Mutagenesis and Repair in Bacillus anthracis: the Effect of Mutators

Krystle Zeibell, Sharon Aguila, Vivian Yan Shi, Andrea Chan, Hanjing Yang, and Jeffrey H. Miller*

Department of Microbiology, Immunology, and Molecular Genetics, and The Molecular Biology Institute, University of California, Los Angeles, California 90095

Received 25 October 2006/Accepted 21 December 2006

We have generated mutator strains of Bacillus anthracis Sterne by using directed gene knockouts to investigate the effect of deleting genes involved in mismatch repair, oxidative repair, and maintaining triphosphate pools. The single-knockout strains are deleted for mutS, mutY, mutM, or ndk. We also made double-knockout strains that are mutS ndk or mutY mutM. We have measured the levels of mutations in the rpoB gene that lead to the Rifr phenotype and have examined the mutational specificity. In addition, we examined the mutational specificity of two mutagens, 5-azaacytidine and N-methyl-N′-nitro-N-nitroso-guanidine. The mutY and mutM single knockouts are weak mutators by themselves, but the combination of mutY mutM results in very high mutation rates, all due to G:C → T:A transversions. The situation parallels that seen in Escherichia coli. Also, mutS knockouts are strong mutators and even stronger in the presence of a deletion of ndk. The number of sites in rpoB that can result in the Rifr phenotype by single-base substitution is more limited than in certain other bacteria, such as E. coli and Deinococcus radiodurans, although the average mutation rate per mutational site is roughly comparable. Hotspots at sites with virtually identical surrounding sequences are organism specific.

Mutational pathways, mutagenesis, and DNA repair systems are an integral part of biology. On the one hand, unchecked mutagenesis can lead to aberrant cellular physiology, cell death, and myriad human diseases, including cancer (for a review, see reference 8). On the other hand, mutational pathways can be a driving force in adaptive evolution, can create sufficient antigenic variation to allow pathogens to overcome host immune responses, and are instrumental in generating antibody diversity (for a review, see reference 19). Surprisingly, mutagenesis and repair have been studied in detail only in a very small number of organisms, such as Escherichia coli and Salmonella, yeast, and humans. As the field of genomics opens up possibilities for examining mutational pathways and repair strategies in a wide range of microorganisms, it is important to study these processes in microorganisms of interest. We will no doubt uncover many new mechanisms and will also learn more about the organism in question. In this study we focus on Bacillus anthracis, a bacterium of great interest for biodefense.

Bacillus anthracis is a gram-positive spore-forming bacillus that can infect humans, causing anthrax (for a review, see reference 15). Two plasmids are responsible for the virulence of B. anthracis. These are pXO1, which encodes the toxin protein and the lethal and edema factors, among other proteins (11, 24), and pXO2, encoding the capsule biosynthesis protein and the lethal and edema factors, among other proteins aimed at reversing UV-induced DNA damage and at reducing oxidative damage, such as bromoperoxidase, thioldioxidase, thioredoxin proteins, and a Cu-Zn superoxide dismutase (25). The latter is involved in the virulence of other bacteria, such as Salmonella, protecting them from nitric oxide killing (5). These findings underscore the need for examining mutational pathways and DNA repair in B. anthracis rather than in E. coli. In any case, repair systems have essentially not been studied in B. anthracis and only sparingly in B. subtilis (see, however, references 27 and 28).

In this study we used directed gene knockouts to derive a set of mutator strains of B. anthracis deficient in mismatch repair, as well as oxidative repair, and characterize these strains with regard to mutation rates and mutational specificity. We also examine the specificity of several mutagens in this organism and extend the rpoB/Rifr mutational analysis system that has been used with other bacteria (e.g., see references 9 and 14), as well as with B. anthracis (31), and use this system to measure mutation rates at specific sites.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, antibiotics, and growth conditions. All of the B. anthracis strains used in this study are derivatives of the wild-type B. anthracis Sterne 7022, a gift from K. Bradley (University of California, Los Angeles). The plasmids pUTE618, pUTE619, and pUTE583 are gifts from T. M. Koehler (The University of Texas Health Science Center Medical School). Unless described elsewhere, bacterial cultures were grown in standard LB medium supplemented with kanamycin (100 μg/ml), spectinomycin (100 μg/ml), and erythromycin (5 μg/ml) when appropriate.

Construction of B. anthracis single- and double-knockout mutants. The procedure for making single- or double-knockout mutants is described in the text, in

* Corresponding author. Mailing address: Department of MIMM/ UCLA, 1602 Molecular Sciences Bldg., 405 Hilgard Ave., Los Angeles, CA 90095. Phone: (310) 825-8460. Fax: (310) 206-3088. E-mail: jhmiller@microbio.ucla.edu.

Published ahead of print on 12 January 2007.
The mutS null allele. The mutS coding region was replaced by a spectinomycin resistance-encoding cassette through recombination of homologous flanking sequences. A white box represents the mutS-coding region. Black boxes represent homologous sequences flanking the mutS gene. Gray boxes represent the spectinomycin resistance-encoding cassette. (B) PCR analysis of genomic DNA isolated from the wild type (lanes 1, 3, and 5) and mutS knockout mutant (lanes 2, 4, and 6). Lanes 1 and 2, primers F1 and R1 for the B. anthracis rpoB gene (31); lanes 3 and 4, primers BAmutS7 and BAmutS8 (see panel A); lanes 5 and 6, primers BAmutS7 and SP3 (see panel A); lane M, 1-kb DNA ladder standard (sizes are marked to the left).

RESULTS

Construction of a set of DNA repair gene knockout mutants. We used the following procedure, as exemplified by the construction of a mutS knockout, shown in Fig. 1. A DNA construct for making a mutS null mutant by precise ORF replacement was engineered by a slightly modified procedure of Koehler and coworkers (3; also T. Koehler, personal communication). Briefly, a 1.2-kb upstream flanking region (starting before the mutS start codon) and a 0.9-kb downstream flanking region (starting after the mutS stop codon) were PCR amplified and cloned into separate pCR2.1 TOPO vectors (Invitrogen). Then, the downstream fragment was subcloned behind the upstream fragment, and subsequently a Ω-Sp cassette, from the plasmid pUTE619, which confers spectinomycin resistance (Fig. 1A), was inserted between the two fragments.

The mutS knockout construct was then subcloned into a shuttle vector (pUTE853) that contains an erythromycin resistance determinant but is unstable in B. anthracis when grown in liquid medium without selection (3; also T. Koehler, personal communication). The DNA of the resulting mutS knockout construct, pUTE853ΔmutS, was prepared from a Dam− E. coli strain (GM2163) and electroporated into B. anthracis wild-type cells. Erythromycin-resistant transformants were selected and purified. To allow reciprocal recombination to take place and
a subsequent loss of the shuttle vector, the purified transformants were grown in liquid medium for several days without erythromycin. The mutants were grown in liquid medium for several days without a subsequent loss of the shuttle vector, the purified transformants were grown in liquid medium for several days without erythromycin sensitive. The disruption of the wild-type mutS allele generated by precise ORF replacement with the ΔΩ-Sp cassette was verified by PCR, demonstrating the absence of the wild-type mutS allele with the primer pair BAmutS7 and BAmutS8 (Fig. 1B, lane 4; the locations of both primers are shown in Fig. 1A) and the presence of the ΔΩ-Sp DNA cassette with the primer pair BAmutS7 and SP3 (Fig. 1B, lane 6; the locations of both primers are shown in Fig. 1A). The corresponding wild-type controls are also shown in Fig. 1B (lanes 3 and 5). Table 1 depicts the single-knockout mutants we constructed.

**Double knockouts.** We also completed the construction of two double knockouts, mutS ndk and mutY mutM (Table 1). The procedure for making the double-knockout mutants was similar to the procedure used for making the single knockouts, except that the second gene in the double-knockout mutant (such as ndk or mutM, respectively) was replaced by an antibiotic resistance-encoding cassette that was different from the antibiotic resistance cassette, ΔΩ-Sp, used to replace the first gene, as depicted in Fig. 2. We used ΔΩ-Km, from the plasmid pUTE618, which confers kanamycin resistance (3; also T. Koehler, personal communication). The double-knockout mutants were identified as those colonies that were resistant to both spectinomycin and kanamycin but sensitive to erythromycin. The knockout mutations were further verified by PCR, demonstrating the loss of two wild-type genes and the presence of both ΔΩ-Sp and ΔΩ-Km. Together, these knockout strains were used for mutagenesis studies with *B. anthracis* and for further development of the rapB/RifR system, as described below.

**Measuring mutation frequencies and rates.** We measured the RifR frequency in cultures grown in LB at 37°C and calculated the mutation rates per generation (see Materials and Methods), as described previously (see reference 9 and references therein). Table 2 shows the results for the wild type and for the knockout mutants that we constructed, including mutY, mutM, mutS, and ndk, as well as the double-knockout mutants mutY mutM and mutS ndk. It can be seen that whereas mutM and mutY strains are weak mutators by themselves, the combination of mutY and mutM, which lacks the ability to repair 8-oxoguanine, generates an enormous mutation rate per generation (μ) that is 269 times that of the wild type and results in frequencies in overnight cultures (approximately 30 generations) that are 1,000-fold higher than those of the wild type. Mutants that are mutS and lack mismatch repair have RifR frequencies 178-fold higher than the wild type in overnight cultures. Although ndk strains (with altered nucleoside triphosphate pools) show up as weak mutators, the combination of mutS ndk results in frequencies that are 786-fold higher than the wild type.

**Use and extension of the rapB/RifR system.** Table 3 shows the distribution of spontaneous mutations in rapB leading to the RifR phenotype; those from mutS strains, mutY strains, mutY mutM strains; and those occurring after treatment with the mutagens 5AZ and MNNG. (Note that the location numbers

<table>
<thead>
<tr>
<th>Knockout constructed</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td></td>
</tr>
<tr>
<td>mutS (BAS3618)</td>
<td>DNA mismatch repair protein</td>
</tr>
<tr>
<td>mutY (BAS0491)</td>
<td>A/G-specific adenine glycosylase</td>
</tr>
<tr>
<td>mutM (BAS4481)</td>
<td>Formamidopyrimidine-DNA glycosylase</td>
</tr>
<tr>
<td>ndk (BAS1425)</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>Double</td>
<td></td>
</tr>
<tr>
<td>mutS ndk</td>
<td></td>
</tr>
<tr>
<td>mutY mutM</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2. B. anthracis rapB mutation frequencies (f) and rates (μ)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>f (10^-5)</th>
<th>μ (10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.8 (1.7–4.7)</td>
<td>1.6 (1.2–2.3)</td>
</tr>
<tr>
<td>mutM</td>
<td>6.1 (2.8–8.9)</td>
<td>3.2 (2.0–4.1)</td>
</tr>
<tr>
<td>mutY</td>
<td>33 (31–56)</td>
<td>9.4 (9.0–14)</td>
</tr>
<tr>
<td>mutY mutM</td>
<td>2,800 (2,500–3,300)</td>
<td>430 (390–500)</td>
</tr>
<tr>
<td>ndk</td>
<td>8.0 (5.0–13)</td>
<td>3.7 (2.8–5.3)</td>
</tr>
<tr>
<td>mutS</td>
<td>500 (480–550)</td>
<td>87 (84–94)</td>
</tr>
<tr>
<td>mutS ndk</td>
<td>2,200 (1,500–4,800)</td>
<td>360 (260–710)</td>
</tr>
</tbody>
</table>

| a Values in parentheses are 95% confidence limits (6). |
| b Mutation rates are calculated by the method of Drake (7). |

**FIG. 2. Schematic representation of the targeting strategy for generating a mutS ndk double-knockout mutant.** The ndk-coding region in the mutS single-knockout mutant was replaced by a kanamycin resistance-encoding cassette through reciprocal recombination to generate a mutS ndk double-knockout mutant. White boxes represent coding regions of mutS and ndk. Light gray boxes represent the spectinomycin resistance-encoding cassette. Dark gray boxes represent the kanamycin resistance-encoding cassette.
for each base pair are 42 bp higher than previously reported (31). It can be seen that there are distinct hotspots for each mutator and for each mutagenic treatment. The spontaneous mutations are almost exclusively at 3 of the 14 sites detected in the \textit{rpoB} gene so far. Previous work by Vogler and coworkers (31) detected 11 of these sites, and the work described here has resulted in three new sites being uncovered. The second column in the spontaneous category presents the totals when the spontaneous mutations found by Vogler and coworkers (31) are added to these. The \textit{mutY} and \textit{mutY \textit{mutM}} strains have increased levels only of the G:C $\rightarrow$ T:A changes, while strains defective in \textit{mutS} have increases mainly at the A:T $\rightarrow$ G:C sites. MNNG induces G:C $\rightarrow$ A:T changes; 5AZ induces only G:C $\rightarrow$ C:G changes and only at one site. These results are similar in form to those we found in the \textit{rpoB} gene of \textit{E. coli} (9). However, the frequency of Rif$^\text{R}$ mutations, as first noted by Keim and coworkers, is an order of magnitude lower than that seen in \textit{E. coli}. Is this because of an inherently lower mutation rate, or because of a distinctly lower number of mutational sites (14 so far in \textit{B. anthracis rpoB} versus 77 in \textit{E. coli rpoB})? Some considerations in the following sections point to the latter explanation.

**Comparisons of hotspots at different positions in rpoB in \textit{B. anthracis} and in two other bacteria.** Because of the high degree of sequence conservation in parts of the \textit{rpoB} gene among prokaryotes, we can compare the DNA sequences surrounding

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{B. anthracis site (bp)} & \textbf{Amino acid change} & \textbf{Base pair change} & \textbf{Spontaneous} & \textbf{mutS} & \textbf{mutS ndk} & \textbf{mutY} & \textbf{mutY mutM} & \textbf{5AZ} & \textbf{MNNG} \\
\hline
1403 & Q454R & AT$\rightarrow$GC & 15 & 24$^\text{‡}$ & 19 & 8 & 0 & 0 & 0 & 0 & 0  \\
1442 & H467R & AT$\rightarrow$GC & 17 & 23$^\text{‡}$ & 20 & 10 & 0 & 0 & 0 & 2  \\
1441 & H467Y & GC$\rightarrow$AT & 20 & 23$^\text{‡}$ & 1 & 0 & 0 & 0 & 0 & 31  \\
1391 & S450F & GC$\rightarrow$AT & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
1457 & S472F & GC$\rightarrow$AT & 1 & 4$^\text{‡}$ & 0 & 0 & 0 & 0 & 8  \\
1457$^*$ & S472Y & GC$\rightarrow$TA & 1 & 1 & 0 & 0 & 10 & 0 & 0  \\
1402 & Q454K & GC$\rightarrow$TA & 1 & 2$^\text{‡}$ & 0 & 0 & 27 & 30 & 0 & 0  \\
1403$^*$ & Q454L & AT$\rightarrow$TA & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
1417 & T459S & AT$\rightarrow$TA & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
1444 & K468Q & AT$\rightarrow$CG & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
1442$^*$ & H467P & AT$\rightarrow$CG & 1 & 1 & 0 & 0 & 0 & 0 & 0  \\
1391 & S450C & GC$\rightarrow$CG & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
1441$^*$ & H467D & GC$\rightarrow$CG & 0 & 0 & 0 & 0 & 0 & 17 & 0  \\
1400 & S453C & GC$\rightarrow$CG & 0 & 0 & 0 & 0 & 0 & 0 & 0  \\
\hline
\textbf{Total (14)} & & & 56 & 78$^\text{‡}$ & 40 & 18 & 37 & 30 & 17 & 41  \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Symbols: *, new mutational changes in \textit{B. anthracis} detected in this study; †, site detected after analysis of additional mutations from the same cultures (see Materials and Methods) as those included in the first column of spontaneous mutations.

\textsuperscript{b} ‡, includes data from Vogler and coworkers (31).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{rpoB_sequences.png}
\caption{DNA sequences surrounding specific sites in \textit{rpoB}. Portions of the \textit{rpoB} genes from \textit{D. radiodurans}, \textit{E. coli}, and \textit{B. anthracis} are aligned. Five sites are designated by Roman numerals. Identical bases are indicated in boldface type. Mutation rates at these sites are given in Table 4.}
\end{figure}
mutational sites in rpoB in several bacteria for which we have measured the mutation rates that lead to Rif r. Figure 3 aligns the corresponding regions of rpoB near some of the most frequently mutated sites in Deinococcus radiodurans (14), Escherichia coli (9, 32), and Bacillus anthracis (31; also this work). Figure 4 displays the relative frequency among rpoB mutations in each organism for five selected sites (sites I to V in Fig. 3). Here, arrows depict sites at which no occurrences were detected. Despite extensive identities in surrounding sequences, there are dramatic differences in the relative mutation frequencies in the three bacteria. Table 4 converts these relative frequencies to actual mutation rates per site. (At site I, no occurrences were found in E. coli among 444 spontaneous mutations [32], although this site has been found in different mutator strains. The numbers in parentheses for this site represent 95% confidence limits.) Site I is the best example of a clear difference in mutation rates at the same base in a sequence context that is identical for the three or four nearest neighbors on each side, in the case of B. anthracis and E. coli, with only slightly less identity in the case of D. radiodurans. Site II also shows an apparent difference between D. radiodurans and E. coli, as does site V between E. coli and B. anthracis. The question of whether the failure to find mutations at sites II and III in B. anthracis and at sites III and V in D. radiodurans is due to very low mutagenicity (cold spots) or the failure of the respective base change to result in a rifampin-resistant phenotype requires additional experiments. We have done these in the case of B. anthracis, using site-directed mutagenesis (described below) to show that sites II and III do not yield the Rif r phenotype when changed from G to A (site II) or A to G (site III).

Table 4 also shows the average mutation rate per site in rpoB, based on 33 sites in D. radiodurans, 77 in E. coli, and 14 in B. anthracis. D. radiodurans and E. coli display identical rates, whereas that of B. anthracis is almost twofold lower. Whether this apparent difference will hold when many more sites are considered remains to be seen. The vast majority of the spontaneous base substitutions in rpoB that lead to the Rif r phenotype are transitions in D. radiodurans and B. anthracis, although to a lesser degree in E. coli. Therefore, we have charted at the bottom of Table 4 the average transition rate per site in the three bacteria. Keeping in mind that this average rate is based on fewer sites, we see that in all three cases they are within a range of from \(2.3 \times 10^{-10}\) to \(3.1 \times 10^{-10}\). Here, the rates for B. anthracis are not lower than for E. coli.

**Site-directed mutagenesis of the B. anthracis rpoB gene.** The failure to find mutations in the B. anthracis rpoB gene, in cases where the corresponding location in E. coli or D. radiodurans rpoB is a hotspot, led us to ask whether mutations at these sites are rare in B. anthracis or simply do not lead to a viable Rif r organism. We chose to construct two rpoB mutants, each of which contains a single-base substitution mutation at one of

![FIG. 4. Comparison of relative mutation frequencies observed at specific sites in rpoB. The values for five sites in rpoB in three different bacteria are shown as the percentage of all detected rpoB mutations that lead to Rif r mutants. Arrows point to sites where no occurrences were detected. In some but not all cases, this is due to the failure of the mutation to generate a viable Rif r mutant (see Table 4).](image-url)
the following locations, G→A at position 1411 and A→G at position 1412, both of which have not yet been detected in B. anthracis rpoB mutants, although the corresponding positions in the D. radiodurans (G→A at position 1546) or E. coli (A→G at position 1547) rpoB gene are hotspots (see Fig. 3 and 4 and Table 4). We also decided to construct an additional rpoB mutant, A→G at position 1403, which naturally occurs among B. anthracis rpoB mutants, as a positive control.

The procedures for generating a single-base substitution in the rpoB gene are summarized as follows (Fig. 5). A DNA fragment of the B. anthracis rpoB gene (~2 kb in length, with the base pair targeted for the base substitution, e.g., A/T at position 1403, placed in the center) was subcloned into the cloning vector pCR2.1TOPO (Invitrogen). Then, a single-base substitution was introduced into the cloned rpoB gene fragment by site-directed mutagenesis using a QuickChange mutagenesis kit (Stratagene). In order to distinguish the base substitution we introduced from spontaneous occurrences of the same mutation, we also introduced a second silent point mutation, T→C at position 1426, that leads to TTA to CTA (both encode the amino acid residue leucine), as a tag. The rpoB gene fragment that contains the designed base substitution and the tag was subcloned into pUTE583 (Ery r), the same shuttle vector that we used for constructing gene knockouts. The plasmid DNA was prepared from a Dam E. coli strain and electroporated into B. anthracis wild-type cells. The Ery' transformants were selected and purified. The purified transformants were grown in the absence of antibiotic selection for several days to allow reciprocal recombination and subsequent loss of the shuttle vector. The colonies that were resistant to rifampin but sensitive to erythromycin were further studied.

We analyzed the frequency of Rif' and determined the nature of the base substitution mutations in the rpoB gene among those Rif' colonies (which also lost the shuttle vector). The results are shown in Table 5. The Rif' frequency for the positive control (A→G at position 1403), a naturally occurring site, was $10^{-9}$. Note that this frequency is generated by site-directed mutagenesis and is thus different from that for spontaneously occurring mutations. Among 19 Rif' colonies analyzed, all except 1 contained both A→G at position 1403 and the tag CTA, demonstrating that the procedure for making the site-directed rpoB mutation on the B. anthracis chromosome worked. The Rif' frequencies of the other two base substitution samples (intended for G→A at position 1411 and A→G at position 1412) are $0.4 \times 10^{-9}$ and $0.5 \times 10^{-9}$, respectively, which is about 20- to 25-fold lower than the Rif' frequency obtained from the positive control and even somewhat below the background spontaneous mutation frequency (see Table 2). Moreover, among 16 Rif' colonies analyzed, none of them contain the mutation we intended to introduce. Our results very strongly indicate that the specific base substitutions we

![FIG. 5. Schematic representation of the strategy for generating a single point mutation in the B. anthracis rpoB gene. After cloning of a portion of the rpoB gene (~2 kb in length), a single point mutation (A→G at position 1403) was introduced into the coding region of the rpoB gene along with a tag (T→C at position 1426, a silent mutation) by using the QuickChange mutagenesis kit (Stratagene). The wild-type rpoB DNA sequence on the chromosome was replaced by the mutated DNA sequence through reciprocal recombination. Gray boxes represent a portion of the rpoB gene. White boxes represent the original codons (CAG and TTA) in the wild-type rpoB gene. Black boxes represent the mutated codons (CGG and CTA) in the rpoB mutant.

<table>
<thead>
<tr>
<th>Intended base substitution</th>
<th>Rif' frequency (10^-9) (95% confidence limit)</th>
<th>No. of correct base substitutions/total no. of substitutions</th>
<th>Adjusted Rif' frequency (10^-9) (95% confidence limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A→G at 1403 (naturally occurring site)</td>
<td>10 (8.0–12.9)</td>
<td>18/19</td>
<td>9.5 (7.6–12.2)</td>
</tr>
<tr>
<td>G→A at 1411 (not yet detected)</td>
<td>0.4 (0.0–0.8)</td>
<td>0/8</td>
<td>0.03 (0.0–0.15)</td>
</tr>
<tr>
<td>A→G at 1412 (not yet detected)</td>
<td>0.5 (0.0–0.6)</td>
<td>0/8</td>
<td>0.03 (0.0–0.15)</td>
</tr>
</tbody>
</table>
made at positions 1411 and 1412 on the B. anthracis rpoB gene do not lead to Rifr in B. anthracis.

**DISCUSSION**

Recent advances in developing methods for recombinant shuttle vectors, for generating gene knockouts, and for using transposon mutagenesis in Bacillus anthracis (2, 3, 16, 26, 29; also T. Koehler, personal communication), as well as the knowledge of the complete B. anthracis genome sequence (25), now make it feasible to begin a serious program aimed at fully characterizing in vivo mutational pathways, mutational avoidance pathways, and DNA repair in this important microorganism. There is surprisingly little variation among B. anthracis isolates (13), but Keim and coworkers have used rare single-nucleotide polymorphisms and variable-number tandem repeats to develop forensic analyses crucial to aiding law enforcement and national security (13). Understanding the forces behind this low but visible diversity can only aid in continued refinement of forensic studies.

In this work, we created mutator strains by constructing the following knockout mutants: mutS, mutY, mutM, ndk, and the double knockouts mutY mutM and mutS ndk. Each corresponding mutant of E. coli displays a specific base substitution spectrum in the rpoB gene (reference 9 and unpublished data). We therefore utilized the rpoB gene of B. anthracis. The RNA polymerase β subunit, and particularly that portion involved in rifampin binding, is highly conserved in prokaryotes (22), and mutations conferring Rifr have been found in numerous microorganisms; in most cases, these affect positions in the protein corresponding to those found in E. coli (see references 9, 22, and 31 and references therein). We have developed an extensive rpoB/Riff mutation analysis system for both Escherichia coli (9, 32) and Deinococcus radiodurans (14). Fortunately, Vogler and coworkers have carried out a pilot study with B. anthracis and have shown that Rifr mutants of this organism indeed result from mutations at expected sites in the rpoB gene (31). They analyzed 22 spontaneous and 23 UV-induced mutations in an attenuated B. anthracis strain and found that they fell into 10 mutational sites, all within a small cluster, making them amenable to PCR amplification and sequencing with a single primer pair in each case. We have already extended this analysis here, using the B. anthracis Sterne 7702 strain, increasing the number of sites to 14.

We measured mutation rates in B. anthracis and compared the specificity of mutations with those seen in the rpoB gene in E. coli and in some cases in Deinococcus radiodurans (14). We supplemented this work by analyzing mutations generated with 5AZ and MNNG. The mutation rates for each mutator are depicted in Table 2. The increases relative to the wild type follow the same pattern as seen with E. coli (1, 9, 17, 20, 21, 23). Thus, mutators that are either mutM or mutY give small increases, whereas the double mutant mutY mutM gives very large increases. Strains that are mutS are strong mutators, and ndk results in a weak mutator, although it does enhance the effect of mutS when part of a mutS ndk double mutant. In all cases, the frequencies and rates are 5- to 20-fold lower than the corresponding values for E. coli. Moreover, we find that the rpoB spontaneous mutation rate in B. anthracis (1.6 × 10⁻⁹ per replication) is 10-fold lower than we measured for E. coli (20), Vogler and coworkers (31) first reported the rpoB rate in B. anthracis, by a different method, and also found it to be 1.6 × 10⁻⁹ per replication. We can show that this apparent lower level of mutagenesis, which is 10-fold less than that for E. coli rpoB, is primarily a result of fewer sites in rpoB that can mutate to yield viable rifampin-resistant colonies (14 found so far compared with almost 80 in E. coli). Table 4 compares the average rate per site in rpoB for E. coli, D. radiodurans, and B. anthracis, based on 77, 33, and 14 sites, respectively. These rates vary only from 1.1 × 10⁻¹⁰ per replication for B. anthracis to 1.9 × 10⁻¹⁶ per replication for both E. coli and D. radiodurans. Moreover, the average transition rate varies from 2.3 × 10⁻¹⁰ to 3.1 × 10⁻¹⁰ per replication, with that of B. anthracis slightly higher than that of E. coli. Interestingly, as Fig. 3 and 4 and Table 4 show, the same sites can have widely varying mutation rates in each of the three organisms, even when the surrounding base sequence is identical. For example sites I and V show 10-fold (or more)-higher rates of mutation in B. anthracis than in E. coli, despite very similar surrounding sequences, particularly for site I. Thus, hotspots are highly species specific and reflect interactions of polymerase, editing, and repair proteins with the mutational site and surrounding sequences. Each protein has its own inherent specificity with regard to sites and surrounding sequences.

Comparative genomics suggests the presence of auxiliary repair pathways for B. anthracis when we compare the B. anthracis genome with the B. subtilis genome in the category of DNA replication, recombination, and repair (see the introduction). Furthermore, when we compare the B. anthracis genome with the E. coli K-12 genome in the same gene category (http://www.ncbi.nlm.nih.gov/sutils/cogtik.cgi?gi=115&cog=L), we find that 17 COGs in B. anthracis, some with multiple orthologs, are absent from E. coli and 15 COGs in B. anthracis contain additional protein members (data not shown). B. anthracis appears to have additional DNA repair capabilities for UV-induced DNA damage (25) and several proteins that have detoxification functions (25). Investigations of their roles in mutation avoidance will be the subject of future studies.

**ACKNOWLEDGMENTS**

We thank Ken Bradley and Theresa Koehler for bacterial strains and plasmids and together with Paul Keim for helpful advice. This work was supported by a grant from the National Institutes of Health (ES010875).

**REFERENCES**

ERRATUM

Mutagenesis and Repair in *Bacillus anthracis*: the Effect of Mutators

Krystle Ziebell, Sharon Aguila, Vivian Yan Shi, Andrea Chan, Hanjing Yang, and Jeffrey H. Miller*

*Department of Microbiology, Immunology, and Molecular Genetics, and The Molecular Biology Institute, University of California, Los Angeles, California 90095*

Page 2333, Table 2, boxheads for columns 2 and 3: “(10^9)” should read “(10^9),”
Page 2335, Table 4, spanner for columns 2 through 4: “(10^9)” should read “(10^9).”