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Characterization of the Operon Encoding the Alternative σ^B Factor from *Bacillus anthracis* and Its Role in Virulence

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The operon encoding the general stress transcription factor σ^B and two proteins of its regulatory network, RsbV and RsbW, was cloned from the gram-positive bacterium *Bacillus anthracis* by PCR amplification of chromosomal DNA with degenerate primers, by inverse PCR, and by direct cloning. The gene cluster was very similar to the *Bacillus subtilis sigB* operon both in the primary sequences of the gene products and in the order of its three genes. However, the deduced products of sequences upstream and downstream from this operon showed no similarity to other proteins encoded by the *B. subtilis sigB* operon. Therefore, the *B. anthracis sigB* operon contains three genes rather than eight as in *B. subtilis*. The *B. anthracis* operon is preceded by a σ^B-like promoter sequence, the expression of which depends on an intact σ^B transcription factor in *B. subtilis*. It is followed by another open reading frame that is also preceded by a promoter sequence similarly dependent on *B. subtilis* σ^B. We found that in *B. anthracis*, both these promoters were induced during the stationary phase and induction required an intact *sigB* gene. The *sigB* operon was induced by heat shock. Mutants from which *sigB* was deleted were constructed in a toxinogenic and a plasmidless strain. These mutants differed from the parental strains in terms of morphology. The toxinogenic *sigB* mutant strain was also less virulent than the parental strain in the mouse model. *B. anthracis* σ^B may therefore be a minor virulence factor.

Bacillus anthracis is the etiological agent of anthrax, a mammalian disease, and is usually regarded as the only pathogen belonging to the *Bacillus* genus (68). We have studied the response of this bacterium to various stresses by isolating its *clpB* and *clpC* genes (57). We have shown that both genes are functional and that the expression of *clpB* is induced in response to heat shock (unpublished observation). In *Bacillus subtilis*, the *clpC* operon is transcribed by a σ^B-containing RNA polymerase (41).

In bacteria, the initiation of transcription is dependent on the sigma factor associated with the RNA polymerase core enzyme. Different promoter specificities are associated with alternative sigma factors and result in a change in the pattern of gene expression. In *B. subtilis*, various environmental stresses induce the synthesis and activation of σ^B (35). σ^B then initiates the transcription of more than 100 stress genes that constitute the σ^B regulon (6, 11, 25, 35, 75). The activation of σ^B itself involves a network of regulatory proteins (Fig. 1). Seven proteins are involved in this process. They are encoded by *rsb* genes (for "regulator of sigma B") belonging to the eight-gene *sigB* operon (8, 12, 22, 39, 79, 84). This network of proteins includes kinases and phosphatases, which transmit signals to an anti-anti-σ and an anti-σ factor (39, 79, 81, 84, 86). Depending on the kind of stress encountered, the signals are transmitted to the upstream or downstream switch module (Fig. 1B) (1, 39, 79, 81, 84). The last four genes are preceded by a σ^B-dependent promoter; thus, σ^B increases its own transcription as a consequence of its activation, further inducing the entire σ^B regulon (Fig. 1A) (38, 84).

Despite improvements in our understanding of the mechanism of expression and activation of σ^B, the physiological function of σ^B has remained unclear. The first surprise came when the first σ^B mutants were constructed and found to have no impairment in growth or sporulation (10, 23). Insertional inactivation of the σ^B-dependent *ctc* gene leads to a sporulation deficiency at high temperature, indicating that genes from the σ^B regulon have physiological functions (71). One possible reason for the lack of effect of deletion of the *sigB* gene is that many of the genes controlled by σ^B also have σ^B-independent induction pathways (36). There also appears to be some gene redundancy, and so a lack of transcription of a σ^B-dependent gene can be compensated by expression of a σ^B-independent gene encoding a similar function. Indeed, multiple-mutant strains have been constructed and shown to be impaired in resistance to a given stress (24). Culture conditions have also been devised to investigate the physiological functions of σ^B, and the data obtained indicate that the σ^B regulon confers multiple stress resistance on nonsporulating cells (36, 80). However, the advantages conferred by σ^B on *B. subtilis* in its natural ecosystem cannot easily be assessed.

One way to investigate the physiological importance of the σ^B regulatory network is to test whether the partner-switching mechanism of signal transduction is widespread. This led to studies of the *sigB* operon of a closely related soil bacterium, *Bacillus licheniformis* (14). In this organism, the organization of the *sigB* operon is identical to that in *B. subtilis* (Fig. 1A). The sequences of the gene products are also extremely similar, with the least highly conserved being RsbX, for which catalytic activity rather than simple protein-protein interaction is required (14, 78). However, this high conservation does not extend to the *sigB* operons of all gram-positive bacteria.

The *sigB* operon has been characterized in several bacteria including two gram-positive species, *Listeria monocytogenes*, a facultative intracellular pathogenic nonsporulating member of the *Bacillaceae*, and *Staphylococcus aureus*, an extracellular pathogen. The aim of such studies is mainly to define the stress response that these organisms develop upon entry into the host, where they encounter hostile environments (e.g., acidic

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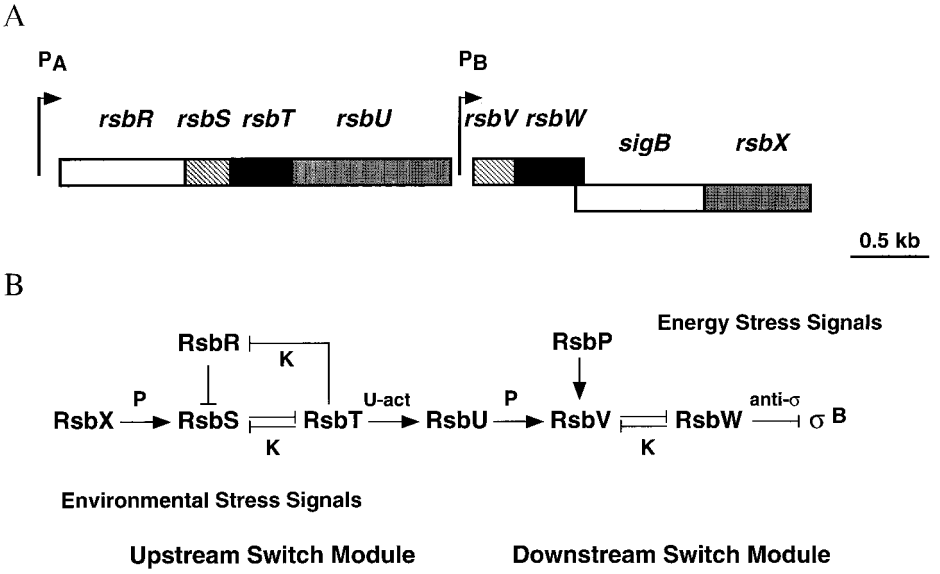

FIG. 1. Organization of the *B. subtilis sigB* operon and current model of σ^B regulation. (A) Schematic diagram of the *B. subtilis sigB* region. P_A is the promoter for the eight-gene operon, and P_B is an internal, σ^B-dependent promoter. (B) Schematic diagram of the partner-switching modules. For the function of each protein, see main text and references therein. Arrows indicate activation, and T-headed arrows indicate inhibition. P and K stand for phosphatase and kinase, respectively, and U-act stands for RsbU activator.

and oxidative shocks). The organization of the *sigB* operon in *L. monocytogenes* is somewhat similar to that in *B. subtilis* (7, 83). It contains the last four genes, at least part of *rsbU*, and the internal σ^B-dependent promoter. As in *B. licheniformis*, the least highly conserved sequence is that of RsbX. The regulatory network therefore contains the complete downstream module (Fig. 1B). It lacks part of the upstream module but contains at least the first protein of the cascade, RsbX (Fig. 1B). However, the σ^B mutant is impaired in acid stress resistance and in its response to signals such as high osmotic strength (7, 83). The genes encoding the other regulatory proteins may be located at other chromosomal loci. However, σ^B does not appear to be essential for the spread of *L. monocytogenes* in an animal model (83).

In *S. aureus*, only the partners of the downstream module are present, because the *sigB* operon contains four genes and lacks that for RsbX. There is a σ^B-dependent promoter between the first (*rsbU*) and second (*rsbV*) genes (42, 52, 85). The *rsbU* gene, the first of the operon, differs according to the strain analyzed, and an 11-bp deletion has been detected in a collection strain (43). Depending on the stress imposed, the various strains have similar or different responses (15, 30, 43). σ^B is a major regulator of the stress response and is involved in the same regulatory network as Sar (15, 16, 20). Sar is one of two global regulatory elements that control the synthesis of the extracellular and cell surface proteins involved in *S. aureus* pathogenesis. However, σ^B mutants do not seem to be less virulent than the wild type (58).

B. anthracis is a sporulating pathogen closely related to *B. subtilis* and *B. licheniformis*. We therefore decided to study its *sigB* operon. Anthrax infection begins after inoculation, ingestion, or inhalation of spores, preventing exposure of the bacteria to stressful conditions immediately after entry into the host (29, 45). Germination is required for establishment of the disease. In the murine inhalation infection model, spores germinate in the alveolar macrophages (33). Fully virulent *B. anthracis* bacilli are toxinogenic and encapsulated. The toxins and probably also the capsule are synthesized in the macrophage (27, 33). The three toxin genes are located on pXO1, and the proteins responsible for capsule synthesis are encoded by genes carried on pXO2 (31, 50). The regulation of expression of these genes has been thoroughly studied (18, 28, 34, 40, 49, 65, 68, 72, 73). They are expressed during the exponential growth phase in response to signals in the host environment (bicarbonate and temperature). Septicemia occurs later in the development of the disease, when the bacilli are under conditions of nutrient limitation.

In this paper, we report the characterization of the *B. anthracis sigB* operon and analysis of the regulation of its expression. Deletion mutants were constructed, and the toxinogenic derivative was found to be less virulent than its parental strain.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture media. *Escherichia coli* TG1 (46) was used as a host for the cloning experiments. *E. coli* HB101 harboring pRK24 (69) was used for mating experiments. The *B. subtilis* and *B. anthracis* strains used and constructed in this work are listed in Table 1.

For cloning experiments, pUC19 was routinely used (87). For mating experiments, DNA fragments were subcloned from pUC19, in which the initial constructions were made, or were directly cloned and inserted into pATΔS28 (57) or pAT113 (70). pDL was used for β-galactosidase assays in *B. subtilis* or as a source of the *bgaB* gene (88). More specific plasmids used or constructed in this work are listed in Table 1.

E. coli were cultured in Luria (L) broth or on L agar plates (51). *B. subtilis* cells were grown in L broth, on L agar plates, or in 121J medium with or without added glucose (55). *B. anthracis* cells were grown in brain heart infusion (BHI) broth (Difco) or on BHI agar plates, in L broth, or on NBY agar (31). Antibiotics were used at the following concentrations: 100 μg of ampicillin ml^{-1} and 40 μg of kanamycin ml^{-1} for *E. coli*, 100 μg of spectinomycin ml^{-1} for both *E. coli* and *B. anthracis*, 5 μg of erythromycin ml^{-1} for *B. anthracis*, and 5 μg of chloramphenicol ml^{-1} for *B. subtilis*.

DNA manipulation and sequencing. Methods for plasmid extraction, endonuclease digestion, ligation, and agarose gel electrophoresis were as described by Maniatis et al. (46). PCR amplification and the filling in of the ends of DNA molecules, using Vent DNA polymerase, were performed as indicated by the manufacturer (New England Biolabs). If bacterial colonies were used instead of DNA, the polymerase was added after an initial incubation for 5 min at 100°C. Chromosomal DNA was extracted as described by Delecluse et al. (19). Sequences were determined either from PCR products or from double-stranded DNA by the dideoxy chain termination procedure (62) using Sequenase kits (Amersham/USB) or the PRISM AmpliTaq dye primer sequencing kit (Applied

TABLE 1. *Bacillus* strains and plasmids used and constructed in this study

Organism or plasmid	Trait or relevant genotype[a]	Source or reference[b]
B. subtilis strains		
SMY	Prototroph	A. L. Sonenshein
QB4919	*trpC2 sigB*::*aphA3*	54
GL100	Δ*amyE*::*gsiB-bgaB*	pGL100→SMY
GL200	Δ*amyE*::*rsbV-bgaB*	pOSB17→SMY
GL300	Δ*amyE*::p*orf4-bgaB*	pGL300→SMY
GLQ100	*trpC2 sigb*::*aphA3* Δ*amyE*::Φ(*gsiB-bgaB*)	pGL100→QB4919
GLQ200	*trpC2 sigb*::*aphA3* Δ*amyE*::Φ(*rsbV-bgaB*)	pOSB17→QB4919
GLQ300	*trpC2 sigb*::*aphA3* Δ*amyE*::Φ(*orf4-bgaB*)	pGL300→QB4919
B. anthracis strains		
9131	pXO1$^-$ pXO2$^-$	Laboratory stock
7702	pXO1$^+$	Laboratory stock
GSB10	Δ*sigB*::*erm*	CP51 (SSB10)[c] × 9131
SSB10	Δ*sigB*::*erm* pXO1$^+$	pON12→7702
GSB1	Φ*sigB-bgaB*	pSGB10→9131
GSB2	Δ*eag*::Φ(*rsbV-bgaB*)	pOSB27→9131
GSB12	Δ*sigB*::*erm* Δ*eag*::Φ(*rsbV-bgaB*)	pOSB27→GSB10
SSB2	Δ*eag*::Φ(*rsbV-bgaB*)	pOSB27→7702
SSB12	Δ*sigB*::*erm* Δ*eag*::Φ(*rsbV-bgaB*)	pOSB27→SSB10
SSB3	Δ*eag*::Φ(*orf4-bgaB*)	pON300→7702
SSB13	Δ*sigB*::*erm* Δ*eag*::Φ(*orf4-bgaB*)	pON300→SSB10
Plasmids		
pATΔS28	*spc tra*$^+$ *B. anthracis* suicide vector	57
pB5	*bgaB bla* (4.8)	57
pDL	*amyE*::*bgaB B. subtilis* suicide vector	T. Msadek (88)
pGL100	*amyE*::Φ(pg*siB-bgaB*) *bla cat* (10.4)	This work
pGL300	*amyE*::Φ(p*orf4-bgaB*) (10.3)	This work
pJPM70	Φ(pg*siB-lacZ*) *bla cat* (8.4)	A. L. Sonenshein (55)
pON12	3′ *rsbW-sigB*::*erm-orf4 spc tra*$^+$ (7.7)	This work
pON3.12	3′ *rsbW*-5′ *sigB-orf4 spc tra*$^+$ (6.5)	This work
pON30	p*orf4-bgaB bla* (5.2)	This work
pON300	*eag*::Φ(p*orf4-bgaB*) *spc erm kan tra*$^+$ (11.6)	This work
pOSB10	*rsbV rsbW sigB*::*erm orf4 bla* (7.5)	This work
pOSB17	*amyE*::Φ(p*rsbV-bgaB*) *bla cat* (10.1)	This work
pOSB27	*eag*::Φ(p*rsbV-bgaB*) *spc erm kan tra*$^+$ (10.9)	This work
pRswA4	3′ *rsbW*-5′ *sigB spc* (5.85)	This work
pSAL322	*eag*::*spc erm kan tra*$^+$ (8.7)	47
pSB	3′*rsbW*-5′ *sigB*′ *bla* (3.4)	This work
pSigB2	*rsbW sigB orf4 bla* (4.9)	This work
pSBG2	*sigB spc tra*$^+$ (5.6)	This work
pSBG4	*sigB-bgaB spc tra*$^+$ (7.6)	This work
pSBG10	Φ(*sigB-bgaB*) *erm spc tra*$^+$ (9.5)	This work

[a] Sizes of plasmids (in kilobases) are given in parentheses.
[b] An arrow indicates construction by transformation for *B. subtilis* strains and by mating for *B. anthracis* strains.
[c] CP51-mediated transduction.

Biosystems) with an Applied Biosystems PRISM 373A sequencer. Nucleotide and deduced amino acid sequences were analyzed using the Wisconsin package (Genetics Computer Group Inc.).

General methods. *E. coli* cells were made competent as described by Chung and Miller (17). *B. subtilis* strains were transformed using the method of Kunst and Rapoport (44). Recombinant plasmids were transferred from *E. coli* to *B. anthracis* by a heterogramic conjugation procedure (69). Allelic exchange was carried out as described previously (60). Transduction experiments with bacteriophage CP51 were performed as described by Green et al. (31).

Cloning of the *sigB* locus and disruption of the *sigB* gene. The initial DNA fragment (about 750 bp) was amplified by PCR using the degenerate oligonucleotides rsbW52 and sigB147 and inserted into pUC19 (Table 2; Fig. 2) (pSB; see also Results). A fragment comprising the insert in pSB was cloned by inverse PCR. Chromosomal DNA was digested with *Eco*RI, for which there are no known sites in the target sequence, ligated, and used as a template for amplification with the divergent primers rsbW82 and rsbW135 (Table 2; Fig. 2A). The amplicon, a 2.05-kb fragment, was digested with *Cla*I, immediately 5′ to rsbW82, and inserted into pUC19, giving rise to pSigB2 (Fig. 2B). The sequence analysis indicated that the *sigB* operon was not complete. We decided to clone the genes preceding *rsbW* by using a direct cloning and selection procedure. Since the SSB10 (Δ*sigB*) strain had been constructed with an erythromycin resistance cassette inserted into *sigB*, an erythromycin-resistant clone could be selected after digesting SSB10 chromosomal DNA with an enzyme for which there was a site either within *orf4* or 3′ to it and no site in either the resistance cassette or the rest of the known sequence. Various restriction enzymes were used alone or in combination (*Eco*RV, *Alw*NI, and *Hpa*I). *Eco*RV digestion gave rise to the 4.9-kb DNA fragment of pOSB10 (Fig. 2B; Table 1).

Δ*sigB* strains were constructed as follows. A fragment overlapping the 3′ end of *rsbW* and the 5′ end of *sigB* was amplified using rsbW135 and sigB662 as primers (Table 2; Fig. 2A). The fragment was digested with *Sma*I and inserted into pATΔS28, giving rise to pRswA4 (Fig. 2B). A DNA fragment overlapping *orf4* was amplified with sigB1280 and sigB1953 as primers (Table 2; Fig. 2A), digested with *Sma*I and *Bam*HI and inserted into pRswA4. Plasmid pON3.12 (Fig. 2B) was digested with *Sma*I, and an erythromycin cassette was inserted into it, giving rise to pON12 (Fig. 2B; Table 1). The cassette therefore replaces the DNA fragment between oligonucleotides sigB662 and sigB1280. HB101(pRK24) was transformed with pON12, and the transformant was used in mating experiments with *B. anthracis* 7702 (pXO1$^+$) to produce SSB10, the Sterne Δ*sigB* derivative. To obtain the plasmidless Δ*sigB* strain, GSB10, a phage transduction experiment using CP51 was carried out with SSB10 as the donor and 9131 as the recipient (Table 1).

Construction of pg*siB-bgaB*, p*rsbV-bgaB*, p*orf4-bgaB*, and *sigB-bgaB* transcriptional fusions. The *gsiB* promoter was obtained by digesting pJPM70 with *Eco*RI

TABLE 2. Primers used in the construction of plasmids

Primer name	Sequence (5′ to 3′)[a]
rsbW52	ACI AAY GCD GTD MAR CAY GCD TAY AAR GAR
sigB147	CAT YTC CAT IGY YTC HAR HAC YTC YTC YTC
rsbW82	TTG GCG CGC CAA AGC TAA CCC CAT TAT CAG CAA C
rsbW135	TTG GCG CGC CGT ATG ATA TTA GTA AAC CTG TAG
sigB662	TAA CCC GGG TAA CAT GCC TAC TTG TAT AAT ATC C
sigB1280	CAA CCC GGG GAT GTT TAA AAC ATG AGA AAA GGG GTA C
sigB1953	CGG GAT CCG GAT TAT CAT CTA CAA TTA AAA TGG AC
rsbV−80	GTG TTA AGC TGA GAA AGA TAT AGA AAA
rsbV+20	GCA AAA TAT TTA TTC CCA AAT TCA TCA
orf1030	TTG AAT CTG TAG GTG AAG TAG AGC AAG G
orf1361	TTC CGC TAA ATC TTC ATT CAA TCC TTC G
sig266	GGG AAT TCG GAT ATT ATA CAA GTA GGC ATG TTA GG
sig1238	GGG GTA CCT TAT GTA TCT AAA AAT GCG GCT TGT TTC

[a] The code used is as follows: D, G or A or T; H, A or T or C; M, A or C; R, A or G; Y, C or T. The restriction sites included in the oligonucleotide sequences, for use during the cloning experiments, are underlined (see Materials and Methods).

and *Hin*dIII (55). The 370-bp fragment was blunted and inserted into pDL that had previously been cut with *Sna*BI, giving rise to pGL100 (Table 1).

pOSB17, harboring the *rsbV-bgaB* fusion, was constructed by amplifying the *rsbV* promoter region with primers rsbV−80 and rsbV+20 (Table 2; Fig. 2) and inserting this fragment into pDL digested with *Sna*BI. The 2.25-kb fragment containing the fusion was purified after digesting pOSB17 with *Eco*RI and *Ecl* 136II. This fragment was blunted and inserted into pSAL322 (48), which had been digested with *Bam*HI and treated with Vent DNA polymerase. The resulting plasmid was pOSB27 (Fig. 2B; Table 1).

The *orf4-bgaB* fusion was constructed by inserting the amplified orf1030-orf1361 fragment (Table 2; Fig. 2A) into pDL digested with *Sna*BI, giving rise to pGL300 (Fig. 2B). pON30 was obtained by inserting the DNA fragment used to construct pGL300 into pB5 digested with *Sna*BI (Fig. 2B) (57). pON300 was constructed by inserting the pON30 2.85-kb *Pvu*II fragment into pSAL322 (Fig.

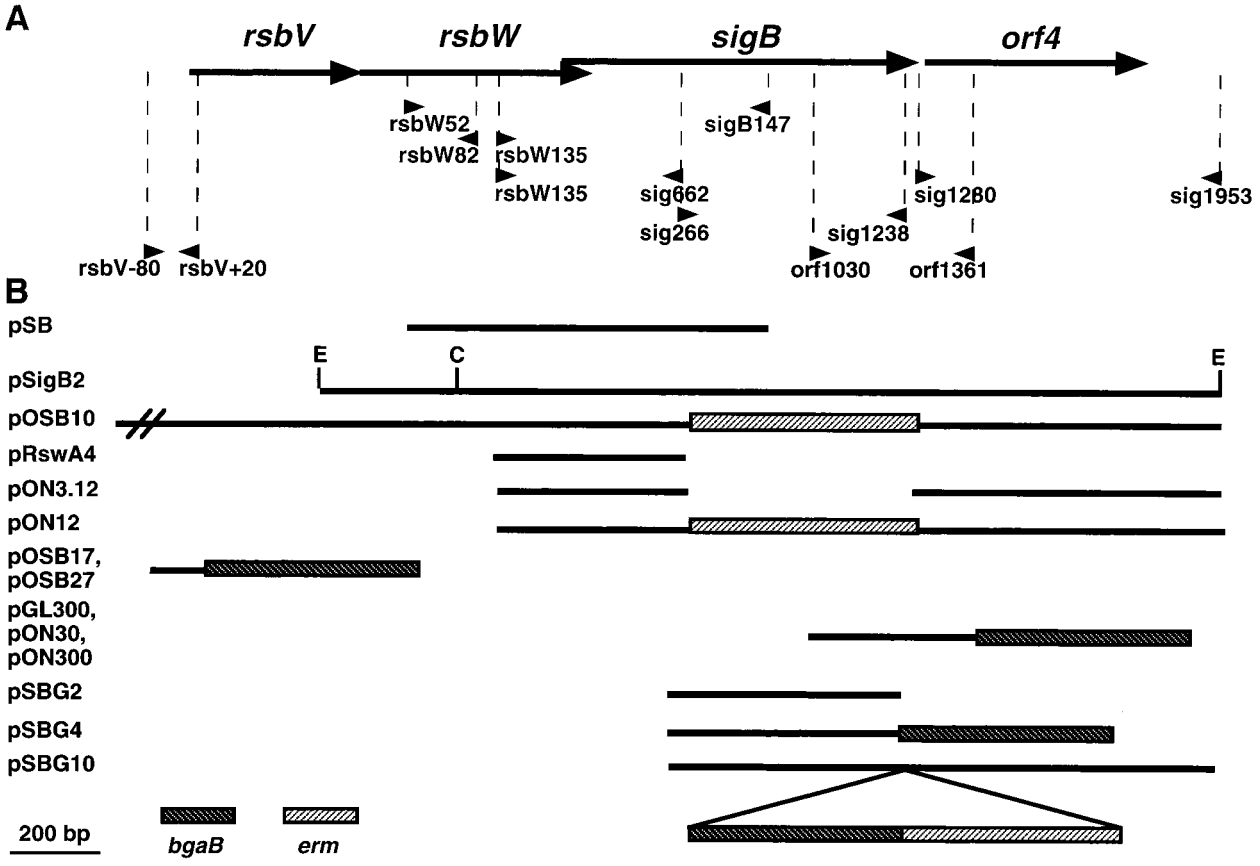

FIG. 2. Schematic diagram of the *B. anthracis sigB* region. (A) The *sigB* operon and the following ORF are represented by long arrows indicating the size and direction of transcription of the genes identified from sequence data. The arrowheads at the ends of the dashed lines indicate the position and orientation of binding of the oligonucleotides used for the cloning experiments described in this work. For the sake of clarity, they have been aligned and sometimes duplicated to indicate the fragments obtained using the various pairs. The 1.8 kb 5′ and 0.3 kb 3′ to the four indicated genes, which were cloned and sequenced from pOSB10, are not represented. (B) Schematic representation of the *B. anthracis* chromosomal fragments cloned in different vectors during this work. The *bgaB* and *erm* cassettes are also represented. The only restriction sites indicated are those used for chromosome walking by inverse PCR: E, *Eco*RI; C, *Cla*I.

2B). The vector was digested with *Bam*HI, and all the fragments were treated with Vent DNA polymerase before ligation (Table 1).

The pDL derivatives were used to transform *B. subtilis* SMY and QB4919 (Table 1). The corresponding inserts were integrated into the chromosome within the α-amylase gene by double crossover. The inactivation of the α-amylase gene was demonstrated by the absence of a halo of starch hydrolysis on TBAB (Difco)-starch plates stained with 1% iodine.

The pSAL322 derivatives were transferred by mating into *B. anthracis* strains (Table 1). The corresponding inserts were integrated into the chromosome within the *eag* gene by double crossover. Integration was demonstrated by the loss of the erythromycin resistance provided by the vector and was checked by appropriate PCR amplifications.

The nondisruptive *sigB-bgaB* transcriptional fusion, inserted into the *sigB* locus, was constructed as follows. A DNA fragment was amplified using sig266 and sig1238 as primers (Table 2; Fig. 2A), digested with *Eco*RI and *Kpn*I, and ligated into pATΔS28, giving rise to pSBG2 (Fig. 2B). The *bgaB* gene was extracted from pDL by *Kpn*I-*Ecl*136II double digestion and inserted into pSBG2 digested with *Kpn*I and *Sma*I. The resulting plasmid carrying the fusion was called pSBG4 (Fig. 2B). To construct pSBG10, the *erm* gene and *orf4* were simultaneously amplified using sig266 and sig1953 as primers and pON12 as template (Fig. 2; Table 1). The amplicon was inserted into pSBG4 that had been digested with *Bam*HI and blunted. The orientation of the insert (*sigB-bgaB-erm-orf4*) was checked by PCR. pSBG10 was then transferred into *B. anthracis* 9131, and correct insertion by double crossover into *sigB* and *orf4* was checked (GSB1) (Table 1).

Enzyme assay. β-Galactosidase activity was determined as described by Dingman et al. (21), except that the assay temperature used was 55°C instead of 37°C. The protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce). The curves show results from a typical experiment; each experiment was carried out at least three times.

Infection of mice. Pathogen-free 6-week-old female Swiss mice were supplied by IFFA-CREDO. Groups of 10 mice were subcutaneously injected with different spore doses (10^4 to 10^8) of strain 7702 or SSB10, and mortality was monitored as described previously (32).

Nucleotide sequence accession number. The sequence in this paper has been deposited under accession number AJ272497.

RESULTS

Cloning of the *sigB* locus from *B. anthracis*. We first looked for well-conserved amino acid sequences in RsbW and σ^B from *B. subtilis* (10, 23) and *S. aureus* (85). Using published Bestfit comparisons, we identified residues 52 to 61 for RsbW and 147 to 156 for σ^B (*S. aureus* numbering) and used these sequences to design degenerate oligonucleotides (rsbW52 and sigB147 [Table 2]) (85). If the two sequences were not identical for a particular residue, we used the residue from the *B. subtilis* sequence because *B. anthracis* is phylogenetically closer to this organism.

We used these primers to amplify and clone an initial DNA fragment, giving rise to pSB (see Materials and Methods). Sequence analysis indicated that the correct fragment had been isolated. The closest matches for the two incomplete open reading frame (ORF) products were with *B. subtilis* RsbW and σ^B, respectively. Inverse PCR was successfully carried out with oligonucleotides rsbW82 and rsbw135 to expand the isolated region (pSigB2; see Materials and Methods). pSigB2 starts 30 bp 5′ to *rsbW* and ends 280 bp 3′ to *orf4* (Fig. 2B). We were unable to clone the 5′ sequence of the σ^B operon using this approach. An erythromycin resistance cassette was therefore introduced into the *sigB* sequence, replacing the DNA fragment located between oligonucleotides sig662 and sig1280 (Fig. 2A, pON12; Fig. 2B, SSB10 [see Materials and Methods]). Using a restriction enzyme that did not cut the known sequence, we cloned a fragment covering the entire region (Fig. 2B, pOSB10 [see Materials and Methods]). pOSB10 contains the four genes shown in Fig. 2 and also approximately 1.8 kb 5′ to *rsbV* and 280 bp 3′ to *orf4*.

Sequence analysis for the *B. anthracis sigB* locus. The sequence of pOSB10 was determined and analyzed (Fig. 2). Since the completion of this part of the work, The Institute for Genomic Research (TIGR) has begun sequencing the *B. anthracis* genome. We regularly compared our sequence with their data and found that the contigs identified by the BLASTN search are 100% identical to the sequence overlapping the four genes presented. The DNA sequence of the initial fragment harbored three ORFs that could be organized into an operon. A BLASTP (version 2.0.10) search was carried out with each translation product (5). The first, 112 amino acids long, hereafter referred to as RsbV, was most similar to the *S. aureus* and *B. subtilis* RsbV factors (*E* values, 4×10^{19} and 2×10^{18}, respectively). Similarly, the predicted product of the second ORF, a 161-residue polypeptide, was most similar to *B. subtilis* and *B. licheniformis* RsbW factors (7×10^{47} and 3×10^{46}), and that of the third ORF, a deduced 257-amino-acid protein, was most similar to *L. monocytogenes* and *B. subtilis* σ^B (2×10^{74} and 6×10^{73}). ORF2 and ORF3 were therefore called *rsbW* and *sigB*. As expected, from the high level of similarity between the sequences of the proteins encoded by *B. subtilis sigB* and *sigF*, the three deduced amino acid sequences showed various levels of similarity to the products of the *sigF* operon (*E* values, 3×10^{12}, 2×10^{7}, and 7×10^{33} for SpoIIAA, SpoIIAB, and σ^F, respectively; 23 to 33% identity and 51 to 60% similarity). The *rsbV* ORF is preceded by a consensus *B. subtilis* σ^B recognition sequence (GTTTAA 13 bp GGGTAa) (35, 67).

No putative ORF was found immediately 5′ to *rsbV*. In fact, there are multiple translation stop codons in all frames covering the 600 bp preceding *rsbV*. Furthermore, the ORF downstream from *sigB* showed no similarity to *B. subtilis rsbX*. Thus, unlike the *B. subtilis sigB* operon, which contains eight genes with an upstream σ^A consensus sequence and an internal σ^B-dependent promoter, the *sigB* operon of *B. anthracis* has only three genes, with a single putative promoter (84). The absence of *rsbX*, whose product acts early in the σ^B regulatory cascade, has already been reported for *S. aureus* (9, 11, 42, 85). However, in *S. aureus*, *rsbV* is preceded by *rsbU*. A three-gene operon is also encountered in the sporulation factor σ^F-encoding operon of *B. subtilis* (*spoIIA*). However, sequence comparisons suggested that the *B. anthracis* operon studied does not encode σ^F. The second-best matches identified by a BLASTN (2.0a19MP-Wash-U) search with the incomplete TIGR sequence were translated and used to screen SubtiList (4, 53). The second-best match identified for RsbV was SpoIIAA, suggesting that the *sigF* operon also exists in *B. anthracis* but is not the operon studied here. No second *sigB*-like operon was identified, and biological data confirmed that the locus studied was not the *sigF* operon (see below).

It has been suggested that, for physiological reasons, additional regulators may be encoded elsewhere on the chromosome of *S. aureus* (14). We therefore searched for equivalents of the *rsb* genes in the *B. anthracis* sequence available on the TIGR site, as well as for other homologs as a control (66). We found sequences with high scores for similarity to *B. subtilis* SpoIIAA, SpoIIAB, and SpoIIE but found no sequences similar to RsbR or RsbS. Thus, the closest match, as expected in the absence of a true homolog, was with SpoIIAA. We also found no sequences similar to RsbT or RsbU; the closest match was, as expected, the end of SpoIIE. We also found no sequence similar to RsbX. Recently, another positive regulator of *B. subtilis* σ^B, RsbP, has been characterized (74). A BLAST search of the TIGR sequence with this PP2C phosphatase sequence suggested the existence of a homolog in *B. anthracis*. The sequence identified showed 44% identity and 76% similarity over the 100 central residues (residues 156 to 240) (*E* value, 4×10^{17}). This rather low score may be due to the small size of the contig pulled out (542 nucleotides). A 428-amino-acid homolog of Obg was also identified (*E* value, 7.4×10^{187}). Obg is an essential GTP-binding protein, which is required for

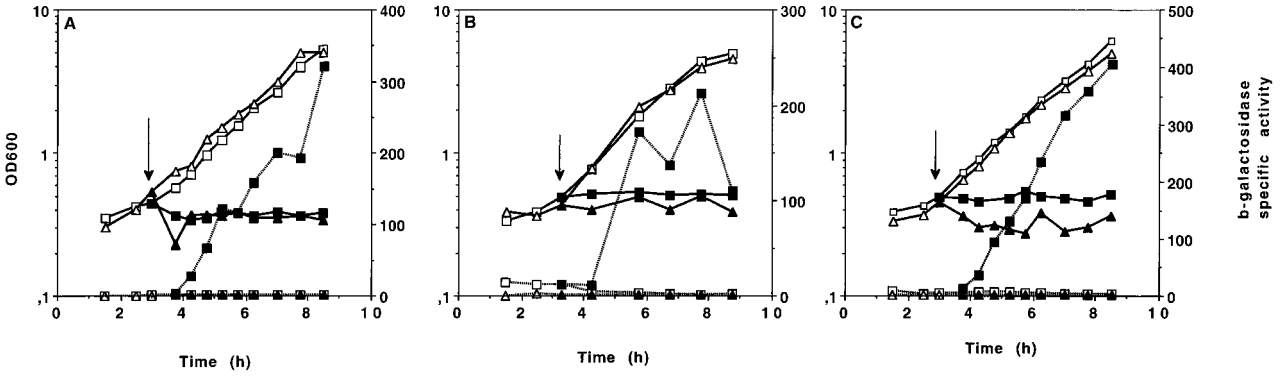

FIG. 3. Expression of β-galactosidase from p*gsiB-bgaB* (A), p*rsbV-bgaB* (B), and pORF4-*bgaB* (C) fusions in parental (squares) and Δ*sigB* (triangles) *B. subtilis* strains. Samples were assayed at the times indicated for growth (continuous lines) and for β-galactosidase activity (dotted lines). The bacteria were cultured in 121J medium (open symbols) and 121J medium from which glucose was depleted at the time indicated by the arrows (solid symbols). OD600, optical density at 600 nm.

the stress activation of *B. subtilis* σ^B but does not belong to the *sigB* operon (64).

The product of the ORF just downstream from *sigB* (designated *orf4*) is approximately 30% identical and 50% similar (depending on the bacterial origin of the protein [*E* values, 3×10^6 to 0.001]) to various bacterioferritin proteins. A chromosome-encoded iron capture system has been found in *B. anthracis* (T. M. Koehler, R. Pasha, and R. P. Williams, Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992, abstr. B-125, 1992). The *orf4* gene product is also 27% identical and 40% similar to a nutrient starvation-induced DNA-binding protein (encoded by the *dpsA* gene) from *Synechococcus* strain PCC7949 and its homolog from *Synechococcus* strain PCC6301 (*E* values, 0.002 and 7×10^4, respectively). This ORF is preceded by a sequence similar to the *B. subtilis* σ^B consensus recognition sequence (GTTTAA 13bp GGGTAc) (35, 67). The synthesis of the protein encoded by this ORF may therefore be responsive to stress conditions, making it a candidate for membership of the putative *B. anthracis* σ^B regulon.

The clear difference in σ^B operon organization between *B. anthracis* and other *Bacillus* species led us to investigate whether the organization of this operon was unique to this pathogen. We used Southern blotting to analyze the chromosome region harboring *sigB* in various bacteria from the *Bacillus cereus* group closely related to *B. anthracis*, namely, *Bacillus thuringiensis* (III-BL, III-BS, and subsp. *konkukian* 97-27) and *Bacillus cereus* (II4, T6/9778, S8553, and PC1) (37, 59, 61). In all strains tested, including *B. anthracis* 9131, the same DNA fragment of 5 kb hybridized with the *sigB* and *orf4* probes, obtained by PCR amplification with sig266 plus sig1238 and with sig1280 plus sig1953, respectively. This suggested that there is no other *sigB* operon in *B. anthracis* and that a similar chromosomal organization is shared by other closely related organisms. To unambiguously test the absence of *rsbX* immediately 3′ to *sigB* in these bacteria from the *B. cereus* group, PCR amplification was carried out on these chromosomal DNAs with convergent oligonucleotides, one internal to *sigB* and the other one internal to *orf4* (orf1030 and orf1361) (Fig. 2A). The same, approximately 300-bp, DNA fragment was obtained in all cases (data not shown). There is therefore no space for *rsbX* immediately 3′ to *sigB*. All these members of the *B. cereus* group therefore seem to lack *rsbX* and probably have a *sigB* operon similar to that of *B. anthracis*.

Characterization of a *sigB* deletion mutant of *B. anthracis*. The sequence data showed the *sigB* operon to be the most similar to the operon studied, but the genetic organization of the operon was more like that of the operon encoding σ^F, a sporulation transcription factor. To discriminate between these two possibilities, we constructed mutants in which *sigB* was deleted and assayed the sporulation efficiency of these mutants. In liquid BHI medium and on NBY agar, the mutants sporulated over the same period as and with similar efficiency to the parental strains. Thus, this operon does not encode a transcription factor that is necessary for sporulation, as σ^F is in *B. subtilis*.

The *sigB* deletion mutant and the parental strain differed in morphology. The mutant produced smaller colonies on BHI agar plates, flocculated during growth in liquid medium, and was more difficult to harvest by centrifugation, building up as cotton-like rather than sand-like pellets. These phenotypic differences became clearer with advancing cultures. Optical microscopy showed that the mutant was present as longer filaments than the parental strain. The observed phenotype was very similar to that observed for the *S. aureus* Δ*sigB* strain, except for the obvious differences due to one bacterium being a bacillus (long filaments) and the other being a coccus (aggregates) (43). The observed morphological modifications indicated that this gene is usually expressed.

***B. subtilis* σ^B-dependent expression of two putative *B. anthracis* promoters.** We studied the σ^B dependence of the *sigB* promoter-like sequences by monitoring their transcriptional response to various environmental conditions in the bacterium in which σ^B was first described (*B. subtilis*). We made three different constructs. The first, a positive control, contained the *B. subtilis gsiB* promoter fused to *bgaB*, which encodes a thermostable β-galactosidase (55, 56). *gsiB* responds to multiple stimuli in a σ^B-dependent manner and is one of two genes well characterized as being solely under the control of σ^B (2, 47). In the other two constructs, *bgaB* was preceded by one of the two *B. anthracis* σ^B promoter-like sequences, that upstream from *rsbV* or that upstream from *orf4*. These constructs, pGL100, pOSB17, and pGL300 (see Materials and Methods) (Table 1), respectively, were integrated into the chromosome of wild-type *B. subtilis* and of a *sigB* deletion mutant (GL100, GL200, and GL300, and GLQ100, GLQ200, and GLQ300, respectively [Table 1]). The effect of glucose depletion was then analyzed (Fig. 3). As expected, *gsiB* was expressed at low levels during exponential growth in medium containing excess glucose and was induced rapidly in response to glucose limitation in the wild-type background (Fig. 3A). No induction was observed if the same experiment was carried out in the Δ*sigB* background (Fig. 3A). Similar results were obtained with the strains har-

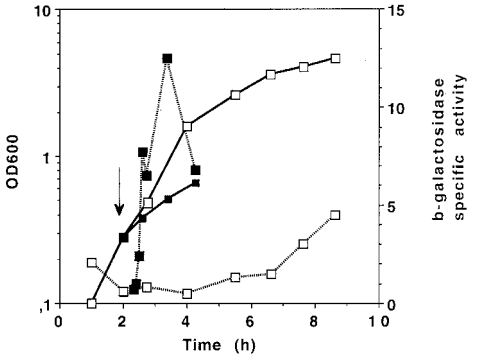

FIG. 4. Expression of β-galactosidase from a *sigB-bgaB* fusion in *B. anthracis* GSB1 in the stationary phase and in response to heat shock. Samples were assayed at the times indicated for growth (continuous lines) and β-galactosidase activity (dotted lines). Bacteria were cultured in L broth at 37°C (open symbols) or subjected to heat shock (arrow) and then cultured further at 44°C (solid symbols). OD600, optical density at 600 nm.

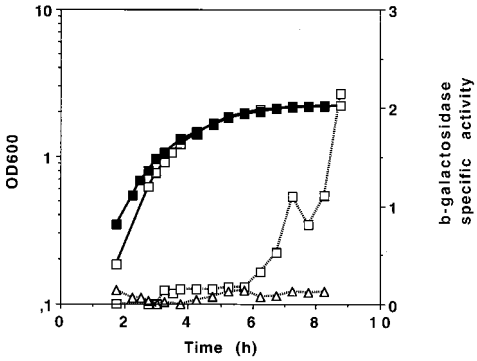

FIG. 5. Expression of β-galactosidase from a p*orf4-bgaB* fusion in parental (squares) and Δ*sigB* (triangles) *B. anthracis* strains SSB3 and SSB13, respectively, during growth. Samples were assayed at the times indicated for growth (continuous lines) and β-galactosidase activity (dotted lines). Bacteria were cultured in L broth. OD600, optical density at 600 nm.

boring the promoters preceding *rsbV* and *orf4* (Fig. 3B and C, respectively). This indicates that the sequences are efficiently recognized by *B. subtilis* RNA polymerase and that, like the *gsiB* promoter, they are dependent on *B. subtilis* σ^B for their transcription.

Expression of the *B. anthracis sigB* operon. The morphological changes induced by the deletion of *sigB* suggested that this gene is normally transcribed in *B. anthracis*. To confirm this and to study the regulation of expression of the *B. anthracis sigB* operon, the *bgaB* gene was inserted between the translational stop codon of *sigB* and the beginning of *orf4* (Fig. 2B, strain GSB1 [see Materials and Methods]). We monitored the transcriptional response of the *sigB-bgaB* fusion during growth by assessing β-galactosidase activity (Fig. 4). β-Galactosidase specific activity increased during the stationary phase, starting shortly after T_0 (end of exponential phase). However, this increase in activity was low and persisted throughout the stationary phase (Fig. 4). The highest values reached were consistently those for overnight cultures, with values of 6.5 ± 1 units. To assess the response to stress of this σ factor, we subjected the culture to heat shock (Fig. 4). The β-galactosidase specific activity rose immediately. The transcription of this operon is therefore stress inducible.

To determine whether the σ^B consensus sequence upstream from *rsbV*, the probable promoter of the three-gene operon containing *sigB*, and the promoter preceding *orf4* were indeed *B. anthracis* σ^B dependent, we constructed two plasmids homologous to those used to assay the *rsbV* and *orf4* promoters in *B. subtilis* (pOSB27 and pON300 [Fig. 2B; Table 1] [see Materials and Methods]). They were inserted into the *B. anthracis* chromosome, in the independent *eag* locus, in the parental strains (9131 and 7702) and *sigB*-deleted derivatives (GSB10 and SSB10) (forming GSB2, SSB2, SSB3, GSB12, SSB12, and SSB13, respectively [Table 1]). Figure 5 shows the results obtained with SSB3 and SSB13, the strains harboring the *orf4* promoter-*bgaB* transcriptional fusion. The β-galactosidase specific activity rose during growth, as in GSB1 (Fig. 4), in the parental background, SSB3, but not in the Δ*sigB* mutant, SSB13 (Fig. 5). This indicates that the *orf4* promoter is *B. anthracis* σ^B dependent. The *rsbV* promoter was also found to be *B. anthracis* σ^B dependent from a comparison of the β-galactosidase specific activity values obtained for late-stationary-phase and overnight cultures in parental (GSB2 and SSB2) and Δ*sigB* (GSB12 and SSB12) backgrounds (1 and 0.15 U, respectively).

In vivo role of the *B. anthracis* σ^B factor. We injected groups of 10 mice with different doses (10^4 to 10^8) of spores of the 7702 strain or its Δ*sigB* derivative, SSB10. Repeatedly, the number of deaths with given doses of the Δ*sigB* strain were similar to those obtained with the 1-log-unit lower doses of the parental strain, suggesting a 1-log-unit difference in the 50% lethal dose (LD_{50}). Consequently, for a given dose, the number of deaths was smaller with the Δ*sigB* strain than with the parental strain. Because there is a certain variability, the determination of a precise LD_{50} for the Δ*sigB* strain has been hampered. We have therefore chosen to represent, as an example, the cumulative mortality with a dose equivalent to 1 LD_{50} for the parental strain (10^5 spores) for both strains (Fig. 6). Thus, the Δ*sigB* strain was less virulent than the parental strain. To rule out an effect on toxin syntheses, the in vitro production of protective antigen, i.e., the binding domain common to both toxins, was assayed. It was found to be identical in the mutant and parental strains (data not shown). This is consistent with previous results showing that the three toxin genes are transcribed during the exponential phase of growth, i.e., before the synthesis of σ^B in the absence of stress (65). In addition, no σ^B consensus recognition sequence has been identified upstream from the promoters of the toxin genes (13, 18, 26, 40, 82).

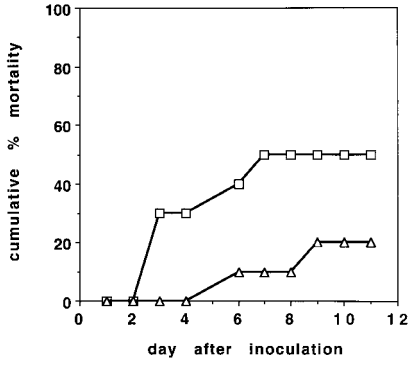

FIG. 6. Virulence of *B. anthracis* SSB10 (triangles) and 7702 (squares) strains. Swiss mice were inoculated subcutaneously with 10^5 spores per mouse (groups of 10 mice). Mortality was recorded daily and plotted as the cumulative number of deaths.

DISCUSSION

In this study, we identified the operon encoding σ^B in *B. anthracis*. The genetic organization of the σ^B operon is identical in *B. subtilis* and *B. licheniformis* and differs from the organization of those in *L. monocytogenes* and *S. aureus*, which also differ from one another. In *L. monocytogenes*, the first four genes are thought to be present because part of the fourth gene (*rsbU*) has been shown to precede the fifth and because, most importantly, the last, *rsbX*, whose product belongs to the upstream module, is also present (Fig. 1) (7, 85). *rsbX* is absent from the *S. aureus sigB* operon, which contains four genes (42, 83). Since *B. anthracis* belongs to the genus *Bacillus*, we thought that its *sigB* operon would probably be identical to that of the other two *Bacillus* species studied. In fact, its organization, with three genes, *rsbV*, *rsbW*, and *sigB*, that seem to be conserved in strains from the *B. cereus* group, is closer to that of the *B. subtilis sigF* operon than to that of any *sigB* operon. However, this is not the *B. anthracis sigF* operon. Our data therefore suggest that neither phylogeny nor physiological similarity (the capacity to sporulate under given growth-limiting conditions) imposes conservation of the genetic organization of the operon encoding the general stress σ factor.

We assessed the expression of the studied σ factor operon in *B. anthracis*. To that end, we constructed a *sigB* deletion mutant. This mutant differed morphologically from the parental strain but sporulated normally. We further analyzed whether this operon encoded a stress response transcription factor by studying the regulation of its expression after imposing stresses on strains containing appropriate transcriptional fusions. Stationary-phase and heat shock inductions of the operon were observed. The integration of fusions between the *rsbV* or *orf4* promoter and a reporter gene, into an independent locus, indicated that the stationary phase-induced initiation of transcription at these promoters was effectively dependent on the *B. anthracis* σ factor, hereafter called σ^B.

The σ^B-dependent, stationary-phase-induced expression of *orf4* is of interest. Our data and analysis of the sequences in the vicinity and upstream from the promoter-like sequence of *orf4* strongly suggested that this gene was solely under the control of σ^B. In *B. subtilis*, in which the σ^B regulon has been thoroughly studied, only two genes, *gsiB* and *csbC*, have been shown to be good candidates for strict dependence (2, 47). The *gsiB* gene was isolated because it is induced by glucose starvation. Its product, GsiB, seems to be involved in protection against osmotic stress, and CbsC belongs to a family of proteins containing symporters that transport sugars from the environment (2, 36, 56). The rationale for studying *csbC* was that elucidation of the regulation and function of strictly σ^B-dependent genes would provide clues to the role of the *B. subtilis* σ^B regulon (2). Similarly, the function of the *orf4* gene product needs to be defined. Weak similarities were found between the sequence of this protein and those of bacterioferritins and nutrient starvation-induced DNA-binding proteins. If the product of *orf4* were shown to have the same function as either of these types of protein, this would increase our understanding of anthrax physiopathology.

We found that *B. anthracis* and *B. subtilis* σ^B operons do not respond to the same stresses. Glucose starvation could not be achieved because no minimal media from which glucose could be depleted are available for this organism. Stationary phase was induced by addition of azide, but, in contrast to what is described in *B. subtilis*, this did not induce the expression of the *B. anthracis* σ^B operon. During noninduced stationary phase, this operon was transcribed later than that of *B. subtilis*. The σ^B operon of *B. subtilis* is transcribed from T_0 and reaches a steady state around T_1 (38). Transcription of the *B. anthracis* σ^B operon begins, albeit slowly, at the same time point but is still increasing at T_5. A similar situation has been described for the *S. aureus* σ^B operon (42). Analysis of the expression of the *B. subtilis sigB* operon under various growth conditions, including slow growth, and using various mutants indicated that neither RsbX nor RsbU is required for the energy stress response (3, 63, 76, 77, 80, 81). The *sigB* induction pattern observed in *B. anthracis* resembles that described in an *rsbU* mutant suppressor strain derived from a *B. subtilis* $RsbX^-$ strain (66). In *B. subtilis*, stationary-phase induction seems to involve a specific RsbV-P phosphatase, RsbP, with σ^B being activated when RsbV is in a dephosphorylated state (3, 74, 77). Sequence comparison with the available *B. anthracis* sequence suggests that a gene encoding such a phosphatase is also present in *B. anthracis*. It has also been suggested that *S. aureus* contains additional regulators because the synthesis of its σ^B homolog responds to both energy and environmental stress (14). The *B. anthracis* σ^B homolog also responds to heat shock. However, we have identified no RsbR, RsbS, RsbT, RsbU, or RsbX homolog in the available *B. anthracis* sequence. Therefore, if other regulators exist, they have little sequence similarity to their *B. subtilis* homologs.

The recognized role of *L. monocytogenes* σ^B in osmotolerance led to the suggestion that the role of the *B. subtilis* σ^B regulon may have diminished partly due to the development of other adaptative responses such as sporulation (7). One of our goals when we began working on the *B. anthracis sigB* operon was to determine whether it was more similar to those of other *Bacillus* species or to those of other pathogenic bacteria. In fact, with the absence of *rsbX*, it seems to be most similar to that of the most distant bacterium, *S. aureus*, because *L. monocytogenes*, although nonsporulating, belongs to the *Bacillaceae*. The stresses encountered by these pathogenic bacteria, one intracellular and the other extracellular, are probably different. Since they enter the host as vegetative cells, the stresses they encounter may also differ from those experienced by *B. anthracis*. Indeed, the currently accepted life cycle of *B. anthracis* stipulates that it has no multiplication cycle outside the host and that its infecting form is the highly resistant spore. It was therefore unclear why this bacterium has a general stress regulon. However, our data indicate that the σ^B mutant was less virulent than the parental strain, suggesting that under physiological conditions σ^B may confer an advantage and indicating that σ^B is a minor virulence factor. This may not be the most important contribution of this transcription factor to the persistence of *B. anthracis*. The last stage of anthrax is septicemia, and the bacilli do not sporulate unless they have access to external oxygen (in outflowing body fluids or if the carcass is opened). These bacteria therefore have to survive as nongrowing vegetative cells, and σ^B may be important at this stage. We therefore suggest that the *B. anthracis* and *B. subtilis* σ^B regulons may play similar roles. The stress-resistant state of growth-restricted cells in the mammalian environment for *B. anthracis* and under certain soil conditions for *B. subtilis* would constitute the alternative survival mechanism if sporulation was hampered, although the stresses experienced are different (36, 80). Thus, in *B. anthracis*, σ^B is probably a minor virulence factor and a persistence factor.

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REFERENCES

1. **Akbar, S., C. M. Kang, T. A. Gaidenko, and C. W. Price.** 1997. Modulator protein RsBR regulates environmental signalling in the general stress pathway of *Bacillus subtilis*. Mol. Microbiol. **24:**567–578.
2. **Akbar, S., S. Y. Lee, S. A. Boylan, and C. W. Price.** 1999. Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor σ^B. Microbiology **145:**1069–1078.
3. **Alper, S., A. Dufour, D. A. Garsin, L. Duncan, and R. Losick.** 1996. Role of adenosine nucleotides in the regulation of a stress-response transcription factor in *Bacillus subtilis*. J. Mol. Biol. **260:**165–177.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
6. **Antelmann, H., S. Engelmann, R. Schmid, A. Sorokin, A. Lapidus, and M. Hecker.** 1997. Expression of a stress- and starvation-induced *dps*/*pexB*-homologous gene is controlled by the alternative sigma factor σ^B in *Bacillus subtilis*. J. Bacteriol. **179:**7251–7256.
7. **Becker, L. A., M. S. Cetin, R. W. Hutkins, and A. K. Benson.** 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. **180:**4547–4554.
8. **Benson, A. K., and W. G. Haldenwang.** 1993. *Bacillus subtilis* σ^B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase. Proc. Natl. Acad. Sci. USA **90:**2330–2334.
9. **Benson, A. K., and W. G. Haldenwang.** 1992. Characterization of a regulatory network that controls σ^B expression in *Bacillus subtilis*. J. Bacteriol. **174:** 749–757.
10. **Binnie, C., M. Lampe, and R. Losick.** 1986. Gene encoding the σ^{37} species of RNA polymerase σ factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83:**5943–5947.
11. **Boylan, S. A., A. R. Redfield, M. S. Brody, and C. W. Price.** 1993. Stress-induced activation of the σ^B transcription factor of *Bacillus subtilis*. J. Bacteriol. **175:**7931–7937.
12. **Boylan, S. A., A. Rutherford, S. M. Thomas, and C. W. Price.** 1992. Activation of *Bacillus subtilis* transcription factor σ^B by a regulatory pathway responsive to stationary-phase signals. J. Bacteriol. **174:**3695–3706.
13. **Bragg, T. S., and D. L. Robertson.** 1989. Nucleotide sequence and analysis of the lethal factor gene (*lef*) from *Bacillus anthracis*. Gene **81:**45–54.
14. **Brody, M. S., and C. W. Price.** 1998. *Bacillus licheniformis sigB* operon encoding the general stress transcription factor σ^B. Gene **212:**111–118.
15. **Chan, P. F., S. J. Foster, E. Ingham, and M. O. Clements.** 1998. The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J. Bacteriol. **180:**6082–6089.
16. **Cheung, A. L., Y.-T. Chien, and A. S. Bayer.** 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. Infect. Immun. **67:**1331–1337.
17. **Chung, C. T., and R. H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16:** 3580.
18. **Dai, Z., J.-C. Sirard, M. Mock, and T. M. Koehler.** 1995. The *atxA* gene product activates transcription of the anthrax toxin genes and is essential for virulence. Mol. Microbiol. **16:**1171–1181.
19. **Delecluse, A., J.-F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
20. **Deora, R., T. Tseng, and T. K. Misra.** 1997. Alternative transcription factor σ^B of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar*. J. Bacteriol. **179:**6355–6359.
21. **Dingman, D. W., M. S. Rosenkrantz, and A. L. Sonenshein.** 1987. Relationship between aconitase gene expression and sporulation in *Bacillus subtilis*. J. Bacteriol. **169:**3068–3075.
22. **Dufour, A., and W. G. Haldenwang.** 1994. Interactions between a *Bacillus subtilis* anti-σ factor (RsbW) and its antagonist (RsbV). J. Bacteriol. **176:** 1813–1820.
23. **Duncan, M. L., S. S. Kalman, S. M. Thomas, and C. W. Price.** 1987. Gene encoding the 37,000-dalton minor sigma factor of *Bacillus subtilis* RNA polymerase: isolation, nucleotide sequence, chromosomal locus, and cryptic function. J. Bacteriol. **169:**771–778.
24. **Engelmann, S., and M. Hecker.** 1996. Impaired oxidative stress resistance of *Bacillus subtilis sigB* mutants and the role of *katA* and *katE*. FEMS Microbiol. Lett. **145:**63–69.
25. **Engelmann, S., C. Lindner, and M. Hecker.** 1995. Cloning, nucleotide sequence, and regulation of *katE* encoding a σ^B-dependent catalase in *Bacillus subtilis*. J. Bacteriol. **177:**5598–5605.
26. **Escuyer, V., E. Duflot, O. Sezer, A. Danchin, and M. Mock.** 1988. Structural homology between virulence-associated bacterial adenylate cyclases. Gene **71:**293–298.
27. **Ezzell, J. W., and T. G. Abshire.** 1996. Encapsulation of *Bacillus anthracis* spores and spore identification, p. 42. *In* P. C. B. Turnbull (ed.), Proceedings of the International Workshop on Anthrax. Salisbury Medical Bulletin Special Supplement 87.
28. **Fouet, A., and M. Mock.** 1996. Differential influence of the two *Bacillus anthracis* plasmids on regulation of virulence gene expression. Infect. Immun. **64:**4928–4932.
29. **Friedlander, A. M., R. Bhatnagar, S. H. Leppla, L. Johnson, and Y. Singh.** 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect. Immun. **61:**245–252.
30. **Gertz, S., S. Engelmann, R. Schmid, K. Ohlsen, J. Hacker, and M. Hecker.** 1999. Regulation of σ^B-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains. Mol. Gen. Genet. **261:**558–566.
31. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. **49:** 291–297.
32. **Guidi-Rontani, C., Y. Pereira, S. Ruffié, J.-C. Sirard, M. Weber-Levy, and M. Mock.** 1999. Identification and characterization of a germination operon on the virulence plasmid pXO1 of *Bacillus anthracis*. Mol. Microbiol. **33:**407–414.
33. **Guidi-Rontani, C., M. Weber-Levy, E. Labruyère, and M. Mock.** 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. **31:**9–17.
34. **Guignot, J., M. Mock, and A. Fouet.** 1997. AtxA activates the transcription of genes harbored by both *Bacillus anthracis* virulence plasmids. FEMS Microbiol. Lett. **147:**203–207.
35. **Hecker, M., W. Schumann, and U. Völker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417–428.
36. **Hecker, M., and U. Völker.** 1998. Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the σ^B regulon. Mol. Microbiol. **29:**1129–1136.
37. **Hernandez, E., F. Ramisse, J.-P. Ducoureau, T. Cruel, and J.-D. Cavallo.** 1998. *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. J. Clin. Microbiol. **36:**2138–2139.
38. **Kalman, S., M. L. Duncan, S. M. Thomas, and C. W. Price.** 1990. Similar organization of the *sigB* and *spoIIA* operons encoding alternate sigma factors of *Bacillus subtilis* RNA polymerase. J. Bacteriol. **172:**5575–5585.
39. **Kang, C. M., M. S. Brody, S. Akbar, X. F. Yang, and C. W. Price.** 1996. Homologous pairs of regulatory proteins control activity of *Bacillus subtilis* transcription factor σ^B in response to environmental stress. J. Bacteriol. **178:** 3846–3853.
40. **Koehler, T. M., Z. Dai, and M. Kaufman-Yarbray.** 1994. Regulation of the *Bacillus anthracis* protective antigen gene: CO_2 and a *trans*-acting element activate transcription from one of two promoters. J. Bacteriol. **176:**586–595.
41. **Krüger, E., T. Msadek, and M. Hecker.** 1996. Alternate promoters direct stress-induced transcription of the *Bacillus subtilis clpC* operon. Mol. Microbiol. **20:**713–723.
42. **Kullik, I., and P. Giachino.** 1997. The alternative sigma factor σ^B in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. Arch. Microbiol. **167:**151–159.
43. **Kullik, I., P. Giachino, and T. Fuchs.** 1998. Deletion of the alternative sigma factor σ^B in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J. Bacteriol. **180:**4814–4820.
44. **Kunst, F., and G. Rapoport.** 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. J. Bacteriol. **177:** 2403–2407.
45. **Laforce, F. M., F. H. Bumford, J. C. Feeley, S. L. Stokes, and D. B. Snow.** 1969. Epidemiologic study of a fatal case of inhalation anthrax. Arch. Environ. Health **18:**798–805.
46. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Maul, B., U. Völker, S. Riethdorf, S. Engelmann, and M. Hecker.** 1995. σ^B-dependent regulation of *gsiB* in response to multiple stimuli in *Bacillus subtilis*. Mol. Gen. Genet. **248:**114–120.
48. **Mesnage, S., E. Tosi-Couture, M. Mock, P. Gounon, and A. Fouet.** 1997. Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell-associated antigen. Mol. Microbiol. **23:** 1147–1155.
49. **Meynell, E., and G. G. Meynell.** 1964. The roles of serum and carbon dioxide in capsule formation by *Bacillus anthracis*. J. Gen. Microbiol. **34:**153–164.
50. **Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39:** 371–376.
51. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Miyazaki, E., J.-M. Chen, C. Ko, and W. R. Bishai.** 1999. The *Staphylococcus*

aureus rsbW (*orf159*) gene encodes an anti-sigma factor of SigB. J. Bacteriol. **181:**2846–2851.
53. **Moszer, I., P. Glaser, and A. Danchin.** 1995. SubtiList: a relational database for the *Bacillus subtilis* genome. Microbiology **141:**261–268.
54. **Msadek, T., V. Dartois, F. Kunst, M. L. Herbaud, F. Denizot, and G. Rapoport.** 1998. ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. Mol. Microbiol. **27:**899–914.
55. **Mueller, J. P., G. Bukusoglu, and A. L. Sonenshein.** 1992. Transcriptional regulation of *Bacillus subtilis* glucose starvation-inducible genes: control of *gsiA* by the ComP-ComA signal transduction system. J. Bacteriol. **174:**4361–4373.
56. **Mueller, J. P., C. Mathiopoulos, F. J. Slack, and A. L. Sonenshein.** 1991. Identification of *Bacillus subtilis* adaptative response genes by subtractive differential hybridization. Res. Microbiol. **142:**805–813.
57. **Namy, O., M. Mock, and A. Fouet.** 1999. Co-existence of *clpB* and *clpC* in the *Bacillaceae*. FEMS Microbiol. Lett. **173:**297–302.
58. **Nicholas, R. O., T. Li, D. McDevitt, A. Marra, S. Sucoloski, P. L. Demarsh, and D. R. Gentry.** 1999. Isolation and characterization of a *sigB* deletion mutant of *Staphylococcus aureus*. Infect. Immun. **67:**3667–3669.
59. **Patra, G., J. Vaissaire, M. Weber-Levy, C. Le Doujet, and M. Mock.** 1998. Molecular characterization of *Bacillus* strains involved in outbreaks of anthrax in France in 1997. J. Clin. Microbiol. **36:**3412–3414.
60. **Pezard, C., P. Berche, and M. Mock.** 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. **59:**3472–3477.
61. **Ramisse, V., G. Patra, J. Vaissaire, and M. Mock.** 1999. The Ba813 chromosomal DNA sequence effectively traces the whole *Bacillus anthracis* community. J. Appl. Microbiol. **87:**224–228.
62. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
63. **Schweder, T., A. Kolyschkow, U. Völker, and M. Hecker.** 1999. Analysis of the expression and function of the σ^B-dependent general stress regulon of *Bacillus subtilis* during slow growth. Arch. Microbiol. **171:**439–443.
64. **Scott, J. M., and W. G. Haldenwang.** 1999. Obg, an essential GTP binding protein of *Bacillus subtilis*, is necessary for stress activation of transcription factor σ^B. J. Bacteriol. **181:**4653–4660.
65. **Sirard, J.-C., M. Mock, and A. Fouet.** 1994. The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. J. Bacteriol. **176:**5188–5192.
66. **Smirnova, N., J. Scott, U. Voelker, and W. G. Haldenwang.** 1998. Isolation and characterization of *Bacillus subtilis sigB* operon mutations that suppress the loss of the negative regulator RsbX. J. Bacteriol. **180:**3671–3680.
67. **Tatti, K. M., and C. P. Moran, Jr.** 1984. Promoter recognition by σ^{37} RNA polymerase from *Bacillus subtilis*. J. Mol. Biol. **175:**285–297.
68. **Thorne, C. B.** 1993. *Bacillus anthracis*, p. 113–124. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
69. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
70. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1991. An integrative vector exploiting the transposition properties of Tn*1545* for insertional mutagenesis and cloning of genes from Gram-positive bacteria. Gene **106:**21–27.
71. **Truitt, C. L., E. A. Weaver, and W. G. Haldenwang.** 1988. Effects on growth and sporulation of inactivation of a *Bacillus subtilis* gene (*ctc*) transcribed in vitro by minor vegetative cell RNA polymerases. Mol. Gen. Genet. **212:**166–171.
72. **Uchida, I., J. M. Hornung, C. B. Thorne, K. R. Klimpel, and S. H. Leppla.** 1993. Cloning and characterization of a gene whose product is a *trans*-activator of anthrax toxin synthesis. J. Bacteriol. **175:**5329–5338.
73. **Vietri, N. J., R. Marrero, T. A. Hoover, and S. L. Welkos.** 1995. Identification and characterization of a *trans*-activator involved in the regulation of encapsulation by *Bacillus anthracis*. Gene **152:**1–9.
74. **Vijay, K., M. S. Brody, E. Fredlund, and C. W. Price.** 2000. A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the σ^B transcription factor of *Bacillus subtilis*. Mol. Microbiol. **35:**180–188.
75. **Voelker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Voelker, R. Schmid, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology **140:**741–752.
76. **Voelker, U., T. Q. Luo, N. Smirnova, and W. Haldenwang.** 1997. Stress activation of *Bacillus subtilis* σ^B can occur in the absence of the σ^B negative regulator RsbX. J. Bacteriol. **179:**1980–1984.
77. **Voelker, U., A. Voelker, and W. G. Haldenwang.** 1996. Reactivation of the *Bacillus subtilis* anti-σ^B antagonist, RsbV, by stress- or starvation-induced phosphatase activities. J. Bacteriol. **178:**5456–5463.
78. **Voelker, U., A. Voelker, and W. G. Haldenwang.** 1996. The yeast two-hybrid system detects interactions between *Bacillus subtilis* σ^B regulators. J. Bacteriol. **178:**7020–7023.
79. **Völker, U., A. Dufour, and W. G. Haldenwang.** 1995. The *Bacillus subtilis rsbU* gene product is necessary for RsbX-dependent regulation of σ^B. J. Bacteriol. **177:**114–122.
80. **Völker, U., B. Maul, and M. Hecker.** 1999. Expression of the σ^B-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. J. Bacteriol. **181:**3942–3948.
81. **Völker, U., A. Völker, B. Maul, M. Hecker, A. Dufour, and W. G. Haldenwang.** 1995. Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stress. J. Bacteriol. **177:**3771–3780.
82. **Welkos, S. L., J. R. Lowe, F. Eden-McCutchan, M. Vodkin, S. H. Leppla, and J. J. Schmidt.** 1988. Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene **69:**287–300.
83. **Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor.** 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. **180:**3650–3656.
84. **Wise, A. A., and C. W. Price.** 1995. Four additional genes in the *sigB* operon of *Bacillus subtilis* that control activity of the general stress factor σ^B in response to environmental signals. J. Bacteriol. **177:**123–133.
85. **Wu, S. W., H. De Lencastre, and A. Tomasz.** 1996. σ^B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J. Bacteriol. **178:**6036–6042.
86. **Yang, X. F., C. M. Kang, M. S. Brody, and C. W. Price.** 1996. Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. Genes Dev. **10:**2265–2275.
87. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
88. **Yuan, G., and S.-L. Wong.** 1995. Regulation of *groE* expression in *Bacillus subtilis*: the involvement of the σ^A-like promoter and the roles of the inverted repeat sequences (CIRCE). J. Bacteriol. **177:**5427–5433.