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ARTICLE

# Antibiotic resistance mutations induced in growing cells of *Bacillus*-related thermophiles

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## Abstract

Stress-induced mutagenesis can assist pathogens in generating drug-resistant cells during antibiotic therapy; however, if and how antibiotics induce mutagenesis in microbes remains poorly understood. A non-pathogenic thermophile, *Geobacillus kaustophilus* HTA426, efficiently produces derivative cells resistant to rifampicin and streptomycin via *rpoB* and *rpsL* mutations, respectively. Here, we examined this phenomenon to suggest a novel mutagenic mode induced by antibiotics. Fluctuation analysis indicated that mutations occurred via spontaneous mutations during culture. However, mutations were much more frequent in growing cells than stationary cells, and mutation sites were varied through cell growth. These observations suggested that growing cells induced mutagenesis in response to antibiotics. An in-frame deletion of *mfd*, which governs transcription-coupled repair to correct DNA lesions on the transcribed strand, caused mutations that were comparable between growing and stationary cells; therefore, the mutagenic mechanism was attributable to DNA repair defects where growing cells depressed *mfd* function. Mutations occurred more frequently at optimal growth temperatures for *G. kaustophilus* than at a higher growth temperature, suggesting that the mutagenesis relies on active cellular activities rather than high temperature-associated DNA damage. In addition, the mutagenesis may involve a mutagenic factor targeting these sites, in addition to *mfd* depression, because *rpoB* and *rpsL* mutations were dominant at thymine and guanine sites on the transcribed strand. A similar mutagenic profile was observed for other *Geobacillus* and thermophilic *Bacillus* species. This suggests that *Bacillus*-related thermophiles commonly induce mutagenesis in response to rifampicin and streptomycin to produce resistant cells.

## Introduction

Pathogens often generate drug-resistant cells during antibiotic therapy via spontaneous gene mutations that primarily arise from DNA damage (e.g., deamination, depurination, guanine oxidation) and replication errors [1]. Deamination spontaneously occurs to convert cytosine and adenine into uracil and hypoxanthine, respectively, which then form base pairs with adenine and cytosine, thereby causing C:G→T:A and A:T→G:C transitions via genome replication [2, 3]. Guanine oxidation mainly arises from active oxygen species, producing the aberrant base 8-oxo-7,8-dihydroguanine (8-oxoG). Because 8-oxoG forms base pairs with adenine and cytosine, this damage elicits C:G→A:T transversions [4]. Guanine oxidation also produces 8-oxo-7,8-dihydroguanosine 5′-triphosphate. This product can be used for genome replication, causing A:8-oxoG mispair formation that results in A:T→C:G transversions [5]. Depurination and replication errors cause many types of transitions and transversions through nucleotide misincorporation during genome replication.

Spontaneous mutations may lead to favorable adaptation, but are generally deleterious for microbes; therefore, microbes essentially seek to suppress mutations using several systems for DNA repair. In *Bacillus subtilis* and *Escherichia coli*, mispairs that arise from replication errors are corrected using *mutSL*-dependent and *mutSLH*-dependent mismatch repairs, respectively [6–8]. *E. coli* also corrects DNA damage using base excision repair, in which aberrant bases are excised by

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DNA glycosylases, such as the *ung* product that excises uracil from the uracil:G mispair [9] and the *mutM* and *mutY* products that excise 8-oxoG from C:8-oxoG and A:8-oxoG, respectively [5, 10]. In addition, *E. coli* utilizes *mutT* and *mfd* to decrease spontaneous mutations. The *mutT* product degrades 8-oxo-7,8-dihydroguanosine 5′-triphosphate to prevent 8-oxoG incorporation during genome replication [5]. Transcription-coupled repair, which corrects DNA lesions on the transcribed strand, is governed by the *mfd* product that works to remove RNA polymerases that are stalled at a DNA lesion, while recruiting DNA repair proteins to the lesion on the transcribed strand [11, 12]. Because deamination and depurination rates are known to increase at high temperatures [13–16], thermophiles presumably undergo frequent DNA damage. However, it has been shown that spontaneous mutations in the hyperthermophile *Sulfolobus acidocaldarius* are comparable to those in mesophiles, probably due to its efficient systems for DNA repair [17]. Intriguingly, some microbes employ stress-induced mutagenesis [18–20]. Whereas spontaneous mutations constitutively occur during cell divisions, induced mutagenesis generates mutations in response to environmental stressors but not under stress-free conditions, thereby serving as a convenient strategy for microbial adaptation.

The genus *Bacillus* comprises gram-positive, aerobic or facultative anaerobic, and endospore-forming bacilli. The members of this genus have been identified from diverse and often extreme environments. Several thermophilic members of the genus have been reclassified into the new genus *Geobacillus* [21]. The thermophiles of the genera *Bacillus* and *Geobacillus* (termed *Bacillus*-related thermophiles) preferentially grow at 55–65 °C and are non-pathogenic [22–24]. We have studied thermoadaptation-directed enzyme evolution using a strain, *Geobacillus kaustophilus* HTA426 [25–28]. In this connection, the strain was previously characterized for genetic mutability using rifampicin (Rif) and streptomycin (Str) as indicators and was demonstrated to produce substantial Rif-resistant ($Rif^R$) and Str-resistant ($Str^R$) via *rpoB* and *rpsL* mutations, respectively [27]. This phenomenon may be due simply to frequent spontaneous mutations during cell division, but is also possible via stress-induced mutagenesis. In this study, we examined this phenomenon in detail and identified stress-induced mutagenesis that was invoked notably in growing cells in response to antibiotics. We also showed that a similar type of mutagenesis, termed growing cell-specific stress-induced mutagenesis (GroSIM), was observed in diverse *Bacillus*-related thermophiles.

## Materials and methods

### Bacterial strains and culture conditions

Table 1 summarizes the *Bacillus*-related thermophiles used in this study. *G. kaustophilus* HTA426 (JCM 12893) was obtained from the RIKEN BioResource Center (Tsukuba,

**Table 1** *Bacillus*-related thermophiles used in this study

| Strain | Relevant description | Reference |
|---|---|---|
| *G. kaustophilus* HTA426 | Wild-type | 22 |
| *G. kaustophilus* MK370 | HTA426 derivative; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) *GK0707*::$P_{gk704}$-*bgaB trpE*:: pGKE70 | 27 |
| *G. kaustophilus* Δ*mutSL* | MK370 derivative having in-frame deletion in *mutSL* gene | 27 |
| *G. kaustophilus* Δ*mutM* | MK370 derivative having in-frame deletion in *mutM* gene | 27 |
| *G. kaustophilus* Δ*mutY* | MK370 derivative having in-frame deletion in *mutY* gene | 27 |
| *G. kaustophilus* Δ*mutT* | MK370 derivative having in-frame deletion in *mutT* gene | 27 |
| *G. kaustophilus* Δ*ung* | MK370 derivative having in-frame deletion in *ung* gene | 27 |
| *G. kaustophilus* Δ*mfd* | MK370 derivative having in-frame deletion in *mfd* gene | 27 |
| *G. subterraneus* DSM 13552 | Isolate from formation water of the Liaohe oil field | 23 |
| *G. stearothermophilus* ATCC 12980 | Type strain of *G. stearothermophilus* | 23 |
| *G. thermoleovorans* DSM 5366 | Isolate from soil near hot water effluent | 23 |
| *G. uzenensis* DSM 13551 | Isolate from formation water of the Liaohe oil field | 23 |
| *B. caldolyticus* DSM 405 | Isolate from hot natural pool | 24 |
| *B. caldotenax* DSM 406 | Isolate from superheated pool water | 24 |
| *B. caldovelox* DSM 411 | Isolate from superheated pool water | 24 |

*G. kaustophilus* MK370 lacks genes related to pyrimidine biosynthesis (*pyrF* and *pyrR*) and DNA restriction–modification ($hsdM_1S_1R_1$, $mcrB_1$, $mcrB_2$, $hsdM_2S_2R_2$, and *mrr*) but integrates the pGKE70 plasmid [27] and *bgaB* expression cassette ($P_{gk704}$-*bgaB*) at the *trpE* and *GK0707* loci, respectively. Because pGKE70 contains *TK101* gene, which encodes thermostable kanamycin nucleotidyltransferase [29], *G. kaustophilus* MK370 and its derivatives are resistant to kanamycin

Japan). Other thermophiles and *B. subtilis* 168 were purchased from the Bacillus Genetic Stock Center (Columbus, OH, USA). If not otherwise specified, the thermophiles were grown at 60 °C in Luria–Bertani (LB) medium. *G. kaustophilus* MK370 and its derivatives were constructed previously [27]. These strains were resistant to kanamycin due to *TK101* gene [29], so they were cultured in the presence of kanamycin (5 mg $l^{-1}$). $Rif^R$ and $Str^R$ cells were identified on LB plates supplemented with Rif and Str (10 mg $l^{-1}$), respectively; however, a higher concentration of Str (100 mg $l^{-1}$) was used for *B. subtilis* because of its intrinsic resistance to lower concentration of Str.

### Luria–Delbrück fluctuation test

*G. kaustophilus* MK370 was precultured overnight with rotary shaking at 180 rpm in LB medium. Aliquots ($10^3$ cells containing <1 $Rif^R$ and $Str^R$ cells stochastically) then were inoculated into 11 shaking flasks (500 ml) with LB medium (120 ml). These cultures were incubated for 12 h (until stationary phase) with reciprocal shaking at 150 rpm; subsequently, ten culture aliquots from one flask (for control tests; $10^9$ cells each) and individual aliquots from the other ten flasks (for fluctuation tests; $10^9$ cells each) were incubated independently on LB plates with Rif or Str. After incubation for 24 h, colonies were counted exactly to determine the mean and variance for each test. This process was performed without a dilution series because colonies were $<10^3$ on each plate, and thus countable.

### Mutation frequency assay

In this experiment, thermophiles and *B. subtilis* were grown at 60 and 37 °C, respectively. Bacterial cells were cultured overnight in LB medium at 180 rpm and mixed with glycerol to a 20% final concentration. The mixture was divided into aliquots and stored at −80 °C until further use. The stock was thawed, and an aliquot ($10^5$ cells) was inoculated into LB medium (20 ml) in an Erlenmeyer flask (100 ml) with a silicon plug. In each experiment, four flasks were incubated with rotary shaking at 180 rpm; optical density at 600 nm ($OD_{600}$) was monitored using an infrared-dependent $OD_{600}$ detector (OD-MonitorA; Taitec, Saitama, Japan). We confirmed that this instrument can detect $OD_{600}$ below 2.4 without correction. Upon reaching $OD_{600} = 1$ (proliferative phase; PR), cells in one flask were concentrated by centrifugation. Large portions of the resultant cells were spread on LB plates with Rif and Str to isolate $Rif^R$ and $Str^R$ cells, respectively. Because portions appropriate for spreading onto plates were determined preliminarily, the process was performed without a dilution series. The remaining cells were incubated on LB plates without Rif or Str to determine viable cell concentrations. This analysis was performed for all cultures, along with a dilution series. The colonies grown on plates ($<10^3$ colonies) were counted to calculate the ratio of adaptive ($Rif^R$ or $Str^R$) cells to viable cells incubated, which was defined as the mutation frequency. This frequency also was determined for cells in three other flasks after additional 2 (early stationary phase; ES), 4 (stationary phase 1; S1), and 6 h of incubation (stationary phase 2; S2) from the PR phase. The analysis was repeated more than three times ($n = 3$–5).

### Temperature effect assay

This analysis was performed in accordance with the procedure for mutation frequency assay, as described above. *G. kaustophilus* MK370 was precultured in LB medium, and then an aliquot ($10^5$ cells) was cultured in LB medium (20 ml). Upon reaching PR phase, cells were spread onto LB plates with and without Rif or Str and further incubated for 24 h at 50–70 °C. Colonies grown on plates were then counted to determine the mutation frequency. This analysis was repeated four times ($n = 4$).

### Mutation site analysis

*G. kaustophilus* MK370 was cultured in LB medium (100 ml) in a single shaking flask. Upon reaching PR, ES, S1, and S2 phases, culture aliquots were recovered successively from the flask and incubated on LB plates with Rif and Str to isolate $Rif^R$ and $Str^R$ cells, respectively. From the $Rif^R$ and $Str^R$ cells isolated, the *rpoB* and *rpsL* genes were amplified, respectively, and sequenced to analyze mutations. The *rpoB* gene was amplified using primers 5′-GGAACGCGTCATTGTTTCCC-3′ and 5′-TCCGATAGTTGATTGTCTCC-3′, whereas *rpsL* was amplified using 5′-CGATCATCGAAAAAGTGACC-3′ and 5′-TGCGAGTCTTTTCCAAGGAG-3′.

## Results

### Fluctuation tests using stationary cells

We previously observed that *G. kaustophilus* MK370, which was derived from the wild-type strain HTA426, efficiently provided $Rif^R$ and $Str^R$ cells via *rpoB* and *rpsL* mutations, respectively [27]. To see whether the phenomenon arose from spontaneous or induced mutations, MK370 cells in late stationary phase were subjected to fluctuation tests. In the test, induced mutations theoretically exhibit the mean equal to the variance, whereas spontaneous mutations exhibit a variance much higher than the mean [30, 31]. These tests exhibited variances that were higher than the means (mean ± variance: $Rif^R$ colonies, 280 ± 12,000; $Str^R$

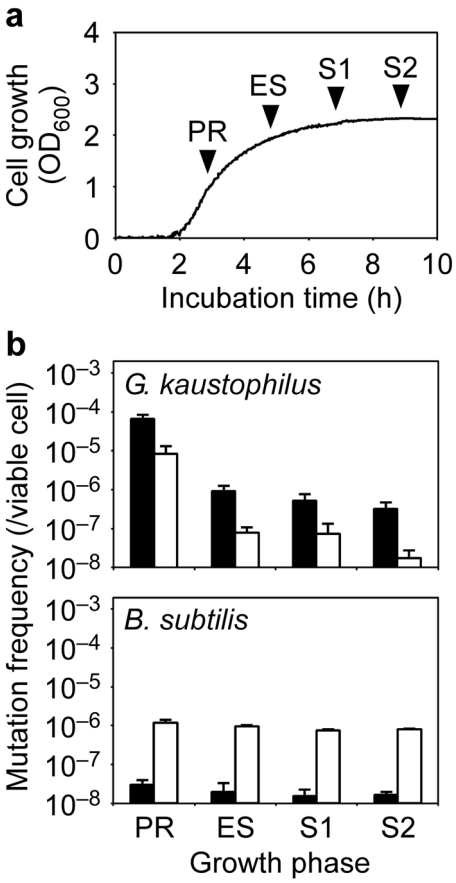


**Fig. 1** GroSIM of *G. kaustophilus* MK370. **a** Growth curve of *G. kaustophilus* MK370 in LB medium at 60 °C. The growth phases at $OD_{600} = 1$ and 2, 4, and 6 h later following $OD_{600} = 1$ were defined as PR, ES, S1, and S2 phases, respectively. **b** $Rif^R$ (solid bars) and $Str^R$ (hollow bars) mutations in *G. kaustophilus* MK370 (upper panel) and *B. subtilis* 168 (lower panel) in each growth phase. *G. kaustophilus* and *B. subtilis* were incubated at 60 °C and 37 °C, respectively. Data are presented as the mean ± standard error ($n = 3$–5)

colonies, 40 ± 900). Variances were even higher than those for the control tests ($Rif^R$ colonies, 510 ± 1900; $Str^R$ colonies, 31 ± 56). Thus, it was likely that $Rif^R$ and $Str^R$ mutations in stationary cells resulted from spontaneous mutations during preculture.

## $Rif^R$ and $Str^R$ mutations in each growth phase

The fluctuation test indicated spontaneous mutations (see above); however, $Rif^R$ and $Str^R$ mutations seemed to be less frequent in the fluctuation test that used late stationary cells than in the previous observations for early stationary cells [27], leading us to hypothesize that growth phases may affect mutation frequency. To test this hypothesis. *G. kaustophilus* MK370 was precultured to the respective growth phases, which were systematically divided into four stages (Fig. 1a), and then evaluated for $Rif^R$ and $Str^R$ mutations based on the mutation frequency (Fig. 1b). As expected, the analysis showed that $Rif^R$ mutations were more frequent in the PR phase than in the stationary phases with >71-fold increase ($p < 0.01$; one-way ANOVA; $n = 5$), and the same phenomenon was observed for $Str^R$ mutations with >100-fold increase ($p < 0.10$; one-way ANOVA; $n = 5$). In theory, a spontaneous mutation generated in earlier phases is conserved throughout the subsequent phases in a large population, unless the mutation slows cell growth. Because $Rif^R$ and $Str^R$ cells could grow as rapidly as the parent strain MK370, it was unlikely that $Rif^R$ and $Str^R$ cells were present substantially in the PR phase via spontaneous mutations and then were decreased drastically during the stationary phases. Instead, frequent mutations in growing cells (in the PR phase) could be explained by induced mutations specific to growing cells (i.e., GroSIM). In *B. subtilis*, $Rif^R$ and $Str^R$ mutations were equally common in the growth phases (Fig. 1b); therefore, GroAIM is not common in bacteria.

## Mutations generated in $Rif^R$ and $Str^R$ cells

A spontaneous mutation generated in earlier phases is conserved throughout the subsequent phases, but an induced mutation is not always conserved. To confirm induced mutations in growing cells, *G. kaustophilus* MK370 was cultured in one flask and cells in each growth phase were used to generate $Rif^R$ and $Str^R$ cells, followed by *rpoB* and *rpsL* mutation analyses, respectively. The analyses were carried out twice (experiments 1 and 2). Table 2 summarizes *rpoB* and *rpsL* mutations identified in respective experiments. Altogether, 11 *rpoB* mutations were identified between codons 469 and 487. In *rpsL*, four mutations were identified at either codon 56 or 101. Several $Str^R$ cells had no mutation in *rpsL*, probably because of an *rrn* mutation responsible for $Str^R$ as well as *rpsL* mutations [32]. The *rpoB* and *rpsL* mutations were similar to those observed for *B. subtilis* [33–36]; however, mutations were more frequent and diversified in *G. kaustophilus* than in *B. subtilis*, suggesting more active mutagenesis in *G. kaustophilus*. On the transcribed strand, most mutations were observed at the thymine and guanine sites. Because A302G in *rpsL* (experiment 1) were conserved throughout growth phases, the mutation probably arose from spontaneous mutations prior to the PR phase. However, several mutations generated in growing cells were not identified in stationary cells, as exemplified by G1414T (experiment 1), C1444T (experiment 2), and A1445G (experiment 1) in *rpoB* and A301G (experiment 2) in *rpsL*. Because all mutants grew at comparable rates, these data confirmed that mutations in the PR phase were mainly generated via GroSIM following antibiotic exposure on LB plates (Fig. 2).

**Table 2** *rpoB* and *rpsL* mutations generated in MK370 cells at each growth phase

| Mutations | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | PR | ES | S1 | S2 | PR | ES | S1 | S2 |
| *rpoB* in Rif$^R$ cells | | | | | | | | |
| T1393C | | 3/6 | | | 1/6 | 1/6 | 1/6 | |
| A1406G | | | 1/6 | 2/6 | | 1/6 | | |
| A1406T | | 1/6 | | 1/6 | | 1/6 | | |
| G1414T | 1/6 | | | | | | | |
| A1415T | 3/6 | | | 2/6 | | | 1/6 | |
| C1433A | | 1/6 | 2/6 | | | | 1/6 | |
| C1433T | 1/6 | 1/6 | 2/6 | 1/6 | 1/6 | 2/6 | 1/6 | 4/6 |
| C1444T | | | | | 4/6 | | | |
| A1445G | 1/6 | | | | | 1/6 | 1/6 | |
| C1460A | | | 1/6 | | | | | |
| C1460T | | | | | | | 1/6 | 2/6 |
| *rpsL* in Str$^R$ cells | | | | | | | | |
| A167C | | | | | | 1/6 | | |
| A167G | | | | | | 2/6 | | |
| A301G | | | | | 4/6 | | 4/6 | |
| A302G | 6/6 | 6/6 | 6/6 | 6/6 | | 3/6 | 2/6 | |

*G. kaustophilus* MK370 was precultured in one flask until PR, ES, S1, and S2 phases were reached and used to generate Rif$^R$ and Str$^R$ cells. The *rpoB* and *rpsL* genes were subsequently sequenced to determine mutations in Rif$^R$ and Str$^R$ cells, respectively. The mutation indicates substitutions on the non-transcribed strand. The fraction represents the number of sequences carrying the mutation per total number of sequences determined. The analysis was carried out twice (experiments 1 and 2). Several Str$^R$ clone had no mutation in *rpsL*, probably because of an *rrn* mutation [32]

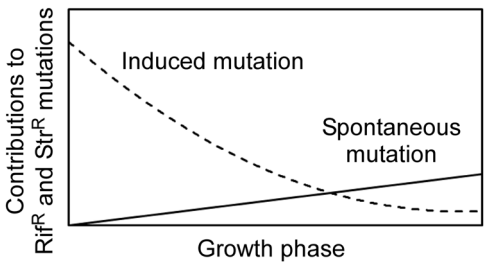


**Fig. 2** Proposed contributions of spontaneous (solid line) and induced mutations (broken line) to Rif$^R$ and Str$^R$ mutations in *G. kaustophilus* MK370. Spontaneous mutations occur during cell divisions while accumulating Rif$^R$ and Str$^R$ mutants, whereas induced mutations preferentially occur in the PR phase following Rif and Str exposure

## Temperature effects on GroSIM

It is known that increased temperatures can accelerate certain types of DNA damage [13–16]. To determine whether GroSIM relies on high growth temperatures of *G. kaustophilus*, Rif$^R$ and Str$^R$ mutations at 50–70 °C were analyzed using MK370 cells in the PR phase (Fig. 3a). Overall, these

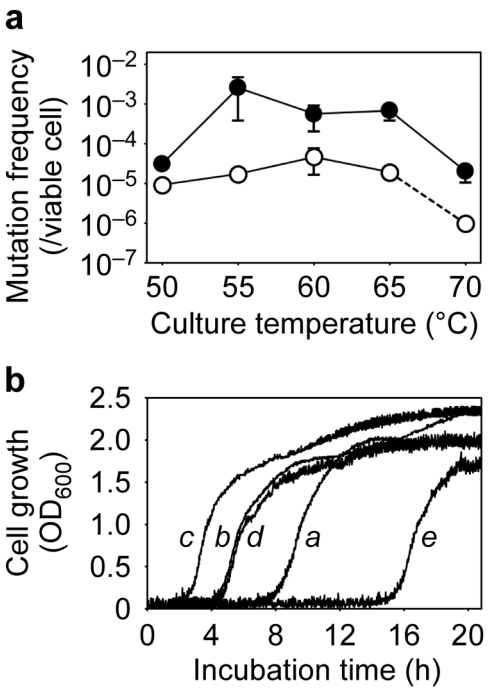


**Fig. 3** Effects of culture temperature on GroSIM. **a** *G. kaustophilus* MK370 in the PR phase was incubated at 50–70 °C on LB plates supplemented with Rif or Str to analyze temperature effects on GroSIM. The frequency of Rif$^R$ (solid circles) and Str$^R$ (hollow circles) mutations is presented as the mean ± standard error ($n = 4$). Str$^R$ cells were not observed at 70 °C ($<1 \times 10^{-6}$). **b** Growth curves of *G. kaustophilus* MK370 in LB medium at 50 °C (*a*), 55 °C (*b*), 60 °C (*c*), 65 °C (*d*), and 70 °C (*e*)

mutations occurred frequently between 55 and 65 °C, exhibiting bell-shaped profiles, in accordance with the optimal growth temperatures of *G. kaustophilus* (Fig. 3b). This suggested that GroSIM relied on active cellular activities (e.g., biological mutagenic factors and rapid genome replication to fix DNA damage as mutations) rather than high temperature-associated DNA damage for increasing gene mutations.

## Δmfd causes Rif$^R$ and Str$^R$ mutations irrespective of growth phases

We previously constructed MK370 mutants that deleted either *mutSL*, *mutM*, *mutY*, *mutT*, *ung*, or *mfd* in frame [27]. To determine the involvement of these genes in GroSIM, the mutants in PR and S2 phases were analyzed for Rif$^R$ and Str$^R$ mutations at 60 °C (Fig. 4). All mutants showed an increase in mutation frequency, but with different rates between the PR and S2 phases. Mutation frequency by Δ*mutSL*, Δ*mutM*, and Δ*mutY* was enhanced more in the PR phase than in the S2 phase, whereas Δ*mutT* and Δ*ung* enhanced mutation frequency in both phases. Consequently, the five mutants still exhibited GroSIM. However, Δ*mfd* caused comparable mutation frequencies between the two

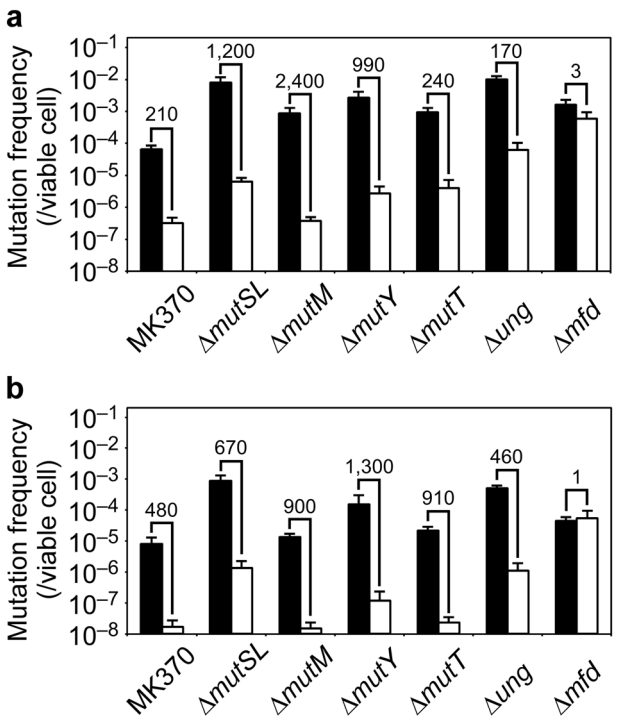


**Fig. 4** Effects of Δ*mutSL*, Δ*mutM*, Δ*mutY*, Δ*mutT*, Δ*ung*, and Δ*mfd* on GroSIM. Null mutants (Δ*mutSL*, Δ*mutM*, Δ*mutY*, Δ*mutT*, Δ*ung*, and Δ*mfd*) were precultured until the PR (solid bars) and S2 (hollow bars) phases and then incubated at 60 °C on LB plates supplemented with Rif or Str to analyze GroSIM in the mutants. The frequency of $Rif^R$ (**a**) and $Str^R$ (**b**) mutations is presented as the mean ± standard error ($n = 4$). The inset number indicates the fold change of the mutation frequency in the PR phase to that in the S2 phase

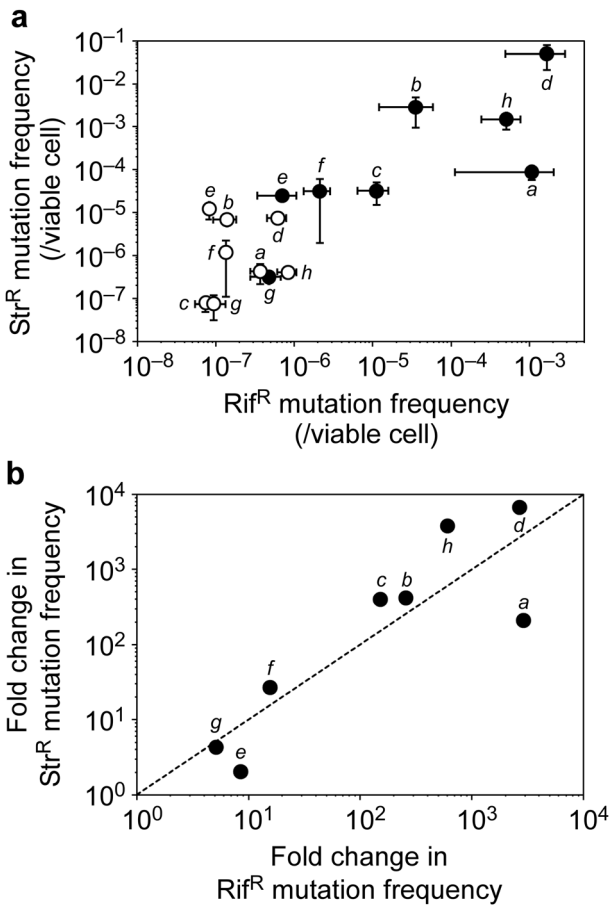


**Fig. 5** GroSIM distributed in *Bacillus*-related thermophiles. **a** *Bacillus*-related thermophiles in the PR (solid circles) and the S1 (hollow circles) phases were incubated at 60 °C on LB plates supplemented with Rif or Str to analyze GroSIM. The frequencies of $Rif^R$ and $Str^R$ mutations are shown as two-dimensional plots of the mean ± standard error ($n = 4$). Thermophiles examined are *G. kaustophilus* HTA426 [22] (*a*), *G. subterraneus* DSM 13552 [23] (*b*), *G. stearothermophilus* ATCC 12980 [23] (*c*), *G. thermoleovorans* DSM 5366 [23] (*d*), *G. uzenensis* DSM 13551 [23] (*e*), *B. caldolyticus* DSM 405 [24] (*f*), *B. caldotenax* DSM 406 [24] (*g*), and *B. caldovelox* DSM 411 [24] (*h*). **b** The plots show the fold change of the mean mutation frequency in the PR phase to that in the S1 phase

phases as a result of more enhancement in the S2 phase than in the PR phase, suggesting that *mfd* is intrinsically less functional in the PR phase than in the S2 phase.

## Bacillus-related thermophiles share GroSIM

$Rif^R$ and $Str^R$ mutations were analyzed for several *Bacillus*-related thermophiles in the PR and S1 phases (Fig. 5a). Although *G. kaustophilus* MK370 was derived from the wild-type strain HTA426 through artificial genetic modifications [22], GroSIM was observed even in the wild-type strain; therefore, GroSIM is not artificial because of genetic modifications but is an intrinsic phenomenon of *G. kaustophilus* HTA426. All other thermophiles, regardless of the genera *Bacillus* and *Geobacillus*, also exhibited GroSIM. This suggested that *Bacillus*-related thermophiles commonly employed the mutagenic mode. The fold change, which is the ratio of mutation frequencies in the PR phase to those in the S1 phase, was varied among thermophiles, but was approximately equal between $Rif^R$ and $Str^R$ mutations in a particular thermophile (Fig. 5b).

## Discussion

Stress-induced mutagenesis is invoked specifically by environmental stressors, such as antibiotics and starvation, and thereby serves as a convenient strategy for microbial adaptation [18–20]. Among the various stressors that trigger mutagenesis, nutrient starvation to elicit auxotroph reversion has been relatively well studied [19]. Intriguingly, although mutations are fixed through genome replication, whether because of a replication error or DNA damage, starvation-induced mutagenesis can be achieved even in non-dividing cells that barely replicate genomes. One possible explanation for this is retromutagenesis [37, 38], in which RNA polymerase transcribes a gene with a DNA

lesion along with the ribonucleotide misincorporation. The resulting transcript with a mutation potentially yields a mutant protein that functions to overcome environmental stressors. When one such protein is yielded, cells can proliferate and replicate the genome while fixing the lesion as a mutation for constitutive production of the mutant protein. This theory is consistent with the observation that DNA lesions on the transcribed strand are preferentially fixed as adaptive mutations [37], and that adaptive mutations increase in parallel with increased transcription in non-dividing cells [39].

This study demonstrated that *G. kaustophilus* MK370 in the PR phase efficiently generated $Rif^R$ and $Str^R$ mutations in response to antibiotic exposure. This mutagenesis, GroSIM, has never been reported for other microbes and was not observed for *B. subtilis* (Fig. 1b). Moreover, *Pseudomonas putida* has been reported to invoke stress-induced mutagenesis more efficiently in stationary cells than in growing cells [40]. Thus, in the growth phase, GroSIM is distinguishable from the stress-induced mutagenesis hitherto reported for other microbes, providing a new insight into microbial mutagenic modes. It is noteworthy that $Rif^R$ cells were rapidly generated from *G. kaustophilus* in the PR phase with a much higher frequency ($6.6 \times 10^{-5}$/cell) than *B. subtilis* ($3.0 \times 10^{-8}$/cell). The frequency was even higher when compared with those for other modes of stress-induced mutagenesis. For example, *P. putida* generated phenol-utilizing mutants with a frequency of $<10^{-5}$ after 10 days [40], and *E. coli* generated lactose-utilizing revertants with a frequency of $<10^{-6}$ after 5 days [41]. The growing cell-specific, rapid, and highly frequent mutations of GroSIM implied the presence of a novel molecular mechanism for stress-induced mutagenesis.

Stress-induced mutagenesis is observed often, along with the induction of error-prone DNA polymerases [19, 41–43], and the depletion of mismatch repair proteins [44]. In *B. subtilis*, *mutY* and *mfd* play mutagenic roles in stress-induced mutagenesis, despite their primary roles for DNA repair [39, 45–47]. In *G. kaustophilus*, Δ*mutY* and Δ*mfd* decrease $Rif^R$ and $Str^R$ mutations (Fig. 4); therefore, *mutY* and *mfd* function to decrease mutations but not play mutagenic roles. Of note, however, Δ*mfd* enhanced mutations less often in the PR phase than in the S2 phase, causing the apparent loss of GroSIM. This result suggests that the *mfd* function was depressed in the PR phase, most likely by inhibiting *mfd* expression and/or Mfd activity, when growing cells were exposed to antibiotics. In addition, considering that the *mfd* product is important to correct DNA lesions on the transcribed strand and that *rpoB* and *rpsL* mutations were dominantly identified at thymine and guanine sites on the transcribed strand, GroSIM may involve thymine and guanine damage via induction of mutagenic factors, such as a glycosidase that excises thymine and guanine. This is not surprising given that apurinic/apyrimidinic sites are accumulated during stress-induced mutagenesis in *B. subtilis* [48]. Moreover, it is possible that GroSIM involves retromutagenesis, which exploits transcriptional errors from DNA lesions, and thus, probably is inhibited by transcription-coupled repair (i.e., the *mfd* product). This idea can explain why GroSIM was achieved even in non-dividing cells and why *mfd* depression remarkably increases mutations.

GroSIM was exhibited in several *Bacillus*-related thermophiles but not in *B. subtilis*. This suggests that *Bacillus*-related thermophiles exclusively share a biological mechanism to induce mutations when exposed to Rif and Str. Because changes in mutation frequency were approximately equivalent between $Rif^R$ and $Str^R$ mutations in one particular thermophile (Fig. 5b), it is possible that the mechanism involves functional depression of DNA repair systems. Although further analyses are required to clarify how these antibiotics invoke GroSIM, *Bacillus*-related thermophiles may commonly depress *mfd* function in response to Rif and Str.

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