FILE S1. SUPPLEMENTAL MATERIAL AND METHODS

Bacterial strains and media: The bacterial strains used and the methods of their construction are given in Supplemental Table S1. All strains used in MA experiments are derivatives of *E. coli* K12 strain PFM2 (LEE *et al.* 2012). Strains constructed by P1 phage transduction (MILLER 1992) from the Keio knockout strains (BABA *et al.* 2006), followed by FLP recombination to remove the Kn^R element, have an in-frame scar sequence (DATSENKO AND WANNER 2001). Out of concern that these scars could promote recombination during the MA experiments, we used recombineering with the *cat*-I-SceI cassette (BLANK *et al.* 2011) to construct some of the strains. These "clean" deletions were then moved by P1 transduction into recipient strains using nearby genes with selectable phenotypes. However, we did not observe any such recombination events. MA experiments with these "clean" deletion mutant strains also demonstrated that the Kn^R scars themselves did not have mutational phenotypes. Out of concern that the highly mutating strains could accumulate second-site mutations that would modify mutation rates or spectra, we also reconstructed several mutant strains and repeated the MA experiments. One Δ*mutS* mutant strain proved to have a mutation rate and spectrum inconsistent with 3 MA experiments with other Δ*mutS* mutant strains, and it was eliminated. Genetic constructions were confirmed by PCR analyses using the oligonucleotides listed in Supplemental Table S2. The mutational phenotype of the mutant strains was confirmed by determining the mutation rate using fluctuation assays (FOSTER 2006; HALL *et al.* 2009).

During the course of these studies, we discovered that some of the sequence reads of the MA lines mapped to the genome of bacteriophage Phi80. Thus, some of our strains are lysogenized with this phage, which has been reported to be ubiquitous among laboratory

strains of *E. coli* (ROTMAN *et al.* 2010). The occurrence of the phage sequences was sporadic, and we have been unable to build a coherent history of the introduction and spread of the contamination. However, the phage had little or no impact on the results of our experiments for the following reasons. First, we have never observed lysis or plaques in our cultures, most likely because the incubation temperature of all but one of our experiments was 37°C, which is nonpermissive for Ph80 growth (ROTMAN *et al.* 2012). Second, strains that were lysogens and those that were not had the same mutational phenotype. For example, of the MMR defective strains, only PLFM144, PFM567, and PFM568 yielded a substantial number of reads that mapped to Phi80; yet the rate and mutational spectrum of these strains differed little from the other MMR defective strains (Supplemental Tables S3 and S4). The only other strain used in this study that appeared to be a Phi80 lysogen was PFM666.

The following media were used for both liquid cultures and agar plates. Rich medium was Miller Luria Broth (LB) (Difco); minimal medium was Vogel-Bonner minimal (VB min) medium with 0.2% glucose (MILLER 1992). Buffered LB was LB plus 100 mM HEPES adjusted to pH 7. Dilute LB was 20 volumes LB broth plus 80 volumes 10 g/L NaCL in dH_2O. Supplemented minimal was 10g Bacto tryptone and 5 g Bacto yeast extract (both Difco) added to a final volume of 1 L Vogel-Bonner minimal medium plus 0.2% glucose. For agar plates, Bacto agar (Difco) was added at 15 g/L. When required the antibiotic concentrations in rich medium were: anhydrotetracycline (AHT), 500 μg/mL; carbenicillin (Carb), 100 μg/mL; and kanamycin (Kn), 50 μg/mL; nalidixic acid (Nal), 40 μg/mL, chloramphenicol (Cam), 30 μg/mL; rifampicin (Rif), 100 μg/mL. Half of these concentrations was used in minimal medium.

Mutation accumulation experiments: The MA procedure was as described (LEE *et al.* 2012; FOSTER *et al.* 2015). The MA lines originated from single colonies isolated from two or more founder colonies that were isolated from freezer stocks by streaking onto LB or VBmin agar plates. Originally we initiated the first passages of the MA lines by streaking from one or two well isolated colonies from the founder plate. However, since 40 or 50 MA lines needed to be initiated, we subsequently found it more efficient to excise colonies from the founder plate, soak them for 30 minutes in 0.85% NaCl + 0.01% gelatin, vortex the mixture for 60 seconds, and plate appropriate dilutions to obtain well-isolated colonies. Each MA line was periodically streaked for single colonies on agar plates. Lines were streaked every 24 hrs if on LB medium incubated at 37^{o}C, every 48 hrs if on VBmin medium incubated at 37^{o}C, and every 48 hrs if on LB medium incubated at 28^{o}C (one experiment). The number of passes required was determined by the preliminary mutation rate obtained from fluctuation assay.

The number of generations that each line had undergone was estimated as described (LEE *et al.* 2012). Briefly, during an experiment the diameter of each colony that was picked for streaking was measured and recoded. Then, other colonies of various appropriate sizes were excised, soaked for 30 minutes in 0.85% NaCl + 0.01% gelatin, the mixture vortexed for 60 seconds, and appropriate dilutions plated to obtained the number of cells in the colonies. The $\log_2$ of this number gave the number of generations that gave rise colonies of each size. The diameters of the colonies recorded during the experiment was then converted to generations and summed.

All but one of the MA experiments were done at 37oC. In one experiment we wished to slow the growth of the colonies on LB to that of colonies on VB min medium. By measuring

colony growth at various temperatures, we determined that at 28°C the cells required 48 hrs to obtain the same size as when growing at 37°C.

Estimation of mutation rates by fluctuation tests: Mutation rates to rifampicin resistance (Rif^R) or to nalidixic acid resistance (Nal^R) were estimated using fluctuation tests as described (FOSTER 2006). Each test consisted of thirty to forty parallel 100 μl cultures incubated overnight in well agitated microtiter plates. Appropriate dilutions of aliquots from 3 cultures were plated on LB plates to determine cell numbers, and the rest of the cultures were plated on LB plus Nal or LB plus Rif. Nal was generally used for high-mutating strains. The mutation rates were calculated using the Ma-Sandri-Sarkar maximum likelihood method (SARKAR *et al.* 1992) implemented by the FALCOR web tool found at www.mitochondria.org/protocols/FALCOR.html (HALL *et al.* 2009).

Genomic DNA preparation, library construction, sequencing, and SNP calling: Freezer stocks made after the last passage of each MA line were used to inoculate LB or VBmin cultures from which DNA was extracted using PureLink Genomic DNA purification kit (Invitrogen). DNA concentrations were measured on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.). Before library construction the identity of the MA lines containing gene deletions was confirmed using diagnostic PCR of the gDNA with the oligonucleotides listed in Supplemental Table S2. Paired end libraries were made by the Indiana University Center for Genomics and Bioinformatics and were sequenced at the University of New Hampshire Hubbard Center for Genome Studies using the Ilumina HiSeq 2500 platform. Insert size was between 350 and 600 base pairs. SNP and indel calling were as described (LEE *et al.* 2012). The

reference genome used was *E. coli* MG1655 NCBI reference sequence NC_000913.2, as this version matches our MG1655 parental strain (FOSTER *et al.* 2015).

MA lines with poor sequence coverage were eliminated. In addition, occasionally lines from the same experiment shared mutations, either because the mutations occurred during growth of the founder colony or because of cross-contamination during streaking. If MA lines from the same experiment shared over 50% of their mutations, one line was eliminated; if less than 50% were shared, the mutations were randomly assigned to one line and eliminated from the other. Lines were also eliminated if sequencing revealed known mutators or antimutators had appeared, or if the mutation rate was more than 2 standard deviations from the mean for the experiment, indicating that unknown mutators or antimutators had appeared.

REFERENCES

Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura *et al.*, 2006 Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular Systems Biology 2**:** 2006.

Blank, K., M. Hensel and R. G. Gerlach, 2011 Rapid and highly efficient method for scarless mutagenesis within the *Salmonella enterica* chromosome. PLoS One 6**:** e15763.

Datsenko, K. A., and B. L. Wanner, 2001 One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences, USA 97**:** 6640-6645.

Foster, P. L., 2006 Methods for determining spontaneous mutation rates. Methods in Enzymology 409**:** 195-213.

Foster, P. L., H. Lee, E. Popodi, J. P. Townes and H. Tang, 2015 Determinants of spontaneous mutation in the bacterium *Escherichia coli* as revealed by whole-genome sequencing. Proc Natl Acad Sci U S A 112**:** E5990-E5599.

Hall, B. M., C. X. Ma, P. Liang and K. K. Singh, 2009 Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis. Bioinformatics. 25**:** 1564-1565.

Lee, H., E. Popodi, H. Tang and P. L. Foster, 2012 Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing. Proc Natl Acad Sci U S A 109**:** E2774-E2783.

Miller, J. H., 1992 *A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Rotman, E., L. Amado and A. Kuzminov, 2010 Unauthorized horizontal spread in the laboratory environment: the tactics of Lula, a temperate lambdoid bacteriophage of Escherichia coli. PLoS One 5**:** e11106.

Rotman, E., E. Kouzminova, G. Plunkett, 3rd and A. Kuzminov, 2012 Genome of Enterobacteriophage Lula/phi80 and insights into its ability to spread in the laboratory environment. J Bacteriol 194**:** 6802-6817.

Sarkar, S., W. T. Ma and G. v. H. Sandri, 1992 On fluctuation analysis: a new, simple and efficient method for computing the expected number of mutants. Genetica 85**:** 173-179.