the segregational stability of the *PyfIT*-*gfp_{Bte}* plasmid in $\Delta sigB$ mutant cells *in vitro* (see Table S2 in the supplemental material). Altogether, these data show that the SigB factor is active *in vivo*, regardless of the infection route.

SigB is required for the pathogenicity of *B. thuringiensis* via the oral infection route. Along with the assessment of the *PyfIT*-*gfp_{Bte}* fusion activation, we recorded the mortality of the larvae following force-feeding and intrahemocoelic injection (Fig. 3). No difference in mortality was measured following intrahemocoelic infection with wt or $\Delta sigB$ mutant cells (Fig. 3A). With both strains, the mortality appeared rapidly after infection (less than 18 h postinfection), and the mean survival reached a plateau of about 10% at 24 h postinoculation. Conversely, the results show that the mean survival

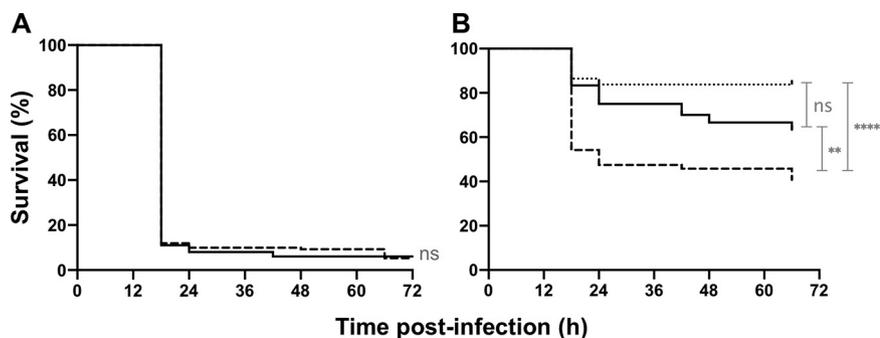


FIG 3 Effect of the *sigB* deletion on the virulence of *B. thuringiensis*. wt and $\Delta sigB$ mutant cells were inoculated to *G. mellonella* larvae via force feeding (A) or via intrahemocoelic injection (B). Inocula were a suspension of bacteria and Cry1C toxin (A) or bacteria alone (B). The assay was performed in parallel with the experiment described for Fig. 1; the bacteria thus harbored the pPy β IT-gfp $_{Bte}$ plasmid. The larvae were incubated at 30°C, and the number of dead larvae was recorded over time. The data are the result of at least 2 independent experiments. In total, 151 and 100 larvae were injected with wt and $\Delta sigB$ mutant cells, respectively (A), and 59, 60, and 37 larvae were force-fed with wt and $\Delta sigB$ mutant cells (along with the Cry1Ac toxin) or with the Cry1C toxin alone, respectively (B). The survival curves were compared using the Gehan-Breslow-Wilcoxon test. ns, not significantly different; **, $P = 0.0022$; ***, $P < 0.0001$. Dashed line, wt bacteria; solid line, $\Delta sigB$ mutant bacteria; dotted line, Cry1C toxin alone.

of larvae force-fed with $\Delta sigB$ mutant cells was similar to that of larvae force-fed with Cry1C toxins alone 18 h postinfection and was about 80%, whereas only 54% of the larvae force-fed with wt cells survived (Fig. 3B). At 66 h postinfection, 63% of the larvae remained alive following ingestion of $\Delta sigB$ mutant cells, whereas survival of larvae force-fed with wt cells decreased to 41%. No effect of the Cry1C toxin was detected on the survival of the $\Delta sigB$ mutant bacteria *in vitro* (Table S2). These data show that SigB is critical for the oral infection route, whereas it is dispensable when the bacterial cells are directly injected into the hemocoelic cavity of the larvae.

The $\Delta sigB$ mutant is able to survive and sporulate efficiently *in vivo*. We then examined the capacity of $\Delta sigB$ mutant cells to complete the infection cycle by assessing their ability to survive in the insect cadaver as well as to form heat-resistant spores (Fig. 4). The bacteria were injected into the hemocoel of the larvae and were recovered from the cadavers and then plated before and after being subjected to a heat treatment to kill the cells that did not form a spore. The results show that the $\Delta sigB$ mutant cells were as able as the wt cells to survive in the insect cadaver for the duration of the experiment as indicated by the solid symbols. At 18 h postinfection, the mean values of the total population reached about 6×10^8 and 1×10^9 CFU/larva for the

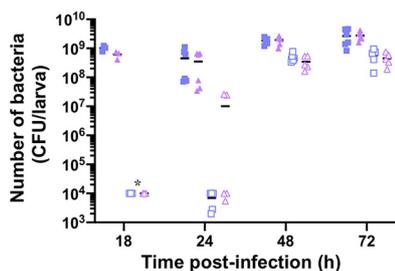


FIG 4 Effect of the *sigB* deletion on the survival and sporulation of *B. thuringiensis* *in vivo*. wt and $\Delta sigB$ mutant cells were inoculated to *G. mellonella* larvae via intrahemocoelic injection. The larvae were then incubated at 30°C. Bacteria were isolated from the cadavers of the larvae at each time point and enumerated on plates. The cells were either plated directly after harvest or were subjected to heating at 80°C for 13 min before plating to select for heat-resistant spores. The data are the results of 2 independent experiments; each symbol represents bacteria isolated from one larva. Violet squares, wt cells; pink triangles, $\Delta sigB$ mutant cells. Solid symbols, total cells; open symbols, heat-resistant spores. Black bars represent the mean. Statistical analysis performed using a Mann-Whitney test showed that the differences between the wt and $\Delta sigB$ mutant CFU/larva at each time point were not significantly different. The asterisk indicates that the CFU numbers at this time point were inferior to 10^4 .

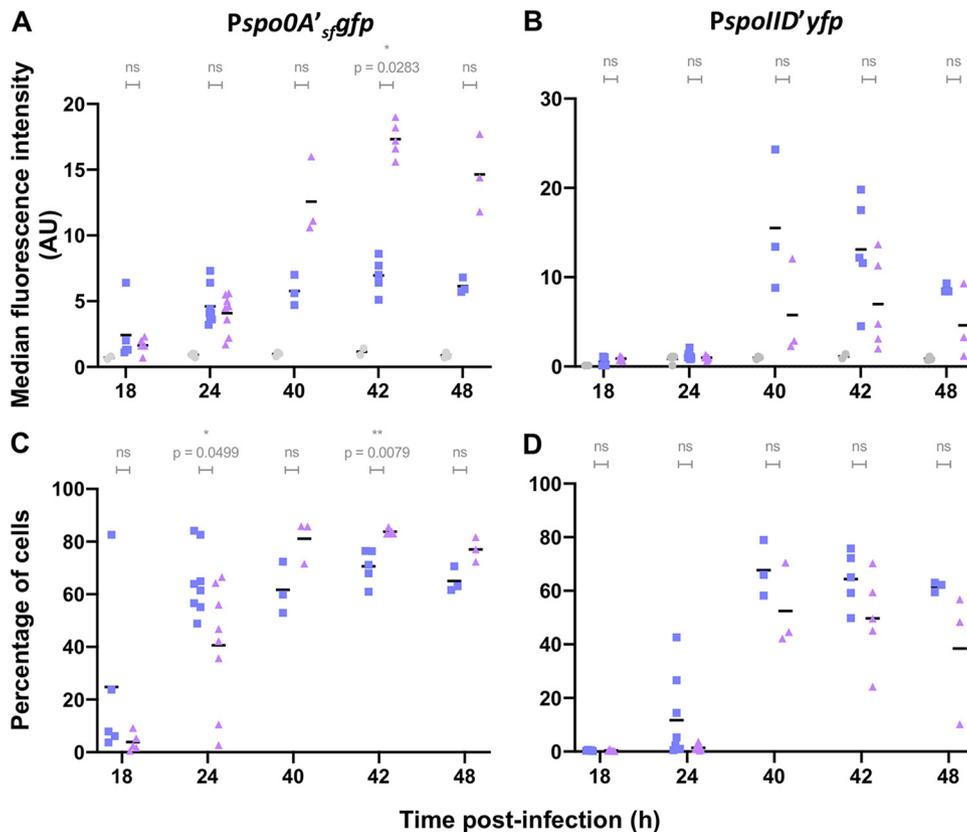


FIG 5 Effect of the *sigB* deletion on *spo0A* and *spoIID* transcription *in vivo*. wt and $\Delta sigB$ mutant cells harboring a transcriptional fusion between the *Pspo0A* promoter and the reporter gene *sgfp* (A, C) or the *PspoIID* promoter and the reporter gene *yfp* (B, D), and wt cells harboring the empty vector pHT304.18 (A, B), were inoculated to *G. mellonella* larvae via intrahemocoelic injection. The larvae were then incubated at 30°C. Bacteria were isolated from the cadavers of the larvae at each time point and subjected to flow cytometry analysis. The data are the results of 2 independent experiments, with each symbol representing bacteria isolated from one larva. The data were analyzed using a Kruskal-Wallis test followed by an uncorrected Dunn's test (A, B) or using a Mann-Whitney test (C, D). Gray dots, wt cells harboring the empty vector pHT304.18 as a control for autofluorescence; violet squares, wt cells harboring pPsp0A'-*sgfp* (A, C) or pPspIID'-*yfp* (B, D); pink triangles, $\Delta sigB$ mutant cells harboring pPsp0A'-*sgfp* (A, C) or pPspIID'-*yfp* (B, D); black bars represent the mean.

ΔsigB strain and wt strain, respectively. The total population increased up to 48 h and stayed constant until the end of the experiment with about 2.5×10^9 CFU/larva for the $\Delta sigB$ mutant and wt cells at 72 h postinoculation. No spores were detected for either strain 18 h postinoculation. At the 48- and 72-h time points, the number of heat-resistant spores was similar for both strains and ranged from 3.5×10^8 to 6×10^8 CFU/larva, as indicated on Fig. 4. However, at 24 h postinoculation, a higher number of heat-resistant spores was enumerated for the $\Delta sigB$ mutant cells in two larvae compared to that of the wt cells (mean values of 1×10^7 CFU/larva and 7×10^3 CFU/larva, respectively). However, a statistical analysis did not reveal a significant difference between these two mean values. Altogether, these results indicate that SigB is dispensable for the survival and sporulation of *B. thuringiensis* in the cadaver of *G. mellonella* larva. Survival and sporulation were also assessed *in vitro* in two standard *B. thuringiensis* cultivation media and showed that there was no difference in total CFU number and heat-resistant spores between the wt and $\Delta sigB$ mutant cells in 72-h cultures at 30 or 42°C (see Fig. S4 in the supplemental material).

Transcription of *spo0A* is higher in the $\Delta sigB$ mutant cells. To confirm the *in vivo* data and assess if SigB may affect early stationary and sporulation processes, we assayed the kinetics of expression of *Pspo0A*'-*sgfp* and *PspoIID*'-*yfp* following injection of the bacteria in *G. mellonella* (Fig. 5; see also Fig. S4). Spo0A is a pleiotropic regulator that controls various stationary-phase pathways including the initiation of sporulation

(28), and the expression of *PspolID* reflects the commitment of the bacteria toward the sporulation pathway (22, 29). Figure S4 represents the histograms obtained after flow cytometry analysis that were used to produce Fig. 5. The results show that there was an increase in the fluorescence of the cells harboring the *Pspo0A'*-*gfp* fusion between 18 and 24 h postinfection with a maximum at 42 h (Fig. 5A). Interestingly, the mean of the fluorescence intensity was higher in the $\Delta sigB$ mutant cells than in the wt cells at the late time points (notably significant at 42 h with a mean of 17 and 7 AU, respectively). The *PspolID'*-*yfp* fusion was expressed later during infection, and fluorescence was detected in the cells from 40 h postinoculation (Fig. 5B). Contrary to what was observed with the *Pspo0A* promoter, the wt cells seemed to present a higher mean fluorescence intensity than the $\Delta sigB$ mutant cells (mean of 15 and 6 AU at 40 h, 13 and 7 AU at 42 h, and 9 and 5 AU at 48 h, respectively). However, a statistical analysis revealed that these differences were not significant. We also analyzed the data with a complementary representation by plotting the percentage of the population that was positive for the expression of each fusion as a function of time (Fig. 5C and D). In the case of the *PspolID'*-*yfp* fusion, this allows visualization of the proportion of bacterial cells that actually entered the sporulation process. The data show that there was an increase in the number of bacteria that expressed *Pspo0A'*-*gfp* and *PspolID'*-*yfp* between the 18- and 24-h time points and the 24- and 40-h time points, respectively. The *Pspo0A'*-*gfp* fusion was expressed in more wt cells than $\Delta sigB$ mutant cells at 24 h postinoculation (mean of 65% and 41%, respectively), whereas it was the contrary at 40, 42, and 48 h (mean of 62% and 81%, 71% and 84%, and 65% and 77%, respectively). Statistical analysis of the results showed that the differences between the means in the proportion of fluorescent cells between the wt and $\Delta sigB$ mutant strains were significant at 24 and 42 h for the *Pspo0A'*-*gfp* fusion (Fig. 5C). The percentage of cells that expressed *PspolID'*-*yfp* were not significantly different (Fig. 5D), indicating that the differences observed for *Pspo0A'*-*gfp* did not seem to impact the number of cells that actually entered the irreversible stage of the sporulation process. Segregational stability was assessed for plasmid p*Pspo0A'*-*gfp* and showed that a small percentage of cells had lost the plasmid after 72 h of infection (see Table S3 in the supplemental material). However, this does not impact the conclusions drawn from Fig. 5.

DISCUSSION

In the *B. cereus* group, the regulation and the function of the alternative sigma factor SigB has been investigated mainly in *B. cereus sensu stricto* and in *in vitro* conditions (for reviews, see references 6 and 8). Only one study reported its role *in vivo* in *B. anthracis* in which the virulence of the $\Delta sigB$ mutant was attenuated compared to that of the parental strain when subcutaneously injected into mice (18). Here, we assessed the requirement for SigB during the life cycle of *B. thuringiensis* in a larva model of infection.

We observed that a SigB-dependent reporter was activated *in vivo* and that this activation occurred earlier in the bacteria that were force-fed to the larvae than after direct injection into the hemocoel, suggesting that the bacteria are confronted to SigB-inducing cues earlier in the insect gut than in the hemolymph. Interestingly, our results show that the virulence of the $\Delta sigB$ mutant strain was significantly lower than that of the wt strain when the bacteria were inoculated via the oral route. In contrast, the $\Delta sigB$ strain injected into the hemocoel induced the same mortality as the parental strain. These results suggest that SigB-regulated factors are required to allow the bacteria to resist the insect gut environment and/or to damage the intestinal barrier and gain access to the hemocoel in order to induce septicemia. Indeed, insect larvae are protected against ingested pathogenic bacteria thanks to anatomical and physiological barriers, such as the peritrophic membrane, which lines the intestine, or the alkaline pH of the gut (30, 31). Additionally, they are equipped with a cellular and humoral innate immune response that allows them to phagocytose intruders as well as synthesize defense molecules, such as reactive oxygen species (ROS), antimicrobial peptides (AMPs), lysozyme, and a cascade of enzymes leading to melanization. These defense molecules can be produced in the gut or in the hemolymph of the larva; however, it is

difficult to evaluate their level of production in either compartment in our conditions. In *B. thuringiensis* and *B. cereus*, PlcR regulates the transcription of the main nonspecific virulence factors and is required for the bacteria to cross the epithelial barrier when ingested by larvae (21, 32). Previous work has reported that there was no major alteration in the production of the virulence factors controlled by PlcR in a *B. cereus* $\Delta sigB$ strain (17). Similarly, the attenuation in virulence observed in the *B. anthracis* $\Delta sigB$ mutant was found to be toxin independent, suggesting a role in the resistance to stresses encountered during infection (18). This is in contrast with *L. monocytogenes* in which the main regulator of virulence gene expression, PrfA, and some virulence-factor-encoding genes are under the direct control of SigB (33–35). SigB was found to be critical for the virulence of this bacterium through cell invasion but not for the later stages of infection (36, 37). We can hypothesize that the difference in the mortality caused by the *B. thuringiensis* $\Delta sigB$ mutant following force-feeding and intrahemocoelic injection is that when the bacterial cells enter via the oral route, the insect has more time to mount a proper immune response, which would be deleterious to weakened cells unable to respond to stress, than when the bacteria are directly injected into the hemocoel where they can rapidly develop to induce septicemia. Transcriptomic analysis comparing the RNA profile of the *B. cereus* ATCC 14579 $\Delta sigB$ mutant and wt cells revealed that, in addition to genes encoding general stress proteins, the regulon includes genes whose product could be involved in the bacterial development in the host and its response to the host immune system, such as Orf4 (a putative bacterioferritin) and KatE (a catalase) (8, 26). However, many of the SigB-dependent genes encode proteins of unknown function.

The SigB-dependent response has been proposed as an alternative to sporulation in *B. subtilis* when the conditions did not support the commitment into this costly and irreversible process (8). We observed that SigB was activated between 18 and 24 h after intrahemocoelic injection in the larvae. This corresponds to a time where *B. thuringiensis* enters the necrotrophic stage in its host cadaver (22, 23). During the necrotrophic phase of the infectious cycle, the *B. thuringiensis* population is subdivided into sporulating and nonsporulating bacteria (22). We hypothesized that SigB could be involved in the survival properties of *B. thuringiensis* in the host cadaver. However, we could not detect any difference in the ability of the $\Delta sigB$ mutant cells to survive in the insect cadaver after they killed the host compared to that of the wt strain or in their capacity to form spores in these conditions. The higher number of spores in some of the larvae infected with the $\Delta sigB$ mutant might not be statistically significant considering the size of the sample. We could nevertheless observe a significantly higher expression of *spo0A* in the $\Delta sigB$ mutant than in the wt. This did not reflect on the number of spores formed by this strain. However, it is known that Spo0A is a pleiotropic regulator that controls pathways other than sporulation in *B. subtilis* and in bacteria of the *B. cereus* group (38–40). Although activation of SigB was observed in the cadavers of the larvae, we could not determine the role of this sigma factor during this stage of the infection. One hypothesis is that SigB activation at this stage was selected to facilitate the transition from the cadaver to the environment, either as nonsporulated cells by preventively producing general stress response proteins or as fully proficient spores. It was indeed demonstrated that $\Delta sigB$ mutant spores were deficient for germination in *B. cereus* (19).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The acrySTALLIFEROUS *B. thuringiensis* 407 Cry⁻ strain (*B. thuringiensis* 407⁻) (41) was used as the parental strain to create all of the strains used in this study. *Escherichia coli* strain DH5 α (42) was used as the host strain for plasmid construction. *E. coli* strain ET12567 (43) was used to prepare DNA prior to electroporation in *B. thuringiensis*. Cells were grown under shaking at 30°C or 37°C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) or HCT medium (0.7% casein hydrolysate, 0.5% tryptone, 0.68% KH₂PO₄, 0.012% MgSO₄·7H₂O, 0.00022% MnSO₄·4H₂O, 0.0014% ZnSO₄·7H₂O, 0.008% ferric ammonium citrate, 0.018% CaCl₂·4H₂O, pH 7.2) to which 0.3% glucose was added when indicated (44). The bacteria were stored at -80°C in LB containing 15% glycerol.

The antibiotic concentrations used for selection of *B. thuringiensis* were erythromycin (10 μ g/ml) and kanamycin (200 μ g/ml). Ampicillin (100 μ g/ml) was used for selection of *E. coli*.

TABLE 1 Plasmids used in this study

Plasmid name	Relevant information	Reference or source
pHT304.18	Replicative multicopy <i>E. coli</i> / <i>B. thuringiensis</i> shuttle vector	46
pPxyI	Replicative multicopy <i>E. coli</i> / <i>B. thuringiensis</i> shuttle vector harboring the xylose-inducible promoter PxyI	47
pMAD Δ sigB	<i>sigB</i> flanking regions were amplified by PCR from the chromosome of <i>B. thuringiensis</i> 407 ⁻ using primer pairs DsigB1/DsigB2 and DsigB3/DsigB4 followed by overlap extension PCR and then cloned between the PstI and Ascl restriction sites of the thermosensitive plasmid pMAD (48).	This study
pPxyI- <i>sigB</i>	The <i>sigB</i> coding region was amplified by PCR using primer pair sigmaB1/sigmaB2 and the chromosome of <i>B. thuringiensis</i> 407 ⁻ as a template. The resulting fragment was then cloned between the BamHI and XmaI restriction sites of pPxyI.	This study
pPyfIT'- <i>gfp</i> _{Bte}	The <i>yfIT</i> promoter region was amplified by PCR using primer pair PyfIT1/PyfIT2 and the chromosome of <i>B. thuringiensis</i> 407 ⁻ as a template. The resulting fragment was then cloned between the PstI and Ascl restriction sites of <i>pgfp</i> _{Bte} (24).	This study
pPspo0A'- <i>gfp</i>	The <i>gfp</i> coding region was amplified by PCR using primer pair SG30/SG31 and the pCM11 plasmid DNA as a template. The resulting fragment was then cloned between the SphI and HindIII restriction sites of pHT304 (49), generating vector <i>p_sgfp</i> . Then, the <i>spo0A</i> promoter region was PCR amplified using primer pair SG61/SG62 and the chromosome of <i>B. thuringiensis</i> 407 ⁻ as a template, and the resulting fragment was cloned between the EcoRI and XbaI restriction sites of <i>p_sgfp</i> .	This study
pPspolID'- <i>yfp</i>	Multicopy vector harboring a transcriptional fusion between the promoter of <i>spolID</i> and the <i>yfp</i> reporter gene	22

When required, xylose was used at a concentration of 20 mM.

Plasmid and strain constructions. DNA manipulations are detailed in the supplemental material. All of the plasmids and strains used in this study are indicated in Tables 1 and 2. Oligonucleotide primers are listed in Table S1 in the supplemental material.

Heat shock assay. Cells were grown overnight at 30°C on HCT agar plates supplemented with glucose and the appropriate antibiotics. Liquid HCT was then inoculated to an optical density at 600 nm (OD₆₀₀) of 0.05, and the cultures were incubated at 30°C under shaking until an OD₆₀₀ of 0.4 ± 10%. At this time point (designated T₀), cells were harvested, and xylose was added to the remaining cultures that were then split in half. One of the flasks was transferred to a water bath shaker and incubated at 42°C for 30 min, whereas the other flask was incubated at 30°C before the bacteria were harvested. Each aliquot was fixed essentially as described in reference 22. Briefly, the cells were centrifuged, fixed for 7 min in saline-formaldehyde (4%), and then washed in saline. The pellet was then resuspended in glucose-Tris-EDTA (GTE) buffer (45) and kept at 4°C until further analysis.

Infections. *In vivo* experiments with *G. mellonella* were carried out essentially as described previously (21). Briefly, 20 to 40 last instar larvae were each either injected with 2 × 10⁴ bacteria or were force-fed

TABLE 2 Strains used in this study

Strain	Relevant information	Source
<i>B. thuringiensis</i> Δ sigB	The 657-bp internal deletion in the <i>sigB</i> coding region was obtained by homologous recombination between the flanking regions of <i>sigB</i> on the chromosome of strain <i>B. thuringiensis</i> 407 ⁻ and plasmid pMAD Δ sigB. The deletion and integrity of the flanking regions were verified using primer pair VDisigB5/VDisigB6.	This study
<i>B. thuringiensis</i> Δ sigB(pPxyI)(pHT304.18)	<i>B. thuringiensis</i> Δ sigB strain carrying the empty pPxyI and pHT304.18 vectors	This study
<i>B. thuringiensis</i> (pPxyI)(pPyfIT'- <i>gfp</i> _{Bte})	<i>B. thuringiensis</i> 407 ⁻ carrying the empty pPxyI vector and in which we measure the activity of the <i>yfIT</i> gene promoter using Gfp _{Bte} fluorescence	This study
<i>B. thuringiensis</i> Δ sigB(pPxyI)(pPyfIT'- <i>gfp</i> _{Bte})	<i>B. thuringiensis</i> Δ sigB strain carrying the empty pPxyI vector and in which we measure the activity of the <i>yfIT</i> gene promoter using Gfp _{Bte} fluorescence	This study
<i>B. thuringiensis</i> Δ sigB(pPxyI'- <i>sigB</i>)(pPyfIT'- <i>gfp</i> _{Bte})	<i>B. thuringiensis</i> Δ sigB strain complemented for <i>sigB</i> deletion and in which we measure the activity of the <i>yfIT</i> gene promoter using Gfp _{Bte} fluorescence	This study
<i>B. thuringiensis</i> (pHT304.18)	<i>B. thuringiensis</i> 407 ⁻ carrying the empty pHT304.18 vector	This study
<i>B. thuringiensis</i> (pPyfIT'- <i>gfp</i> _{Bte})	<i>B. thuringiensis</i> 407 ⁻ strain in which we measure the activity of the <i>yfIT</i> gene promoter using Gfp _{Bte} fluorescence	This study
<i>B. thuringiensis</i> Δ sigB(pPyfIT'- <i>gfp</i> _{Bte})	<i>B. thuringiensis</i> Δ sigB strain in which we measure the activity of the <i>yfIT</i> gene promoter using Gfp _{Bte} fluorescence	This study
<i>B. thuringiensis</i> (pPspo0A'- <i>gfp</i>)	<i>B. thuringiensis</i> 407 ⁻ strain in which we measure the activity of the <i>spo0A</i> gene promoter using <i>gfp</i> fluorescence	This study
<i>B. thuringiensis</i> Δ sigB(pPspo0A'- <i>gfp</i>)	<i>B. thuringiensis</i> Δ sigB strain in which we measure the activity of the <i>spo0A</i> gene promoter using <i>gfp</i> fluorescence	This study
<i>B. thuringiensis</i> (pPspolID'- <i>yfp</i>)	<i>B. thuringiensis</i> 407 ⁻ strain in which we measure the activity of the <i>spolID</i> gene promoter using Yfp fluorescence	This study
<i>B. thuringiensis</i> Δ sigB(pPspolID'- <i>yfp</i>)	<i>B. thuringiensis</i> Δ sigB strain in which we measure the activity of the <i>spolID</i> gene promoter using Yfp fluorescence	This study

with a mixture of Cry1C toxin (0.3 µg) and 5×10^6 bacteria. The bacterial counts in the inocula were verified by CFU enumeration following plating of the suspensions. The larvae were kept at 30°C for the duration of the experiment.

Harvest of the bacteria from the insect cadavers for flow cytometry analysis. At each time point, *B. thuringiensis* cells were harvested from insects as described previously (24). Briefly, the larva was cut open, transferred to a 1.5-ml Eppendorf tube containing 1 ml of phosphate-buffered saline (PBS) and vortexed. The suspension was pipetted into a new 1.5-ml Eppendorf tube (leaving behind most of the large insect debris). The sample was centrifuged, and the pellet was resuspended in saline-formaldehyde 4%, fixed for 7 min, and then washed in saline. This suspension was filtered onto a cotton pad in a 1-ml syringe in order to retain the cadaver debris and recover the bacterial cells in the filtrate. These were then concentrated by centrifugation, resuspended in GTE buffer (45) and kept at 4°C until further analysis.

Flow cytometry settings. We analyzed the samples using a CyFlow Space flow cytometer (Sysmex, France). For Gfp- and Yfp-based fluorescence, the solid blue laser emitting at 488 nm was combined to a 500-nm long-pass dichroic mirror and a 527-nm band-pass filter (512 to 542) (FL1 channel). The analyses were performed using logarithmic gains and detector settings, adjusted on a sample of reporterless cells, to define cellular autofluorescence. Gating on forward scatter and side scatter (FSC/SSC) was used to discriminate bacteria from the background. For each sample, 20,000 gated events were measured. Data were collected and analyzed with the FloMax software (Sysmex, France) and were further analyzed with the Weasel 3.3.3 software to generate overlays (WEHI, USA). For each time point, to identify positive and negative populations on histograms, we set the threshold on the control strain so that 99% of the population presented a fluorescence intensity below the threshold.

Survival and sporulation of the bacteria in the insect cadaver. Each cadaver was ground in 4 ml saline on ice using an T10 Ultra-Turaxx apparatus. The volume was then adjusted to 10 ml. To determine the number of total cells and the number of heat-resistant spores, the bacteria were enumerated by plating on LB agar plates following serial dilutions before or after heat treatment at 80°C for 12 min.

Statistical analysis. The data were analyzed using the Prism 8 software (GraphPad).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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