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The Alternative Sigma Factor σ^H Is Required for Toxin Gene Expression by *Bacillus anthracis*▽

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Expression of the structural genes for the anthrax toxin proteins is coordinately controlled by host-related signals, such as elevated CO_2, and the *trans*-acting positive regulator AtxA. In addition to these requirements, toxin gene expression is under growth phase regulation. The transition state regulator AbrB represses *atxA* expression to influence toxin synthesis. During the late exponential phase of growth, when AbrB levels begin to decrease, toxin synthesis increases. Here we report that toxin gene expression also requires the presence of *sigH*, a gene encoding the RNA polymerase sigma factor associated with development in *Bacillus subtilis*. In the well-studied *B. subtilis* system, σ^H is required for sporulation and other post-exponential-phase processes and is part of a feedback control pathway for *abrB* expression. Our data indicate that a *Bacillus anthracis sigH*-null mutant is asporogenous and toxin deficient. Yet the sigma factor is required for toxin gene expression in a manner that is independent of the pathway leading to post-exponential-phase gene expression. σ^H positively controls *atxA* in an AbrB-independent manner. These findings, combined with previous observations, suggest that the steady-state level of *atxA* expression is critical for optimal toxin gene transcription. We propose a model whereby, under toxin-inducing growth conditions, control of toxin gene expression is fine-tuned by the independent effects of σ^H and AbrB on the expression of *atxA*.

Expression of the structural genes for the anthrax toxin proteins PagA (protective antigen), Cya (edema factor), and Lef (lethal factor) is coordinately controlled by host-related signals and *trans*-acting regulatory genes. The toxin genes are located noncontiguously within a 30-kb region of the 182-kb *Bacillus anthracis* virulence plasmid pXO1. Two pXO1-encoded regulators of toxin gene expression have been reported, PagR and AtxA. The *pagR* gene encodes a weak repressor of the bicistronic *pagAR* operon (25) and negatively affects the expression of the chromosomal S-layer genes *sap* and *eag* (34). Purified PagR has been shown to bind sequences within the *pagA*, *sap*, and *eag* promoter regions; however, alignment of the protected sequences does not reveal a PagR consensus sequence (34).

AtxA is a positive regulator of *pagAR*, *cya*, and *lef* as well as a number of other plasmid- and chromosome-carried genes (6, 24, 29). AtxA controls gene expression in *trans*, but the molecular mechanism for this regulation is unknown. AtxA is predicted to be a 56-kDa basic protein with a weak helix-turn-helix motif located at the amino terminus. Yet specific nucleic acid binding activity has not been ascribed to this protein, and there are no obvious similarities in the promoter regions of the AtxA-controlled genes. Moreover, consensus sequences for recognition by RNA polymerase sigma factors are generally not apparent for AtxA-dependent transcription start sites.

In addition to the pXO1-encoded regulators, a chromosomal gene, *abrB*, has been reported to affect anthrax toxin gene expression. Extensive studies in *Bacillus subtilis* have revealed that AbrB is a transition state regulator that plays a critical role in the suppression of post-exponential-phase gene expression during the logarithmic phase of growth (42). Optimal toxin synthesis by *B. anthracis* occurs during growth at 37°C in Casamino Acids medium containing bicarbonate (11, 48). In these conditions, toxin gene expression reaches a maximum during the late exponential phase of growth, coinciding with a decrease in *abrB* transcription. A *B. anthracis abrB* deletion mutant produces higher levels of all three toxin proteins, and toxin gene expression peaks earlier during growth (46).

The *abrB* effect on toxin gene expression may be due in part to the repression of *atxA* transcription. Saile and Koehler (46) demonstrated elevated transcripts of *atxA* in an *abrB*-null mutant of *B. anthracis*, and Strauch et al. (54) reported recently that *B. anthracis* AbrB binds to specific DNA sequences in the *atxA* promoter region. Nevertheless, there is evidence that *abrB* controls toxin gene expression in an *atxA*-independent manner. Baillie et al. (3) reported that a *B. subtilis abrB*-null mutant harboring the cloned *pagA* gene produced elevated levels of protective antigen. Considering that *B. subtilis* does not appear to contain an *atxA* homologue, this effect is independent of *atxA*.

The *B. subtilis* AbrB is a pleiotropic regulator and binds to the promoter regions of multiple target genes (42). One AbrB target is *sigH* (19, 53), a gene encoding the alternative sigma factor σ^H, which plays an important role in post-exponential-phase gene expression. The σ^H RNA polymerase holoenzyme recognizes and transcribes genes associated with the transition to the stationary phase of growth, including genes for cytochrome biogenesis, generation of potential nutrient sources, transport, and cell wall metabolism (7), as well as genes im-

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TABLE 1. *B. anthracis* strains used in this study

Strain[a]	Phenotype or genotype	Relevant characteristics[b]	Source or reference
UM44	Ind^-	Weybridge strain; $pXO1^+$ $pXO2^-$	C. Thorne
UT53	*atxA*	Tox^- Km^r	15
UT133	*cya*::*lacZ*	Em^r Km^r; UM44 transduced with CP51 (55) propagated on RBAF 144 (48)	This work
UT147	*pag*::*lacZ*	Em^r Km^r; UM44 transduced with CP51 (55) propagated on RBAF 140 (48)	This work
UT148	*lef*::*lacZ*	Em^r Km^r; UM44 transduced with CP51 (55) propagated on RBAF 143 (48)	This work
UT157	*spo0A*	Spo^- Spc^r	46
UT166	*abrB*	Spc^r	46
UT198	*sigH*	Spo^- Km^r	This work
UT199	*sigH pagA*::*lacZ*	Spo^- Km^r Em^r; UT147 transduced with CP51 (55) propagated on UT198	This work
UT200	*sigH lef*::*lacZ*	Spo^- Km^r Em^r; UT148 transduced with CP51 (55) propagated on UT198	This work
UT201	*sigH cya*::*lacZ*	Spo^- Km^r Em^r; UT133 transduced with CP51 (55) propagated on UT198	This work
UT285	*abrB spo0A*	Spo^- Spc^r Km^r; *abrB* was deleted in UT157 and is replaced by Ω-*km2*	This work
UT290	*spo0A sigH*	Spo^- Spc^r Km^r; UT157 was transduced using CP51 phage (55) propagated on UT198	This work
UT291	*abrB sigH*	Spo^- Spc^r,Km^r; UT66 was transduced using CP51 phage (55) propagated on UT198	This work
UT301	*sigH plcR*::*sigH*	Spo^+ Spc^r Km^r; *sigH* gene introduced in the *plcR* locus using pUTB1	This work

[a] All UT mutants listed are derivatives of Weybridge strain UM44.
[b] Abbreviations: Ind^-, indole auxotrophy; Tox^-, toxin production deficiency; Em^r, erythromycin resistance; Km^r, kanamycin resistance; Spc^r, spectinomycin resistance; Spo^-, sporulation deficiency.

portant for competence and sporulation initiation (16). In *B. subtilis*, a complex signal transduction phosphorelay system keeps the initiation of sporulation under stringent control by regulating the phosphorylation of the master response regulator, Spo0A (23, 38–41).

σ^H, AbrB, and Spo0A are all part of a feedback mechanism that ultimately controls the expression of each regulator and is critical for sporulation initiation. During the logarithmic phase of growth, *sigH* expression is repressed by AbrB, resulting in relatively low-level expression of σ^H targets (19, 53). As a culture transitions into the stationary phase, phosphorylation of Spo0A increases, leading to gradual activation of Spo0A (50). Phosphorylated Spo0A represses the transcription of *abrB* (21, 52), resulting in increased levels of σ^H. The *spo0A* gene has two promoters, one recognized by the housekeeping sigma factor, σ^A, and the other recognized by σ^H (43). The elevated Spo0A levels in the stationary phase are attributed to enhanced *spo0A* transcription when σ^H levels are high. Therefore, *abrB* expression in *B. subtilis* is increased in a *sigH*-null mutant that produces low levels of Spo0A (54).

In *B. anthracis*, AbrB represses the toxin regulator *atxA* (46, 54). As predicted from the *B. subtilis* model in which *abrB* expression is subject to control by σ^H and Spo0A, the activity of a reporter gene driven by the *atxA* promoter is reduced in *B. subtilis spo0A* and *sigH* mutants (54). Moreover, the increased *atxA* promoter activity exhibited by a *B. subtilis abrB* mutant is comparable to the activities of double mutants with *abrB* and *sigH* or *abrB* and *spo0A* deleted (54).

In the work presented here, we further explored the relationship between anthrax toxin gene expression and the multicomponent system controlling growth phase-specific gene expression and development that is well studied in *B. subtilis*. As is true for σ^H function in *B. subtilis*, the *B. anthracis sigH* gene is required for sporulation, demonstrating a role for this alternative sigma factor in post-exponential-phase gene regulation. However, our data show that SigH controls toxin gene expression independently of AbrB, indicating an additional function for SigH in *B. anthracis*. Thus, SigH joins AbrB as another key player in the disparate processes of sporulation and toxin synthesis by *B. anthracis*.

MATERIALS AND METHODS

Growth conditions. *Escherichia coli* strains JM109 and GM2163 (New England Biolabs, Beverly, MA) were grown in Luria-Bertani (LB) broth (47) and used as hosts for cloning. *B. anthracis* strains were grown in LB broth to obtain cells for transductions, electroporations, and DNA extraction. *B. anthracis* culture supernatants and cell extracts for Western hybridization and β-galactosidase assays were obtained from cells grown as follows. A sample from an overnight culture grown at 30°C with agitation in LB broth containing 0.5% glycerol and appropriate antibiotics was used to inoculate 45 ml of $CACO_3$ medium (CA medium [56] buffered with 100 mM HEPES [pH 8.0] and 0.8% [wt/vol] sodium bicarbonate) in a 250-ml Erlenmeyer flask. Cultures were incubated at 37°C in an atmosphere containing 5% CO_2. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) and added to media (concentrations are indicated in parentheses) when appropriate: ampicillin (100 μg/ml), erythromycin (150 μg/ml for *E. coli*; 5 μg/ml for *B. anthracis*), kanamycin (20 μg/ml for *E. coli*; 100 μg/ml for *B. anthracis*), and spectinomycin (50 μg/ml for *E. coli*; 100 μg/ml for *B. anthracis*). All other chemicals were purchased from Sigma Aldrich unless indicated otherwise.

DNA isolation and manipulation. Extraction of chromosomal DNA from *B. anthracis* cultures was carried out using a Mo Bio genomic isolation kit (Mo Bio Laboratories, Solana Beach, CA). Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were performed using standard procedures (2). *B. anthracis* was electroporated with unmethylated plasmid DNA from *E. coli* GM2163 as described elsewhere (29). Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI) and Fisher Scientific, and *Taq* DNA polymerase was purchased from New England Biolabs (Beverly, MA).

Strain construction. Table 1 contains a complete list of *B. anthracis* strains used, including relevant characteristics. The *B. anthracis sigH*-null mutant UT198 was constructed by replacing the *sigH* gene (NC003997.3; nucleotides [nt] 102990 to 103646) with the Ω-*km2* element, using a previously described protocol (29). *B. anthracis* strain UT301 was created by double-crossover recombination of *sigH* and upstream regulatory elements into the chromosomal *plcR* locus (NC003997.3; nt 1133897 to 1134781). To create UT301, plasmid pUTE719, containing the Ω-*spc* element flanked by DNA sequences found upstream and downstream of the *plcR* gene, was constructed. The *plcR*-flanking sequences were amplified with primer pairs CR123 (5′-GAGCTCGGATCCCGATTCAA TTCGGCTCACTT-3′)/ES40 (5′-AACTCCAGTGTTGCGGAAACGTTAAA GA-3′) and CR124(5′-GAGCTCTTGAAAACGCAATTGCAAAC-3′)/ES43 (5′-ACGCGTCGACTCGTATCTCCTGCCCAATTC-3′) and *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). The *plcR*-flanking regions and the Ω-*spc* element were cloned into pUTE583 (12) such that a unique SacI site was located between the Ω-*spc* element and the downstream flanking region. Primers MH187 (5′-CCCGAGCTCGGAAGAATCAAACTGCAGATG-3′) and MH188 (5′-CCC GAGCTCGTATCTTTCATCGTAGAG-3′) were used to amplify a 1,209-bp fragment containing the *sigH* gene and its predicted promoter region. The PCR product was digested with SacI and cloned into pUTE719. The resulting construct, pUTB1,

was electroporated into UT198, and the resulting strain was cultured to facilitate recombination as described previously (46).

Microscopy. *B. anthracis* cells were visualized using a Labophot Nikon microscope, and pictures were obtained using a COOLPIX995 digital camera.

Western blot analysis. Culture supernatants were filtered through 0.2-μm-pore-size syringe filters (Corning, Corning, NY). For detection of protective antigen (PA), lethal factor (LF), and edema factor (EF), samples were treated and visualized as described previously (46). Equal volumes of culture supernatants were used to compare toxin levels in the parent and mutant strains. Cell extracts were prepared and probed for the presence of σ^H as follows. Cell pellets from 1-ml samples were resuspended in 1× phosphate-buffered saline (PBS) and mechanically sheared by using a mini-bead beater 8 (MBB8) (Biospec Products, Bartlesville, OK), incubated for 5 min at 65°C, and sheared again for 1 min. Cell debris was pelleted at 16,000 × *g* for 10 min. Supernatant fractions were treated with 40 μl of 25× stock protease inhibitor cocktail (Roche, Indianapolis, IN). The protein concentration was determined by using a BCA assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard, following the manufacturer's instructions. Samples containing 4 μg of total protein were resolved on 15% sodium dodecyl sulfate-polyacrylamide gels, transferred, and blocked in 1× TBS-T (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]) containing 5% milk as described previously (46). The membranes were probed using rabbit antisera raised against *B. subtilis* σ^H and SigA, at dilutions of 1:500 and 1:1,000, respectively, in 1× TBS-T–5% milk (46).

β-Galactosidase assays. One-milliliter culture samples were collected hourly from the early logarithmic (3 h) to the 3-h post-stationary phase of growth (10 h). β-Galactosidase assays were performed according to Miller (36). Cell extracts were prepared as described for Western blot analysis (above). At least three independent cultures were assayed for enzyme activity. The figures show data from representative experiments.

Bioinformatics analyses. The predicted amino acid sequences of σ^H from *B. anthracis* and *B. subtilis* were aligned using ClustalW. The NCBI BlastP program was used to identify *B. anthracis* homologs of *B. subtilis* genes known to be transcribed by σ^H RNA polymerase. The predicted amino acid sequences for the *B. subtilis* Spo0A, Spo0F, Spo0M, CitG, KinA, PhrC, PhrI, Spo0M, SpoVG, SpoVS, and SigA proteins were compared by BLAST analysis against the sequence of the *B. anthracis* Ames genome (NC003997). Genes encoding the proteins with the highest percent identity were considered for analysis. Upon identification of a candidate gene, the sequence of the 500 nucleotides upstream of the predicted translational start was examined for the presence of a σ^H consensus. Wherever possible, the promoter regions of the *B. subtilis* genes were aligned with the *B. anthracis* sequences using ClustalW.

Purification of *B. anthracis* σ^H. Sequences corresponding to the *B. anthracis sigH* locus were amplified from genomic DNA using PCR with primers YC85 (5′-CACCATGGAAGCAGGCTTCGTAAGTG-3′) and YC86 (5′-ATTTGAA GTGGTACTCTCTCTC-3′). The resulting product was cloned into the protein expression vector pET101/D-TOPO (Invitrogen, Carlsbad, CA) to give plasmid pUTE493. The plasmid was constructed such that C-terminally His-tagged σ^H was expressed from a T7 promoter. pUTE493 was introduced into *E. coli* BL21(DE3), and the recombinant *B. anthracis* protein was expressed and purified from the strain under denaturing conditions according to the manufacturer's instructions (QIAexpress; QIAGEN, Valencia, CA). Recombinant σ^H was dialyzed overnight in refolding buffer following the protocol described by Haldenwang et al. (22).

In vitro runoff transcription assays. To create templates for in vitro runoff transcription reactions, blunt-ended DNA fragments corresponding to the *pagA*, *lef*, and *cya* promoter regions from positions −90 to +88, −70 to +132 and −122 to +63, respectively, relative to the main transcriptional start sites, and to the *spoVG* and *atxA* promoter regions from positions −243 to +1 and −870 to +1, respectively, relative to the translational start, were amplified by PCR using Deep Vent DNA polymerase. Each PCR product was purified using Zymoclean reagents (Orange, CA), concentrated 4 times and quantified by measuring absorbance at an optical density of 260 nm.

Recombinant *B. anthracis* σ^H was used in combination with *E. coli* core RNA polymerase purchased from Epicenter (Madison, WI) for in vitro transcription reactions. For each reaction, 0.1 pmol of template DNA was added to 1× in vitro transcription buffer (Promega) supplemented with 200 mM KCl and containing 77.25 nM *E. coli* core RNA polymerase and 480 nM σ^H in a total volume of 10 μl. Following incubation at 37°C for 30 min, ribonucleotides and [α-^{32}P]UTP were added to a final concentration of 0.5 mM in a 20-μl volume. After incubation for an additional 15 min at 37°C, reactions were terminated by purification through G-30 columns (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Eluates were mixed with equal volumes of RNA loading buffer (0.05% bromophenol blue, 20 mM EDTA in deionized formamide [Sigma-Aldrich]) and heated at 80°C for 10 min prior to electrophoresis. Samples (7 μl) were electrophoresed at 1,400 V for 2.5 h in 8% acrylamide–7 M urea denaturing gels. Gels were dried for 4 h using a model 583 Bio-Rad gel dryer prior to exposure to photographic film at −80°C overnight. Molecular size markers were generated by in vitro transcription of an RNA century marker template set (Ambion, Austin, TX), following the manufacturer's instructions.

RESULTS

Creation of a *B. anthracis sigH*-null mutant. We identified the *sigH* gene of *B. anthracis* (NC003997.3; nt 102990 to 103646) on the basis of sequence homology to *B. subtilis sigH* (NC000964.2; nt 116597 to 117253). A protein BLAST search (BlastP) revealed that BA0093 is predicted to encode a protein (NP_842661.1) that bears an 81% identity to the *B. subtilis* σ^H (NP_387979). We constructed a *sigH*-null mutant, UT198, in which the *sigH* gene was replaced by a kanamycin cassette using methods described previously (46). As expected, the *sigH* mutant, UT198, was unable to sporulate (Fig. 1A). The *sigH*-null mutation did not affect the growth rate when cells were cultured in $CACO_3$ medium at 37°C in an atmosphere containing 5% CO_2, conditions that are optimal for toxin gene expression (11, 48) (Fig. 1B). The mutation in UT198 was complemented by placement of the *sigH* gene in another chromosomal locus, *plcR* (NC003997.3; nt 1133897 to 1134781). The *B. anthracis plcR* gene is predicted to encode a truncated, nonfunctional protein (1, 35, 49). Recombinant strain UT301 was derived from UT198 and harbors *sigH* under the control of its native promoter in the *plcR* locus. Sporulation and σ^H synthesis by UT301 are comparable to those of the parent strain, UM44 (Fig. 1A and C).

Deletion of *sigH* results in a toxin-deficient phenotype. Considering that *abrB* and *spo0A* affect toxin gene expression in *B. anthracis* (46), and considering the relationships between *sigH* and these regulators in *B. subtilis* (50), we assessed toxin synthesis by the *B. anthracis* parent and the *sigH*-null mutant. Equal volumes of culture supernatant fractions of strains grown in conditions that promote toxin gene expression were probed for the relative amounts of each toxin protein. As shown in Fig. 2A, at the late exponential growth phase the levels of PA, LF, and EF in the supernatant of the UT198 (*sigH*) strain were significantly lower than those in the supernatant of the UM44 (parent) culture. The toxin deficiency of UT198 was complemented by the addition of *sigH* in *trans* (UT301). UT301 produced greater amounts of PA and LF than UT198, but less PA and LF than UM44 (Fig. 2A, lane 4). This result was surprising, given that σ^H synthesis by UT301 was comparable to that of the parent strain (Fig. 1B).

Transcriptional analysis also revealed significantly reduced expression of the toxin genes in the *sigH*-null mutant compared to that in the parent strain. UM44 (parent)- and UT198 (*sigH*)-derived strains harboring the individual transcriptional fusions *pagA*::*lacZ*, *lef*::*lacZ*, and *cya*::*lacZ* at the toxin gene loci were created, and toxin gene promoter activity was assessed as the β-galactosidase activity of cells grown in conditions optimal for toxin synthesis. Throughout growth, the enzyme activities associated with the *pag-lacZ*, *cya-lacZ*, and *lef-lacZ* reporter genes in *sigH*-null strains were 4% or less of those observed for the parent strains (Fig. 2B). These results demonstrate that *sigH* affects toxin gene expression at the transcriptional level.

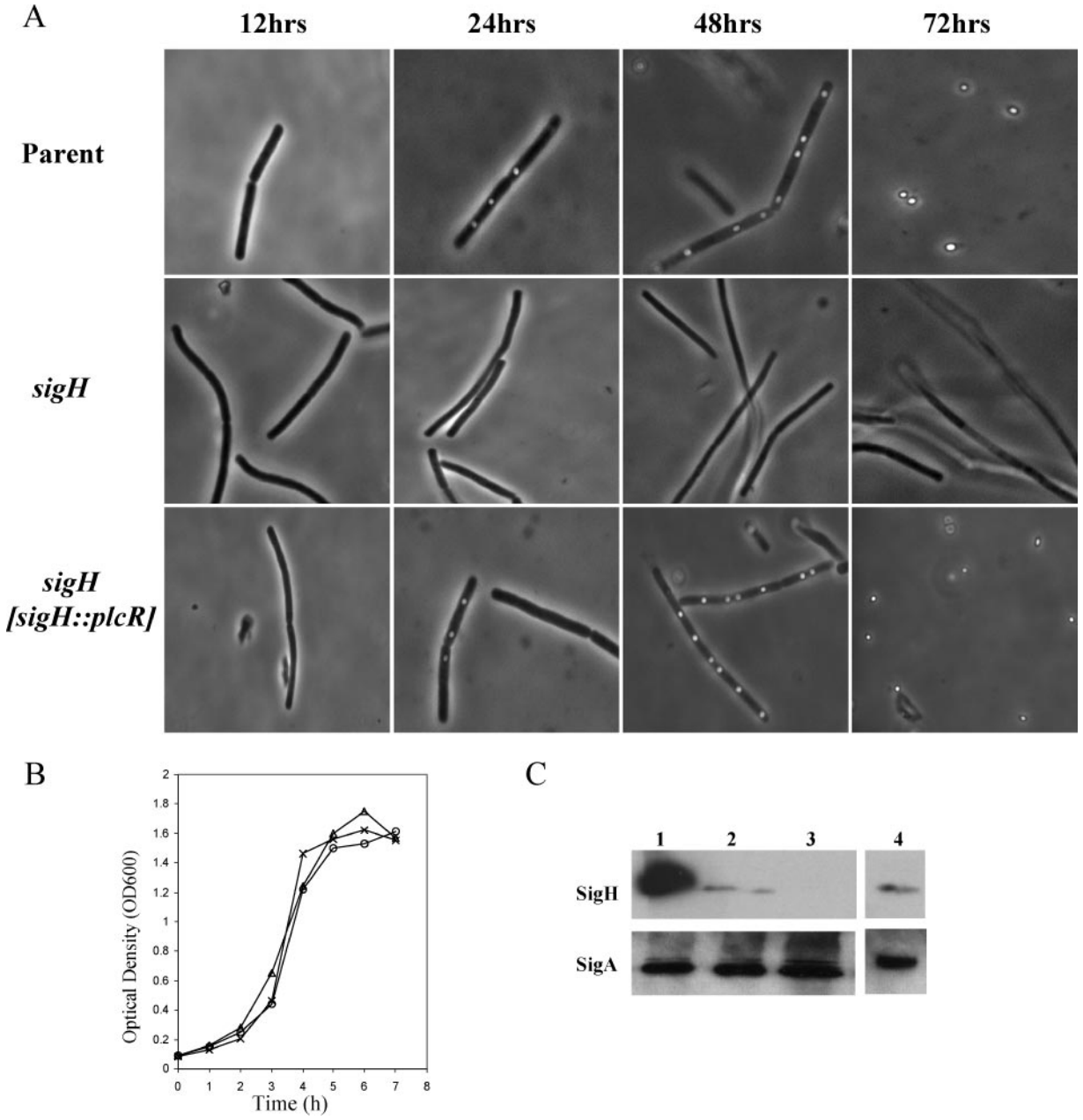

FIG. 1. Creation of a *B. anthracis sigH*-null mutant. (A) Sporulation of the parent, mutant, and complemented strains grown in PA broth (20) at 30°C. Samples were taken at the times indicated and assessed for the presence of spores using light microscopy. (B) Growth of UM44 (parent), UT198 (Δ*sigH*), and UT301 (Δ*sigH sigH*::*plcR*) in $CACO_3$ broth in 5% CO_2 at 37°C. OD600, optical density at 600 nm. (C) Synthesis of σ^H by the parent and mutant strains. The culture samples were obtained during the late exponential phase of growth (5 h). Solubilized cellular protein (4 μg) was subjected to Western blot analysis using rabbit anti-σ^H antibody raised against *B. subtilis* σ^H or rabbit anti-σ^A antibody raised against *B. subtilis* σ^A (as a loading control). Lane 1, recombinant protein; lane 2, UM44; lane 3, UT198; lane 4, UT301.

σ^H affects toxin synthesis independently of AbrB. The *B. anthracis abrB* gene exerts a negative effect on toxin gene expression. An *abrB*-null mutant expresses *cya*, *lef*, and *pagA* earlier than the parent strain during growth in batch culture, indicative of growth phase-dependent control (46). This phenotype has been attributed to specific binding of the AbrB protein to the promoter of the toxin gene regulator *atxA* (54). AtxA is a strong positive regulator of the toxin genes; the toxin-deficient phenotype of an *atxA*-null mutant is comparable to that of the *sigH* mutant (15). In *B. subtilis*, steady-state levels of AbrB are modulated by a feedback mechanism whereby AbrB represses *sigH*, σ^H RNA polymerase transcribes *spo0A*, and phosphorylated Spo0A represses *abrB*. In *B. subtilis*, deletion of *sigH* results in elevated levels of AbrB during the stationary phase of growth (19, 43, 50).

We reasoned that if the toxin-deficient phenotype of the *B. anthracis sigH*-null mutant was due solely to elevated AbrB levels, then the toxin phenotype of a *sigH abrB* double mutant

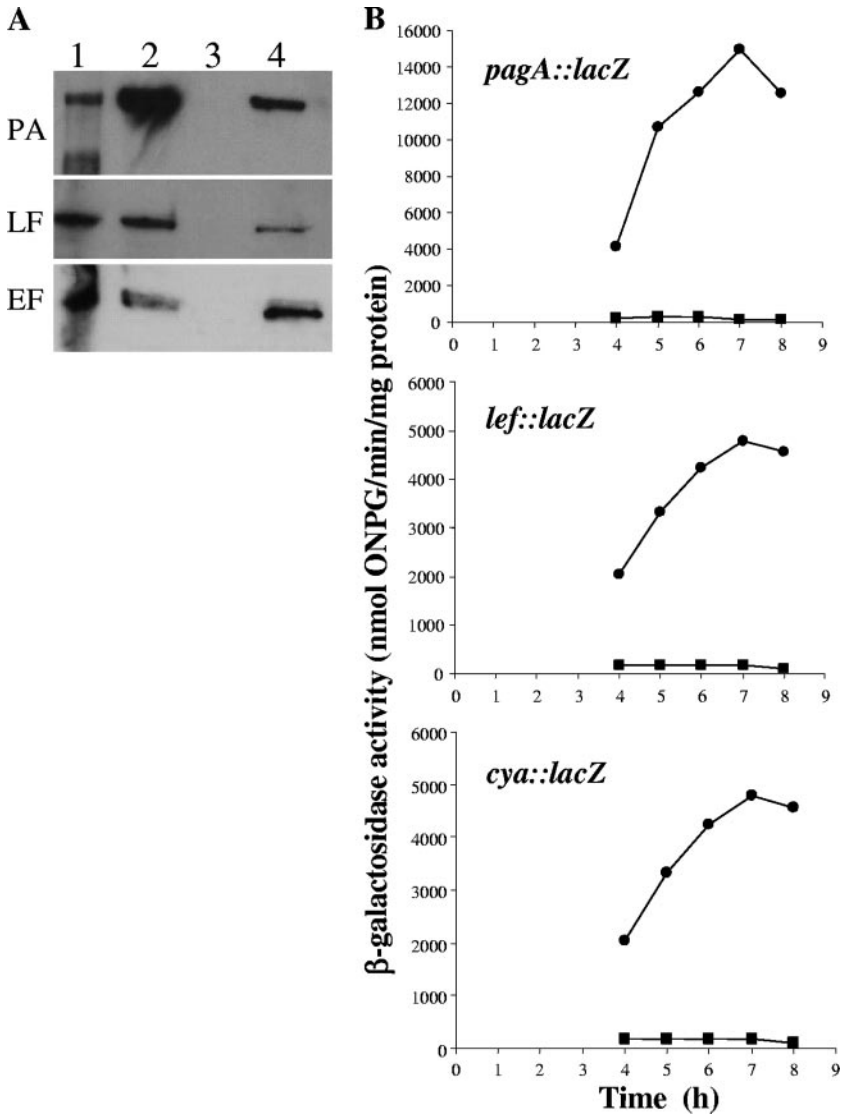

FIG. 2. Reduced toxin gene expression by a *B. anthracis sigH*-null mutant. (A) Synthesis of the toxin proteins. Supernatant samples for Western hybridization analysis were obtained during the late exponential phase of growth (5 h). Equal volumes of supernatant were probed using rabbit antisera raised against the toxin proteins as indicated. Lane 1, recombinant protein; lane 2, UM44 (parent); lane 3, UT198 (*sigH*); lane 4, UT301 (*sigH plcR*::*sigH*). (B) β-Galactosidase activities of promoter-*lacZ* fusions in parent (circle) and *sigH* (square) backgrounds. *pagA*::*lacZ*, UT147 (parent) and UT199 (*sigH*); *lef*::*lacZ*, UT148 (parent) and UT200 (*sigH*); *cya*::*lacZ*, UT133 (parent) and UT201 (*sigH*). Specific β-galactosidase activity (nmol *o*-nitrophenyl-β-D-galactopyranoside [ONPG]/min/mg protein) is shown.

should match that of an *abrB* mutant. We assessed toxin protein levels in culture supernatants of *sigH*, *abrB*, and *abrB sigH* mutants. Figure 3 shows the results of Western hybridizations comparing LF production by the parent and mutant strains. As reported previously (46), LF production by the *abrB*-null mutant was elevated relative to that of the parent strain. However, the *sigH abrB* double mutant exhibited an LF-deficient phenotype comparable to that of the *sigH*-null mutant. Similar results were obtained when supernatants were probed for the other toxin components, EF and PA (data not shown). These data indicate that the SigH effect on toxin synthesis cannot be attributed exclusively to SigH control of *abrB*.

σ^H positively regulates *atxA*. Other than AbrB, no *trans*-acting regulators have been associated with expression of the *atxA* gene. To assess the effect of SigH on *atxA* expression, we examined *atxA* promoter activity in the parent and mutant strains. As shown in Fig. 4, *atxA* promoter activity was highest during the mid-exponential growth phase and elevated in the *abrB*-null mutant, consistent with repression of *atxA* by AbrB

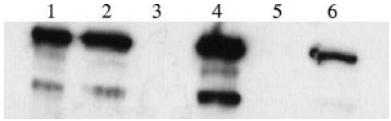

FIG. 3. Synthesis of LF by parent and mutant strains. Supernatant samples for toxin analysis were taken during the late exponential phase of growth (5 h). Lane 1, recombinant protein; lane 2, UM44 (parent); lane 3, UT198 (*sigH*); lane 4, UT166 (*abrB*); lane 5, UT291 (*abrB sigH*); lane 6, UT301 (*sigH sigH*::*plcR*).

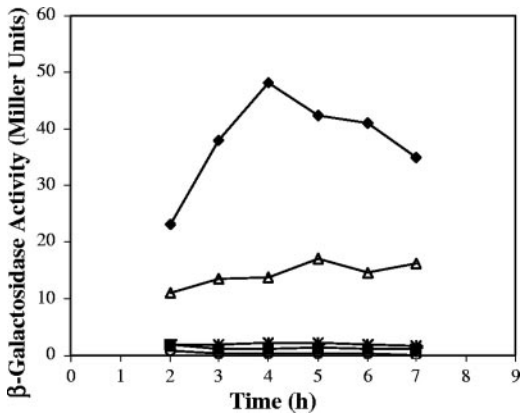

FIG. 4. β-Galactosidase activities of *atxA-lacZ* fusions in parent and mutant strains during growth in toxin-inducing conditions. The low-copy-number plasmid pUTE411 containing a P*atxA-lacZ* transcriptional fusion (46) was introduced into UM44 (parent; triangle), UT198 (*sigH*; square), UT166 (*abrB*; diamond), UT291 (*abrB sigH*; asterisk), and UM44(pHT304-18Z) (empty vector control; circle).

(46, 54). Expression of *atxA* was barely detectable in the *sigH* mutant, in agreement with the low level of toxin synthesis by this mutant. Nevertheless, the *atxA* promoter activity of the *abrB sigH* double mutant was comparable to that of the *sigH* mutant, indicating that SigH positively controls *atxA* independently of AbrB.

The σ^H consensus sequence is absent from the *atxA* and toxin gene promoters. In *B. subtilis*, genes that are recognized by σ^H RNA polymerase are preceded by a consensus sequence (4, 7, 31, 33, 44). Dai et al. (15) previously reported a single transcriptional start site for the *atxA* gene that is preceded by a consensus sequence for the housekeeping sigma factor, σ^A. We examined DNA sequences up to 1,000 nucleotides upstream of the predicted translational start site of *atxA* and detected no apparent σ^H consensus sequences. Sequences corresponding to the σ^H consensus are also not apparent in the toxin gene promoter regions. In fact, no consensus sequences for recognition by any known sigma factor are apparent in the *cya* and *lef* gene promoters or upstream of the major *atxA*-regulated transcription start site, P1, of the *pagA* gene. Weak, constitutively expressed apparent start sites for *pagA*, P2, and other RNAs with 5′ ends mapping downstream of P2 have been described (15, 29). A consensus sequence for σ^A is located upstream of P2 (15).

We questioned whether the promoter regions of *B. anthracis* genes transcribed by σ^H RNA polymerase contained sequences resembling the *B. subtilis* σ^H consensus. We identified *B. anthracis* homologues of *B. subtilis* genes known to be transcribed by σ^H RNA polymerase. Analysis of sequences upstream of such genes revealed the presence of sequences that closely resembled the σ^H consensus sequence established in *B. subtilis* (Table 2). Each promoter was aligned with its *B. subtilis* counterpart using ClustalW (13), to compare sequence similarities and positioning of the σ^H consensus. In the cases of *spoVS*, *sigA*, *spoVG*, and *phrC*, the distances between the consensus sequences and the predicted translational start sites for the genes were very similar in the two species. The *B. subtilis* consensus sequence for the −10 region (RxxGAATww; R indicates A or G; w indicates A or T [7]) was present in all of the *B. anthracis* promoters investigated. On the other hand, the −35 regions of the *B. anthracis* promoters deviated from the *B. subtilis* σ^H consensus in the third and last positions (Table 2). The −10 and −35 regions for σ^H recognition in *B. subtilis* are typically separated by an 11- to 12-nucleotide spacer. The *B. anthracis* promoter regions investigated here revealed spacers that ranged from 10 to 14 nucleotides.

In vitro transcription of *B. anthracis* promoters by σ^H RNA polymerase. To determine if *atxA* or the toxin genes are transcribed directly by σ^H RNA polymerase, we performed in vitro transcription experiments using recombinant *B. anthracis* σ^H, *E. coli* core RNA polymerase, and *B. anthracis* promoter templates (Fig. 5). *B. subtilis* σ factors have been reported to function in vitro with the *E. coli* core enzyme (9, 17). The *B. anthracis spoVG* promoter, which bears the *B. subtilis* σ^H consensus and is strongly SigH dependent in *B. subtilis* (10, 16), was tested as a positive control. Reactions with the *spoVG* DNA template yielded an abundant RNA product of the predicted size for a runoff transcript initiated at the *spoVG* promoter (Fig. 5A). Thus, the purified recombinant *B. anthracis* σ^H protein can function with core RNA polymerase to direct

TABLE 2. *B. anthracis* homologues of *B. subtilis* σ^H RNA polymerase-transcribed genes[a]

B. anthracis gene	*B. subtilis* homolog[b]	Predicted −35 region	Spacing (nt)	Predicted −10 region
BA1767	*citG*	**A**G**GGA**ATT	13	**A**TA**GAAT**AT
BA4223	*kinA*	ND[c]		**A**AA**GAAT**TA
BA1018	*phrC*	TC**AGGA**GTA	10	TTAGCAGTG
BA3285	*phrI*	**A**TG**GGA**ATA	13	**G**CC**GAAT**TA
BA4394	*spo0A*	**A**TC**GGA**AAG	14	**A**GG**GAAT**TT
BA5581	*spo0F*	**A**T**AGGA**AAC	11	**A**AA**GAAT**AG
BA2308	*spo0M*	TTT**GGA**AAA	20	TGC**GAAT**GT
		ATG**GGA**GAA	16	**A**TG**GAAT**AT
BA0047	*spoVG*	**A**AG**GGA**AAA	10	**G**TG**GAAT**TT
BA2154	*spoVS*	**G**C**AGGA**AGA	11	**A**TC**GAAT**GA
BA4515	*sigA*	**G**A**AGGA**TTC	11	**G**TA**GAAT**AC
B. anthracis consensus		**R**xx**GGA**w**W**w	10 to 14	**RxxGAATww**

[a] Boldface indicates a conserved nucleotide. R is an A or a G, and W is an A or a T. A lowercase letter denotes a position in the consensus that is not always conserved.
[b] The *B. subtilis* consensus is as follows: **RxAGGAwWW** (−35 region), 11 to 12 spaces, **RxxGAAT**ww (−10 region).
[c] ND, not determined.

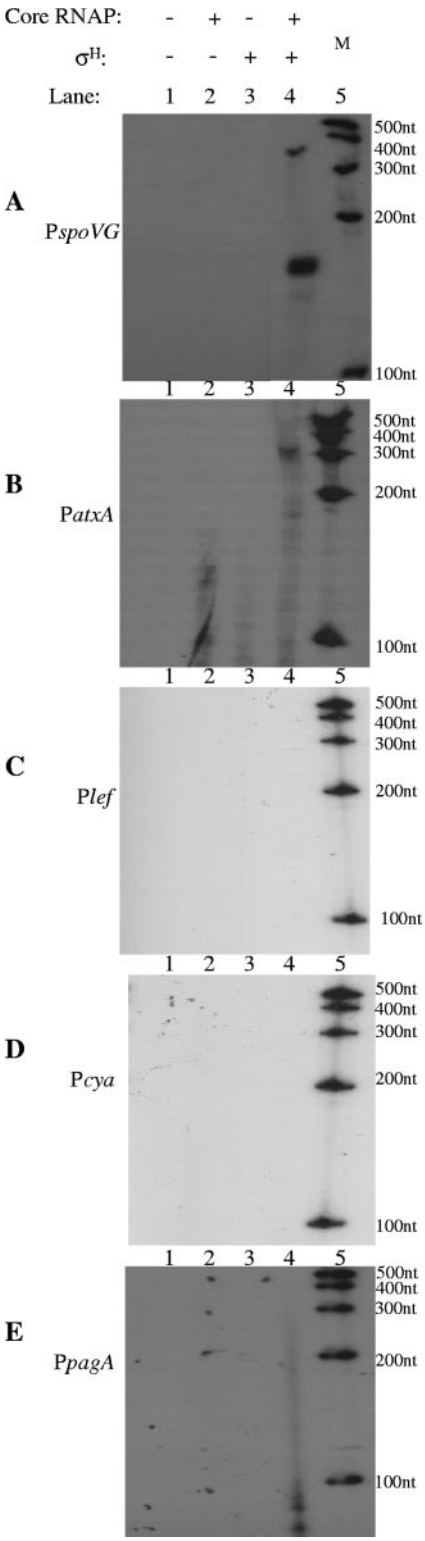

FIG. 5. In vitro transcription of *B. anthracis* promoters using recombinant σ^H RNA polymerase holoenzyme. C-terminally His-tagged σ^H purified from *B. anthracis* was used with core RNA polymerase (RNAP) from *E. coli* as described in Materials and Methods. The compositions of the reaction mixtures and DNA templates are as indicated. +, present; −, absent. Molecular size markers (M) shown in lane 5 are as indicated on the right.

transcription from a *B. anthracis* gene promoter harboring a σ^H consensus sequence.

Given the apparent lack of σ^H recognition sequences in the *atxA*, *pagA*, *lef*, and *cya* gene promoters, we predicted that transcripts would not be generated in reactions using these templates. As expected, the toxin promoter templates did not yield transcripts (Fig. 5C, D, and E), indicating that σ^H affects *pagA*, *lef*, and *cya* gene expression indirectly. In some reactions, faint bands of a size indicating nonspecific end-to-end transcription of the templates were observed (data not shown). The small species detected in some reactions containing the *pagA* template (Fig. 5E, lane 4) are most likely stable degradation products of end-to-end transcripts.

As shown in Fig. 5B, lane 4, an RNA transcript was detected in reactions with the *atxA* promoter template. The size of the in vitro-generated RNA product indicated a transcript initiating approximately 250 nt upstream of the previously reported transcriptional start site for *atxA* (14). This result was surprising because, as discussed above, sequences in this region do not resemble the σ^H consensus established for *B. subtilis*. Nevertheless, data from reverse transcription-PCRs employing RNA from cultured cells confirmed the presence of an RNA transcript in this region (data not shown).

DISCUSSION

The alternative sigma factors of bacteria provide mechanisms for shifting gene expression patterns in response to a changing environment or phase of growth (27). Among the large number of genes with promoters recognized by alternative sigma factors are genes encoding virulence or virulence-associated proteins. Examples include the *inlA* gene of *Listeria monocytogenes*, the *ica* operon in *Staphylococcus aureus* (27), and the exotoxin genes of various clostridial species (17). In some cases, sigma factor homologues possess species-specific functions. For example, SigN RNA polymerase transcribes genes associated with virulence in *Pseudomonas aeruginosa* and *Pseudomonas syringae*, but SigN is not required for the transcription of virulence genes in *Xanthomonas campestris* (26, 27). Similarly, lipase gene expression is differentially regulated by the alternative sigma factor SigB in the closely related species *Staphylococcus aureus* and *Staphylococcus epidermidis* (27). While lipase secretion is elevated in an *S. aureus sigB*-null mutant (30), secretion of processed lipase in an *S. epidermidis sigB*-null mutant is reduced significantly (28).

In the nonpathogenic *Bacillus* species *B. subtilis*, the alternative sigma factor σ^H is required for transcription of a large number of genes that are essential for sporulation (7, 50, 51). As expected, deletion of the *sigH* homolog in *B. anthracis* abolished the ability of the mutant to sporulate, but surprisingly, this sigma factor gene was also required for anthrax toxin gene transcription. Thus, the *sigH* gene, well studied in *B. subtilis* for its role in development, has an additional role in *B. anthracis* as a key player in virulence gene expression.

In *B. subtilis*, the promoters of genes transcribed by σ^H RNA polymerase contain a conserved DNA sequence that is recognized by σ^H. A sequence closely resembling the *B. subtilis* σ^H consensus is present in the promoter regions of *B. anthracis* homologues of *B. subtilis* genes known to be transcribed by σ^H RNA polymerase. For the *B. anthracis* homologues, the se-

```
B.s: 15  FCQLEDEQVIEKVHVGDSDALDYLITKYRNFVRAKARSYFLIGADREDIVQEGMIGLYKS 74
         F  LEDE ++E V  G++DAL+YLI KY+NFVRAK+RSYFL+GADREDIVQEGMIGL+K+
B.a: 13  FRDLEDEAIVELVRKGNTDALEYLIHKYKNFVRAKSRSYFLVGADREDIVQEGMIGLFKA 72

B.s: 75  IRDFKEDKLTSFKAFAELCITRQIITAIKTATRQKHIPLNSYASLDKPIFDEESDRTLLD 134
         IRD+KEDKL+SFKAFAELCITRQIITAIKTATRQKHIPLNSY SLDKPI+DEESDRTLLD
B.a: 73  IRDYKEDKLSSFKAFAELCITRQIITAIKTATRQKHIPLNSYVSLDKPIYDEESDRTLLD 132

B.s: 135 VISGAKTLNPEEMIINQEEFDDIEMKMGELLSDLERKVLVLYLDGRSYQEISDELNRHVK 194
         VIS AK  +PEEMII+QEE+ DIE K+ ELLSDLERKVL LYLDGRSYQEIS++LNRHVK
B.a: 133 VISEAKVTDPEEMIISQEEYTDIESKISELLSDLERKVLSLYLDGRSYQEISEQLNRHVK 192

B.s: 195 SIDNALQRVKRKLEKYLEIRE 215
         SIDNALQRVKRKLE+Y+E+RE
B.a: 193 SIDNALQRVKRKLERYMEMRE 213
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FIG. 6. Amino acid sequence comparison of the *B. anthracis* (B.a) and *B. subtilis* (B.s) homologs. Alignment was performed using the ClustalW web-based alignment program. The underlined sequence marks the position of region 4. Region 4 makes contact with the −35 box on the target promoter. Two significant amino acid differences are boxed.

quence in the −35 region differs slightly from the consensus established for the *B. subtilis* genes. RNA transcripts were obtained from in vitro reactions using the promoter of one representative σ^H RNA polymerase-transcribed homologue, the *B. anthracis spoVG* gene, as the template. The promoter region of *atxA*, the major regulator of anthrax toxin gene transcription, does not bear sequence similarity to the σ^H consensus sequence established for *B subtilis*. Nevertheless, in in vitro reactions, σ^H RNA polymerase generated transcripts from the *atxA* promoter template. No in vitro transcripts were detected in comparable reactions employing toxin gene templates, indicating that σ^H RNA polymerase-mediated transcription from the *atxA* promoter was specific. Taken together, these data indicate that σ^H controls toxin gene expression indirectly, via its direct control of *atxA* gene transcription. Moreover, the *B. anthracis* sigma factor appears to recognize promoters lacking the σ^H consensus sequence established for *B. subtilis* σ^H.

The biochemical activity of the *B. anthracis* σ^H protein may not be identical to that of its *B. subtilis* counterpart. The predicted amino acid sequences of the *B. anthracis* and *B. subtilis* σ^H homologues differ by two residues within region 4.2, the domain responsible for recognition and binding of the −35 region (Fig. 6). The *B. anthracis* σ^H contains serine residues in positions 160 and 172 that are occupied by glycine and valine residues, respectively, in the *B. subtilis* σ^H protein. In addition to these differences, the 13 amino-terminal residues of the two proteins exhibit relatively low amino acid sequence similarity. Comparable differences among other sets of sigma factor homologues can result in altered target specificity and binding affinity (45).

Our investigation reveals newly discovered regulatory relationships between *sigH*, *atxA*, and another anthrax toxin gene regulator, *abrB*, when *B. anthracis* is grown in conditions that favor toxin gene expression. AbrB is a transition state regulator, best characterized in *B. subtilis*, that affects the transcription of multiple genes in a growth phase-dependent manner (42, 50). Steady-state levels of anthrax toxin gene transcripts are increased in a *B. anthracis abrB*-null mutant (46), and in vitro footprint analyses have revealed AbrB binding sites within the *atxA* promoter region (54). Thus, AbrB appears to affect toxin gene expression by controlling the transcription of *atxA*.

In experiments designed to model AbrB control of *atxA* in *B. subtilis*, Strauch et al. (54) showed that the activity of an *atxA* promoter-*lacZ* transcriptional fusion is increased in a *B. subtilis abrB*-null mutant. In *B. subtilis*, AbrB is one of the many factors that affect σ^H synthesis (54, 57). The *sigH* gene is subject to stringent transcriptional, posttranscriptional, and posttranslational control (32, 57). During the logarithmic phase of growth, when conditions are not conducive to sporulation, levels of σ^H are relatively low. Upon entry into the stationary growth phase, σ^H levels rise as a result of *abrB* repression by phosphorylated Spo0A, the master regulator of sporulation initiation. The *spo0A* gene has two transcriptional start sites, one of which is σ^H dependent. Thus, as a culture enters the stationary phase, the levels of Spo0A and σ^H, as members of the same positive feedback loop, rise (50). Strauch et al. (54) demonstrated that, when *B. subtilis* was cultured in conditions conducive to sporulation, the activity of an *atxA* promoter-*lacZ* transcriptional fusion was reduced in a *sigH* single mutant and increased in an *abrB sigH* double mutant. The change in *atxA* expression by the *B. subtilis sigH* mutant was attributed to σ^H control of *abrB* via Spo0A and AbrB control of *atxA* transcription. In contrast, our experiments investigating gene expression in *B. anthracis* revealed that the *sigH* effect on *atxA* expression is not dependent upon *abrB*. We note that the *atxA* promoter-*lacZ* transcriptional fusion used by Strauch et al. (54) contained only 200 nt that were upstream of the previously reported transcriptional start site for *atxA* and was thus devoid of the upstream region we have associated with σ^H RNA polymerase-mediated transcription. Moreover, there could be species-specific or growth condition-dependent differences in σ^H function. For our investigations, we grew *B. anthracis* in conditions favorable for toxin synthesis, $CACO_3$ medium in 5% CO_2. When cultured in this manner, *B. anthracis* cells produce high levels of the anthrax toxin proteins yet sporulate poorly even after prolonged growth (M. Hadjifrangiskou and T. M. Koehler, unpublished data) (37).

The implication of genes associated with the complex network of developmental control in *B. anthracis* toxin gene expression is one of a few intriguing insights into relationships between *B. anthracis* sporulation and virulence. Perego and coworkers (5, 8) recently reported genetic and biochemical analyses of components of the phosphorelay signal transduction system controlling development in *Bacillus* species. Nine sporulation kinase genes were identified in *B. anthracis*. Two of these contained frame shifts in all *B. anthracis* strains investi-

gated, and one of them was also inactivated in a pathogenic strain of *B. cereus* harboring the *B. anthracis* toxin plasmid pXO1 (8). Their results suggest that acquisition of pXO1 and, possibly, virulence genes is associated with loss of sporulation sensor histidine kinase activities. Interestingly, in *B. subtilis* the sensor kinase genes *kinA* and *kinE* have σ^H-regulated promoters. We examined the promoter regions of the *B. anthracis* kinase genes for the σ^H consensus sequence. With the exception of BA4223, which has an apparent −10 sequence matching that of the σ^H consensus, none of the *B. anthracis* kinase genes are predicted on the basis of sequence data to be transcribed by σ^H RNA polymerase. Despite the lack of a σ^H consensus sequence in the promoter regions of these genes, the kinases may be transcribed directly by σ^H RNA polymerase in *B. anthracis*, as we have found for *atxA*. Alternatively, if the kinase genes are not recognized by σ^H RNA polymerase, this may be another indication that the pathway for *B. anthracis* development deviates from the pathway established for *B. subtilis*.

Additional evidence implicating pXO1 in *B. anthracis* development is the apparent incompatibility between *atxA* and *plcR*, a pleiotropic regulator of virulence genes in *B. cereus* and *B. thuringiensis* (1). The *atxA* gene, located on pXO1, is not found in most *B. cereus* and *B. thuringiensis* strains. The *plcR* gene is found in all three species but contains a nonsense mutation in all *B. anthracis* strains examined (49). Mignot et al. (35) reported that coexpression of *plcR* and *atxA* in a pXO1$^+$ background prevented *B. anthracis* from sporulating efficiently. The sporulation defect was rescued in an *atxA*-null mutant, suggesting that the *plcR* and *atxA* regulons cannot successfully coexist in *B. anthracis*.

The results reported here, combined with previously published data, point to multiple means of control of *atxA* expression. Stringent control of *atxA* expression is in agreement with the results of a previous study indicating that steady-state levels of AtxA in *B. anthracis* are critical for optimal toxin synthesis. A recombinant strain harboring multiple copies of *atxA* and producing elevated levels of AtxA exhibited reduced *pagA* expression (14). We have found that a recombinant strain that overexpresses *sigH* produces reduced levels of the toxin proteins. Furthermore, as is true for Spo0A in *B. subtilis* (18), overexpression of *sigH* results in a delay in the onset of sporulation when *B. anthracis* is cultured in conditions that favor sporulation (Hadjifrangiskou and Koehler, unpublished data). The regulatory relationships between *sigH* and other genes controlling development may be species specific and/or vary with respect to growth conditions. Altered expression and/or function of these developmental regulators in *B. anthracis* may enable the bacterium to maximize toxin production during growth within a host where sporulation does not occur, while maintaining the ability to initiate sporulation upon a change in environment.

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