JOURNAL OF BACTERIOLOGY, Nov. 2009, p. 6683–6693 Vol. 191, No. 21
0021-9193/09/$12.00 doi:10.1128/JB.00691-09

An Extracytoplasmic Function Sigma Factor Controls β-Lactamase Gene Expression in *Bacillus anthracis* and Other *Bacillus cereus* Group Species▽†

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Received 27 May 2009/Accepted 11 August 2009

The susceptibility of most *Bacillus anthracis* strains to β-lactam antibiotics is intriguing considering that the closely related species *Bacillus cereus* and *Bacillus thuringiensis* typically produce β-lactamases and the *B. anthracis* genome harbors two β-lactamase genes, *bla1* and *bla2*. We show that β-lactamase activity associated with *B. anthracis* is affected by two genes, *sigP* (BA2502) and *rsiP* (BA2503), predicted to encode an extracytoplasmic function sigma factor and an anti-sigma factor, respectively. Deletion of the *sigP-rsiP* locus abolished β-lactamase activity in a naturally occurring penicillin-resistant strain and had no effect on β-lactamase activity in a prototypical penicillin-susceptible strain. Complementation with *sigP* and *rsiP* from the penicillin-resistant strain, but not with *sigP* and *rsiP* from the penicillin-susceptible strain, conferred constitutive β-lactamase activity in both mutants. These results are attributed to a nucleotide deletion near the 5′ end of *rsiP* in the penicillin-resistant strain that is predicted to result in a nonfunctional protein. *B. cereus* and *B. thuringiensis sigP* and *rsiP* homologues are required for inducible penicillin resistance in these species. Expression of the *B. cereus* or *B. thuringiensis sigP* and *rsiP* genes in a *B. anthracis sigP-rsiP*-null mutant confers inducible production of β-lactamase activity, suggesting that while *B. anthracis* contains the genes necessary for sensing β-lactam antibiotics, the *B. anthracis sigP* and *rsiP* gene products are not sufficient for *bla* induction.

Bacillus anthracis, *Bacillus cereus*, and *Bacillus thuringiensis* are the best-studied members of the *B. cereus* group. Extensive genomic studies, including DNA-DNA hybridization, 16S and 23S rRNA sequence comparisons, multilocus sequence typing, multilocus enzyme electrophoresis, and amplified fragment length polymorphism analysis, have revealed a high degree of phylogenetic relatedness among these organisms, leading to the proposal that *B. anthracis*, *B. cereus*, and *B. thuringiensis* may be viewed as a single species (7, 8, 12, 23, 28, 46–48, 51, 56). Despite the remarkably high degree of DNA sequence similarity and synteny among these species, there are notable species-specific phenotypes, many of which are attributed to plasmid content. *B. anthracis*, the causative agent of anthrax, typically contains two virulence plasmids, pXO1 and pXO2, which harbor genes encoding toxins and capsule biosynthetic enzymes, respectively (57, 73). Large, transmissible plasmids associated with the insect pathogen *B. thuringiensis* usually contain genes encoding insect toxins, which are produced as large parasporal inclusions (40, 77). Strains of *B. cereus*, a ubiquitous soil bacterium and opportunistic pathogen, can harbor a variety of extrachromosomal elements, including plasmids with high levels of similarity to pXO1 (44, 52).

In addition to plasmid-associated traits, some species-specific phenotypes are due to differential expression of chromosomal genes. *B. cereus* and *B. thuringiensis* have certain phospolipase, hemolysin, protease, and β-lactamase activities that are generally not associated with *B. anthracis*. The pleiotropic transcriptional regulator PlcR activates expression of genes associated with some of these phenotypes in *B. cereus* and *B. thuringiensis*. The *B. anthracis plcR* gene contains a nonsense mutation that most likely results in a protein product that cannot activate expression of target genes, leading to dramatic reductions in lecithinase, protease, and hemolytic activities associated with this species (2, 37, 38, 60, 80).

Penicillin susceptibility is a characteristic of *B. anthracis* that is commonly used to distinguish this species from *B. cereus* and *B. thuringiensis*, which typically exhibit inducible penicillin resistance (15, 82). The genetic basis for β-lactam resistance in this group of organisms is unknown (16). Interestingly, all sequenced strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* contain β-lactamase genes. Previous work in our laboratory showed that the two β-lactamase genes, *bla1* and *bla2*, of a prototypical penicillin-susceptible strain of *B. anthracis* are transcriptionally silent, while the *bla* genes of a penicillin-resistant *B. anthracis* clinical isolate, strain 32, are expressed constitutively. Our studies revealed that differential expression of the *bla* genes is dependent on strain background and that *bla1* is the major contributor to the high-level resistance to ampicillin and piperacillin in strain 32 (25).

In work presented here, we show that β-lactamase activity in *B. anthracis* and its close relatives *B. cereus* and *B. thuringiensis* is associated with an extracytoplasmic function (ECF) sigma factor. The ECF sigma factors are a subfamily of alternative sigma factors found in a diverse array of bacteria. The products of ECF sigma factor-regulated genes are associated with a wide range of functions, including responses to heat, osmotic

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 28 August 2009.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
B. anthracis strains		
32	pXO1$^+$ pXO2$^+$, Ap^r	61
UT308	Derived from 32, pXO1$^-$ pXO2$^-$, Ap^r	This study
UT335	Derived from UT308, Δ*sigP*(r) *rsiP*(r), Ap^s	This study
9131	pXO1$^-$ pXO2$^-$, Ap^s	34
UT334	Derived from 9131, Δ*sigP*(s) *rsiP*(s), Ap^s	This study
B. cereus strains		
569	Ap^r	11
UTC4	Derived from 569, Δ$sigP_{Bc}$ $PrsiP_{Bc}$, Ap^s	This study
B. thuringiensis strains		
AW43	Ap^r	87
UTT4	Derived from AW43, Δ$sigP_{Bt}$ $rsiP_{Bt}$, Ap^s	This study
E. coli strains		
TG1	Cloning host	76
JM109	Cloning host	76
GM2163	*dam dcm*	74
Plasmids		
pGEM-T Easy	T/A cloning vector	Promega
pHT304	Low-copy-number vector	6
pUTE692	pHT304 carrying *sigP*(s) and *rsiP*(s) from 9131	This study
pUTE693	pHT304 carrying *sigP*(r) and *rsiP*(r) from UT308	This study
pUTE697	Derived from pUTE693, contains *sigP*(r) from UT308 and *rsiP*(s) from 9131	This study
pUTE698	Derived from pUTE692, contains *sigP*(s) from 9131 and *rsiP*(r) from UT308	This study
pUTE751	pHT304 carrying *gfpmut3a*	This study
pUTE705	pHT304 carrying *sigP*(r) from UT308 and *gfpmut3a* fused to first ATG of *rsiP*(r)	This study
pUTE707	pHT304 carrying *sigP*(r) from UT308 and *gfpmut3a* fused to second ATG of *rsiP*(r)	This study
pUTE708	pHT304 vector carrying *sigP*(s) from 9131 and *gfpmut3a* fused to second ATG of *rsiP*(s)	This study
pUTE728	pHT304 carrying $sigP_{Bc}$ and $rsiP_{Bc}$ from *B. cereus* 569	This study
pUTE729	pHT304 carrying $sigP_{Bt}$ and $rsiP_{Bt}$ from *B. thuringiensis* AW43	This study
pHT304-18Z	Low-copy-number vector containing promoterless *lacZ* gene	6
pUTE461	pHT304-18Z carrying *bla1*::*lacZ* transcriptional fusion	24
pUTE462	pHT304-18Z carrying *bla2*::*lacZ* transcriptional fusion	24
pUTE869	pHT304 containing *sigP*(s) from 9131 and $rsiP_{Bc}$ from *B. cereus* 569	This study
pUTE871	pHT304 containing *sigP*(r) from UT308 and and $rsiP_{Bc}$ from *B. cereus* 569	This study

[a] Ap^r, ampicillin resistant; Ap^s, ampicillin sensitive. Subscripts associated with genes indicate gene origin, as follows: r, penicillin-resistant *B. anthracis*; s, penicillin-susceptible *B. anthracis*. *Bc*, *B. cereus*; *Bt*, *B. thuringiensis*.

and oxidative stress, and synthesis of alginate or carotenoids. ECF sigma factors have also been shown to mediate resistance to antimicrobial compounds in *Bacillus subtilis* (4, 5, 13, 14, 17, 18, 20–22, 72, 81). We establish that an ECF sigma factor and its cognate anti-sigma factor are associated with inducible resistance to β-lactam antibiotics in *B. cereus* and *B. thuringiensis*, but the ECF sigma factor and anti-sigma factor genes are not sufficient for β-lactamase gene expression in *B. anthracis*. We also show that the constitutive β-lactamase activity of an unusual penicillin-resistant clinical isolate of *B. anthracis* is likely due to the production of a truncated anti-sigma factor which cannot sequester its cognate ECF sigma factor. Our data reveal yet another phenotype commonly used to differentiate *B. anthracis* from *B. cereus* and *B. thuringiensis* that can be attributed to differences in *trans*-acting factors produced by these species.

MATERIALS AND METHODS

Media and growth conditions. *B. anthracis*, *B. cereus*, and *B. thuringiensis* were cultured in LB medium (Difco, Detroit, MI) containing 0.5% glycerol at 30°C with shaking (200 rpm). Following overnight incubation, cultures were transferred to fresh LB medium containing 0.5% glycerol such that the starting optical density at 600 nm (OD_{600}) was 0.06 to 0.08. Cultures were incubated at 37°C with shaking, and samples were obtained at the late exponential growth phase (OD_{600}, 2.9 to 3.6). When appropriate, erythromycin (Fisher, Pittsburgh, PA) was used at a final concentration of 5 μg/ml.

To assess *bla1* induction, strains were inoculated into LB medium such that the starting OD_{600} was 0.1. Prior to incubation, cultures were divided into two equal portions. Water or ampicillin (0.1 μg/ml) was added. Following growth at 37°C for 2 h, samples of culture supernatants were assessed for *bla1* expression.

DNA isolation and manipulation. A Mo Bio genomic isolation kit (Mo Bio Laboratories, Solana Beach, CA) was used to obtain chromosomal DNA from *B. anthracis*, *B. cereus*, and *B. thuringiensis* according to the manufacturer's instructions. All PCRs were performed using the high-fidelity cloning enzymes Easy-A (Stratagene, La Jolla, CA) and Phusion (New England Biolabs, Ipswich, MA). The PCR products obtained using the Phusion enzyme were incubated for 30 min at 72°C in the presence of 1 U of *Taq* polymerase (New England Biolabs, Ipswich, MA) and 200 nM dATP (Invitrogen, Carlsbad, CA) before the clean-up step. The PCR products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA). Transformation of *Escherichia coli* and subsequent extraction of plasmid DNA were performed using standard procedures (9). Unmethylated plasmid DNA was electroporated into *Bacillus* species using a method described previously (58).

A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to create plasmids pUTE697 and pUTE698 (Table 1). Primer pairs CR95/CR97 and CR104/CR105 (see Table S1 in the supplemental material) annealed

to opposite strands of template vectors and created new plasmids that contained the desired point mutations as described previously (75).

Strain and plasmid construction. Strains and plasmids are listed in Table 1. Plasmids pXO1 and pXO2 were cured from *B. anthracis* 32 during growth in the presence of novobiocin, as described previously (41), to create strain UT308. The *sigP-rsiP* (BA2502-BA2503) gene pairs from the prototypical penicillin-susceptible strain *B. anthracis* 9131 [designated *sigP*(s) and *rsiP*(s)] and the penicillin-resistant strain UT308 [designated *sigP*(r) and *rsiP*(r)] and the homologues from *B. cereus* 569 and *B. thuringiensis* AW43 were replaced with an Ω*km* cassette to create strains UT334, UT335, UTC4, and UTT4, respectively, using a protocol described previously (29). DNA sequences upstream from the *sigP* genes from nucleotide (nt) −809 to nt 4 relative to the translational start were amplified using CR139 (*B. anthracis*) or CR141 (*B. cereus* and *B. thuringiensis*) in combination with primer sigR4 (see Table S1 in the supplemental material). DNA sequences from nt −615 to nt 928 relative to the translational stop of the *rsiP* genes were amplified using primer CR162 in combination with sigR8 (*B. anthracis*) or CR143 (*B. cereus* and *B. thuringiensis*) (see Table S1 in the supplemental material). PCR products were cloned in pUTE583 such that upstream and downstream regions flanked the *aph3A* gene encoding kanamycin resistance. The *aph3A* gene was derived from pUC4, which was kindly provided by Gary Dunny. Constructs were electroporated into *B. cereus* 569, *B. thuringiensis* AW43, and *B. anthracis* 9131 and UT308.

Plasmids pUTE728, pUTE729, pUTE692, and pUTE693 contain the *sigP-rsiP* gene pairs and promoter regions from *B. cereus* 569, *B. thuringiensis* AW43, *B. anthracis* 9131, and *B. anthracis* UT308, respectively. PCR products spanning the region from 249 nt upstream of the predicted translational start site of *sigP* to 97 nt downstream of the apparent *rsiP* translational stop codon were amplified using oligonucleotide primers CR103 and rsrA2 and template DNA extracted from *B. anthracis* 9131 and UT308 (accession number EU979636). Similar regions were amplified from *B. cereus* 569 DNA (accession number EU979634) and *B. thuringiensis* AW43 DNA (accession number EU979635) using primers CR153 and CR155. The PCR products were purified, ligated into pGEM-T Easy (Promega, Madison, WI), and cloned into *E. coli* TG1 or JM109. PstI/SphI fragments containing the gene pairs were subcloned into pHT304 (6).

The cloning strategy described above was used to create plasmids pUTE705, pUTE707, pUTE708, and pUTE751. Briefly, primers CR103 and CR134 and DNA from UT308 were used to amplify DNA containing *sigP*(r), the first 9 nt of *rsiP*(r), and the upstream promoter region from UT308 DNA. This fragment, digested with PstI and SphI, was simultaneously ligated into pHT304 with an SphI/HindIII fragment containing the *gfpmut3a* gene to create plasmid pUTE705 (84). Similarly, primer CR103 was used in combination with CR106 to create a PCR fragment containing *sigP* and 53 nt of *rsiP* amplified from UT308 and 9131 DNA to create plasmids pUTE707 and pUTE708, respectively. Finally, plasmid pUTE651 was created by introducing the same *gfpmut3a* gene, amplified from plasmid pAD123 (33) using primers CR126 and CR127, into pHT304 previously digested with SphI and HindIII.

Plasmids pUTE864 [*sigP*(s) $rsiP_{Bc}$] and pUTE866 [*sigP*(r) $rsiP_{Bc}$] were created using overlap extension PCR. Briefly, DNA corresponding to the *sigP* genes in *B. anthracis* 9131 and UT308 were amplified using primers CR103 and CR176 (see Table S1 in the supplemental material), while DNA corresponding to the *rsiP* gene of *B. cereus* was amplified using primers CR175 and CR155 (see Table S1 in the supplemental material). PCR products corresponding to *sigP* from either *B. anthracis* strain were mixed with the PCR product corresponding to the *B. cereus rsiP* gene without additional primers in a PCR for two cycles, during which the homologous regions of primers CR176 and CR175 were allowed to anneal. Finally, primers CR103 and CR155 were added to the PCR mixture for an additional 25 cycles to create PCR products containing *sigP* from the *B. anthracis* penicillin-susceptible or -resistant strain in combination with the *rsiP* gene from *B. cereus* 569. These PCR products were cloned into pHT304 using the procedure described above.

The DNA associated with recombinant loci in mutant isolates and all cloned DNA were sequenced to confirm the fidelity of the constructs.

5′ end mapping. RNA was extracted from UT308 (Ap^r) cells grown to the late exponential phase as described previously (43). The 5′ ends of the *bla1* and *sigP* transcripts were determined using the 5′ rapid amplification of cDNA ends system (version 2.0; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, with the following modifications. The *bla1* and *sigP* primers (CR129 and CR132, respectively [see Table S1 in the supplemental material]) were incubated at 70°C for 10 min and then hybridized to 1 to 2 μg of RNA overnight at 30°C in S1 hybridization buffer. Following ethanol precipitation of the RNA-primer complex, the Invitrogen protocol was followed.

β-Lactamase assays. Supernatants from cultures in the late exponential growth phase were tested for β-lactamase activity using the chromogenic substrate nitrocefin (Clabiochem, San Diego, CA, and BD, San Jose, CA). Supernatant samples were diluted 10-fold. Ten microliters of diluted supernatant was added to a 100 mM nitrocefin solution according to the manufacturer's instructions. Following incubation for 30 min at 37°C, the absorbance at 486 nm was assessed using a Thermo Electron Multiskan Spectrum and the SkanIt 2.2 software. The β-lactamase activity of the parent strain, UT308, was defined as 100 arbitrary units. Samples from three independent cultures were tested.

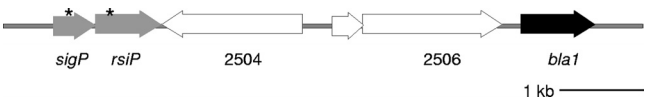

FIG. 1. Schematic representation of the *bla1* locus. The *sigP* (BA2502) and *rsiP* (BA2503) genes are located approximately 5 kb upstream of the *bla1* gene. Asterisks indicate the locations of the sequence differences between the *sigP-rsiP* gene pairs from the penicillin-resistant strain *B. anthracis* 32 and the prototypical penicillin-susceptible strain 9131.

Fluorescence microscopy. Samples from cultures in the late exponential growth phase were placed on a glass slide, covered with a coverslip, and visualized using a fluorescein isothiocyanate filter with a Nikon Eclipse E600 microscope.

β-Galactosidase assays. Samples were collected from *B. cereus* and *B. thuringiensis* cultures during late exponential growth. The 1-ml samples were pelleted, resuspended in 1 ml of Z-buffer, and homogenized using a Mini-bead Beater 8 (Biospec Products, Bartlesville, OK). The beads were removed by centrifugation (15,000 × g for 3 min), and an aliquot of the remaining sample was assayed for β-galactosidase activity as described by Miller (67). Three independent cultures were assessed for β-galactosidase activity. For *B. anthracis*, cultures were grown in the presence or absence of ampicillin and 1-ml samples were collected after 4 h of growth at 37°C. β-Galactosidase activity was assessed using the procedure described above.

Determination of MICs of ampicillin. *B. anthracis*, *B. cereus*, and *B. thuringiensis* spores were streaked onto Mueller-Hinton (Fluka-Biochemica, Buchs, Switzerland) agar plates and incubated overnight at 37°C. Colonies were used to inoculate phosphate-buffered saline to obtain turbidity equivalent to 0.5 McFarland standard. Per the instructions of the Etest strip manufacturer (AB-Biodisk, Piscataway, NJ), cell suspensions were applied to 1-day-old 90-mm plates containing Mueller-Hinton agar at a depth of 4.0 ± 0.5 mm and erythromycin at a final concentration of 5 μg/ml. Sterile swabs were used to inoculate the plates for confluent growth, and Etest strips were placed individually onto the freshly swabbed plates. The MICs were determined after incubation at 37°C for 20 h.

RESULTS

***bla* gene expression by a penicillin-resistant *B. anthracis* strain is associated with an ECF sigma factor.** We reported previously that prototypical penicillin-susceptible *B. anthracis* strains harbor two β-lactamase genes, *bla1* and *bla2*, which are transcriptionally silent even in the presence of subinhibitory levels of β-lactam antibiotics. We also demonstrated that the β-lactamase activity produced by the atypical clinical isolate *B. anthracis* strain 32 is due to high-level, constitutive expression of *bla1* and *bla2*. Although sequence differences immediately upstream of both *bla* genes are apparent, they are not responsible for the differences in *bla* gene expression in the penicillin-susceptible and -resistant strains (25).

In an effort to identify *trans*-acting regulators of the *bla* genes, the predicted functions of neighboring open reading frame (ORFs) were investigated. Located approximately 5 kb upstream from the *bla1* gene are two overlapping ORFs predicted to encode an ECF sigma factor (BA2502) and an anti-sigma factor (BA2503) (Fig. 1). As is true for other ECF sigma factors, the predicted amino acid sequence of BA2502 contains well-conserved domains, region 2 and region 4, that interact with the β′ and β subunits of RNA polymerase and recognize

TABLE 2. Expression of the *B. anthracis bla1* and *bla2* promoters in the penicillin-resistant strain UT308 and the isogenic *sigP-rsiP*-null strain UT335[a]

Strain	Expression (Miller units)		
	bla1::*lacZ* (pUTE461)	*bla2*::*lacZ* (pUTE462)	*lacZ* (pHT304-18Z)
UT308	510.36 ± 116.84	728.16 ± 36.37	1.64 ± 0.46
UT335	1.19 ± 0.19	1.68 ± 0.58	1.87 ± 0.37

[a] The plasmids contained *bla1* and *bla2* promoters fused to a promoterless *lacZ* gene as indicated. The values are means ± standard deviations.

the −10 and −35 promoter elements, respectively (19, 71, 85, 89). In addition, the apparent BA2502 amino acid sequence lacks region 3, a domain that is present in other subclasses of sigma factors but is not present in ECF sigma factors (62). The location of the BA2503 ORF and its predicted cotranscription and cotranslation with BA2502 make it an ideal candidate for an anti-sigma factor-encoding gene. Furthermore, the predicted amino acid sequence appears to contain cytoplasmic, membrane-spanning, and extracytoplasmic domains, consistent with the sequences of other anti-sigma factors (31, 69, 70).

To determine if the BA2502-BA2503 locus affects *bla* gene expression, plasmids harboring *bla1*- and *bla2-lacZ* transcriptional fusions were introduced individually into UT308, a pXO1$^-$ pXO2$^-$ derivative of the penicillin-resistant strain *B. anthracis* 32, and UT335, an isogenic mutant lacking both ORFs. Only the UT308 strains containing the *bla1* or *bla2* transcriptional fusions exhibited β-galactosidase activity, demonstrating that the locus is required for *bla* gene expression in the penicillin-resistant strain background (Table 2). We also determined that deletion of the two ORFs abolished β-lactamase activity (Fig. 2) and resistance to the β-lactam antibiotic ampicillin (Table 3).

To establish the effects of the BA2502-BA2503 locus on *bla* gene expression in a prototypical *B. anthracis* strain, the tandem ORFs were deleted from *B. anthracis* 9131 (34), a prototypical penicillin-susceptible strain lacking pXO1 and pXO2, to create UT334. We compared the β-lactamase activities of equal volumes of culture supernatants from the parent strains 9131 (Pen^s) and UT308 (Pen^r) and the BA2502-BA2503 deletion mutants UT334 and UT335, respectively, using the chromogenic substrate nitrocefin. As indicated above, deletion of the gene pair from the penicillin-resistant strain abolished β-lactamase activity (Fig. 2), whereas the same mutation in the penicillin-susceptible strain did not alter β-lactamase activity; β-lactamase activity remained undetectable in culture supernatants. Furthermore, complementation of UT334 and UT335 with pUTE693, a low-copy-number vector containing BA2502 and BA2503 from penicillin-resistant strain UT308, conferred β-lactamase activity similar to that of UT308 in both recombinant strains. Complementation of UT334 and UT335 with pUTE692, which contains the corresponding ORFs from penicillin-susceptible strain 9131, did not result in enzyme activity (Fig. 2). These data indicate that the products of BA2502 and/or BA2503 in the penicillin-susceptible and penicillin-resistant strains 9131 and UT308 are functionally different.

Considering the availability of sigma factor gene (*sig*) designations in *Bacillus* gene nomenclature and our data indicating that the presence of BA2502 and BA2503 affects resistance to the β-lactam antibiotic penicillin, we designated the two genes *sigP* and *rsiP* (*r*epressor of *sigP*), respectively.

Conserved sequences 5′ of the *bla* and *sigP* transcriptional start sites. Genes transcribed by specific sigma factors have conserved DNA recognition sequences 5′ of their transcription start sites. Since ECF sigma factor genes are typically autoregulated, the specific recognition sequences are also in the promoter regions of the ECF sigma factor genes (49). We compared the DNA sequences 5′ of the *bla1*, *bla2*, and *sigP* coding regions and discovered two common sequences, as shown in Fig. 3. A conserved 10-bp sequence, 5′-ATGGAAC AAA-3′, includes an upstream G region and an AAC motif, features typically found in the −35 binding elements recognized by ECF sigma factors (59). A common 7-bp consensus sequence, 5′-TTGTCTA-3′, is located 13 bp from the putative −35 binding element, consistent with a potential −10 element.

We attempted to map the 5′ ends of *bla1*, *bla2*, and *sigP* gene transcripts using RNA from the β-lactamase-positive penicillin-resistant strain *B. anthracis* 32. We detected transcripts with 5′ ends mapping 8 to 9 nt from the putative −10 element of the *sigP* promoter and 8 and 15 nt from the putative −10 element of the *bla1* promoter (Fig. 3 and data not shown). Despite repeated attempts, we were unable to determine the 5′ end(s) of *bla2* transcripts. Nevertheless, the locations of the putative transcriptional start sites relative to the consensus sequences indicate that the conserved DNA sequences are likely sigma factor-specific −35 and −10 binding elements.

The penicillin-resistant *B. anthracis* strain produces a truncated anti-sigma factor. To further investigate the different β-lactamase phenotypes conferred by the *sigP* and *rsiP* genes of the penicillin-susceptible and penicillin-resistant strains, we compared the nucleic acid sequences of the gene pairs and the amino acid sequences of the predicted gene products. DNA sequencing results and subsequent alignments of the genes

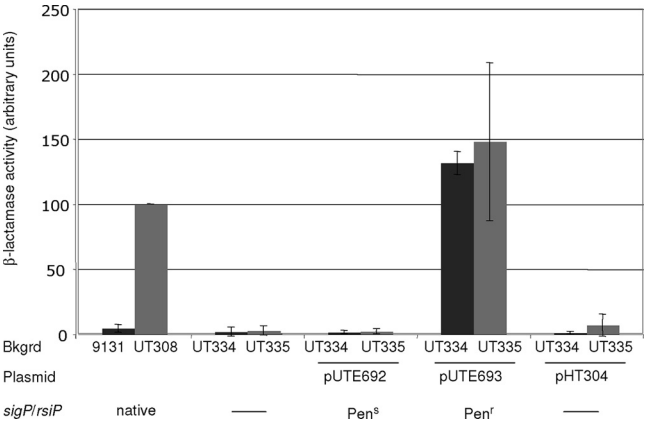

FIG. 2. β-Lactamase production by *B. anthracis* strains in the presence and absence of *sigP-rsiP* gene pairs. β-Lactamase activities were determined using culture supernatants from parent strain 9131 (Pen^s) and the corresponding Δ*sigP* Δ*rsiP* mutant UT334, parent strain UT308 (Pen^r) and the corresponding Δ*sigP* Δ*rsiP* mutant UT335, and Δ*sigP* Δ*rsiP* mutants complemented with pUTE692 (*sigP* and *rsiP* from the Pen^s strain), pUTE693 (*sigP* and *rsiP* from the Pen^r strain), and pHT304 (empty vector). Black bars indicate a Pen^s strain (9131) background, and gray bars indicate a Pen^r strain (UT308) background. The error bars represent standard deviations. Bkgrd, background.

TABLE 3. MICs of ampicillin for *B. anthracis*, *B. cereus*, and *B. thuringiensis* parent and *sigP-rsiP*-null strains

Strain	MICs of ampicillin (μg/ml) with the following sources of *sigP* and *rsiP*:				
	Vector only (pHT304)	*B. anthracis* 9131(pUTE692)	*B. anthracis* UT308(pUTE693)	*B. cereus* 569(pUTE728)	*B. thuringiensis* AW43(pUTE729)
B. anthracis 9131 (parent)	0.032–0.047				
B. anthracis UT334 (Δ*sigP* Δ*rsiP*)	0.023–0.047	0.032–0.047	192–256	1.5–6	1–3
B. anthracis UT308 (parent)	192–>256				
B. anthracis UT335 (Δ*sigP* Δ*rsiP*)	0.032	0.032–0.047	128–>256	0.023–0.047	0.023–0.047
B. cereus 569 (parent)	32–48				
B. cereus UTC4 (Δ*sigP* Δ*rsiP*)	0.047–0.064	0.047–0.064	64–128	48–96	16
B. thuringiensis AW43 (parent)	32–96				
B. thuringiensis UTT4 (Δ*sigP* Δ*rsiP*)	0.047–0.064	0.25–2	64–128	32–48	3–8

from the penicillin-susceptible and penicillin-resistant strains revealed two nucleotide sequence differences (Fig. 1; see Fig. S1 in the supplemental material). One difference occurs within *sigP* and is predicted to result in a single amino acid difference; amino acid 24 of σ^P from the penicillin-susceptible strain is aspartic acid, while amino acid 24 of σ^P from the penicillin-resistant strain is glycine (see Fig. S2 in the supplemental material). This amino acid difference could affect protein activity since it occurs within region 2, a conserved domain in all sigma factors that contains residues important for interactions with the β′ subunit of polymerase, as well as the −10 promoter element (42, 53, 54, 71, 79, 85, 89). For clarity, we designated the *sigP* gene from the penicillin-susceptible strain "*sigP*(s)" and the *sigP* gene from the penicillin-resistant strain "*sigP*(r)."

The other nucleic acid sequence difference is located 17 nt downstream from a predicted translational start codon of *rsiP* (Fig. 1; see Fig. S1 in the supplemental material). In the penicillin-resistant strain, a nucleotide deletion results in a frameshift mutation predicted to result in a truncated, 12-amino-acid protein (see Fig. S3 in the supplemental material). Only the first 5 amino acids of the 12-amino-acid protein would resemble the protein produced by the prototypical penicillin-susceptible strain initiating translation from the same position. However, in sequences from these strains we also noted a methionine codon located 51 and 50 nt downstream from the first predicted translational start codon in the penicillin-susceptible and penicillin-resistant strains, respectively. The presence of a ribosome binding site upstream from either ATG codon was not apparent. In both strains, translation initiation from the second ATG, which is in frame with the first ATG, would result in an amino-terminally truncated protein lacking the first 17 amino acids. Typically, the amino-terminal domains of this class of anti-sigma factors reside in the cytoplasm, where they bind and sequester the cognate ECF sigma factor under normal growth conditions (31, 69). Moreover, expression of the cytoplasmic N terminus alone can prevent the ECF sigma factor from interacting with polymerase. In the case of σ^E and RseA, an ECF sigma factor and anti-sigma factor pair found in *E. coli*, only the first 97 amino acids of the anti-sigma factor are required to inhibit the interaction of the sigma factor and RNA polymerase (69).

To determine the translation initiation codon for *rsiP*, we created translational fusions in which the green fluorescent protein (GFP) gene *gfpmut3* lacking its native start codon was cloned downstream of the first ATG (pUTE705) and downstream of the second ATG using sequences from the penicillin-susceptible strain (pUTE708) and from the penicillin-resistant strain (pUTE707). Since each translational fusion construct contains the *sigP* coding region, GFP synthesis was assessed in the *sigP-rsiP*-null strains UT334 (derived from the Pen^s parent) and UT335 (derived from the Pen^r parent), using fluorescence microscopy and Western hybridization. In both mutants, the presence of pUTE705 or pUTE708 resulted in GFP production, while the presence of pUTE707 did not appear to result in synthesis of GFP (Fig. 4). A translational fusion similar to the one in pUTE705 containing *sigP* from the penicillin-sus-

```
                    -35                 -10
bla1  TTTTTAAGTTTATGGAACAAATTTGTTTCGGTGATTGTCTATTATGTGTACGTATAAAAA
bla2  AGCAAGTAAAGATGGAACAAACATTTGAGCTGAATTGTCTAATATAGGTAATAAATATTT
sigP  ACTCGAACTAAATGGAACAAATGAAACTTGTATCTTGTCTATATTGAACCATAATAA
                  ATGGAACAAA          TTGTCTA
```

FIG. 3. Conserved sequences in the *bla1*, *bla2*, and *sigP* promoter regions. The putative −35 and −10 sequences are indicated by boldface type. The conserved G residue and AAC motif of the putative −35 region are underlined in the consensus sequence. Putative transcriptional start sites of *bla1* and *sigP*, as determined from the results of 5′-end mapping, are indicated by boldface type and underlining.

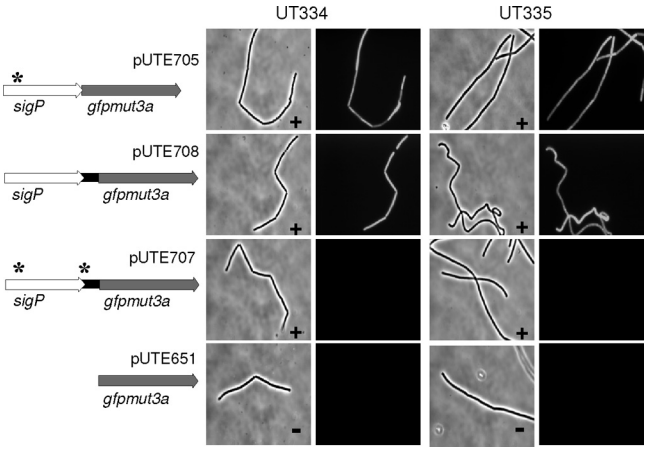

FIG. 4. Fluorescence microscopy of strains UT334 (Δ*sigP* Δ*rsiP*) and UT335 (Δ*sigP* Δ*rsiP*) harboring GFP translational fusions, observed using light and a fluorescein isothiocyanate filter. The fusions contained *sigP* genes from a Pen^s or Pen^r (asterisk) background and the 5′ end of the *rsiP* gene from Pen^s and Pen^r (asterisk) backgrounds. Plus and minus signs indicate that the strains are β-lactamase positive and β-lactamase negative, respectively.

TABLE 4. β-Lactamase activity of UT334 (*sigP-rsp*-null strain) containing *sigP* and *rsiP* alleles[a]

Background	Plasmid	Cloned *sigP* allele	Cloned *rsiP* allele	β-Lactamase activity
UT308 (Pen^r)	pHT304			+
9131 (Pen^s)	pHT304			−
UT334	pUTE692	*sigP*(s)	*rsiP*(s)	−
UT334	pUTE693	*sigP*(r)	*rsiP*(r)	+
UT334	pUTE697	*sigP*(r)	*rsiP*(s)	−
UT334	pUTE698	*sigP*(s)	*rsiP*(r)	+

[a] The sequences of the cloned *sigP* and *rsiP* alleles are identical to the sequences in the penicillin-resistant [*sigP*(r) or *rsiP*(r)] or penicillin-susceptible [*sigP*(s) or *rsiP*(s)] strains. β-Lactamase activity in culture supernatants is positive or negative.

ceptible strain instead of the resistant strain was also created, but we were unable to introduce it into the UT335 strain. Polyclonal anti-GFP serum reacted with cell extracts from strains that exhibited fluorescence, but it did not react with extracts from nonfluorescent cells, even when increasing amounts of protein extract were used (data not shown). These results indicate that translation initiation occurs at the first ATG in both strain backgrounds and predict that a full-length and functional anti-sigma factor is present in the prototypical penicillin-susceptible *B. anthracis* strain and a 12-amino-acid peptide is present in the penicillin-resistant strain. For clarity, we designated the *rsiP* gene from the penicillin-susceptible strain "*rsiP*(s)" and the *rsiP* gene from the penicillin-resistant strain "*rsiP*(r)."

Finally, we used site-directed mutagenesis to create plasmids containing hybrid *sigP-rsiP* gene pairs. As indicated in Table 4, pUTE697 harbors sequences corresponding to *sigP*(r) and *rsiP*(s), while pUTE698 harbors *sigP*(s) and *rsiP*(r). The plasmids were introduced into UT334 [*sigP*(s)-*rsiP*(s)-null strain], and β-lactamase activity was measured. Several attempts were made to introduce these constructs into the UT335 strain, but a UT335 derivative containing *sigP* from the penicillin-susceptible strain could not be obtained. As expected, the recombinant strain containing *rsiP*(r) exhibited β-lactamase activity, while strains harboring *rsiP*(s) did not exhibit β-lactamase activity (Table 4). This result is consistent with the synthesis of a truncated nonfunctional anti-sigma factor by the penicillin-resistant strain.

***bla* gene induction and expression in *B. cereus* and *B. thuringiensis* require homologous *sigP* and *rsiP* loci.** The penicillin susceptibility of prototypical *B. anthracis* and constitutive β-lactamase synthesis by the unusual penicillin-resistant *B. anthracis* strain are interesting, considering that the closely related species *B. cereus* and *B. thuringiensis* commonly exhibit inducible β-lactamase activity. A protein BLAST search (BlastP) revealed that most sequenced *B. cereus* and *B. thuringiensis* strains contain a gene pair that is nearly identical in sequence and location to the *B. anthracis sigP* and *rsiP* genes. We cloned and sequenced the *sigP* and *rsiP* loci of the common laboratory strains *B. cereus* 569 and *B. thuringiensis* AW43 (see Fig. S2 and S3 in the supplemental material). To determine if these homologues affect β-lactamase expression, we replaced the *B. cereus* 569 and *B. thuringiensis* AW43 *sigP-rsiP* gene pairs with an *aph3* gene, encoding kanamycin resistance, to create strains UTC4 and UTT4, respectively. These mutants were unable to grow on media containing a β-lactam antibiotic, a phenotype that was complemented by introducing the native *B. cereus* 569 and *B. thuringiensis* AW43 *sigP* and *rsiP* genes in *trans* (Table 3).

To establish whether *B. anthracis bla* gene expression could be induced in these species, we introduced *B. anthracis bla1*- and *bla2-lacZ* transcriptional fusions into *B. cereus* 569, *B. thuringiensis* AW43, and the *sigP-rsiP*-null strains UTC4 and UTT4. The presence of the *B. anthracis bla1*- and *bla2-lacZ* transcriptional fusions in the parent strains, but not their presence in the mutant strains, resulted in significantly increased β-galactosidase activity following the addition of sublytic concentrations of a β-lactam antibiotic (Table 5), indicating that *sigP* and *rsiP* play roles in *bla* gene induction and expression in *B. cereus* and *B. thuringiensis*.

The *B. cereus* and *B. thuringiensis sigP* and *rsiP* homologues are sufficient for penicillin resistance in prototypical *B. anthracis*. Given that the *B. anthracis bla* genes could be induced in *B. cereus* and *B. thuringiensis* strains harboring *sigP* and *rsiP* homologues, we questioned the lack of β-lactamase induction in prototypical *B. anthracis* strains during growth in the presence of β-lactam antibiotics. We considered three possible reasons for the lack of induction: (i) *B. anthracis* cannot sense the presence of a β-lactam antibiotic in the environment, (ii) *B. anthracis* lacks the ability to transduce the signal generated by the presence of a β-lactam antibiotic to the anti-sigma factor, and (iii) in *B. anthracis* the protein product of *rsiP* cannot respond to the upstream signal.

To investigate these hypotheses, we complemented *B. anthracis* UT334 [*sigP*(s)-*rsiP*(s)-null strain] with pUTE728 and pUTE729, plasmids that contained *sigP* and *rsiP* from *B. cereus* and *B. thuringiensis*, respectively. The recombinant strains ex-

TABLE 5. Expression of the *B. anthracis bla1* and *bla2* promoters in *B. cereus* and *B. thuringiensis* grown in the presence and absence of ampicillin (1 μg/ml)[a]

Organism	Expression (Miller units)					
	bla1::*lacZ* (pUTE461)		*bla2*::*lacZ* (pUTE462)		*lacZ* (pHT304-18Z)	
	Without ampicillin	With ampicillin	Without ampicillin	With ampicillin	Without ampicillin	With ampicillin
B. cereus	0.43 ± 0.61	46.03 ± 18.42	0.30 ± 0.12	134.19 ± 53.92	0.11 ± 0.15	0.12 ± 0.04
UTC4	0.10 ± 0.07	0.08 ± 0.07	0.02 ± 0.03	0.18 ± 0.17	0.03 ± 0.04	0.18 ± 0.21
B. thuringiensis	0.34 ± 0.12	13.70 ± 7.46	0.75 ± 0.04	60.43 ± 17.27	0.58 ± 0.10	0.60 ± 0.16
UTT4	0.26 ± 0.02	0.28 ± 0.09	0.35 ± 0.11	0.39 ± 0.19	0.50 ± 0.09	0.53 ± 0.29

[a] The plasmids contained *bla1* and *bla2* promoters fused to a promoterless *lacZ* gene as indicated. The values are means ± standard deviations.

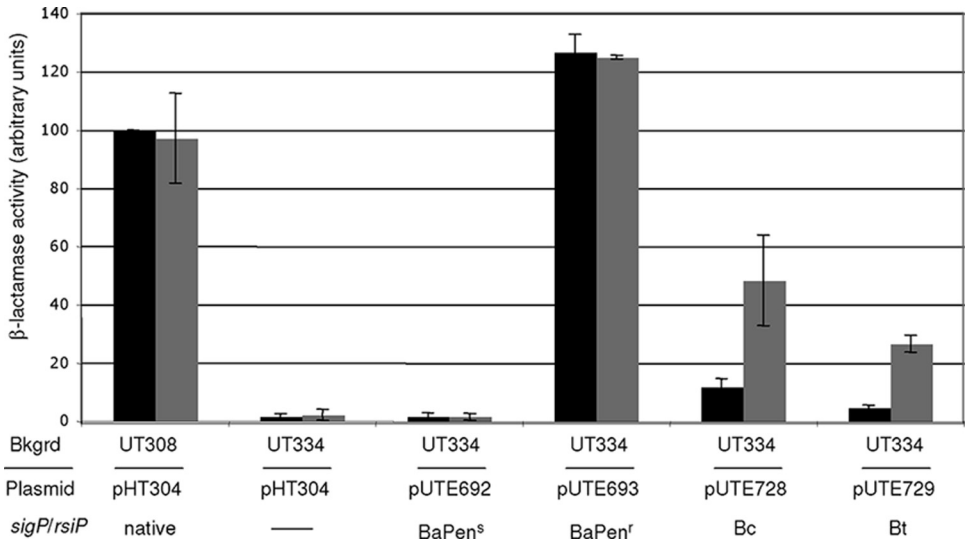

FIG. 5. β-Lactamase activities of *B. anthracis* strains containing the *sigP-rsiP* gene pairs from *B. anthracis*, *B. cereus*, and *B. thuringiensis* grown in the presence and absence of ampicillin. The β-lactamase activities of the *sigP-rsiP*-null strain UT334 complemented in *trans* with the *sigP-rsiP* gene pairs from penicillin-susceptible *B. anthracis* (pUTE692), penicillin-resistant *B. anthracis* (pUTE693), *B. cereus* (pUTE728), and *B. thuringiensis* (pUTE729) and with the empty vector (pHT304) were compared to the β-lactamase activity of the parent penicillin-resistant strain, UT308. Supernatants from the indicated strains were sampled 4 h after induction with 0.1 μg ampicillin/ml (gray bars) or water (black bars). The error bars represent standard deviations. Bkgrd, background; Ba, *B. anthracis*; Bc, *B. cereus*; Bt, *B. thuringiensis*.

hibited increased MICs of ampicillin compared to the penicillin-susceptible parent strain 9131 and UT334 containing only the vector (Table 3). Interestingly, introduction of the *B. cereus* and *B. thuringiensis* genes into *B. anthracis* UT335 [*sigP*(r)-*rsiP*(r)-null strain] did not result in increased resistance to ampicillin, illustrating further differences between the two *B. anthracis* strains. To determine if the increased β-lactam resistance of the *B. anthracis sigP-rsiP*-null strains containing the *B. cereus* and *B. thuringiensis sigP* and *rsiP* genes was a result of *bla* induction, we compared the β-lactamase activities of recombinant strains grown in the presence and absence of ampicillin. The β-lactamase activities of culture supernatants from *B. anthracis sigP-rsiP*-null strain UT334 containing the *B. cereus* and *B. thuringiensis sigP* and *rsiP* genes were elevated following the addition of ampicillin (Fig. 5). As expected, the β-lactamase activity of UT334 complemented with the *B. anthracis sigP* and *rsiP* genes from the resistant or susceptible strains was not affected by the addition of a β-lactam antibiotic. These results indicate that the increased MICs of ampicillin for the *B. anthracis sigP-rsiP*-null strain (UT334) complemented with the *B. cereus* and *B. thuringiensis sigP* and *rsiP* genes results from *bla* induction.

We also compared the ampicillin resistance of the *sigP-rsiP*-null mutants *B. cereus* UTC4 and *B. thuringiensis* UTT4 complemented with *sigP-rsiP* gene pairs from the three species (Table 3). We found that the gene pairs from *B. cereus* and *B. thuringiensis* could restore some level of resistance to ampicillin in both species backgrounds. However, pUTE692 [harboring *B. anthracis sigP*(s) and *rsiP*(s)] was able to increase resistance to ampicillin only in the UTT4 background. Taken together, these data indicate that protypical Pen^s *B. anthracis* strains like 9131 contain the proteins necessary to sense the presence of a β-lactam antibiotic and transduce the signal to the anti-sigma factor, as evidenced by the increased resistance and β-lactamase activity of *B. anthracis* UT334 (*sigP-rsiP*-null strain) harboring the *B. cereus* or *B. thuringiensis sigP* and *rsiP* homologues. However, the product of the *rsiP*(s) gene from *B. anthracis*, when produced in *B. anthracis* 9131 or *B. cereus* 569, does not respond to an upstream signal, resulting in continued sequestration of the ECF sigma factor.

Functional differences in the *B. anthracis sigP*(s) and *sigP*(r) alleles. We were unable to introduce the *sigP*(s) gene alone (in the absence of an *rsiP* allele) into UT335 [*sigP*(r)-*rsiP*(r)-null strain]. Moreover, the presence of *sigP*(s) (without *rsiP*) in UT334 [*sigP*(s)-*rsiP*(s)-null strain] yielded a recombinant strain that exhibited a growth defect (data not shown). Notably, the *sigP*(r) gene was readily electroporated into either strain background, and the recombinant strains exhibited growth rates similar to those of the parent strains. To circumvent the problems associated with introducing *sigP*(s) into *B. anthracis* without its cognate negative regulator, we created hybrid gene pairs consisting of the *B. anthracis sigP*(s) or *sigP*(r) gene fused to the *B. cereus rsiP* gene ($rsiP_{Bc}$) and introduced them into the *B. cereus* and *B. anthracis sigP-rsiP*-null strains UTC4 and UT334, respectively. The recombinant *B. anthracis* and *B. cereus* strains containing pUTE869 [*sigP*(s) $rsiP_{Bc}$] exhibited MICs of ampicillin that were higher than those exhibited by strains containing pUTE871 [*sigP*(r) $rsiP_{Bc}$] (Table 6). These results strongly

TABLE 6. MICs of ampicillin for *B. anthracis* and *B. cereus* *sigP-rsiP*-null strains containing hybrid *sigP-rsiP* gene pairs

Strain	MICs of ampicillin (μg/ml)		
	sigP(s) $rsiP_{Bc}$ (pUTE869)	*sigP*(r) $rsiP_{Bc}$ (pUTE871)	Empty vector (pHT304)
B. anthracis UT334	2–12	0.047–1	0.023–0.047
B. cereus UTC4	16–24	2–6	0.047–0.064

suggest that the σ^P produced by the penicillin-susceptible strain exhibits greater activity than the σ^P produced by the penicillin-resistant strain, a difference most likely attributed to the amino acid change (D24G) in region 2.

DISCUSSION

The susceptibility of *B. anthracis* to β-lactam antibiotics is well established, and as a result, penicillin has historically been the drug of choice for anthrax treatment worldwide (83; M. N. Jones, R. J. Beedham, P. C. B. Turnbull, and R. J. Manchee, presented at the International Workshop on Anthrax, Winchester, England, 1995). However, treatment of anthrax with penicillin is not always successful, and the use of penicillin for prophylaxis and treatment of anthrax in experimental animals has produced various outcomes (36, 39, 65; A. M. Friedlander, presented at the Third International Conference on Anthrax, Plymouth, England, 1998). *B. cereus* and *B. thuringiensis*, two *Bacillus* species that are closely related to *B. anthracis*, commonly exhibit inducible penicillin resistance, and some strains of *B. cereus* and *B. thuringiensis* have been associated with human disease. *B. thuringiensis* has been implicated in burn wound infections (30). Reports of *B. cereus* infections have included reports of necrotic enteritis, liver failure, endocarditis, meningitis, bacteremia, and lethal pneumonia (10, 26, 27, 50, 52, 63, 64, 68). Investigations of the genetic basis for differential expression of the β-lactamase genes in these species should contribute to the taxonomy of these bacteria.

In this work we demonstrated that constitutive expression of the *bla* genes in a naturally penicillin-resistant clinical isolate of *B. anthracis* is associated with the ECF sigma factor–anti-sigma factor gene pair, *sigP-rsiP*. In our model, a nucleotide deletion in the 5′ end of the *rsiP* gene most likely results in a nonfunctional, 12-amino-acid peptide that is unable to sequester its cognate ECF sigma factor, σ^P. As a result, σ^P is available to interact with RNA polymerase, promoting expression of *bla1* and *bla2*. In a prototypical *B. anthracis* strain, σ^P is sequestered by a full-length anti-sigma factor, RsiP, resulting in the familiar penicillin-susceptible phenotype. *bla* gene expression by the prototypical strain is not induced by the presence of a β-lactam antibiotic. Our data also suggest that the single predicted amino acid difference in region 2 of σ^P from the penicillin-resistant strain is associated with decreased σ^P activity since the MICs of ampicillin for *B. cereus* and *B. anthracis sigP-rsiP*-null strains containing *sigP*(s) in combination with *B. cereus rsiP* were higher than those of the same strains containing *sigP*(r) paired with the *B. cereus rsiP* gene.

Initially, we tried to compare the σ^P proteins produced by the penicillin-susceptible and -resistant strains by introducing the *sigP* genes into the *B. anthracis sigP-rsiP*-null mutants on a low-copy-number vector. However, we were unable to obtain a UT335 strain containing *sigP*(s), and a UT334 strain complemented with *sigP*(s) exhibited growth defects and almost no β-lactamase activity. Surprisingly, addition of the 5′ end of *rsiP*(s) to constructs containing *sigP*(s) resulted in strains with increased β-lactamase activity. For example, complementing UT334 [*sigP*(s)-*rsiP*(s)-null strain] with a construct containing *sigP*(s) and the 5′ end of *rsiP*, corresponding to the first 5 amino acids of RsiP(s), increased the β-lactamase activity from 0 to approximately 45 arbitrary units. If the 5′ end of *rsiP*(s) encoding the first 17 amino acids of RsiP(s) was included with *sigP*(s), the recombinant strain exhibited β-lactamase activity of approximately 100 arbitrary units, which is similar to the activity of the parent Pen^r strain UT308 (data not shown). Notably, a similar construct, pUTE708, was used to assess the translational start of RsiP (Fig. 4). Recombinant strain UT335(pUTE708) had significant growth defects (data not shown), suggesting that constitutive expression of σ^P from the penicillin-susceptible strain is detrimental to *B. anthracis*.

Given that all sequenced *B. anthracis* strains contain *bla1* and *bla2* genes and the *sigP-rsiP* gene pair, we wondered why β-lactamase activity is not induced when *B. anthracis* is grown in the presence of a β-lactam antibiotic. Using commonly used laboratory strains of *B. cereus* and *B. thuringiensis*, we demonstrated that the inducible β-lactamase expression exhibited by these species is dependent on the presence of their native *sigP* and *rsiP* genes. Furthermore, complementation of *B. anthracis* UT334 [*sigP*(s)-*rsiP*(s)-null strain] with the *B. cereus* and *B. thuringiensis sigP-rsiP* gene pairs resulted in MICs of ampicillin ranging from 1 to 6 μg/ml. This is significantly higher than the MICs of ampicilin for UT334 complemented with its native *sigP-rsiP* gene pair (0.032 to 0.047 μg/ml). In addition, these strains exhibited increased β-lactamase activity when they were grown in the presence of a β-lactam antibiotic. These results indicate that prototypical *B. anthracis* strains are able to sense the presence of a β-lactam antibiotic and transduce the signal to the *B. cereus* and *B. thuringiensis* anti-sigma factors, ultimately resulting in expression of the *bla* genes.

In well-studied systems of other bacteria, sequestration of ECF sigma factors is alleviated when the cognate anti-sigma factor is degraded through a process called regulated intramembrane proteolysis. In *E. coli*, the ECF sigma factor, σ^E, is regulated by the anti-sigma factor, RseA. Under normal growth conditions, RseA tethers σ^E to the cytoplasmic membrane. In response to unfolded proteins in the periplasmic space, DegS, a periplasmic serine protease, carries out site 1 cleavage of RseA. The cleaved RseA is subject to site 2 cleavage by the protease YaeL/RseP (1, 3, 55, 86, 88). The remaining amino-terminal portion of RseA is then subject to further degradation by ClpXP, ultimately resulting in free σ^E (35). The *B. subtilis* ECF sigma factor, σ^W, is regulated by the anti-sigma factor RsiW, which is also subject to site 1 and site 2 cleavage by PrsW and YluC (also referred to as RasP), respectively (32, 45, 78). After the remaining RsiW fragment is degraded by the cytoplasmic proteases ClpXP, σ^w associates with core RNA polymerase and directs transcription of target genes (90).

Based on our data, we considered the following models for regulation of *bla* gene expression in *B. cereus*, *B. thuringiensis*, and *B. anthracis*. We propose that prototypical *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains can sense the presence of a β-lactam antibiotic in the environment. The *B. cereus* and *B. thuringiensis* RsiP protein responds to this stress, and σ^P is released, resulting in transcription of the *bla* genes. However, in prototypical penicillin-susceptible *B. anthracis* strains, RsiP does not respond to the signal, possibly because the anti-sigma factor is not recognized and/or is degraded by proteases or because RsiP is defective in the ability to receive a signal from an upstream factor. In either case, σ^P remains sequestered, and the *bla* genes are not expressed.

Overall, our studies of SigP-RsiP function in the *B. cereus*

group support the emerging idea that phenotypic differences between *B. anthracis*, *B. cereus*, and *B. thuringiensis* are in large part due to altered gene expression rather than to the gain or loss of coding regions. The *sigP* and *rsiP* gene products can be added to the growing list of *trans*-acting factors that differentially affect transcription of genes common to the *B. cereus* group species. The best example of this is the PlcR regulon (2, 37, 60). In *B. cereus* and *B. thuringiensis*, PlcR controls expression of multiple genes, some of which are associated with pathogenesis. Although many of these genes are present in the *B. anthracis* genome, the *B. anthracis plcR* gene contains a nonsense mutation resulting in a nonfunctional protein product. Multiple phenotypic differences between *B. anthracis* and the other two species are attributed to low-level expression of the PlcR regulon in *B. anthracis*. Another *trans*-acting regulator, AtxA, is located on a *B. anthracis* plasmid and controls expression of *B. anthracis*-specific virulence genes and some chromosomal genes common to the *B. cereus* group species. *B. cereus* and *B. thuringiensis* strains do not carry the plasmid harboring *atxA* and therefore exhibit differential expression of the AtxA-controlled chromosomal genes. Interestingly, expression of a *B. thuringiensis plcR* gene in a *B. anthracis* strain containing *atxA* resulted in a significant decrease in sporulation, a phenotype that was rescued by deletion of *atxA* (66). These results suggest that the *plcR* and *atxA* regulons in *B. anthracis* are not compatible and that the nonsense mutation in the *B. anthracis plcR* gene provided a selective advantage.

What, if any, is the selective advantage of an apparently noninducible SigP-RsiP system in *B. anthracis*? We have not found culture conditions that allow induction of the *B. anthracis bla* genes, and our data suggest that constitutive expression of *sigP* is detrimental to *B. anthracis* growth. In the case of a penicillin-resistant strain, this toxicity appears to be alleviated by an amino acid change in σ^P, which reduces the activity of the sigma factor. It is possible that the *sigP-rsiP* system could be induced by a non-β-lactam signal in the host, adversely affecting growth. Regulatory relationships between σ^P and other genes involved in pathogenesis and development of *B. anthracis* are unknown. Future experiments will define the σ^P regulon.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI33537 from the National Institutes of Health and by the U.S. Army Medical Research and Materiel Command under award W81XWH-04-2-0035.

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

We are grateful to Yahua Chen for technical assistance and helpful discussions.

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