Dual Promoters Control Expression of the *Bacillus anthracis* Virulence Factor AtxA

Cristina Bongiorni,§ Tatsuya Fukushima, Adam C. Wilson, Christina Chiang, M. Cecilia Mansilla,¶ James A. Hoch, and Marta Perego*  
Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037  

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The AtxA virulence regulator of *Bacillus anthracis* is required for toxin and capsule gene expression. AtxA is a phosphotransferase system regulatory domain-containing protein whose activity is regulated by phosphorylation/dephosphorylation of conserved histidine residues. Here we report that transcription of the *atxA* gene occurs from two independent promoters, P1 (previously described by Dai et al. [Z. Dai, J. C. Sirard, M. Mock, and T. M. Koehler, Mol. Microbiol. 16:1171–1181, 1995]) and P2, whose transcription start sites are separated by 650 bp. Both promoters have −10 and −35 consensus sequences compatible with recognition by σ^~^-containing RNA polymerase, and neither promoter depends on the sporulation sigma factor SigH. The dual promoter activity and the extended untranslated mRNA suggest that as-yet-unknown regulatory mechanisms may act on this region to influence the level of AtxA in the cell.

The virulence of *Bacillus anthracis*, the causative agent of anthrax, results from the production of a tripartite toxin and an antiphagocytic poly-β-glutamic acid capsule (21). The toxin consists of three proteins: protective antigen (PA), encoded by the *pagA* gene; edema factor, encoded by the *cya* gene; and lethal factor, encoded by the *lef* gene. The *pagA*, *cya*, and *lef* genes are located noncontiguously on the pXO1 virulence plasmid, and their expression requires the product of another plasmid gene, *atxA* (9, 17, 22, 32). The AtxA regulator is also required for transcription of the *capBCADE* operon for capsule production, which is located on the second virulence plasmid of *B. anthracis*, pXO2 (7, 12, 19, 33). The requirement for AtxA for *cap* operon transcription is mediated by the products of two pXO2 genes, *acpA* and *acpB*, which share some similarity with AtxA (11).

In addition to being required for virulence gene expression, AtxA has also been shown to be a global regulator of gene expression in *B. anthracis*. Expression profiling indicated that the transcription of a variety of genes, located either on the chromosome or on the virulence plasmids, is affected by AtxA (4).

AtxA belongs to the wide family of phosphotransferase system regulatory domain (PRD)-containing proteins (31). PRDs are structural domains generally found in RNA-binding anti-terminators or in DNA-binding transcription factors. The activity of PRD-containing proteins is generally regulated by phosphorylation on conserved histidine residues. The phosphorylation is carried out by enzymes of the phosphoenolpyruvate:sugar phosphotransferase system (for a review, see reference 10). Phosphorylation of the His199 residue in PRD1 of AtxA was shown to be necessary for its activity, while phosphorylation of His379 in PRD2 was inhibitory. This suggested that regulation of virulence factor production in *B. anthracis* may be linked to carbohydrate metabolism (31).

Virulence factor gene expression is also affected by growth under specific conditions; capsule synthesis and toxin protein synthesis are induced when strains are grown in defined media with bicarbonate at elevated atmospheric CO₂ concentrations (≥5%). Although this induction requires AtxA, the transcription of *atxA* itself did not appear to be affected by growth under CO₂/bicarbonate conditions compared to growth in air (8, 18).

The *atxA* gene was reported to be transcribed from a promoter (referred to here as P1) (9) located 99 bp upstream from the ATG translational start site (Fig. 1). The AbrB protein, characterized in *Bacillus subtilis* as a transition state regulator that suppresses postexponential-phase gene expression during the exponential phase of growth, was shown to bind to the *atxA* promoter region (30) and to repress its transcription (28). In agreement with these results, deletion of the *abrB* gene in *B. anthracis* resulted not only in elevated *atxA* expression in exponential phase but also in earlier transcription and higher levels of transcription of *pagA*, *cya*, and *lef* compared to the parental strain (28).

Deletion of *sigH*, encoding the sigma factor for sporulation initiation in *B. anthracis*, was shown to result in a sporulation defect and to completely inhibit *atxA* and toxin gene expression. However, Strauch et al. (30) observed that transcription of the *atxA* P1 promoter did not require SigH when the *B. subtilis* model system was used.

Here we report that transcription of *atxA* initiates at an additional promoter (P2) located within the coding sequence of a gene on pXO1, *orf118* (referred to here as the pXO1-118 gene), which is upstream of and divergently transcribed from...
the atxA gene. This study showed that, although the Orf118 protein was not required for atxA expression, its coding sequence contained promoter elements critical for full activation of this gene. Furthermore, our analysis revealed that SigH is not required for atxA transcription under either air or CO₂/bicarbonate growth conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. B. anthracis strain 34F2 (pXO1−/pXO2−) was used throughout this study. Cells were grown in LB medium in air or in R medium (27) in an atmosphere containing 5% CO₂. Antibiotics were added at the following concentrations: kanamycin, 7.5 μg/ml; chloramphenicol, 7.5 μg/ml; spectinomycin, 50 μg/ml; erythromycin, 5 μg/ml; and lincomycin, 25 μg/ml. Escherichia coli strains DH5α and TG1 were used for plasmid construction and propagation. E. coli strains C600 and SCS110 were used for the production of unmethylated DNA for transformation in B. anthracis. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; and spectinomycin, 100 μg/ml.

Electrocompetent cells of B. anthracis were prepared as described by Koehler et al. (17).

Construction of pXO1-118, atxA, and sigH deletion strains. The sequences of the oligonucleotide primers used in this study are shown in Table S1 in the supplemental material. Plasmid pORICm carrying a temperature-sensitive replication origin and a chloramphenicol resistance marker was used for construction of the pXO1-118 gene deletion strain (5). A 720-bp fragment downstream of the pXO1-118 gene was PCR amplified using oligonucleotides Delta118Kpn and Delta118Bam and cloned in pORICm at the KpnI and BamHI sites. An 860-bp fragment upstream of the pXO1-118 gene was also PCR amplified using oligonucleotides Delta118Sal and Delta118Pst and cloned in the plasmid indicated above at the SalI-PstI sites. Finally, a blunt-ended spectinomycin cassette (5) was cloned at the HincII site located between the two cloned fragments in the vector multiple cloning site. The resulting plasmid (pORICm-Δ118) was transformed into strain 34F2 and used to generate a deletion-spectinomycin replacement of the pXO1-118 gene essentially as described previously (5).

Plasmid pORICm was also used for construction of the atxA deletion strain. A fragment containing the entire atxA coding region and approximately 500 bp upstream was PCR amplified using oligonucleotides Ba118delta and AtxA3Bam and cloned in the EcoRI-BamHI sites of pORICm. The resulting plasmid, after transformation in dam E. coli strain C600, was digested with BclI and EcoRV, and the 670-bp excised fragment was replaced by the spectinomycin cassette as a BamHI-HincII fragment. The resulting plasmid (pORI-ΔatxA) (Fig. 1) was used to transform strain 34F2 and generate a deletion replacement of the atxA gene essentially as described previously (5).

A deletion in the sigH gene (BA0093 in the B. anthracis Ames strain; accession number NC_003997) was generated by using the protocol of Janes and Stibitz (16) and the temperature-sensitive plasmid pORICm-SceI (3). A 1,000-bp fragment generated by PCR amplification using oligonucleotide primers Basig HpEco and BasigH3 Bsm2 and digested with BamHI was cloned in the HincII-
BamHI sites of pORICm-Scl. A spectinomycin cassette, purified as an EcoRV-EcoRV fragment, was cloned in the pORICm-Scl-SigH plasmid digested with BglII and treated with T4 DNA polymerase (New England Biolabs). The resulting plasmid, designated pORI-SigH, generated a spectinomycin cassette insertion site of 84 bp of strain 34F2, giving rise to a 347-bp fragment.

Each mutant construct was verified by diagnostic PCR using genomic DNA. All cloned fragments were fully sequenced to ensure the fidelity of the amplification reaction. A diagnostic PCR was also carried out with genomic DNA using atxA-specific primers to ensure that the pXO1 plasmid was not lost during the process.

Construction of lacZ fusion plasmids. The pTVCV transcriptional fusion vector was used for construction of the atxA-lacZ reporter plasmids (24). A map of the cloned fragments is shown in Fig. 1. The fragment in pAtxA10 was obtained by PCR amplification using oligonucleotide primers AtxA5’promEco and AtxA3’Bam. The fragment was digested with EcoRI and EcoRV (the latter naturally occurring within the atxA sequence), and the purified 200-bp fragment was cloned in the EcoRI and SmaI sites of pTVCV. Similarly, fragments in pAtxA13, pAtxA15, and pAtxA12 were generated with oligonucleotide primers Delta 118’Eco2, Alt118delta, and AtxA5’upEcoRI, respectively, as the forward primers and primer AtxA3’Bam as the reverse primer. The fragments were digested with EcoRI and EcoRV and cloned in the EcoRI-SmaI-digested pTVCV vector. The fragment in pAtxA12 was also cloned in the pHT315 vector digested with EcoRI and SmaI to generate pAtxA3 for in trans complementation. The fragment in pAtxA10 was generated with oligonucleotide primers AtxA5’upEcoRI and AtxApromBam2. The fragment was digested with EcoRI and BamHI and cloned in similarly digested pTVCV. Fragments in pAtxA20 and pAtxA21 were generated with oligonucleotides AtxA5’upEcoRI and delta118Eco2, respectively, as the forward primers and AtxApromBam3 and AtxApromBam2, respectively, as the reverse primers. The approximately 500- and 200-bp digested fragments, respectively, were cloned in the EcoRI-BamHI-digested pTVCV vector.

Plasmid pAtxA18 was constructed in three steps. First, the product of PCR amplification carried out with oligonucleotide primers AtxA5’upEcoRI and 1183’Kpn was cloned in the EcoRI-Kpn site of the pUC19-derived multiple cloning site of plasmid pHT315 (2). The resulting plasmid was digested with KpnI and BamHI and ligated to the KpnI-BamHI-digested PCR product obtained with oligonucleotide primers 1185’Kpn and AtxA3’Bam. The resulting plasmid contained the entire pXO1 fragment shown in Fig. 1. The fragment in pAtxA10 was also cloned in the EcoRI and SmaI sites of pTVCV. Similarly, fragments in pAtxA13, pAtxA15, and pAtxA12 were generated with oligonucleotide primers Delta 118’Eco2, Alt118delta, and AtxA5’upEcoRI, respectively, as the forward primers and primer AtxA3’Bam as the reverse primer. The fragments were digested with EcoRI and EcoRV and cloned in the EcoRI-SmaI-digested pTVCV vector. The fragment in pAtxA12 was also cloned in the pHT315 vector digested with EcoRI and SmaI to generate pAtxA3 for in trans complementation. The fragment in pAtxA10 was generated with oligonucleotide primers AtxA5’upEcoRI and AtxApromBam2. The fragment was digested with EcoRI and BamHI and cloned in similarly digested pTVCV. Fragments in pAtxA20 and pAtxA21 were generated with oligonucleotides AtxA5’upEcoRI and delta118Eco2, respectively, as the forward primers and AtxApromBam3 and AtxApromBam2, respectively, as the reverse primers. The approximately 500- and 200-bp digested fragments, respectively, were cloned in the EcoRI-BamHI-digested pTVCV vector.

In order to ensure that orf118 was no longer translated in this construct, a translational lacZ fusion construct was generated by cloning a PCR fragment obtained with oligonucleotide primers P1183’Eco and 1183’Kpn into the BamHI site of plasmid pHT315 (2). The resulting plasmid was digested with KpnI and BamHI and ligated to the KpnI-BamHI-digested PCR product obtained with oligonucleotide primers 1185’Kpn and AtxA3’Bam. The resulting plasmid contained the entire pXO1 fragment shown in Fig. 1 with a KpnI site and a base pair deletion engineered at the ATG translational start codon of orf118. The fragment was cloned in the EcoRI and SmaI sites of pTVCV. Similarly, fragments in pAtxA13, pAtxA15, and pAtxA12 were generated with oligonucleotide primers Delta 118’Eco2, Alt118delta, and AtxA5’upEcoRI, respectively, as the forward primers and primer AtxA3’Bam as the reverse primer.
gene encodes a virulence plasmid sensor domain with a high level of similarity to the sensor domain of the BA2291 sporulation histidine kinase (5, 34). Both the pXO1-118 protein and its paralogue encoded on the pXO2 virulence plasmid, the pXO2Δ118 protein, were shown to inhibit the activity of the pXO1-118 gene region, including the putative BA2291 kinase and thereby inhibit sporulation initiation in B. anthracis. A deletion of the pXO1-118 gene was generated by replacing the entire coding sequence with a spectinomycin resistance cassette, resulting in strain 34F2/H9252. While analyzing the role of the pXO1-118 gene in the physiology of B. anthracis, we found that deletion of this gene resulted in a 50% decrease in PA production in the culture supernatant (Fig. 2B, compare lanes 1 and 3). Cells were grown in LB medium containing kanamycin. Time zero is the time of transition between logarithmic growth and the stationary phase. (B) Western blot analysis of the PA levels in culture supernatants of parental and 34F2Δ118 mutant strains complemented with the pXO1-118 gene on the multicopy vector pHT315. Samples were collected at the time point indicated by the double arrow in panel A. Lane 1, 34F2/pHT315; lane 2, 34F2/pAtxA3; lane 3, 34F2Δ118/pHT315; lane 4, 34F2Δ118/pAtxA3; lane 5, purified PA (0.01 μg). Lane M contained Western blot standard Magic Mark XP (Invitrogen). The molecular masses of bands (in kDa) are indicated on the left. The bands were quantitated with the Image Quant software (Amersham-Molecular Dynamics), and the pixel value for each lane is indicated below the gel.

FIG. 2. PA expression in the pXO1-118 gene deletion strain. (A) β-Galactosidase assay of the pagA-lacZ reporter in parental strain 34F2 (●) and in the 34F2Δ118 mutant strain (▲). Cells were grown in LB medium containing kanamycin. Time zero is the time of transition between logarithmic growth and the stationary phase. (B) Western blot analysis of the PA levels in culture supernatants of parental and 34F2Δ118 mutant strains complemented with the pXO1-118 gene on the multicopy vector pHT315. Samples were collected at the time point indicated by the double arrow in panel A. Lane 1, 34F2/pHT315; lane 2, 34F2/pAtxA3; lane 3, 34F2Δ118/pHT315; lane 4, 34F2Δ118/pAtxA3; lane 5, purified PA (0.01 μg). Lane M contained Western blot standard Magic Mark XP (Invitrogen). The molecular masses of bands (in kDa) are indicated on the left. The bands were quantitated with the Image Quant software (Amersham-Molecular Dynamics), and the pixel value for each lane is indicated below the gel.

FIG. 3. Transcriptional analysis of atxA-lacZ fusion constructs in parental strain 34F2. Cultures used for β-galactosidase assays were grown in LB medium, and samples were taken at hourly intervals before and after the transition (time zero) from exponential phase to stationary phase. Symbols: +, pTCVlac; □, pAtxA10; ○, pAtxA12; ○, pAtxA13; ▲, pAtxA15; ▼, pAtxA18; ●, pAtxA19; ▲, pAtxA20; ●, pAtxA21; △, growth curve for a representative strain (34F2/pAtxA15). OD525, optical density at 525 nm.

Full expression of atxA requires the DNA region containing the pXO1-118 gene. Because the expression of pagA was reduced by deletion of the pXO1-118 coding sequence but not by the absence of the pXO1-118 protein, we reasoned that deletion of this DNA region could have affected the expression of atxA, whose product is required for pagA expression. Thus, we constructed an atxA-lacZ transcriptional fusion in vector pTCVlac (pAtxA10) and tested the level of atxA transcription in the 34F2Δ118 strain. The fragment in pAtxA10 contains the P1 promoter previously identified as the atxA promoter (Fig. 1) (9). The results (data not shown) did not reveal any difference in the level of transcription between the parental 34F2 strain and the pXO1-118 gene mutant strain. Therefore, we considered the possibility that DNA regions upstream of the fragment cloned in pAtxA10 and encompassing the pXO1-118 gene could be involved in atxA transcription.

To examine this, a series of atxA-lacZ fusion plasmids were constructed that contained fragments extending upstream of atxA to include the entire pXO1-118-atxA intergenic region (pAtxA13), the intergenic region and part of the pXO1-118 gene (pAtxA15), and the entire intergenic region and the pXO1-118 gene region, including the putative rho-independent terminator that follows it (pAtxA12). When β-galactosidase assays were carried out with the 34F2 strains carrying these atxA-lacZ fusion constructs grown in LB medium (Fig. 3A), it was observed that the fusion carrying the entire pXO1-118 gene, pAtxA12, had atxA transcriptional activity that was approximately threefold higher than that of the pAtxA10 fusion. Using the pAtxA13 and pAtxA15 fusions, which contained the intergenic region without and with a portion of the pXO1-118
gene, respectively, resulted in a level of atxA transcription that was intermediate between that of the fusion with the short fragment (pAtxA10) and that of the fusion with the long fragment (pAtxA12) (Fig. 3A). Similar patterns of transcription were observed when cells were grown in R medium with CO2/bicarbonate, but significantly smaller differences in the level of activity were observed between the pAtxA12 construct and the pAtxA10 construct (see Fig. 7B and data not shown).

The levels of AtxA expression observed with each fusion construct (pAtxA10, pAtxA12, pAtxA13, and pAtxA15) were identical in the parental 34F2 strain and the 34F2Δ118 strain (data not shown), confirming the lack of a role for the pXO1-118 gene product in controlling atxA transcription. Transcription from the pAtxA10 and pAtxA12 constructs was also not affected by deletion of atxA itself (strain 34F2ΔatxA) (see Fig. S1A in the supplemental material), ruling out the possibility of an autoregulatory mechanism. Furthermore, the level of β-galactosidase activity measured in the 34F2 strain carrying plasmid pAtxA22, which contained the long fragment but in which the pXO1-118 gene was replaced by the spectinomycin resistance cassette, was twofold lower than the activity measured in the strain carrying the pAtxA12 construct (data not shown).

The findings just described again suggested that the DNA containing the pXO1-118 gene was required for full atxA transcription. In order to confirm this, an additional atxA-lacZ fusion construct was generated in pTCVlac carrying the long fragment like pAtxA12 but with the start codon of the pXO1-118 gene replaced by the GTA sequence in a KpnI restriction site and a base pair deletion that shifted the translational frame (see Materials and Methods). β-Galactosidase assays carried out with the 34F2 strain containing the resulting plasmid, pAtxA18 (Fig. 1), showed that the translational frameshift mutation in the pXO1-118 gene, which eliminated expression of the pXO1-118 protein, did not affect the level of transcription of atxA, which remained identical to the level obtained with plasmid pAtxA12 (Fig. 3B).

Altogether, these results indicated that full transcription of atxA requires the ~900-bp fragment upstream of its translation start codon, which contains the divergently transcribed pXO1-118 gene.

The atxA 900-bp upstream region contains an additional promoter. In order to distinguish whether the 900-bp sequence upstream of atxA was required for full expression because it contained a binding site for an activator or contained an additional promoter(s), two more atxA-lacZ fusions were generated with the same 5′ end of pAtxA12 but with truncation at the 3′ end (Fig. 1); plasmid pAtxA19 lacked approximately 170 bp at the 3′ end and the P1 promoter was deleted, while in pAtxA20 the entire pXO1-118-atxA intergenic region was deleted. A third plasmid, pAtxA21, was also constructed and contained the intergenic region without the P1 promoter. β-Galactosidase assays carried out with the 34F2 strains containing these plasmids in LB medium showed that the transcription generated from the pAtxA21 reporter was not significantly different from the transcription observed with the control vector pTCVlac alone, indicating that this fragment is unlikely to carry an active promoter (Fig. 3A). In contrast, efficient transcription was observed with the pAtxA19 and pAtxA20 constructs, suggesting that promoter activity independent of the P1 promoter was present. The transcription of atxA from the pAtxA19 and pAtxA20 constructs initiated at the same time during growth, as observed with the pAtxA10 and pAtxA12 constructs but with different rates. The initial rate of transcription was only 20% lower for pAtxA19 than for pAtxA12, but it was 80% lower for pAtxA20 than for pAtxA12. After 4 h of growth, the level of transcription from the fragment in pAtxA19 was approximately the same as the level obtained with the long fragment in pAtxA12, while the level of transcription from pAtxA20 was approximately the same as the level of transcription from the short fragment in pAtxA10. The same pattern of gene expression was observed for all constructs when cells were grown in R medium under CO2/bicarbonate conditions (data not shown).

Altogether, these results suggest that the 900-bp region upstream of the atxA gene, which includes the pXO1-118 gene, most likely contains an additional promoter for atxA expression. Furthermore, the pAtxA21 results indicate that this putative promoter is in the pXO1-118 gene coding region and not in the intergenic region between the pXO1-118 gene and atxA.

The atxA transcript starts within the pXO1-118 gene. In order to confirm that an atxA mRNA transcript initiated within the sequence of the pXO1-118 gene, RT-PCRs were carried out with DNase-treated mRNA from strain 34F2 grown in LB medium using primer AtxART2 in the RT reaction and the oligonucleotide primer pairs AtxART2-AtxA5’upEcoRI and AtxA5’upEcoRI, respectively, using 34F2 genomic DNA as the template. Lane M contained the 1 kb Plus DNA ladder (New England Biolabs). Molecular sizes (in base pairs) are indicated on the right.

FIG. 4. RT-PCR analysis of the atxA mRNA. RT-PCR was carried out with mRNA extracted from strain 34F2 and treated with RNase-free DNase. The mRNA was first incubated with (+) or without (−) RT and oligonucleotide primer AtxART2 (RT2 in Fig. 1). An aliquot of the reaction mixture was then incubated with oligonucleotide primers AtxART2 and AtxART3 (RT3 in Fig. 1) (lanes 1 and 2) or oligonucleotide primers AtxART2 and AtxA5’upEcoRI (5’up in Fig. 1) (lanes 4 and 5). Lanes 3 and 6 contained the products of PCR amplifications carried out with oligonucleotides AtxART2 and AtxA5’upEcoRI, respectively, using 34F2 genomic DNA as the template. Lane M contained the 1 kb Plus DNA ladder (New England Biolabs). Molecular sizes (in base pairs) are indicated on the right.

These results confirmed that atxA mRNA extended into the pXO1-118 gene region and supported the hypothesis that there was a promoter within the gene that contributed to atxA transcription in addition to the P1 promoter identified by Dai et al. (9).
Identification of the additional **atxA** transcription start site.

In order to precisely determine the additional start site of **atxA** transcription, RT reactions were carried out with mRNA extracted from parental strain 34F2. Using the oligonucleotide primer AtxART2, whose sequence was identical to that of the primer used by Dai et al. (9), we noticed that, in addition to an approximately 180-bp product that defined the P1 start site, a very weak but consistent approximately 700-base product was also generated (see Fig. S2 in the supplemental material). A primer extension reaction was then carried out with primer AtxART1, which overlaps the P1 start site (Fig. 1). A slightly stronger, defined product consisting of two approximately 600-base bands was consistently observed on 4% acrylamide-8 M urea gels. This result was obtained with mRNA extracted from cells grown in either LB medium or R medium (see Fig. S2 in the supplemental material; data not shown). This confirmed again that the **atxA** mRNA extended upstream of the P1 start site. With a third oligonucleotide primer, AtxART4, located within the pXO1-118 gene, two bands at approximately 180 and 250 bases were also clearly obtained (see Fig. S2 in the supplemental material).

The product of the RT reaction carried out with the AtxART4 primer was then run on a sequencing gel alongside the product of a sequencing reaction carried out with the same primer (Fig. 5A). This allowed identification of the start site of transcription of the longer product as an adenine base (P2), which is preceded at the expected distance by a −10 canonical sequence for σ^70-containing RNA polymerase (TATAAT). A possible −35 consensus sequence (ATGAAAT) was also identified upstream and was separated by 17 bp from the −10 sequence. The shorter and weaker product of the RT reaction identified a guanine nucleotide as a putative start site (P3). This residue is also preceded by a putative −10 consensus sequence (AATTAC), whose first four residues could suggest involvement of a σ^70-containing RNA polymerase in the transcription initiating at this site. However, sequences with similarity to consensus sequences for σ^70 and σ^32 sites could be identified (ATGATC−18 bp−TTCAAT) upstream of this site, raising the possibility that the product is indeed a product of the RT reaction and not an artifact (Fig. 5B).

Altogether, these results showed that a second promoter, P2, and perhaps a third promoter, P3 (Fig. 1), in addition to the P1 promoter identified by Dai et al. (9) contribute to full expression of the **atxA** gene.

**Transcription of **atxA** is independent of the SigH sigma factor.**

A requirement for the SigH sporulation sigma factor for transcription of **atxA** was recently reported (14). Our finding that multiple promoters contribute to **atxA** expression prompted us to analyze whether the SigH requirement applied to any specific **atxA** promoter. An inactivation/spectinomycin insertion of the **sigH** gene was generated in the 34F2 strain (see Materials and Methods), and, as previously reported (14), the resulting 34F2Δ**sigH** strain was sporulation deficient (data not shown).
shown) and did not produce any SigH protein as determined by Western blot analysis (Fig. 6).

The atxA-lacZ fusion constructs pAtxA10, pAtxA12, pAtxA13, pAtxA15, pAtxA19, pAtxA20, and pAtxA21 were introduced by electroporation into the 34F2ΔsigH mutant strain, and β-galactosidase assays were carried out in LB medium in air or in R medium in a 5% CO₂ atmosphere. β-Galactosidase assays carried out with the resulting strains grown in LB medium showed no inhibition of atxA expression; rather, they showed a slight, but consistent, increase in expression (compare Fig. 3A with Fig. 7A and Fig. S3 in the supplemental material). An increase in β-galactosidase activity in the sigH mutant compared to the parental strain was also observed with the pAtxA10 and pAtxA12 constructs when cells were grown in R medium with CO₂/bicarbonate, indicating that this effect was not specific to one growth condition (Fig. 7B). The same results were obtained with a sigH mutant generated in a different background, Sterne strain 7702, indicating that the effect was not strain specific (data not shown).

Notably, neither the time of induction of atxA transcription (approximately 1 h before the transition from the vegetative to stationary phase) nor the initial level of activity in R medium (Fig. 7B) was affected by the sigH mutation in either the LB or R medium growth conditions.

AtxA and PA protein analysis by Western blotting carried out with cell lysates and culture supernatants, respectively, confirmed that sigH inactivation did not eliminate the production of these proteins (Fig. 6).

Altogether, these results indicate that inactivation of the sigH gene in B. anthracis blocks sporulation initiation but does not affect atxA gene transcription and, consequently, toxin gene production.

**DISCUSSION**

Toxin and capsule synthesis in B. anthracis requires the product of the atxA gene. Consistently, an atxA-null mutant was shown to be avirulent in mice and to elicit a lower antibody response to toxin proteins than the parental strain (9). The relevance of AtxA in the virulence of B. anthracis is likely to be associated with complex mechanisms regulating its expression and/or activity. The evidence that this is the case includes the finding that AtxA is phosphorylated in vivo and the finding that its activity is regulated by opposing effects of phosphorylation on two conserved histidine residues within PRDs. Phosphorylation on His199 of PRD1 is required for AtxA activity, while phosphorylation on two conserved histidine residues within PRDs. Phosphorylation on His199 of PRD1 is required for AtxA activity, while phosphorylation of His379 inhibits the regulator (31).

Notably, the virulence regulator Mga in the group A streptococcus (Streptococcus pyogenes) shares structural domain similarities with B. anthracis AtxA as it also contains two DNA-binding domains at the amino terminus and two PRDs in the central domain. Expression of mga is autoregulated, and its promoter has two transcriptional start sites, one of which is activated by the carbon catabolite regulator protein CcpA (1, 20, 21).

In this work, previous studies on transcription regulation of the atxA gene were extended in order to better understand the
possible contribution of regulation of gene expression in *B. anthracis* virulence.

The first promoter identified as a promoter responsible for *atxA* transcription (P1) (9) is approximately 100 bp upstream of the translational start site and is presumably dependent on RNA polymerase containing the $\sigma^E$ sigma factor. This promoter is also subject to repression by the AbrB transition state regulator, as shown by $\beta$-galactosidase assays and DNase footprinting analysis (28, 30). The region protected from DNase digestion by the AbrB protein overlaps the putative −35 region of P1 (Fig. 5B), and derepression of *atxA* expression in an *abrB* mutant strain was observed in the *B. subtilis* model system using a lacZ-reporter fragment extending as little as 200 bp upstream of the P1 start site (30). This is consistent with the hypothesis that AbrB acts as a preventer of RNA polymerase binding to the *atxA* P1 promoter (15). The same study also showed that this promoter was not affected by the absence of the SigH sigma factor because, in the absence of the repressor effect of AbrB, the P1 promoter was transcribed in a *B. subtilis* *abrB*-sigH double mutant at the level observed in the *abrB* single-mutant strain. This is in contrast to the later report by Hadjifrangiskou et al. (14), which described the requirement for the SigH sporation sigma factor in *atxA* transcription in *B. anthracis*.

Our analysis of *atxA* transcription in parental strain 34F2 and the sigH mutant revealed that two promoters are responsible for this transcription, the proximal previously described promoter P1 (9), whose start site is located 101 bp upstream of the translational start, and a distal promoter, P2, which is located 744 bp upstream of the ATG start codon. A possible third promoter, P3, whose start site is 686 bp upstream of the *atxA* first codon, may also contribute to *atxA* transcription because a consistent, although weaker-than-P2, 5′ end of mRNA was detected with two different oligonucleotides in the RT primer extension assay. P2 and P3 are within the coding sequence of the divergently transcribed pXO1-118 gene. As shown previously for the P1 promoter (9), P2 and P3 were not affected by growth under CO$_2$/bicarbonate conditions (see Fig. S1B in the supplemental material). Nonetheless, the expression of *atxA* is consistently three- to fourfold higher in cells grown in R medium than in cells grown in LB medium (see Fig. S1 in the supplemental material; data not shown).

The presence of a canonical −10 consensus sequence and a −35 region with three bases conserved with the canonical sequence TTGACA strongly suggests that P2 is also most likely transcribed by $\sigma^E$-containing RNA polymerase. Consensus sequences for P3 are more divergent but still compatible with $\sigma^E$ recognition. Neither the P1 promoter nor the P2 or P3 promoters were found to be dependent on SigH for transcription regardless of the bacterial growth conditions used for the assay (LB medium in air or R medium in CO$_2$/bicarbonate) (Fig. 7 and data not shown).

These conclusions contradict the conclusions reached by Hadjifrangiskou et al. (14) but are in line with the observations made by Strauch et al. (30). Several lines of reasoning support our conclusions. (i) The weak product of an in vitro transcription assay carried out for the *atxA* promoter region with purified *B. anthracis* SigH protein and *E. coli* core RNA polymerase suggested to Hadjifrangiskou et al. (14) that a possible *atxA* sigH-dependent promoter could be located approximately 250 bp upstream of the translational start site, in the pXO1-118-*atxA* intergenic region, despite the absence of canonical −35 and −10 consensus sequences for $\sigma^H$-containing RNA polymerase. Our studies did not identify any 5′ mRNA end in this intergenic region and did not detect any significant transcriptional activity greater than the activity of the negative control plasmid pTCVlac when this intergenic region was tested using construct pATxA21 (Fig. 3A; see Fig. S2 in the supplemental material). Nevertheless, the presence of this intergenic region in the pATxA13 and pATxA15 constructs resulted in higher $\beta$-galactosidase activity than in the pATxA10 construct containing the P1 promoter alone in the short fragment. Similarly, the construct in pATxA19, which contained P2 and part of the intergenic region but did not contain P1, generated more expression of the *ataA-lacZ* fusion than the construct in pATxA20 containing only P2. Whether the effect exerted by this approximately 170-bp region is intrinsic to the DNA sequence or results from the presence in it of the pXO1-118 gene promoter determinants (unpublished data) has not been determined. The presence of a binding site of an activator that can act on either the upstream or downstream promoter is another possibility.

(ii) The seemingly total dependence of *atxA* transcription on SigH reported by Hadjifrangiskou et al. (14) using an *ataA-lacZ* reporter extending from the 5′ end of *atxA*, like our pATxA12 construct, implies that all promoters (P1, as well as P2 and P3) are recognized by this sigma factor. This would be a very unusual situation as to, our knowledge, tandem SigH promoters have never been identified. The activities of the P1 and P2/P3 promoters of *atxA* are additive but independent of each other, as each promoter can be transcribed in the absence of the other (cf. the activity of pATxA10 and the activity of pATxA19, as shown in Fig. 3A), ruling out the possibility that in the absence of SigH the lack of transcription from one of the *atxA* promoters reduces transcription of the others.

(iii) The hypothesis proposed by Hadjifrangiskou et al. (14) to justify the dependence of *atxA* transcription from SigH despite the absence of canonical −35 and −10 consensus sequences similar to those identified in *B. subtilis* implied that the *B. anthracis* orthologue may recognize different binding sequences and/or have a more relaxed specificity. Although this hypothesis cannot be completely ruled out, the current knowledge concerning sigma factors, and SigH in particular, suggests that it is highly unlikely. The SigH proteins of *B. subtilis* and *B. anthracis* share 75% identical residues and 20% conserved substitutions. The lowest level of similarity was found in the N-terminal 15 amino acids, suggesting to Hadjifrangiskou et al. (14) that the divergence resulted in altered target specificity and binding affinity in a manner similar to the one proposed by Ramirez-Romero et al. (26) for $\sigma^70$ of *Rhizobium etli*. However, lux promoter recognition was proposed by Ramirez-Romero et al. to be associated with the amino acid differences in amino-terminal region 1 between *R. etli* $\sigma^70$ (685 amino acids) and *E. coli* $\sigma^70$ (613 amino acids), but this region is absent in $\sigma^41$ (213 amino acids), making the argument inconsistent. Furthermore, two divergent residues in SigH of *B. subtilis* and *B. anthracis* (G160S and V172S, *B. anthracis* numbering) were also proposed to perhaps affect promoter specificity (14). These residues are in region 4 of the sigma factor, but they are not in helix-turn-helix region 4.2 that directly binds the −35
sequence and provides sigma factor specificity; thus, they are unlikely to affect the interaction with the DNA based on the structure of region 4 of *Thermus aquaticus* σ70, a member of the σ70 family (6).

Although the reason for the conflicting results concerning the role of SigH is unknown, it is clear that at least in the genetic backgrounds used in this study (*B. anthracis* Sterne strains 34F2 and 7702) the absence of the SigH protein results in the expected sporulation-deficient phenotype but does not affect AtxA and toxin gene expression.

Nevertheless, in the sigH mutant, the atxA gene is transcribed at a consistently higher level than it is in the parental strain, particularly when cells are grown in a rich medium (such as LB medium) (see Fig. S3 in the supplemental material). This may be due to the fact that in a rich medium, the parental strain may have a regulatory mechanism that reduces atxA expression if conditions conducive to sporulation are present. It is becoming more apparent, in fact, that efficient sporulation negatively affects the virulence potential of *B. anthracis* (5, 23). In the sigH mutant this negative regulation might be missing because the cells cannot commit to spore formation, resulting in higher levels of atxA expression. In view of the hypothesis that efficient sporulation is detrimental to virulence gene expression (23), the requirement for the SigH sigma factor, which is necessary for cell commitment to the developmental process, in toxin production seems paradoxical (14).

In the spoH mutant, a negative effect on atxA expression by the AcrB repressor is not expected because in *B. anthracis*, as well as in *B. subtilis*, the lack of SigH does not have a significant impact on the level of transcription of *abrB* and its product in the cell (Fig. 6 and data not shown). This is because the level of phosphorylated Spo0A protein necessary to repress the transcription of *abrB* does not require the induction of spo0A transcription brought about by SigH at the onset of sporulation (25; M. Perego, unpublished data).

As observed for the Mga protein of *S. pyogenes*, the presence of multiple promoters transcribing the atxA gene is not surprising. However, the distance of the P2 and P3 promoters (650 bp upstream of P1 and within a divergently transcribed open reading frame) is considerably greater than the distance in the mga promoter (295 bp) (21), possibly providing additional space for a regulatory effector(s) acting at the DNA or RNA level.

In contrast to the Mga system of *S. pyogenes*, the AtxA protein does not have autoregulatory functions (see Fig. S1A in the supplemental material), and it does not appear to require the CcpA carbon catabolite regulator protein for full induction (1, 21; unpublished data). Nevertheless, evidence of additional regulatory mechanisms for atxA expression is emerging from a transposon mutagenesis analysis (35; A. C. Wilson, M. Perego, and J. A. Hoch, unpublished data), and characterization of these mechanisms is under way in our laboratory.

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