The Spectrum of Spontaneous Rifampicin-Resistance Mutations in the *Bacillus subtilis* rpoB Gene Depends on the Growth Environment

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Abstract

Results from previous investigations into spontaneous rifampicin-resistance (RifR) mutations in the Bacillus subtilis rpoB gene suggested that the spectrum of mutations may depend on the growth environment. However, these studies were limited by low sample numbers, allowing for the potential distortion of the data by the presence of 'jackpot' mutations which may have arisen early in the growth of a population. Here we addressed this issue by performing fluctuation analyses to assess both the rate and spectrum of RifR mutations in two distinct media: LB, a complete laboratory medium and SMM\textsubscript{Asn}, a minimal medium utilizing L-asparagine as the sole carbon source. We cultivated 60 separate populations under each growth condition and determined that the mutation rate to Rif\textsuperscript{R} to be slightly but significantly higher in LB cultures. We then sequenced the relevant regions of rpoB to map the spectrum of Rif\textsuperscript{R} mutations under each growth condition. We found a distinct spectrum of mutations in each medium; LB cultures were dominated by the H482Y mutation (27/53 or 51\%) whereas SMM\textsubscript{Asn} cultures were dominated by the S487L mutation (24/51 or 47\%). Furthermore, we found through competition experiments that the relative fitness of the S487L mutant was significantly higher in SMM\textsubscript{Asn} than in LB medium. We therefore conclude that both the spectrum of Rif\textsuperscript{R} mutations in the B. subtilis rpoB gene and the fitness of resulting mutants to be influenced by the growth environment.
Importance

The rpoB gene encodes the beta subunit of RNA polymerase, and mutations in rpoB are key determinants of resistance to the clinically important antibiotic rifampicin. We show here that the spectrum of mutations in Bacillus subtilis rpoB depends on the medium in which the cells are cultivated. The results show that not only does the growth environment play a role in natural selection and fitness, but it also influences the probability of mutation at particular bases within the target gene.

Introduction

Because genetic variation is the raw material of evolution, understanding how microbial genomes respond and adapt to changing environments is a fundamental issue in microbiology. In their natural habitats, microorganisms rarely, if ever, encounter optimal growth conditions, due to nutrient limitations or the presence of environmental stressors; these environmental limitations comprise the selective pressures which drive evolution. In the classical view, random mutations generate the genetic variability upon which the environment selects. But do mutations occur randomly? Experiments dating back to Benzer's investigations of the phage T4 rII region in the 1950's indicate that the answer is no. Along the rII region, mutations were far from randomly distributed; certain "hotspots" accumulated mutations at a much higher frequency than others (1). While to this day the exact reason for this is unknown, it is suspected that the sequence context, regional DNA conformation, or DNA supercoiling state may influence the probability of a particular nucleotide sustaining a mutation (2, 3). Because DNA architecture changes in
response to the external environment (4, 5), it stands to reason that the rate of mutation at a particular nucleotide may also respond to environmental changes.

A growing body of evidence indicates that the environment not only serves as the agent of selection, but also plays a role in determining what range of mutations are possible within a gene, i.e., its mutational spectrum. This phenomenon has been eloquently demonstrated recently in a series of experiments using *Escherichia coli* grown under six different nutritional environments, either unlimited or limited for carbon, oxygen, phosphate, iron, or nitrogen (6). It was observed that the frequency of mutation only increased in two of the six nutritionally depleted stress regimes. However, growth in each medium produced a unique spectrum of mutations in the *cycA* gene encoding cycloserine resistance (6). In another recent study, the yeast *Saccharomyces cerevisiae* was cultivated in seven different environments that maintained both relatively high growth rates and minimal cell death, and the rates and spectra of mutation were measured using whole genome sequencing (7). The authors found significant differences in the ratio of transitions to transversions and in the AT mutational bias (i.e. the extent to which GC-to-AT mutations were more frequent than AT-to-GC mutations) in different media (7). These studies lend credence to the notion that a 'spontaneous' mutation is simply a mutation where the environmental influence is unknown (Friedberg et al., 2006).

Based on extensive experimental testing, it was concluded that the spectrum of mutations arising in the *E. coli* genome in response to environmental or genetic factors could be monitored accurately by analyzing mutations leading to resistance to the antibiotic rifampicin (Rif) (8). Rif is a potent inhibitor of prokaryotic transcription initiation (9) and has a history of use both in bacterial transcription studies and in treatment of various bacterial infections, particularly the mycobacterial diseases tuberculosis and leprosy. Resistance to Rif (Rif\(^R\)) arises from mutations
in the *rpoB* gene encoding the β subunit of RNA polymerase (RNAP) (Jin and Gross, 1988).

Mutations conferring Rif^R^ are generally localized to 4 regions within the *rpoB* gene designated the N-cluster and Clusters I, II, and III (Severinov et al., 1993), most mutations occurring within Cluster I. From three-dimensional crystal structures of RNAP-Rif complexes, it is seen that most Rif^R^ mutations occur at conserved residues within the Rif binding pocket, localized to the RNA exit channel, where binding of Rif physically blocks progression of the nascent transcript through the exit channel (10, 11).

We have been investigating how the external environment affects the spectrum of mutations leading to Rif^R^ in the *rpoB* gene of *Bacillus subtilis*. To date we have observed alterations in the mutational spectrum of *rpoB* after cultivation under environmental conditions including: vegetative growth vs. sporulation (Nicholson and Maughan, 2002) and aerobic vs. anaerobic growth (Nicholson and Park, 2015). Moreover, we recently observed an alteration in the spectrum of Rif^R^ mutations in the *rpoB* gene of *B. subtilis* grown under microgravity conditions on the International Space Station compared to matched ground controls (12).

A potential drawback of our early studies measuring the spectrum of Rif^R^ *rpoB* mutations in *B. subtilis* derived from the rather small numbers of mutants examined, which could have led to statistical artifacts. To remedy this situation, in the present study we have measured the frequency and spectrum of mutation to Rif^R^ in the *B. subtilis rpoB* gene in 120 total populations cultivated under two different environmental conditions, a complex laboratory medium (LB) vs. Spizizen Minimal Medium containing the amino acid L-asparagine (Asn) as the sole carbon source (SMM_{Asn}). This minimal medium was chosen for two reasons. First, it was considered more representative of conditions that *B. subtilis* might encounter in its natural soil habitat, given the role of root exudate Asn in influencing the composition of the rhizosphere microbiome (13).
Second, metabolic profiling of various Rif\textsuperscript{R} \textit{B. subtilis} \textit{rpoB} mutants revealed that a particular \textit{rpoB} mutant carrying the S487L substitution exhibited significantly increased utilization of Asn over the wild-type strain (14). To bring greater statistical power to the experiment, we examined using fluctuation analysis the occurrence of Rif\textsuperscript{R} \textit{rpoB} mutations in 60 independent cultures of \textit{B. subtilis} cultivated in LB vs. 60 independent cultures in SMM\textsubscript{Asn}. Sequencing of the resulting Rif\textsuperscript{R} regions of \textit{rpoB} from the Rif\textsuperscript{R} mutant collections resulted in the finding that the spectrum of Rif\textsuperscript{R} mutations indeed differed significantly between the two growth conditions. And finally, we tested the competitive fitness of the predominant \textit{rpoB} mutations under each growth condition. With these experiments we were able to contrast the spectrum of mutations observed in a typical laboratory environment (LB) with one that more closely approximates the natural soil habitat of \textit{B. subtilis} (SMM\textsubscript{Asn}).

\textbf{Results}

\textbf{Growth in LB and SMM\textsubscript{Asn}.} For fluctuation analysis, in each of three separate experiments we inoculated 20 individual 2-mL cultures in either LB or SMM\textsubscript{Asn}, incubated the cultures for 24 hours, and determined final cell densities as described in Materials and Methods. Because it had previously been determined that the frequency of mutation to Rif\textsuperscript{R} in \textit{B. subtilis} was on the order of \textasciitilde10\textsuperscript{-8} to \textasciitilde10\textsuperscript{-9} (15), we chose the initial population to be \textasciitilde10\textsuperscript{5} cells per culture, to ensure that less than 1 out of 1000 cultures would be predicted to carry a pre-existing Rif\textsuperscript{R} mutant. The final collection of data consisted of 6 datasets, \textit{n} = 20 each, for a total of 120 separate cultures. We determined the final population sizes for each culture, performed a log\textsubscript{10} transformation of the data, then screened the datasets for outliers. The final cell density of only one culture (SMM\textsubscript{Asn} Experiment 1, Culture 16) was anomalously high and found to be a
statistical outlier, which was discarded. All 6 screened data sets were next determined to be normally distributed, thus amenable to normal statistics. The average 24-hour cell densities were calculated to be $1.10 \times 10^9 \pm 1.42 \times 10^8$ and $8.06 \times 10^8 \pm 2.68 \times 10^8$ CFU/mL for LB and SMM<sub>Asn</sub> cultures, respectively. Testing of the data sets by ANOVA revealed that the difference in growth between LB and SMM<sub>Asn</sub> cultures was slight (1.36-fold), but highly statistically significant ($P < 0.0001$) (Fig. 1).

**Mutation frequencies to Rif<sup>R</sup>.** Each culture was concentrated and plated on medium containing rifampicin to determine mutation frequencies to Rif<sup>R</sup>. Screening of log<sub>10</sub>-transformed datasets revealed that cultures containing zero Rif<sup>R</sup> mutants were outliers, thus were removed. The screened datasets were found to be normally distributed. The average frequencies for mutation to Rif<sup>R</sup> were calculated to be $1.10 \times 10^8 \pm 6.25 \times 10^7$ for LB cultures and $4.22 \times 10^8 \pm 6.08 \times 10^7$ for SMM<sub>Asn</sub> cultures, respectively. The log<sub>10</sub>-transformed datasets were next tested for statistical differences by Two-way ANOVA with Tukey’s hsd test. Neither the three replicate cultures in LB nor the three replicate cultures in SMM<sub>Asn</sub> were significantly different among themselves ($P > 0.803$) (Fig. 2). However, all three LB datasets were significantly different than all three SMM<sub>Asn</sub> datasets ($P < 0.0001$) (Fig. 2). By this analysis it appeared that strain 168 mutated to Rif<sup>R</sup> at a slightly (~2.6-fold) but significantly higher frequency in LB than in SMM<sub>Asn</sub>.

**Mutation rates to Rif<sup>R</sup>.** Although the mutation frequency is a relatively simple parameter to calculate, it is inherently inaccurate because the number of mutants in any culture depends on when during the growth period the initial mutation occurred (16). We therefore took advantage of the large number of cultures ($n = 60$ from each growth condition) to perform fluctuation analysis and calculate the mutation rate $\mu$, the probability of Rif<sup>R</sup> mutation per
generation, under each growth condition. In prior experiments we established that in *B. subtilis* 152 the Minimum Inhibitory Concentration (MIC) for Rif was < 0.1 µg/mL (data not shown), thus 153 the selective concentration used in the experiment (5 µg/mL) was ~50x the MIC. From the initial 154 (N₁ ~10⁵) and final (N₂ ~10⁹) number of cells in each culture and the growth equation (\(\log N₂ - \log N₁\)/0.313, it was estimated that each population had passed through ~13 generations. The 157 number of Rif⁺ mutants and total cells were measured from each culture and fluctuation analysis 158 was performed using an online calculator, bz-rates (17). The data were observed to exhibit 159 reasonable goodness-of-fit to the Luria-Delbrück distribution; the distributions and population 160 statistics are shown in Fig. 3. The mutation rates \(\mu\) were calculated to be 3.33 x 10⁻⁹ and 1.66 x 161 10⁻⁹ for cells cultivated in LB and SMMₐn respectively, approximately a 2-fold difference 162 between the two growth conditions.

**Spectrum of Rif⁺ rpoB mutations in LB and SMMₐn cultures.** To ensure that each 163 Rif⁺ mutation had arisen independently, a single Rif⁺ mutant was selected from each culture for 164 mutation spectrum analysis. The N-cluster and Clusters I, II, and III of the rpoB gene were PCR 165 amplified from Rif⁺ mutants obtained from each LB and SMMₐn culture. Nucleotide sequences 166 were determined from a total of 53 LB and 51 SMMₐn Rif⁺ mutants (Table 2) and the data are 167 summarized graphically in Figure 4. All mutations were located in Cluster I, which forms the 168 Rif-binding pocket in *Escherichia coli* RpoB (18) and by analogy in *B. subtilis* RpoB (12). This 169 observation is consistent with prior studies indicating that most mutations leading to Rif⁺ occur 170 in Cluster I in *E. coli* (19, 20).

Prior studies of mutations in *B. subtilis* rpoB leading to Rif⁺ have documented that the 173 most common amino acid changes occur in Cluster I at amino acids Q469, H482, and S487 (12, 14, 15, 21-23). The mutation spectrum data corresponding to these three positions (Table 2) was
normalized as percentages of total point mutations that occurred within clusters N, I, II, or III of the \textit{rpoB} gene, which were determined to be distributed normally and analyzed by ANOVA (Fig. 5). Statistical analysis showed no significant difference between the percentage of mutations at Q469 in cells cultivated in LB vs. SMM\textsubscript{Asn} (Fig. 5). However, a noteworthy shift was observed in the proportion of mutations occurring at H482 and S487, depending on the growth medium used (Fig. 5). In cells cultivated in LB, most Rif\textsuperscript{R} mutations in \textit{rpoB} occurred at residue H482 (68.5 \pm 3.2\% of total). In contrast, cells cultivated in SMM\textsubscript{Asn} accumulated mostly the S487L mutation (47.9 \pm 8.1\% of total) (Fig. 5). When mutations at H482 were examined more closely, it was seen that the H482Y allele predominated under both growth conditions (Table 2).

In addition to mutations at Q469, H482, and S487, additional Rif\textsuperscript{R} mutations were observed in minor proportions at S465, D472, and A478, as have been noted previously. Two new Rif\textsuperscript{R} alleles were observed in this study, S465P and D472N. The discovery of these new alleles is likely due to the lower concentration of Rif (5 \mu g/mL) used to select for Rif\textsuperscript{R} in this study than was used in most previous studies (50 \mu g/mL). In six of the 111 Rif\textsuperscript{R} mutants sequenced, we did not find mutations in Clusters N, I, II, or III. This phenomenon has been reported previously (12, 24) and is likely to have occurred for two potential reasons. First, the mutations may have occurred in \textit{rpoB}, but were outside of the canonical Rif\textsuperscript{R} clusters. Considering that the regions we sequenced in this study only cover \textasciitilde 38\% of the entire \textit{rpoB} sequence, this is entirely possible. Second, the mutations may be located elsewhere in the genome outside the \textit{rpoB} gene. For example, low-level Rif\textsuperscript{R} has been previously reported in mutants with altered permeability or efflux (25).

Transitions and transversions in LB vs. SMM\textsubscript{Asn}. With the exception of one mutation, a +3 bp insertion (labeled as “Other” in Table 2), all mutations corresponding to \textit{rpoB} clusters N,
I, II, or III were found to be single-base changes leading to single amino acid substitutions. Given the different spectra of mutations seen in LB vs. SMM\textsubscript{Asn} cultures (Table 2 and Fig. 5), we wondered if the types of mutations seen (transitions vs. transversions) also differed in cells grown under the two conditions. The results of this analysis (Fig. 6) showed that cells suffered mostly (~85%) transition mutations, with the vast majority being C to T transitions (Fig. 6). The datasets were found to be normally distributed and were analyzed using ANOVA; no significant differences in the frequency of transition or transversion mutations were measured under either growth condition (Fig. 6).

**Relative Fitness of Predominant rpoB Alleles.** As reported above, a dramatic difference in the frequency of Rif\textsuperscript{R} mutations at H482 and S487 was observed in LB vs. SMM\textsubscript{Asn} medium. Given that the methodology we used for determining the spectrum of mutation in the \textit{rpoB} gene captured only one mutant per population, mutants that exhibit a less severe fitness deficit without selective pressure would be more likely to be detected. To test whether relative fitness might contribute to the difference in the observed frequency of a given mutation, we devised a series of pairwise competition experiments. Strains harboring either the wild-type \textit{rpoB} allele, the H482Y, or the S487L mutation were each competed against a congenic wild-type strain (strain WN1651) in either LB or SMM\textsubscript{Asn} medium for 21 generations in triplicate independent experiments (Fig. 7). The data were found to be normally distributed, thus amenable to analysis by normal statistics.

In LB medium, strains carrying either the H482Y or S487L mutation were significantly less fit than the wild-type strain, and there was no significant difference in fitness between the two mutant strains (Fig. 7). In marked contrast, in SMM\textsubscript{Asn} medium we observed that the S487L mutant exhibited a significantly less severe fitness deficit when compared to the H482Y mutant.
Visual inspection of the data suggested that in $\text{SMM}_{\text{Asn}}$ medium the S487L mutant was less fit than wild type, but the difference in their fitnesses was found to be not significant at the $P < 0.05$ level (Fig. 7). This increase in the fitness of the S487L mutant in $\text{SMM}_{\text{Asn}}$ medium may be related to its increased ability to metabolize Asn, as measured by Omnilog Phenotype Microarray analysis (Perkins and Nicholson, 2008). However, further studies would be required to confirm the specific mechanism resulting in the increased fitness of S487L relative to H482Y in $\text{SMM}_{\text{Asn}}$.

**Discussion**

In this communication we showed that the medium in which *B. subtilis* cells are cultivated exerts an effect on the resulting spectrum of mutations in the $rpoB$ gene to RifR. There may be several reasons underlying this observation. First, different external environments may impact the internal chemical environment within a *B. subtilis* cell in a distinct manner. For example, it has been shown that acid stress lowers the cytoplasmic pH (Martinez et al., 2012); osmotic stress increases the internal concentrations of compatible solutes such as proline, glycine betaine, ectoine, potassium, etc. (26); and the core of dormant spores has a dramatically lower water content and contains a high concentration of calcium dipicolinic acid (27). One could argue that such differences in the internal chemical environment engendered by cultivation in complex LB vs. minimal $\text{SMM}_{\text{Asn}}$ medium may differentially affect the reactivity of individual bases in DNA, resulting in different spectra of mutations (Nicholson and Maughan, 2002).

Second, three-dimensional nucleoid architecture (5, 28) and DNA supercoiling (Dorman and Dorman, 2016) are essential components of genetic regulation in prokaryotes, and both are sensitive to growth rate and the external environment. Because the transcription of different sets of genes is required for optimal growth in LB vs. $\text{SMM}_{\text{Asn}}$, it follows that changes in
chromosome architecture and supercoiling may also alter the reactivity of particular DNA bases, thus altering the possible spectrum of mutations in these two environments, in agreement with previous experiments using the yeast *S. cerevisiae* (7). While the experiments described here clearly show that the mutational spectrum of *rpoB* responds to the growth environment, they do not directly address which possible mechanism(s) which may be involved. This question could be addressed experimentally by measuring 3-dimensional genome-wide chromosome architecture via chromosome conformation capture (Hi-C) experiments (5) or DNA supercoiling of proxy plasmid molecules by 2-dimensional electrophoresis (29).

Under either growth environment, we noted that the preponderance of mutations (70-80%) observed in *rpoB* consisted of C-to-T transitions (**Fig. 6**). In a classic study of mutational hotspots in the *Escherichia coli lacI* gene it was shown that 5-methylcytosine (5mC) was a hotspot for C-to-T transitions (30). This observation prompted us to search for 5mC sites in the *B. subtilis rpoB* gene. In an unrelated project we had previously determined the *B. subtilis* methylome (data not shown). Searching this dataset revealed 37 5mC sites in the *rpoB* sequence; however, none of them coincided with the location of the Rif\(^R\) mutations reported here (**Fig. 4**).

In bacteria, RNAP is the central macromolecular machine responsible for contacting every promoter and transcribing every gene in the cell. A large body of evidence indicates that the identity and location of particular Rif\(^R\) mutations in *B. subtilis rpoB* can exert a wide variety of pleiotropic effects including: alterations in exponential growth rate and developmental events such as competence for DNA-mediated transformation, temperature-sensitive sporulation, temperature-sensitive germination (31) and catabolite repression-resistant sporulation and spore resistance properties (32); altering patterns of substrate metabolism and enhanced utilization of uncommon nutrient substrates (14); activation of a cryptic antibiotic biosynthetic operon (33);
and altered transcription termination through enhancement of the action of Rho and sensitivity to the elongation factor NusG (34); [reviewed in (35)]. Thus it is clear that single amino acid changes in RpoB can exert profound effects on the global transcriptome.

Under standard laboratory conditions, mutations in *B. subtilis* rpoB leading to a RifR phenotype have been associated with a fitness burden (23, 31, 36), and indeed RifR mutants carrying the predominant rpoB alleles isolated from LB and SMMAsn, H482Y and S487L respectively, were both significantly less fit than wild type when competed in LB medium (Fig. 7). However, when competed in SMMAsn minimal medium, fitness of the S487L mutant, but not the H482Y mutant, was statistically indistinguishable from wild type (Fig. 7). Thus, cultivation of *B. subtilis* in SMMAsn medium favored production of the S487L mutation (Table 2, Fig. 4), and the S487L mutant exhibited increased fitness in SMMAsn medium (Fig. 7). This situation may have a clinically relevant analog in the evolution of rifampicin resistance in *Mycobacterium tuberculosis* (Mtb), the causative agent of the disease tuberculosis. Numerous studies have documented that globally 60% of all RifR Mtb clinical isolates carry the RpoB mutation S531L, the equivalent of the *B. subtilis* S487L mutation (Supplemental Table 1). It is tempting to speculate that environmental factors within the host (e.g. anaerobiosis, nutritional status, immune attack) may alter the spectrum of spontaneous RifR mutations in the Mtb rpoB gene to favor production of the S531L mutation over others, which in turn may confer a fitness advantage in the host. Elucidating the specific details underlying these phenomena will advance our understanding of how microbes evolve to optimally function within such diverse environments.

**Materials and Methods**
**Strains, media, and culture conditions:** A list of all bacterial strains used in this study is provided in Table 1. Media used were Miller LB medium (37) and Spizizen Minimal Medium (38) supplemented with 95 mM Asn in place of glucose (SMM\textsubscript{Asn}). Both media were prepared using ultra-purified (Nanopure) water and were supplemented with the following final concentration of trace minerals: 50µM CaCl\textsubscript{2} \cdot 2H\textsubscript{2}O, 5 µM MnCl\textsubscript{2} \cdot 4H\textsubscript{2}O, 12.5 µM ZnSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 2µM CuSO\textsubscript{4} \cdot 5H\textsubscript{2}O, 2.5 µM CoCl\textsubscript{2} \cdot 6H\textsubscript{2}O, 2.5µM Na\textsubscript{2}MoO\textsubscript{4} \cdot 2H\textsubscript{2}O, 0.5 µM FeCl\textsubscript{3}, and 3.5 µM sodium citrate dihydrate. For fluctuation tests, 20 separate cultures (2 mL each in 16-mm test tubes) in either LB or SMM\textsubscript{Asn} were each inoculated with ~10\textsuperscript{5} cells from an overnight LB culture. All tubes were incubated on a slant for 24 hours in a rotary water bath set to 37°C and 200 rpm, and samples were processed as described below. Each experiment was repeated on three separate occasions, for a total of \(n = 60\) individual cultures per growth condition.

**Mutation frequency determinations:** Following 24 hours of growth, from each 2-mL culture a 10 µL aliquot was removed and diluted serially tenfold in Phosphate Buffered Saline, pH 7.5 (PBS) to determine total colony-forming units (CFU)/mL. A 1.5-mL aliquot from each remaining culture was pelleted via centrifugation, resuspended in a total volume of 0.15 mL, and plated on LB or SMM\textsubscript{Asn} agar plates supplemented with Rif (5 µg/mL final concentration) to select for Rif\textsuperscript{R} mutants.

**Mutation rate determinations.** For fluctuation analyses, data from sample sets from each growth condition were entered into the online mutation rate calculator bz-rates (http://www.lcqb.upmc.fr/bzrates) (17).

**Mutation spectrum determinations:** One Rif\textsuperscript{R} colony from each individual culture was picked, resuspended in 0.01 mL nuclease-free sterile water and heat-lysed for five minutes at 98°C. Cell debris was pelleted via centrifugation. The supernatant was used as template for PCR.
amplification of two ~700 base pair amplicons within the *rpoB* gene spanning the N-cluster and Clusters I, II, and III (Fig. 4) using the primer pairs and PCR conditions described in detail previously (12). An aliquot of each PCR product was assessed via 0.8% agarose gel electrophoresis for appropriate amplification and length, and the rest of each sample was column-purified using a Qiagen PCR purification kit and shipped to Genewiz LLC (South Plainfield, NJ) for Sanger sequencing using the same PCR primers.

**Competition Assays.** Strains to be competed in pairwise combinations each carried a distinct antibiotic resistance marker (Table 1). Individual overnight cultures of the wild-type strain and each congenic Rif<sup>R</sup> strain were grown in fresh 2-mL LB cultures under antibiotic selection. Upon entering stationary phase, the OD<sub>660</sub> of each seed culture was measured and a volume corresponding to ~10<sup>8</sup> cells of each strain was combined into a single mixed culture which was then diluted 1:100 into 10 mL of either LB or SMM<sub>Asn</sub> medium lacking selective antibiotics in 125 mL Erlenmeyer flasks. Cultures were grown at 37 °C in a temperature-controlled rotary shaking bath with 200 rpm shaking. Every 24 hours cultures were diluted 1:100 into fresh nonselective media and cultivation continued. Under these conditions, cultures progress through ~7 generations per day (39). At 24-hour intervals, an aliquot from each culture was diluted serially tenfold in PBS and plated on two different selective LB plates, each supplemented with the appropriate selective antibiotic. These LB plates were then incubated overnight at 37 °C to determine CFU/mL of each strain. Relative fitness was quantified using a previously described selection coefficient model (40).

**Statistics.** CFU/mL and Mutation Frequency data sets from each replicate experiment were log<sub>10</sub> transformed and screened for outliers. Following removal of outliers from data, all data sets were tested for normality using the Shapiro-Wilk method (41). Data sets passing the
normality test were analyzed for differences by Analysis of Variance (ANOVA) (42) with Tukey’s Honestly Significant Difference (hsd) test (43). All statistical analyses were performed with the statistical graphing software Prism (GraphPad Software). Differences with $P < 0.05$ were considered statistically significant.

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Figure Legends

Figure 1. Twenty four-hour cell densities of *B. subtilis* cells cultivated in LB and in SMM<sub>Asn</sub>. Data are averages ± standard deviation. Averages of each set of 3 replicate experiments (Rep), each consisting of *n* = 20, are indicated by dashed lines. No significant difference (ns) was observed between replicates in the same media (Two-way ANOVA with Tukey’s hsd test, *P* > 0.05) Comparison of all experiments in LB vs. SMM<sub>Asn</sub> (Two-way ANOVA with Tukey’s hsd test, *P* < 0.0001) is denoted.

Figure 2. Frequency mutation to Rif<sup>R</sup> in *B. subtilis* cultivated in LB vs. SMM<sub>Asn</sub>. Data are averages ± standard deviation. Dashed lines indicate the average of 3 separate replicates (Rep). No significant difference (ns) was observed between replicates in the same media (Two-way ANOVA with Tukey’s hsd test, *P* > 0.05). Mutation frequency was significantly higher in LB than in SMM<sub>Asn</sub> cultures (Two-way ANOVA with Tukey’s hsd test, *P* < 0.0001).

Figure 3. Mutation rate analysis. Fit of data (black dots) to the Luria-Delbrück distribution (LD Model; dashed line) and statistics for 60 cultures grown in either LB (A) or SMM<sub>Asn</sub> (B). Note different scales on X-axes.

Figure 4. (Top) Schematic diagram of the *B. subtilis* RpoB protein sequence (red bar). Yellow boxes denote the positions of the N-cluster and Clusters I, II, and III where Rif<sup>R</sup> mutations have been located (Severinov et al., 1993). Gray bars denote the regions amplified by PCR and sequenced using the corresponding primers (black arrows). (Bottom) Expanded graphic summary of the distribution of Rif<sup>R</sup> mutations within Cluster I of the *B. subtilis rpoB* gene. The center row depicts the RpoB amino acid sequence from amino acids 465-489, flanked above and
below by the wild-type *rpoB* nucleotide sequence. Mutations identified in LB and SMM$_{Asn}$
cultures are denoted above and below the central line, respectively. Each small, colored box
represents an independently sequenced mutation, and the corresponding amino acid alteration is
indicated in a large white box above. Three shades of gray and blue are used to represent the
three separate replicate cultures grown in LB and SMM$_{Asn}$ respectively.

**Figure 5.** Percentage of total Rif$^R$ mutations observed at Q469, H482, and S487 within RpoB in
*B. subtilis* cells cultivated in LB (black bars) vs. SMM$_{Asn}$ (blue bars). Data are averages +
standard deviations ($n = 3$) and were analyzed by Two-way ANOVA with Tukey’s hsd test. ns,
not significantly different.

**Figure 6.** Distribution of transition and transversion mutations in *B. subtilis* cells grown in either
LB (black bars) or SMM$_{Asn}$ (blue bars). **X-axis:** The bottom letter represents original wild-type
nucleotide in *rpoB*; the top letter indicates the mutant nucleotide which replaced the wild-type
nucleotide in *rpoB*. Data are averages ± standard deviations from $n = 3$ replicates. No significant
differences in the percentage of a given type of transition or transversion mutation were observed
in different growth environments (Two-way ANOVA with Tukey’s hsd test, $P > 0.9999$)

**Figure 7.** Pairwise competition experiments. Wild-type (w.t.) strain WN1675 (black bars) and
Rif$^R$ strains carrying the variants H482Y (strain WN760; blue bars) or S487L (strain WN761;
yellow bars) were each competed against congenic wild-type strain WN1651 in LB or SMM$_{Asn}$
medium for 21 generations and fitness coefficients ($S$) were calculated as described previously
(Woods et al., 2011). Data are averages ± standard deviation ($n = 3$) and were analyzed by Two-
way ANOVA with Tukey’s hsd test. Degree of statistical significance is denoted with asterisks

\( ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001 \). Non-statistically significant differences are
denoted with ns \( (P > 0.05) \).
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype; phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis subsp. subtilis strain 168</td>
<td>trpC2</td>
<td>(44)</td>
</tr>
<tr>
<td>WN547</td>
<td>trpC2 pheA1 cat 6His-rpoC; CmR</td>
<td>(45)</td>
</tr>
<tr>
<td>WN760</td>
<td>trpC2 pheA1 rpoB-H482Y cat 6His-rpoC; RifR CmR</td>
<td>(31)</td>
</tr>
<tr>
<td>WN761</td>
<td>trpC2 pheA1 rpoB-S487L cat 6His-rpoC; RifR CmR</td>
<td>(31)</td>
</tr>
<tr>
<td>WN1651</td>
<td>trpC2 pheA1 amyE::spc cat 6His-rpoC; CmR SpcR</td>
<td>This study</td>
</tr>
<tr>
<td>WN1675</td>
<td>trpC2 pheA1 amyE::erm cat 6His-rpoC; CmR ErmR</td>
<td>This study</td>
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### Table 2. Summary of rpoB mutations leading to Rif$^R$ in *B. subtilis* LB and SMM$_{Asn}$ cultures.

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<th>Replicate</th>
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<th>3</th>
<th>Total</th>
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<td>SMM$_{Asn}$</td>
<td>LB</td>
<td>SMM$_{Asn}$</td>
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<tr>
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<tr>
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<td>0</td>
</tr>
<tr>
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<td>19</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

522
% of Total Point Mutations

- Q469
- H482
- S487

- LB
- SMM_{Asn}

$P = 0.0004$

$P < 0.0001$

$P = 0.700$ ns