

## Control of Anthrax Toxin Gene Expression by the Transition State Regulator *abrB*

Elke Saile and Theresa M. Koehler\*

Department of Microbiology and Molecular Genetics, The University of Texas-Houston Health Science Center  
Medical School, Houston, Texas 77030

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*Bacillus anthracis* produces the anthrax toxin proteins protective antigen (PA), lethal factor (LF), and edema factor (EF) in a growth phase-dependent manner when cultured in liquid medium. Expression of the toxin genes *pagA*, *lef*, and *cya* peaks in late log phase, and steady-state levels of the toxin proteins are highest during the transition into stationary phase. Here we show that an apparent transition state regulator negatively regulates toxin gene expression. We identified two orthologues of the *B. subtilis* transition state regulator *abrB* in the *B. anthracis* genome: one on the chromosome and one on the 182-kb virulence plasmid pXO1. The orthologue located on the chromosome is predicted to encode a 94-amino-acid protein that is 85% identical to *B. subtilis* AbrB. The hypothetical protein encoded on pXO1 is 41% identical to *B. subtilis* AbrB but missing 27 amino acid residues from the amino terminus compared to the *B. subtilis* protein. Deletion of the pXO1-encoded *abrB* orthologue did not affect toxin gene expression under the conditions tested. However, a *B. anthracis* mutant in which the chromosomal *abrB* gene was deleted expressed *pagA* earlier and at a higher level than the parent strain. Expression of a transcriptional *pagA-lacZ* fusion in the *abrB* mutant was increased up to 20-fold during early exponential growth compared to the parent strain and peaked in mid-exponential rather than late exponential phase. In contrast to the strong effect of *abrB* on *pagA* expression, *lef-lacZ* and *cya-lacZ* expression during early-log-phase growth was increased only two- to threefold in the *abrB* null mutant. Western hybridization analysis showed increased PA, LF, and EF synthesis by the mutant. As is true in *B. subtilis*, the *B. anthracis* *abrB* gene is negatively regulated by *spo0A*. Our findings tie anthrax toxin gene expression to the complex network of postexponential phase adaptive responses that have been well studied in *B. subtilis*.

Bacteria adjust their physiological state in response to environmental cues, shifting from periods of growth and multiplication to quiescent states and vice versa. In liquid batch culture, changes in growth rate are reflected as the different phases of the bacterial growth curve. Many of the nutrient factors and other environmental parameters that determine growth rate and cause the transition from one growth phase to another are known. However, the regulatory genes and networks controlling gene expression that is specific to certain growth phases are not well understood. Moreover, for pathogenic bacteria multiplying in the environment of a host organism, the significance of growth phase-specific gene expression with regard to pathogenesis is not clear.

Toxin synthesis by *Bacillus anthracis*, the causative agent of anthrax, peaks during the transition from exponential phase to stationary phase during growth in batch culture. *B. anthracis* produces three different toxin proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), encoded by *pagA*, *lef*, and *cya*, respectively (10, 23). The anthrax toxin genes are not part of an operon but are coordinately controlled. Toxin gene expression is highest when the bacterium is cultured at 37°C in a highly buffered synthetic medium containing bicarbonate. In this medium, toxin gene transcript lev-

els and toxin protein levels peak at the end of exponential growth (21, 24, 25, 35).

For an increasing number of pathogenic bacteria, production of toxins and other virulence factors at the end of exponential growth when cells are at a high density has been attributed to quorum sensing. The individual cells of a bacterial population produce a diffusible signal molecule which, after crossing a concentration threshold, interacts with a histidine kinase sensor and a transcriptional activator to induce gene expression (11, 17, 48). To date, there are no reports of *B. anthracis* toxin gene control by quorum sensing. Under all growth conditions tested in our laboratory, addition of cell-free, spent media to *B. anthracis* cultures did not affect toxin gene expression or expression of the *trans*-acting virulence gene regulator *atxA* (7, 8, 44; T. M. Koehler, unpublished data).

Growth rate-dependent gene expression that appears to be unlinked to diffusible chemical signals has been studied in only a few bacteria. In *Streptococcus pyogenes*, the *trans*-acting regulatory protein Mga activates transcription of several genes, including *emm* (which encodes M protein) and *scpA* (which encodes C5a peptidase). Genes regulated by Mga are expressed in the exponential but not in the stationary phase. Transcription of *mga* itself is growth rate dependent: expression is maximal in exponential phase but decreased upon entry into stationary phase (26). The gene encoding the nucleoid-associated protein Fis in *Escherichia coli* has a very similar expression profile (5) and has recently been implicated in the regulation of *Salmonella enterica* serovar Typhimurium SPI-1 invasion genes (30, 49). In the nonpathogenic soil bacterium

\* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas-Houston Health Science Center Medical School, 6431 Fannin St., JFB 1.765, Houston, TX 77030. Phone: (713) 500-5450. Fax: (713) 500-5499. E-mail: Theresa.M.Koehler@uth.tmc.edu.

*Bacillus subtilis*, regulatory proteins that control growth phase-dependent gene expression have been termed "transition state regulators" (31, 40). The best studied of these is AbrB (37). AbrB is a pleiotropic regulator of gene expression with a role in mediating the transition from a quiescent state to one of growth and cell division and the transition from active growth into stationary phase (29).

In the current model for *B. anthracis*-host interaction during inhalation anthrax disease, bacterial spores enter the lungs via normal respiration. Once in the lung, the spores are phagocytosed by alveolar macrophages (32). Some *B. anthracis* spores survive the hostile environment of the macrophage and are transported to the mediastinum, which is the initial site of infection. The spores then germinate in the macrophages, becoming metabolically active vegetative cells. Ultimately, vegetative cells enter the bloodstream, reaching concentrations of up to  $10^8$  cells per ml (12, 13). Guidi-Rontani et al. (15) have reported that the toxin genes, *pagA*, *lef*, and *cya*, and the positive regulator *atxA* are expressed by vegetative *B. anthracis* cells shortly after germination in cultured macrophages. In studies using the murine macrophage-like cell line RAW264.7 infected with *B. anthracis* strains harboring transcriptional *lacZ* fusions, the toxin genes and *atxA* are expressed within 3 h after infection. The role of the toxin proteins at this very early stage of infection is not clear, but a report by Dixon et al. (9) indicates that toxin synthesis in the macrophage may have a role in release of the bacterium from the eukaryotic cell.

Anthrax toxin gene expression shortly after *B. anthracis* spores are phagocytosed by macrophages indicates that toxin synthesis occurs when the bacterium is transitioning from the dormant spore state to one of active growth. We hypothesized that the timing of toxin gene expression by *B. anthracis* is controlled by a transition state regulator, such as AbrB of *B. subtilis*. We searched the *B. anthracis* plasmid sequence databases and the incomplete chromosome sequence database of The Institute for Genomic Research (TIGR) for *abrB* orthologues in *B. anthracis*. We identified two potential *abrB* genes, one located on the pathogenicity island of pXO1 and another on the *B. anthracis* chromosome. Here we show that the chromosomal *abrB* gene is involved in timing of toxin gene expression in *B. anthracis* during batch culture. Evidence for the involvement of an *abrB* orthologue in the regulation of toxin synthesis in *B. anthracis* is the first report tying virulence factor expression to the regulatory network controlling the transition of bacterial cells into and out of exponential growth.

## MATERIALS AND METHODS

**Growth conditions.** *E. coli* strains were grown in Luria-Bertani (LB) broth (2) and used as hosts for cloning. For extraction of chromosomal DNA, the *B. anthracis* strains were grown in brain heart infusion medium (BHI) (Difco, Detroit, Mich.). For extraction of pXO1 DNA, BHI was amended with 10% horse serum. For transductions and electroporations, *B. anthracis* strains were grown in BHI containing 0.5% glycerol. Culture supernatant samples and cell extracts for Western hybridization experiments were obtained from *B. anthracis* cultures grown in LB broth buffered with 100 mM HEPES (pH 8.0) and 0.8% (wt/vol) sodium bicarbonate, in an atmosphere containing 5% CO<sub>2</sub> at 37°C. Cells for  $\beta$ -galactosidase assays were grown in buffered LB at 37°C in a 5% CO<sub>2</sub> atmosphere. The following antibiotics were purchased from Sigma-Aldrich (St. Louis, Mo.) or Fisher Scientific and added to media (concentrations are indicated in parentheses) when appropriate: ampicillin (100  $\mu$ g/ml), erythromycin (5

$\mu$ g/ml), kanamycin (20  $\mu$ g/ml), lincomycin (25  $\mu$ g/ml), spectinomycin (100  $\mu$ g/ml), and tetracycline (5  $\mu$ g/ml).

**DNA isolation and manipulation.** A method for extraction of plasmid DNA from *B. anthracis* cultures has been described (14). For isolation of chromosomal DNA, the protocol was slightly modified by omitting the centrifugation step that is intended to remove chromosomal DNA from plasmid DNA. Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures (2). *B. anthracis* was electroporated with unmethylated plasmid DNA from *E. coli* GM1684 as described elsewhere (21). Restriction enzymes, T4 ligase, and *Taq* polymerase were purchased from Promega (Madison, Wis.) or Gibco BRL (Rockville, Md.).

**Strain construction.** Strains, plasmids, and their relevant characteristics are listed in Table 1. Oligonucleotide primers are listed in Table 2. *B. anthracis* *abrB* (UT166), pXO1 open reading frame (ORF) 105 (UT141), and *spo0A* (UT157) mutants were constructed by replacement of coding sequences with an omega element conferring spectinomycin resistance,  $\Omega$ -*sp*, using a method described previously (8). Generally, the  $\Omega$ -*sp* element and DNA sequences directly upstream and downstream of the target gene were cloned into the vector pUTE29 such that *B. anthracis* sequences flanked the  $\Omega$ -*sp* element. pUTE29 replicates in *E. coli* and *B. anthracis* and confers tetracycline resistance to both species but is unstable in *B. anthracis* when strains are grown without selection. pUTE29-derived constructs were electroporated into *B. anthracis* with selection for tetracycline resistance. Electroporants were inoculated into LB broth without antibiotics, incubated at 37°C, and transferred to fresh medium two times daily for several days. The cultures were screened for spectinomycin-resistant tetracycline-sensitive clones. Isolates in which a double-crossover recombination event had occurred were confirmed by PCR.

Mutations were transduced between *B. anthracis* strains using a temperature-sensitive mutant of the generalized transducing phage CP51 as described previously (19, 43). The genotypes of transductants were confirmed by PCR.

**Cloning of the *abrB* locus.** When this work was initiated, only partial sequence of the *abrB* locus was available in the TIGR database (<http://www.tigr.org>). The database revealed a DNA sequence containing 215 nucleotides (nt) of the 3' end of the *abrB* coding sequence and 512 nt downstream of the gene. We cloned the 5' end of the *abrB* gene and upstream sequences using the inverse PCR. *B. anthracis* chromosomal DNA was digested with *EcoRI*, for which there are no restriction sites in the target sequence; ligated; and used as a template for amplification with the oligonucleotides ES2 and ES3 (Table 2). The PCR product was cloned into pGEM-T Easy, giving rise to pUTE387, and sequenced with an Applied Biosystems PRISM 377 sequencer. A *SacII-PstI* fragment was subcloned into pBluescript II KS(+), generating pUTE474. pUTE474 was used as a template for PCR amplification with oligonucleotides ES8 and M13 forward. The PCR product was digested with *BamHI* and *SacI*. This fragment and the  $\Omega$ -*sp* element from pJRS312 were cloned into pUTE417, giving rise to pUTE416. Thus, in pUTE416 the 2.3-kb  $\Omega$ -*sp* cassette is flanked by 1.9 kb of DNA upstream of *abrB*, including 73 nt from the 5' start of the *abrB* gene, and 1.6 kb of DNA downstream of *abrB*, including 61 nt from the 3' end of the gene (Fig. 2A).

**$\beta$ -Galactosidase assays.** Strains were cultured overnight at 28°C, with shaking at 250 rpm, in 50 ml of LB broth with appropriate antibiotics and 0.5% glycerol to suppress sporulation. One milliliter of the overnight culture was pelleted, and the cells were resuspended in 1 ml of LB broth. A 50-ml flask containing buffered LB broth was inoculated with this bacterial suspension to achieve an initial optical density at 600 nm (OD<sub>600</sub>) of 0.04 to 0.08. Cultures were incubated at 37°C in 5% CO<sub>2</sub> with stirring (400 rpm). OD<sub>600</sub> was measured at 1-h intervals, and duplicate samples were collected for  $\beta$ -galactosidase assays. The samples were frozen at -20°C until assaying for enzyme activity.  $\beta$ -Galactosidase assays were performed as described by Miller (27), using toluene to permeabilize the cells. At least three independent cultures were assayed for enzyme activity. The figures show data from representative experiments.

**Western blot analysis.** Strains were cultured overnight and diluted into buffered LB broth the following morning as described above. Culture supernatant samples were collected and filtered through 0.2- $\mu$ m-pore-size syringe filters. The samples were frozen immediately at -20°C. For detection of PA, LF, and EF, samples were applied to nitrocellulose membranes using vacuum blotting with a slot blot apparatus (Hoefer Scientific, San Francisco, Calif.). The membranes were blocked for 2 h with TBS-T (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]) containing 5% milk at 4°C. Subsequently, the membranes were incubated with rabbit anti-PA, anti-LF, or anti-EF serum diluted in TBS-T-5% milk overnight at 4°C. Membranes were washed in TBS-T and reacted with donkey anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (diluted 1:5,000 in TBS-T-5% milk) for 1 h at room temperature. Immunoreactive material was visualized on autoradiographs with enhanced

TABLE 1. Plasmids and strains used in this study

Plasmid or strain		Relevant characteristic(s) <sup>a</sup>	Source or reference
<b>Plasmids</b>			
<i>B. anthracis</i> pXO1	Tox <sup>+</sup>		42
<i>E. coli</i>			
pBSIIKS+	Ap <sup>r</sup>		Stratagene
pGEM-T Easy	Ap <sup>r</sup>		Promega
pJRS312		pUC18 carrying an $\Omega$ element with spectinomycin resistance marker <i>aad9</i> ( $\Omega$ - <i>sp</i> ); Sp <sup>r</sup>	J. R. Scott
pUTE387		1.9-kb product of inverse PCR (with ES2-ES3) containing 5' end of <i>abrB</i> and upstream sequences cloned into pGEM-T Easy; Ap <sup>r</sup>	This work
pUTE460		556-bp PCR product (with ES4-ES16) containing <i>abrB</i> cloned into pGEM-T Easy; Ap <sup>r</sup>	This work
pUTE474		1.9-kb <i>SacII-PstI</i> fragment from pUTE387 containing 5' end of <i>abrB</i> and upstream sequences cloned into pBSIIKS+; Ap <sup>r</sup>	This work
pUTE475		556-bp fragment from pUTE460 containing <i>abrB</i> cloned into pBSIIKS+; Ap <sup>r</sup>	This work
<b>Bifunctional</b>			
pHT304-18Z		Contains promoterless <i>lacZ</i> ; Ap <sup>r</sup> in <i>E. coli</i> , Em <sup>r</sup> in <i>B. anthracis</i>	1
pUTE29		Ap <sup>r</sup> in <i>E. coli</i> , Tc <sup>r</sup> in <i>B. anthracis</i>	21
pUTE379		2.3-kb <i>BamHI</i> fragment containing $\Omega$ - <i>sp</i> from pJRS312 flanked by (i) a 1.2-kb PCR product containing 3' end of ORF 105 and downstream sequences (EB1-EB2) and (ii) a 1.3-kb PCR product containing 5' end of ORF 105 and upstream sequences (EB3-EB4) cloned into pUTE29; Sp <sup>r</sup> Tc <sup>r</sup> ; used to construct UT141 $\Delta$ ORF 105 <sub>pXO1</sub>	This work
pUTE408		2.3-kb <i>BamHI</i> fragment containing $\Omega$ - <i>sp</i> from pJRS312 flanked by (i) a 0.8-kb PCR product containing 3' end of <i>spo0A</i> and downstream sequences (YC24/YC25) and (ii) a 1.0-kb PCR product containing 5' end of <i>spo0A</i> and upstream sequences (YC22-YC23) cloned into pUTE29; Sp <sup>r</sup> Tc <sup>r</sup> ; used to construct UT157 $\Delta$ <i>spo0A</i>	This work
pUTE411		1.2-kb PCR product containing promoter region of <i>atxA</i> (YC9-YC10) cloned into multiple cloning site of pHT304-18Z; <i>atxA::lacZ</i> ; Ap <sup>r</sup> Em <sup>r</sup>	This work
pUTE416		2.3-kb <i>BamHI</i> fragment containing $\Omega$ - <i>sp</i> from pJRS312 and 1.9-kb PCR product (ES8-M13fw, pUTE474 template) containing 5' end of <i>abrB</i> and upstream sequences cloned into pUTE417; Sp <sup>r</sup> Tc <sup>r</sup> ; used to construct UT166 $\Delta$ <i>abrB</i>	This work
pUTE417		1.6-kb <i>BamHI-PstI</i> PCR product (ES9-ES10) containing 3' end of <i>abrB</i> and downstream sequences cloned into pUTE29; Ap <sup>r</sup> Tc <sup>r</sup>	This work
pUTE441		559-bp PCR product containing promoter region of <i>abrB</i> (ES21-ES22) cloned into multiple cloning site of pHT304-18Z; <i>abrB::lacZ</i> ; Ap <sup>r</sup> Em <sup>r</sup>	This work
pUTE448		<i>BamHI-SacI</i> fragment from pUTE475 containing <i>abrB</i> cloned into pUTE29; Ap <sup>r</sup> Tc <sup>r</sup>	This work
<b>Strains</b>			
<i>B. anthracis</i>			
RBAF140 <sup>c</sup>		<i>pagA-lacZ</i> integrated adjacent to <i>pagA</i> locus; Em <sup>r</sup> Km <sup>r</sup>	35
RBAF143 <sup>c</sup>		<i>lef-lacZ</i> integrated adjacent to <i>lef</i> locus; Em <sup>r</sup> Km <sup>r</sup>	35
RBAF144 <sup>c</sup>		<i>cya-lacZ</i> integrated adjacent to <i>cya</i> locus; Em <sup>r</sup> Km <sup>r</sup>	35
UM44 <sup>b</sup>		pXO1 <sup>+</sup> Tox <sup>+</sup> Ind <sup>-</sup>	42
UM44-1C9 <sup>b</sup>		Plasmid-cured derivative of UM44; pXO1 <sup>-</sup> Tox <sup>-</sup> Ind <sup>-</sup> Str <sup>r</sup>	C. Thorne
UT53 <sup>b</sup>		UM44 $\Delta$ <i>atxA</i> ; <i>atxA</i> is replaced by $\Omega$ <i>km-2</i> ; Km <sup>r</sup>	8
UT141 <sup>b</sup>		UM44 electroporated with pUTE379; $\Delta$ ORF 105 <sub>pXO1</sub> ; Ind <sup>-</sup> Sp <sup>r</sup>	This work
UT157 <sup>b</sup>		UM44 electroporated with pUTE408; $\Delta$ <i>spo0A</i> ; Ind <sup>-</sup> Sp <sup>r</sup>	This work
UT166 <sup>b</sup>		UM44 electroporated with pUTE416; $\Delta$ <i>abrB</i> ; Ind <sup>-</sup> Sp <sup>r</sup>	This work
UT168 <sup>c</sup>		RBAF140 transduced with CP51 propagated on UT166; <i>pagA::lacZ</i> , $\Delta$ <i>abrB</i> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
UT169 <sup>c</sup>		RBAF143 transduced with CP51 propagated on UT166; <i>lef::lacZ</i> , $\Delta$ <i>abrB</i> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
UT170 <sup>c</sup>		RBAF144 transduced with CP51 propagated on UT166; <i>cya::lacZ</i> , $\Delta$ <i>abrB</i> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
UT182 <sup>c</sup>		RBAF140 transduced with CP51 propagated on UT141; <i>pagA::lacZ</i> , $\Delta$ ORF 105 <sub>pXO1</sub> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
UT183 <sup>c</sup>		RBAF143 transduced with CP51 propagated on UT141; <i>lef::lacZ</i> , $\Delta$ ORF 105 <sub>pXO1</sub> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
UT184 <sup>c</sup>		RBAF144 transduced with CP51 propagated on UT141; <i>cya::lacZ</i> , $\Delta$ ORF 105 <sub>pXO1</sub> ; Em <sup>r</sup> Km <sup>r</sup> Sp <sup>r</sup>	This work
<i>E. coli</i>			
DH5 $\alpha$ F <sup>r</sup>		F <sup>r</sup> <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoR</i> [ $\phi$ 80 <i>dlac</i> $\Delta$ ( <i>lacZ</i> )M15]	50
GM1684		F <sup>r</sup> F- <i>lacI</i> <sup>ts</sup> DM15 <i>pro</i> <sup>+</sup> / <i>dam-4</i> $\Delta$ ( <i>lac-pro</i> ) <sub>X111</sub> <i>thi-1 glnV44</i> ( <i>relA1</i> )	R. Kolter
JM109		F <sup>r</sup> <i>traD36 proAB<sup>+</sup> lacI<sup>ts</sup> lacZ</i> $\Delta$ M15/ <i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> $\Delta$ ( <i>lac-proAB</i> ) <i>mcrA</i>	53
TG1		F <sup>r</sup> <i>traD36 lacI<sup>ts</sup> \Delta</i> ( <i>lacZ</i> )M15 <i>proA<sup>+</sup>B<sup>+</sup>/supE</i> $\Delta$ ( <i>hsdM-mcrB</i> )5 ( $\tau_K^-$ m <sub>K</sub> <sup>+</sup> <i>mcrB</i> ) <i>thi</i> $\Delta$ ( <i>lac-proAB</i> )	33

<sup>a</sup> Abbreviations: Ap<sup>r</sup>, ampicillin resistant; Em<sup>r</sup>, erythromycin resistant; Ind, indole; Km<sup>r</sup>, kanamycin resistant; Sp<sup>r</sup>, spectinomycin resistant; Str<sup>r</sup>, streptomycin resistant; Tc<sup>r</sup>, tetracycline resistant; Tox, anthrax toxin; pBSIIKS+, pBluescript II KS(+).

<sup>b</sup> Weybridge strain derivative.

<sup>c</sup> Sterne strain derivative.

chemiluminescence immunoblotting (Amersham). The autoradiographs were analyzed with the Scion Image Beta 4.02 software.

To investigate the accumulation of AbrB protein during growth, extracts were prepared from cells harvested at early exponential phase (OD<sub>600</sub>, 0.08 to 0.2), mid-exponential phase (OD<sub>600</sub>, 0.5 to 0.7), and stationary phase (OD<sub>600</sub>, 1.3 to 1.5). Cells were boiled for 10 min in sample buffer (63 mM Tris OH, 10% glycerol

[vol/vol], 1% SDS [wt/vol]), 5%  $\beta$ -mercaptoethanol [vol/vol] [pH 6.8]). Protein concentrations were determined by using the Bio-Rad protein assay reagent with bovine serum albumin (Sigma) as the standard. Samples containing 15  $\mu$ g of protein were resolved on 4 to 20% Tris-HCl Ready Gels (Bio-Rad) and transferred with a Semi-Dry Electrobloater (CLP) to Optitran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using Towbin buffer (0.025 M

TABLE 2. Primers used in this study<sup>a</sup>

Primer	Sequence (5'-3') <sup>b</sup> (location)	Relevant property	Restriction site
EB1	TTAGGACATGAGCTCATTATGACGACGG (from bp 1138 to 1111 after ORF 105 stop codon)	Outer primer used to amplify ORF 105 3' region	<i>SacI</i>
EB2	GCGGATCCGAAATCCAGCAACTTAGTGAAGTAA (from bp 26 to 0 before ORF 105 stop codon)	Inner primer used to amplify ORF 105 3' region	<i>BamHI</i>
EB3	GCGGATTCCTACAATTCCTCATGTTCTTC (from bp 11 to -7 from ORF 105 start codon)	Inner primer used to amplify ORF 105 5' region	<i>BamHI</i>
EB4	CATTAAGTCTTCACGTCTGAAGATCAGC (from bp -1335 to -1308 from ORF 105 start codon)	Outer primer used to amplify ORF 105 5' region	
ES2	CACCAGTTACTTGCAAGTCATG (from bp 174 to 152 after <i>abrB</i> start codon)	Outward facing primer used for inverse PCR to amplify <i>abrB</i> 5' region	
ES3	CATCGCGTTTTGACAAATCTCG (from bp 55 to 76 after <i>abrB</i> stop codon)	Outward facing primer used for inverse PCR to amplify <i>abrB</i> 5' region	
ES4	CCTGCTCCAGAAGAACAATGGT (from bp 190 to 169 after <i>abrB</i> stop codon)	Used to amplify <i>abrB</i>	
ES8	ATGGATCCGTACGGCGTAATTCGATTGG (from bp 73 to 54 after <i>abrB</i> start codon)	Inner primer used to amplify <i>abrB</i> 5' region	<i>BamHI</i>
ES9	GCCGGATCCAAGCAAAGAAGGCGCTGA (from bp 61 to 44 before <i>abrB</i> stop codon)	Inner primer used to amplify <i>abrB</i> 3' region	<i>BamHI</i>
ES10	ATACTGCAGATCCGCCTTATTTCAAAACG (from bp 1523 to 1504 after <i>abrB</i> stop codon)	Outer primer used to amplify <i>abrB</i> 3' region	<i>PstI</i>
ES16	GTCCATAGGGTATTTTGTCGAA (from bp -84 to -52 to <i>abrB</i> start codon)	Used to amplify <i>abrB</i>	
ES21	GCCAAGCTTGTTAATGCGTGATAAAA (from bp -320 to -300 to <i>abrB</i> start codon)	Used to amplify <i>abrB</i> promoter	<i>HindIII</i>
ES22	ATGGATCCCTCAGCGCCTTCTTTGCTTA (from bp 239 to 220 after <i>abrB</i> start codon)	Used to amplify <i>abrB</i> promoter	<i>BamHI</i>
M13 Fw (-20)	GTAAAACGACGGCCAGTG		
YC9	AAAAAAGCTTATAACCCCTTACAATC (from bp -870 to -843 to <i>atxA</i> start codon)	Used to amplify <i>atxA</i> promoter	<i>HindIII</i>
YC10	ATAAGGATCCTACTTGTAAGTGGAG (from bp 364 to 340 to <i>atxA</i> start codon)	Used to amplify <i>atxA</i> promoter	<i>BamHI</i>
YC22	ATCGGTACCATGACATTTGTTTCATCC (from bp -884 to -859 from <i>spo0A</i> start codon)	Outer primer used to amplify <i>spo0A</i> 5' region	<i>KpnI</i>
YC23	ATAGGATCCAATACGAGTACATCCG (from bp 209 to 185 from <i>spo0A</i> start codon)	Inner primer used to amplify <i>spo0A</i> 5' region	<i>BamHI</i>
YC24	TTCGGATCCGCAACAACAGTAGATGG (from bp 386 to 361 before <i>spo0A</i> stop codon)	Inner primer used to amplify <i>spo0A</i> 3' region	<i>BamHI</i>
YC25	TTCTGCAGTATACTCTCAATCCG (from bp 454 to 430 after <i>spo0A</i> stop codon)	Outer primer used to amplify <i>spo0A</i> 3' region	<i>PstI</i>

<sup>a</sup> Purchased from Sigma Genosys or IDT.

<sup>b</sup> Restriction enzyme recognition sites are underlined.

Tris base, 0.192 M glycine, 20% methanol [pH 8.3]). The membranes were treated as described above using rabbit antiserum raised against *B. subtilis* AbrB at a dilution of 1:1,000 in TBS-T-5% milk.

## RESULTS

**The *B. anthracis* genome harbors two apparent *abrB* genes.** BLAST searches of the preliminary *B. anthracis* chromosome sequence released by TIGR (<http://www.tigr.org>) and of the *B. anthracis* virulence plasmids pXO1 (28) and pXO2 (GenBank accession no. AF188935) revealed two predicted peptides highly homologous to *B. subtilis* AbrB. The hypothetical protein encoded by ORF 105 of pXO1, located 2.2 kb upstream of *lef* and within the boundaries of a pathogenicity island, is 41% identical and 64% similar to AbrB in *B. subtilis*. The predicted protein encoded by a chromosomal ORF is 85% identical and 93% similar to *B. subtilis* AbrB. Figure 1 shows an alignment of the predicted amino acid sequences of AbrB from several *Bacillus* species. All of the chromosomally encoded proteins (from *B. subtilis*, *B. anthracis*, *Bacillus stearothermophilus*, *Bacillus halodurans*, and *Bacillus cereus*) are highly conserved, whereas the hypothetical protein encoded by ORF 105 of *B.*

*anthracis* plasmid pXO1 is lacking the first 27 residues of the N-terminal domain. In the *B. subtilis* protein, residues 1 to 53 are involved in DNA binding (46, 47, 52) and mutations of Arg23 and Arg24 result in loss of DNA-binding activity (39, 51). Since the hypothetical peptide encoded by ORF 105 is truncated in its potential DNA-binding domain, we surmise that a protein resulting from expression of ORF 105 would be nonfunctional.

In the *B. subtilis* genome, the *abrB* gene is flanked by the divergently transcribed *metS* and *yabC* genes (22). The sequence information from the *B. anthracis* chromosome shows identical organization (Fig. 2A). The DNA sequence immediately upstream of *abrB* on the chromosome of *B. anthracis* is comparable to the promoter region of the *B. subtilis* *abrB* gene (31). The region has the elements of a  $\sigma^A$ -dependent promoter and contains Spo0A boxes identical to the ones found in *B. subtilis* (Fig. 2B) (18).

***abrB* is a negative regulator of toxin gene expression.** To determine whether the *B. anthracis* *abrB* orthologues affect anthrax toxin gene expression, we created *abrB* null mutations in strains harboring toxin gene promoter-*lacZ* transcriptional

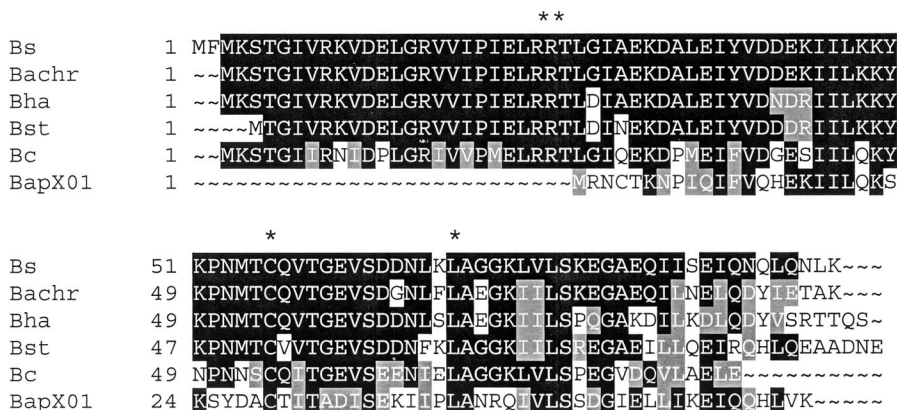


FIG. 1. Comparative analysis of AbrB peptide sequences. Black background indicates identical residues. Gray background signifies similar residues. The sequences were aligned with the procedure PILEUP in the Genetics Computer Group software package and plotted using BOXSHADE. Asterisks indicate amino acids that have been assigned a critical role following mutational analysis of the *B. subtilis* gene. Abbreviations: Bs, *B. subtilis* 168; Bachr, *B. anthracis* Ames chromosome; Bha, *B. halodurans* C-125; Bst, *B. stearothermophilus* ATCC 7953; Bc, *B. cereus*; BapXO1, *B. anthracis* Sterne pXO1.

fusions. Internal *abrB* sequences were deleted and replaced with  $\Omega$ -*sp*. The  $\Omega$ -*sp* DNA cassette harbors the spectinomycin resistance gene *aad9* flanked on both sides by T4 transcription terminators and translational stop codons in all three reading frames. We measured  $\beta$ -galactosidase activity of the parent

and *abrB* null mutants following growth at 37°C in a 5% CO<sub>2</sub> atmosphere.

*B. anthracis* RBAF140 (35) carries a *pagA::lacZ* transcriptional fusion adjacent to the intact *pagA* locus. Strain UT168 was derived from RBAF140 and carries the chromosomal *abrB*

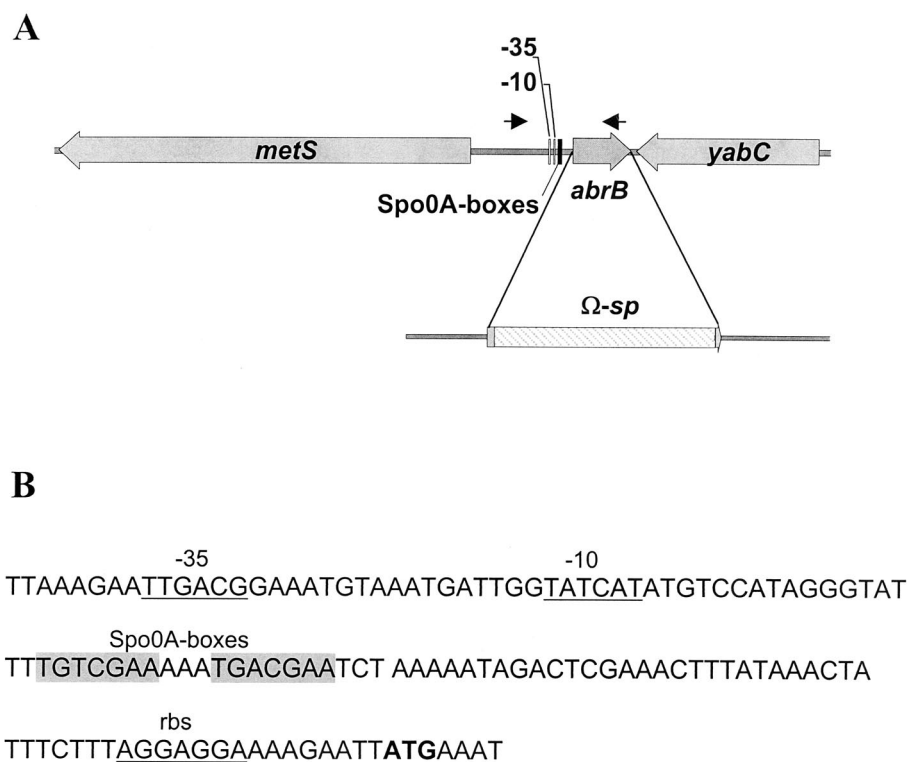


FIG. 2. (A) Schematic representation of the *abrB* region of the *B. anthracis* chromosome and replacement of 148 nt in the central portion of the 282-nt *abrB* coding region. The 73 nt at the 5' end and 61 nt at the 3' end of the gene are separated by an  $\Omega$ -*sp* element. Black arrows mark the position and orientation of the primers used for PCR amplification of the region which was cloned into pHT304-18Z to generate a transcriptional *abrB::lacZ* fusion. (B) Nucleotide sequence at the 5' end of the *abrB* coding sequence on the *B. anthracis* chromosome. Predicted areas of contact between the promoter sequence and the  $\sigma^A$  subunit of RNA polymerase (-35, -10) and the ribosome binding site (rbs) are underlined. The predicted translational start codon is shown in bold type. The hypothesized binding sites for Spo0A are shaded.

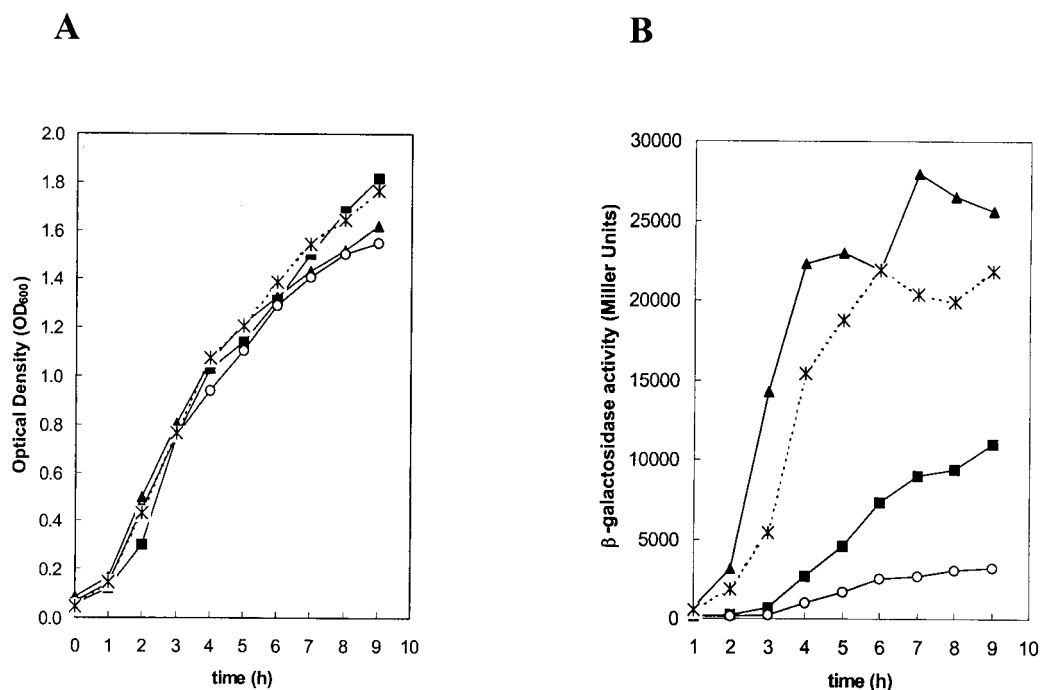


FIG. 3. Growth curves (A) and  $\beta$ -galactosidase activity of *pagA::lacZ* (B) for parent strain RBAF140 (■), *abrB* mutant UT168 (▲), *abrB*-complemented strain UT168(pUTE448) (○), and control UT168(pUTE29) (\*).

null mutation depicted in Fig. 2A. As shown in Fig. 3, the parent and mutant strains grew similarly, yet expression of the reporter gene fusion in the *abrB* null mutant was up to 20-fold greater than that of the parent strain. The mutant exhibited a large increase in *pagA::lacZ* expression during early- and mid-exponential-phase growth, peaking in mid-exponential phase.  $\beta$ -Galactosidase activity of the parent strain increased more gradually, and peak levels were obtained upon entry into stationary phase. When a plasmid-encoded copy of *abrB* was introduced into UT168, *pagA::lacZ* expression was reduced to levels lower than those of the parent strain. The overcompensation was most likely due to the high copy number of the plasmid.

The chromosomal *abrB* null mutation exhibited a similar but less pronounced effect on expression of *lef::lacZ* and *cya::lacZ* transcriptional fusions. Figure 4 shows the  $\beta$ -galactosidase activities of the reporter strains RBAF143 (*lef::lacZ*) and RBAF144 (*cya::lacZ*) and the respective *abrB* mutants UT169 and UT170. The increase in reporter gene expression in the *abrB* mutants was only two- to threefold compared to the parent strains. However, we found consistently that the maximum difference between chromosomal *abrB* mutant and parent strains occurred during the first 5 h of growth, subsequent to dilution of stationary-phase cells into fresh medium. Thus, similar to the effect of the chromosomal *abrB* null mutation on expression of the *pagA-lacZ* fusion, *lef-lacZ* and *cya-lacZ* expression in *abrB* mutants was most elevated during early and mid-exponential growth phases.

To determine whether the *abrB* orthologue ORF 105 on pXO1 affected toxin gene expression, we constructed an ORF 105 deletion mutation in the toxin gene promoter-*lacZ* fusion strains. ORF 105 null mutants harboring a 166-nt internal

deletion (Table 1) and carrying the wild-type chromosomal *abrB* gene were unaffected for toxin gene expression under the conditions tested (data not shown). Thus, of the two *abrB* orthologues found in the genome, only the chromosomal gene affects toxin gene expression.

***abrB* affects toxin protein levels.** When *B. anthracis* is grown in buffered LB broth at 37°C in 5% atmospheric CO<sub>2</sub>, toxin production by this bacterium is growth phase dependent. Levels of PA, LF, and EF peak during late exponential and early stationary phase (21, 24, 25). To determine whether elevated toxin gene transcription in the *abrB* null mutant resulted in a change in toxin protein synthesis, we assessed toxin levels in supernatants from cultures of parent and *abrB* null strains. Levels of the toxin proteins in supernatants from mid-exponential-phase cultures were examined using immunoblotting. Figure 5 shows that supernatant from a UT166 culture (*abrB* null mutant) contained significantly more PA, LF, and EF than supernatant from a culture of the parent strain, UM44. When plasmid pUTE448, harboring *abrB*, was introduced into the *abrB* null mutant, toxin protein levels were reduced to the amount detected for the parent strain UM44. UT166 containing the vector alone (pUTE29) produced toxin protein levels slightly below the ones for UT166 without the plasmid. Supernatant from a culture of the toxin-negative, pXO1-cured strain UM44-1C9 showed only very low levels of cross-reactive material.

**Negative regulatory effect of *abrB* on *atxA::lacZ* expression.** The main *trans*-acting regulator of anthrax toxin gene expression is the pXO1-encoded gene *atxA*. Transcription of all three toxin genes is decreased dramatically in an *atxA* null mutant (8); however, the molecular basis for *atxA*-dependent gene

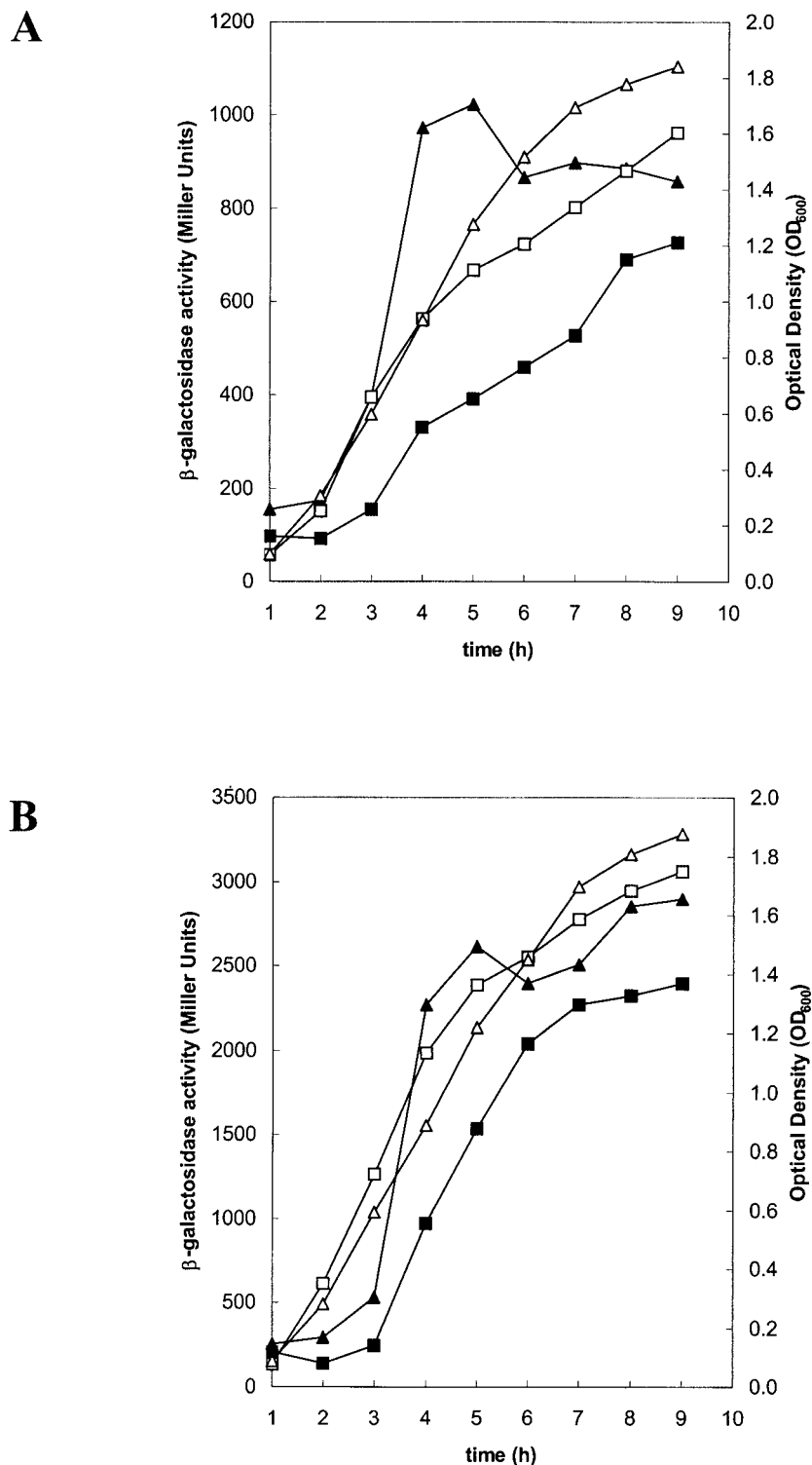


FIG. 4. Growth curves (open symbols) and  $\beta$ -galactosidase activity of toxin gene promoter-*lacZ* fusions (solid symbols). (A) Growth of parent RBAF143 ( $\square$ ) and *abrB* mutant UT169 ( $\triangle$ ) and *lef::lacZ* expression of parent RBAF143 ( $\blacksquare$ ) and *abrB* mutant UT169 ( $\blacktriangle$ ). (B) Growth of parent RBAF144 ( $\square$ ) and *abrB* mutant UT170 ( $\triangle$ ) and *cya::lacZ* expression of parent RBAF144 ( $\blacksquare$ ) and *abrB* mutant UT170 ( $\blacktriangle$ ).

expression is not known. To determine whether an epistatic relationship exists between *abrB* and *atxA*, we monitored expression of *atxA::lacZ* and *abrB::lacZ* transcriptional fusions borne on a low-copy-number plasmid ( $4 \pm 1$  copies/chromo-

some) in parent and mutant strains grown under the same conditions used above.

Expression of the *abrB::lacZ* transcriptional fusion was unaffected by the presence of *atxA*. In repeated experiments, the

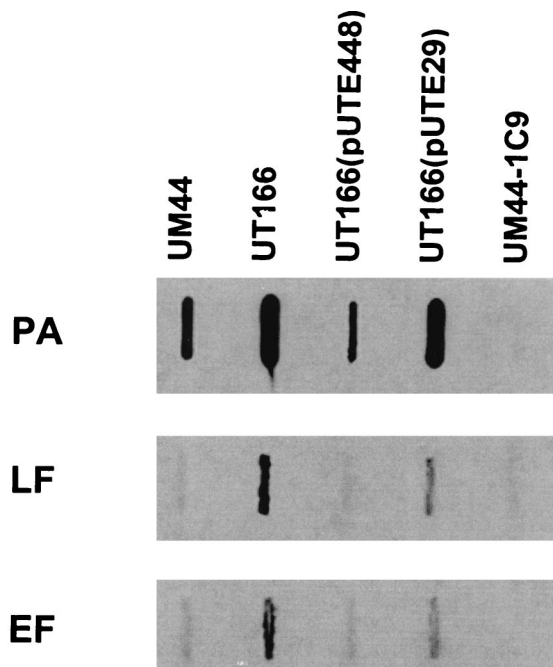


FIG. 5. Western blot analysis demonstrating *abrB* control of toxin production. Supernatant samples from mid-exponential-phase cultures were probed with antitoxin antisera as indicated. Lanes: UM44, parent; UT166, *abrB* mutant; UT166(pUTE448), *abrB*-complemented strain; UT166(pUTE29), *abrB* mutant; UM44-1C9, pX01<sup>-</sup>.

$\beta$ -galactosidase activity of the parent and *atxA* null strains harboring the plasmid-borne reporter (pUTE441) did not differ significantly throughout growth (Fig. 6A). Thus, *abrB* expression is independent of *atxA*.

Expression of the *atxA::lacZ* fusion (pUTE411) was slightly elevated in the *abrB* null mutant compared to the parent strain (Fig. 6B). The expression profile was highly reproducible, with a distinct peak 4 h after diluting cells of an overnight culture into fresh medium. The peak at the 4-h time point coincides with the strong derepression of *pagA* observed during early exponential growth of the  $\Delta$ *abrB pagA::lacZ* mutant. We conclude that *abrB* has a negative regulatory effect on *atxA*.

***abrB* is *spo0A* regulated.** In *B. subtilis*, *abrB* mRNA can be detected during the lag phase of growth in batch culture. Transcript levels peak early in exponential phase and become undetectable in mid-exponential phase. The repression of *abrB* expression, beginning in exponential phase, is primarily due to negative control by phosphorylated Spo0A. As Spo0A-P levels increase, AbrB protein levels decrease. *spo0A* mutants exhibit high levels of *abrB* mRNA and AbrB protein in exponential phase and during the transition into stationary phase (29, 41).

Expression of the *abrB::lacZ* fusion was observed throughout growth in *B. anthracis* batch culture (Fig. 7). The presence of two well-conserved apparent Spo0A-binding sites upstream of the *abrB*-coding sequence suggested that the *B. anthracis* gene may be regulated in a manner similar to that of the *B. subtilis abrB* (4, 31, 36). The *B. anthracis spo0A* gene was identified previously by Brown et al. (6). We constructed a *B. anthracis spo0A* null mutant, UT157, by replacing an internal fragment of the *spo0A* gene with  $\Omega$ -*sp*. As shown in Fig. 7,

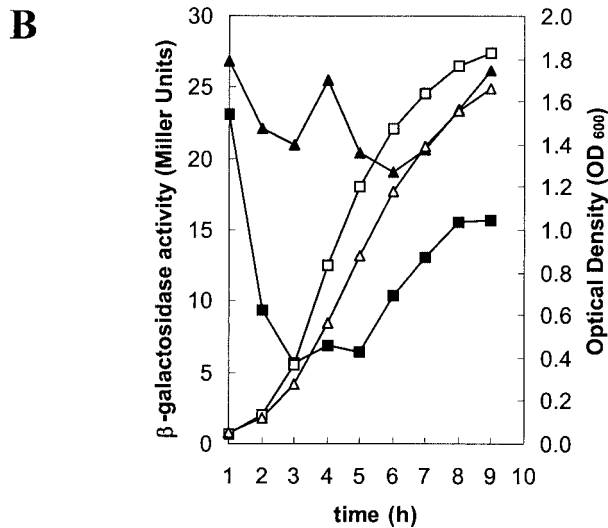
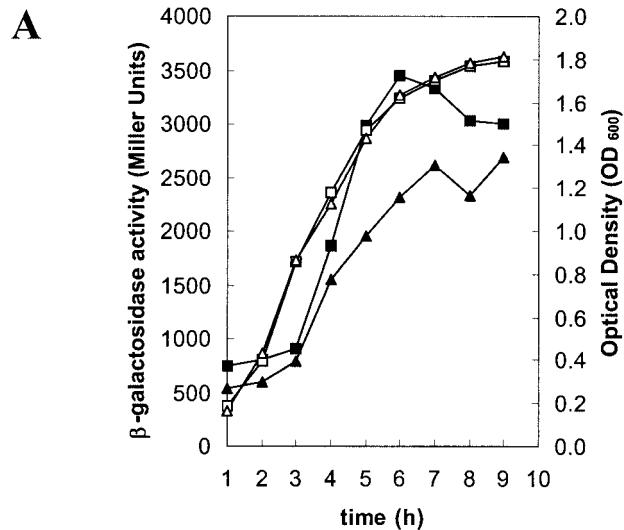


FIG. 6. (A) Growth curves for parent strain UM44 ( $\square$ ) and *atxA* mutant UT53 ( $\Delta$ ), carrying a plasmid-encoded *abrB::lacZ* fusion, pUTE441, and  $\beta$ -galactosidase activity of UM44(pUTE441) ( $\blacksquare$ ) and UT53(pUTE441) ( $\blacktriangle$ ). (B) Growth curves for parent strain UM44 ( $\square$ ) and *abrB* mutant UT166 ( $\Delta$ ), carrying a plasmid-encoded *atxA::lacZ* fusion, pUTE411, and  $\beta$ -galactosidase activity of UM44(pUTE411) ( $\blacksquare$ ) and UT166(pUTE411) ( $\blacktriangle$ ).

*abrB::lacZ* expression by UT157 did not decrease in mid-exponential and stationary phase, analogous to the situation described for a *B. subtilis*  $\Delta$ *spo0A* mutant (29).

Relative AbrB protein levels in the *B. anthracis* strains were assessed using immunoblotting. Whole-cell extracts from cultures grown to early exponential, mid-exponential, and stationary phase were probed using antiserum generated in rabbits against purified *B. subtilis* AbrB (Fig. 8). In the parent strain UM44, the maximum amount of AbrB was present in mid-

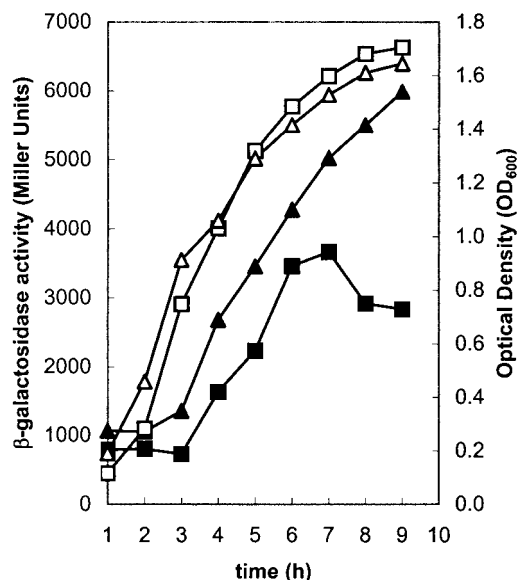


FIG. 7. Growth curves (open symbols) and *abrB::lacZ* expression (solid symbols) of parent strain UM44(pUTE441) (squares) and *spo0A* null mutant UT157(pUTE441) (triangles).

exponential phase, while only very low levels were found in early-log- and in stationary-phase cultures. In the  $\Delta spo0A$  mutant (UT157), high levels of AbrB protein persisted in stationary-phase cultures. No AbrB protein was detected in the *abrB* null mutant, UT166.

Taken together, these results indicate that the chromosomal

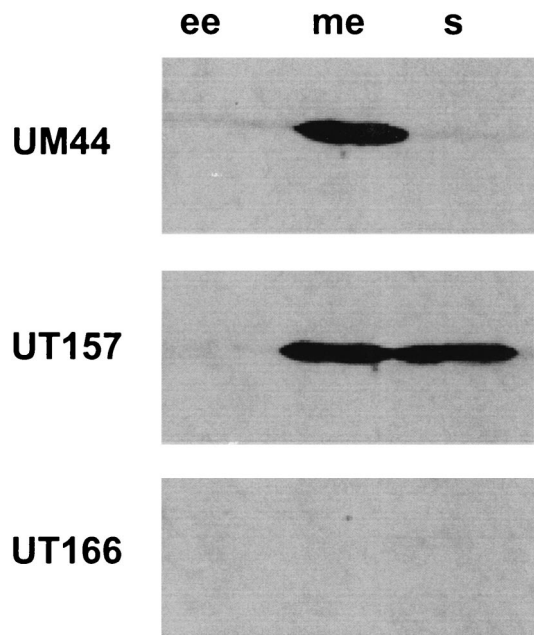


FIG. 8. Detection of AbrB in *B. anthracis* total cell extracts using anti-AbrB serum raised against *B. subtilis* AbrB. Samples from cultures of *B. anthracis* strains UM44, UT157 ( $\Delta spo0A$ ) and UT166 ( $\Delta abrB$ ) were taken at early exponential phase (ee), mid-exponential phase (me), and stationary phase (s). Approximately 15  $\mu$ g of total protein was loaded into each well.

*abrB* gene of *B. anthracis* is transcribed and regulated in a manner similar to that of the *B. subtilis* gene.

## DISCUSSION

In this study, we have shown that the chromosomally encoded *abrB* gene of *B. anthracis* negatively regulates expression of the three anthrax toxin genes. The toxin genes, *pagA*, *lef*, and *cya*, are located noncontiguously on a 30-kb region of virulence plasmid pXO1 (28). Expression of the toxin genes was measured by monitoring the  $\beta$ -galactosidase activity of reporter strains harboring toxin gene promoter-*lacZ* fusions at the normal genetic loci on pXO1. In addition, toxin protein levels in culture supernatants were assessed using immunoblotting. For all three genes, promoter activity and protein levels were increased in the absence of a functional *abrB* gene. The *pagA* gene was the most strongly affected by *abrB*.

The *B. anthracis* *abrB* gene is an orthologue of the well-studied *abrB* gene of *B. subtilis*. AbrB has been called a "transition state regulator," a term coined to describe regulatory proteins that repress or activate genes during the transition from active growth into stationary phase (37, 40). In *B. subtilis*, *abrB* affects expression of numerous genes upon entry of a culture into stationary phase and has also been implicated in altered gene expression during the transition from lag to exponential phase growth (29). Our results indicate that the *B. anthracis* *abrB* gene regulates toxin gene expression upon entry of the culture into exponential phase. In *B. anthracis* strains harboring toxin gene promoter-*lacZ* fusions,  $\beta$ -galactosidase activity increased gradually throughout exponential phase and reached maximum activity in late exponential phase. In contrast, the enzyme activity of reporter strains carrying an *abrB* null mutation increased sharply in early exponential phase and reached maximum activity in mid-exponential phase.

The chromosomal *abrB* genes of *B. anthracis* and *B. subtilis* seem to encode proteins of similar structure. The predicted amino acid sequences of the AbrB proteins are 85% identical and 93% similar. We found that antiserum raised against the *B. subtilis* AbrB protein cross-reacts with AbrB from *B. anthracis*. Moreover, the genes appear to have functional similarity. In a report by Baille et al. (3) the *B. anthracis* *pagA* gene was cloned in different *B. subtilis* mutants and toxin synthesis was assessed. A *B. subtilis* *abrB* mutant harboring *pagA* on a multicopy plasmid exhibited an eightfold increase in PA synthesis compared to the parent strain harboring the same plasmid construct (3).

In addition to the structural and functional similarity of the *B. anthracis* and *B. subtilis* AbrB proteins, the *abrB* genes of these species seem to be regulated similarly. In *B. subtilis*, expression of the *abrB* gene is transient and highly growth phase dependent. Highest levels of *abrB* transcript and protein are found during the transition from lag phase into exponential growth (29). After this early peak, levels drop sharply due to negative regulation by the phosphorylated form of response regulator Spo0A (29, 37). Our data show that the *B. anthracis* *abrB* gene is also *spo0A* regulated. The  $\beta$ -galactosidase activity of a *B. anthracis* strain harboring an *abrB::lacZ* fusion increased throughout growth, reaching maximum activity in late exponential phase. In a *spo0A* null *B. anthracis* mutant,  $\beta$ -galactosidase activity continued to increase in late exponential

and stationary phase. The steady-state levels of AbrB protein in the parent and *spo0A* mutant *B. anthracis* strains echo the reporter gene fusion results. In the parent strain, AbrB levels decrease after mid-exponential phase. In the *spo0A* mutant, AbrB levels remain high during late exponential and stationary-phase growth. These data indicate that the *B. anthracis* *abrB* gene is negatively regulated by *spo0A*.

We are presently investigating the molecular mechanism for *abrB*-controlled anthrax toxin gene expression. The simplest model for negative regulation by AbrB is direct binding of the protein to each of the toxin gene promoters. In *B. subtilis*, AbrB has been shown to bind to the promoter regions of over 40 different genes (37). A DNA consensus sequence for AbrB binding in *B. subtilis* has been put forth by Strauch (38). The sequence, WAWWTTTWCAAAAAAW, is extremely AT rich, similar to the 67% AT in the *B. anthracis* genome (20, 28). Using only the 182-kb DNA sequence of the pXO1 plasmid, we found 16 potential AbrB binding sites when we allowed one mismatch and 122 potential sites when we allowed two mismatches. Although some potential binding sites are in the toxin gene promoter regions, we cannot predict with confidence whether AbrB directly binds to these sequences. Recently, Vaughn et al. (46) speculated that the unique structure of the N terminus of the *B. subtilis* AbrB protein confers DNA-binding specificity in the absence of a consensus sequence. These authors reported that the amino-terminal domain of the protein possesses a looped-hinge helix DNA-binding topology, representing a novel class of DNA binding proteins (46, 47).

Whether or not AbrB binds directly to a specific nucleic acid sequence or structure, the differential effect of *abrB* on regulation of the three toxin genes may be indicative of divergent mechanisms for control of these genes. Previous studies of the toxin gene promoter regions have not revealed any obvious similarities in sequences or predicted structure (8). In *B. subtilis*, some of the effects of AbrB on gene expression are indirect, resulting from repression of additional regulatory genes by AbrB. For example, AbrB represses expression of the *sigH* (*spo0H*) gene, encoding the alternative sigma factor  $\sigma^H$ . Consequently, genes transcribed by RNA polymerase containing  $\sigma^H$  are affected in an *abrB* mutant (37). It is conceivable that in *B. anthracis*, *abrB* controls expression of another regulator important for expression of one or more of the toxin genes. It should be noted, however, that consensus sites for recognition by alternative sigma factors are not apparent in any of the toxin gene promoters.

Prior to this study, only one *trans*-acting regulator of the toxin genes was known. The *atxA* gene on pXO1 is essential for expression of all three toxin genes and also positively affects expression of the capsule biosynthesis genes (8, 16, 44, 45). *atxA* null mutants are avirulent in a mouse model for anthrax (8). The mechanism for *atxA*-mediated activation of gene expression is not known. The predicted amino acid sequence of the 56-kDa cytoplasmic AtxA protein does not indicate that it is a DNA-binding protein, and sequence-specific interactions between AtxA and the promoter regions of the toxin and capsule genes have not been demonstrated.

We tested for an epistatic relationship between *abrB* and *atxA* by monitoring expression of transcriptional *atxA::lacZ* and *abrB::lacZ* fusions in *atxA* and *abrB* null mutants. Our results indicate that *abrB* has a small negative effect on *atxA* expres-

sion. It is tempting to hypothesize that repression of toxin gene expression by *abrB* is due solely to negative regulation of *atxA*. However, results of previous studies render that model unlikely. Strains carrying multiple copies of the *atxA* gene produce elevated levels of AtxA but do not show increased PA synthesis (7). Also, the addition of the *pagA* promoter on a multicopy vector to a strain harboring the wild-type *pagA* gene on pXO1 does not affect PA synthesis (34). These data indicate that steady-state levels of AtxA are not limiting for toxin gene expression. Thus, the fourfold effect of *abrB* on *atxA* expression cannot explain the differences in toxin gene expression observed in an *abrB* mutant.

The role of regulatory genes for anthrax toxin synthesis during infection is critical to understanding *B. anthracis* pathogenesis. In inhalation anthrax, *B. anthracis* spores are phagocytosed by alveolar macrophages and can germinate intracellularly (15, 32). Ultimately, vegetative cells proliferate in the blood and other body tissues. The toxin proteins are synthesized and secreted by cells growing in the blood, and recently, evidence for *atxA* and toxin gene expression by newly vegetative cells in macrophages has been reported (15). Not surprisingly, in a mouse model for anthrax, *atxA* null mutant-infected animals show a significantly decreased antibody response to all three toxin proteins and *atxA* mutants are avirulent (8). The contribution of *abrB* to virulence has not been established. The newly discovered players in anthrax toxin gene expression, *abrB* and *spo0A*, may have a role in the timing of toxin synthesis during *B. anthracis* infection. Our findings link anthrax toxin gene expression to the multicomponent phosphorelay signaling system, well-studied in *B. subtilis*. Future experiments will address whether this signaling system plays a role in environmental sensing by *B. anthracis* during infection.

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