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Deletion of *dnaN1* generates a mutator phenotype in *Bacillus anthracis*

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Abstract

The *dnaN* gene in eubacteria is an essential gene that encodes the β subunit of replicative DNA polymerase. Nearly all eubacterial genomes sequenced to date predict a single copy of the *dnaN* gene in a well-conserved neighboring gene context. However, 19 genomes out of 348 scanned, including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*, predict more than one *dnaN* gene. In most cases, these genomes appear to maintain a copy of the *dnaN* homolog in its usual neighboring gene context (designated as *dnaN1*) in addition to a second copy (designated as *dnaN2*) in an entirely different gene context. We used *B. anthracis* as our model system to investigate the role of these DnaNs. We constructed a single knockout mutant of *dnaN1* and of *dnaN2*; however, we could not make a viable double knockout mutant of *dnaN1* and *dnaN2*. The *dnaN1* knockout mutant displays a markedly reduced colony size. It also displays a significantly increased mutation rate, which is similar to that of a mismatch repair deficient strain and to a strain deficient both in *dnaN1* and mismatch repair. The *dnaN2* knockout mutant, however, has a similar growth rate and a comparable mutation rate to that of the wild type. This is the first study demonstrating the existence of two functional DnaN homologs in the *B. anthracis* genome, with DnaN1 appearing to be more crucial than DnaN2. Our results also suggest the direct involvement of DnaN1 in the DNA mismatch repair process, which is consistent with previous findings.

1. Introduction

The *dnaN* gene of *Escherichia coli* encodes the β subunit of *E. coli* DNA polymerase III [1–4]. The β subunit dimerizes to form a ring-shaped sliding clamp, which encircles an intact duplex DNA and moves freely on it [5,6]. This ring-shaped topology is also observed in the homotrimer (or heterotrimer) of proliferating cell nuclear antigen (PCNA), a eukaryotic and archaeal counterpart of the β subunit, despite their unrelated sequences [7–11]. Owing to this unique topology, the sliding clamp acts as a processivity factor for the replicative DNA polymerase by tethering the core polymerase to its respective DNA template [5,12]. For example, in the presence of the β sliding clamp the DNA synthesis rate and the processivity of the *E. coli* core polymerase increases from less than 20 nucleotides s^{-1} to $1\text{ kb } s^{-1}$ and less than 10 base pairs to over 50 kb, respectively [2]. The high processivity of the replicative DNA polymerase is vital for survival.

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Recent studies have shown, however, that the function of the sliding clamp is not limited to serving as a subunit of the replicative DNA polymerase [1–4,13]. In *E. coli*, the β sliding clamp is found to also play a role in okazaki fragment maturation by interacting with DNA ligase and DNA polymerase I [14]. Additionally, it is also involved in various DNA repair processes by interacting with DNA polymerase II, translesion DNA polymerase IV and V [3,15–17], and DNA mismatch repair proteins MutS and MutL [14,15,18]. Studies also show that PCNA from eukaryotic replisome interacts with many protein components that are involved in DNA replication, repair and recombination, and cell cycle control [13,19]. In summary, the sliding clamp plays a significant role in the protein interaction network associated with various DNA processing activities.

DNA mismatch repair machinery is one of several important DNA repair systems that encounter errors arising during DNA replication. MutS and MutL are the two central protein components in mismatch repair in bacteria. MutS directly recognizes and binds to the mismatched base pair, which then recruits MutL to trigger the subsequent repair events [20]. López de Saro and O'Donnell first reported the involvement of β clamp in mismatch repair by demonstrating protein-protein interactions between β clamp and MutS [14]. Later, Dalrymple et al identified a pentapeptide motif (QL[SD]LF) that enables MutS and several other proteins to interact directly with the β clamp [15]. Recently, López de Saro et al examined in detail the interactions between MutS (MutL) and the β clamp. They showed that MutS and MutL with mutated β clamp binding sites are defective in both β clamp binding and mismatch repair [18]. They proposed that the β clamp is an essential player in orchestrating the sequence of events in this multi-step repair pathway in the cell.

The β sliding clamp is essential for cell viability. With the exception of several genomes that will be described below, only one gene encoding the β subunit homolog, *dnaN*, is predicted in nearly all eubacterial genomes that have been sequenced thus far [21]. Thus far, a viable *dnaN* knockout mutant has not been reported in the genomes with only one *dnaN* gene. However, point mutation mutants as well as temperature sensitive mutants of the β clamp have been constructed and studied, and have provided insights into β clamp structure and function [22–25]. In this work, we report that 19 genomes, including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*, predict more than one *dnaN* gene. We used *B. anthracis* as a model system to investigate the role of two annotated *dnaNs*, *dnaN1* and *dnaN2*. We were able to construct the *dnaN1* and *dnaN2* single knockout mutants in *B. anthracis*. We also determined the mutation rates and mutational specificities in the *dnaN1* and *dnaN2* knockout mutants. We discuss the relationship of the β clamp with regard to the DNA mismatch repair machinery.

2. Materials and methods

2.1 Phylogenetic study of *DnaN* homologs

The protein sequences of DnaN homologs were obtained from the BLAST database [26,27]. A single complete genome of each species was chosen in the study, although there are frequently multiple genomes from different subspecies available within the same species. A total of 348 genomes were analyzed in this study and 75 genomes within the phylum of Firmicutes were used in the phylogenetic analysis. The phylogenetic distribution of the predicted DnaN homologs within the phylum of Firmicutes was constructed using a multiple sequence alignment program ClustalW at the website www.ebi.ac.uk. To investigate the possible conservation of the location “context” for the additional copy (or copies) of the predicted *dnaN* genes from 19 bacterial genomes, the protein sequences encoded by the two open reading frames immediately upstream and the two open reading frames immediately downstream of the predicted *dnaN* gene were compared among 19 bacterial genomes.

2.2 Media and growth conditions

B. anthracis strains were grown non-selectively in LB medium. Erythromycin-resistant transformants were selected on LB agar plates supplemented with 5 µg/ml erythromycin. Spectinomycin or kanamycin resistant colonies were selected on LB agar plates supplemented with 100 µg/ml spectinomycin or 100 µg/ml kanamycin. All growth occurred at 37°C.

To measure the growth rate in liquid culture, a culture containing 2 ml LB medium with inoculation of a single colony was grown overnight in a 37°C incubator. Half milliliter of the overnight culture was used to inoculate 50 ml LB medium in a 500 ml flask. The culture was first warmed up for 30 min in a 37°C waterbath without agitation followed by 30 min on a 37°C shaker with agitation before any measurement was taken place. The optical density of the culture at 600 nm was measured at a 15-min interval for a period of four and half hours. The growth curve was generated by plotting the duration vs. the optical density at 600 nm on a semi-log scale. The doubling time was estimated within the linear range of the plot, which was typically expanded over 1.5 hour for the wild type and *dnaN2* knockout mutant, and 2.5 hour for the *dnaN1* knockout mutant. The doubling time of each strain was obtained as an average value calculated from two to seven independent experiments.

2.3 *B. anthracis* strain constructions

The protocols used for constructing single and double knockout mutants were as previously published [28–31]. The primer sequences are available upon request.

2.4 Detection of Rif^r mutants and base substitution spectrum in the *rpoB* gene

Mutation frequencies (*f*) were determined using the protocols as described previously [31]. Mutation rate (*μ*) per replication was calculated by the method of Drake [32]. The 95% confidence limits were determined following the guidelines dictated by the method of Dixon and Massey [33]. The procedures used for the detection of Rif^r mutants and determination of the base substitution spectrum in the *rpoB* gene were as previously published [31,34].

3. Results and discussion

3.1 Two β subunit homologs (*dnaN1* and *dnaN2*) in the *B. anthracis* genome

Nearly all eubacterial genomes sequenced to date predict only one gene (*dnaN*) that encodes the β subunit homolog of DNA polymerase. The *dnaN* locus is strongly linked to several other genes such as *dnaA*, *recF*, and *gyrB* [21]. This conservation of location “context” throughout many bacterial species infers the functional linkage between DnaN, DnaA, RecF and GyrB, all of which are involved in DNA replication process [35–37]. Interestingly, nineteen genomes out of 348 scanned predict more than one *dnaN* gene: a copy of a *dnaN* homolog in its usual gene context (in most cases), and a second copy of a *dnaN* homolog flanked by genes without any obvious conservation among most of the 19 genomes analyzed. The exceptions are two cyanobacteria genomes including *Anabaena variabilis* and *Nostoc sp.*, which do have a similar neighboring gene context surrounding the second predicted *dnaN* gene. Also, four bacillus genomes including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*, have a different conserved set of flanking genes. Examples of the gene-neighboring context of single *dnaN* genes from genomes of *Escherichia coli* and *Streptococcus pyogenes*, and multiple *dnaN* genes predicted from 19 genomes are shown in Figure 1. The four bacillus genomes, *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis*, predict two chromosomal copies of *dnaN* genes. They were designated as *dnaN1* and *dnaN2*, where *dnaN1* is linked to *dnaA*, *recF*, *gyrA* and *gyrB* (*B. cereus* genome predicts an additional *dnaN* gene, *dnaN3*, originated from a plasmid). We used *B. anthracis* as our model system to investigate the role of these DnaNs. We speculate that in *B. anthracis* DnaN1 may behave

similarly as DnaNs from *E. coli* and *S. pyogenes*, since DnaN1 is in the conserved *dnaN* gene surroundings.

We analyzed the sequence divergence between *B. anthracis* DnaN1 and DnaN2 through a phylogenetic analysis of the DnaN homologs of 75 complete genomes (with the exception of the *Bacillus weihenstephanensis*) that are in the order of Bacillales, Lactobacillales, Clostridia, and Mollicutes within the phylum of Firmicutes. It can be seen that DnaN homologs within each order are clustered together, including those DnaN1 homologs from the genomes of *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* (The only exception being a DnaN homolog from the *Symbiobacterium thermophilum* genome). However, DnaN2 homologs are not clustered with their corresponding DnaN1 homologs, but instead, are clustered together to form a separate clade (Figure 2).

The protein sequences of *B. anthracis* DnaN1 and DnaN2 were analyzed in detail with respect to that of *Streptococcus pyogenes* DnaN, and of *Escherichia coli* DnaN (both of which have a resolved crystal structure [6,39], Figure 3). *B. anthracis* DnaN1 and DnaN2 share 40% protein sequence identity amongst themselves. Both homologs also share a similar extent of sequence identity with *S. pyogenes* DnaN (39% and 33%, respectively). However, they are less homologous to DnaNs from gram-negative bacteria (25% and 24%, respectively, with *E. coli* DnaN). Highly conserved amino acid residues [39,40] are present in both *B. anthracis* DnaN1 and DnaN2 (Figure 3), suggesting that both proteins may be functional in the cell.

3.2 *B. anthracis* *dnaN1* and *dnaN2* single knockout mutants are viable

We asked whether DnaN1 or DnaN2 could function independently in *B. anthracis*. Since having at least one *dnaN* gene is essential, we investigated whether a single knockout mutant of *dnaN1* or *dnaN2* is viable in *B. anthracis*. We engineered two knockout constructs aimed at replacing the open reading frame of *dnaN1* or *dnaN2* with an antibiotic resistance encoding cassette for spectinomycin or kanamycin, respectively (Figure 4A and 4B). Using previously published protocols [31] we were able to obtain *dnaN1* and *dnaN2* single knockout mutants. The gene replacement in each knockout mutant was verified by PCR, indicating the absence of a *dnaN1* coding region in the *dnaN1* knockout mutant and the absence of a *dnaN2* coding region in the *dnaN2* knockout mutant (Figure 4C and 4D). The fact that both *dnaN1* and *dnaN2* single knockout mutants are viable indicates that DnaN1 and DnaN2 are capable of forming a functional β -sliding clamp in the absence of the other homolog in *B. anthracis*.

The colony size of the *dnaN2* knockout mutant is similar to that of the wild-type strain on a LB agar plate; however, a significantly reduced colony size was observed in the *dnaN1* knockout mutant (Figure 4E). The growth rate of *dnaN1* and *dnaN2* knockout mutants in the liquid culture was also assessed. The doubling time for wild type and *dnaN2* knockout mutant was 32.0 min and 30.5 min, respectively. However, the doubling time of *dnaN1* knockout mutant was 41.8 min, which was about 1/3 slower than that of the wild type and *dnaN2* (Figure 4F). Thus, *dnaN1* appears to be more crucial than *dnaN2*.

We failed to construct a *dnaN1* and *dnaN2* double mutant, despite the fact that we were able to make double knockout mutants with *dnaN1* and other genes such as *mutM* or *mutS* (data not shown). Failure to construct the *dnaN1* and *dnaN2* double knockout mutant strongly infers that the deletion of both *dnaN1* and *dnaN2* is lethal for *B. anthracis*.

3.3 DNA mismatch repair defect in *dnaN1* knockout mutant

Recent studies show that the β sliding clamp not only serves as a part of the DNA replication machinery to offer processivity to DNA replicative polymerases, but also physically interacts with many other proteins that are involved in different processes of DNA metabolism [13]. We

focused on the role of the β sliding clamp in the DNA mismatch repair process. It has been proposed that the β sliding clamp orchestrates the sequence of events that leads to mismatch repair in the cell [18]. We asked whether deletion of *dnaN1* or *dnaN2* affects mutation rate in *B. anthracis*. Cultures of the two knockout mutant strains and a wild-type strain were grown in LB liquid medium and were determined for the mutation levels in the *rpoB* gene leading to the Rif^r phenotype. Our results indicate that the *dnaN2* knockout strain and the wild-type strain have similar levels of spontaneous *rpoB* mutations (frequencies of 3.8×10^{-9} and 3.0×10^{-9} , respectively; and rates per replication of 2.4×10^{-9} and 2.0×10^{-9} , respectively), which is similar to our previously published work [31]. The *dnaN1* knockout strain, however, has a spontaneous *rpoB* mutation frequency of 95 times that of the wild type and a mutation rate of 29 times that of the wild type (Table 1).

To eliminate the possibility that the mutator phenotype observed in the *dnaN1* knockout mutant resulted from an unrelated mutation introduced into the genome during the process of constructing the *dnaN1* knockout mutant, we constructed an independent *dnaN1* knockout mutant in which the *dnaN1* open reading frame was replaced by a kanamycin resistance encoding cassette. In this *dnaN1* knockout mutant, a similar growth phenotype and mutation rate was observed (data not shown), which supports the idea that the mutator phenotype observed in the *dnaN1* knockout mutant must indeed be attributed to the absence of *dnaN1*.

We asked whether (1) an increased level of replication errors generated by DNA polymerase or (2) reduced efficiency of DNA mismatch repair system causes this apparent mismatch repair deficiency phenotype observed in the *dnaN1* knockout mutant. To distinguish between these two possibilities, we constructed a double-knockout mutant of *dnaN1* and *mutS*, and measured the mutation rate in this double knockout mutant. If the first possibility were correct, a significantly increase in mutation rate would be observed in the *dnaN1*, *mutS* double knockout strain. On the other hand, if the second possibility were correct, a mutational rate similar to that of the mismatch repair deficient strain would be observed. The results are shown in Table 1. The level of the mutation rate in the double knockout strain was closely to that of the mismatch repair deficient strain. Our results indicate that in the absence of *dnaN1*, the mismatch repair efficiency is significantly reduced. This finding suggests that DnaN1 plays an essential role in DNA mismatch repair in *B. anthracis*.

Our previous work has shown that the base substitution spectrum in the *rpoB* gene leading to the Rif^r phenotype contains distinct hotspots for each mutator [31] and is therefore a useful diagnostic tool in the analysis of mutational pathways responsible for the apparent mutator phenotypes. We investigated the base substitution spectrum of the *rpoB* gene in the *dnaN1* knockout mutant background. The results are shown in Table 2. It can be observed that the mutations in the *rpoB* gene are dominated by two A→G hotspots at 1403 bp and 1442 bp, which is similar to the fingerprint of a mismatch repair deficient strain [31].

This is the first study demonstrating the existence of two functional DnaN homologs in the *B. anthracis* genome, with DnaN1 appearing to be more crucial than DnaN2. Our results suggest the direct involvement of DnaN1 in the DNA mismatch repair process, which is consistent with previous findings [18]. A possible explanation for the growth and mismatch repair deficiency in *dnaN1* knockout strain is that DnaN2 homolog functions much less efficiently than DnaN1. We speculate that the rate of DNA replication and the rate of DNA mismatch repair process are reduced in the *dnaN1* mutant, which contribute to decreased growth and increased mutation rate. However, the fidelity of the replicative DNA polymerase apparently remains unchanged. Further biochemical analysis is necessary to address the phenotypical difference between DnaN1 and DnaN2 at the molecular level. The *B. anthracis* *dnaN1* knockout mutant can serve as a valuable model system for studying other β clamp related DNA processing pathways.

Nineteen genomes predict more than one *dnaN* gene (Figure 1). It is unclear whether these additional *dnaN* genes are vestigial remnants from the past or the landmarks of specific horizontal gene transfer events. Whether these *dnaN* genes behave similarly to the *B. anthracis* *dnaN1* and *dnaN2* remains to be studied. The presence of the *dnaN2* gene in the four bacillus genomes, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*, suggests that these species were closely related during evolution [41].

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References

1. Wijffels G, Dalrymple B, Kongsuwan K, Dixon NE. Conservation of eubacterial replicases. *IUBMB Life* 2005;57:413–419. [PubMed: 16012050]
2. Johnson A, O'Donnell M. Cellular DNA replicases: components and dynamics at the replication fork. *Annu Rev Biochem* 2005;74:283–315. [PubMed: 15952889]
3. Lehmann AR. Clubbing together on clamps: The key to translesion synthesis. *DNA Repair (Amst)* 2006;5:404–407. [PubMed: 16427367]
4. Pomerantz RT, O'Donnell M. Replisome mechanics: insights into a twin DNA polymerase machine. *Trends Microbiol* 2007;15:156–164. [PubMed: 17350265]
5. Stukenberg PT, Studwell-Vaughan PS, O'Donnell M. Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. *J Biol Chem* 1991;266:11328–11334. [PubMed: 2040637]
6. Kong XP, Onrust R, O'Donnell M, Kuriyan J. Three-dimensional structure of the beta subunit of *E. coli* DNA polymerase III holoenzyme: a sliding DNA clamp. *Cell* 1992;69:425–437. [PubMed: 1349852]
7. Krishna TS, Fenyo D, Kong XP, Gary S, Chait BT, Burgers P, Kuriyan J. Crystallization of proliferating cell nuclear antigen (PCNA) from *Saccharomyces cerevisiae*. *J Mol Biol* 1994;241:265–268. [PubMed: 7914545]
8. Gulbis JM, Kelman Z, Hurwitz J, O'Donnell M, Kuriyan J. Structure of the C-terminal region of p21 (WAF1/CIP1) complexed with human PCNA. *Cell* 1996;87:297–306. [PubMed: 8861913]
9. Matsumiya S, Ishino Y, Morikawa K. Crystal structure of an archaeal DNA sliding clamp: proliferating cell nuclear antigen from *Pyrococcus furiosus*. *Protein Sci* 2001;10:17–23. [PubMed: 11266590]
10. Dore AS, Kilkenny ML, Jones SA, Oliver AW, Roe SM, Bell SD, Pearl LH. Structure of an archaeal PCNA1-PCNA2-FEN1 complex: elucidating PCNA subunit and client enzyme specificity. *Nucleic Acids Res* 2006;34:4515–4526. [PubMed: 16945955]
11. Williams GJ, Johnson K, Rudolf J, McMahon SA, Carter L, Oke M, Liu H, Taylor GL, White MF, Naismith JH. Structure of the heterotrimeric PCNA from *Sulfolobus solfataricus*. *Acta Crystallograph Sect F Struct Biol Cryst Commun* 2006;62(Pt 10):944–948.
12. Lamers MH, Georgescu RE, Lee SG, O'Donnell M, Kuriyan J. Crystal structure of the catalytic alpha subunit of *E. coli* replicative DNA polymerase III. *Cell* 2006;126:881–892. [PubMed: 16959568]
13. Vivona JB, Kelman Z. The diverse spectrum of sliding clamp interacting proteins. *FEBOS Lett* 2003;546:167–172.
14. López de Saro FJ, O'Donnell M. Interaction of the beta sliding clamp with MutS, ligase, and DNA polymerase I. *Proc Natl Acad Sci U S A* 2001;98:8376–8380. [PubMed: 11459978]
15. Dalrymple BP, Kongsuwan K, Wijffels G, Dixon NE, Jennings PA. A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems. *Proc Natl Acad Sci U S A* 2001;98:11627–11632. [PubMed: 11573000]
16. Becherel OJ, Fuchs RP, Wagner J. Pivotal role of the beta-clamp in translesion DNA synthesis and mutagenesis in *E. coli* cells. *DNA Repair (Amst)* 2002;1:703–708. [PubMed: 12509274]
17. Burnouf DY, Olieric V, Wagner J, Fujii S, Reinbolt J, Fuchs RP, Dumas P. Structural and biochemical analysis of sliding clamp/ligand interactions suggest a competition between replicative and translesion DNA polymerases. *J Mol Biol* 2004;335:1187–1197. [PubMed: 14729336]

18. López de Saro FJ, Marinus MG, Modrich P, O'Donnell M. The beta sliding clamp binds to multiple sites within MutL and MutS. *J Biol Chem* 2006;281:14340–14349. [PubMed: 16546997]
19. Maga G, Hübscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 2003;116(Pt 15):3051–3060. [PubMed: 12829735]
20. Iyer RR, Pluciennik A, Burdett V, Modrich PL. DNA mismatch repair: functions and mechanisms. *Chem Rev* 2006;106:302–323. [PubMed: 16464007]
21. Bruck I, O'Donnell M. The ring-type polymerase sliding clamp family. *Genome Biol* 2001;2:REVIEWS3001. [PubMed: 11178284]
22. Sakakibara Y, Mizukami T. A temperature-sensitive *Escherichia coli* mutant defective in DNA replication: *dnaN*, a new gene adjacent to the *dnaA* gene. *Mol Genet* 1980;178:541–553.
23. Duzen JM, Walker GC, Sutton MD. Identification of specific amino acid residues in the *E. coli* beta processivity clamp involved in interactions with DNA polymerase III, UmuD and UmuD'. *DNA Repair (Amst)* 2004;3:301–312. [PubMed: 15177045]
24. Sutton MD, Duzen JM, Maul RW. Mutant forms of the *Escherichia coli* beta sliding clamp that distinguish between its roles in replication and DNA polymerase V-dependent translesion DNA synthesis. *Mol Microbiol* 2005;55:1751–1766. [PubMed: 15752198]
25. Sutton MD, Duzen JM. Specific amino acid residues in the beta sliding clamp establish a DNA polymerase usage hierarchy in *Escherichia coli*. *DNA Repair (Amst)* 2006;5:312–323. [PubMed: 16338175]
26. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402. [PubMed: 9254694]
27. Cummings L, Riley L, Black L, Souvorov A, Resenchuk S, Dondoshansky I, Tatusova T. Genomic BLAST: custom-defined virtual databases for complete and unfinished genomes. *FEMS Microbiol Lett* 2002;216:133–138. [PubMed: 12435493]
28. Koehler TM, Dai Z, Kaufman-Yarbray M. Regulation of the *Bacillus anthracis* protective antigen gene: CO2 and a trans-acting element activate transcription from one of two promoters. *J Bacteriol* 1994;176:586–595. [PubMed: 8300513]
29. Saile E, Koehler TM. Control of anthrax toxin gene expression by the transition state regulator *abrB*. *J Bacteriol* 2002;184:370–380. [PubMed: 11751813]
30. Chen Y, Tenover FC, Koehler TM. Beta-lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. *Antimicrob Agents Chemother* 2004;48:4873–4877. [PubMed: 15561870]
31. Ziebell K, Aguila S, Yan Shi V, Chan A, Yang H, Miller JH. Mutagenesis and repair in *Bacillus anthracis*: the effect of mutators. *J Bacteriol* 2007;189:2331–2338. [PubMed: 17220233] [Erratum in: *J. Bacteriol.* 189 (2007) 3932]
32. Drake JW. A constant rate of spontaneous mutation in DNA-based microbes. *Proc Natl Acad Sci USA* 1991;88:7160–7164. [PubMed: 1831267]
33. Dixon, WJ.; Massey, FJ, Jr. Introduction to statistical analysis. McGraw-Hill; New York, NY: 1969.
34. Garibyan L, Huang T, Kim M, Wolff E, Nguyen A, Nguyen T, Diep A, Hu K, Iverson A, Yang H, Miller JH. Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. *DNA Repair (Amst)* 2003;2:593–608. [PubMed: 12713816]
35. Dandekar T, Snel B, Huynen M, Bork P. Conservation of gene order: a fingerprint of proteins that physically interact. *Trends Biochem Sci* 1998;23:324–328. [PubMed: 9787636]
36. Overbeek R, Fonstein M, D'Souza M, Pusch GD, Maltsev N. The use of gene clusters to infer functional coupling. *Proc Natl Acad Sci U S A* 1999;96:2896–2901. [PubMed: 10077608]
37. Eisenberg D, Marcotte EM, Xenarios I, Yeates TO. Protein function in the post-genomic era. *Nature* 2000;405:823–826. [PubMed: 10866208]
38. Bessman MJ, Frick DN, O'Handley SF. The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. *J Biol Chem* 1996;271:25059–25062. [PubMed: 8810257]
39. Argiriadi MA, Goedken ER, Bruck I, O'Donnell M, Kuriyan J. Crystal structure of a DNA polymerase sliding clamp from a Gram-positive bacterium. *BMC Struct Biol* 2006;6:2. [PubMed: 16403212]

40. Neuwald AF. Evolutionary clues to DNA polymerase III beta clamp structural mechanisms. *Nucleic Acids Res* 2003;31:4503–4516. [PubMed: 12888511]
41. Huang J, Gogarten JP. Ancient horizontal gene transfer can benefit phylogenetic reconstruction. *Trends Genet* 2006;22:361–366. [PubMed: 16730850]
42. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680. [PubMed: 7984417]

asterisks mark the genomes, in which the predicted *dnaN* genes appear to be not linked to *dnaA*, *recF*, *gyrA*, or *gyrB*.

Figure 2.

Phylogenetic distribution of DnaN protein sequences from 75 genomes within the phylum of Firmicutes. The clades of DnaN1 and DnaN2, and DnaN3 (originated from a plasmid) are indicated. The number of genomes analyzed in each order is indicated in the parentheses. The asterisk marks the genome that belongs to the order of *Lactobacillales*.

Figure 4.

B. anthracis *dnaN1* and *dnaN2* knockout mutants. A. Strategy for generating the *dnaN1* null allele. The location for each primer E1, F1, K, L, and SP3 is as marked. B. Strategy for generating the *dnaN2* null allele. The location for each primer E2, F2, M, N, and Kan2 is as marked. C. PCR analysis of genomic DNA isolated from wild type, *dnaN1* and *dnaN2* knockout mutants using primer set E1-F1 (for wild-type *dnaN1*), K-L (for wild-type *dnaN1*), and E1-SP3 (for Ω -sp cassette). D. PCR analysis of genomic DNA isolated from wild type, *dnaN1* and *dnaN2* knockout mutants using primer set E2-F2 (for wild-type *dnaN2*), M-N (for wild-type *dnaN2*), and E2-Kan2 (for Ω -km cassette). E. Photograph of LB agar plates streaked

with wild type, *dnaN1* knockout, and *dnaN2* knockout mutant, after an overnight growth at 37°C.

Table 1*B. anthracis rpoB* mutation frequencies (*f*) and rates (*μ*)

Genotype	<i>f</i> ($\times 10^9$) ^a	<i>μ</i> ($\times 10^9$) ^b
wild type	3.8 (1.1 – 7.7)	2.4 (1.2–3.8)
<i>dnaN1</i>	360 (290 – 480)	70 (59–90)
<i>dnaN2</i>	3.0 (0.92 – 19)	2.0 (1.0–7.0)
<i>mutS</i>	740 (670 – 810)	140 (130–150)
<i>dnaN1 mutS</i>	700 (590 – 820)	120 (110–140)

^aValues in parentheses are 95% confidence limits [33].

^bMutation rates, *μ*, are calculated by the method of Drake [32].

Table 2
Distribution of mutations leading to Rif^r in *B. anthracis rpoB* gene

<i>B. anthracis</i> site (bp)	Amino acid change	Base-pair change	Spontaneous ^a	duaN1	<i>mutS</i> ^b
1403	Q454R	AT→GC	20	13	19
1442	H467R	AT→GC	30	30	20
1441	H467Y	GC→AT	33	2	1
1391	S450F	GC→AT	0	0	0
1457	S472F	GC→AT	1	0	0
1457	S472Y	GC→TA	1	0	0
1402	Q454K	GC→TA	4	1	0
1403	Q454L	AT→TA	0	0	0
1417	T459S	AT→TA	0	0	0
1444	K468Q	AT→CG	0	0	0
1442	H467P	AT→CG	1	0	0
1391	S450C	GC→CG	0	0	0
1441	H467D	GC→CG	0	1	0
1600	S453C	GC→CG	0	0	0
Total 14			90	47	40

^aIncludes data from ref. 31.
^bFrom ref. 31.