

Spontaneous Deletion Formation within the β -Galactosidase Gene of *Lactobacillus bulgaricus*

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To investigate the genetic stability of the dairy organism *Lactobacillus bulgaricus*, we have analyzed 107 spontaneous mutations of the β -galactosidase gene of this organism. Ten of these mutations were DNA rearrangements giving rise to different deletions, located predominantly within a small hot spot area. The DNA sequences of the different deletion junctions have been determined. The analysis showed that the deletions can be divided into two classes, depending on the presence of short direct-repeat sequences at the deletion endpoints and on the length of the deleted sequences. Possible mechanisms of these deletion formations and the involvement of inverted-repeat sequences that may enhance slipped DNA mispairing are discussed.

Lactobacillus bulgaricus, a member of the large and economically important genus *Lactobacillus*, is a subspecies of *Lactobacillus delbrueckii* (19). *L. bulgaricus* itself is a gram-positive, facultative anaerobe dairy microorganism, perhaps best known for its ability to ferment milk to yogurt. It is a fastidious bacterium, growing only in rich growth media such as milk, and it can ferment only lactose, glucose, and fructose (8, 19). In the natural habitat, milk, lactose is the only sugar and serves as a unique carbon source.

Despite the use of *L. bulgaricus* for hundreds of years, very little is known about its genetic properties. Only recently have attempts to introduce DNA into *L. bulgaricus* by transfection or conjugation been reported (2, 24). The only genes isolated and analyzed so far are the β -galactosidase (β -gal) gene (44; P. Leong-Morgenthaler and M.-C. Zwahlen, personal communication) and several tRNA genes (16, 36, 37).

To investigate the genetic stability of *L. bulgaricus*, we searched for DNA rearrangements by isolating and characterizing spontaneous mutations. As target gene for our work, we chose the β -gal gene.

MATERIALS AND METHODS

Media. *L. bulgaricus* was grown in Difco litmus milk and Difco lactobacillus MRS broth (8). MRS agar plates contained MRS with 10 g of agar per liter. MRS X-Gal agar plates contained X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) at 25 μ g/ml. Luria broth (LB) to grow *Escherichia coli* was prepared according to Maniatis et al. (27). For maintenance of plasmid pMZ2, ampicillin (50 μ g/ml) was added to the growth medium.

Bacteria and plasmid. *L. bulgaricus* type strain N123 was obtained from B. Marchesini (Nestlé Collection) and is identical to strain NCDO 1489 (National Collection of Dairy Organisms, Food Research Institute, Reading, Great Britain). N123 was plated onto MRS agar plates and incubated under normal aerobic conditions at 43°C overnight. Single colonies were transferred to MRS medium, grown at 43°C, restreaked onto MRS agar plates, and grown again under aerobic conditions. This procedure was repeated four times

to obtain a good plating efficiency under aerotolerant growth conditions for N123. One of the isolates was N299.

Plasmid pMZ2, obtained from M.-C. Zwahlen, contains the entire β -gal gene of N123 on a 4.3-kilobase (kb) *SalI*-*BamHI* fragment cloned into pBR322 (Leong-Morgenthaler and Zwahlen, personal communication). The *E. coli* strain used to propagate pMZ2 was JM105-8, a derivative of JM105 that has been cured of F' (28; Zwahlen, personal communication); plasmid extraction of pMZ2 was done as described by Maniatis et al. (27).

Screening for β -gal mutants. To screen for β -gal mutants, we used the X-Gal plate assay as described for *E. coli* (31). N299 was subcultured from litmus milk cultures into MRS medium and grown overnight at 43°C. Appropriate dilutions were plated onto MRS X-Gal plates and incubated under aerobic conditions at 43°C. White colonies were picked after 48 h and restreaked twice on MRS X-Gal plates. The purified cultures were stored in MRS medium supplemented with glycerol to 15% (vol/vol) at -70°C. To ensure isolation of independent mutants, only one white colony per MRS subculture was picked and purified. We observed that the β -gal gene of *L. bulgaricus* is expressed constitutively and does not need to be induced by isopropyl- β -D-thiogalactopyranoside (IPTG).

Analysis of β -gal mutants. *L. bulgaricus* strains were grown in 10 ml of MRS broth at 43°C, and the chromosomal DNA was extracted as described previously (7). The chromosomal DNA was digested to completion with restriction enzymes, separated on agarose gels, and transferred to GeneScreen hybridization transfer membrane. Southern blot hybridization was then performed as described by Southern (46). The DNA probe used for the hybridization was the agarose gel-purified 4.3-kb *SalI*-*BamHI* fragment of pMZ2 containing the entire β -gal gene. The probe was ³²P labeled by the random priming method (48). Hybridization and washing of the blots were performed under stringent conditions at 65°C.

DNA amplification and sequence analysis. The regions containing the deletions were amplified from the chromosomal DNA by the polymerase chain reaction method (40, 41). The synthetic oligonucleotides used as primers were 5'-TACCAACAGCTCATCTTA (primer 1) and 5'-TACAGCAATCTTGCCCCGTCC (primer 2) for isolates *lac135*,

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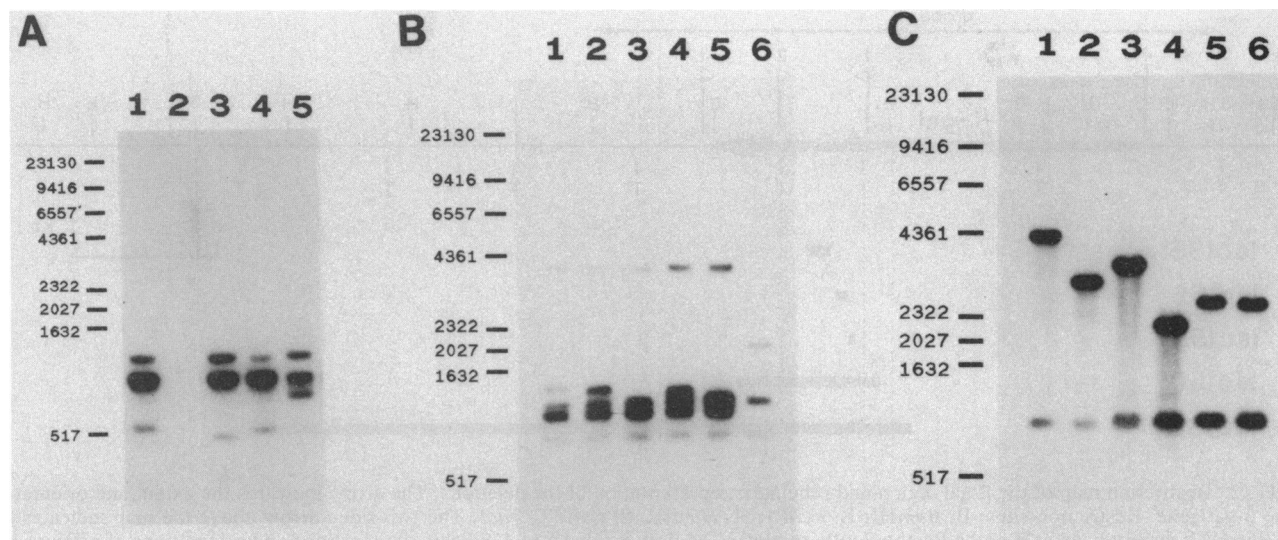


FIG. 1. Southern blots of total chromosomal DNA of mutant strains probed with the β -gal gene from pMZ2. (A) *TaqI* digest of N299, *lac104*, *lac156*, *lac139*, and *lac150* (lanes 1 to 5, respectively). (B) *EcoRI* digest of N299, *lac139*, *lac150*, *lac156*, *lac166*, *lac186* (lane 1 to 6, respectively). (C) *HindIII* digest of N299, *lac150*, and *lac186* (lanes 1, 3, and 5, respectively); *HindIII-BamHI* double digest of N299, *lac150*, and *lac186* (lanes 2, 4, and 6, respectively). The DNA quantities per lane varied. Smaller DNA fragments ran off the gel and are not visible on the blots. Size markers are indicated in base pairs.

lac139, *lac154*, *lac156*, *lac159*, *lac166*, and *lac181*. 5'-TCATCCTGTTAAGTCAATTGTAGCA (primer 3) and 5'-GTTAGAAGGGAAGAATTAGAAAATG (primer 4) were used for *lac150*. The primers were synthesized with an Applied Biosystems 380B DNA synthesizer and purified on NAP-10 gel filtration columns (Pharmacia LKB). The amplified DNA fragments were run on 1.0% agarose gels and isolated therefrom. They were purified by phenol-chloroform extractions and used for DNA sequencing. The dideoxy chain termination method of Sanger et al. (42) was applied, using fragment internal 17-mer oligonucleotides as sequencing primers. We used the T7 DNA polymerase sequencing kit from Pharmacia LKB. The resulting DNA sequences were compared with the sequence of the *L. bulgaricus* wild-type β -gal gene (Leong-Morgenthaler and Zwahlen, personal communication; 44). For DNA sequence analysis, the University of Wisconsin Genetics Computer Group software package was used (9).

RESULTS

Screening for spontaneous β -gal mutants. A total of 107 independent spontaneous β -gal mutants were isolated as described above. The observed mutation frequency, i.e., white over blue colonies, was 3.5×10^{-5} . After two purification steps, 90 mutants still showed white colonies on MRS X-Gal plates, whereas 17 mutants formed light blue colonies. In these latter cases, the β -gal activity is not completely lost after the mutation event.

Restriction mapping of the mutants. Chromosomal DNA of all isolated mutants was extracted and digested with *HindIII* and in separate reactions with *TaqI*. The digested DNA was then analyzed on Southern blots as described in Materials and Methods, using a homologous β -gal gene as probe.

The results indicated that the chromosomal DNA isolated from 97 of the 107 mutants showed a hybridization pattern of the restriction digests identical to that of wild-type strain N299. There were no obvious DNA rearrangements, insertions, or deletions within the β -gal gene or its immediate

flanking regions, as determined by Southern blot analysis. It is noteworthy that all mutants forming light blue colonies on MRS X-Gal plates fell into this category of mutants.

Ten of the analyzed mutants showed a hybridization pattern clearly different from that of N299. The smallest *TaqI* fragment (558 base pairs [bp]) of these mutants was changed or lost (Fig. 1A). In the case of *lac104*, there was no hybridization with the β -gal probe at all. To further characterize these mutants, we made Southern blots with *EcoRI* and *HindIII-BamHI* double-digested chromosomal DNA. Figures 1B and C show some of the alterations observed with these Southern blots. The hybridization of the *EcoRI*-digested DNA showed that the second-largest fragment (1,194 bp) was shifted for all mutants, and *lac186* had only two bands unchanged in comparison with N299, the 1,017- and 711-bp fragments. The *HindIII* and *HindIII-BamHI* digests of *lac150* and *lac186* indicated that in the latter case, the *BamHI* site downstream of the β -gal gene was deleted.

Analysis of the blots and mapping of the restriction sites indicated that we had isolated 10 deletions of variable sizes. Seven of these isolated deletions were very small (i.e., smaller than 200 bp) and located entirely within the 558-bp *TaqI* fragment of the β -gal gene. Two deletions, *lac150* and *lac186*, were of distinctly larger sizes and completely deleted the 558-bp *TaqI* fragment. Deletion *lac186* was about 4.5 kb in size and extended beyond the cloned β -gal region of pMZ2 (Fig. 2). The longest deletion isolated was *lac104*. It had the entire β -gal region of pMZ2 deleted and gave no signal on the hybridization blots. Southern blots using DNA probes of sequences further up- and downstream also showed no hybridization (data not shown). We were therefore not able to identify and map the junction site of this deletion. From the total length of the DNA probes used, we estimate that the *lac104* deletion exceeded 17 kb in size.

DNA sequence analysis of the deletion junction sites. By making use of the polymerase chain reaction technique, the regions containing deletions *lac135*, *lac139*, *lac154*, *lac156*, *lac159*, *lac166*, and *lac181* were amplified from total chro-

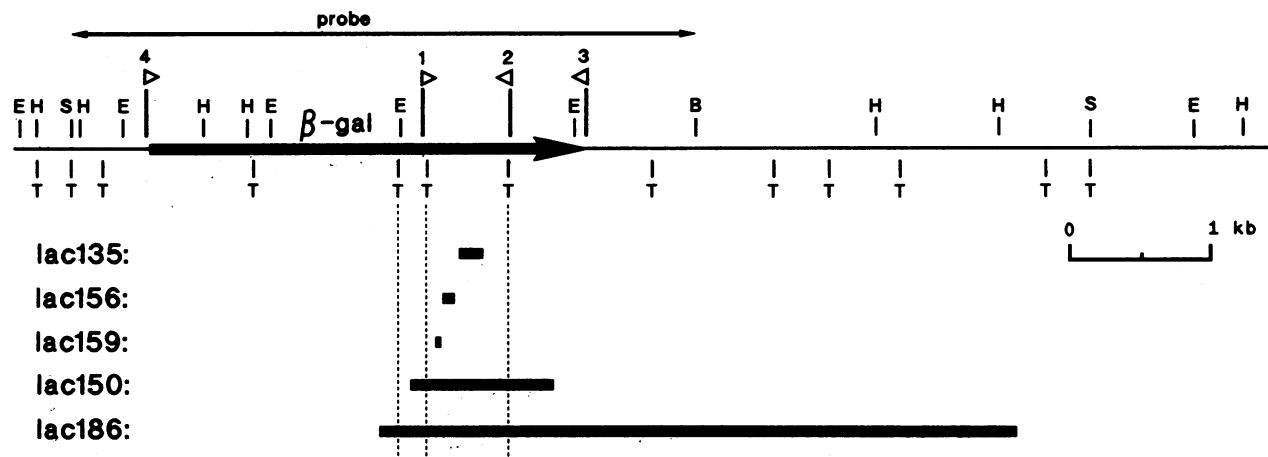


FIG. 2. Restriction map of the β -gal region and schematic representation of the deletions. The arrow indicates the extent and orientation of the β -gal gene. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I; T, *Taq*I. The two-sided arrow above the map indicates the *Sal*I-*Bam*HI fragment used as a probe for the Southern blots. The flags labeled 1 to 4 indicate the locations and orientations of primers 1 to 4, respectively. The heavy bars underneath the β -gal gene indicate the locations and extents of the deletions.

mosomal DNA. The relative positions of the primers applied for the polymerase chain reaction are shown in Fig. 2. The amplified DNA fragments were then used directly for sequencing, and the analysis thereof showed the following.

The four deletions *lac135*, *lac154*, *lac166*, and *lac181* were identical. A region flanked by a 14-bp-long perfect direct repeat was deleted, leaving just one copy of the repeat in the mutated β -gal gene. The deletion removed 150 bp (Fig. 3). A similar situation was observed for *lac156*. The 72-bp deletion was flanked by a 13-bp direct repeat. The two smallest deletions, *lac139* and *lac159*, were identical and also flanked by a 13-bp direct-repeat sequence. The extent of the deletion was 30 bp (Fig. 3). The region deleted in mutant *lac150* was much larger than the above-described deletions (933 bp), and it was flanked by a very short direct repeat of only 4 bp. Interestingly, in this case the two repeats did not fuse in a homologous way to leave one copy of the repeat in the mutant genome. In addition to the one copy, a part of the second copy of the repeat also remained on the chromosome (Fig. 3). The locations of these deletions within the β -gal gene are shown in Fig. 2.

DISCUSSION

To understand the genetic stability of *L. bulgaricus*, we have isolated spontaneous mutations of the β -gal gene and analyzed them for DNA rearrangements. For our study, we have chosen the β -gal gene as a target because it is relatively large and its DNA sequence is known. Furthermore, β -gal activity represents an absolute essential function for this bacterium in its natural environment, milk, where the only fermentable carbohydrate is lactose.

The frequency of spontaneous mutations. The frequency of spontaneous mutations in *L. bulgaricus* N299, derived from the parent type strain N123, is relatively high. A screening for β -gal-minus mutants by using another, nontype *L. bulgaricus* strain indicated that the frequency of spontaneous mutations was considerably lower in this second strain than in N299 (our unpublished observation). The reason for this high mutational frequency of N299 is still unclear.

Spectrum of mutations. We were able to isolate at least two different classes of mutational events, which presumably represent a variety of different mutational mechanisms. The

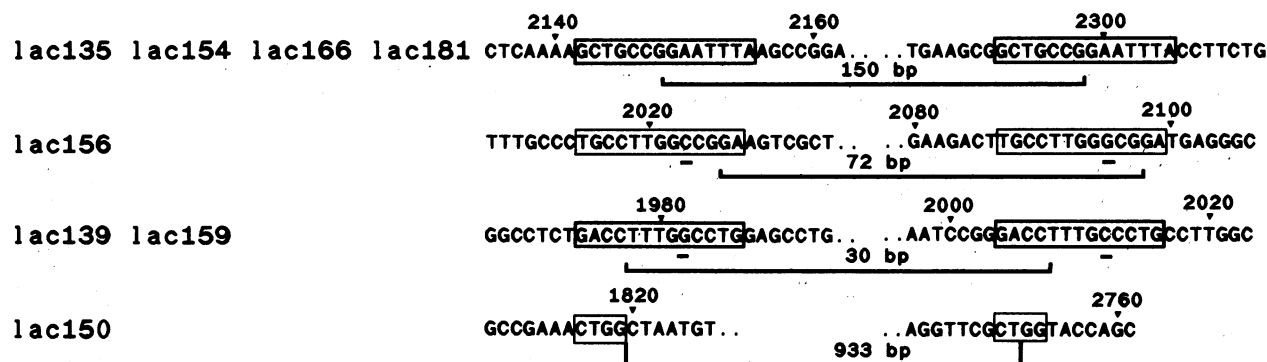


FIG. 3. Junction sites of the deletions. Direct repeats are boxed. Mismatches within the direct repeats are underlined. The brackets underneath the sequences represent the junction of the deletions. For the deletions represented by *lac135*, *lac156*, and *lac139*, the exact junction site cannot be determined and is within the homologous part of the direct repeat. Numbers above the brackets indicate the extents of the deletions in base pairs. Numbers above the sequences indicate positions within the β -gal gene, starting with 1.

major class of events, comprising 91% of all observed mutations, consists of mutations that do not give noticeable differences in the restriction map of the mutated β -gal gene as compared with the wild-type gene. They are probably transitions, transversions and frameshifts giving rise to nonsense and missense mutations within the gene or affecting its promoter region. The observation that a few mutations still had some residual β -gal activity on MRS X-gal plates would tend to support this assumption.

The second class of events consists of mutations giving rise to alterations in the restriction pattern due to DNA rearrangements. We were able to isolate 10 independent mutants belonging to this class of events. All of these isolates had deletions of various sizes within the β -gal gene or, in one case, a deletion of the entire gene. The frequency with which we observed these spontaneous deletion formations was very high. Furthermore, most of these deletions were located within a small *TaqI* fragment (Fig. 2). It is likely that this region, because of its specific primary sequence, is very unstable and prone to undergo deletion formations. It is also interesting that we did not find any other type of spontaneous DNA rearrangements such as duplications, inversions, or insertion sequences (17, 20, 43). One can imagine that such a high frequency of deletion formation may mask the occurrence of other recombinational events. We therefore cannot exclude the existence of active transposable elements in *L. bulgaricus* or the possibility that there is translational exclusion of insertion mutagenesis as has been observed for *Salmonella typhimurium* (4).

Spectrum of deletions. We isolated a total of 10 independent deletions. The largest deletion isolated was *lac104*. It spans at least 17 kb, and we were not able to determine its endpoints. The second largest deletion, *lac186*, is 4.5 kb long; and its endpoints have been mapped and are shown in Fig. 2. All other identified deletions are of considerably smaller size and located entirely within the β -gal gene. The junction sites of these deletions were sequenced and compared with the wild-type β -gal gene sequence. Several of the deletions, which have been isolated independently, are identical. They can be distinguished as four different deletion events giving rise to two different classes of mutations.

The first class of mutations consists of three different deletion events. The most frequent event is a 150-bp deletion flanked by a 14-bp perfect repeated sequence. Four of the analyzed deletions belong to this particular event. Two other deletion events represented, by *lac156* and the two identical

TABLE 1. Direct-repeat sequences within the β -gal gene

Matches/size ^a (bp)	Positions ^b	Distance between repeats (bp)	Deletion
14/14	2142–2292	150	<i>lac135</i> , 154, 166, 181
13/14	859–2107	1,248	
13/14	501–2925	2,424	
13/14	13–3058	3,045	
12/13	2015–2087	72	<i>lac156</i>
12/13	1974–2004	30	
12/13	1199–2443	1,244	<i>lac139</i> , 159
12/13	1095–2350	1,255	
12/13	915–2988	2,073	
12/13	781–2152	1,371	
12/13	504–2421	1,917	
12/13	51–177	126	

^a Matches versus size of the direct repeats.

^b Indicates position of the first nucleotide of each direct repeat within the β -gal gene.

deletions *lac139* and *lac159*, also belong to this class of mutations. They all have small deletions and are flanked by short direct-repeat sequences. Similar deletion events have been observed earlier, mainly through mutational studies of the *lacI* gene of *E. coli* (1, 11, 13, 43), bacteriophages T4 (38, 45) and T7 (35, 47), and chimeric M13 plasmids and pBR322 (3, 6, 18, 29, 30) and on excision of transposable elements (10, 12). In the gram-positive organism *Bacillus subtilis*, similar deletion events were discovered on plasmids (15, 23, 25, 34).

The second class of mutations consists of deletion *lac150*. The deletion is relatively large, and there is only a 4-bp direct-repeat flanking sequence. Similar deletions between very small repeats have also been observed in *E. coli* and at the *aprt* locus of hamster cells (11, 32, 33). From our observation, it seems that mutations of the first class occur with much higher frequency than do those of the second class.

Mechanisms of deletion formation. The mechanisms of deletion formation involving short repeated sequences at the deletion endpoints have been the subject of much discussion in the recent literature. Several models involving inter- and intramolecular homologous recombination, strand slippage during replication due to DNA mispairing, and excision-repair mechanisms have been proposed (1, 3, 18, 35, 43, 45). Since most of the deletion events that we observed in *L. bulgaricus* are due to the presence of direct-repeat se-

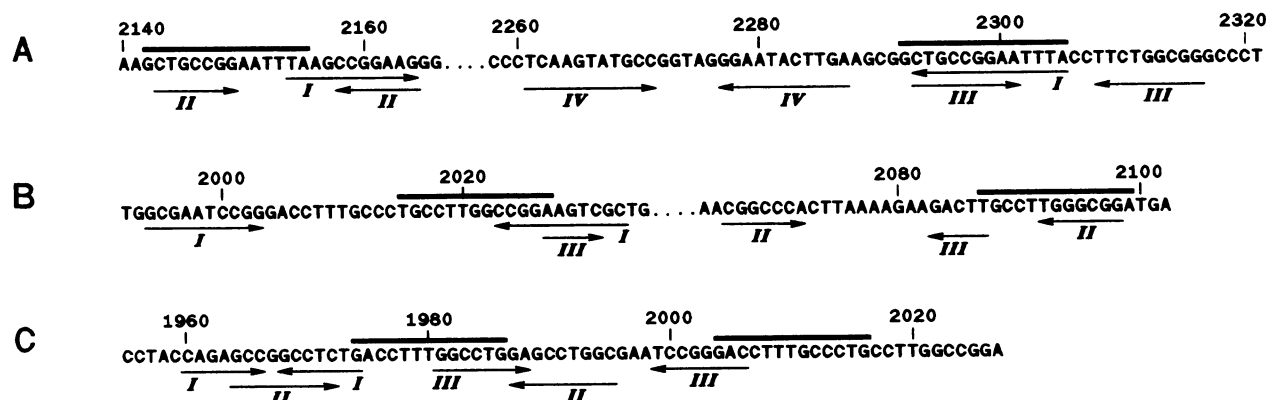


FIG. 4. Inverted-repeat sequences at the deletion junction sites. Shown are deletion sites of *lac135* (A), *lac156* (B), and *lac139* (C). The direct repeats are indicated by bars; the inverted repeats are indicated by arrows. Sister inverted repeats are labeled with the same roman numeral.

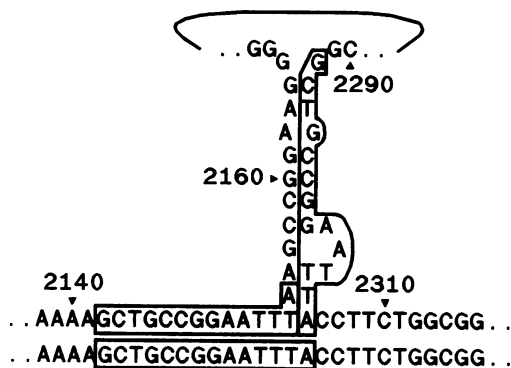


FIG. 5. Suggested DNA sequence misalignment of the inverted repeat I at the *lac135* deletion locus. The direct repeated sequence is boxed. The DNA sequence underneath the misalignment represents the sequence of the *lac135* deletion. Numbers indicate base pair positions within the β -gal gene.

quences, we searched the entire β -gal gene and its immediate flanking region for the occurrence and distribution of direct repeats (Table 1). We found a total of 12 direct repeats of at least 13 bp in length, allowing one mismatch. The most prominent repeat found was the 14-bp perfect repeat, which gave rise to the most frequent deletion event. The other two deletion events were found associated with the lesser repeats but clearly favoring the repeats with the least separation. This analysis indicates that although the possibility of formation of different deletions between repeats of similar quality is high, only a few events have been recovered repeatedly. The deletion events seem not to occur randomly between the different directly repeated sequences. We can assume, therefore, that the models involving exclusively homologous recombination do not represent the entire story of these deletion formations.

It has been reported that slipped mispairing between homologous stretches of DNA during replication or repair is enhanced by the presence of palindromic sequences (6, 39, 49). A closer look at the DNA sequence of the β -gal gene indicates that the hot spot region, where most of the deletions occurred, is in fact rich in inverted-repeat sequences. Some of these repeats, located around the endpoints of the three major deletion events, are shown in Fig. 4. At the endpoints of the most frequent deletion event (represented by *lac135*), a prominent inverted repeat is found. It brackets the whole sequence to be deleted and overlaps entirely with one of the 14-bp direct repeats (Fig. 4A, I). A possible two-dimensional structure of this region is illustrated in Fig. 5. The recovered deletion may have been formed by slipped mispairing, whereby the newly synthesized repeat dissociates from the original template and misaligns to the complement of the other copy of the repeat. This misalignment may have been mediated by the secondary structure of the template strand (Fig. 6A).

Furthermore, it is possible that one of the inverted repeats II and III (Fig. 4A), each of which brackets a copy of the direct repeat, helped to mediate strand slippage during replication or repair. By forming hairpin structures, the inverted repeats may destabilize the newly synthesized DNA double strand and enhance the possibility that the direct repeat misaligns to the complement of its sister copy. A very similar situation is also found around the other two deletion sites (Fig. 4B, I and II; Fig. 4C, II). Internal stem-and-loop structures may thereby shorten distances

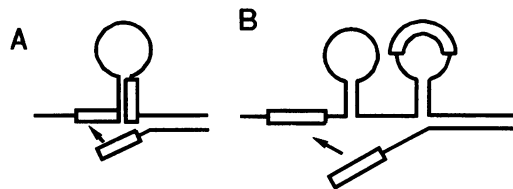


FIG. 6. Schematic model of strand slippage. (A) Model for strand slippage as presented in Fig. 5. (B) Model for slippage involving destabilization of just one direct repeat. The boxes represent direct repeats, and the arrows indicate the misalignment of the newly synthesized repeat to its complementary sister copy on the template strand. After misalignment, DNA replication will proceed.

between the direct repeats (Fig. 4A, IV; Fig. 4B, III; Fig. 4C, III). A possible model is outlined in Fig. 6B.

The deletion *lac150* must have been formed by a different mechanism. There is no direct repeat that could be joined in a homologous way. Therefore, the models of homologous recombination or slipped mispairing, as discussed above, are not applicable for this mutation event. One should also bear in mind that this class of mutation is much less frequent than the other deletions and that we have isolated just this one. A close look at the DNA sequence indicated that the two ends of this relatively large deletion may have been brought together by a misalignment, as shown in Fig. 7. Such a secondary DNA structure may have been formed on single-stranded DNA during replication or a gap repair process, after which the deletion was fixed by DNA synthesis of the other strand across the misalignment. Similar observations have been reported by Glickman and Ripley (14). It is interesting that the entire region of instability, as observed for the above-discussed hot spot deletions, is located within the deleted part of *lac150*. The presence of this multitude of direct and inverted repeats may have enhanced the formation of single-stranded DNA in this area.

Concluding remarks. In this study, we investigated the genetic stability of the β -gal gene of *L. bulgaricus* N299. We found that the frequency of spontaneous mutations is relatively high. Furthermore, there is a specific region within the

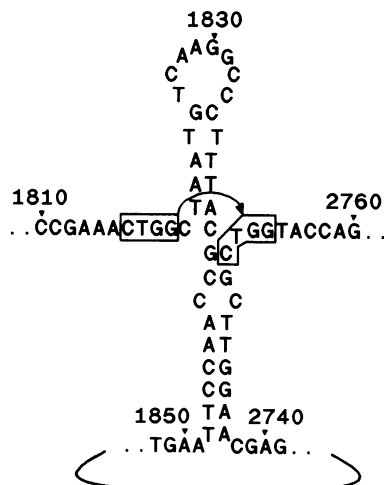


FIG. 7. Potential secondary structure of the DNA sequence involved in the deletion formation of *lac150*. The short direct repeat is boxed. The arrow indicates the junction that formed the *lac150* deletion. Numbers indicate base pair positions within the β -gal gene.

gene which is prone to deletion formation. Short direct repeats of 14 and 13 bp in length in concert with short inverted repeated sequences flanking or even overlapping the direct repeats are responsible for this event.

Preliminary studies indicate that other strains of *L. bulgaricus* do not show this same high mutation frequency. It remains to be seen whether these differences in frequency are due to actual differences in the DNA sequence of the β -gal gene (e.g., disruption of some of the repeated sequences) or whether still unknown host factors which influence the frequency of deletion formation are involved, similar to what has been described for *E. coli* (5, 21, 22, 26). Furthermore, analysis of spontaneous mutations in more stable *L. bulgaricus* strains may reveal other types of spontaneous mutations that we did not observe in this study, such as duplications and insertions.

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