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A High-Frequency Mutation in *Bacillus subtilis*: Requirements for the Decryptification of the *gudB* Glutamate Dehydrogenase Gene

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Common laboratory strains of *Bacillus subtilis* encode two glutamate dehydrogenases: the enzymatically active protein RocG and the cryptic enzyme GudB that is inactive due to a duplication of three amino acids in its active center. The inactivation of the *rocG* gene results in poor growth of the bacteria on complex media due to the accumulation of toxic intermediates. Therefore, *rocG* mutants readily acquire suppressor mutations that decryptify the *gudB* gene. This decryptification occurs by a precise deletion of one part of the 9-bp direct repeat that causes the amino acid duplication. This mutation occurs at the extremely high frequency of 10^{-4}. Mutations affecting the integrity of the direct repeat result in a strong reduction of the mutation frequency; however, the actual sequence of the repeat is not essential. The mutation frequency of *gudB* was not affected by the position of the gene on the chromosome. When the direct repeat was placed in the completely different context of an artificial promoter, the precise deletion of one part of the repeat was also observed, but the mutation frequency was reduced by 3 orders of magnitude. Thus, transcription of the *gudB* gene seems to be essential for the high frequency of the appearance of the *gudB1* mutation. This idea is supported by the finding that the transcription-repair coupling factor Mfd is required for the decryptification of *gudB*. The Mfd-mediated coupling of transcription to mutagenesis might be a built-in precaution that facilitates the accumulation of mutations preferentially in transcribed genes.

As the central amino group donor for nearly all biosynthetic pathways in any living cell, glutamate plays a key role in the biochemistry and physiology of all organisms (15). Investigations with *Escherichia coli* demonstrate that glutamate is by far the most abundant metabolite in these bacteria, accounting for ca. 40% of the internal metabolite pool (60). Moreover, glutamate is one of the most highly embedded metabolites. In the Gram-positive soil bacterium *Bacillus subtilis*, at least 37 reactions make use of this amino acid (42).

In *B. subtilis*, glutamate is exclusively synthesized from 2-oxoglutarate and glutamine by the activity of glutamate synthase in the absence of exogenous glutamate or other sources of glutamate. 2-Oxoglutarate is replenished in the citric acid cycle, whereas glutamine can be synthesized with ammonium as the nitrogen source and one of the two molecules of glutamate that are generated by glutamate synthase as the acceptor. Glutamate does also serve as a precursor for proline biosynthesis and, under conditions of osmotic stress, molar concentrations of proline have to be produced (28). Thus, it is not surprising that glutamate synthesis has to be a highly efficient process and, indeed, interactions between enzymes of the branch of the citric acid cycle that generates 2-oxoglutarate and glutamate synthase have been reported (39). Glutamate can also serve as source of carbon and nitrogen. Its utilization is initiated by an oxidative deamination catalyzed by the glutamate dehydrogenase. The expression of the genes encoding glutamate biosynthetic and catabolic enzymes is subject to complex control mechanisms that allow the adjustment of the intracellular glutamate concentration to the actual requirement (6, 7, 16, 44, 51).

B. subtilis encodes two glutamate dehydrogenases, GudB and RocG (5). However, the *gudB* gene experienced an inactivating mutation during domestication, resulting in an inactive pseudogene in the laboratory strain *B. subtilis* 168. In contrast, the *gudB* gene encodes an active enzyme in wild isolates and in nondomesticated strains such as NCIB3610 (61). The inactivation of *gudB* is caused by a duplication of nine base pairs of the coding sequence resulting in a duplication of three amino acids in the active center of the protein. The glutamate dehydrogenase RocG catalyzes the final step of the catabolic pathway for arginine, ornithine and citrulline. Accordingly, its expression is strongly induced in the presence of arginine (5). Interestingly, the glutamate dehydrogenases are not only required for glutamate utilization, but they are also involved in the control of glutamate biosynthesis: in the presence of glutamate they inhibit the transcription activator GltC that is necessary for the expression of the glutamate synthase operon, *gltAB* (8, 16, 18, 25). In the active state, the two glutamate dehydrogenases are very similar to each other, both at the level of the amino acid sequence and also concerning their structures. In contrast, the inactive GudB protein seems to misfold and is subject to rapid degradation (23, 25).

The importance of glutamate for the cellular physiology is underlined by the observation that any mutation that disturbs the glutamate homoeostasis results in the accumulation of suppressing mutations. This is true for both *E. coli* and *B. subtilis* (19, 59). In the laboratory strain of *B. subtilis*, the inactivation of the *rocG* gene encoding the only active glutamate dehydrogenase results in the appearance of mutants with an active GudB enzyme (these alleles are designated *gudB1*) (5). Moreover, *rocG gudB* double mutants easily acquire suppressive mutations affecting the glutamate synthase (19). The *rocG gudB* double mutants are unable to

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utilize glutamate as the single source of carbon. However, cultivation of such mutants in the presence of glutamate or its precursors such as arginine results in the selection of suppressor mutants that catabolize glutamate by a pathway that is not operative in wild-type bacteria. An analysis of one such mutant revealed constitutive expression of the aspartase pathway due to the inactivation of the repressor of the corresponding *ansAB* operon, AnsR (21).

The accumulation of mutations that restore growth of mutants or that allow faster growth is a common phenomenon in bacteria. Several studies suggest that mutations that overcome the specific limitation are preferentially acquired (3, 13); however, the underlying mechanisms have not yet been elucidated.

Mutations can be acquired during replication. Most of the errors are eliminated by DNA mismatch repair, including the MutSL system which contributes to genome stability (22, 40). Some errors can escape from repair and may be beneficial for the organism. Many bacteria, including *B. subtilis*, possess systems for the induction of mutations in the stationary phase (55). The emergence of these mutations is associated with transcription rather than with DNA replication and plays an important role in the generation of diversity in nondividing populations of *B. subtilis*. The process of transcription-coupled DNA repair is crucial for the accumulation of mutations in the stationary phase, and this involves the transcription repair coupling factor Mfd (2). The Mfd protein targets DNA lesions during transcription that provoked a roadblock of transcription. Subsequently, Mfd may displace the RNA polymerase and recruit the nucleotide excision repair system to resolve the lesion (12, 56). It was suggested that this process favors the acquisition of beneficial mutations of highly transcribed genes (45, 46).

We are interested in the mechanism by which the decryptification of the *gudB* gene occurs in *rocG* mutants. The *gudB1* mutation appears during growth and requires a deletion of 9 bp. Therefore, *gudB* provides a unique system to study the emergence of mutations. Our results suggest that the decryptification of *gudB* requires the presence of a perfect direct repeat. Moreover, a part of this repeat is preferably deleted with a high frequency in the context of a transcribed gene, and this deletion requires the Mfd transcription repair coupling factor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains used in the present study are derived from the laboratory wild-type strain 168. They are listed in Table 1. *E. coli* DH5α (48) was used for cloning experiments. *B. subtilis* was grown in SP medium, in LB medium, or in C minimal medium supplemented with tryptophan (at 50 mg liter^{-1}) (58). CSE medium is C minimal medium supplemented with sodium succinate (6 g liter^{-1}) and potassium glutamate (8 g liter^{-1}). C-Glc is C minimal medium supplemented with glucose (1 g liter^{-1}), and CS is supplemented with sodium succinate (6 g liter^{-1}) (58). Additional sources of carbon and nitrogen were added as indicated. *E. coli* was grown in LB medium, and transformants were selected on plates containing ampicillin (100 μg/ml). LB, SP, and CS plates were prepared by the addition of 17 g of Bacto agar (Difco)/liter to LB, SP, or CS medium, respectively.

DNA manipulation, transformation, and phenotypic analysis. Transformation of *E. coli* and plasmid DNA extraction were performed according to standard procedures (48). Restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. Phusion DNA polymerase was used for the PCR as recommended by the manufacturer. All primer sequences are provided as supplementary material (see Table S1 in the supplemental material). DNA sequences were determined using the dideoxy chain termination method (48). All plasmid inserts derived from PCR products were verified by DNA sequencing. Chromosomal DNA of *B. subtilis* was isolated as described previously (32).

E. coli transformants were selected on LB plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA according to the two-step protocol described previously (32). Transformants were selected on SP plates containing kanamycin (10 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (150 μg/ml), or erythromycin-lincomycin (2 and 25 μg/ml, respectively).

In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g/liter), 17 g of Bacto agar (Difco)/liter, and 5 g of hydrolyzed starch (Connaught)/liter. Starch degradation was detected by sublimating iodine onto the plates.

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows. Cells were grown in CSE medium supplemented with different carbon and nitrogen sources as indicated. The cells were harvested at an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 for cultures in CSE medium and an OD_{600} of 0.8 to 1.0 for cultures in CSE medium with sugar. β-Galactosidase specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (32). One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol of *o*-nitrophenol per min at 28°C.

Ectopic expression of *gudB* variants. To express the *gudB* gene at an ectopic site, we used plasmid pAC5 (37). This plasmid allows integration of the cloned fragments into the *amyE* site of the *B. subtilis* chromosome. Briefly, the *gudB* gene was amplified with its natural promoter using the oligonucleotides ST1 and KG92 with the chromosomal DNA of *B. subtilis* 168 as the template. The PCR product was digested with EcoRI and BamHI and cloned into pAC5 linearized with the same enzymes. The resulting plasmid pGP900 was used to introduce the *gudB* allele into the chromosome.

The direct repeat of *gudB* present in pGP900 was subjected to site-directed mutagenesis by a modified PCR protocol, the combined chain reaction (9). Primers ST1 and KG92 were used as outer primers. The primers KG119, KG120, and KG133 were used to introduce point mutations into the *gudB* coding region. These primers were phosphorylated at their 5′ ends and allowed ligation of the nascent elongation product initiated from ST1. The resulting products carrying the mutations were cut with EcoI and BamHI and cloned into pAC5 digested with the same enzymes. The resulting plasmids were pGP1714 (G3T G9T), pGP1715 (G3T G9T G12T G18T), and pGP1721 (G12T G18T). The plasmids were linearized with PstI and used to transform *B. subtilis* (see Table 1).

Design and construction of a mutagenesis reporter system. In order to analyze the occurrence of the deletion of the repeat in a nonrelated sequence context, we developed a reporter system that is based on a promoter that is only active upon deletion of one part of the *gudB*-derived direct repeat. This artificial *alf* promoter controls the expression of genes coding for a kanamycin-resistant determinant (*aphA3*) and *E. coli* β-galactosidase. To obtain the reporter strain, we first constructed plasmid pGP655 as follows. The promoterless *aphA3* gene was amplified from pDG780 (24) by using the primer pair ST4 and ST9. These oligonucleotides attached restriction sites for EcoRI and BamHI (ST9) and for BglII (ST4) to the PCR product. The fragment was digested with EcoRI and BglII and cloned into the integration vector pAC6 (54), linearized with EcoRI and BamHI. The resulting plasmid pGP653 contained a promoterless *aphA3-lacZ* operon. The *alf* promoter fragment was obtained by hybridization of the complementary oligonucleotides ST7 and ST8. It was cloned between the EcoRI and BamHI sites of pGP653, resulting in plasmid pGP655.

Construction of mutant strains. Deletion of the *recJ*, *exoA*, *nfo*, *uvrAB*, *mutSL*, *sbcDC*, *gudB*, and *mfd* genes was achieved by transformation with PCR products constructed using oligonucleotides (see Table S1 in the supplemental material) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes (24), as described previously (57).

TABLE 1 *B. subtilis* strains used in this study

Strain	Genotype	Source or reference[a]
168	*trpC2*	Laboratory collection
BG427	*trpC2 metB5 amyE sigB xin-1 attSP recU::cat*	20
BP12	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB1*$_{mut1}$ *lacZ cat*)	Spontaneous mutation of GP1179 on SP
BP13	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB1*$_{mut3}$ *lacZ cat*)	Spontaneous mutation of GP1197 on SP
GP747	*trpC2 rocG::*Tn*10 spc*	17
GP753	*trpC2 rocG::*Tn*10 spc gudB1*	Spontaneous mutation of GP747 on SP
GP754	*trpC2 rocG::cat amyE::*(*gltA-lacZ aphA3*)	16
GP804	*trpC2 gudB1 amyE::*(*gltA-lacZ aphA3*)	19
GP891	*trpC2 recU::cat*	BG427→168
GP892	*trpC2 rocG::spc recU::cat*	BG427→GP747
GP894	*trpC2* Δ*sbcDC::aphA3*	See Materials and Methods
GP895	*trpC2* Δ*recJ::aphA3*	See Materials and Methods
GP896	*trpC2 rocG::spc* Δ*sbcDC::aphA3*	GP747→GP894
GP897	*trpC2 rocG::spc* Δ*recJ::aphA3*	GP747→GP895
GP898	*trpC2* Δ*exoA::aphA3*	See Materials and Methods
GP900	*trpC2 rocG::spc* Δ*exoA::aphA3*	GP747→GP898
GP1101	*trpC2 amyE::*(*gudB-lacZ cat*)	pGP651→168
GP1102	*trpC2 gudB1 amyE::*(*gudB-lacZ cat*)	pGP651→GP804
GP1103	*trpC2 rocG::*Tn*10 spc recA::erm cat*	IRN444→GP747
GP1104	*trpC2 rocG::*Tn*10 spc amyE::*(*gudB-lacZ cat*)	pGP651→GP747
GP1105	*trpC2 rocG::*Tn*10 spc amyE::*(*gudB-lacZ cat*) *gudB1*	Spontaneous mutation of GP1104 on SP
GP1106	*trpC2* Δ*addAB::spc*	HVS666→168
GP1107	*trpC2* Δ*addAB::spc rocG::cat amyE::*(*gltA-lacZ aphA3*)	GP1106→GP754
GP1123	*trpC amyE::*(*alf-aphA3 lacZ cat*)	pGP655→168
GP1127	*trpC2 amyE::*(*alf1-aphA3 lacZ cat*)	Spontaneous mutation of GP1123 on SP-Km
GP1160	*trpC2* Δ*gudB::aphA3*	See Materials and Methods
GP1161	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc*	GP1160→GP747
GP1163	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB lacZ cat*)	pGP900→GP1161
GP1167	*trpC2* Δ*mfd::ermC*	See Materials and Methods
GP1168	*trpC2* Δ*mfd::ermC amyE::*(*alf-aphA3 lacZ cat*)	GP1167→GP1123
GP1169	*trpC2 rocG::*Tn*10 spc* Δ*mfd::ermC*	GP1167→GP747
GP1175	*trpC2* Δ*uvrAB*::Erm^r	See Materials and Methods
GP1176	*trpC2* Δ*uvrAB*::Erm^r *rocG::*Tn*10 spc*	GP1175→GP747
GP1177	*trpC2* Δ*gudB::aphA3 amyE::*(*gudB*$_{mut1}$ *lacZ cat*)	pGP1714→GP1160
GP1178	*trpC2* Δ*gudB::aphA3 amyE::*(*gudB*$_{mut2}$ *lacZ cat*)	pGP1715→GP1160
GP1179	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB*$_{mut1}$ *lacZ cat*)	GP747→GP1177
GP1180	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB*$_{mut2}$ *lacZ cat*)	GP747→GP1178
GP1190	*trpC2* Δ*mutSL::aphA3*	See Materials and Methods
GP1191	*trpC2* Δ*mutSL::aphA3 rocG::*Tn*10 spc*	GP747→GP1190
GP1192	*trpC2* Δ*mutSL::aphA3* Δ*uvrAB*::Erm^r *rocG::*Tn*10 spc*	GP1176→GP1190
GP1197	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB*$_{mut3}$ *lacZ cat*)	pGP1721→GP1161
GP1198	*trpC2* Δ*gudB::aphA3 rocG::*Tn*10 spc amyE::*(*gudB1*$_{mut3}$ *lacZ cat*)	Spontaneous mutation of GP1197 on SP
GP1502	*trpC2* Δ*nfo::cat*	See Materials and Methods
GP1503	*trpC2* Δ*exoA::aphA3* Δ*nfo::cat*	GP1502→GP898
GP1504	*trpC2* Δ*exoA::aphA3* Δ*nfo::cat rocG::spc*	GP747→GP1503
HVS666	*trpC2* Δ*addAB::spc*	14
IRN444	*trpC2 recA::erm cat*	34

[a] Arrows indicate construction by transformation.

Construction of a *gudB-lacZ* fusion. To determine the activity of the *gudB* promoter, a translational fusion of the *gudB* promoter to a promoterless *lacZ* gene encoding β-galactosidase was constructed as follows. A DNA fragment containing the *gudB* promoter region was generated by PCR using the primers ST1 and ST2, digested with BamHI and EcoRI, and cloned into the plasmid pAC5. The plasmid pAC5 contains a promoterless *lacZ* gene and allows the introduction of translational fusions into the *amyE* locus of *B. subtilis* (37). The resulting plasmid pGP651 was used to introduce the fusion into different *B. subtilis* mutants (see Table 1).

Determination of mutation frequencies. The mutation frequencies were determined by the method of the median (33). Briefly, 11 cultures in CSE-Glc were inoculated to a density of 100 cells/ml with an overnight culture grown in the same medium. The cultures were incubated at 37°C to an OD_{600} of 2.0. For the analysis of culture titers appropriate dilutions of four cultures were plated on SP medium containing glucose to allow growth of the *rocG* mutant strains. To screen for *gudB1* mutations, appropriate dilutions of each culture were plated on SP medium. After 24 h, the colonies showing the *gudB1* phenotype (wild type-like colonies on SP plates) were counted. To be sure of the identity of the mutations, the *gudB* allele was sequenced for at least three independent suppressor mutants in each experiment. In every single case, the correct excision of one part of the repeat (i.e., the *gudB1* mutation) was observed. For the determination of mutation frequencies of the *alf* promoter present in the strains GP1123 and GP1168, the bacteria were plated on SP medium containing kanamycin (60 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 80 μg/ml).

Northern blot analysis. Preparation of total RNA and Northern blot analysis were carried out as described previously (35). Digoxigenin (DIG) RNA probes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated DNA fragments as templates. The primer pairs used to amplify DNA fragments specific for *gudB* and *gapA* are listed in Table S1 in the supplemental material. The reverse primers contained a T7 RNA polymerase recognition sequence. *In vitro* RNA labeling, hybridization, and signal detection were carried out according to the instructions of the manufacturer (DIG RNA labeling kit and detection chemicals; Roche Diagnostics). To determine the size of the *gudB* mRNA, we used the transcripts observed with a *gapA* probe as the standard. RNA stability was analyzed as described previously (38). Briefly, rifampin was added to logarithmically growing cultures (final concentration, 100 μg/ml), and samples were taken at the time points indicated. The quantification was performed using ImageJ software v1.42 (1).

Western blotting. For Western blot analysis, proteins were separated by SDS–12.5% PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad) by electroblotting. Rabbit anti-RocG (1:15,000) (17) served as the primary antibody. The antibodies were visualized by using anti-rabbit immunoglobulin G-alkaline phosphatase secondary antibodies (Promega) and the CDP-Star detection system (Roche Diagnostics), as described previously (16).

RESULTS

The *gudB1* suppressor mutation appears at an extremely high frequency. Previous studies revealed that the *gudB* gene readily acquired the *gudB1* suppressor mutation if the *rocG* gene was inactivated (5, 17, 19). In order to describe this mutation event in a quantitative way, the frequency of the *gudB* reversion was determined. For this purpose, 11 independent cultures of the *rocG* mutant strain *B. subtilis* GP747 were inoculated with approximately 100 cells/ml to reduce the likelihood of very early mutants. The cultures were grown under nonselective conditions (in CSE medium supplemented with glucose) for 20 generations and plated on complex medium (SP medium), which is toxic for the *rocG* mutant but not for emerging *rocG gudB1* suppressor strains. Suppressor mutants were recognized since they grew as solid colonies (like the wild-type strain *B. subtilis* 168), whereas the *rocG* mutant strain GP747 formed only very small opaque colonies on complex medium. To ascertain that the mutation had appeared during the cultivation and not as a result of selection on the plates, only suppressor mutants that were present after 24 h were taken into consideration. The mutation frequency was 10^{-4}. To the best of our knowledge, such a high mutation frequency has never been reported for *B. subtilis*.

Role of chromosomal location and direct repeat for the high reversion frequency of *gudB*. The extremely high frequency of reversion of *gudB* might result from the presence of a direct repeat of 9 bp. However, the chromosomal arrangement might play a role as well. To distinguish between these possibilities, we decided to address the role of the chromosomal location of the *gudB* gene first. For this purpose, we used the *B. subtilis* strain GP1163. In this strain, the chromosomal *gudB* gene was deleted, and another copy of *gudB* under the control of its own promoter was inserted ectopically at the *amyE* site of the chromosome. The ectopic copy of the *gudB* allele was orientated in the same orientation as the native copy. The mutation frequency of this strain was 0.49×10^{-4}. Sequence analysis of three randomly selected suppressor mutants revealed that all contained the *gudB1* mutation. This observation suggests that the chromosomal location has no major impact on the occurrence of the *gudB1* suppressor mutation (see Table 3).

As shown above, the *gudB* gene can be decryptified by the deletion of one part of the direct repeat irrespective of the chromosomal location of the *gudB* allele. Next, we wanted to address the relevance of this repeat in the decryptification process by a mutational analysis. Since the direct repeat is located within the coding sequence of *gudB*, any mutation to be introduced into the direct repeat had to conserve the *gudB* open reading frame. In order to destroy the direct repeat, we replaced two G residues by T (at positions 3 and 9 of the repeat, corresponding to wobble bases of the codons for valine and alanine). This mutation was introduced into both the first part and the second part of the direct repeat; the corresponding strains are *B. subtilis* GP1179 and GP1197, respectively. Moreover, we restored a direct repeat, albeit with a sequence that deviates from the original repeat by introducing the same mutations in both parts of the repeat. This strain was GP1180 (Fig. 1A). A comparison of the mutation frequencies revealed that the perfect repeat was a prerequisite for efficient accumulation of *gudB1* suppressor mutants. In the absence of a perfect direct repeat, the mutation frequency was reduced by a factor of about 15 (0.036×10^{-4} and 0.034×10^{-4} for GP1179 and GP1197, respectively, versus 0.49×0^{-4} for strain GP1163 carrying the wild-type repeat; see Table 3). The introduction of compensatory mutations that restore the direct repeat did also restore the high frequency of the appearance of the *gudB1* mutation (0.89×10^{-4} for GP1180). These results clearly demonstrate that the presence of the direct repeat is the decisive factor for the high *gudB1* mutation frequency.

Selective excision of the first part of the direct repeat. In all experiments to determine mutation frequencies, we analyzed the nucleotide sequence of the *gudB* suppressor mutations. As stated above, a precise deletion of the direct repeat was observed in all

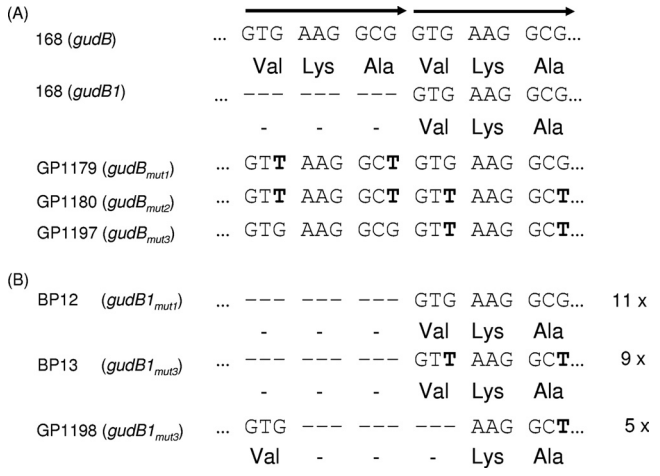

FIG 1 Crucial role of the direct repeat for the decryptification of *gudB*. (A) The wild-type *gudB* sequence was mutated without changing the amino acid sequence. In GP1179 two G residues were replaced by T in the first half of the repeat (positions 3 and 9 of the repeat). In GP1197 these mutations were introduced in the second part of the direct repeat (positions 12 and 18). The perfect direct repeat was restored in the strain GP1180. This study served to analyze the role of a perfect direct repeat in the rapid decryptification of the *gudB* allele. (B) Selective deletion of the first part of the direct repeat in the *gudB* gene. In all *gudB1* mutants (designated as BP12) derived from the strain GP1179, the first part of the imperfect repeat was excised. Of 14 *gudB1* mutants derived from the strain GP1197, in 9 (designated as BP13) the first half of the imperfect repeat was deleted, whereas in 5 (designated as GP1198) an internal excision had occurred. The numbers indicate the number of occurrences of the particular mutations in a selected set of mutants that were analyzed.

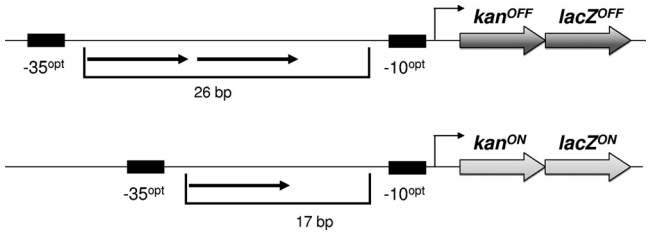

FIG 2 Mutagenesis test system. The direct repeat originating from the *gudB* allele of *B. subtilis* was placed as the spacer between an optimal −10 and −35 region (upper part). An operon consisting of a kanamycin resistance gene (*aphA3*) and the β-galactosidase gene (*lacZ*) was placed under the control of the artificial promoter. Due to the long spacer, the promoter is not active. By the precise deletion of 9 bp in the spacer region, the promoter gains function, and the kanamycin resistance and the β-galactosidase are highly expressed (lower part).

cases. However, the presence of a perfect repeat precluded the identification of the nucleotides that had actually been excised. This question became tractable with the availability of the suppressor mutants of *B. subtilis* GP1179 and GP1197 in which the repeat is not perfect. The sequence analysis of the *gudB1* alleles of 11 suppressor mutants (designated as BP12) derived from GP1179 (mutated in the first part of the repeat) revealed that the first half of the repeat was deleted in all cases (see Fig. 1B). This strong bias might indicate that either the first part of the repeat is preferentially excised or that the naturally occurring sequence is retained with preference. This question was addressed by analysis of the suppressor mutants derived from GP1197 (mutations in the second part of the repeat). In this case, of 14 analyzed mutants, 9 (designated as BP13) had a deletion of the first part of the repeat. Moreover, five mutants (designated as GP1198) exhibited internal deletions of the repeat that restored a sequence coding for the active GudB protein (see Fig. 1B). Thus, none of the mutants derived from GP1197 restored the original nucleotide sequence of the remainder of the repeat. Instead, we observed again a strong bias toward deletion of the first part of the repeat, suggesting that this selective deletion is inherent to the mutagenesis process that decryptifies the *gudB* gene.

Construction and analysis of a deletion reporter system. The results presented above demonstrate that the deletion of one part of the *gudB* repeat occurs at a very high frequency both in the native context and in a nonrelated genomic context as long as the repeat is intact. These findings prompted us to ask whether the deletion would also take place as efficiently in a completely different sequence context as it does in the *gudB* gene. For this purpose, we constructed a reporter system consisting of an *aphA3-lacZ* operon encoding a resistance to kanamycin and β-galactosidase under the control of an artificial (*alf*) promoter. This promoter was designed to have perfect recognition sequences for the housekeeping sigma factor of the RNA polymerase (−10 and −35); however, the spacing between the two boxes was 26 bp rather than the canonical 17 to 18 bp. The perfect repeat of the *gudB* gene should be a part of this spacer. This promoter is not likely to be recognized by the RNA polymerase unless one part of the repeat is deleted and the optimal 17-bp spacing is restored (Fig. 2). Such a reporter system was constructed as described in Materials and Methods and introduced into the genome of *B. subtilis*, resulting in strain GP1123 (Table 1). *B. subtilis* GP1123 was unable to grow in the presence of kanamycin and formed white colonies on plates containing X-Gal, suggesting that neither kanamycin resistance nor β-galactosidase was expressed by these bacteria. These findings demonstrate that the *alf* promoter was inactive, as expected. However, we observed the sporadic appearance of kanamycin-resistant blue colonies that might result from the activation of the *alf* promoter. Indeed, a sequence analysis of the promoter for several colonies revealed the deletion of one part of the repeat resulting in a promoter (*alf1*) with perfect −10 and −35 regions separated by the preferred distance of 17 bp. Thus, the deletion of one part of the repeat occurs also in an unrelated sequence context.

Next, we sought to determine whether the deletion of the repeat in the *alf* promoter took place with a similar high frequency as observed for the decryptification of *gudB*. For this purpose, the frequency of appearance of kanamycin-resistant suppressor mutants of GP1123 was determined. It was found to be 1.3×10^{-7}. This mutation frequency is in the range typically observed in bacteria (31), but 3 orders of magnitude lower than the frequency found for the deletion event in the *gudB* gene context. Thus, there seems to be a relevant difference between the sequence contexts of the *gudB* gene and the *alf* promoter that results in drastically changed mutation frequencies (see Table S2 in the supplemental material).

Expression of *gudB* gene and stability of cryptic and active glutamate dehydrogenases. While the direct repeat is part of a putatively expressed coding region in the *gudB* gene, it is present in the nontranscribed spacer in the artificial *alf* promoter. This difference might contribute to the different mutation frequencies observed in the two sequence contexts. Therefore, we decided to study first the expression of the *gudB* gene to some detail. Previous studies have shown that *gudB* expression is not modulated by the source of nitrogen present in the medium (5). We have studied the activity of the *gudB* promoter by determining the expression of a *gudB-lacZ* fusion in wild-type, Δ*rocG*, and *gudB1* genetic backgrounds. As shown in Table 2, the fusion was highly expressed irrespective of the genetic background or medium analyzed. The expression level of about 500 U/mg of protein is rather high for translational *lacZ* reporter fusions (50). Thus, even the cryptic *gudB* gene coding for an inactive protein is expressed at high levels in *B. subtilis*.

To allow the action of selective pressure on the decryptification of *gudB*, the accumulation of the active protein is required. However, the inactive GudB protein was reported to be one of the most unstable proteins of *B. subtilis* (23). In contrast, preliminary evidence suggested that the active GudB1 protein is much more stable (25). The issue of stability might apply not only at the level of the protein, but may also be relevant for the *gudB* mRNA. To

TABLE 2 Analysis of *gudB* expression

Strain	Relevant genotype	β-Galactosidase activity (U mg of protein^{-1})[a]				
		C-Glc	CE	CE-Glc	CR	CR-Glc
GP1101	Wild type	357	NG	504	573	415
GP1102	*gudB1*	182	384	268	415	242
GP1104	*rocG*::Tn*10*	422	NG	557	NG	394
GP1105	*rocG*::Tn*10 gudB1*	225	658	478	410	295

[a] Bacteria were grown in C minimal medium. Glucose (Glc), glutamate (E), and arginine (R) were added to final concentrations of 0.5% (Glc and R) or 0.8% (E). Experiments were carried out at least 3-fold. The maximum deviation of the series of representative data shown here was <30%. NG, no growth.

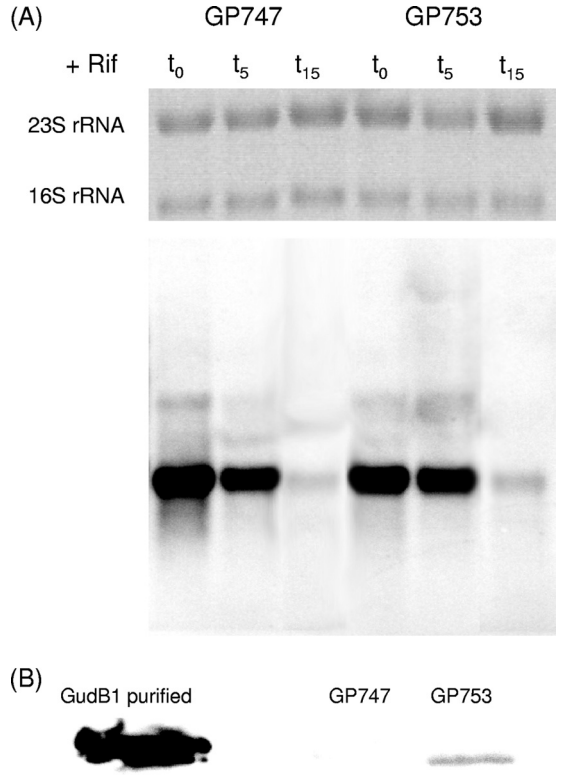

FIG 3 Expression of *gudB* gene and GudB protein level. (A) Northern blot analysis was performed to determine the stability of the *gudB* mRNA of *B. subtilis* GP747 and the isogenic *gudB1* mutant GP753. Both mRNAs do not differ in stability and half-life, implying that the direct repeat does not influence mRNA levels. (B) A Western blot analysis was performed to compare the protein levels of GudB with the level of GudB1. Crude extracts of *B. subtilis* GP747 and the isogenic *gudB1* mutant GP753 were used, and the GudB protein was detected by using antibodies raised against RocG that cross-react with the GudB protein.

address these problems, we first determined the stability of the *gudB* mRNA of *B. subtilis* GP747 and the isogenic *gudB1* mutant GP753 by a Northern blot analysis. As shown in Fig. 3A, we detected a single transcript of ~1,300 bp for *gudB*. This corresponds to a monocistronic transcript and is in good agreement with previous suggestions based on genome analysis (5). The quantitative evaluation of the mRNA stability revealed a half-life of ~4 min. The stability of the mRNA was similar in both strains, demonstrating that it is not affected by the presence of the direct repeat.

The accumulation of the glutamate dehydrogenase GudB was studied by Western blot analysis. For this purpose, we used the cell extracts of the *rocG* mutant GP747 and its isogenic *gudB1* derivative GP753 that were prepared for the determination of the mRNA stability (just prior to rifampin addition, lane t_0 in Fig. 3A). To detect the GudB protein, we used an antibody raised against RocG. Both proteins are very similar and the antibody recognizes GudB as well. Since both strains used for this experiment are *rocG* mutants, the only signal is obtained with GudB (17). As shown in Fig. 3B, the active enzyme GudB1 could be detected in the extract of GP753. In contrast, no signal was observed for the cryptic GudB protein. Since the mRNA amounts are similar for both strains (see Fig. 3A), we may conclude that the inactive GudB protein is highly unstable, as suggested by a previous study (23). In contrast, the active glutamate dehydrogenase GudB1 is a stable protein that accumulates in the cell. Thus, the decryptification of *gudB* is sufficient for the cell to obtain immediately an active glutamate dehydrogenase that may help to overcome the metabolic imbalance of the *rocG* mutant.

Implication of repair and recombination proteins in the decryptification of *gudB*. The extremely high frequency at which the *gudB* decryptification occurs and the fact that the frequency is much higher in the *gudB* locus compared to the *alf* mutagenesis reporter system suggest the involvement of proteins in the mutagenesis process. The sequence of the direct repeat in *gudB* is somewhat similar to the *chi* sequence that is recognized and bound by the AddAB helicase/nuclease, a component of the recombination machinery of the cell. Since the recombination protein RecA is also involved in the generation of mutations, we determined the *gudB* mutation frequency of the *addAB* and *recA* mutant strains GP1107 and GP1103, respectively. The frequencies were similar to those observed with the isogenic *rocG* mutants (0.9×10^{-4} and 1.1×10^{-4} for the *addAB* mutant and the wild-type strain GP754; 0.3×10^{-4} versus 1.3×10^{-4} for the *recA* mutant and the wild-type strain GP747; Table 3). Therefore, AddAB and RecA do not seem to play a major role in the deletion of the direct repeat in the *gudB* gene. We also tested the effect of mutations in the genes *recJ*, *recU*, *exoA*, *nfo*, *uvrAB*, *mutSL*, and *sbcDC*. Moreover, we tested the effect of the combined *exoA nfoA* (GP1504) and *mutSL uvrAB* (GP1192) mutations. Similarly to the *addAB* and *recA* deletions, we did not observe an effect of the mutations on the decryptification of the *gudB* gene (see Table 3).

If a mutation in any of the genes encoding enzymes of DNA repair and recombination would have played a role in the deletion of the direct repeat in *gudB*, we would have expected that they are not selective for the *gudB* gene context compared to the context of the *alf* promoter. Thus, the genetic context plays a decisive role in the decryptification of *gudB*. As shown above, the *gudB* gene is

TABLE 3 Frequency of *gudB1* mutation

Strain	Relevant genotype	Mutation frequency (avg ± SD)[a]
GP747	*rocG*::Tn*10*	$(1.3 \times 10^{-4}) \pm (40 \times 10^{-4})$
GP754	*rocG*::*cat*	$(1.1 \times 10^{-4}) \pm (0.9 \times 10^{-4})$
GP1103	*rocG*::Tn*10 recA*	$(0.3 \times 10^{-4}) \pm (0.4 \times 10^{-4})$
GP1107	*rocG*::*cat* Δ*addAB*	$(0.9 \times 10^{-4}) \pm (0.4 \times 10^{-4})$
GP1123	*amyE*::(*alf-aphA3 lacZ cat*)	$(1.3 \times 10^{-7}) \pm (0.61 \times 10^{-7})$
GP1163	*rocG*::Tn*10* Δ*gudB*::*aphA3* *amyE*::(*gudB cat*)	$(0.49 \times 10^{-4}) \pm (0.4 \times 10^{-4})$
GP1168	*amyE*::(*alf-aphA3 lacZ cat*) Δ*mfd*::*ermC*	$(2.3 \times 10^{-7}) \pm (0.48 \times 10^{-7})$
GP1169	*rocG*::Tn*10* Δ*mfd*::*ermC*	1.0×10^{-6}[b]
GP1176	*rocG*::Tn*10* Δ*uvrAB*::*ermC*	$(0.1 \times 10^{-4}) \pm (0.02 \times 10^{-4})$
GP1179	*rocG*::Tn*10* Δ*gudB*::*aphA3* *amyE*::($gudB_{mut1}$ *cat*)	$(3.6 \times 10^{-6}) \pm (2.7 \times 10^{-6})$
GP1180	*rocG*::Tn*10* Δ*gudB*::*aphA3* *amyE*::($gudB_{mut2}$ *cat*)	$(0.89 \times 10^{-4}) \pm (0.14 \times 10^{-4})$
GP1191	*rocG*::Tn*10* Δ*mutSL*::*aphA3*	$(0.2 \times 10^{-4}) \pm (0.07 \times 10^{-4})$
GP1192	*rocG*::Tn*10* Δ*mutSL*::*aphA3* Δ*uvrAB*::*ermC*	$(0.14 \times 10^{-4}) \pm (0.03 \times 10^{-4})$
GP1197	*rocG*::Tn*10* Δ*gudB*::*aphA3* *amyE*::($gudB_{mut3}$ *cat*)	$(3.4 \times 10^{-6}) \pm (8 \times 10^{-6})$

[a] Mutation frequencies were determined at least three times.
[b] Due to the low mutation frequency of the *gudB* allele in GP1169, the determination of the precise frequency was limited by the experimental procedure.

constitutively expressed. In contrast, the core promoter of the mutagenesis reporter system is a nontranscribed region. The transcription-repair coupling factor Mfd might therefore participate in the deletion of the *gudB* repeat. To test this idea, we constructed the *mfd* deletion mutant GP1169 and compared the mutation frequency in this strain to that of the isogenic *rocG* mutant GP747. In this case, we detected a 100-fold reduction in the frequency of *gudB1* mutants (1.25×10^{-6} versus 1.3×10^{-4}). Next, we investigated the impact of the *mfd* mutation on the deletion of the repeat in the *alf* promoter. In this case, the mutation frequencies of the wild-type strain (GP1123) and the isogenic *mfd* mutant strain GP1168 were very similar (1.3×10^{-7} versus 2.3×10^{-7}). Thus, the *mfd* mutation affects the deletion of the direct repeat only in the context of the transcribed gene. This observation strongly supports the idea that transcription of the *gudB* gene is essential for obtaining the high frequency of decryptification.

DISCUSSION

High-fidelity DNA synthesis is very important for maintaining genetic information over many generations of a bacterial population. Indeed, the frequency of single base pair substitutions during DNA replication is very low in *E. coli*. The frequency of these mutagenic events was estimated to be in the range of 10^{-7} to 10^{-8} in the absence of internal or external stress (49). Beneficial mutations occur even 2 orders of magnitudes less frequently (27, 29, 31). However, research in the last few years suggests that selective pressure may somehow favor the appearance of beneficial mutations (13, 47). A recent long-term study with *E. coli* suggested that the cells acquire the most beneficial mutations early during starvation (i.e., mutations that have the highest positive impact on fitness) and that independent bacterial cultures are likely to accumulate the same beneficial mutations (3). However, in contrast to base pair substitutions that occur during DNA synthesis, the occurrence of other genetic events such as transpositions, RecA-dependent deletions and inversions may vary from moderately frequent to very frequent.

The *gudB1* mutation studied here appeared with a frequency of about 10^{-4}. To the best of our knowledge, this is the highest mutation frequency for a specific allele that has been observed in *B. subtilis*. A particular feature of the *gudB* gene is the presence of a tandem repeat. As shown in the present study, the tandem repeat is essential for the high frequency of *gudB* decryptification, and any mutation that impairs the integrity of one of the tandem repeat units resulted in a reduced mutation frequency.

In bacteria, tandem repeats, often termed contingency loci, can be located within an open reading frame or in promoter regions (41). The longest tract of 57 tandem repeats was identified in the *licA1* gene of *H. influenzae* (26). Moreover, tandem repeats are usually very instable since they are prone to high frequencies of mutations through slipped DNA strand mispairing (11, 41, 53). Well-studied tandem repeats such as the *lgtC* repeat in *Haemophilus influenzae* or the *nadA* repeat in *Neisseria meningitidis* are hot spots to generate phenotypic variation, thereby allowing the bacteria to adapt to changing environmental conditions (4, 36). The frequencies of the phenotypic variations range from 10^{-2} to 10^{-5} (41). Thus, the frequency of *gudB* decryptification is in the range observed for other tandem repeats.

In contrast to the well-studied examples, the tandem repeat in the *gudB* gene in the domesticated strain 168 possesses only two repeat units composed of nine nucleotides each. Moreover, the *gudB* repeat is surprisingly quite stable in a nonrelated, nontranscribed genomic context under laboratory growth conditions. Indeed, a derivative of the strain 168 with an active GudB glutamate dehydrogenase could only be selected on minimal medium with glutamate as the single carbon source (5, 19). The situation is completely different when the *rocG* gene that encodes the final enzyme of the arginine degradation pathway is inactivated. These bacteria form only small translucent colonies on complex medium and rapidly acquire the *gudB1* mutation. The occurrence of the *gudB1* mutation at such a high frequency suggests the existence of a strong selective pressure exerted on the *rocG* mutant. The *rocG* gene product, the glutamate dehydrogenase, converts glutamate to 2-oxoglutarate. This suggests that glutamate or one of its precursors in the arginine degradation pathway might accumulate in the *rocG* mutant, and this might be problematic for the cell. We have tested the growth of mutants affected in the different steps of arginine degradation on complex medium; however, the strong growth defect was unique to the *rocG* mutant (our unpublished results). Thus, the accumulation of glutamate may be toxic for the cell. This idea is in good agreement with the observation that a strain with a constitutive high-level expression of the glutamate synthesizing enzyme glutamate synthase acquired a mutation that inactivates this enzyme when grown in the presence of glutamate (19). This leaves us with the question why glutamate should be toxic for the cell when it is the most abundant metabolite anyway. The enzyme glutamate racemase (encoded by the essential gene *racE* in *B. subtilis*) catalyzes the conversion of L-glutamate to D-glutamate that is a building block for peptidoglycan biosynthesis (30, 52). Indeed, the accumulation D-glutamate was shown to be toxic for *B. subtilis* (30). In the presence of very high intracellular amounts of L-glutamate due to the strong induction of the enzymes of the arginine degradation pathway, RacE probably generates higher concentrations of D-glutamate than are tolerated by the cell. The activation of the normally cryptic glutamate dehydrogenase GudB might then bring the glutamate concentration to a level that does not longer result in the accumulation of harmful D-glutamate.

The high frequency and the high precision of the *gudB* decryptification imply that the molecular tools to generate the mutation must be present in *B. subtilis*. It has been reported that tandem repeat deletions occur in *E. coli* rather during chromosome replication by slipped DNA strand mispairing than via the RecA-dependent homologous recombination pathway (10, 11). This observation is in good agreement with our results that the mutations in the *addAB* and *recA* genes do not influence the decryptification of the *gudB* gene. Similarly, the absence of the proteins RecJ, RecU, ExoA, Nfo, UvrAB, MutSL, and SbcDC that are involved in DNA repair, recombination, and stationary-phase mutagenesis did not affect the high-frequency *gudB* mutation. However, we cannot exclude that other factors involved in the excision of the tandem repeat in the *gudB* gene escaped our attention. Moreover, the observed bias for selective excision of the first part of the direct repeat might be explained by the slipped DNA strand mispairing model (11). To the best of our knowledge, this was not yet shown for tandem repeat deletions in bacteria. Thus far, there are no reports available that describe the underlying molecular mechanism and the players involved in tandem repeat deletions in *B. subtilis* and any other Gram-positive species. However, short sequence tandem repeats may play a role in the adaptation of

clinical isolates of *Streptococcus pneumoniae* as a result of selective pressure exerted by the human immune system (43).

Mfd is a multifunctional protein that can play different roles in the cell, e.g., Mfd is involved in transcription-coupled DNA repair and the removal of stalled transcription complexes from DNA (12). However, previous observations indicate that Mfd may facilitate the acquisition of beneficial mutations in the stationary growth phase in *B. subtilis* (45, 46). Our work shows that the Mfd protein is essential for the high-frequency decryptification of the *gudB* gene. The decryptification of the gene occurs by deleting one part of a direct repeat that is located in a transcribed region. The identification of other enzymes that are required for the decryptification of *gudB* and the underlying molecular mechanism will be the subject of further analyses.

The Mfd-mediated coupling of transcription to DNA repair and mutagenesis can be regarded as a built-in precaution that facilitates the accumulation of mutations preferentially in transcribed genes. This has several implications. (i) The coupling allows that the mutations occur in genes that are expressed at the given time point; therefore, the mutant variants of the encoded proteins might help to overcome the actual limitation. (ii) Nontranscribed genes that may be required under different conditions are in this way protected from potentially harmful mutations. Both effects facilitate the adaptation of bacteria to all kind of challenges that limit their growth and are therefore crucial for bacterial evolution.

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