Antibiotic treatment enhances the genome-wide mutation rate of target cells

Hongan Long\textsuperscript{a}, Samuel F. Miller\textsuperscript{a}, Chloe Strauss\textsuperscript{a}, Chaoxian Zhao\textsuperscript{b}, Lei Cheng\textsuperscript{c}, Zhiqiang Ye\textsuperscript{a}, Katherine Griffin\textsuperscript{a}, Ronald Te\textsuperscript{b}, Heewook Lee\textsuperscript{d}, Chi-Chun Chen\textsuperscript{e}, and Michael Lynch\textsuperscript{a,1}

\textsuperscript{a}Department of Biology, Indiana University, Bloomington, IN 47405; \textsuperscript{b}Department of Biology, School of Life Sciences, East China Normal University, Shanghai, China 200241; \textsuperscript{c}Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin, Heilongjiang Province, China 150001; and \textsuperscript{d}School of Informatics and Computing, Indiana University, Bloomington, IN 47405

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Although it is well known that microbial populations can respond adaptively to challenges from antibiotics, empirical difficulties in distinguishing the roles of de novo mutation and natural selection have left several issues unresolved. Here, we explore the mutational properties of \textit{Escherichia coli} exposed to long-term sublethal levels of the antibiotic norfloxacin, using a mutation accumulation design combined with whole-genome sequencing of replicate lines. The genome-wide mutation rate significantly increases with norfloxacin concentration. This response is associated with enhanced expression of error-prone DNA polymerases and may also involve indirect effects of norfloxacin on DNA mismatch and oxidative-damage repair. Moreover, we find that acquisition of antibiotic resistance can be enhanced solely by accelerated mutagenesis, i.e., without direct involvement of selection. Our results suggest that antibiotics may generally enhance the mutation rates of target cells, thereby accelerating the rate of adaptation not only to the antibiotic itself but to additional challenges faced by invasive pathogens.

Significance

The evolution of antibiotic resistance by pathogenic bacteria poses a major challenge for human health. Whereas it is clear that natural selection promotes resistance-conferring mutations, our understanding of the response of the mutation rate to antibiotics is limited. With hundreds of \textit{Escherichia coli} cell lines evolving in a near-neutral scenario under exposure to the fluoroquinolone norfloxacin, this study reveals a significant linear relationship between the mutation rate and antibiotic concentration, while also demonstrating that antibiotic treatment compromises the efficiency of DNA oxidative-damage repair and postreplicative mismatch repair. Thus, antibiotics not only impose a selective challenge to target and off-target bacteria but also accelerate the rate of adaptation by magnifying the rate at which advantageous mutations arise.
These types of limitations can be avoided entirely by whole-genome sequencing (WGS) of lines generated in mutation accumulation (MA) experiments. MA experiments using bacteria are performed by repeatedly passing large numbers of initially identical lines through single-cell bottlenecks, a procedure that prevents natural selection from promoting or eradicating nearly all mutations, except the small subset with extremely large effects (31). This MA/WGS procedure provides an essentially unbiased, genome-wide view of the rate and full molecular spectrum of mutations, and has yielded accurate estimates of these features in a wide variety of prokaryotic and eukaryotic microbes (27, 28, 32–34).

Here, we apply the MA/WGS strategy to characterize the rate and molecular spectrum of genomic mutations produced by three sets of E. coli MA lines exposed to a wide range of sublethal norfloxacin concentrations. In addition to performing assays on wild-type K-12 MG1655, which has a representative background mutation rate for a prokaryote (27, 35), we performed parallel assays with a MMR-deficient mutator strain (ΔmutS), as well as with an adenine DNA glycosylase-deficient strain (ΔmutY) to evaluate the potential role of oxidative damage of nucleotides. Our results provide quantitative insight into the relationship between antibiotic concentration and the rate and molecular spectra of mutations in the target species, the degree to which DNA oxidation is involved, and the extent to which elevated mutation rates accelerate the rate of resistance acquisition.

**Results**

We explored how E. coli mutation rates change when treated with sublethal levels of norfloxacin after first predetermining the range of applicable sublethal norfloxacin concentrations. We exposed the three progenitor strains with 10 concentrations of norfloxacin, incubating ~1,500 cells on LB plates at 37 °C for 24 h, then counting the colony-forming units to estimate the efficiency of plating (EOP) by dividing treatment colony-forming units by that without norfloxacin. The EOP, which reflects the efficiency of plating (EOP) by dividing treatment colony-forming units per group on LB plates with a standard daily streak-and-plate procedure. The norfloxacin doses applied (0, 12.5, 25, 37.5, 50, 62.5, 75, and 87.5 ng/mL) are orders of magnitude lower than peak norfloxacin concentrations in the serum of patients after taking a clinical dosage (37). After ~2 mo of daily single-colony transfers for each of the 384 lines, we sequenced whole genomes and ascertained the acquired mutations in each final evolved line (Table 1 and Dataset S1, Tables S1–S3). Mutation rates involving single base pair substitutions (BPSs) across the genome exhibit a strong linear increase with norfloxacin dose (Pearson’s product-moment correlation coefficient \( r = 0.94, P = 0.0006 \)), with the highest concentration yielding ~4.0 times elevation relative to the control (Fig. 2B). The mutation rates to small-sized insertions/deletions (>19 bp) also increase with norfloxacin treatment in both the wild-type and ΔmutS lines (Fig. S1), although as is usually seen in bacterial mutation assays (27), such mutations are only on average 11% as abundant as BPS mutations.

Genetic drift in single-cell bottlenecked lines of E. coli is strong enough to prevent the operation of selection on all but mutations with very large effects (31), and numerous prior studies of this sort have validated the effectively nonselective nature of MA experiments (27, 28, 33, 38, 39). However, to directly test whether selection might have biased the mutation rate/spectrum (e.g., by enriching for mutations conferring norfloxacin resistance), we examined the synonymous and nonsynonymous status of each coding-region BPS (Dataset S1, Table S2). For no treatment is the nonsynonymous/synonymous BPS ratio significantly different from that of the nontreatment control (\( \chi^2 \) test, \( P > 0.05 \) in all cases), indicating that the vast majority of acquired amino acid-altering BPSs were not selectively promoted by norfloxacin treatment but simply accumulated in a neutral fashion. As noted below, this conclusion is also confirmed by the very low incidence of enriched BPSs in resistance-associated genes.

Because nucleotide changes at fourfold degenerate sites do not cause an amino acid change, whereas most resistance mutations likely involve amino acid changes, as a final check for selection bias, we calculated BPS mutation rates exclusively at fourfold degenerate sites. These rates strongly correlate with norfloxacin concentration (\( r = 0.97, P = 6.34 \times 10^{-5} \)); Fig. 2A), yielding a pattern not significantly different from that for rates derived from all genomic sites (two-sample Kolmogorov–Smirnov test, \( D = 0.25, P = 0.98 \)). Examination of the spectrum of norfloxacin-induced BPS mutations shows that the mutation rates of all possible BPS categories increase significantly with norfloxacin doses (Fig. 2B and Dataset S1, Table S4), which contrasts with the transversion-dominant spectra of previously reported SOS-dependent mutagenesis (40, 41), implying the operation of mutagenesis mechanisms beyond a simple SOS response during norfloxacin treatment. Thus, we conclude that genome-wide BPS mutation rates increase with sublethal concentrations of norfloxacin. Moreover, the linear relationship between the BPS mutation rate and dosage indicates an absence of saturation in the response for the range of concentrations applied, suggesting that higher doses will elicit still higher mutation rates.

Because the MMR pathway repairs multiple types of mutations in wild-type lines (27, 42), the contrast between mutation spectra in MMR-deficient and wild-type strains can be used to reveal early-stage mutagenic effects (i.e., before MMR removal). Therefore, we performed a MA/WGS analysis of lines originating from a single MMR− cell with the critical mutS gene deleted, which yields ~104 times BPS mutation rate elevation over the wild-type level (Dataset S1, Tables S1 and S5–S7).
strong correlation between the BPS mutation rate and the norfloxacin concentration remains in the mutator lines, with a ~1.85 times increase over the full range of concentrations within the MMR− lines (Fig. 2C and Dataset S1, Table S4).

As seen in previous work on background mutational features in E. coli (27), there are substantial differences in the mutation spectra of wild-type and MMR− lines (Fig. 2B and D). Most notably, the transition/transversion ratio of mutations is much higher in the latter, and also scales negatively with norfloxacin concentration, whereas there is no correlation detected in the wild-type lines (Fig. 3A). The pattern observed in MMR− lines is likely shaped by norfloxacin induction of the SOS response (see next section), which elevates the relative incidence of transversion mutations (43).

The ratio of mutation rates in MMR+ and MMR− backgrounds, a measure of the fraction of premutations not eliminated by MMR, is positively correlated with norfloxacin concentration ($r = 0.86$, $P = 0.007$), increasing from 0.009 in the absence of norfloxacin to 0.019 at the highest dose. Although this concentration ($nated by MMR, is positively correlated with norfloxacin

Table 1. Summary of MA line information

<table>
<thead>
<tr>
<th>Strains</th>
<th>Concentration</th>
<th>N</th>
<th>Cell divisions</th>
<th>Ts</th>
<th>Tv</th>
<th>$N_e$</th>
<th>Insertions</th>
<th>Deletions</th>
<th>MIC (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0 (A)</td>
<td>46</td>
<td>1,682</td>
<td>42</td>
<td>37</td>
<td>14.0</td>
<td>1</td>
<td>8</td>
<td>121.38 (2.08)</td>
</tr>
<tr>
<td>+</td>
<td>12.5 (B)</td>
<td>48</td>
<td>1,687</td>
<td>54</td>
<td>41</td>
<td>14.0</td>
<td>10</td>
<td>8</td>
<td>125.00 (0)</td>
</tr>
<tr>
<td>+</td>
<td>25 (C)</td>
<td>48</td>
<td>1,612</td>
<td>60</td>
<td>58</td>
<td>13.5</td>
<td>5</td>
<td>15</td>
<td>125.43 (2.36)</td>
</tr>
<tr>
<td>+</td>
<td>37.5 (D, XD)</td>
<td>47</td>
<td>1,254</td>
<td>68</td>
<td>62</td>
<td>12.5</td>
<td>6</td>
<td>16</td>
<td>142.73 (5.91)</td>
</tr>
<tr>
<td>+</td>
<td>50 (E)</td>
<td>47</td>
<td>1,277</td>
<td>98</td>
<td>76</td>
<td>11.5</td>
<td>11</td>
<td>12</td>
<td>177.75 (15.26)</td>
</tr>
<tr>
<td>+</td>
<td>62.5 (F)</td>
<td>45</td>
<td>1,274</td>
<td>97</td>
<td>95</td>
<td>11.5</td>
<td>9</td>
<td>21</td>
<td>202.78 (15.08)</td>
</tr>
<tr>
<td>+</td>
<td>75 (G)</td>
<td>46</td>
<td>1,221</td>
<td>117</td>
<td>111</td>
<td>11.0</td>
<td>12</td>
<td>22</td>
<td>229.17 (26.57)</td>
</tr>
<tr>
<td>+</td>
<td>87.5 (H)</td>
<td>48</td>
<td>1,229</td>
<td>102</td>
<td>79</td>
<td>11.0</td>
<td>9</td>
<td>24</td>
<td>205.73 (12.62)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>0 (5A)</td>
<td>12</td>
<td>763</td>
<td>950</td>
<td>19</td>
<td>14.0</td>
<td>105</td>
<td>79</td>
<td>149.31 (27.72)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>12.5 (5B)</td>
<td>12</td>
<td>750</td>
<td>1,070</td>
<td>25</td>
<td>14.0</td>
<td>116</td>
<td>58</td>
<td>133.68 (17.48)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>25 (5C)</td>
<td>12</td>
<td>702</td>
<td>1,072</td>
<td>40</td>
<td>13.5</td>
<td>118</td>
<td>90</td>
<td>151.07 (16.45)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>37.5 (5D)</td>
<td>12</td>
<td>1,526</td>
<td>2,249</td>
<td>139</td>
<td>12.5</td>
<td>184</td>
<td>143</td>
<td>262.89 (47.38)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>50 (5E)</td>
<td>12</td>
<td>696</td>
<td>1,221</td>
<td>49</td>
<td>13.5</td>
<td>139</td>
<td>108</td>
<td>395.83 (73.16)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>62.5 (5F)</td>
<td>12</td>
<td>667</td>
<td>955</td>
<td>39</td>
<td>13.0</td>
<td>103</td>
<td>62</td>
<td>338.92 (60.93)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>75 (5G)</td>
<td>12</td>
<td>662</td>
<td>1,437</td>
<td>65</td>
<td>13.0</td>
<td>182</td>
<td>101</td>
<td>322.94 (51.59)</td>
</tr>
<tr>
<td>ΔmutT</td>
<td>87.5 (5H)</td>
<td>12</td>
<td>572</td>
<td>1,114</td>
<td>51</td>
<td>12.0</td>
<td>153</td>
<td>112</td>
<td>361.11 (36.02)</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>0 (YA)</td>
<td>12</td>
<td>2,023</td>
<td>23</td>
<td>211</td>
<td>14.5</td>
<td>2</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>3.125 (YB)</td>
<td>19</td>
<td>1,972</td>
<td>26</td>
<td>276</td>
<td>14.0</td>
<td>2</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>12.5 (YC)</td>
<td>19</td>
<td>2,008</td>
<td>30</td>
<td>286</td>
<td>14.5</td>
<td>4</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>25 (YD)</td>
<td>18</td>
<td>1,735</td>
<td>85</td>
<td>245</td>
<td>12.5</td>
<td>11</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>37.5 (YE)</td>
<td>46</td>
<td>713</td>
<td>31</td>
<td>336</td>
<td>13.0</td>
<td>4</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>50 (YF)</td>
<td>18</td>
<td>1,291</td>
<td>104</td>
<td>1,403</td>
<td>13.5</td>
<td>2</td>
<td>14</td>
<td>—</td>
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<tr>
<td>ΔmutY</td>
<td>62.5 (YG)</td>
<td>46</td>
<td>603</td>
<td>53</td>
<td>343</td>
<td>11.5</td>
<td>6</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>75 (YG)</td>
<td>46</td>
<td>603</td>
<td>53</td>
<td>343</td>
<td>11.5</td>
<td>6</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>ΔmutY</td>
<td>87.5 (YH)</td>
<td>40</td>
<td>622</td>
<td>90</td>
<td>292</td>
<td>11.5</td>
<td>10</td>
<td>19</td>
<td>—</td>
</tr>
</tbody>
</table>

Norfloxacin concentration ("Concentration") and MIC are in units of nanograms per milliliter norfloxacin. Cell divisions, the average number of cell divisions per line; Insertion and Deletion, the total numbers of insertions/ deletion (<19 bp) mutations detected across all lines in the group; MIC, minimum inhibitory concentration of the final evolved lines; N, number of lines in the group; $N_e$, effective population size; SEM, standard error of the mean; Ts, number of transitions in the group; Tv, number of transversions; +, wild type. The letters in parentheses in the Concentration column are group labels.
generated by the major polymerases Pol I and III (47) and the intrinsic transition repair bias of MMR (48).

**Norfloxacin Affects DNA Oxidative Damage Repair.** The reduced efficiency of MMR associated with increasing norfloxacin treatment inspires the idea that other DNA repair pathways may also be compromised by antibiotic treatment. Motivated by the observation that the wild-type G:C→T:A transversion rate is strongly elevated by norfloxacin treatment (49), we conducted MA experiments with a ΔmutY strain to determine whether antibiotic treatment affects DNA oxidative-damage repair. The ΔmutY strain is dysfunctional in recognizing adenines mispaired with 8-oxo-guanines (49), and it is known that the failure to recognize and remove 8-oxo-guanines residing in DNA elevates the postreplication G:C→T:A transversion mutation rate (50–52).

We performed two sets of MA experiments using lines originating from a single-cell ΔmutY progenitor, encompassing the full range of norfloxacin concentrations noted above as well as a treatment of 20 mM H2O2 (but no norfloxacin). The latter treatment was imposed to verify that high oxidative conditions do indeed elevate the mutation rate; if the resultant mutation rate is elevated relative to that under norfloxacin treatment (which it is), a lack of elevated G:C→T:A mutation rate must not be due to baseline saturation with oxidation damage. The highest norfloxacin concentration used in the first experiment was 50 ng/mL, and the second experiment started at 25 ng/mL and expanded the treatment range to the highest levels of norfloxacin concentrations used in earlier experiments.

G:C→T:A mutation rates in ΔmutY lines do not covary with norfloxacin concentrations (two-way ANOVA with experimental set and concentration as the two categories, Pconcentration = 0.25; Dataset S1, Tables S14–S17). This suggests that, although there is a positive response of the rate of G:C→T:A transversion mutations to norfloxacin dosage in wild-type lines, this is not a consequence of the direct promotion of DNA oxidative damage by norfloxacin, but rather an indirect effect of the influence of norfloxacin on the repair systems that normally inhibit G:C→T:A mutations, i.e., DNA oxidative-damage repair. This hypothesis is supported by the observation that MutY is down-regulated by antibiotic-promoted oxidative stress in *E. coli* (23, 53), perhaps because this attenuates the deleterious effect of MutY activity on
damaged DNA (36) (Fig. 1). Interestingly, the G:C→T:A transversion mutations in ΔmutY lines distribute in a nonrandom wave-like pattern across the genome (Fig. S3).

Very Few Genes Are Enriched with Mutations. Although we have shown that selection for norfloxacin resistance has a negligible influence on our mutation rate estimates, given the number of mutations harvested in this analysis (Table 1), there remains a possibility of enrichment of mutations in a small number of genes, including those that are relevant to antibiotic resistance.

To determine whether any changes in sensitivity to norfloxacin occurred in the MA lines, we measured the minimum inhibitory concentration (MIC) for all wild-type and MMR⁻ lines (Dataset S1, Tables S1 and S5). Despite the extreme reduction in the efficiency of selection under the MA design, there is a trend toward increasing resistance with norfloxacin concentration to the point of an approximate doubling at the highest dosage (Table 1), and the MMR⁻ lines develop stronger resistance than wild-type lines at higher concentrations (Fig. 4). Notably, two MMR⁻ lines (SA6 and SA12; Dataset S1, Table S5) grown in the absence of norfloxacin exhibited a ≥2 times increase in MIC, showing that resistance can sometimes be enhanced solely by an accelerated mutation rate without any direct involvement of selection. In both the wild-type and MMR⁻ MA lines, we also observe some MICs lower than that of the progenitor MIC, all in <37.5 ng/mL treatments (Dataset S1, Tables S1 and S5), showing that mutations have arisen that both increase and decrease norfloxacin resistance.

To determine whether specific genes were enriched with mutations conferring resistance, we pooled the BPS mutations in the coding regions for all genes in the wild-type lines treated with norfloxacin (787 BPSs in 674 genes), as well as those within the treated MMR⁻ lines (8,229 BPSs in 3,144 genes). Then, for each gene, we calculated the Poisson probability of seeing greater than or equal to the number of observed mutations in the gene given the expected mutation rate of the gene with norfloxacin treatment. The expected mutation rate of the gene was calculated as the product of the average per base mutation rate in

![Fig. 3. Mutation and gene expression patterns across norfloxacin treatments.](https://www.pnas.org/cgi/doi/10.1073/pnas.1601208113)
norfloxacin-treated lines (4.54 × 10−10 for wild type; 3.19 × 10−8 for MMR−) and the gene length (Dataset S1, Tables S18 and S19). After Bonferroni correction for multiple comparisons (corrected P values: wild type, 7.42 × 10−3; MMR−, 1.59 × 10−5), significant mutation rate elevation is suggested in three [multiple antibiotic resistance operon repressor (marR); DNA gyrase subunit B (gyrB); DNA gyrase subunit A (gyrA)] and two genes [positive regulator of the AcrAB multidrug resistance efflux pump (sdiA); gyrA] in the wild-type and MMR− lines, respectively (Dataset S1, Tables S18 and S19; mdaB) was removed from the wild-type gene list because its mutations reside only in lines with nonelevated MICs). In sum, only 2.2% of wild-type and 0.4% of MMR− BPSs are in genes that possibly experienced selection for resistance.

Only gyrA appears on the candidate lists for both the wild-type and MMR− lines, suggesting that either these two types of lines experience different paths to resistance, or that there are some false positives in this analysis. Most mutated gyrase genes contain only a single mutation (Dataset S1, Table S20), but lines containing at least one gyrase mutation have an average MIC increase of 2.5 times, suggesting that such mutations do specifically confer resistance, although two or more mutations are usually required for resistance to high doses (54). Another known norfloxacin target, topoisomerase IV (consisting of parC and parE) (55), does not exhibit elevated mutation numbers in either the wild-type or the MMR− lines.

Because most of the MA lines acquired multiple mutations, direct links between specific mutations and norfloxacin resistance will need to be empirically tested with constructs developed by site-specific alterations, but the candidate mutations identified here at least provide a logical starting point for such work. In the next section, as a case study using site-directed mutagenesis, we confirm that one point mutation in the candidate resistance gene marR (the top gene in the wild-type candidate list) directly confers multidrug resistance.

Multidrug Resistance Caused by a Base Substitution Mutation. A G→A substitution in the wild-type line H43 (grown at 87.5 ng/mL) occurred at the third position of the start codon GTG of marR (genomic position 1,619,122; causing a fMet→valine amino acid change), possibly causing translation initiation failure of the gene, and this line has an evolved MIC for norfloxacin 4 times the ancestral level (Dataset S1, Table S2). marR is the repressor of the marAB operon, expression of which up-regulates the AcrAB efflux pump responsible for multidrug resistance in E. coli (56). Because there are two additional nonsynonymous mutations in other genes in this line (Dataset S1, Table S2), it is possible that this marR mutation is not the cause of the elevated resistance. However, introduction of the G→A point mutation into the wild-type progenitor by “scarless” site-directed mutagenesis (to produce the constructed line HL1) recapitulates the 4 times MIC elevation.

To evaluate whether this nonsynonymous mutation has general effects, we tested the MICs of HL1 to two other antibiotics: carbenicillin and chloramphenicol. This line has MICs of 32 μg/mL to both antibiotics, whereas parallel assay of the progenitor line reveals MICs of 16 μg/mL for chloramphenicol and 8 μg/mL for carbenicillin. The single G→A base substitution mutation in marR thus confers multiple-drug resistance to E. coli.

Coincidence of ISS-Mediated Duplications and emrE Efflux Pump Gene Expression Decrease. Using coverage analysis and quantitative PCR, we detected and validated large duplications in 21 lines, all in the 75 and 87.5 ng/mL treatments of wild-type lines (Fig. S4 and Dataset S1, Table S21). All duplications locate between genomic position 1 and 689,000, and most of these are 114 and 415 kb in size, with one exception of 689 kb in line G37. All of these duplications are flanked by ISS elements, which are known to mediate chromosomal rearrangements by unequal homologous recombination (57) (Fig. S4), and it is notable that expression of the homologous-recombination pathway is significantly enriched upon norfloxacin treatment (Dataset S1, Table S12).

We find that all duplicated regions contain or flank an efflux pump gene E. coli multidrug resistance E (emrE) (Fig. S4, blue line), which belongs to the small multidrug resistance (SMR) family (58). Gene expression of wild-type emrE shows a negative correlation with norfloxacin concentration (r = −0.81, P = 0.02), and at concentrations ≥37.5 ng/mL is reduced to about half of the untreated expression level (Dataset S1, Table S8). It is plausible that the duplications of emrE may compensate for the expression decrease upon norfloxacin treatment, but knocking out or overexpressing emrE in E. coli in previous studies did not elicit norfloxacin resistance change (59, 60). Thus, the decrease in emrE expression may just be a coincidence, although there are numerous known cases of gene duplication/amplification related to the development of antibiotic resistance (61).

Discussion

This study has systematically evaluated the total pool of genomic mutations arising in 737 E. coli lines subjected to daily single-cell bottlenecks for 1–2 mo. After WGS, we detected thousands of mutational events, including point mutations, indels, prophage deletions, and large duplications, as well as their responses to sublethal concentrations of norfloxacin with or without the presence of DNA repair systems such as MMR and DNA oxidative-damage repair. Our findings demonstrate the power and resolution of MA techniques for ascertaining the consequences of exogenous factors for replication fidelity and damage repair, paving the way for future work on the mutagenic consequences of other antibiotics and other means of microbial intervention.

Numerous checks on the nature of mutations accumulated in this MA setting indicate that this experimental design cleanly separates the response of the mutation rate to antimicrobial dosage from the downstream issue of which specific mutations confer resistance. Because our mutation rate estimates experience negligible changes even after excluding the small subset of potential resistance-associated mutations, it appears clear that antibiotic exposure does not simply encourage the establishment of resistance by natural selection, but specifically increases the likelihood of the de novo emergence of the mutational fuel essential to such adaptation.
The large pool of mutations identified in our MA experiments may include a small subset of candidate resistance loci useful for future targets for drug discovery. For example, many mutations that we observed in known resistance-related genes such as gyrA and gyrB (Dataset S1, Table S20) are not in known quinolone resistance determining regions (QRDRs) (62), and they thus reveal a larger mutational space of the known target genes at low levels of norfloxacin treatments.

The fact that norfloxacin treatment globally elevates the mutation rates of all types of BPSs while also increasing the expression of low-fidelity polyphenases suggests that antibiotic-associated mutation rate enhancement is at least part an indirect consequence of the cellular physiological response to stress (63). If this interpretation is correct, it implies that the application of all antimicrobials, regardless of their mode of action, may indirectly accelerate the rate of appearance of mutations conferring resistance simply by increasing the incidence of error-prone DNA replication. This raises the additional issue that stress-induced mutagenesis associated with the application of antibiotics may in the long run elevate the rate of appearance of adaptive mutations associated with other challenges confronted by pathogenic bacteria, e.g., host cell invasion and avoidance of host cell immune responses. The contribution of the SOS response to mutation elevation during norfloxacin treatment will be quantified by a running MA project using the wild-type and SOS-uninducible strains.

Comparisons of mutation rate responses to norfloxacin doses in wild-type lines with those of MMR− and DNA oxidative-damage repair-deficient lines suggest that this drug also affects the efficiency of both postreplicative DNA mismatch and oxidative-damage repair. The influence of norfloxacin treatment on MMR efficiency corresponds well with a recent molecular genetic study on the negative effect of antibiotics on replication fidelity. Gutierrez et al. (64) showed that antibiotic treatment elevates the expression level of the global transcription regulator RpoS, which then induces expression of a small RNA (SdrS) that binds and represses the mRNA of MutS, a critical component of the MMR pathway, thereby causing mutation rate elevation. The mechanism by which norfloxacin affects DNA oxidative-damage repair needs further exploration.

Our results show that mutation-driven appearance of antibiotic resistance phenotypes increases more rapidly with norfloxacin concentration in mutator strains (the MMR− lines) than in wild-type lines, with resistance mutations occasionally being acquired even in the absence of antibiotic treatment. Such observations correspond well with the widespread presence of MMR− lines in clinical isolates of antibiotic-resistant strains (9). The maximum resistance level reached in this study (8 times ancestral MIC level or 1,000 ng/mL; Dataset S1, Tables S1 and S5) is the same in both the wild-type and MMR− lines, which approximates the level of concentration following a typical clinical dosage: after orally taking 200 mg of norfloxacin, the peak concentration of this drug in serum is 750 ng/mL (37). Thus, the level of concern attached to the frequency of use and dosage levels of antibiotics is certainly justified (65, 66).

Although Kohanski et al. (23) found that mutation rates of norfloxacin-treated E. coli lines in liquid medium (estimated from fluctuation tests) correlate with an increase in ROS, we do not observe an elevation in the types of mutations expected to be elicited by ROS, e.g., G:C → T:A transversions in oxidation-sensitive mutant cells (ΔmutY) treated with norfloxacin. This discrepancy may be a result of the cells growing in different physiological conditions in the experiment of Kohanski et al. (23) and the current study, which may lead to different levels of oxygen exposure and/or degrees of ROS production, i.e., log-phase cells receiving high aeration in liquid medium shaken at high speed vs. heterogeneous cells in colonies on agar plates.

In summary, by revealing a large number of mutations in an unbiased way, the MA/WGS strategy provides a powerful approach to evaluating in a full genomic context the mutagenic consequences of antibiotics and other exogenous chemicals in ways not possible with reporter constructs, and in doing so provides a clear delineation between the separate issues of mutation and selection. Because the modes of action of antibiotics are diverse and microbes differ in their mechanisms of DNA repair, evaluation of the generality of our results will require the incorporation of such diversity in future research. Finally, the fact that antibiotics can act indirectly as bacterial mutagens raises the additional question of whether such exotic reagents may also have negative consequences for DNA stability in the cells of patients undergoing antibiotic treatment.

Materials and Methods
We ran single-colony daily transfers on three sets of E. coli K-12 MG1655 MA lines (wild-type, ΔmutS, ΔmutY), ancestor strains were kindly provided by Pat Foster’s Laboratory (Indiana University, Bloomington, IN) treated with gradients of norfloxacin for 1–2 mo (Table 1). We then constructed illumina genome libraries and applied HiSeq 2500 WGS on each final evolved cell line. Mutations were then analyzed from MA lines after mapping reads to the reference genome (NC_000913.3). We also did RNA-seq on progenitor lines of wild-type and ΔmutS treated with or without norfloxacin, and measured resistance of the progenitor and the evolved lines by testing the MIC. Raw reads of genome sequencing and RNA-seq concern in this study have been deposited in National Center for Biotechnology Information Sequence Read Archive with BioProject no. PRJNA301160 (study no. SRP066119). Details are in SI Materials and Methods.

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5. Lazarus B, Paterson DL, Mollinger JL, Rogers BA (2015) Do human extraintestinal Escherichia coli MRSA have higher resistance rates of all types of BPSs while also increasing the expression of low-fidelity polyphenases suggests that antibiotic-associated mutation rate enhancement is at least part an indirect consequence of the cellular physiological response to stress (63). If this interpretation is correct, it implies that the application of all antimicrobials, regardless of their mode of action, may indirectly accelerate the rate of appearance of mutations conferring resistance simply by increasing the incidence of error-prone DNA replication. This raises the additional issue that stress-induced mutagenesis associated with the application of antibiotics may in the long run elevate the rate of appearance of adaptive mutations associated with other challenges confronted by pathogenic bacteria, e.g., host cell invasion and avoidance of host cell immune responses. The contribution of the SOS response to mutation elevation during norfloxacin treatment will be quantified by a running MA project using the wild-type and SOS-uninducible strains.

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