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# β-Lactamase Genes of the Penicillin-Susceptible *Bacillus anthracis* Sterne Strain

Yahua Chen,[1] Janice Succi,[1] Fred C. Tenover,[2] and Theresa M. Koehler[1]*

*Department of Microbiology and Molecular Genetics, The University of Texas-Houston Health Science Center Medical School, Houston, Texas 77030,[1] and Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]*

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**Susceptibility to penicillin and other β-lactam-containing compounds is a common trait of *Bacillus anthracis*. β-lactam agents, particularly penicillin, have been used worldwide to treat anthrax in humans. Nonetheless, surveys of clinical and soil-derived strains reveal penicillin G resistance in 2 to 16% of isolates tested. Bacterial resistance to β-lactam agents is often mediated by production of one or more types of β-lactamases that hydrolyze the β-lactam ring, inactivating the antimicrobial agent. Here, we report the presence of two β-lactamase (*bla*) genes in the penicillin-susceptible Sterne strain of *B. anthracis*. We identified *bla1* by functional cloning with *Escherichia coli*. *bla1* is a 927-nucleotide (nt) gene predicted to encode a protein with 93.8% identity to the type I β-lactamase gene of *Bacillus cereus*. A second gene, *bla2*, was identified by searching the unfinished *B. anthracis* chromosome sequence database of The Institute for Genome Research for open reading frames (ORFs) predicted to encode β-lactamases. We found a partial ORF predicted to encode a protein with significant similarity to the carboxy-terminal end of the type II β-lactamase of *B. cereus*. DNA adjacent to the 5′ end of the partial ORF was cloned using inverse PCR. *bla2* is a 768-nt gene predicted to encode a protein with 92% identity to the *B. cereus* type II enzyme. The *bla1* and *bla2* genes confer ampicillin resistance to *E. coli* and *Bacillus subtilis* when cloned individually in these species. The MICs of various antimicrobial agents for the *E. coli* clones indicate that the two β-lactamase genes confer different susceptibility profiles to *E. coli*; *bla1* is a penicillinase, while *bla*2 appears to be a cephalosporinase. The β-galactosidase activities of *B. cereus* group species harboring *bla* promoter-*lacZ* transcriptional fusions indicate that *bla1* is poorly transcribed in *B. anthracis*, *B. cereus*, and *B. thuringiensis*. The *bla2* gene is strongly expressed in *B. cereus* and *B. thuringiensis* and weakly expressed in *B. anthracis*. Taken together, these data indicate that the *bla1* and *bla2* genes of the *B. anthracis* Sterne strain encode functional β-lactamases of different types, but gene expression is usually not sufficient to confer resistance to β-lactam agents.**

Penicillin G, doxycycline, and ciprofloxacin have long been the drugs of choice for treatment of all forms of anthrax disease in humans (27). Combinations of these drugs were used to treat the recent U.S. cases (28, 13); yet, historically, penicillin has been the antimicrobial agent most commonly used for treating anthrax worldwide (29, 62). Susceptibility to penicillin and other β-lactam agents is often listed as a defining characteristic of *B. anthracis* (48, 62). However, antimicrobial susceptibility profiles of clinical and nonclinical isolates have revealed various prevalences of penicillin G-resistant strains. Tests of 50 historical *Bacillus anthracis* isolates and 15 isolates from the recent human anthrax cases in the United States indicate widespread susceptibility to β-lactam-containing compounds with one exception. Of the 65 strains tested, one strain, isolated from a human case of anthrax in 1974, was β-lactamase positive and penicillin resistant (47). In another survey, 7 of 44 isolates from carcasses and soil in an area of South Africa to which anthrax is endemic were resistant to penicillin G (51). A third survey of isolates recovered in France, including 1 isolate from a human, 28 from animal sources, and 67 from other environmental sources, revealed resistance to penicillin G and amoxicillin in 11.5% of the isolates (12).

* Corresponding author. Mailing address: University of Texas Medical School, Department of Microbiology and Molecular Genetics, 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.

The susceptibility of most *B. anthracis* strains to β-lactam agents is intriguing considering that closely related species are characteristically β-lactamase positive (10). *B. anthracis* is a member of the *Bacillus cereus* group species, which includes *B. anthracis*, *B. cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. Numerous phylogenetic studies, including 16S rRNA gene comparisons, multienzyme electrophoresis, and amplified fragment length polymorphism analysis, indicate that *B. anthracis* is most closely related to *B. cereus* (6, 24, 61). The most obvious phenotypic differences between *B. anthracis* and *B. cereus* are related to pathogenicity. *B. anthracis*, the causative agent of anthrax, is a highly virulent mammalian pathogen (46), while *B. cereus* is better known as a causal agent of mild food poisoning (21). The virulence of *B. anthracis* has been attributed primarily to the presence of two plasmids that are not found in *B. cereus*. *B. anthracis* plasmid pXO1 carries the structural genes for the anthrax toxin proteins, while plasmid pXO2 harbors the biosynthetic genes for the antiphagocytic poly-D-glutamic acid capsule (42, 52).

Unlike those of *B. anthracis*, *B. cereus* strains commonly exhibit resistance to penicillin and other β-lactam agents. Three different β-lactamases, named β-lactamase I, II, and III, have been reported for various *B. cereus* strains. *B. cereus* strain 569/H is resistant to penicillins and cephalosporins and contains genes encoding all three enzymes. According to the classification scheme proposed by Bush (11), which groups β-lactamases on the basis of specific substrate and inhibitor profiles,

TABLE 1. Plasmids and strains used in this study

| Plasmid or strain | Relevant characteristic(s)[a] | Source or reference |
|---|---|---|
| Plasmids | | |
| *E. coli* | | |
| pACYC184 | $Cm^r$ $Tc^r$ | 14 |
| pGEM-T Easy | $Ap^r$ | Promega |
| pBC | $Cm^r$ | Stratagene |
| pUC18::Ωkm-2 | $Ap^r$ $Km^r$ | 55 |
| Bifunctional | | |
| pGC13 | $Ap^r$ in *E. coli*, $Em^r$ in bacilli | 35 |
| pHT304-18Z | Contains promoterless *lacZ*; $Ap^r$ in *E. coli*, $Em^r$ in bacilli | 3 |
| pUTE461 | 0.7-kb PCR product containing upstream region of *bla1* (YC71-YC72) cloned into pHT304-18Z; *bla1*::*lacZ*; $Ap^r$ $Em^r$ | This work |
| pUTE462 | 0.9-kb PCR product containing upstream region of *bla2* (YC73-YC74) cloned into pHT304-18Z; *bla2*::*lacZ*; $Ap^r$ $Em^r$ | This work |
| pUTE488 | Contains a transcriptional terminator adjacent to multiple cloning site, $Cm^r$ in *E. coli*, $Km^r$ in *E. coli* and *Bacillus* | This work |
| pUTE523 | 1.6-kb PCR product containing *bla1 orf* and upstream region (YC81-YC82) cloned downstream of transcriptional terminator in pUTE488; $Km^r$ $Cm^r$ | This work |
| pUTE490 | 1.6-kb PCR product containing *bla2 orf* and upstream region (YC83-YC84) cloned downstream of transcriptional terminator in pUTE488; $Km^r$ $Cm^r$ | This work |
| Strains | | |
| *B. anthracis* | | |
| UM44[b] | Sterne strain derivative, $Ap^s$ | 60 |
| *B. cereus* | | |
| 569 | $Ap^r$ | 9 |
| *B. thuringiensis* | | |
| AW43 | $Ap^r$ | 63 |
| *B. subtilis* | | |
| 168 | $Ap^s$ | BGSC[c] |
| *E. coli* | | |
| TG1 | Cloning host, $Ap^s$ | 56 |
| GM2163 | *dam dcm* | 54 |

[a] Abbreviations: $Ap^r$, ampicillin resistant; $Ap^s$, ampicillin sensitive; $Cm^r$, chloramphenicol resistant; $Em^r$, erythromycin resistant; $Km^r$, kanamycin resistant; $Tc^r$, tetracycline resistant.
[b] Weybridge (Sterne) strain derivative.
[c] BGSC: Bacillus Genetic Stock Center, Columbus, Ohio.

*B. cereus* β-lactamase I and β-lactamase III are group 2A enzymes that prefer penicillin substrates and are inhibited by clavulanic acid (15, 16, 33, 50). *B. cereus* β-lactamase II is a group 3 enzyme, a heat-stable metallo-β-lactamase, that is not inhibited by clavulanic acid (8, 18, 25, 33, 34, 39). A β-lactamase I enzyme is also produced by the *B. cereus* group species *B. thuringiensis* (40, 65). Most *B. mycoides* strains are resistant to penicillin (J. Mahillon, personal communication), and a β-lactamase I gene has been cloned and sequenced (GenBank, accession no. X62244).

In work presented here, we report the presence of two β-lactamase genes in the Sterne strain of *B. anthracis*. The Sterne strain is a well-studied attenuated strain that serves as a live vaccine for animals (58). The Sterne strain does not produce capsule due to the absence of pXO2. With the exception of being cured of pXO2, this strain is considered to be a prototypical *B. anthracis* strain (59). It is sensitive to penicillin and does not exhibit β-lactamase activity. Our data indicate that the β-lactamase genes carried by this strain are not expressed in the Sterne strain but encode functional enzymes when cloned in other species.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani medium (LB) (7) containing antimicrobial agents at the following concentrations, when appropriate: for *Escherichia coli*, ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin, 25 μg/ml; for *Bacillus* species, erythromycin, 5 μg/ml; and kanamycin, 100 μg/ml.

**DNA isolation and manipulation.** Standard techniques for plasmid and chromosomal DNA preparation and cloning were those described by Ausubel et al. (7). Plasmid DNA was introduced into *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. subtilis* strains using electroporation as described previously (31). DNA for electroporation was obtained from the *dam dcm E. coli* strain GM2163 (54).

PCRs were performed using a PE Applied Biosystems (Norwalk, Conn.) PCR system 9700. A typical PCR mixture (50 μl) included 1× PCR buffer, primer (2 μM each), $MgCl_2$ (1.5 mM), deoxynucleoside triphosphates (0.2 mM), DNA template (0.1 μg), and *Taq* DNA polymerase (2.5 U). The reactions were run for 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. Primers are listed in Table 2. For inverse PCR, *B. anthracis* DNA was digested with a variety of restriction enzymes. Following ligation with T4 DNA ligase, the DNA was used as a template in PCRs with primers reading outward from known sequences. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) for sequencing and further constructions.

Restriction enzymes, T4 DNA ligase, and *Taq* polymerase were purchases from Promega or Gibco BRL (Rockville, Md.). DNA oligonucleotides were purchased from Sigma Genosys (The Woodlands, Tex.) or IDT (Coralville, Iowa).

**Plasmid constructions.** In order to make plasmid constructions for antimicrobial susceptibility testing, a bifunctional vector was constructed. The 4-kb *Eco*RI fragment containing an erythromycin resistance gene and the *B. thuringiensis* plasmid replicon from pGC13 (35) were cloned into pBC (Stratagene, La Jolla, Calif.). Subsequently, the erythromycin resistance gene of the plasmid was replaced with a kanamycin resistance gene by replacing a *Kpn*I fragment with a PCR product amplified from pUC18::Ω*km-2* using primers YC75 and YC76 (Table 2). The resulting plasmid, pUTE488 (see Fig. 2C), contained a T4 transcriptional terminator next to the multiple cloning sites, a chloramphenicol

TABLE 2. Primers used in this study

| Primer | Sequence (5′-3′),[a] location | PCR amplification | Restriction site |
|---|---|---|---|
| YC71 | AAGCTTCGGATTGATATGAAGACAGAA, from −624 to −604 nt upstream of *bla1* start codon | Forward primer for *bla1* upstream region | *Hin*dIII |
| YC72 | GGATCCTTGCAATGCACCTCCTGTA, from 105 to 87 nt downstream of *bla1* start codon | Reverse primer for *bla1* upstream region | *Bam*HI |
| YC73 | AAGCTTCATTCCGGATCAAATAAGTGC, from −790 to −770 nt upstream of *bla2* start codon | Forward primer for *bla2* upstream region | *Hin*dIII |
| YC74 | GGATCCTGCTCTACCTTTCGTTCTGC, from 104 to 85 nt downstream of *bla2* start codon | Reverse primer for *bla2* upstream region | *Bam*HI |
| YC75 | GGTACCTGCAGTCGACGAATTCCCGGGGATCCGGTG | Forward primer for kanamycin-resistant gene and transcriptional terminator | *Kpn*I |
| YC76 | GGTACCAGACATCTAAATCTAGGTAC | Reverse primer for kanamycin-resistant gene and transcriptional terminator | *Kpn*I |
| YC81 | GGATCCCGGATTGATATGAAGACAGAA, from −624 to −604 nt upstream of *bla1* start codon | Forward primer for *bla1* gene | *Bam*HI |
| YC82 | AAGAATCGCTTTCCAAGTTCA, from 55 to 38 nt downstream of *bla1* stop codon | Reverse primer for *bla1* gene | |
| YC83 | GGATCCCATTCCGGATCAAATAAGTGC, from −790 to −770 nt upstream of *bla2* start codon | Forward primer for *bla2* gene | *Bam*HI |
| YC84 | GTAAAGTATGCATAGCTTCGC, from 72 to 52 nt downstream of *bla2* stop codon | Reverse primer for *bla2* gene | |
| BLA1-5 | CATTGCAAGTTGAAGCGAAA, from 98 to 117 nt downstream of *bla1* start codon | Forward primer for RT-PCR of *bla1* transcript | |
| BLA1-6 | TGTCCCGTAACTTCCAGCTC, from −142 to −161 nt upstream of *bla1* stop codon | Reverse primer for RT-PCR of *bla1* transcript | |
| BLA2-5 | TTGTCGATTCTTCTTGGGATG, from 248 to 268 nt downstream of *bla2* start codon | Forward primer for RT-PCR of *bla2* transcript | |
| BLA2-6 | CCCCTACTTCTCCATGACCA, from −39 to −58 nt upstream of *bla2* stop codon | Reverse primer for RT-PCR of *bla2* transcript | |

[a] Restriction enzyme recognition sites are underlined.

resistance gene for selection in *E. coli*, and a kanamycin resistance gene for selection in *Bacillus*. To construct plasmids for antimicrobial resistance tests and MIC assays, DNA sequences containing the *bla1* and *bla2* open reading frames (ORFs) including the upstream regions indicated above were amplified using the PCR with primers YC81 and YC82 (*bla1*) and YC83 and YC84 (*bla2*) (see Fig. 2 and Table 2). The PCR products were first cloned into the pGEM-T Easy vector and then subcloned into the *Bam*HI and *Eco*RI sites of pUTE488 to generate pUTE523 and pUTE490.

To construct plasmids harboring promoter-*lacZ* transcriptional fusions, DNA fragments containing the 5′ ends and upstream sequences of *bla1* and *bla2* were amplified using the primers YC71 and YC73 (*bla1*) and YC73 and YC74 (*bla2*) (see Fig. 2 and Table 2). The 729-nucleotide (nt) PCR product for the *bla1-lacZ* construct included 624 nt upstream of the translational start codon for *bla1*. The PCR product for the *bla2-lacZ* construct included 790 nt upstream of *bla2*. PCR products were first cloned into the pGEM-T Easy vector and ultimately cloned into pHT304-18Z in the correct orientation to generate pUTE461 and pUTE462.

**Selection of clones and antimicrobial susceptibility testing.** *E. coli* and *B. subtilis* isolates were grown overnight at 28°C with shaking at 200 rpm in LB containing appropriate antibiotics: chloramphenicol (20 μg/ml) for *E. coli* and kanamycin (100 μg/ml) for *B. subtilis*. One-hundred-microliter samples of cultures were spread on LB plates containing 100, 200, 400, or 800 μg of ampicillin per ml. Growth was assessed following 24 h at 37°C.

The MICs of various antimicrobial agents for *E. coli* isolates harboring the cloned *bla* genes were assessed by the NCCLS broth microdilution method using Mueller-Hinton broth (BD BioSciences, Sparks, Md.) as described previously (47).

**RT-PCR.** All reagents for reverse transcription-PCR (RT-PCR) were purchased from Ambion (Austin, Tex.). Total RNA was isolated from mid-exponential-phase cultures (optical density of 1.0 at 600 nm) grown in LB medium at 37°C with shaking at 200 rpm. Cells were collected on GP Express membranes (Millipore, Bedford, Mass.) (pore size, 0.22 μm). RNAWIZ was used to isolate total RNA according to the manufacturer's instructions. Purified RNA was treated with the DNA-free kit. The treated RNA (1.0 μg) was subjected to RT using a RETROscript kit. The RT mixtures (1 to 5 μl) were used as templates for PCRs. Positive controls were genomic DNA from *B. anthracis* UM44. Negative controls included a no-RT sample and a no-template sample assembled for each reaction set. Internal primers were BLA1-5/-6 for *bla1* and BLA2-5/-6 for *bla2*.

**β-galactosidase assays.** Strains were grown at 37°C with shaking in LB containing erythromycin. Overnight cultures were diluted 1:100 in fresh medium and incubated as before. Samples were collected at various time points, and enzyme assays were performed as described previously (45), using toluene to permeabilize the cells. At least three independent cultures were assayed for enzyme activity. Figures show data from representative experiments.

**Nucleotide sequence accession numbers.** DNA sequences of the *bla1* and *bla2* genes have been deposited in GenBank (AF367983 and AF367984).

## RESULTS

**The penicillin-susceptible Sterne strain of B. anthracis encodes two β-lactamase genes.** We used two different approaches to find β-lactamase (*bla*) genes in *B. anthracis*. First, we selected for functional *bla* genes by cloning random fragments of chromosomal DNA from *B. anthracis* UM44 (an auxotrophic mutant of the Sterne strain) into *E. coli* and selecting for ampicillin-resistant clones. *B. anthracis* DNA was digested with a variety of restriction enzymes and ligated into plasmid pACYC184, an *E. coli* cloning vector that does not carry a *bla* gene (14). The ligation mixtures were transformed into *E. coli* TG1, and clones were selected on agar containing ampicillin. One ampicillin-resistant transformant was obtained. This clone carried a 5.5-kb *Eco*RI fragment from *B. anthracis*. Sequence analysis revealed that the cloned DNA

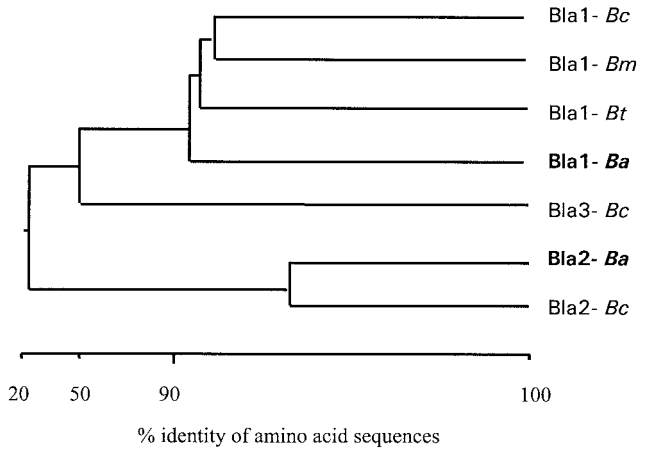


FIG. 1. Relatedness of β-lactamases from *B. cereus* group species. The dendrogram is based on the alignment of the predicted amino acid sequences. The GCG software of the Genetic Computing Group (Madison, Wis.) was used to generate the phylogenetic tree. Bla1 = type I; Bla2 = type II; Bla3 = type III. *Bc*, *B. cereus*; *Bm*, *B. mycoides*; *Bt*, *B. thuringiensis*; *Ba*, *B. anthracis*.

contained a 927-nt ORF predicted to encode a protein that is 87 to 93% identical to the β-lactamase I enzymes of *B. cereus*, *B. thuringiensis*, and *B. mycoides*. This ORF was designated *bla1*.

A second apparent *bla* gene was discovered by searching the unfinished *B. anthracis* genome database of The Institute for Genome Research (TIGR) (http://tigrblast.tigr.org/ufmg/) for ORFs predicted to encode β-lactamases. In addition to finding sequences representing *bla1*, we found a partial ORF predicted to encode a protein with strong sequence similarity to the carboxy-terminal end of the *B. cereus* β-lactamase II enzyme. DNA adjacent to the 5′ end of this *B. anthracis* ORF was cloned from UM44 using inverse PCR. DNA sequence analysis revealed a 768-nt ORF predicted to encode a protein that is 92% identical to *B. cereus* β-lactamase II. We designated this ORF *bla2*. The relatedness of the predicted *B. anthracis* β-lactamases to the enzymes from *B. cereus*, *B. mycoides*, and *B. thuringiensis* strains is depicted in Fig. 1.

The structural organization of the *bla1* and *bla2* genes from *B. anthracis* is shown in Fig. 2. The *bla* genes are located at distinct loci on the *B. anthracis* chromosome. The orientations of flanking ORFs and the potential for stem-loop structures followed by U tracks indicate that expression of *bla1* and *bla2* would result in monocistronic transcripts. An ORF predicted to encode a 529-amino acid protein with 69% similarity to PbpA, a 716-amino-acid penicillin-binding protein of *B. subtilis* (49), is located upstream of *bla1*. An ORF predicted to encode a 198-amino acid protein with 58% similarity to *B. subtilis* YwoA, a 193-amino-acid protein similar to bacteriocin transport permease (32), is downstream of *bla1* and in the opposite orientation. The *bla2* gene is downstream of an ORF predicted to encode a 229-amino-acid protein with 41% similarity to the *Brucella melitensis* lysozyme M1 (17). An ORF predicted to encode a 353-amino-acid protein of unknown function is located downstream of *bla2* and in the opposite direction.

**The *B. anthracis bla* genes confer ampicillin resistance to *E. coli* and *B. subtilis*.** To further test whether the *bla1* and *bla2* genes encode functional β-lactamases, we subcloned *bla1* and *bla2*, including upstream sequences (as indicated in Fig. 2), individually into the bifunctional vector pUTE488. This vector has a transcription termination sequence immediately upstream from the cloning site, so transcription of cloned genes is dependent upon native promoters. *E. coli* TG1 and *B. subtilis* 168 harboring pUTE488 (vector), pUTE523 (*bla1*), and pUTE490 (*bla2*) were tested for the ability to grow on LB agar containing different concentrations of ampicillin. *E. coli* clones harboring either *bla1* or *bla2* grew on agar medium containing up to 800 μg of ampicillin per ml. *B. subtilis* clones carrying *bla1* or *bla2* grew on medium containing up to 200 μg of ampicillin per ml. In contrast, *B. anthracis* UM44 isolates harboring the constructs were unable to grow on medium containing 100 μg of ampicillin per ml. These results indicate that the *B. anthracis* Sterne *bla1* and *bla2* genes encode functional proteins and have endogenous promoters that are active in *E. coli* and *B. subtilis*.

**The *B. anthracis bla* genes are transcribed in other *B. cereus* group species.** We could not assess *B. anthracis bla* gene function in the closely related species *B. cereus* and *B. thuringiensis* by testing for ampicillin resistance because these species harbor their own *bla* genes (see Fig. 1) and are already resistant to β-lactam antibiotics. Therefore, we compared *bla* promoter activity in *B. anthracis* and other *Bacillus cereus* group species by measuring β-galactosidase activity produced by isolates harboring plasmid-borne *bla1*- and *bla2-lacZ* transcriptional fusions. The reporter fusions were constructed by cloning upstream regions of the genes (as indicated in Fig. 2) adjacent to a promoterless *lacZ* gene in pHT304-18Z (2). This vector has an estimated copy number of three to five in *B. thuringiensis* (5). The vector and reporter plasmids, pUTE461 (*bla1*) and pUTE462 (*bla2*), were electroporated into *B. anthracis* UM44, *B. cereus* 569, and *B. thuringiensis* AW43.

As expected, relatively little β-galactosidase activity was produced by *B. anthracis* UM44 isolates harboring the vector alone or the vector-borne *bla-lacZ* fusions. In multiple experiments, less than one Miller unit of enzyme activity was detected throughout growth in batch culture. These results were consistent with the inability to detect *bla* gene transcripts in RNA preparations from *B. anthracis* UM44 using RT-PCR (data not shown). Higher levels of enzyme activity were observed in cultures of the other species. For each isolate tested, enzyme activity was highest at late-exponential phase. A representative comparison of β-galactosidase activities at late-exponential phase for different strains harboring the *bla-lacZ* fusion plasmids is shown in Fig. 3. No significant *bla1* expression was observed with *B. cereus* and *B. thuringiensis* isolates carrying the *bla1-lacZ* fusion. The low levels of β-galactosidase activity (1.2 to 1.6 Miller units) produced by isolates harboring the *bla1-lacZ* fusion were comparable to those for the same isolates harboring the vector alone. In contrast to *bla1* expression, the β-galactosidase activities of *B. cereus* and *B. thuringiensis* isolates harboring *bla2-lacZ* fusions were significantly higher than those for isolates containing the vector. In multiple experiments, the β-galactosidase activities of the *B. cereus* strain carrying the *bla2-lacZ* fusion was 4.5- to 8.1-fold higher than the background activity. The enzyme activity of the *B. thuringiensis* strain carrying the *bla2-lacZ* fusion was 6.9- to 8.9-fold higher than the background level. These data indicate that while the *B. anthracis bla* genes are poorly transcribed in

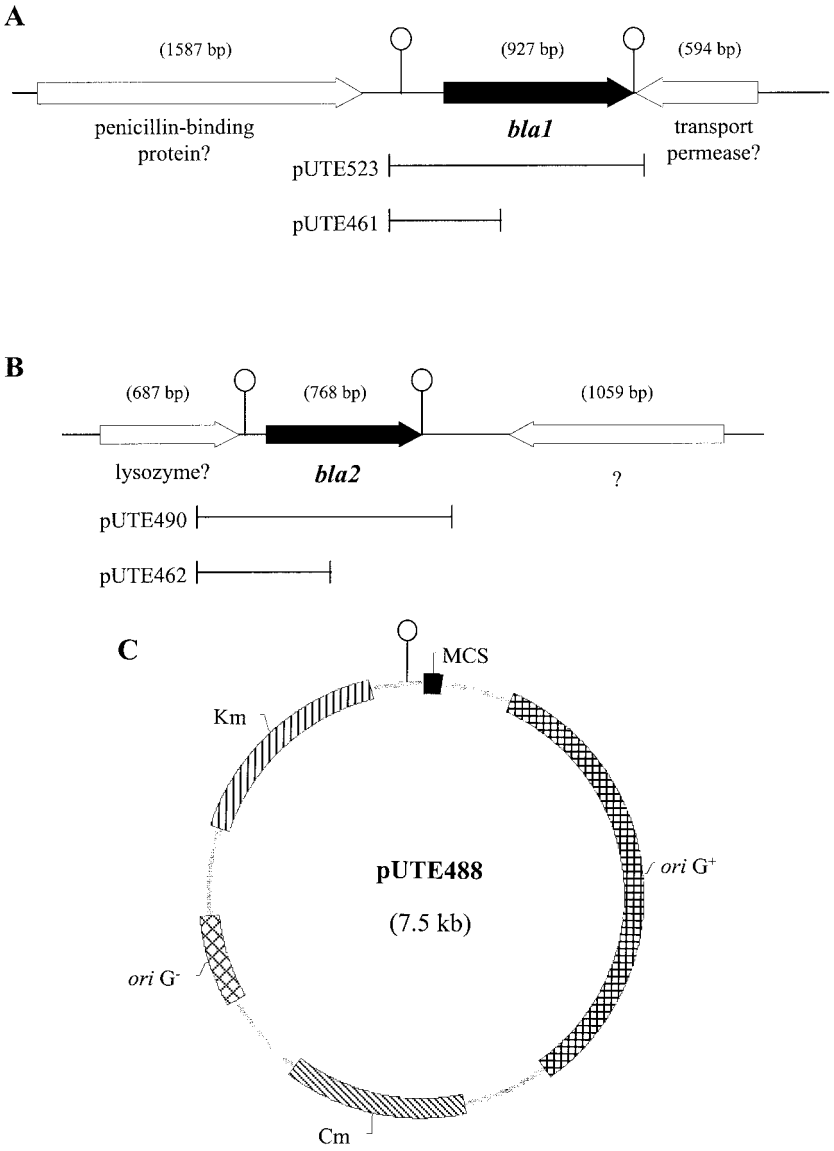


FIG. 2. Structural organization of the *B. anthracis bla* loci and cloning vector. (A) *bla1* locus; (B) *bla2* locus. Putative functions of proteins encoded by flanking ORFs are shown. Vertical lines represent potential stem-loop structures with $\Delta G$ values of −19.4 kcal/mol (upstream from *bla1*), −19.2 kcal/mol (downstream from *bla1*), −15.0 kcal/mol (upstream from *bla2*), and −21.8 kcal/mol (downstream from *bla2*). Cloned DNA regions are indicated. (C) Schematic representation of vector pUTE488. *ori*$G^+$ and *ori*$G^-$ indicate origins functioning in gram-positive and gram-negative species, respectively.

the parent strain, they are highly expressed in other *Bacillus* species.

**The antimicrobial susceptibility profiles of *E. coli* clones harboring *B. anthracis bla1* and *bla2* differ.** *E. coli* clones harboring plasmid-encoded *bla1* and *bla2* were tested for the ability to grow in the presence of a number of β-lactam agents. Results are shown in Table 3. The MICs of ceftazidime, cefpodoxime, cefotaxime, and ceftriaxone were significantly higher for *E. coli* carrying *bla2* than for *E. coli* harboring *bla1*. In addition, the MIC of ampicillin for the *bla1* clone was higher than that for the *bla2* clone. These data indicate that the enzymes encoded by *bla1* and *bla2* have different substrate specificities.

## DISCUSSION

Our studies indicate that the penicillin-susceptible Sterne strain of *B. anthracis* harbors two genes, *bla1* and *bla2*, that encode functional β-lactamases when cloned in other species. An *E. coli* clone harboring *bla1* shows enhanced activity only against ampicillin, suggesting that this enzyme is a penicillinase. This is consistent with the activity reported for the Class A β-lactamase, *bla1*, of *B. cereus* (33). An *E. coli* clone harboring *bla2*, however, shows increased resistance to several cephalosporins, including cefpodoxime, cefotaxime, and ceftriaxone. The *bla2*-associated activity is not inhibited by clavulanic acid, which is consistent with the presence of a class B or

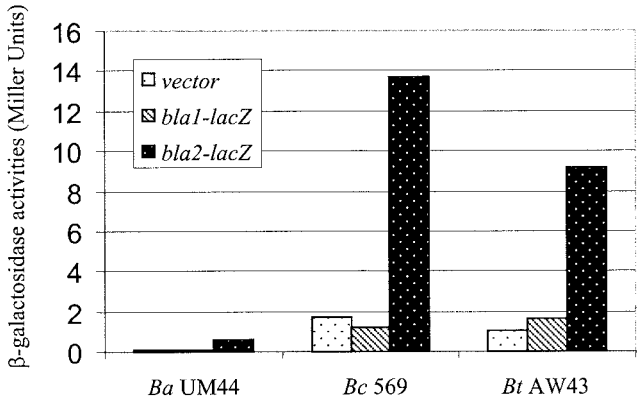


FIG. 3. *bla1* and *bla2* transcription in *B. cereus* group species. Isolates harbored pHT304-18Z (vector), pUTE461 (*bla1*::*lacZ*), or pUTE462 (*bla2*::*lacZ*) as indicated. *Ba*, *B. anthracis*; *Bc*, *B. cereus*; *Bt*, *B. thuringiensis*. β-galactosidase activity was determined following 7 h of incubation at 37°C with shaking at 200 rpm, when the cultures were in late log phase. Data shown are representative of at least three experiments.

class C cephalosporinase (11). Interestingly, the MICs of the cephamycins, cefotetan and cefoxitin, for the *bla2* clone are unchanged, which is unusual for cephalosporinases. Taken together, our data indicate that the activities of the *bla1* and *bla2* β-lactamases differ, and the *bla2* product may have novel features.

The *bla* genes are most likely a common feature of the *B. anthracis* genome and not simply an anomaly of the Sterne strain chosen for this study. Keim et al. have reported an exceedingly low degree of strain diversity for *B. anthracis* strains from numerous geographic locations (30). A comprehensive search for the presence of the *bla* genes in *B. anthracis* isolates has not been performed. However, we have found the *bla1* and *bla2* genes in the penicillin-susceptible Pasteur strain of *B. anthracis* using PCR amplification (data not shown). Also, current sequences in the unfinished *B. anthracis* genomic sequence database of TIGR (http://tigrblast.tigr.org/ufmg) indicate the presence of *bla1* and *bla2* sequences in the genomic sequence of the penicillin-susceptible Ames strain. The sequence of both *bla* genes of Ames is identical to those from Sterne. The annotated genome of *B. anthracis* Ames also shows that there are more than 10 proteins with similarity to metallo-β-lactamases. However, none of them are predicted to be secreted proteins.

Why are the *bla* genes of *B. anthracis* Sterne not expressed? Induction of *bla* gene expression in the β-lactamase-positive *B. cereus* 569 has long been recognized. However, the genetic basis has not been investigated. Imsande suggested a universal mechanism for regulation of β-lactamase synthesis in gram-positive bacteria (26). Recently, studies of regulation systems for β-lactamase synthesis in *Bacillus licheniformis* and staphylococci have yielded substantial breakthroughs (19, 64). For these organisms, three genes (*blaI*, *blaR1*, and *blaR2*) are involved in the regulation of the β-lactamase structural gene. With the exception of *blaR2*, whose presence rests only on genetic evidence, these genes are at a common chromosomal locus, forming a divergeon. Production of β-lactamase is regulated by the sensor-transducer, BlaR1, and the repressor, BlaI, which blocks transcription of the β-lactamase gene. When a β-lactam agent binds to the extracellular sensor domain of BlaR1, the cytoplasmic transducer domain is proteolytically cleaved. The transducer is then free to cleave and inactivate the BlaI repressor, and the transcription of the β-lactamase gene ensues (37, 64). A *blaI* homolog and a truncated *blaR1* gene are present in *B. anthracis* genome; however, they are not linked to either *bla* gene. The potential relationship of these genes to *bla* gene control for *B. anthracis* will be addressed in future investigations.

The presence of silent β-lactamase genes in *B. anthracis* is intriguing in the context of the *B. anthracis* genome as a whole. Preliminary analysis of the *B. anthracis* genomic sequence database of TIGR and several published reports indicate the presence of numerous ORFs in *B. anthracis* that have the potential to encode proteins associated with distinct phenotypes of other *B. cereus* group species (T. D. Read et al., submitted for publication). However, these ORFs appear to be nontranscribed in *B. anthracis*. For example, flagellar genes are located in the *B. anthracis* genome, yet unlike *B. cereus* and *B. thuringiensis*, *B. anthracis* is nonmotile. ORFs predicted to encode proteins with significant similarity to certain *B. cereus* virulence factors, such as enterotoxins (4, 23), can be found in the *B. anthracis* genome, but there are no reports indicating synthesis of these proteins by natural isolates of *B. anthracis.* For some *B. anthracis* genes, lack of expression has been attributed to the presence of a nonfunctional transcriptional activator, PlcR. For *B. cereus* and *B. thuringiensis*, PlcR controls numerous genes with known or predicted roles in virulence (1, 36, 41, 53). The *plcR* gene of *B. anthracis* contains a frameshift mutation that results in synthesis of a truncated protein (1). Mignot et al. (44) reported that expression of a functional *plcR* gene in *B. anthracis* results in transcriptional activation of genes weakly expressed in the absence of the regulator. Nevertheless, the absence of functional *plcR* in *B. anthracis* is most likely not the reason for low-level expression

TABLE 3. Minimal inhibitory concentrations[a] of antibiotics for *E. coli* TG1 harboring cloned *B. anthracis bla1* or *bla2* genes

| Antibiotic/inhibitor (concn) | MIC of drug for bacteria with: | | |
|---|---|---|---|
| | Vector (pUTE488) | *bla1* (pUTE523) | *bla2* (pUTE490) |
| Ampicillin | 2 | >64 | 8 |
| Amoxicillin/clavulanate | 4 | 32 | 32 |
| Cefazolin | 1 | 1 | 2 |
| Cefepime | ≤0.03 | ≤0.03 | ≤0.03 |
| Cefoxitin | 2 | 2 | 4 |
| Cefotetan | <1 | <1 | <1 |
| Ceftazidime | 0.12 | 0.12 | 0.5 |
| Cefpodoxime | 0.5 | 0.5 | 4 |
| Cefpodoxime/clavulanate (4 μg/ml) | 0.25 | 0.25 | 4 |
| Cefotaxime | ≤0.03 | ≤0.03 | 1.0 |
| Ceftriaxone | ≤0.06 | ≤0.06 | 0.25 |
| Imipenem | ≤0.12 | ≤0.12 | ≤0.12 |
| Meropenem | ≤0.25 | ≤0.25 | ≤0.25 |
| Piperacillin | ≤2 | 8 | ≤2 |
| Piperacillin/tazobactam (4 μg/ml) | ≤1 | ≤1 | ≤1 |
| Chloramphenicol | >32 | >32 | >32 |

[a] Given in micrograms/milliliter.

of the *B. anthracis bla* genes. DNA sequences upstream of *bla1* and *bla2* do not appear to contain PlcR binding sites.

The genetic basis for β-lactamase activity in uncommon penicillin-resistant *B. anthracis* isolates is not known. For some penicillin-resistant strains, synthesis of β-lactamase appears to be constitutive. However, there are reports of induction of β-lactamase activity in vitro in certain strains. Lightfoot and colleagues (38) reported β-lactamase activity for three penicillin-susceptible strains following growth in subinhibitory concentrations of flucloxacillin. It is not clear to what extent, if any, strains can be induced to produce β-lactamase activity in vivo. Notably, treatment of anthrax with penicillin is not always successful (22, 43), and the use of penicillin for prophylaxis and treatment of anthrax in experimental animals has had various outcomes (20, 57). Further investigations of *bla* gene expression in *B. anthracis* will likely impact future revisions in treatment guidelines for anthrax. Moreover, studies of *bla* gene expression with *B. anthracis* and the other *B. cereus* group species will provide insight into the different gene expression patterns of these closely related bacteria.

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