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Novel Roles of the Master Transcription Factors Spo0A and σ^B for Survival and Sporulation of *Bacillus subtilis* at Low Growth Temperature

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Spore development and stress resistance in *Bacillus subtilis* are governed by the master transcription factors Spo0A and σ^B, respectively. Here we show that the coding genes for both regulatory proteins are dramatically induced, during logarithmic growth, after a temperature downshift from 37 to 20°C. The loss of σ^B reduces the stationary-phase viability of cold-adapted cells 10- to 50-fold. Furthermore, we show that σ^B activity is required at a late stage of development for efficient sporulation at a low temperature. On the other hand, Spo0A loss dramatically reduces the stationary-phase viability of cold-adapted cells 10,000-fold. We show that the requirement of Spo0A for cellular survival during the cold is independent of the activity of the key transition state regulator AbrB and of the simple loss of sporulation ability. Furthermore, Spo0A, and not proficiency in sporulation, is required for the development of complete stress resistance of cold-adapted cells to heat shock (54°C, 1 h), since a loss of Spo0A, but not a loss of the essential sporulation transcription factor σ^F, reduced the cellular survival in response to heat by more than 1,000-fold. The overall results argue for new and important roles for Spo0A in the development of full stress resistance by nonsporulating cells and for σ^B in sporulation proficiency at a low temperature.

The exposure of bacteria to diverse growth-limiting conditions induces the synthesis of a large set of proteins (called general stress proteins) that protect the cell against internal (metabolic) or external (environmental) stresses (22, 23, 29, 32, 33). In the gram-positive, endospore-forming bacterium *Bacillus subtilis*, the general stress response is controlled mainly by σ^B, the alternative transcription factor of the RNA polymerase that brings about a special physiological state which significantly enhances bacterial survival (11, 20, 22, 23, 29, 32, 33, 37). It is estimated that over 200 genes (5% of the coding capacity of the genome) are directly or indirectly under σ^B control, and the loss of σ^B function leads to multiple-stress sensitivity, compromising the survival of the σ^B null mutant strain (23, 29, 32). Besides having this very important, rapid, reversible, and plastic adaptive response (22, 29), *B. subtilis* is also able to differentiate into dormant spores when nutritional conditions become so extreme that the σ^B-dependent response would not be adequate to guarantee the survival of the cell (19, 21, 24, 30, 31). While σ^B is the key regulatory protein involved in the reversible adaptive stress response of vegetative cells, the master transcription factor Spo0A is the key regulator responsible for the decision of a vegetative cell to differentiate into a dormant and highly resistant new cell, i.e., the spore (31). It is accepted that these responses, general stress adaptation and sporulation, are important for the survival of *B. subtilis* in its natural environment, i.e., soil (29–33). Furthermore, high levels of expression of general stress proteins provide stressed or starved cells with multiple, nonspecific, protective functions for future or unexpected insults (37). In particular, soil is subject to important fluctuations of the environmental temperature, which can vary from mesophyllic values at midday to chilling temperatures at night (28, 41). Taking these observations into consideration, we considered it to be of interest to analyze whether σ^B and/or Spo0A might play a role in the stress adaptation and survival of *B. subtilis* during growth and permanence at a low growth temperature.

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MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The experiments conducted in this study were performed with wild-type reference strain JH642 and isogenic derivatives (38). Mutations and gene reporter fusions were introduced into strain JH642 by transformation of competent cells as previously described (3). The parent strain and the resulting isogenic derivatives are described in Table 1. Bacteria were routinely grown under vigorous agitation (220 rpm) in Spizizen minimal medium (MM), with 0.5% (wt/vol) glucose as the carbon and energy source and L-tryptophan (50 μg/ml) and L-phenylalanine (50 μg/ml) (14). Where indicated below, the strains were grown in Schaeffer sporulation medium or Luria-Bertani (LB) medium. For sporulation efficiency, cells were grown in MM for the times indicated in Tables 2 and 3 and the figure legends and then treated with $CHCl_3$ or heated at 80°C for 15 min before being plated (38). For drug resistance selection in *B. subtilis*, antibiotics were used at the following final concentrations: 5 μg/ml for chloramphenicol, 1 μg/ml for erythromycin, 2 μg/ml for kanamycin, and 25 μg/ml for spectinomycin.

Determination of β-galactosidase activity. For the determination of the β-galactosidase activity of the *lacZ* gene reporter fusions, cultures were propagated as described in the figure legends. At appropriate times, triplicate 1-ml aliquots were removed and harvested by centrifugation at 4°C. β-Galactosidase enzyme assays were conducted as described previously (3).

Western blot analysis. *B. subtilis* cultures of the parent strain JH642 and its isogenic derivate Sik31 (Δ*spo0A*::Ery^r P_{spac}-*spo0Asad67*) (Table 1) were grown in MM and LB medium, respectively. Aliquots of 20 ml of each culture were collected by centrifugation and washed three times in disruption buffer {50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], 50 mM NaCl, 5 mM dithiothreitol, 10% glycerol, 1 mM EDTA (pH 7.5), and protease inhibitor cocktail (Complete; Boehringer)}. Cells were finally resuspended in 0.5 ml of

TABLE 1. *B. subtilis* strains used in this work

Strain	Relevant phenotype	Comments and/or source
GS37	*amyE*::pGES 35 (*fabHlF-lacZ*)	G. Schujman (34)
JH642	*trpC2 pheA1*	Laboratory stock (3)
JH646	*spoAA12*	Laboratory stock
RG12607	Δ*abrB*::*spc*	Laboratory stock
JH16302	*amyE*::*spoIIA-lacZ kan*	Laboratory stock
JH16304	*amyE*::*spoIIG-lacZ kan*	Laboratory stock
JH19005	*amyE*::*spo0A-lacZ cat*	Laboratory stock
MR100	*amyE*::*sigB-lacZ cat*	PB286 (C. Price) → JH642
MR101	*amyE*::*ctc-lacZ cat*	PB198 (C. Price) → JH642
MR102	*amyE*::*spo0H-lacZ cat*	GBS151 (G. Spiegelman) → JH642
MR110	*amyE*::*sigB-lacZ cat* Δ*spo0A*::*ery*	SS955 (T. Leighton) → MR100
MR200	*amyE*::*spo0A-lacZ cat* Δ*spo0H*::*ery*	UOT1850 (F. Kawamura) → JH19005
MR348	*amyE*::*sspB-lacZ cat* Δ*sigB*::*ery*	PS348 (P. Setlow) → MR744
MR644	Δ*sigB*::*cat*	ML6 (W. Schumann) → JH642
MR744	Δ*sigB*::*ery*	pCm::Erm → MR644
MR851	*spoIID-lacZ spc* Ω *spoIID* Δ*sigB*::*cat*	MR644 → RG851
MR2051	*amyE*::*spoIIQ-lacZ spc* Δ*sigB*::*cat*	MR644 → RG2051
MR12607	Δ*abrB*::*spc*Δ*spo0A*::*ery*	SS955 (T. Leighton) → JH12607
MR19005	*amyE*::*spo0A-lacZ cat* Δ*sigB*::*ery*	MR644 → JH19005
NR10	*amyE*::*spoIIA-lacZ kan* Δ*sigB*::*cat*	Laboratory stock
NR11	*amyE*::*spoIIG-lacZ kan* Δ*sigB*::*cat*	Laboratory stock
RG348	*amyE*::*sspB-lacZ cat*	PS348 (P. Setlow) → JH642
RG19148	Δ*sigF*::*kan*	Laboratory stock
RG851	*spoIID-lacZ spc* Ω *spoIID*	Laboratory stock
RG2051	*amyE*::*spoIIQ-lacZ spc*	Laboratory stock
Sik31	*amyE*:: P_{spac}-*spo0Asad67 spo0A*::*ery*	Laboratory stock (A. Grossman)
SS955	Δ*spo0A*::*ery*	Laboratory stock (T. Leighton)

disruption buffer and disrupted by sonication (six times, 10 s each time) using a model 200 sonifier (Branson). Cell debris was removed by centrifugation (10 min, 13,000 rpm at 4°C [Marathon 16 Km; Fisher Scientific]). Protein concentrations in crude extract were determined by using the Bradford protein assay (Bio-Rad) (8a). The samples were subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, transferred to an Immobilon membrane (Millipore, Bedford, Mass.), and revealed by using anti-Spo0A rabbit antibody and an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) (Bio-Rad). For signal quantification, films were scanned and analyzed densitometrically. The Sik31 culture was grown in LB medium at 37°C until the mid-exponential phase. At this point, IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) was added to half of this culture, and growth continued for another 2 h. After this induction period (production of Spo0A-Sad67), the culture was processed for protein analysis.

RESULTS AND DISCUSSION

Expression of *sigB* and *spo0A* at a low growth temperature. It was previously shown that the measurement of β-galactosidase activity from transcriptional *lacZ* fusions constitutes a satisfactory method to analyze the response of *B. subtilis* genes to cold shock (1, 2). Therefore, we assayed the effect of the cold shock (from 37 to 20°C) on the expression of *sigB* (coding for σ^B) and *spo0A* in isogenic *B. subtilis* strains harboring transcriptional *lacZ* fusions to the promoter regions of both regulatory genes (Table 1). Figure 1A shows a typical growth curve of a culture of *B. subtilis* grown in MM (Spizizen salts supplemented with 0.5% glucose, 50 μg of Trp/ml, and 50 μg of Phe/ml) with strong aeration (220 rpm) until the early exponential phase (optical density at 525 nm [OD_{525}], 0.25), half of which was then transferred from 37 to 20°C. The cold-shocked culture did not show a lag phase, and the generation times under these conditions of exponential growth at 37 and 20°C were 2.5 and 13.0 h, respectively (Fig. 1A). Despite the important reduction in the rate of growth, the cold-shocked culture reached the same cellular density as that of the culture that remained at 37°C (Fig. 1A) and reached similar cellular yields at both temperatures (an average of 1.0×10^8 to 4.0×10^8 CFU/ml [data not shown]). Under these experimental conditions, comparing cultures maintained at 37°C, we observed a reproducible 10-fold increase in *sigB* expression and a significant induction (four- to fivefold increase) of *spo0A* expression during the vegetative and stationary phases of the cultures transferred to 20°C (Fig. 1B and C). Interestingly, despite the absence of a lag phase, the cold induction of *sigB* and *spo0A* after the temperature downshift was not immediately perceptible. This delay in gene activation is intriguingly different from what occurs with the widely studied induction of *sigB* that follows the input of metabolic or environmental stress signals at 37°C (7, 8, 23, 29, 33) or the rapid response of the cold shock regulon of *B. subtilis* (6, 16–18, 39). Effectively, all these adaptive responses are fully displayed after the first 15 min following the stress (6, 29, 33). By contrast, in our study, the induction of *sigB* and *spo0A* began to be noticeable only 4 to 5 h following the temperature downshift but many hours before (~40 h) the cold-shocked cultures reached the stationary phase of growth at 20°C (Fig. 1B and C). Interestingly, this apparent delay of σ^B induction during adaptation to the cold had been previously reported to occur during the cold shock response of the psychrotrophic food-borne pathogen *Listeria monocytogenes* (5). In this bacterium, the σ^B-like transcription factor was induced by the cold shock (from 37 to 8°C) only 4 h following the temperature downshift, with a substantial increase in *sigB* expression after 6 h of that treatment (5). Furthermore, as is the case for *sigB* in *B. subtilis*, the induction of the σ^B-like transcription factor of *L. monocytogenes* by other stresses (i.e., osmotic stress) at 37°C occurs during the 10 min that follows the stressing signal (12). Thus, we deduced that

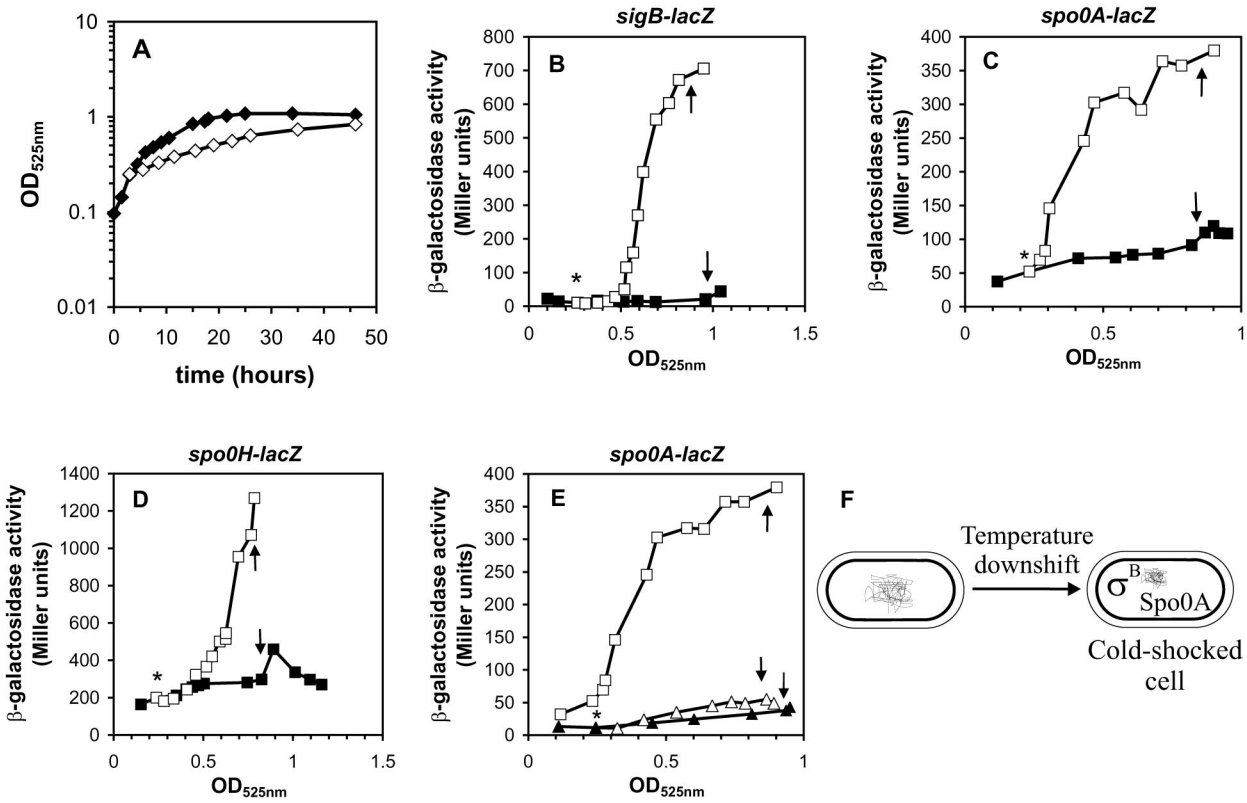

FIG. 1. Growth and gene expression of *B. subtilis* at 37 and 20°C. (A) Growth curve of wild-type strain JH642 in MM at 37°C (filled symbols) or transferred at early exponential phase (OD_{525} of 0.25) to 20°C (open symbols). The growth curves are representative of several independent experiments (see the text for details). (B to E) JH642-derived *B. subtilis* strains harboring the *lacZ* fusions indicated in each panel were grown in MM at 37°C (filled symbols) until early exponential phase. Then, half of each culture was shifted (at the point indicated by the asterisk) to 20°C (open symbols). Samples were collected at different OD_{525}s and assayed for β-galactosidase activity expressed in Miller units. The arrows in each panel indicate the end of the exponential growth of each culture and the beginning of the stationary phase. (E) The curves with open symbols correspond to the Miller units accumulated at 20°C in cultures proficient (□) or deficient (△) in the production of the σ^H transcription factor. In addition, the accumulation of β-galactosidase activity of the same σ^H-deficient culture grown at 37°C for the entire experiment (▲) is shown (see the text for details). The strains used for the experiments were MR100 (B), JH19005 (C), MR102 (D), and JH19005 (□) and MR200 (▲ and △) (E). The β-galactosidase experiments described above were independently repeated five times in triplicate, and a representative set of results is shown in each panel. (F) Diagram showing the appearance of Spo0A and σ^B during the vegetative growth of a cell transferred to a low growth temperature.

although *sigB* and *spo0A* are not components of the rapidly induced cold shock regulon of *B. subtilis* (6), they may constitute a second repertoire of genes induced during the vegetative growth of the adapting cell to the low growth temperature.

Since *spo0A* expression is driven from two promoters, a constitutive and weak σ^A-dependent promoter during vegetative growth and a stronger σ^H-dependent promoter activated at the end of the exponential growth (time zero [T_0]), we wondered on which sigma factor (σ^A or σ^H) the observed induction of *spo0A* expression during the vegetative growth at 20°C depended. Additionally, the transcription factor σ^H, encoded by *spo0H*, is not only essential for the burst of *spo0A* transcription that starts at the beginning of the stationary phase (T_0) at 37°C (4, 21, 24, 31) but also required for the survival of *B. subtilis* under extreme growth conditions (11). Therefore, we first corroborated that the σ^H-coding gene *spo0H* was also expressed during the logarithmic phase of cold-shocked cells (Fig. 1D). Then, we confirmed that the activity of σ^H was essential for the induction of *spo0A* transcription at 20°C since a *spo0H* deletion strain was completely unable to induce the *spo0A-lacZ* fusion after the temperature downshift (Fig. 1E). We also show (Fig. 1) that the levels of β-galactosidase activity that accumulated in σ^H-deficient cells at both temperatures (37 and 20°C) did not significantly differ from one another. This result strongly indicates, as expected (1, 2), that the enhanced β-galactosidase activity of the reporter fusions detected in wild-type cells after the temperature downshift reflected a real induction of the cold-shocked genes and not a passive accumulation of β-galactosidase as a consequence of a conceivably greater stability of the enzyme at the low temperature. Moreover, the levels of β-galactosidase activity accumulated by strain GS37 (Table 1), harboring a transcriptional *lacZ* fusion to the promoter region of the non-cold shock-inducible *fabH* gene (36), were essentially the same at 37 and 20°C (data not shown).

It has been extensively observed that the induction of *sigB* and *spo0A* expression in cultures of *B. subtilis* grown at 37°C under nonstress conditions occurs only at the beginning of the stationary phase of growth (T_0). It is believed that this postexponential induction of *sigB* and *spo0A* expression at 37°C allows the growth-restricted cells to adapt (σ^B-dependent response) or sporulate (Spo0A-dependent response) under the

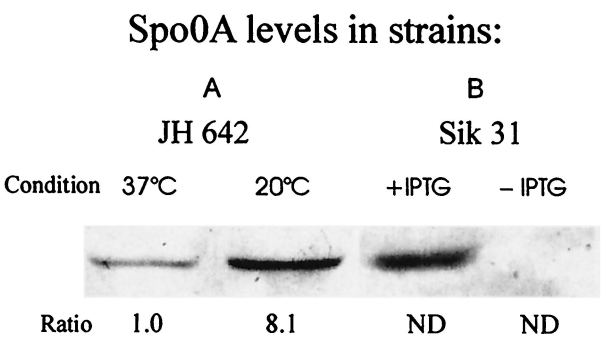

FIG. 2. Western blot analysis of Spo0A levels in vegetative cells grown at 37 and 20°C. (A) Crude protein extracts were prepared from cultures of the wild-type strain JH642 grown in MM at 37 or 20°C until the end of the exponential phase. After separation by sodium dodecyl sulfate-polyacrylamide protein gel electrophoresis and transfer of the proteins to a nitrocellulose membrane, the proteins were reacted against anti-Spo0A antibodies. (B) Specificity and sensitivity of the antibodies used (kindly provided by F. Kawamura and M. Fujita) to put in evidence the level of induction of *spo0A* (see Materials and Methods for details). The 8.1 ratio in panel A indicates the increase in the level of Spo0A (determined by densitometric analysis) that occurred during growth at the low temperature relative to the level produced by the same strain grown at 37°C. ND, not determined.

unfavorable conditions prevailing during the stationary phase (23–25, 29, 33). By contrast, the present results indicate that *sigB* and *spo0A* expression is induced after a temperature downshift from 37 to 20°C and suggest that σ^B and Spo0A would be overproduced in cold-shocked cells of *B. subtilis* many hours before the beginning of the stationary phase of growth. Moreover, the unexpected high levels of induction of *sigB* and *spo0A* expression during the logarithmic growth of *B. subtilis* at 20°C (Fig. 1B and C) leaves open the possibility that σ^B and/or Spo0A plays a previously unrecognized role during the adaptive response of this bacterium to the cold (Fig. 1F).

Spo0A is overproduced and active during the vegetative phase of cold-shocked cells. As we have shown, the expression of the regulatory genes *spo0A* and *spo0H* was highly induced after the temperature downshift (Fig. 1C and D). Since the expression of both genes is controlled by a positive autoregulatory loop (3, 4, 24, 25, 31) that requires high levels of the active phosphorylated form of Spo0A (Spo0A~P), it can be hypothesized that the observed upregulation of β-galactosidase activity derived from the *spo0H-lacZ* and *spo0A-lacZ* fusions at the low temperature reflected an overproduction of Spo0A during the vegetative phase. Hence, it was conceivable to hypothesize that the active form of Spo0A (Spo0A~P) should be present in higher levels at 20°C than at 37°C. Effectively, Western blot experiments using anti-Spo0A antibodies (4) confirmed the overproduction of Spo0A during the logarithmic growth of *B. subtilis* at 20°C (Fig. 2). The vegetative levels of Spo0A at 20°C were severalfold higher than the levels of the regulatory protein found in logarithmic cells of the same strain (JH642) grown at 37°C (Fig. 2). This result confirmed the overproduction of Spo0A and strongly suggested that Spo0A~P was predominant during logarithmic growth at the low temperature. Effectively, this presumption was confirmed since the expression of two exclusive Spo0A~P-dependent sporulation genes (*spoIIA* and *spoIIG*), which are normally activated after T_0 at 37°C, was dramatically induced many hours (~40 h) before the cold-shocked culture reached the stationary phase of growth (T_0) (Fig. 3). These results confirmed the cold induction of *spo0A* and the overproduction of active Spo0A (Spo0A~P) during the vegetative growth of *B. subtilis* at the low temperature (Fig. 3C).

A loss of Spo0A, but not the loss of the sporulation ability, results in diminished survival and a higher sensitivity to stress for cold-shocked vegetative cells. The high levels of *spo0A* expression (Fig. 1C) and Spo0A production (Fig. 2) and the evidence for its activity (Fig. 3) in wild-type cells during exponential growth at 20°C prompted us to analyze a potential improvement of the sporulation ability at the low temperature. To this end, we determined the kinetics of spore formation during the growth of wild-type strain JH642 at 20 and 37°C. As shown in Table 2, the wild-type strain formed low numbers of spores (fewer than 10^5 spores/ml) during the logarithmic and the early stationary phase of growth that were almost identical at both growth temperatures. This result suggested that the high level of Spo0A production (Fig. 2) and its premature activity (Fig. 3) during the logarithmic phase of the cultures grown at 20°C were not related to an improvement of the capacity to make spores at the low temperature. Furthermore, after 2 or 3 days in the stationary phase, the increase in the number of mature spores was more marked for the culture maintained at 37°C (an average of 5×10^7 spores/ml) than for the culture maintained at 20°C (1×10^6 spores/ml) (data not shown). The fact that *spo0A* was highly expressed at 20°C many hours (~70 to 80 h) before a significant number of spores were formed (Table 2) suggested that the activity of the transcription factor was required for a role other than that of premature spore formation. These results induced us to hypothesize that Spo0A plays a role in the survival and/or the development of full stress resistance of cells that were shocked by a temperature downshift. To test this hypothesis of a non-sporulation-related role of Spo0A during the adaptation of vegetative cells to the cold, we first examined the growth features and the survival properties of the reference wild-type strain JH642 and its isogenic *spo0A* mutant strain JH646 (Table 1). The growth of both isogenic strains did not show any significant difference among the lag phases, generation times, and the final cellular yields in MM at 20°C (data not shown). However, after the commencement of the stationary phase at 20°C, a reduction of the OD of the *spo0A* mutant was evident compared with that of the wild-type strain, which suggested that the mutant was dying faster than its parental strain (Fig. 4A). To determine the cellular viability during the stationary phase, we plated serial dilutions of both strains after their permanence during 7 days in the stationary phase at the low temperature. The result was a reproducible 10,000-fold loss of viability of the *spo0A* mutant relative to that of the wild type (data not shown). In fact, after a week at the low temperature, the average counts of viable cells from five independent experiments were 3×10^3 CFU/ml for the *spo0A* mutant and 3×10^7 CFU/ml for the wild-type strain. To address the possibility that this dramatic loss of viability was due to the impaired sporulation ability of the *spo0A* mutant, we tested the viability of a *sigF* null mutant strain grown at 20°C (3). σ^F, the product encoded by *sigF*, is the first sporulation-specific sigma factor that becomes active during sporulation, and the loss of its function completely blocks spore formation but does not impair Spo0A synthesis and its

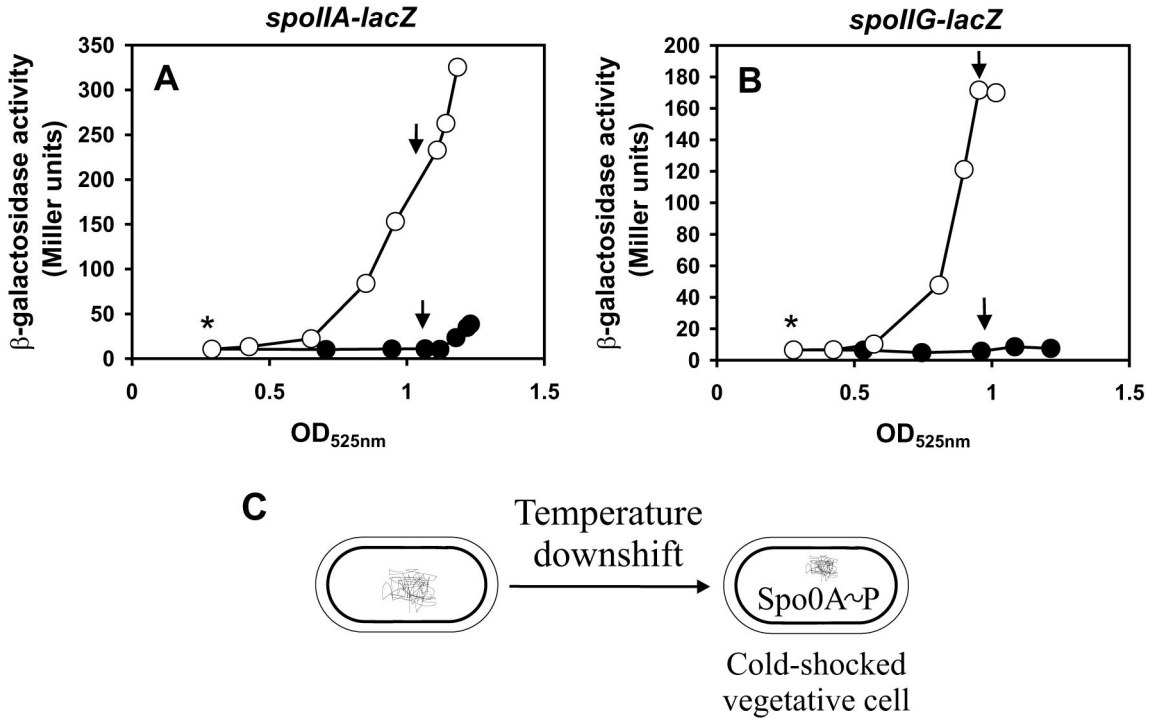

FIG. 3. Active Spo0A is present during the logarithmic phase of cold-shocked cultures. (A and B) Levels of expression of the Spo0A~P-dependent genes *spoIIA* (strain JH16302) (A) and *spoIIG* (strain JH16304) (B) in cultures grown in MM at 37°C (filled symbols) or transferred (at the moment indicated by the asterisk) to 20°C (open symbols) are shown. The arrows indicate the end of the exponential growth of each culture. (C) Diagram of the production of Spo0A~P in a vegetative cell after its transference to a low growth temperature (see the text for details).

activation by the phosphorelay signaling system (3, 24, 31). Figure 4A shows that the survival of the *sigF* null mutant was indistinguishable from that of the wild type after a prolonged incubation at the low growth temperature. After 3 weeks in the stationary phase at 20°C, the viable counts for both sporulation-deficient strains, the *sigF* and *spo0A* mutants, were 5×10^6 and 2×10^2 CFU/ml, respectively. Since the only difference between both asporogenous strains was the ability to produce Spo0A~P, it can be concluded that the diminished viability of the *spo0A* mutant strain at the low temperature was specifically due to the loss of Spo0A function and not to a general loss of sporulation ability. In this regard, one of the first functions of active Spo0A is that of repression (achieved by very low levels of Spo0A~P) of *abrB* (3, 24, 25, 31). AbrB is a key transcription factor responsible, as long as the provision of nutrients is adequate to maintain the vegetative growth, for the repression, direct or indirect, of many stationary-phase genes (24, 25, 31). Since the AbrB levels fall as the Spo0A~P levels increase (3, 31), the possibility existed that the requirement of Spo0A~P for the survival of nonsporulating cells during the stationary phase at a low temperature is indirect due to the upregulated levels of AbrB in the *spo0A* mutant strain (31). In this scenario, AbrB should play a negative role in the prolonged survival of cold-shocked cells instead of Spo0A playing a direct positive role in it. To test this possibility, we studied the survival properties of the wild-type strain and the *spo0A* mutant strain after the introduction of a null *abrB* mutation into both strains (Table 1). As shown in Fig. 4A, the introduction of the *abrB* mutation did not alter the survival properties of either strain. This result indicated that Spo0A~P plays a novel and direct role, apart from sporulation, in the adaptation to the cold and the survival of nonsporulating cells of *B. subtilis* (Fig. 4B). In this respect, one important stress factor that *B. subtilis* cells should frequently confront in the soil, its natural habitat, is the fluctuation of environmental temperature (16–18, 28, 39, 41). Therefore, we tested the ability of *B. subtilis* (the wild type and

TABLE 2. Efficiency of spore formation in wild-type *B. subtilis* cells at 37 and 20°C[a]

Growth phase	OD_{525}	37°C			20°C		
		No. of viable cells ml^{-1}	No. of spores ml^{-1}	% Survival	No. of viable cells ml^{-1}	No. of spores ml^{-1}	% Survival
Exponential	0.45	1.8×10^8	5.0×10^3	0.003	1.2×10^8	3.0×10^3	0.002
T_0	0.85	4.3×10^8	6.0×10^4	0.014	3.8×10^8	3.0×10^4	0.008
Stationary	1.00	3.0×10^8	3.0×10^5	0.1	4.7×10^8	1.0×10^5	0.02

[a] The wild-type strain JH642 was grown in MM from inoculation at 37 and 20°C. Samples of each culture were taken at the indicated times (exponential phase, T_0, and 1 day after the entrance into the stationary phase of growth). The percentage of cells that sporulated was calculated by plating serial dilutions before (viable cells) and after (spores) treatment with 10% $CHCl_3$ for 15 min. The data are the averages of results from five independent experiments.

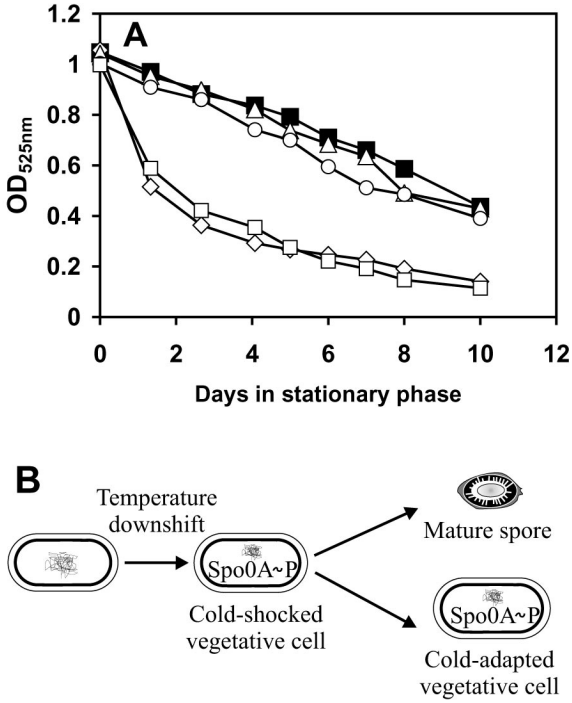

FIG. 4. The transcription factor Spo0A, and not the ability to sporulate, is required for the survival of *B. subtilis* during long permanence at a low growth temperature. (A) Survival of cold-shocked cells during permanence in the stationary phase of growth. The wild-type strain JH642 (■) and its isogenic derivatives; the single mutant strains JH646 (*spo0A* [◇]), RG12607 (*abrB*::*spc* [△]), and RG19148 (*sigF*::*kan* [○]); and the double mutant strain MR12607 (*spo0A*::*ery-abrB*::*spc* [□]) were grown in MM at 20°C until 2 weeks into the stationary phase. Cellular survival during the stationary phase was monitored by measuring the OD_{525} and by plating appropriate dilutions of samples at different times (see the text for details). The data shown are the averages of results from four independent experiments. (B) Diagram showing the two roles (sporulation and stress adaptation) of Spo0A that contribute to the survival of *B. subtilis* at a low environmental temperature.

the *spo0A* and *sigF* mutant strains) grown at 20°C until the beginning of the stationary phase (T_0) to survive the treatment at 54°C for 1 h (heat shock) before being plated. As shown in Table 3, the survival rates of the Spo^+ wild-type strain and the Spo^- *sigF* strain were essentially the same. By contrast, the *spo0A* mutant strain showed a 1,000-fold decrease in the percentage of cells that survived after the heat shock treatment. This result indicated that the inability to synthesize Spo0A, and not the inability to sporulate, was the cause for the higher sensitivity of the cold-shocked cells to heat shock. This result strongly reinforces the hypothesis of a new and important role for Spo0A~P in the development of full stress resistance and the survival of cold-adapted cells under nonsporulation conditions (Fig. 4B).

A loss of σ^B function results in a decreased rate of survival of cells grown at a low temperature. Since σ^B activity is posttranslationally controlled by a cascade of regulatory proteins (32), we wondered whether the *sigB* induction that followed the temperature downshift (Fig. 1B) reflected an upregulated activity of σ^B during the logarithmic phase of the cold-shocked cultures. To check this possibility, we measured the activity of the σ^B-dependent fusion *ctc-lacZ*, which is a traditional reporter of σ^B activity (7, 8, 12). The expression of *ctc* was also dramatically induced after the cold shock (Fig. 5A), suggesting that the alternative transcription factor σ^B was active during the logarithmic growth of *B. subtilis* at 20°C. To test the physiological role of σ^B during growth at the low temperature, we compared the levels of growth of wild-type strain JH642 and its isogenic *sigB* mutant strain MR644 at 20°C (Table 1). Comparison of the growth patterns of *sigB* mutant cells and wild-type cells after the temperature downshift revealed no difference between the two strains (JH642 and MR644) (data not shown). However, as was the case with the *spo0A* mutant, the σ^B-deficient cells started to lyse soon after the commencement of the stationary phase (Fig. 5B). The average viable-cell counts after a week at 20°C were 5×10^7 CFU/ml for the wild-type and 1×10^6 CFU/ml for the *sigB* mutant strain. This moderate but reproducible requirement of σ^B activity for survival during cold temperatures was observed previously when *B. subtilis* confronted other environmental or metabolic stresses (23, 32, 37). Thus, the present results enlarge the horizon of the known scenarios that require the activity of σ^B for a better adaptation of *B. subtilis* after an environmental insult (Fig. 5C).

A loss of σ^B function results in a delayed and decreased sporulation proficiency for *B. subtilis* at a low growth temperature. Interestingly, the *sigB* mutant strain, apart from its decreased viability at 20°C, showed a clear oligosporic phenotype after the growth on sporulation plates during a week at 20°C (Fig. 6A). This result prompted us to further examine whether σ^B was required for survival and/or sporulation of the cold-shocked cells. Therefore, we monitored the cellular viability and the sporulation proficiency of wild-type strain JH642 and its isogenic *sigB* mutant derivate MR644 (Table 1) at 20°C. The cold-shocked *sigB*$^+$ and *sigB* mutant cultures, having similar generation times (data not shown), reached essentially the same number of viable cells at the beginning of the stationary phase (T_0) (4×10^8 CFU/ml for the σ^B-proficient cultures and 5×10^8 CFU/ml for the σ^B-deficient cultures) (Fig. 6B). How-

TABLE 3. Survival of cold-adapted *B. subtilis* cells after heat shock[a]

Strain	Relevant genotype	Relevant phenotype	No. of viable cells ml^{-1} before heat	No. of viable cells ml^{-1} after heat	% Survival
JH642	*spo0A*$^+$	Spo^+	3.0×10^8	1.0×10^7	3.3
JH646	*spo0A* mutant	Spo^-	5.0×10^8	8.0×10^3	0.0016
RG19148	*spo0A*$^+$ *spoIIAC* mutant	Spo^-	2.0×10^8	1.2×10^7	6.0

[a] The wild-type strain JH642 and its isogenic JH646 and RG19148 derivatives were grown until the beginning of the stationary phase at 20°C and challenged by heat shock at 54°C for 1 h. Survival was determined by plating appropriate dilutions before and after the heat shock. The data of a representative set of experiments are presented.

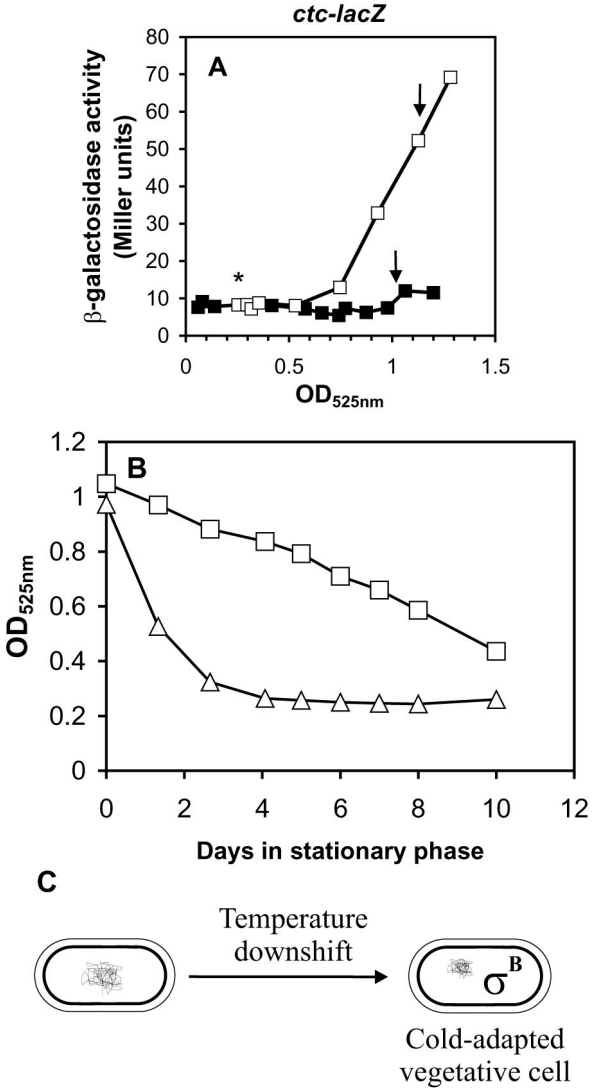

FIG. 5. The transcription factor σ^B is overproduced and required for the cellular survival of *B. subtilis* at a low temperature. (A) Induction of the σ^B-dependent reporter fusion *ctc-lacZ* (strain MR101) after a temperature downshift (indicated by the asterisk) from 37°C (filled symbols) to 20°C (open symbols). The arrows indicate the commencement of the stationary phase of growth. (B) Survival of *sigB*$^+$ and *sigB* mutant *B. subtilis* cells during the stationary phase at 20°C. The wild-type strain JH642 (□) and its isogenic *sigB* mutant derivate MR644 (△) were grown until several days into the stationary phase in MM at 20°C. Cellular survival was determined as described in the legend to Fig. 4A. The data are the averages from four independent experiments. (C) Synthesis and requirement of the σ^B transcription factor for *B. subtilis* survival after a temperature downshift during the logarithmic phase of growth.

ever, soon after T_0, i.e., 1 to 18 h after T_0 (T_1 to T_{18}), there was a reproducible 10- to 15-fold loss of cellular viability for the *sigB* mutant culture (Fig. 6B). As shown earlier (Fig. 5B), this moderate but reproducible loss of cellular viability had previously been observed in σ^B-deficient cells after different challenges (7, 8, 11, 23, 37) and confirms that the loss of σ^B function has a modest but significant effect on cellular viability at the low temperature. In fact, σ^B-deficient cells challenged at the beginning of the stationary phase (T_0) by heat shock (54°C, 1 h) showed a survival percentage of 0.5 (data not shown). This value was between the survival rates obtained for the wild-type (3.3%) and the *spo0A* mutant (0.002%) strains after the heat treatment (Table 3). Remarkably, the σ^B-deficient culture showed a lower capacity to make spores than the culture proficient in σ^B production (Fig. 6B). Throughout the stationary phase at 20°C, which lasted for 3 weeks, and even before or at T_0, when the numbers of viable cells were similar for the two strains, the number of spores formed by the *sigB* mutant culture was always significantly lower than the number of spores obtained with the *sigB*$^+$ counterpart (Fig. 6B) ($T_{-0.5}$ to T_0). Finally, after 20 days in the stationary phase, both cultures (*sigB*$^+$ strain and *sigB* mutant) were completely sporulated, with a 50-fold loss of spore efficiency for the σ^B-deficient culture. This significant and reproducible oligosporic phenotype of the σ^B-deficient culture suggested that the activity of σ^B was necessary, apart from its role in stress survival of vegetative cells, for the complete proficiency in the sporulation of *B. subtilis* grown at a low temperature (Fig. 6A).

The delay in spore formation and the reduced sporulation yield observed for the *sigB* mutant cultures at 20°C (Fig. 6) prompted us to better understand the novel requirement of the σ^B activity for efficient sporulation at a low growth temperature. To determine the stage at which sporulation was delayed and/or affected by the absence of σ^B activity at 20°C, we measured the expression of several sporulation genes that are under temporal and spatial regulation during the normal development of the spore (Fig. 7) (31). Despite the observed delay and the reduced efficiency in spore formation of the σ^B-deficient cultures at 20°C (Fig. 6), the β-galactosidase activities accumulated from the Spo0A∼P-, σ^F-, σ^E-, and σ^G-dependent genes (Fig. 7A to D) were remarkably higher for the *sigB* mutant cultures than for the *sigB*$^+$ parental strains. In contrast, the β-galactosidase activities accumulated from the same strains grown at 37°C were indistinguishable for the *sigB*$^+$ and *sigB* mutant cultures (data not shown). The upregulated accumulation of β-galactosidase activity under Spo0A∼P, σ^F, σ^E, and σ^G control in *sigB* mutant cells at 20°C (Fig. 7A to D) plus the oligosporic phenotype of these cultures at the low temperature (Fig. 6A) strongly suggested that σ^B was required at a late stage of the morphogenesis of the spore beyond σ^G activation (31). In this respect, it is interesting to mention that in the same σ^B-dependent *ctc* operon there is a gene called *spoVC* (29, 33, 42). A thermosensitive *spoVC* mutant strain ceases sporulation at a late stage of development with a deficient spore cortex development and coat formation, both of which are under σ^K control (29, 42). Since *spoVC* is under the control of σ^B activity (29, 33), it is tempting to speculate that the poor sporulation of *sigB* mutant cells at 20°C is a consequence, direct or indirect, of the poor or null activation of the *ctc* and *spoVC* genes in the absence of σ^B activity (Fig. 7E).

***sigB* induction is interconnected with the Spo0A∼P activity in *B. subtilis*.** The dramatic induction of *sigB* and *spo0A* after the temperature downshift (Fig. 1B and C) led us to examine whether there was a connection between the expressions of both regulatory genes (Fig. 8A). Since σ^B is required for the expression of its coding gene *sigB* (29, 33) and the same requirement is valid for Spo0A∼P and the induction of *spo0A* (3, 31), we used *sigB-lacZ* and *spo0A-lacZ* fusions to monitor whether σ^B and/or Spo0A∼P is essential for the cold induction

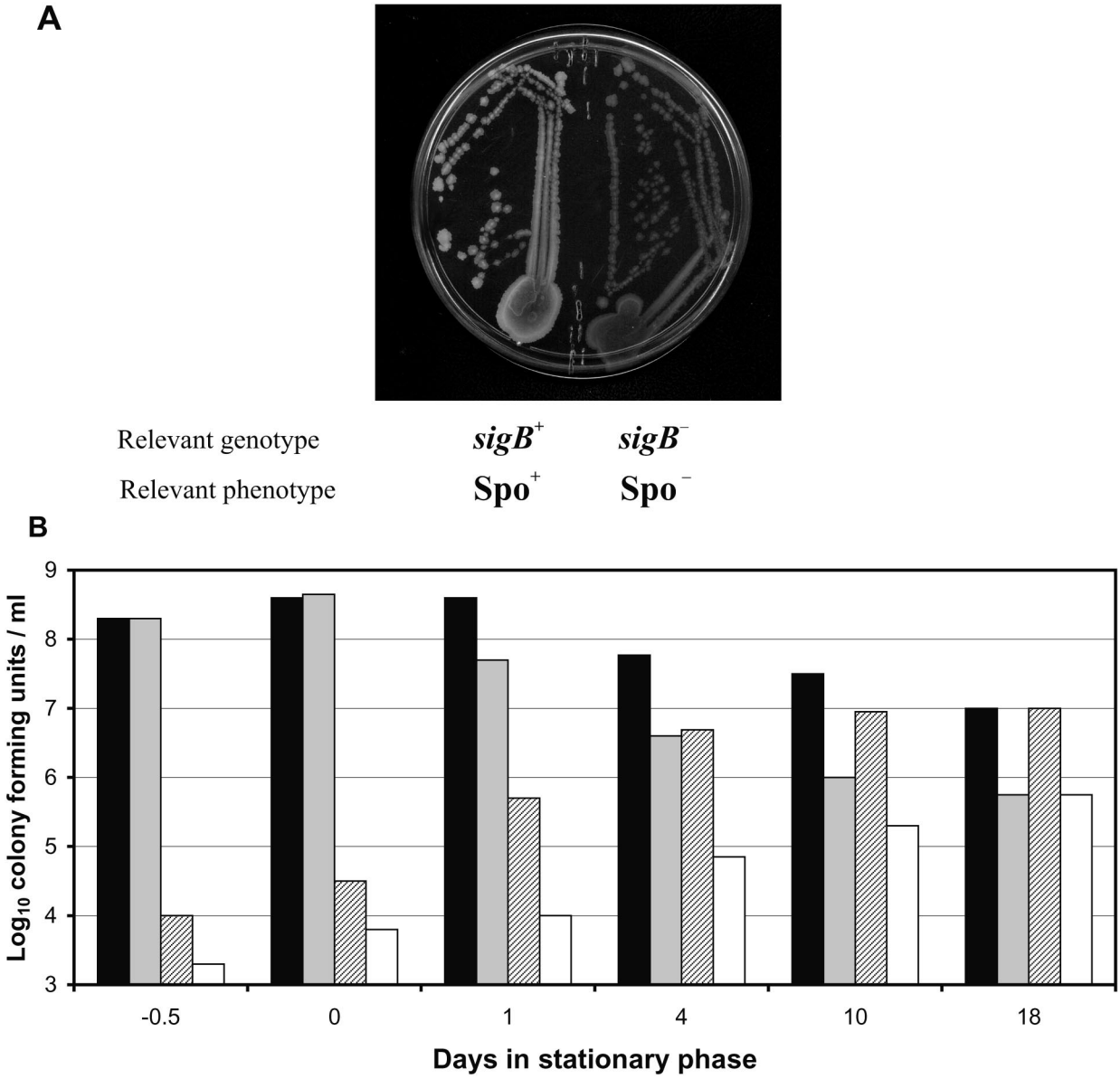

FIG. 6. The transcription factor σ^B is required for an efficient sporulation of *B. subtilis* at low temperatures. (A) Sporulation phenotype of σ^B-proficient (*sigB*$^+$) and σ^B-deficient (*sigB*$^-$) cells (strains JH642 and MR644, respectively) after 1 week of incubation on solid sporulation medium (Schaeffer agar plate) at 20°C. Note that the σ^B-deficient strain (on the right site of the plate) formed translucent and dying colonies but that the wild-type strain (*sigB*$^+$) formed opaque colonies that indicate spore formation. (B) Cellular viability and efficiency of spore formation of *sigB*$^+$ and *sigB* mutant *B. subtilis* cells during growth and maintenance at 20°C. The wild-type and *sigB* strains JH642 and MR644, respectively, were grown in MM at 20°C and assayed for cellular survival and spore formation as described in the text. Black bars, viable JH642 cells; shaded bars, viable MR644 cells; hatched bars, JH642 spores; open bars, MR644 spores). The data are the averages of results from five independent experiments.

of *spo0A* and/or *sigB*, respectively (Fig. 8A). The expression of the Spo0A~P-σ^H-dependent *spo0A-lacZ* fusion was induced, as expected, at 20°C, and this induction was not affected by the absence of σ^B activity (Fig. 8B). In contrast, even though the induction of *sigB* was not affected by the presence or absence of Spo0A~P, there was a significant enhancement of *sigB* expression in a *spo0A* mutant background at both temperatures (20 and 37°C) (Fig. 8C). These results indicated that the cold induction of *sigB* and that of *spo0A* were independent from one another (Fig. 8A, diagram III). In addition, the higher activity of *sigB* in the *spo0A* mutant strain (Fig. 8C) suggested that Spo0A~P has a direct or indirect detrimental effect on the levels of expression of *sigB* (Fig. 8D). Perhaps it is conceivable to hypothesize that in the absence of Spo0A~P activity, the alternative transcription factor σ^B might be upregulated to ameliorate the effects of the absence of Spo0A~P activity in *spo0A* mutant cells under stress conditions (Fig. 8D).

Free-living bacteria have the capacity to react rapidly to sudden upshift and downshift changes of the growth temperature (2, 6, 13, 16–18, 28, 41). In particular, *B. subtilis* has become an

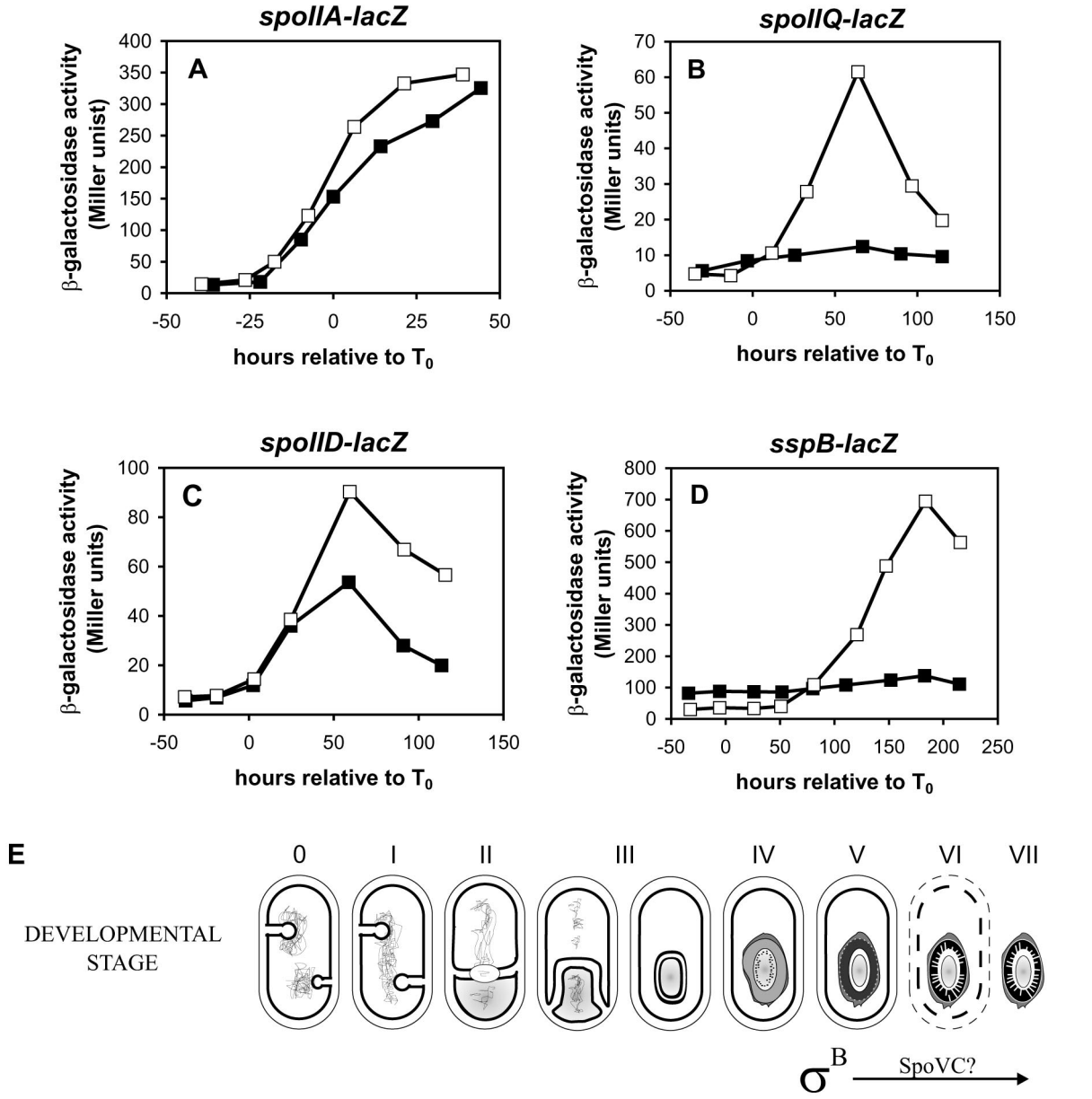

FIG. 7. The transcription factor σ^B is required at a late stage of spore development in cultures grown at a low temperature. (A to D) β-Galactosidase accumulation in σ^B-proficient (filled symbols) and σ^B-deficient (open symbols) cultures grown after inoculation at 20°C. The strains utilized for these experiments harbored transcriptional *lacZ* fusions activated by Spo0A~P (JH16302 and MR16302) (A), σ^F (RG2051 and MR2051) (B), σ^E (RG851 and MR851) (C), and σ^G (RG348 and MR348) (D). (E) Diagram illustrating the novel requirement of σ^B activity to complete the last developmental stages of spore development.

attractive model for study not only because of its capacity to undergo cellular differentiation (31) but also because of our ability to study the bacterium's cold shock response (2, 6, 13, 16–18, 28, 41). In this particular case, *B. subtilis* responds to a decreasing temperature with a rapid induction of a little less than 100 genes that conform to the cold shock regulon (6). The immediate result of this cold induction is a characteristic strong repression of major cellular metabolic activities, whereas only a limited number of processes essential for cold adaptation (principally translation machinery and membrane adaptation) are induced (2, 6, 13, 16–18, 28, 41). Among the relevant members of this regulon whose expression is induced by cold, there is a two-component system (*desK-desR*) that has recently been reported to regulate the expression of another cold-induced gene (*des*) that codes for an acyl-lipid desaturase enzyme (1, 2, 6, 10). With decreasing temperature, the membrane-bound sensor histidine kinase DesK phosphorylates its corresponding response regulator, DesR, which then binds to a specific recognition sequence in the promoter region of the Δ^5 desaturase-coding gene *des* to activate its transcription (1, 2). The activity of the fatty acid desaturase Des, located in the membrane, finally maintains the fluidity of the membrane in the cold (1, 2, 10, 13, 14, 40). Addi-

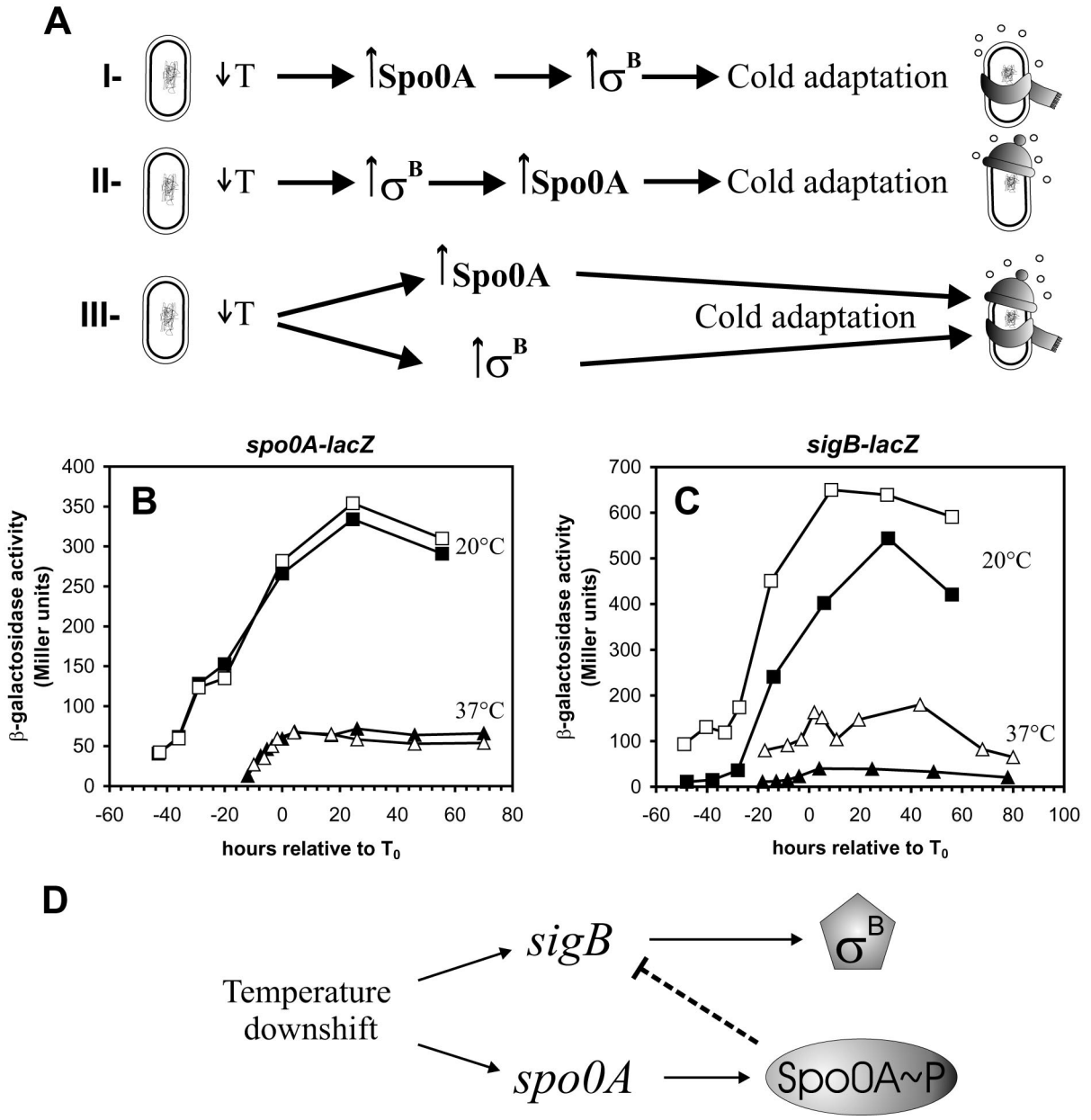

FIG. 8. Spo0A~P downregulates the expression of *sigB* at 37 and 20°C. (A) This diagram shows three possibilities for the complete adaptation of *B. subtilis* to the cold. (I) The temperature downshift induces the production of Spo0A~P, which in turn is essential for *sigB* induction and cold adaptation. (II) The alternative transcription factor σ^B is primarily induced by the cold shock, and its activity is essential for *spo0A* induction and cold adaptation. (III) The inductions of expression of *sigB* and *spo0A*, after the temperature downshift, are independent of one another. The independent induction of Spo0A and σ^B provides the cold-shocked cell with different attributes (the scarf and the wool cap) to confront the low temperature. (B and C) β-Galactosidase accumulation in JH642-derived strains proficient and deficient in σ^B (B) or Spo0A (C) function. The strains harboring a *spo0A-lacZ* (B) or a *sigB-lacZ* (C) fusion were JH19005 (*sigB*$^+$ [filled symbols]) and MR19005 (*sigB* mutant [open symbols]) (B) and MR100 (*spo0A*$^+$ [filled symbols]) and MR110 (*spo0A* mutant [open symbols]) (C). The strains had been grown since inoculation in MM at 37 or 20°C and processed as indicated in the legend to Fig. 1. (D) Shown are the independence of the cold induction of *sigB* and *spo0A* from the activities of Spo0A and σ^B, respectively (possibility III in panel A) and the negative effect, direct or indirect, of Spo0A~P activity on *sigB* expression.

tionally, the expression of the genes coding for the DNA gyrase (*gyrA* and *gyrB*, which increase the negative supercoiling of the DNA) and the DNA topoisomerase I (*topA*, which relaxes the negative supercoiling) is induced and repressed, respectively, after the cold shock (6). This change in the balance of the DNA-modifying enzymes seems to be at least a part of the system regulating membrane fluidity since it has been previously demonstrated that an increment in the negative DNA supercoiling of *B. subtilis* was essential for the transcriptional induction of *des* after cold shock (15, 26).

The main contribution of the present work is the evidence that the very recently characterized cold shock response (6) is

not sufficient for the development of a complete adaptation to low temperatures, and hence of the survival, of *B. subtilis*. In fact, the present study demonstrates that the genes encoding the key transcription factors Spo0A and σ^B are strongly induced after a temperature downshift from 37 to 20°C (Fig. 1). This transcriptional induction of *spo0A* and *sigB* was not immediate, as it was for the genes belonging to the cold shock regulon that were induced during the first minutes following the temperature downshift (6), but occurred during logarithmic growth and many hours (~45 h) before the cold-shocked cultures reached the stationary phase of growth (Fig. 1). This delay in gene activation might explain why the cold induction of *sigB* and *spo0A* was not observed in previous proteome and transcriptome studies that analyzed the initial response of *B. subtilis* to cold shock (6, 16–18, 39). Our results suggest that *spo0A* and *sigB* might correspond, as would be the case for the *sigB*-like gene of *L. monocytogenes* (5), to a second class of cold-induced genes whose function is not related to an immediate adaptation of the cell to cold shock but is related to cellular viability and stress survival during long permanence at the low temperature.

It can be unquestionably asseverated that Spo0A is essential for spore formation at any temperature, but this work has clearly demonstrated that Spo0A has a novel sporulation-independent activity required for the efficient survival of nonsporulating resting cells at a low temperature (Fig. 4). Furthermore, the strong *spo0A* expression during the logarithmic phase at 20°C (Fig. 1C) and the overproduction of active Spo0A (Fig. 2 and 3) were not correlated with an improved ability of the cold-adapted cells to form spores at the low temperature (Table 2). Effectively, the number of spores formed at 20°C did not differ from the number of spores formed at 37°C during either the exponential phase or the early stationary phase of growth (Table 2). This result opened the possibility that the overproduction of Spo0A~P during logarithmic growth at 20°C might be required for the development of full stress resistance before the cold-adapted cells abandon exponential growth. In fact, cold-adapted cells proficient in Spo0A activity at the end of exponential growth (T_0) were >2,000-fold more resistant to heat treatment (54°C, 1 h) than were the equivalent cells deficient in Spo0A production (Table 3). These properties were completely dependent on Spo0A activity and not on the sporulation ability or the activity of the transition state regulator AbrB (Fig. 4A). Therefore, these results indicated a novel and important role for Spo0A in cellular viability and stress survival at a low growth temperature (Fig. 9). This new role of Spo0A might contribute to the preparation of *B. subtilis* cells for future stresses during long-term survival as nongrowing vegetative cells in natural environments (Fig. 4B and 9).

On the other hand, σ^B was also dramatically induced and was required for cellular survival during the stationary phase of cold-adapted *B. subtilis* cells (Fig. 5). Interestingly, during the review process of this work, Brigulla et al. reported the chill induction of *sigB* expression in *B. subtilis* (9). Using a proteome approach in conjunction with Western blot analysis and the measurement of β-galactosidase activity from σ^B-dependent reporter gene fusions, those researchers showed the expression of the σ^B regulon during continuous growth at a low temperature (9). Those researchers showed that the growth of a *sigB* mutant strain was drastically impaired at 15°C but not at 20°C (9). However, the survival and stationary-phase properties of σ^B-deficient cold-shocked cells were not analyzed (9). Effectively, in the present work we show the survival properties and sporulation phenotype of a *sigB* mutant strain grown and maintained at 20°C. σ^B-deficient cultures started to lyse rapidly after the commencement of the stationary phase and were more sensitive to heat shock than were σ^B-proficient cultures at 20°C (Fig. 5 and data not shown). The rapid decline in the OD of the σ^B-deficient cultures in the stationary phase at 20°C (Fig. 5B) was accompanied by a delay and a rate of spore formation lower than that seen with those cultures proficient in σ^B production (Fig. 6). Even though the *sigB*$^+$ and *sigB* mutant cultures grown at 20°C finally reached 100% sporulation, the numbers of spores formed by the σ^B-deficient cultures were 20- to 50-fold lower than the numbers produced by the σ^B-proficient cultures (Fig. 6B). These results reinforce the view that σ^B is required for cellular viability at a low growth temperature and suggest a novel role of this key transcription factor in efficient sporulation at a low growth temperature (Fig. 9). In fact, the moderate but clear negative effect on efficient sporulation of the absence of σ^B (Fig. 6) and the probable exigency of its activity at a late stage of spore development at a low temperature (Fig. 7) were novel and unexpected. As mentioned previously, one gene under σ^B control previously reported to affect spore cortex and coat formation is *spoVC* (42). Recently, it was demonstrated that its product, the protein SpoVC, has peptidyl-tRNA hydrolase activity that is essential for vegetative growth and sporulation (27). Peptidyl-tRNA hydrolase activity is essential for recycling tRNA molecules sequestered as peptidyl-tRNA as a result of premature dissociation from the ribosome during translation (27). In nondividing cells or during the last stages of spore development, in the absence of new tRNA synthesis, the recycling by SpoVC of tRNA sequestered as peptydil-tRNA would acquire high priority. A poor or null activation of the *ctc-spoVC* operon (29, 33) after a temperature downshift in *sigB* cells might be expected from the present results and might

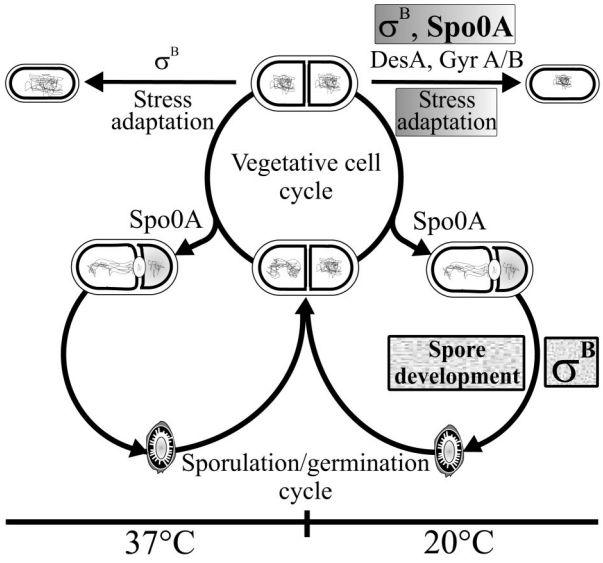

FIG. 9. Workable model marking the novel participation of the transcription factors Spo0A and σ^B in the stress adaptation and sporulation of *B. subtilis* at a growth-restricting temperature (20°C) in comparison to their roles at an optimal laboratory temperature (37°C) (see the text for details).

explain the observed oligosporic phenotype under these conditions (Fig. 7E). In addition, it is interesting to mention that Scott and coworkers have reported that Obg, an essential GTP binding protein required for *B. subtilis* sporulation, was associated with the ribosome fraction and was necessary for the stress activation of σ^B (35, 36). These observations point to the translational machinery and its putative associated proteins (Obg, SpoVC, and Ctc) for a possible coupling between sporulation and σ^B-dependent stress adaptation (35, 36, 43). In fact, we have shown that in the absence of Spo0A activity, *B. subtilis* is unable to sporulate or fully adapt and that there is a significant improvement in *sigB* expression (Fig. 8C). Taking these results into account, we suggest that sporulation and σ^B-dependent stress adaptation are interconnected pathways that allow an adequate response of *B. subtilis* to its fluctuating environment (Fig. 9).

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