Supporting Information

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Gene Knockout Analysis

Escherichia coli. Primers used in the methods to follow are shown in Table S1. The lambda red system for gene replacement by homologous recombination was performed using the DH12S strain. The protocol was as follows: The kanamycin resistance (KanR) cassette was amplified in two steps (primers Kan1/Kan2, and then RnhB1/RnhB2) that, in succession, resulted in a duplex product (60KamR65) bracketed with 60 (5′) and 65 (3′) bp of homology to the region to be deleted in *rnhB*. The first round of PCR with primers Kan1 and Kan2 and plasmid PET28a-LIC as template produced the KanR cassette bracketed by about 30 bp of homology to *rnhB*. The second round of PCR used primers EcRnhB1 and EcRnhB2 as a template the product of previous PCR. The resulting PCR product, 60KamR65, contains sequences homologous to *rnhB* flanking the KamR cassette that contain 60 bp *rnhB* homology at the 5′ end and 65 bp at the 5′ end, which are sufficient lengths for efficient recombination. The 60KamR65 PCR product was introduced into strain DH12S harboring plasmid pKD46 via electroporation. Recombination between short homologous sequences of the construct 60KamR65 and the chromosome were facilitated by inducible λ phage proteins encoded by pKD46. Recombinant clones were selected on LB-agar plates supplemented with 50 μg/mL kanamycin. The RepA protein encoded by plasmid pKD46 is thermosensitive, and thus colonies were grown at 37 °C to eliminate the pKD46 plasmid. The desired deletion in *rnhB* was confirmed by sequencing of the PCR fragment obtained using primers EcRnhBdel1 and EcRnhBdel2, adjacent to the recombination sites. As the result of recombination, the *rnhB* sequence encoding amino acids from 21 to 108 of RNaseH II was substituted for the KanR cassette. This deleted region of RNase HII contains the active center and thus should inactive RNase HII while having minimal effect on the surrounding areas of the chromosome. The resulting strain is DH12SΔ*rnhB*:KmR.

A similar protocol was applied to disrupt the *mutH* gene in both strains DH12S and DH12SΔ*rnhB*:KmR.

The construct containing a chloramphenicol resistance (CamR) cassette bracketed with sequences homologous to *mutH* was assembled as follows: The CamR cassette was amplified from chromosomal DNA from *E. coli* strain ER2925, using primers Cm1 and Cm2. Two additional rounds of PCR were performed to extend the PCR product of the CamR cassette with a sufficient length of terminal sequence homologous to the desired region to be deleted/substituted into the *mutH* gene. Primers CmMutH1 and CmMutH2 were used in the first round, and primers MutH1 and MutH2 were used in the second round. The resulting PCR product, 50CamR57, contained the Cam cassette bracketed with 50 bp homologous to *mutH* on the 5′ end and 57 bp on 3′ end. The PCR product, 50CamR57, was introduced into either strain DH12S or DH12SΔ*rnhB*:KmR, harboring plasmid pKD46 encoding for proteins of the lambda RED recombination system via electroporation. Recombinant clones were selected on LB plates supplemented with 30 μg/mL chloramphenicol at 37 °C. Strains containing the desired *mutH* deletion were confirmed by sequencing the PCR obtained using primers EcMutHdel1 and EcMutHdel2, which are adjacent to recombination sites. The recombinant substitutes the CamR cassette for amino acids 16–140 of the MutH protein, which contains the active center of the enzyme. The resulting strains were DH12SΔ*mutH* and DH12SΔ*rnhBmutH*.

The mutation frequencies in the strains were determined by measuring the frequency of rifampicin resistance (Rif^R) mutations. For each *E. coli* strain (DH12S, DH12SΔ*rnhB*, DH12SΔ*mutH*, and DH12SΔ*rnhBmutH*), 25 cultures were seeded with 10^4 cells from different colonies and grown overnight. The cultures were titrated and plated on LB to determine the cell titer in the culture and on LB+25 mg/L rifampicin to select for rifampicin-resistant mutants. Spontaneous mutation frequencies were calculated from the median number of Rif^R cells present in each of the 25 cultures and dividing by the total number of cells in the corresponding cultures (1).

Bacillus subtilis. The strains and primers used for this protocol are found in Tables S2 and S3. Plasmid pJS40 was used to cleanly delete the *rhnB* locus from *B. subtilis.* The 680 bases upstream (primers oJS262 and 263) of *rnhB* and the 550 bases downstream (primers oJS264 and 265) of *rnhB* were amplified, generating the products PCR1 and PCR2, respectively. PCR1 was digested with SalI and BamHI, and PCR2 was digested with BamHI and KpnI, followed by simultaneous ligation of both inserts into pMiniMAD2 (2) digested with SalI and KpnI. Deletion of the *rnhB* locus with pJS40 was then performed as described (2), and loss of *rnhB* was confirmed by diagnostic PCR using primers oJS280 and oJS281, demonstrating loss of the *rnhB* coding region. The resulting Δ*rnhB* strain is JWS105.

Plasmid pJS81 was built to disrupt the *rnhC* locus by single-crossover integration of pJS81 into the WT strain PY79 at the *rnhC* locus. pJS81 was prepared by PCR amplification of the first 250 bases of the *rnhC* gene with SalI and HindIII engineered in the primers (oJS300 and oJS301). The PCR product was then digested and ligated into pJL74 digested with the same restriction endonucleases (3). This generated plasmid pJS81, which was subsequently used to transform WT *B. subtilis* strain PY79, followed by selection for *spc* resistance (100 μg/mL). The resulting strain is JWS107 (*rnhC::spc*).

A strain bearing a clean deletion of the *mutSL* operon was generated using plasmid pJS66. To construct pJS66, the 500 bases upstream (primers oJS272 and 273) and 500 bases downstream (oJS274 and 275) of *mutSL* were PCR amplified, generating PCR3 and PCR4. PCR3 was digested with SalI and BamHI, whereas PCR4 was digested with BamHI and KpnI. The resulting inserts were simultaneously ligated into the SalI and KpnI sites of pMiniMAD2 digested with the same restriction endonucleases. Plasmid pJS66 was used to transform PY79, followed by screening for loss of the plasmid and deletion of the target locus as described (2). Deletion of the *mutSL* coding region was confirmed by diagnostic PCR amplification of the flanking regions using the following primers: oJS298 and 299. The resulting Δ*mutSL* strain is JWS108.

To build a strain lacking both *rnhB* and *rnhC* activities, we grew JWS105 to competence, followed by transformation with genomic DNA isolated from JWS105, using standard procedures (4). Transformants were selected on LB agar with 100 μg/mL spectinomycin, and the resulting strain (JWS152) was colony purified before use.

Mutation rates were determined essentially as described, applying the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method (5). A single colony of the strains indicated in Fig. 6 was grown in 3 mL LB medium at 37 °C to late-exponential phase growth (OD_{600} ~0.7–1.0). The culture was diluted 1,000-fold into fresh LB medium, from which 3-mL aliquots were transferred each to several tubes. These cultures were allowed to grow at 37 °C to OD_{600} ~1.0–1.2. Cultures were then plated onto LB agar plates containing 150 μg/mL rifampin to select for

mutations in the *rpoB* gene, and dilutions were plated onto LB agar medium without antibiotic to determine the number of viable cells in each culture. The Web tool FALCOR (6) was then used to estimate mutations per culture, and this estimate was then adjusted for plating efficiency. Mutation rate per generation was then calculated as mutations per culture divided by total viable cells per culture. Growth rate of *B. subtilis* strains were conducted by inoculating cultures in LB at a starting OD_{600} = 0.05. Growth was carried out at 37 °C with constant agitation. Culture density was measured in a klett meter at regular intervals, and data were fit using the splines option in the R package grofit (7).

1. Rosche WA, Foster PL (2000) Determining mutation rates in bacterial populations. *Methods* 20(1):4–17.
2. Patrick JE, Kearns DB (2008) MinJ (YvjD) is a topological determinant of cell division in Bacillus subtilis. *Mol Microbiol* 70(5):1166–1179.
3. LeDeaux JR, Yu N, Grossman AD (1995) Different roles for KinA, KinB, and KinC in the initiation of sporulation in Bacillus subtilis. *J Bacteriol* 177(3):861–863.
4. Hardwood CR, Cutting SM (1990) *Molecular Biological Methods for Bacillus* (*Modern Microbiological Methods*) (John Wiley & Sons, Hoboken, New Jersey).
5. Bolz NJ, Lenhart JS, Weindorf SC, Simmons LA (2012) Residues in the N-terminal domain of MutL required for mismatch repair in Bacillus subtilis. *J Bacteriol* 194(19): 5361–5367.
6. Hall BM, Ma CX, Liang P, Singh KK (2009) Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis. *Bioinformatics* 25(12):1564–1565.
7. Kahm M, Hasenbrink G, Lichtenberg-Frate H, Ludwig J, Kschischo M (2010) grofit: Fitting Biological Growth Curves with R. *J Stat Softw* 33(7):1–21.

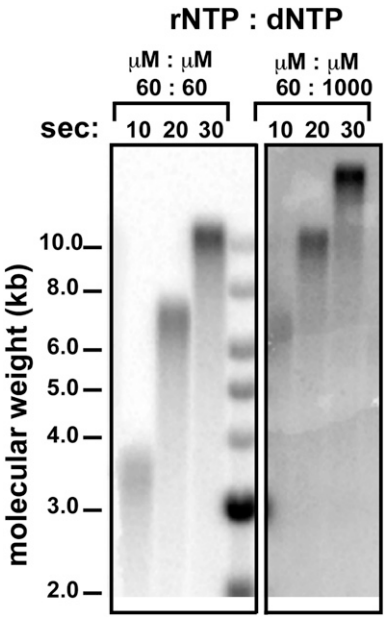

Fig. S1. Additional dNTPs partially reverse the ribonucleoside triphosphate (rNTP)-mediated decrease in replisome rate. Leading strand replication reactions using the synthetic rolling circle substrate were as described in *Experimental Procedures*, with the following exceptions: Each rNTP was present at 60 μM, and reactions contained either 60 μM each dNTP or 1,000 μM each dNTP.

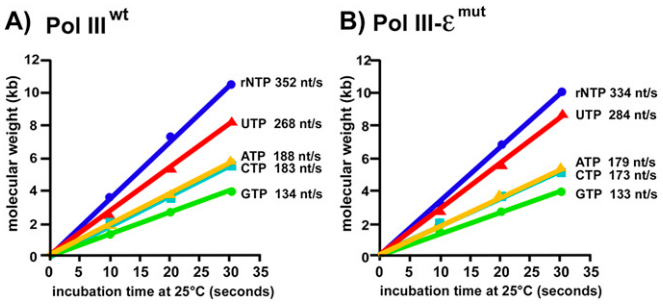

Fig. S2. Exonuclease deficient polymerase (Pol) III*-β is slowed by rNTPs to same extent as WT Pol III-β. Experimental conditions are as described for leading strand replication in *Experimental Procedures*, except reactions used either 7.32 nM WT Pol III* (*A*) or 7.32 nM exonuclease mutant Pol III* (*B*).

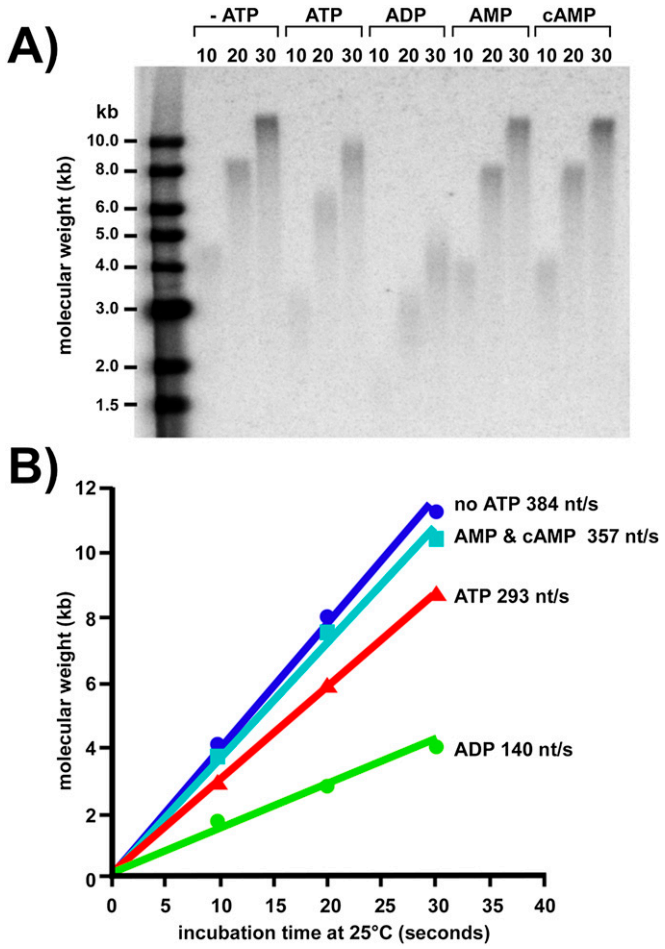

Fig. S3. Comparison of adenine nucleotides in replication inhibition. Coupled leading/lagging strand replication reactions using the synthetic rolling circle substrate was performed as described in *Experimental Procedures*, with the following exceptions: reactions contained either no added rNTP or 2 mM of ATP, ADP, AMP, or cAMP. *A* is an alkaline gel of the products, *B* shows the rate of synthesis obtained from the alkaline gel.

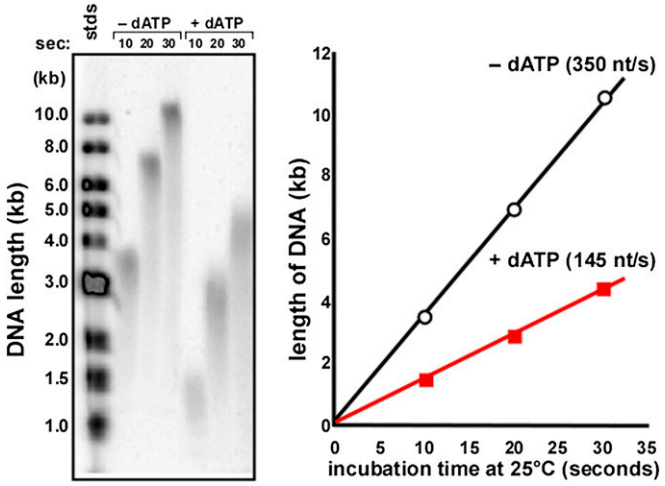

Fig. S4. dATP pool imbalance slows down the replisome similar to an rATP pool imbalance. Leading strand replication reactions are performed as described in *Experimental Procedures*, except no rNTPs are added and the dATP concentration is elevated to 3 mM.

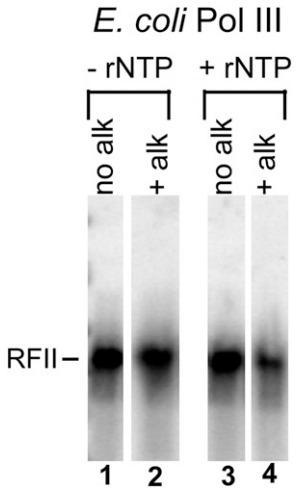

Fig. S5. Cleavage of rNMP residues within DNA requires pretreatment with 300 mM alkali. Reactions were performed using either dNTPs or dNTPs with a mixture of 0.25 mM each rNTP (1 mM total). Reactions were divided, and one half was pretreated with 300 mM NaOH (alk), as described in *Experimental Procedures*; the other half was treated the same but without NaOH. Reactions were then analyzed in an alkaline agarose gel. Relevant lanes from a single gel analysis are shown. Percentage of full-length replicative form II (RFII) product for lanes 1–4 are 100%, 116% 104%, and 62%.

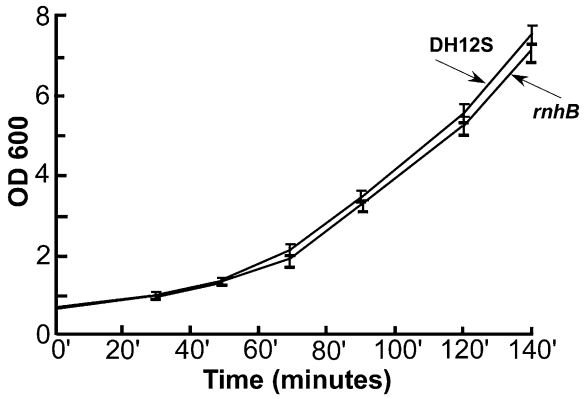

Fig. S6. Growth rates of the WT strain DH12S and the DH12SΔ*rnhB* mutant are similar. Cells from 10 saturated cultures (5 cultures of each strain) were diluted 30 times, giving an OD of 0.066 +/– 0.003. Timed aliquots were initiated on cultures reaching an OD600 of 0.75 (time 0′ in the plot). The OD600 at each time is the average of 5 cultures.

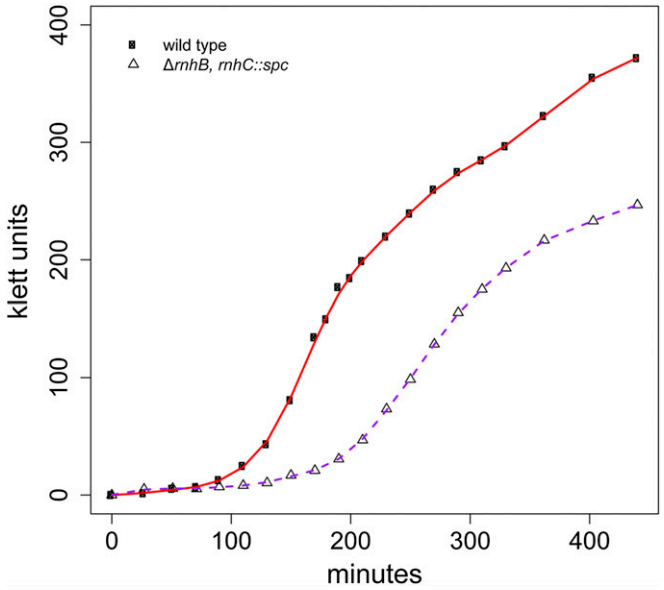

Fig. S7. Loss of *rnhB* and *rnhC* impairs growth. Growth of the indicated strains of *B. subtilis* was monitored at 37 °C. Growth curves were fit with the grofit package in R.

Table S1. Primers used for *E. coli rnhB* and *mutH* deletion strains

Primer	Sequences from 5′ to 3′
Kan1	GGTTGCGGGTGTGGATGAAGTCGGACGCGGGTCACGTTAAGGGATTTTGGTCATGAAC
Kan2	AGCTGCGCCGATGGTGTTCGGTCGCGCCGTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
EcRnhB1	ATCGAATTTGTTTATCCGCACACGCAGCTGGTTGCGGGTGTGGATGAAGTCGGACGCGGG
EcRnhB2	GGACGCAAGTCCCAGTGCGCGTTTGACAGGCCCAAAGCTGCGCCGATGGTGTTCGGTCGCGCCGT
EcRnhBdel1	ATCGAATTTGTTTATCCGCACACGCA
EcRnhBdel2	GGACGCAAGTCCCAGTGCGCGTTTGAC
Cm1	TGAGACGTTGATCGGCACGTAAGAGG
Cm2	TCACTTATTCAGGCGTAGCAACCAGG
CmMutH1	CTCTCCTCCCGAAACTGAAGAACAGTTGTTGAGACGTTGATCGGCACGTAAG
CmMutH2	CACCAATGGCTTCGGTAAGCGCTTTCGTCACTTATTCAGGCGTAGCAACCAGG
MutH1	ATGTCCCAACCTCGCCCACTGCTCTCTCCTCCCGAAACTGAAGAACAG
MutH2	GTGGCAGCGTCAGAATCCGTTCGCCCCGGGCACCAATGGCTTCGGTAAGCGC
EcMutHdel1	CTTCAAATAAAAGCCGCGTGGCAGCGTCAGAATCCGTTCG
EcMutHdel2	GTTTTTTAATCAAGGTATCATGACATGTCCCAACCTCGCCCACTGC

Table S2. List of strains for *B. subtilis* mutagenesis

Strain	Relevant genotype
JWS010	PY79 (WT) SPβ°
JWS105	*ΔrnhB*
JWS107	*rnhCρspec*
JWS152	*ΔrnhB, rnhCρspec*
JWS108	*ΔmutSL*

Table S3. List of primers for *B. subtilis* mutagenesis

Primer	Sequence	Purpose
oJS262	GCGGTCGACGATTATCGGCATTCCAAACGTCGG	Deleting *rnhB*
oJS263	GCGGGATCCTTTCTCTTCTCCCTTACTGTATGTATCGG	Deleting *rnhB*
oJS264	GCGGGATCCTAAATCACCATGGACAAGGAGGC	Deleting *rnhB*
oJS265	GCGGGTACCAATGGGAAGATCACGCTTTAAAGC	Deleting *rnhB*
oJS272	GCGGTCGACATGATATAAACGTATGGTACTCTTACGCG	Deleting *mutSL*
oJS273	CGCGGATCCTGTTTAATCCCTCACTATGTATCAACGTTTC	Deleting *mutSL*
oJS274	GCGGGATCCTAGCGGGGGTGGTAGGC	Deleting *mutSL*
oJS275	CGCGGTACCGTCTATTTATATTCCAGATGTCTTGCTTGTTGTG	Deleting *mutSL*
oJS280	TGTTTGGTCTTCGTTTTCTCGAAGAAC	PCR screen for loss of *rnhB* locus
oJS281	AGACGGCCTGTTTGCTCTG	PCR screen for loss of *rnhB* locus
oJS298	AGTTGTTGAGGTCAATCCTGACTGG	PCR screen for loss of *mutSL* locus
oJS299	ATCATCAAGTTCATCCAAAAAGATAATGATCTTCG	PCR screen for loss of *mutSL* locus
oJS300	GCGGTCGACGTGTCCCATTCAGTGATAAAAGTATCGTTG	Disrupting *rnhC*
oJS301	CGCAAGCTTGGGGATCAGCCGGCTTTTTG	Disrupting *rnhC*