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RESEARCH ARTICLE
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Analysis of Whole-Genome Sequences for the Prediction of Penicillin Resistance and β-Lactamase Activity in *Bacillus anthracis*

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ABSTRACT Penicillin (PEN) is a low-cost option for anthrax treatment, but naturally occurring resistance has been reported. β-Lactamase expression (*bla1*, *bla2*) in *Bacillus anthracis* is regulated by a sigma factor (SigP) and its cognate anti-sigma factor (RsiP). Mutations leading to truncation of RsiP were previously described as a basis for PEN resistance. Here, we analyze whole-genome sequencing (WGS) data and compare the chromosomal *sigP-bla1* regions from 374 *B. anthracis* strains to determine the frequency of mutations, identify mutations associated with PEN resistance, and evaluate the usefulness of WGS for predicting PEN resistance. Few (3.5%) strains contained at least 1 of 11 different mutations in *sigP*, *rsiP*, or *bla1*. Nine of these mutations have not been previously associated with PEN resistance. Four strains showed PEN resistance (PEN-R) by conventional broth microdilution, including 1 strain with a novel frameshift in *rsiP*. One strain that carries the same *rsiP* frameshift mutation as that found previously in a PEN-R strain showed a PEN-susceptible (PEN-S) phenotype and exhibited decreased *bla1* and *bla2* transcription. An unexpectedly small colony size, a reduced growth rate, and undetectable β-lactamase activity levels (culture supernatant and cell lysate) were observed in this PEN-S strain. Sequence analysis revealed mutations in genes associated with growth defects that may contribute to this phenotype. While *B. anthracis rsiP* mutations cannot be exclusively used to predict resistance, four of the five strains with *rsiP* mutations were PEN-R. Therefore, the *B. anthracis sigP-bla1* region is a useful locus for WGS-based PEN resistance prediction, but phenotypic testing remains essential.

IMPORTANCE Determination of antimicrobial susceptibility of *B. anthracis* is essential for the appropriate distribution of antimicrobial agents for postexposure prophylaxis (PEP) and treatment of anthrax. Analysis of WGS data allows for the rapid detection of mutations in antimicrobial resistance (AMR) genes in an isolate, but the presence of a mutation in an AMR gene does not always accurately predict resistance. As mutations in the anti-sigma factor RsiP have been previously associated with high-level penicillin resistance in a limited number of strains, we investigated WGS assemblies from 374 strains to determine the frequency of mutations and performed functional antimicrobial susceptibility testing. Of the five strains that contained mutations in *rsiP*, only four were PEN-R by functional antimicrobial susceptibility testing. We conclude that while sequence analysis of this region is useful for AMR prediction in *B. anthracis*, genetic analysis should not be used exclusively and phenotypic susceptibility testing remains essential.

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Ciprofloxacin, doxycycline, and β-lactam antibiotics (including ampicillin, penicillin G, and penicillin VK) are recommended by the U.S. Centers for Disease Control and Prevention for postexposure prophylaxis (PEP) of inhalation anthrax in adults following exposure to *Bacillus anthracis* (1, 2). In developing countries where anthrax is endemic, penicillin is considered a drug of choice for treatment because it is effective, widely available, and low in cost (3). Penicillin susceptibility is a *B. anthracis* characteristic that is commonly used to differentiate it from *Bacillus cereus* and *Bacillus thuringiensis*, which display inducible β-lactam resistance (4). While most *B. anthracis* strains are susceptible to penicillin, surveys of clinical and environmental isolates indicate that penicillin resistance occurs in 2% to 16% of strains (5). Penicillin treatment failures have been reported for anthrax (6, 7), and use of this antibiotic for PEP in experimental animals had variable results (8, 9). Therefore, antimicrobial susceptibility testing (AST) is recommended prior to treatment with penicillin (1).

All *B. anthracis* strains analyzed to date have two chromosomal β-lactamase genes: *bla1* (which encodes a penicillinase) and *bla2* (which encodes a cephalosporinase) (5). When transferred to other organisms, such as *Escherichia coli*, both genes are complete and functional, but in *B. anthracis*, *bla1* and *bla2* are poorly transcribed and gene expression is not sufficient to confer resistance to β-lactam antibiotics (5, 10). Furthermore, induction of β-lactamase activity or penicillin resistance was not observed following growth in sublethal levels of β-lactam antibiotics (4, 10). β-Lactamase expression and penicillin resistance in *B. anthracis* were characterized in studies of penicillin-resistant (PEN-R) strain 32 (4, 5, 10, 11), which was originally isolated in 1974 from a fatal anthrax case in Northampton, England (12, 13). While the β-lactamase genes of a typical penicillin-susceptible (PEN-S) *B. anthracis* strain are transcriptionally silent, *bla1* and *bla2* are expressed constitutively in strain 32, and *bla1* was identified as the major contributor to PEN resistance (10). Another naturally occurring, PEN-R *B. anthracis* isolate, strain SK57, has been described previously (14) and was isolated in England in November 1975; however, few details about the strain's source are available. DNA sequence analysis and details of the β-lactamase expression of SK57 have also not been published previously. Whole-genome sequencing (WGS) data for strains 32 and SK57 are publicly available under GenBank accession numbers QPKO00000000 and QPKQ00000000, respectively (15).

In *B. anthracis* and other *B. cereus* group species, an extracytoplasmic function (ECF) sigma factor, SigP, and its cognate anti-sigma factor, RsiP, regulate *bla1* and *bla2* transcription (4). ECF sigma factors represent a diverse subfamily of alternative sigma factors that typically activate gene expression in response to extracellular signals, including agents that threaten cell envelope integrity (4, 16, 17). The mechanism of signal perception and the basis for anti-sigma factor inactivation are not well understood for the majority of ECF sigma factor/anti-sigma factor pairs (16), including SigP and RsiP. Although associated with inducible β-lactam resistance in *B. cereus* and *B. thuringiensis*, the presence of SigP and RsiP is not sufficient for β-lactamase gene expression in *B. anthracis* (4). In PEN-R strain 32, a nucleotide deletion that results in a frameshift mutation and an amino-terminally truncated RsiP was described as the basis of high-level PEN resistance (4). *B. anthracis* strain 32 also contains a single nucleotide polymorphism (SNP) in *sigP* (an A-to-G transition at position 183) that results in a single amino acid difference (an aspartic acid in the PEN-S reference strain and a glycine in strain 32 at position 24). This mutation occurs within a conserved sigma factor domain that is important for interactions with both the RNA polymerase and the −10 promoter element and is predicted to affect protein activity (4). Mutations in *rsiP* and *sigP* in several PEN-R strains isolated from cattle following a 2011 anthrax outbreak were also previously described (18). Transcriptome analysis of one of the PEN-R isolates from that study revealed that the frameshift mutation in *rsiP* led to upregulation of five genes,

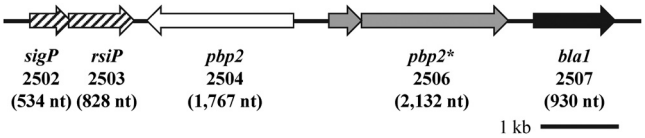

FIG 1 Diagram of the *B. anthracis sigP-bla1* region. The numbers refer to ORFs in the *B. anthracis* Ames Ancestor reference sequence. The asterisk (*) indicates that *pbp2* contains a frameshift that results in two predicted ORFs; the first contains 124 amino acids of the predicted PBP2, and the second contains the remaining 586 amino acids of the predicted PBP2. (Adapted from reference 4 with permission).

rsiP, *sigP*, *bla1*, *bla2*, and a predicted penicillin-binding protein (PBP) transpeptidase gene that is located immediately upstream of *bla1* (18).

Detection of phenotypic penicillin resistance by AST is essential for the distribution of appropriate antimicrobial agents for PEP and treatment during a public health emergency involving anthrax. Conventional broth microdilution (BMD) is considered the gold standard laboratory method for AST and requires a 16- to 20-h incubation period for *B. anthracis* based on Clinical and Laboratory Standards Institute (CLSI) guidelines (19). Several functional phenotypic methods have been developed to reduce the time required for antimicrobial susceptibility profiling of *B. anthracis*, including real-time PCR to detect growth in the presence of antimicrobial agents (20), bioluminescent reporter phage analysis (21), laser light scattering technology (22), and optical screening (23). The analysis of WGS data from a suspect isolate can complement these phenotypic AST methods, as they can detect the introduction of mutations, genes, and/or plasmids associated with antimicrobial resistance and provide details on the mechanisms of resistance that are critical for an effective public health response. For penicillin resistance in *B. anthracis*, the presence of β-lactamase genes cannot predict whether an isolate would be penicillin resistant; however, the analysis of genes that regulate β-lactamase expression may serve as a more accurate predictor. Here, we analyze WGS data for genetic markers that predict penicillin resistance in *B. anthracis*. The chromosomal regions containing *sigP*, *rsiP*, and *bla1* (*sigP-bla1* region) were compared in a collection of *B. anthracis* strains at CDC in order to (i) determine the frequency of mutations (SNPs, insertions, or deletions) in this region; (ii) identify mutations associated with penicillin resistance; and (iii) evaluate the usefulness of WGS for predicting penicillin resistance.

Here, we show that the coding regions of *sigP*, *rsiP*, and *bla1* have low sequence variability among *B. anthracis* strains, with only 3.5% (13/374) of strains containing mutations compared to the Ames Ancestor reference genome. When a mutation was identified in the anti-sigma factor *rsiP*, penicillin resistance was detected in only four of the five strains. Therefore, analysis of the *sigP-bla1* region of *B. anthracis* has shown that it is a useful locus to analyze for prediction of penicillin resistance in *B. anthracis*. However, to accurately assess β-lactam resistance in *B. anthracis*, a conventional method such as BMD remains essential.

RESULTS

BLAST analysis of the *sigP-bla1* region in *B. anthracis* strains. The Ames Ancestor reference sequence contains five predicted open reading frames (ORF) in the *sigP-bla1* region (Fig. 1). Together with *sigP* (ORF 2502), *rsiP* (ORF 2503), and *bla1* (ORF 2507), there are two additional ORFs (2504 and 2506) predicted to encode PBPs within the approximately 5-kb region between *rsiP* and *bla1*. A local BLAST search was performed by querying the *sigP-bla1* region, 6,892 bp (Fig. 1), from the PEN-S Ames Ancestor reference against genomes from 374 *B. anthracis* strains in the CDC WGS database. Forty-two additional *B. anthracis* whole-genome sequences, for which there are no BMD data available, were in GenBank at the time of analysis and included in this screen (total, 416 strains). Over half of the strains, 235/416 (56%), were identical to the Ames Ancestor reference strain, and 185/416 (44%) of the strains had at least one mutation in the *sigP-bla1* region. These mutations were distributed among 42 positions. The

TABLE 1 *B. anthracis* strains identified from the WGS screen as containing mutations within *sigP*, *rsiP*, and *bla1*[a]

Strain	Gene	nt mutation position(s)	Confirmed by Sanger sequencing	Mutation type	Predicted effect	PEN MIC (μg/ml)	Mutation previously described (reference[s])
2002013094	*sigP*	119	Yes	SNP; transition	(G) ▶ (D)	≤0.015	No
2000031021	*sigP*	119	Yes	SNP; transition	(G) ▶ (D)	0.3	No
2000031052	*sigP*	119, 395	Yes	SNP; transitions	(G) ▶ (D), (L) ▶ (S)	0.3	No
2002734089	*bla1*	93	Yes	SNP; transition	Silent mutation (G) ▶ (G)	0.06	No
2000031048	*bla1*	−64†	Yes	SNP; transversion in −35 binding element	5′-ATGGAA**C**AAA-3′ ▶ 5′-ATGGAA**A**AAA-3′	0.3	No
2002013017	*rsiP*	60	No	SNP; transversion	(H) ▶ (Q)	≤0.015	No
2002013007	*rsiP*	827	No	Insertion of AAAAAG	Deletion of (K) + (R)	0.03	No
2002013011	*rsiP*	505	Yes	SNP; transition	(Y) ▶ (H)	0.03	No
2000031038	*rsiP*	39	Yes	SNP; deletion	FS; truncates RsiP to 30 aa	>512	No
2000032823	*rsiP*	471	Yes	SNP; insertion	FS; truncates RsiP to 163 aa	256	Yes (18)
2002734065	*rsiP*	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	>512	Yes (4)
2002734039	*rsiP*	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	>512	Yes (4)
2002013027	*rsiP*	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	0.06	Yes (4)
Pen-R strains not included in screen*							
32	*sigP*	183	Yes	SNP; transition	(D) ▶ (G)	>512	Yes (4, 15)
	rsiP	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	>512	Yes
UT308	*sigP*	183	Yes	SNP; transition	(D) ▶ (G)	>512	Yes (4, 15)
	rsiP	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	>512	Yes (4, 15)
SK57	*rsiP*	10	Yes	SNP; deletion	FS; truncates RsiP to 12 aa	>512	Yes

[a]Nucleotide (nt) mutation positions representing the position within the corresponding gene in the Ames Ancestor reference sequence (NC_007530.2). The last column indicates whether the corresponding mutation has been previously described in the literature. Amino acid abbreviations are as follows: (G), glycine; (D), aspartic acid; (L), leucine; (S), serine, (H), histidine; (Q), glutamine; (Y), tyrosine. †, nt position upstream of the ATG start codon for *bla1*. *, penicillin-resistant strains not included in this screen but included in this table for reference purposes. FS, frameshift.

majority of mutations were synonymous substitutions and/or shared across multiple strains (see Fig. S1 in the supplemental material). *bla2* is located ~900 kb away from *bla1* on the chromosome and was not included in the screen. The sequence of *bla2* and its promoter region were analyzed in all 13 strains from the CDC collection (see below) that contained a *sigP*, *rsiP*, or *bla1* mutation and were identical to those of the Ames Ancestor reference.

Sequence analysis and AST of strains with *sigP*, *rsiP*, *and bla1* coding region mutations. No mutations were identified in the promoter or coding regions of *sigP*, *rsiP*, or *bla1* in the 42 *B. anthracis* genome sequences in GenBank. Analysis of the 374 sequenced *B. anthracis* strains from the CDC collection identified 13 (3.5%) strains with mutations in the *sigP*, *rsiP*, or *bla1* coding region (Table 1). As previously described by Ross et al. in 2009 (4), the promoter regions are highly conserved. These regions were also analyzed for the 13 strains.

***sigP*.** Three of the 13 strains, 2002013094 (*B. anthracis* 3094 [Ba3094]), 2000031021 (Ba1021), and 2000031052 (Ba1052), contained mutations in *sigP*, and all mutations were confirmed by Sanger sequencing. Ba3094, Ba1021, and Ba1052 were shown to belong to clade C by multilocus variable-number tandem-repeat 8 (MLVA-8) genotyping (24) and to share the same transition mutation at nucleotide position 119. Strain Ba1052 contained an additional transition mutation in *sigP* at position 395. None of the strains contained the *sigP* SNP previously described in PEN-R strain 32 that resulted in a single amino acid difference predicted to decrease SigP activity (4). All study strains with *sigP* mutations were PEN-S by conventional BMD AST (Table 1).

***bla1*.** Two of 13 strains contained mutations in the *bla1* coding region, or in the *bla1* promoter region, and all of the mutations were confirmed by Sanger sequencing. Strain 2002734089 (Ba4089) contained a transition mutation and belonged to clade A by MLVA-8 genotyping, and strain 2000031048 (Ba1048) contained a transversion in the −35 promoter element and belonged to clade B. All strains with mutations in the *bla1* coding region or promoter region were PEN-S by conventional BMD AST.

***rsiP*.** Eight of the 13 strains contained mutations in the anti-sigma factor gene, *rsiP* (Table 1). Mutations were identified in each WGS assembly for strains 2002013017 (Ba3017) and 2002013007 (Ba3007), but Sanger sequencing did not confirm these changes. Analysis of the WGS data revealed errors in the WGS assemblies, and, as a result, these strains were removed from further analysis. The remaining six strains with Sanger-confirmed *rsiP* mutations belonged to clade A (MLVA-8). Strain 2002013011 (Ba3011) contained a novel SNP that is predicted to lead to an amino acid substitution in RsiP at position 170 of 275 and was PEN-S by AST. Strain 2000031038 (Ba1038) contained a novel frameshift mutation predicted to result in an amino-terminally truncated RsiP protein (30 amino acids [aa]). Strain Ba1038 was isolated from an environmental surface sample in 1957; however, the source of the sample is unknown. Strain 2000032823 (Ba2823) contained a frameshift mutation predicted to result in an amino-terminally truncated RsiP protein (163 aa). This mutation was also previously described in PEN-R strains isolated in 2011 from cattle (18). Strain Ba2823 was isolated prior to 2011, but additional details about the source of the strain are unknown (25). Both Ba1038 and Ba2823 were PEN-R by conventional BMD AST. Three strains, 2002734065 (Ba4065 or SK57A), 2002734039 (Ba4039 or SK57C), and 2002013027 (Ba3027), contained the same frameshift predicted to result in a 12-amino-acid, amino-terminally truncated RsiP previously described in PEN-R strain 32 (4) and in PEN-R strains isolated from cattle (18). Strains Ba4065 (SK57A) and Ba4039 (SK57C) were collected in England in November 1975. Both are likely related to previously described PEN-R strain SK57 (14). Despite possessing the same *rsiP* mutation as PEN-R strains SK57A, SK57C, and strain 32, conventional AST confirmed that Ba3027 is PEN-S (MIC = 0.06 μg/ml).

ORF 2506 and ORF 2504. In addition to *bla1* and *bla2*, SigP-induced transcription of ORF 2506 in a PEN-R *B. anthracis* strain was described previously (18). BLASTX analysis revealed that ORF 2506 shared similarity with *B. cereus* group PBP2 (99% coverage, 100% identity; accession number WP_000662966.1) and contained the conserved protein domain family FtsI, which is associated with cell cycle control, cell division, chromosome partitioning, and cell wall/membrane/envelope biogenesis. The majority (~93.75%) of strains in this study, including all PEN-R strains, contained a frameshift in a homopolymer region of this gene that is also found in the Ames Ancestor reference strain (NCBI accession number AE017334) and that resulted in 2 predicted ORFs. The first ORF consisted of the N-terminal 124 amino acids of the predicted PBP2 and contained a predicted PBP dimerization domain. The second ORF contained the last 586 amino acids of the predicted PBP2, which includes the transpeptidase domain. Only 6.25% (26/416) of the strains did not contain this frameshift and had a single ORF predicted to represent the full-length PBP2 (710 aa). BLASTX analysis revealed that ORF 2504 also shared similarity with a *B. cereus* group PBP2 (99% coverage, 100% identity; accession number WP_000903320.1). However, ORF 2504 is translated in the opposite direction from the other ORFs in the *sigP-bla1* region and is not regulated by SigP (18).

Sequencing of PEN-R strains and phylogeny based on whole-genome SNP calling. The *de novo* assemblies for all study strains were each ≥99.8% identical to the Ames Ancestor reference genome (data not shown). A phylogeny based on whole-genome SNP calling was created for all strains with mutations in the coding regions of *sigP*, *rsiP*, and/or *bla1* (Fig. 2). Strains 32, UT308, and SK57 (15) contained mutations in *sigP* and/or *rsiP* (Table 1) and were included in the phylogenetic analysis as reference strains. All strains with a mutation in *rsiP* belonged to clade A. Two of the strains identified in this screen with *rsiP* mutations (Ba4065 and Ba4039) fell into the same clade as the three PEN-R reference strains. In comparison to SK57, Ba4065 (SK57A), Ba4039 (SK57C), and strain 32 and its derivative UT308 have an additional mutation in *sigP* that is predicted to affect protein activity (4). PEN-S Ba3027 harbors the same *rsiP* mutation as strains 32, SK57, Ba4065 (SK57A), and Ba4039 (SK57C) but is not in the same branch as the PEN-R strains. Two other PEN-R isolates with *rsiP* mutations,

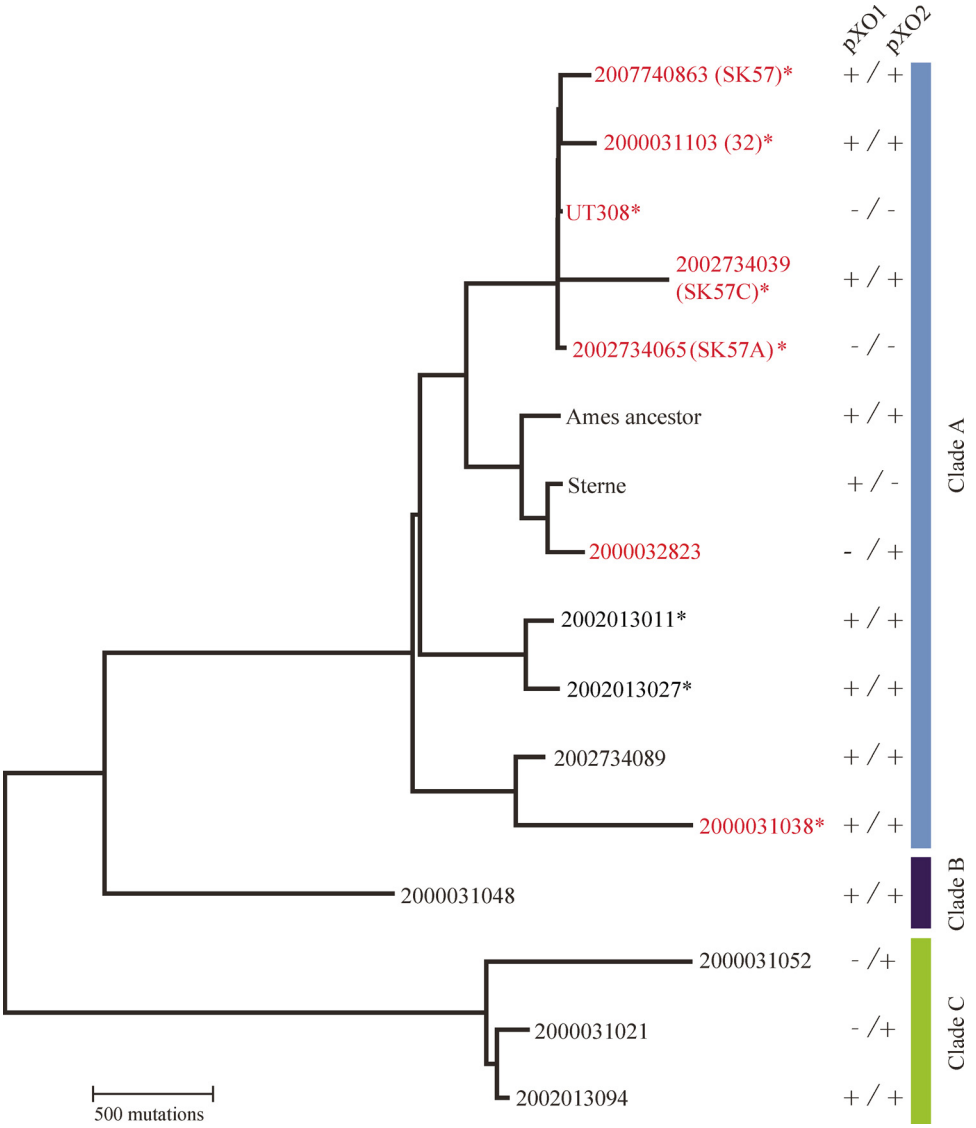

FIG 2 Neighbor-joining tree of *B. anthracis* strains containing mutations within *sigP*, *rsiP*, and *bla1* identified from the WGS screen. Strains from the three major *B. anthracis* clades (A, B, and C) were identified in the WGS screen (color coded) (24). PEN-R strains are colored in red. Asterisks (*) indicate strains containing an *rsiP* mutation. Control strains SK57, UT308, 2000031103 (strain 32), and Sterne, as well as the Ames Ancestor reference strain, were included for comparison.

Ba2823 and Ba1038, were in different branches. The two strains with mutations in *bla1* were members of clade A, and the three strains containing *sigP* mutations fell within clade C.

Characterization of Ba3027: Growth, colony morphology, β-lactamase activity, and *bla* gene expression. Strain Ba3027 exhibited a slower growth rate in broth culture than strain SK57, which contains the same *rsiP* mutation, and 2007740878 (Ba0878), a prototypical PEN-S strain without the *rsiP* mutation (Fig. 3A). After 24 h of culture on agar, single isolated colonies of strain Ba0878 were nearly half the size of strain SK57 colonies, with average colony diameters of 2.82 mm and 5.40 mm, respectively (Fig. 3B). Strain Ba3027 formed smaller colonies (average colony diameter, 0.85 mm) than Ba0878 and SK57. The β-lactamase activity of culture supernatants was measured in a quantitative nitrocefin assay to evaluate extracellular β-lactamase production in Ba3027. Similarly to PEN-S strains Ba0878 and Sterne, β-lactamase activity was not detected in the culture supernatant from Ba3027 (Fig. 4A). A statistically

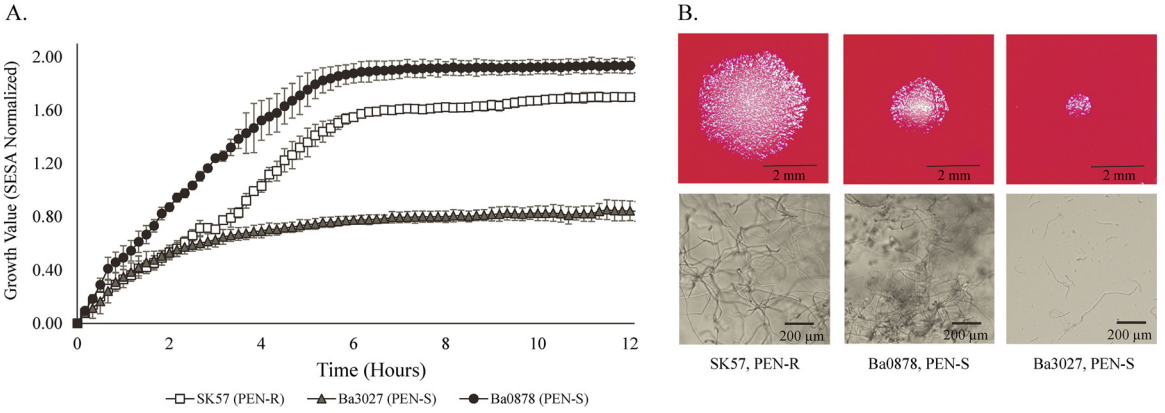

FIG 3 Growth characteristics of Ba3027. (A) Growth kinetics of strains SK57 (PEN-R, *rsiP* 10 mutation), Ba3027 (PEN-S, *rsiP* 10 mutation), and Ba0878 (PEN-S, wild-type strain) were evaluated over a 12-h incubation at 35°C in broth. Growth was measured by the Segmentation and Extraction of Surface Area (SESA) algorithm. Graphs represent the average growth value ± standard deviations from three replicate wells. (B) Microscope images (×8) of single colonies were taken following an 18-h incubation at 35°C in ambient air on SBA (top); optical screen images represent bacterial growth in a 100-μl cell suspension after 7 h (bottom).

significant difference in activity levels ($P = 0.001$ to 0.01) was observed for these three strains compared to β-lactamase-producing strain UT308. SK57 had significantly higher β-lactamase activity (223 ± 4.35 mU/ml) than UT308 (61 ± 3.15 mU/ml) ($P < 0.001$). Whole-cell lysates of SK57, Ba0878, and Ba3027 were prepared and tested using the quantitative nitrocefin assay to determine if β-lactamase is produced in Ba3027 but is not exported to the culture supernatant or associated with the cell wall. β-Lactamase activity was detected in the PEN-R SK57 lysates but not in the PEN-S Ba3027 or Ba0878 lysates (data not shown).

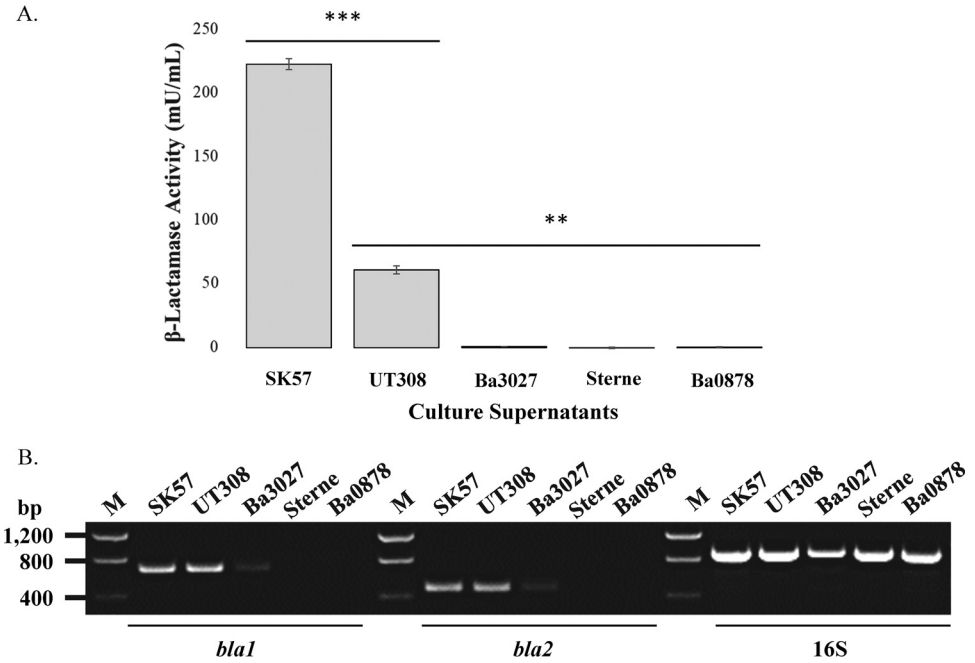

FIG 4 β-Lactamase production and semiquantitative RT-PCR analysis of *bla1*, *bla2*, and 16S transcripts in *B. anthracis* strains. (A) β-Lactamase activity of culture supernatants from strains SK57 (PEN-R, *rsiP* 10 mutation), UT308 (PEN-R, *sigP* 183 mutation, *rsiP* 10 mutation), Ba3027 (PEN-S, *rsiP* 10 mutation), Sterne (PEN-S), and Ba0878 (PEN-S, wild-type strain) was measured using nitrocefin. Error bars represent averages ± standard deviations. **, $P = 0.001$ to 0.01 (statistical significance compared to β-lactamase-producing strain UT308); ***, $P < 0.001$ (statistical significance of UT308 compared to SK57). (B) Expression of SK57, UT308, Ba3027, Sterne, and Ba0878 *bla1*, *bla2*, and 16S genes was analyzed by semiquantitative RT-PCR after 20 cycles. The molecular marker was run in lane M.

Semiquantitative reverse transcriptase PCR (RT-PCR) analysis of *bla1* and *bla2* was performed to measure *bla1* and *bla2* transcription in Ba3027 at the exponential phase of growth in Luria-Bertani (LB) broth. Transcripts of *bla1* and *bla2* were detected in Ba3027 but at lower levels than were seen with PEN-R strains SK57 and UT308. No *bla1* or *bla2* transcript was detected in PEN-S control strains Sterne and Ba0878 (Fig. 4B). Controls for these assays confirmed that no amplification product was detectable in the no-transcriptase or no-template reactions for each reaction set (data not shown).

Genomic mutations unique to Ba3027. Despite harboring the same RsiP truncation mutation as four other PEN-R *B. anthracis* strains, PEN-S strain Ba3027 clustered with one other PEN-S strain in the WGS SNP-based tree (Fig. 2). To identify chromosomal mutations unique to Ba3027, the coding region mutations in Ba3027 were compared to coding region mutations found in the *B. anthracis* isolates used to generate the phylogenetic tree. Forty-six frameshift and missense mutations were unique to Ba3027. These nucleotide differences were found in a variety of genes, including those predicted to be involved in cell growth and cell division, peptidoglycan biosynthesis processes, regulation of transcription, and DNA-directed RNA polymerase activity (see Table S1 in the supplemental material).

DISCUSSION

WGS can be used to detect the mutations and genes most frequently associated with drug resistance for bacterial pathogens such as *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (26–29). For *M. tuberculosis*, the WGS data are valuable because it offers a more rapidly determined AST profile for first-line and second-line anti-TB drugs than the conventional BMD method (28, 30). In the event of a deliberate release of *B. anthracis*, antimicrobial susceptibility results would be essential for the distribution of appropriate antimicrobial agents for PEP and treatment. WGS of the implicated strain(s) could reveal known, novel, and/or engineered genetic modifications, including indels and SNPs related to antibiotic resistance. For example, SNPs located in the quinolone resistance-determining region of *B. anthracis* can be analyzed to predict functional ciprofloxacin resistance (31, 32) and can serve as useful targets during genomic analysis.

The presence of β-lactamase genes is considered predictive of penicillin resistance for many bacterial species (5). However, *B. anthracis* is characteristically susceptible to penicillin despite containing two chromosomal β-lactamase genes. A *B. anthracis* anti-sigma factor (*rsiP*) mutation can lead to β-lactamase gene expression and penicillin resistance (4). Mutations in *sigP* and *rsiP* have been reported only in strain 32 (4) and in PEN-R strains isolated from cattle following a 2011 anthrax outbreak (18). To more accurately predict resistance phenotypes from bacterial sequence data, it is critical to identify mutations that exist among different strains and to determine the impact that these variations have on the observed PEN-R phenotype (33).

In this work, we analyzed the *sigP-bla1* region of 374 strains of *B. anthracis* from a strain collection at the CDC to determine the frequency of mutations across the ~6.9-kb region. This collection includes *B. anthracis* strains isolated from human, animal, and environmental sources worldwide from the 1950s to 2013 (34). Functional antimicrobial susceptibility testing was then performed to determine the accuracy of penicillin resistance prediction. A limitation of this study is that BMD AST was not performed on every study strain, and we therefore do not know if there are any strains in the CDC collection that are PEN-R and lack a mutation in the *sigP-bla1* region. While it was not feasible to perform AST for all 374 strains for which WGS data are available, we screened the WGS data to identify strains containing mutations in the *sigP-bla1* region with the goal of identifying strains similar to strain 32.

Few strains (3.5% [13/374]) contained a mutation(s) (SNPs, insertions, or deletions) in the *sigP*, *rsiP*, or *bla1* coding region. None of the strains with mutations in *sigP* or *bla1* were resistant to penicillin, indicating that the sigma factor and anti-sigma factor supression systems are likely fully functional in these strains. Only 5 strains (1.3% [5/374]) contained a mutation in the *rsiP* coding region that was predicted to result in

a truncation of the anti-sigma factor. Four of those five strains that contained an *rsiP* mutation were resistant to penicillin, indicating that the anti-sigma factor is likely not functional.

A single PEN-S strain with a *rsiP* mutation, Ba3027, displayed a smaller colony size and a slower growth rate than the wild-type PEN-R and PEN-S strains. While β-lactamase activity was not detected by quantitative nitrocefin assays, transcripts of both *bla1* and *bla2* were detected in Ba3027. This indicates that *bla1* and *bla2* are expressed in Ba3027 to some extent but suggests that the production level is not sufficient to detect β-lactamase activity using the nitrocefin assay or resistance by BMD AST. The PEN-S phenotype and lack of β-lactamase activity in Ba3027 were unexpected. To assess whether this phenotype/genotype discrepancy could be explained using WGS data, we compared sequences of all strains included in the phylogenetic tree to the Ames Ancestor reference sequence. All coding region mutations unique to Ba3027 are listed in Table S1 in the supplemental material. A total of 46 frameshift and missense mutations were found, any of which might contribute to the unusual growth characteristics and/or PEN-S phenotype of Ba3027. For example, one mutation was found in *ftsA*, a gene related to the cell cycle. The loss of this gene has been reported to result in impaired cell division and sporulation in *B. subtilis* (35). While the specific genetic basis of this unexpected PEN-S phenotype of Ba3027 was not immediately identified, these unique mutations represent potential candidates for future investigation.

Mutations identified at three positions that led to predicted truncated RsiP proteins of 12, 30, and 163 amino acids were all located in homopolymer regions of the *B. anthracis* chromosome, suggesting that these regions are hot spots for insertion or deletion events (18). We identified two strains with deletions in these homopolymer regions, but Sanger sequencing data did not confirm the presence of these mutations. Subsequent AST testing revealed that both strains were PEN-S. Some next-generation sequencing (NGS) technologies have difficulty resolving homopolymer regions of DNA sequence (36). This emphasizes the importance of sequence accuracy, especially in homopolymer regions, for high-confidence detection of mutations. Depending on the NGS technology used, confirmatory sequencing of these repetitive regions by the Sanger method may be necessary.

While both SK57 and UT308 are PEN-R by AST, the β-lactamase activity in the culture supernatant of SK57 (223 mU/ml) was higher than in that of UT308 (61 mU/ml). β-Lactamases in Gram-positive bacteria are predominantly located extracellularly, but β-lactamases can also adhere to the peptidoglycan layer (or capsule) or diffuse away (37). Whole-cell lysates of Ba3027 were tested using the quantitative nitrocefin assay to assess whether β-lactamase is produced in Ba3027 cells but not exported to the culture supernatant and is instead associated with the cell wall. However, no β-lactamase activity was detected (data not shown). This indicates that the level of production of Bla1 and Bla2 by Ba3027 was too low for detection of cell wall-associated or extracellular β-lactamase activity using the quantitative nitrocefin assay.

The *sigP* sequence of SK57 is identical to that of the Ames Ancestor reference, but UT308 contains the *sigP* mutation that is predicted to be associated with decreased SigP activity (4). The elevated β-lactamase activity in SK57 compared to UT308 is likely due to this predicted decreased activity of SigP in UT308. Ross et al. (4) suggested that constitutive expression of wild-type SigP is detrimental to *B. anthracis* growth and that the strain 32 SigP mutation is acting to reduce the activity of the sigma factor and to alleviate this toxicity. Wild-type SigP activity was noted to be tolerated in the PEN-R strains isolated from the anthrax outbreak in cattle (18). Our findings indicate that SigP activity was also tolerated in the 4 PEN-R strains that contained wild-type SigP in this study. Agren et al. (18) also described a subpopulation that was isolated from the frozen stock of a PEN-R strain that not only contained the *rsiP* mutation but also contained a *sigP* mutation that abolished SigP activity and resulted in a penicillin-susceptible phenotype. This was described as a counteracting mutation. Both Ross et al. (4) and Agren et al. (18) speculated that strains expressing wild-type SigP with *rsiP* mutations

acquired detrimental *sigP* mutations since SigP expression results in growth defects. Downregulation of β-lactamase genes could offer these strains a fitness advantage (4, 18). None of the PEN-R strains isolated in this study contained both a *sigP* mutation and a *rsiP* mutation; therefore, all of the PEN-R strains are presumed to express the wild-type *sigP* gene. Not only was a smaller colony size observed for Ba3027 (Fig. 3), but PEN-R strains Ba1038, Ba2823, and Ba4065 (SK57A) also had smaller colony diameters than PEN-R SK57 and Ba4039 (SK57C) as well as other PEN-S wild-type strains (data not shown). However, it is not uncommon to see variability in growth characteristics and colony morphologies in wild-type *B. anthracis* strains (23). Because not all PEN-R strains in this study exhibited a reduced growth rate and smaller colony size, additional work is needed to establish whether expression of wild-type SigP is directly associated with growth defects in *B. anthracis*.

Work by Ross et al. (4) showed that transforming a *B. anthracis sigP-rsiP* null mutant with *sigP* and *rsiP* genes from PEN-R *B. cereus* or *B. thuringiensis* strains resulted in β-lactamase activity. This suggests that *B. anthracis* contains the genes required for sensing β-lactam antibiotics but that the presence of wild-type (prototypical) SigP and RsiP is not sufficient for *bla* induction. Ross et al. propose that the *B. cereus* and *B. thuringiensis* RsiP proteins can respond to the signal when a β-lactam antibiotic is present and that those species are in turn characteristically PEN-R. The defective anti-sigma factor could explain why RsiP in prototypical PEN-S *B. anthracis* strains does not respond to this signal (4). While the *B. cereus* and *B. thuringiensis rsiP* genes are 91% to 99% identical to the corresponding *B. anthracis* gene on the nucleotide level, future work will determine whether any sequence differences are involved in the inability of *B. anthracis* RsiP to respond to the β-lactam antibiotic signal.

Molecular phylogenies and comparative genome sequencing have revealed that *B. cereus* species group bacteria are closely related and could be classified as a single species (38, 39). The *de novo* assemblies for every strain included in this study were ≥99.8% identical to those in the Ames Ancestor reference genome. Analysis of the whole-genome SNP phylogeny revealed that every strain with a mutation in *rsiP* belonged to clade A; however, the limited number of clade B and clade C strains included in this study precluded a definitive association of *rsiP* mutations or PEN-R genotypes with clade A strains. Two of the strains with *rsiP* mutations (SK57A and SK57C) were located in the same group as the three PEN-R reference strains (strain 32, SK57, and UT308). The results revealing this monophyletic group, along with the common frameshift mutation in *rsiP*, strongly suggest a common origin for all of these isolates. However, three isolates with *rsiP* mutations (Ba3011, Ba3027, and Ba1038) grouped differently, indicating that these isolates evolved independently of the monophyletic group. Strains SK57A and SK57C were isolated from an archival collection of CDC strains that were originally stored on agar slants overlaid with mineral oil at room temperature (40). Both strains were isolated in November 1975, but the CDC records associated with these strains contain few details about the source and there is no clear association with strain 32 (12).

Despite the underlying genomic similarity, *B. anthracis* isolates are phenotypically diverse because of altered gene expression rather than gene content (4, 38). The control of β-lactamase expression by SigP and RsiP is an example of how *trans*-acting factors differentially affect transcription of genes in the *B. cereus* group species. Here, we show that analysis of the *sigP-bla1* region in *B. anthracis* was useful in predicting penicillin resistance in the majority of *B. anthracis* strains that contained a mutation in *rsiP*. This locus should be included in analyses of WGS data to predict antimicrobial resistance of *B. anthracis*. However, it cannot be used exclusively, as only four of the five strains with *rsiP* mutations were PEN-R by conventional BMD testing. There are a limited number of PEN-R *B. anthracis* strains available for analysis, and this makes it difficult to accurately assess concordance between phenotypic susceptibility and resistance genotypes. To accurately assess clinically relevant β-lactamase production in *B. anthracis*, use of a conventional phenotypic method, like BMD AST, remains essential.

TABLE 2 *B. anthracis* strains used in this study[a]

Strain	Alternative ID	Clade/MLVA-8 genotype	Plasmid content	Origin	Source	NCBI accession no.	Reference
Sterne*	34F2	A/—	pX01$^+$, pX02$^-$	South Africa	Animal	CP009541.1	51
UT308*	NA	A/—	pX01$^+$, pX02$^-$	Derived from strain 32	Human	QPKP00000000	4
2007740878*	Ba0878, BA0018	A/7	pX01$^+$, pX02$^+$	Canada	Unknown	NA	52
2000031656	Ba1656, Ames	A/62	pX01$^+$, pX02$^+$	USA (Texas)	Animal	NC_007530.2	53
2000031103	Ba1103, ASC 32, strain 32	A/53	pX01$^+$, pX02$^+$	England	Human	QPKO00000000	12
2007740863	Ba0863, SK57	A/48	pX01$^+$, pX02$^+$	England	Unknown	QPKQ00000000	14
2002734065	Ba4065, SK57A	A/—	pX01$^-$, pX02$^-$	England	Unknown	SRR5811123	This study
2002734039	Ba4039, SK57C	A/116	pX01$^+$, pX02$^+$	England	Unknown	SRR2340304	25
2002013027	Ba3027, AO427	A/4	pX01$^+$, pX02$^+$	Unknown	Unknown	SRR2339620	This study
2000031038	Ba1038, 300, Dole 111	A/71	pX01$^+$, pX02$^+$	Unknown	Environmental	SRR5811163	54
2002013017	Ba3017, AO412	A/4	pX01$^+$, pX02$^+$	Unknown	Unknown	SRR2339614	This study
2000032823	Ba2823, A0048d	A/—	pX01$^-$, pX02$^+$	Unknown	Unknown	SRR5811071	25
2002013011	Ba3011, SPU A0423	A/118	pX01$^+$, pX02$^+$	Unknown	Unknown	SRR2340484	25
2002013007	Ba3007, AO461	A/4	pX01$^+$, pX02$^+$	Unknown	Unknown	SRR2340462	This study
2000031048	Ba1048, 305, tannery 42	B/107	pX01$^+$, pX02$^+$	Unknown	Environmental	SRR5811217	25
2002734089	Ba4089, SK83, C2291	A/71	pX01$^+$, pX02$^+$	USA (New Jersey)	Environmental	SRR2340461	This study
2002013094	Ba3094, 240	C/133	pX01$^+$, pX02$^+$	Unknown	Environmental	SRR5947106	25
2000031021	Ba1021, 239, LA164B	C/—	pX01$^-$, pX02$^+$	USA (Louisiana)	Environmental	SRR5947105	This study
2000031052	Ba1052, 278, #25600	C/—	pX01$^-$, pX02$^+$	USA (Wyoming)	Animal	SRR5811214	This study

[a]Asterisks (*) indicate reference strains not included in the *sigP-bla1* screen. ID, identifier(s); NA, not available; —, MLVA-8 genotype determination not performed due to lack of a plasmid.

MATERIALS AND METHODS

Biosafety procedures. *B. anthracis* is subject to select agent regulations (42 CFR part 73). All procedures using the attenuated, select agent-excluded strains, *B. anthracis* UT308 and Sterne, were performed in a biosafety level 2 (BSL2) laboratory by trained personnel wearing appropriate personal protective equipment (PPE). All procedures involving wild-type *B. anthracis* strains were performed by trained personnel wearing PPE (including a powered air-purifying respirator [PAPR] and protective laboratory clothing) in a class II type A2 biological safety cabinet located in a BSL3 laboratory registered with the U.S. Federal Select Agent Program. Additional information regarding the facility and equipment and procedural guidelines for BSL2 and BSL3 laboratories can be found in the CDC/NIH publication "Biosafety in Microbiological and Biomedical Laboratories," 5th edition (41).

Bacterial strains. All *B. anthracis* strains identified in the *sigP-bla1* screen and control strains used in this study are listed in Table 2. PEN-R strains 32, UT308, and SK57 and the PEN-S Sterne and Ba0878 strains were used as control strains in this study.

Growth conditions. *B. anthracis* strains were cultured on BD BBL Trypticase soy agar II with 5% sheep blood (SBA) (Thermo Fisher Scientific, Waltham, MA, USA) at 35°C in ambient air from glycerol stocks stored at −70°C. For microscopy and sizing of single colony isolates, each strain was subcultured at 35°C in ambient air for 18 h on SBA. For AST, strains were grown overnight (16 to 24 h) at 35°C on SBA in ambient air, following CLSI guidelines. To assess β-lactamase activity (4) and for RNA isolation (5, 10), each strain was cultured at 37°C in ambient air on SBA overnight to replicate the culturing conditions described previously. From an overnight growth culture, a cell suspension equivalent to a 0.5 McFarland density standard was prepared in fresh LB broth (BD Difco Miller Luria-Bertani; Thermo Fisher Scientific, Waltham, MA, USA) for RNA isolations or in LB broth containing 0.5% glycerol for β-lactamase activity assays.

Sequencing and analysis. Genomic DNA was sequenced from 374 *B. anthracis* strains from the CDC collection. DNA from isolates was extracted using one of two technologies, a QIAamp DNA blood minikit (Qiagen, Valencia, CA) or a Maxwell 16 instrument (Promega, Madison, WI). For the QIAamp extraction, one 10-μl loopful of cells from overnight growth on SBA was inoculated in heart infusion broth (Remel, Lenexa, KS) and incubated for 18 to 24 h. Cells were harvested by centrifugation for 10 min at 5,000 $\times$ *g*. After removal of broth, DNA was extracted from remaining cells using a Qiagen QIAamp DNA blood minikit following the manufacturer's protocol for isolating Gram-positive bacteria. For DNA extractions performed on the Maxwell instrument, four to five colonies of overnight growth from SBA were mechanically disrupted by vortex mixing for 2 min in a suspension of silica beads and Tris-EDTA (TE) buffer. The suspension was centrifuged for 30 s at 10,000 $\times$ *g*. A 300-μl volume of the resulting supernatant was used for DNA extraction following the manufacturer's protocol for blood and cells. Sequencing was performed on an Illumina GAIIx system using TruSeq chemistry (Illumina, San Diego, CA). Paired-end reads were trimmed, adaptor sequences were removed, and the reads were subjected to quality checking using SolexaQA++ (42) with a Phred quality score threshold of 20 and minimum length of 50 bp, Scythe with default parameters, and FastQC, respectively. Paired-end reads for which both reads passed the quality control were then assembled using IDBA-UD (43) with precorrection and default parameters, and the resulting scaffolds that were shorter than 500 bp were discarded. A local BLAST (44) search was performed by querying the *sigP-bla1* region (6,892 bp) from a PEN-S reference strain, the Ames Ancestor (accession number AE017334), against the 374 *B. anthracis* strains in the CDC WGS database to identify mutations. Forty-two *B. anthracis* strains in GenBank for which WGS data were available at the time of analysis were also included in the BLAST screen (total, 416 strains). The *sigP-rsiP*,

bla1, and *bla2* promoter regions were also analyzed for the 13 strains that contained a *sigP*, *rsiP*, or *bla1* mutation. Sanger sequencing of the *sigP-bla1* region was performed using an ABI 3130xl or 3500xl Genetic Analyzer (Applied Biosystems, by Thermo Fisher Scientific, Waltham, MA, USA) as described by Hakovirta et al. (45) to confirm mutations. The primers used for PCR amplification and sequencing were as follows: *sigP-rsiP*FwdPCR (5′-GGAGAACTCGAACTAAATGG-3′), *sigP-rsiP*RevPCR (5′-GCTGCTCTCGTTACA TCA-3′), *sigP-rsiP*IntFwd1 (5′-TGATAAACAAACTCTGTCGG-3′), *sigP-rsiP*IntFwd2 (5′-CCTAAAAAGCACCGTG A-3′), *sigP-rsiP*IntFwd3 (5′-CTGCTCAAGATCCAACAT-3′), *sigP-rsiP*IntFwd4 (5′-CTGAACCAAAGCGAGAAT-3′), *sigP-rsiP*IntRev1 (5′-GCCGACAGAGTTTGTTTA-3′), *sigP-rsiP*IntRev2 (5′-AATGGTCTTGTATGTTCCC-3′), *sigP-rsiP*IntRev3 (5′-CTTTTGATTCTCGCTTTGGT-3′), *bla1*FwdPCR (5′-AATAAGAGATAGCAGCGG-3′), *bla1*RevPCR (5′-GGTTTTTCACGTATCTGG-3′), and *bla1*IntRev1 (5′-ACACCTAATCGAGCATCA-3′). BLAST and Sanger sequence data were analyzed using Geneious R8 software, version 8.1.4, and CLC Genomics Workbench software, version 7.5.1. Mutations in wild-type strains were identified by comparing the genome assemblies of the wild-type strains to that of the Ames Ancestor reference sequence using the dnadiff utility from the MUMmer package (46). The SnpEff utility (47) was used to predict the effects of mutations identified as unique to Ba3027. The Harvest suite of tools (48) was used to determine the numbers of SNPs in comparisons between all wild-type genomes. These differences were represented as a distance matrix and used to create a neighbor-joining tree using MEGA 6 (49).

MLVA-8 subtyping. MLVA-8 genotyping was performed as described by Keim et al. (24) and Sue et al. (25). Briefly, six chromosomal loci (*vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, *vrrC2*, and CG3) and two plasmid loci (pXO1-*aat* and pXO2-*at*) were amplified by PCR and the resulting DNA fragments were separated on an ABI 3130xl instrument or an ABI 3500xl instrument (Applied Biosystems, by Thermo Fisher Scientific, Waltham, MA, USA).

Antimicrobial susceptibility testing (AST). Broth microdilution (BMD) was performed to determine antimicrobial susceptibility for penicillin following the CLSI guidelines (19). Cells from four to six isolated colonies of an overnight culture were suspended in saline solution (Beckman Coulter, Brea, CA) and mixed using a vortex mixer to a turbidity equivalent to a 0.5 McFarland standard as measured with a MicroScan turbidity meter (Siemens, Munich, Germany). Each suspension was then diluted 1:20. BMD AST panels prepared in-house with cation-adjusted Mueller-Hinton broth were inoculated and incubated at 35°C in ambient air for 16 to 20 h. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as control strains. The MIC of penicillin for each *B. anthracis* strain was recorded as the concentration at the first well where there was no visible growth.

Microscopy and sizing of single colony isolates. Images of individual colonies on SBA were acquired with a Leica EZ4 HD digital stereo microscope (Leica Microsystems, Wetzlar, Germany). Colony diameters were measured using a Digimatic Solar Caliper (Mitutoyo America, IL, USA).

Imaging and analysis of bacterial growth in broth culture by optical screening. *B. anthracis* cell suspensions equivalent to a 0.5 McFarland density standard were prepared as described previously (23) from colonies grown overnight on SBA. The cell suspension was diluted 1:100 in cation-adjusted Mueller-Hinton broth with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (CAMHBT; Remel Inc., Lenexa, KS). A 100-μl aliquot of each diluted cell suspension was transferred into each well of a 96-well plate. Optical screening images were generated from scans through a fluid sample using digital time-lapse microscopy with an oCelloScope instrument (Biosense Solutions ApS, Farum, Denmark) as described previously (23, 50). The instrument-derived growth values were obtained using the Segmentation and Extraction Surface Area (SESA) normalized algorithm. Growth kinetic data in Fig. 3 represent the means of triplicate values $\pm$ standard deviations.

Nitrocefin β-lactamase assays. Broth cultures in the late-exponential-growth phase (10^5 CFU/ml) of *B. anthracis* strains SK57, Ba3027, Ba0878, Sterne (PEN-S, select agent-excluded strain), and UT308 (PEN-R, select agent-excluded derivative of strain 32) were tested for β-lactamase activity using a nitrocefin-based quantitative β-lactamase activity assay (β-lactamase activity assay kit, MAK221; Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Supernatants from 500 μl of culture from each strain were collected by centrifugation at 8,000 $\times$ *g* for 2 min through a 0.1-μm-pore-size polyvinylidene difluoride (PVDF) Ultrafree-MC spin-filter column (Millipore, Billerica, MA, USA). The absorbance (A_{490}) was measured in a 96-well microplate reader (SpectraMax i3; Molecular Devices, Sunnyvale, CA) in kinetic mode for 60 min at room temperature. Two biological replicates were performed for each sample, and three technical replicates were analyzed for each biological replicate. The statistical significance of differences in levels of β-lactamase production between strains was calculated with a two-tailed *t* test ($n = 3$).

RNA isolation. Total RNA was isolated as previously described (5, 10). Briefly, total RNA was purified from exponential-phase cultures (10^5 CFU/ml) grown in LB broth at 37°C in ambient air, without shaking. Cells were collected on 0.22-μM-pore-size PVDF Ultrafree-MC spin-filter columns (Millipore, Billerica, MA, USA) by centrifugation at 8,000 $\times$ *g* for 2 min, resuspended in RNAprotect bacterial reagent (Qiagen, Hilden, Germany), and stored at −70°C until the time of extraction. RNA was isolated and purified using a RiboPure RNA purification kit for bacteria, including the DNase I treatment to remove traces of contaminating DNA, according to the instructions of the manufacturer (Thermo Fisher Scientific, USA). RNA quantity was assessed using a Qubit RNA HS assay kit and a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quality was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano kit (Agilent, Santa Clara, CA, USA).

Semiquantitative RT-PCR. Purified RNA (1.0 μg) was subjected to reverse transcription with random decamers using a RETROscript kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The resulting reverse transcriptase (RT) mixtures were used as the template for PCRs with internal primers BLA1-5 and BLA1-6 for *bla1*, BLA2-5 and BLA2-6 for *bla2*, and 16S-1 and 16S-2 for the 16S rRNA housekeeping gene, which were previously described by Chen et al. (10).

Semiquantitative PCRs were carried out with a RETROscript Kit as described by the manufacturer with 2 U of SuperTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in 50-μl reaction mixtures containing 100 ng of RT mixture template. PCR amplification conditions were as follows: 1 cycle of denaturation at 94°C for 4 min, followed by 20 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and template extension at 72°C for 1 min, and 1 cycle of final extension at 72°C for 5 min. Genomic DNA extracted from *B. anthracis* Sterne was used as a positive control. A no-RT sample and a no-template sample were included as negative controls for each reaction set. Amplification products were detected by gel electrophoresis on a 1% agarose gel, and size was assessed with a DNA ladder (Invitrogen low-DNA mass ladder; Thermo Fisher Scientific, Waltham, MA, USA).

Accession number(s). All reads were submitted to the NCBI Sequence Read Archive, and the accession numbers are listed in Table 2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00154-18.

FIG S1, TIF file, 2.7 MB.

TABLE S1, PDF file, 0.1 MB.

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