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Indigenous and Environmental Modulation of Frequencies of Mutation in *Lactobacillus plantarum*▽†

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Reliability of microbial (starter) strains in terms of quality, functional properties, growth performance, and robustness is essential for industrial applications. In an industrial fermentation process, the bacterium should be able to successfully withstand various adverse conditions during processing, such as acid, osmotic, temperature, and oxidative stresses. Besides the evolved defense mechanisms, stress-induced mutations participate in adaptive evolution for survival under stress conditions. However, this may lead to accumulation of mutant strains, which may be accompanied by loss of desired functional properties. Defining the effects of specific fermentation or processing conditions on the mutation frequency is an important step toward preventing loss of genome integrity and maintaining the productivity of industrial strains. Therefore, a set of *Lactobacillus plantarum* mutator reporter strains suitable for qualitative and quantitative analysis of low-frequency mutation events was developed. The mutation reporter system constructed was validated by using chemical mutagenesis (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) and by controlled expression of endogenous candidate mutator genes (e.g., a truncated derivative of the *L. plantarum hexA* gene). Growth at different temperatures, under low-pH conditions, at high salt concentrations, or under starvation conditions did not have a significant effect on the mutation frequency. However, incubation with sublethal levels of hydrogen peroxide resulted in a 100-fold increase in the mutation frequency compared to the background mutation frequency. Importantly, when cells of *L. plantarum* were adapted to 42°C prior to treatment with sublethal levels of hydrogen peroxide, there was a 10-fold increase in survival after peroxide treatment, and there was a concomitant 50-fold decrease in the mutation frequency. These results show that specific environmental conditions encountered by bacteria may significantly influence the genetic stability of strains, while protection against mutagenic conditions may be obtained by pretreatment of cultures with other, nonmutagenic stress conditions.

Lactobacillus plantarum is a common inhabitant of the human gastrointestinal tract (3), but it is also encountered in a variety of environmental niches, such as dairy, meat, and vegetable fermentations (25, 39). *L. plantarum* is widely used in industrial and traditional production of fermented plant, food, and feed products, such as sauerkraut, sausage, cabbage, olives, and silage (14, 31, 41). In addition, *L. plantarum* is employed as a starter culture, and it contributes to the conservation, flavor, and texture of fermented foods. The growth performance and robustness of this bacterium are key factors that determine the characteristics of the final products. Moreover, properties that improve consumer health have been attributed to various *L. plantarum* strains, and some strains are marketed as probiotics (10, 14, 35).

Reliability of (starter) strains in terms of quality, functional properties, growth performance, and robustness is very important for industrial applications. In an industrial fermentation process, the bacterium should be able to withstand various adverse conditions during processing, such as acid, osmotic, temperature, and oxidative stresses. In their natural environments bacteria are also constantly exposed to fluctuating environmental conditions, and many of these conditions are potentially detrimental and negatively affect the physiological state and growth rate. Many bacteria possess multiple regulatory networks of stress response systems that allow them to withstand harsh conditions and sudden changes in environmental conditions (49). Moreover, mutations may favor generation of strain variants that are better adapted to survive under these stress conditions (9, 13, 21). However, this process may not be desirable for maintaining the reliability of strains used in industrial processes. Especially when high productivity of strains results in decreased fitness, mutants with decreased productivity and loss of desired functional properties may be readily obtained (46, 53). In addition, DNA instability can even result in a so-called mutator phenotype, where sharply elevated spontaneous mutation rates (transiently) enhance the ability of strains to adapt to radical changes in the environment (19, 20, 51). The lactic acid bacterium *Oenococcus oeni* is an example of an organism that positively adapted to a harsh

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environment due to an elevated mutation rate. *O. oeni* lacks the *mutS* and *mutL* mismatch repair genes, which has been suggested to contribute to its hypermutable status and its accelerated evolution. It is likely that this factor contributed to the unique adaptation of *O. oeni* to acidic and alcoholic environments that made it an ideal organism for the malolactic fermentation during the production of wine (7, 29). However, elevated mutation rates may also result in deterioration of industrial fermentation properties (19, 20, 51). Therefore, defining the effects of conditions encountered during industrial fermentation processes on the mutation frequency is an important step toward preventing loss of genome integrity and maintaining the productivity of industrial strains. It has been shown that adaptation to mild stress conditions (e.g., mild heat shock) can induce cross-protection against more lethal stress conditions, resulting in an increased survival rate. Consequently, establishing the effect of cross-protection on the mutation frequency could also be an important step in maintaining the productivity of industrial strains (11, 49).

In this study we focused on selected environmental challenges that are relevant for fermentation and processing conditions and their effects on the mutation frequency of *L. plantarum*. To this end, a mutation reporter strain set was constructed, which was validated using chemical mutagenesis and controlled expression of candidate genes that may lead to mutator phenotypes upon overexpression. The frequencies of mutation under various environmental conditions were subsequently studied, which revealed that especially oxidative stress challenge (challenge with hydrogen peroxide, which can be produced as a by-product by *L. plantarum* or other lactobacilli during fermentation [4, 30, 36, 40, 49, 52]) increased the mutation frequency. Interestingly, pretreatment of cells under nonmutagenic conditions (42°C) appeared to protect them against subsequent increases in the mutation frequency due to oxidative stress, showing that sequential exposure to stresses may be used to protect industrial strains against elevated mutation rates and in this way may enhance the stability of these strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study and their relevant features are shown in Table 1 (see Table S1 in the supplemental material for a list of the primers used in this study).

L. plantarum was grown at 37°C in MRS medium (Difco, West Molesey, United Kingdom) without aeration. *L. plantarum* was also grown at 30°C in CDM (47) supplemented with 1% maltose, plated on CDM with 1% melibiose and 0.004% bromocresol purple (BCP) (Merck, Darmstadt, Germany) for determination of mutation frequencies, and plated on CDM with 1% maltose for determination of viable counts. The plates were incubated anaerobically at 30°C. *Escherichia coli* strain Top10 (Invitrogen, Breda, the Netherlands) was used as a cloning host for construction of pNZ1800. *E. coli* strain XL1-blue (Stratagene, La Jolla, CA) was used as a cloning host for construction of pNZ1801, pNZ1802, pNZ1803, pNZ1804, pNZ1805, pNZ1806, and pNZ1817. Both *E. coli* strains were grown aerobically at 37°C in TY broth (42). *Lactococcus lactis* MG1363 was used as an intermediate cloning host for plasmids pNZ8154, pNZ1813, and pNZ1814 and was grown without aeration at 30°C in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% glucose. When appropriate, antibiotics were added to the media at the following concentrations: 10 μg/ml chloramphenicol and 10 μg/ml erythromycin for *L. plantarum*, 10 μg/ml chloramphenicol and 50 μg/ml kanamycin for *E. coli*, and 10 μg/ml chloramphenicol and 5 μg/ml erythromycin for *L. lactis*.

DNA techniques. Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (8). Large-scale plasmid DNA isolation was performed using Jetstar columns according to the manufacturer's recommendations (Genomed GmbH, Bad Oberhausen, Germany).

Isolation of *L. lactis* plasmid DNA was performed using Jetstar columns according to the manufacturer's recommendations, with modifications described previously (5). Transformation of *L. lactis* was performed as described by Holo and Nes (23). For DNA techniques with *E. coli* and *L. lactis*, standard procedures were performed as described by Sambrook et al. (42).

L. plantarum total DNA was isolated and strains were transformed as described previously (27). The anticipated genetic organization of the transformants was verified by PCR analysis.

Restriction endonucleases, DNA polymerases, T4 DNA ligase, and the Klenow enzyme were purchased from Invitrogen (Breda, the Netherlands), New England Biolabs (Leusden, the Netherlands), and Stratagene (La Jolla, CA) and used according to the manufacturer's recommendations. Oligonucleotides were purchased from Invitrogen (Breda, the Netherlands). DNA sequencing analysis was performed by BaseClear B.V. (Leiden, the Netherlands).

Construction of *L. plantarum* NZ1800. The regulated promoter of the *melA* α-galactosidase gene of *L. plantarum* NZ7100 was replaced by the strong and constitutive *usp45* promoter from *L. lactis* MG1363 (48). To replace the promoter, a plasmid harboring P*usp45*, *melA*, *lox66*, *cat*, and *lox71* between the upstream and downstream regions of *melA* was constructed. The ori-P*usp45-melA* fragment (5) was isolated from plasmid pNZ5516 by DraI and XbaI digestion and treated with the Klenow enzyme. This fragment was ligated to a Klenow enzyme-treated NruI-SalI fragment from pNZ5340 (27) with the *cat-lox71* downstream region of *melA*, resulting in plasmid pNZ1825. The upstream region of *melA* was PCR amplified with *Pwo* polymerase using primers LacS2forPciI and LacS2revSacI and *L. plantarum* WCFS1 genomic DNA as the template. The PCR fragment was digested with PciI and SacI and ligated in similarly digested pNZ1825. The resulting plasmid (pNZ1826) was digested with DraI and NotI to introduce a *lox66* region (composed of oligonucleotides N-lox66-D and D-lox66-N) by oligonucleotide linker ligation. The resulting plasmid, pNZ1800, was used for double-crossover integration in *L. plantarum* NZ7140. *L. plantarum* NZ7140 is a Δ*melA* derivative of *L. plantarum* NZ7100 that was constructed by double-crossover integration of plasmid pNZ5340 using the method described previously for the *L. plantarum* WCFS1 Δ*melA* derivative NZ5335 (27). P*usp45-melA*-positive colonies were selected by growth on agar with the substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal). The colonies of single-crossover mutants were light blue, and the colonies of double-crossover mutants were dark blue, which was confirmed by PCR analysis (with primers 93 and LK17 and primers LK1 and LK4). To remove the *cat* gene after integration, the Cre expression plasmid pNZ5348 (27) was transformed into the NZ7140 double-crossover mutant. The resulting Cre expression led to recombination of the *lox* sites and excision of the intermediate DNA sequence (1, 22, 27). Since plasmid pNZ5348 is very unstable in *L. plantarum*, it could readily be cured from *L. plantarum* transformants by growth in the absence of erythromycin selection pressure for 10 generations (27). The anticipated genetic structure of the P*usp45-melA* locus in a single colony obtained after excision (designated *L. plantarum* NZ1800) was confirmed by PCR analysis and Southern blotting.

Construction of the mutator reporter strains. Mutations in *melA* in plasmid pNZ1800 were constructed using a QuikChange II mutagenesis kit obtained from Stratagene (La Jolla, CA). Six primer sets were designed to make substitution, deletion, and insertion mutations in the catalytic site of *melA* (Table 2). One primer set was designed to make the mutations necessary to change two amino acid residues to glycine residues and to insert one extra G residue, resulting in a frameshift mutant and a stretch of G residues (Table 2). Sequence analysis (with primers LK34, LK35, and LK36) of the resulting plasmids (pNZ1801, pNZ1802, pNZ1803, pNZ1804, pNZ1805, pNZ1806, and pNZ1817 [Tables 1 and 2]) confirmed the presence of the correct mutations. The plasmids were transformed into *L. plantarum* NZ7100, and double-crossover mutants were selected to obtain a *melA* mutant phenotype. Removal of the *cat* gene and verification of the correct genetic organization of the P*usp45-melA* locus in these strains (strains NZ1801 to NZ1807 [Table 1]) were performed as described above for strain NZ1800. In addition, the sequence of the mutated region of the *melA* gene was confirmed by PCR amplicon (primers LK4 and LK36) sequencing.

Construction of the mutator vector pNZ8154. For controlled expression of potential mutator genes (e.g., *hexA*, *dinP*, and tr-*hexA*, an N-terminally truncated derivative of *hexA* [see below]), a low-copy-number vector with an inducible promoter was constructed. The inducible *nisA* promoter from plasmid pNZ8048 (26) was cloned into the low-copy-number plasmid pIL252 (45). Plasmid pNZ8048 was digested with BglII and XhoI and treated with the Klenow enzyme. The 576-bp P*nisA*-MCS-T*pepN* (where MCS stands for the multiple cloning site and T stands for the terminator sequence derived from the downstream region) fragment was ligated into SmaI-digested pIL252. PCR analysis (with primers Nis1 and RB148) showed that the P*nisA*-T*pepN* fragment was oriented in the

TABLE 1. Strains and plasmids used in this study, their relevant characteristics, and references

Strain or plasmid	Relevant features[a]	Reference
E. coli strains		
Top10	Cloning host	
XL1-blue	Cloning host	
L. lactis MG1363	Cloning host	18
L. plantarum strains		
NZ7100	*L. plantarum* WCFS1 with chromosomal integration of plasmid pEMnisRK	44
NZ7140	*L. plantarum* NZ7100 with Δ*melA* obtained by double-crossover integration of pNZ5340	This study
NZ1800	Integration of *L. lactis usp45* promoter in front of *L. plantarum* NZ7100 *melA*	This study
NZ1801	NZ1800 with substitution mutation in the catalytic site of *melA*, constructed using the pNZ1801 mutagenesis vector	This study
NZ1802	NZ1800 with substitution mutation in the catalytic site of *melA*, constructed using the pNZ1802 mutagenesis vector	This study
NZ1803	NZ1800 with 1-bp deletion in the catalytic site of *melA*, constructed using the pNZ1803 mutagenesis vector	This study
NZ1804	NZ1800 with 2-bp deletion in the catalytic site of *melA*, constructed using the pNZ1804 mutagenesis vector	This study
NZ1805	NZ1800 with 1-bp insertion in the catalytic site of *melA*, constructed using the pNZ1805 mutagenesis vector	This study
NZ1806	NZ1800 with 2-bp insertion in the catalytic site of *melA*, constructed using the pNZ1806 mutagenesis vector	This study
NZ1807	NZ1800 with G stretch and 1-bp insertion in *melA*, constructed using the pNZ1807 mutagenesis vector	This study
Plasmids		
pNZ5340	Cm^r Em^r; integration vector with homologous regions up- and downstream of *L. plantarum* WCFS1 *melA*	27
pNZ5516	Km^r; pCRblunt containing P*usp45-melA*	5
pNZ1825	Cm^r; pNZ5516 derivative containing P*usp45-melA-cat-lox71* downstream region of *melA*	This study
pNZ1826	Cm^r; pNZ1825 derivative containing upstream region of *melA*	This study
pNZ1800	Cm^r; pNZ1826 derivative, insertion of a *lox66* region; integration vector for inserting P*usp45* in front of *melA* based on *cre-lox* system	This study
pNZ5348	Em^r; pGID023 derivative containing *cre* under control of the lp_1144 promoter	27
pNZ1801	Cm^r; pNZ1800 derivative; substitution mutation in the catalytic site of *melA*	This study
pNZ1802	Cm^r; pNZ1800 derivative; substitution mutation in the catalytic site of *melA*	This study
pNZ1803	Cm^r; pNZ1800 derivative; 1-bp deletion in the catalytic site of *melA*	This study
pNZ1804	Cm^r; pNZ1800 derivative; 2-bp deletion in the catalytic site of *melA*	This study
pNZ1805	Cm^r; pNZ1800 derivative; 1-bp insertion in the catalytic site of *melA*	This study
pNZ1806	Cm^r; pNZ1800 derivative; 2-bp insertion in the catalytic site of *melA*	This study
pNZ1817	Cm^r; pNZ1800 derivative; G stretch and 1-bp insertion in *melA*	This study
pIL252	Em^r; low-copy-number vector	45
pNZ8048	Cm^r; high-copy-number vector with inducible P*nisA* from *L. lactis*	12
pNZ8154	Em^r; pIL252 derivative; mutator vector with P*nisA*-T*pepN* fragment of pNZ8048	This study
pCRblunt	Km^r; positive-selection cloning vector	6
pNZ1813	Em^r; pNZ8154 derivative; insertion of *L. plantarum* WCFS1 *hexA*	This study
pNZ1814	Em^r; pNZ8154 derivative; insertion of *L. plantarum* WCFS1 truncated *hexA*	This study
pNZ1820	Em^r; pNZ8154 derivative; insertion of *L. plantarum* WCFS1 *dinP*	This study

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant.

same direction as the replication genes in all of the transformants analyzed. The resulting vector, pNZ8154, was introduced into NZ1800, NZ7140, and the mutator reporter strains. For all experiments performed with one of these strains, the strain harboring plasmid pNZ8154 was used as a control.

Cloning of *hexA*, tr-*hexA*, and *dinP* in pNZ8154. The *L. plantarum* WCFS1 *hexA* gene (lp_2298) (25) was amplified with *Pwo* polymerase using chromosomal DNA as the template and primers LK20 and LK30. An N-terminally truncated derivative of *hexA*, designated tr-*hexA*, which lacks the sequence encoding amino acids 2 to 94 (279 bp) compared to the full-length *hexA*, was amplified with primers LK44 and LK30. The *L. plantarum* WCFS1 *dinP* gene (lp_2280) (25) was amplified with primers LK48 and LK51. The amplicons obtained were cloned directly in pCRblunt (Invitrogen, Breda, the Netherlands), and insert sequences were verified by sequence analyses. The *hexA* and *dinP* fragments were recovered from these plasmids as a NcoI-SpeI fragment and ligated into similarly digested pNZ8154. The resulting plasmids, pNZ1813 (*hexA*), pNZ1814 (tr-*hexA*), and pNZ1820 (*dinP*), were transformed into all seven mutator reporter strains.

Screening of MelA activity. α-Galactosidase can convert the substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) (MP Biomedicals, Amsterdam, the Netherlands), forming a blue color. The *E. coli* transformants and *L. plantarum* mutated strains were screened on agar plates with 40 μg/ml X-α-Gal for white (MelA$^-$) or blue (MelA$^+$) colonies. Alternatively, cells were plated on CDM agar plates with 1% melibiose and 0.004% BCP, and growth was scored by counting melibiose-fermenting clones that appeared as yellow colonies. The latter method was used for quantification of the mutation frequency.

Determination of the mutation frequency by using the mutator reporter system. The frequency of spontaneous revertants of the mutator reporter strains was determined by plating preparations on plates used for selection of MelA activity. The strains were inoculated into CDM with 1% maltose, and after 6 h of growth at 30°C 1% of each culture was inoculated into fresh medium. The resulting cultures were grown for 16 h at 30°C until the optical density at 600 nm (OD_{600}) was approximately 1.0. Based on the OD_{600}, 10^{10}, 10^9, and 10^8 CFU were washed three times with CDM without a carbon source and plated on selective CDM

TABLE 2. Mutations in the reporter strains

Strain	Sequence[a]	Mutation	Primers
NZ7100	ATTGATTATATC AAG TGG GAT ATGAAC	Wild type[b]	
NZ1801	ATTGATTATATC **T**AG TGG GAT ATGAAC	Substitution stop codon	1L, 1R
NZ1802	ATTGATTATATC AAG TGG **T**AT ATGAAC	Substitution Asp → Tyr	2L, 2R
NZ1803	ATTGATTATATC AAG GG GAT ATGAAC	1-bp deletion frameshift	3L, 3R
NZ1804	ATTGATTATATC AAG G GAT ATGAAC	2-bp deletion frameshift	4L, 4R
NZ1805	ATTGATTATATC **G**AAG TGG GAT ATGAAC	1-bp insertion frameshift	5L, 5R
NZ1806	ATTGATTATATC AAG TGG **CT**G ATATGAAC	2-bp insertion frameshift	6L, 6R
NZ7100	AAGTTACCA GGA GGC TTAGCGGATATTAG	Wild type[c]	
NZ1807	AAGTTACCA GG**G** GG**GG** TTAGCGGATATTAG	1-bp insertion frameshift	7L, 7R

[a] Underlining indicates the catalytic site of the *melA* gene; bold type indicates the mutation site.
[b] Positions 1438 to 1464 of the *melA* gene.
[c] Positions 1189 to 1216 of the *melA* gene.

agar plates containing 1% melibiose and 0.004% BCP, as described above for screening of MelA activity. The total viable counts of each culture were also determined by nonselective plating on CDM agar with 1% maltose. The plates were incubated anaerobically at 30°C for 5 days, after which the colonies were counted.

To determine the effect of (over)expression of a gene cloned in pNZ8154 under control of the nisin promoter, a strain containing a specific plasmid (pNZ1813 [*hexA*], pNZ1814 [tr-*hexA*], or pNZ1820 [*dinP*]) was inoculated into CDM with 1% maltose and grown overnight. The culture was diluted in fresh medium to obtain an OD_{600} of 0.1, and 40 ng/ml nisin (Sigma, Zwijndrecht, the Netherlands) was added to induce candidate mutator gene expression. The culture was incubated at 30°C until the OD_{600} was approximately 1.0 and then was inoculated (0.1%) into fresh medium containing 40 ng/ml nisin. After 16 h of growth the OD_{600} was measured, and then 10^9 CFU, as calculated based on the OD_{600} value, was washed three times with CDM without a carbon source and appropriate dilutions were plated on selective CDM agar plates with 1% melibiose and 0.004% BCP. Miller et al. (34) determined the mutation frequency of *E. coli* and observed that plating efficiency was strongly influenced by the number of cells that were actually plated; therefore, a scavenger strain had to be added when fewer than 10^9 cells from the actual culture were plated to prevent measurement artifacts due to so-called crowding effects (34). Our results confirmed the observations made by Miller et al. (34), and 10^8 CFU of the *L. plantarum melA* mutant strain NZ7140 was added routinely to each plate, which allowed accurate and reproducible quantification of MelA-positive revertants without interference by crowding effects. The total viable count of each culture was also determined by nonselective plating on CDM agar with 1% maltose. The plates were incubated anaerobically at 30°C for 5 days, after which the colonies were counted.

Determination of the mutational spectrum of tr-*hexA*. Strains NZ1807 (pNZ1814) (tr-*hexA*) and NZ1807(pNZ8154) (empty vector; spontaneous mutant) were grown overnight in CDM with 1% maltose. The cultures were diluted until the OD_{600} was 0.1, and 40 ng/ml nisin was added. The cultures were incubated at 30°C until the OD_{600} was approximately 1.0 and then were diluted (0.1%) in fresh medium with 40 ng/ml nisin. After 16 h of growth, the OD_{600} was measured, and, based on the OD_{600}, 10^9 CFU was washed three times with CDM without a carbon source and appropriate dilutions were plated on CDM plates with 1% maltose and 50 ng/ml rifampin (Duchefa Biochemie, Haarlem, the Netherlands). The total viable count for each culture was also determined by nonselective plating on CDM agar with 1% maltose. The plates were incubated anaerobically at 30°C for 5 days, after which the colonies were counted. Chromosomal DNA was isolated from individual rifampin-resistant colonies obtained either after induced expression of tr-*hexA* [NZ1807(pNZ1814); 35 colonies] or spontaneously [NZ1807(pNZ8154); 31 colonies].

Garibyan et al. (17) determined the region in the *E. coli rpoB* gene where the main mutations that confer resistance to the antibiotic rifampin occur. The location of this region in the *L. plantarum rpoB* gene was determined by alignment of the sequences of the *E. coli rpoB* gene (b3987; accession no. NC_000913; positions 4179268 to 4183296) and the *L. plantarum* WCFS1 *rpoB* gene (lp_1021; accession no. NC_004567; positions 939961 to 943575). The sequences were aligned using the CLUSTAL program.

The analogous region of the *rpoB* gene (lp_1021; bp 1200 to 1800 of the coding sequence) was amplified with *Pwo* polymerase using the chromosomal DNA of the selected colonies as a template and primers LK54 and LK59 and was subjected to sequence analysis using primer LK54 as a sequencing primer.

Chemical mutagenesis with NTG. To compare the mutation frequency of a mutator gene with the mutagenic capacity of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), a pregrown culture of NZ1807(pNZ8154) with an OD_{600} of 1.0 was centrifuged, and the cells were resuspended in an equal volume of CDM without a carbon source. Then 300 μg/ml NTG (Sigma, Zwijndrecht, the Netherlands) was added, and the culture was incubated for 1 h at 30°C. Subsequently, the cells were washed three times with CDM without a carbon source, and appropriate dilutions were plated on selective plates. The total viable count for each culture was also determined by nonselective plating on CDM agar with 1% maltose. The plates were incubated anaerobically at 30°C for 5 days, after which the colonies were counted.

Effects of environmental conditions on the mutation frequency. To determine whether environmental conditions modulate the mutation frequency of *L. plantarum* NZ1807(pNZ8154), the number of MelA-positive revertant colonies was determined as described above following exposure of the strain to several stress conditions. For all conditions the cultures were washed three times with CDM without a carbon source, and appropriate dilutions were plated after addition of 10^8 CFU of the *melA* mutant strain NZ7140 on selective CDM agar plates with 1% melibiose and 0.004% BCP. The total viable count for each culture was also determined by nonselective plating on CDM agar with 1% maltose. The plates were incubated anaerobically at 30°C for 5 days, after which the colonies were counted.

(i) Temperature. A pregrown culture (OD_{600}, 1.0) was inoculated (1%) into CDM with 1% maltose and incubated at 25°C, 30°C, 37°C and 42°C. After 16 h of growth the cultures were washed and plated.

(ii) High salt concentrations. A pregrown culture was inoculated (1%) into CDM with and without 0.8 M NaCl and incubated at 30°C. After 24 h of growth the cultures were washed and plated.

(iii) Starvation. A pregrown culture was inoculated (1%) into CDM with 0.2% maltose. After 4 days of incubation at 30°C, the culture was washed and plated.

(iv) Stationary phase. A pregrown culture was inoculated (1%) into CDM with 1% maltose. After 4 days of incubation at 30°C, the culture was washed and plated.

(v) Low pH. A pregrown culture was inoculated (1%) into CDM (pH 3.9) and into CDM (pH 5.6) and incubated at 30°C. After 24 h of growth the cultures were washed and plated.

(vi) Hydrogen peroxide. A pregrown culture in CDM with 1% maltose (OD_{600}, 1.0) was incubated with hydrogen peroxide (H_2O_2) at concentrations ranging from 0 to 6 mM for 30 min at 30°C. Subsequently, the cells were washed and plated.

(vii) Cross-protection. A pregrown culture was inoculated (2%) into CDM with 1% maltose and incubated at 42°C. After 16 h of growth at 42°C, the OD_{600} of the culture was approximately 1.0, and culture was then incubated with H_2O_2 at concentrations ranging from 0 to 6 mM for 30 min at 30°C. Subsequently, the cells were washed and plated.

RESULTS

Mutator reporter system. A set of *L. plantarum* mutator reporter strains suitable for qualitative and quantitative analysis of low-frequency mutation events was developed. In these strains the unique chromosomal α-galactosidase-encoding

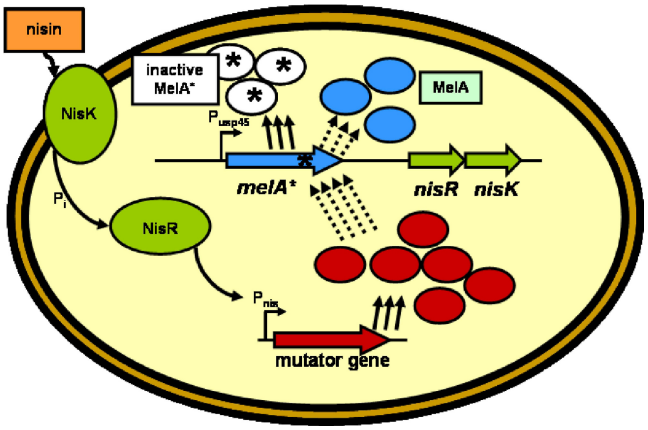

FIG. 1. Mutator reporter system. Mutated *melA*, *nisR*, and *nisK* genes are present in the genome, and the possible mutator gene (or chromosomal fragment) is present in the plasmid. Controlled expression of the mutator gene may enhance recovery of the MelA function by increasing the frequency of random mutations.

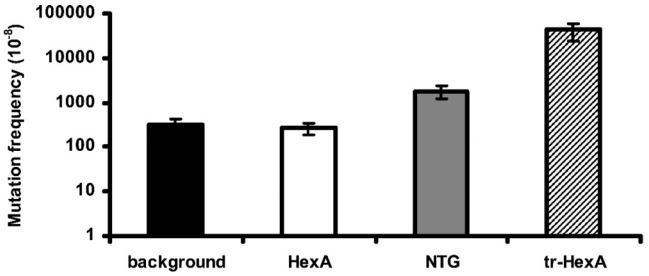

FIG. 2. Mutation frequency. *L. plantarum* strain NZ1807 was used as a G-stretch reporter strain. The mutation frequency is the number of revertants per 10^8 CFU. The background mutation frequency is the mutation frequency at 30°C with the empty vector pNZ8154. The effect of incubation with 300 μg/ml NTG for 1 h at 30°C on the mutation frequency was also determined with the empty vector pNZ8154. Plasmids pNZ1813 and pNZ1814 were used to determine the effects of HexA and tr-HexA, respectively, on the mutation frequency.

gene, *melA*, was replaced by a mutated gene under the control of the strong constitutive *L. lactis usp45* promoter. A versatile set of reporter constructs was obtained in which the *melA* gene is not functional due to a disruptive mutation (see Materials and Methods and Tables 1 and 2) and can be reactivated by a single mutation event, either a base substitution or single-base frameshift. The first six mutants were created by mutation of the region of the *melA* gene that encodes the catalytic site of the cognate enzyme MelA. The location of the mutation in the seventh mutant was chosen because of the possibility of creating a stretch of G residues at this locus without changing the amino acid sequence of the encoded (wild-type) protein (Table 2). In each of the mutants a specific 1-bp mutation can result in a *melA* gene that encodes a functional MelA enzyme, and thus this versatile system should allow growth-based selection of such revertants on the basis of their capacity to grow on media that contain melibiose as a sole carbon source, while nonselective analysis of revertants is possible by identification of blue colonies on nonselective media containing the chromogenic MelA substrate X-α-Gal.

Moreover, a low-copy-number vector carrying the pAMβ1 replicon (45) was constructed, in which the *nisA* promoter was introduced, allowing controlled overexpression of target genes (12). This vector was used for analysis of mutation frequencies as a result of overexpression of potential mutator genes (Fig. 1).

Development of a conditional mutator strain. Yang et al. (51) identified 15 genes in *E. coli* which result in an elevated mutation frequency when they are overexpressed (mutator phenotype). Among these genes were a partially deleted *mutS* gene that generates an N-terminal truncated form of the methyl-directed mismatch repair protein MutS (approximately 940-fold increase in Lac^+ revertant frequency and 5-fold increase in rifampin resistance) and the *dinB* gene encoding error-prone DNA polymerase IV (DinB; approximately 35-fold increase in Lac^+ revertant frequency and 6-fold increase in rifampin resistance) (51). Homologs of the *E. coli mutS* and *dinB* genes were identified in the *L. plantarum* WCFS1 genome. The *dinP* gene (lp_2280) of *L. plantarum* WCFS1 was identified as a homolog of the *E. coli dinB* gene, and the lp_2298 gene of *L. plantarum* WCFS1 (referred to here as *hexA*) was identified as a homolog of the *E. coli mutS* gene.

Based on an alignment of the *E. coli mutS* gene and the *L. plantarum hexA* gene, a truncated form of *hexA* (tr-*hexA*) was constructed. The N-terminal part of *hexA* encoding the sequence from amino acid 2 to amino acid 94 was deleted. Next, the *dinP*, *hexA*, and tr-*hexA* genes were overexpressed in the seven mutation reporter strains by means of the NICE system (33), and mutation frequencies were determined. Incubation with the known chemical mutagen NTG was included for comparison (Fig. 2). Before the effects of mutator genes and NTG on the mutation frequency were determined, the background revertant levels were measured. The background revertant level of *L. plantarum* reporter strain NZ1807 (G-stretch reporter strain) was 1 revertant per 10^6 cells. The other *L. plantarum* reporter strains could not be used to determine the mutator frequency efficiently, because the background revertant levels were below the detection limit, which was 1 revertant per 10^{11} cells. Therefore, the G-stretch reporter strain was used in all subsequent experiments.

DinP and HexA did not have an effect on the mutation frequency compared to the background mutation frequency. Incubation with NTG resulted in an approximately 5-fold increase in the mutation frequency, and overexpression of the truncated *hexA* gene resulted in a >100-fold increase (Fig. 2) (data for DinP are not shown).

Experiments with overexpression of a *hexA* gene in which a larger fragment (encoding 282 amino acids) was deleted, also generating an N-terminally truncated form of HexA, did not result in an increased mutation frequency like that observed with tr-HexA (data not shown). Although overexpression by means of the NICE system is known to provide reliable and stable levels of expression in several Gram-positive bacteria, the possibility that differences in the expression levels between the genes examined could have occurred cannot be ruled out completely (32, 33).

Mutation spectrum of tr-HexA. The truncated form of HexA resulted in a >100-fold increase in the mutation frequency, as determined with the G-stretch reporter strain. The mutated *melA* gene in this mutation reporter strain can be reactivated only by a single-base frameshift mutation. To determine if tr-HexA is also

TABLE 3. Distribution of mutations in *L. plantarum rpoB* gene

Site (bp)	Amino acid change	Base pair change	No. of colonies with mutation	
			Wild type (spontaneous)	tr-HexA
1414–1422		9-bp deletion	3	1
1417–1425		9-bp deletion	8	0
1430	Q477R	A · T → G · C	0	13
1438	D480Y	G · C → T · A	12	0
1468	H490Y	G · C → A · T	3	6
1469	H490R	A · T → G · C	0	1
1478	R493H	G · C → A · T	3	5
1484	S495Y	G · C → A · T	2	9
Total			31	35

capable of inducing base substitution mutations, rifampin resistance experiments were conducted (16, 34).

Garibyan et al. (17) determined the region in the *E. coli rpoB* gene where the main set of mutations that result in resistance to the antibiotic rifampin occur. In their *E. coli* study all sequenced mutations were base pair substitutions. The location of this region in the *L. plantarum rpoB* gene was determined by alignment of the sequences of the *E. coli rpoB* gene and the *L. plantarum* WCFS1 *rpoB* gene. This region of the *rpoB* gene (bp 1200 to bp 1800 of the coding region) was sequenced for 35 individual rifampin-resistant colonies, which were obtained after overexpression of the tr-HexA strain NZ1807(pNZ1814), and 31 spontaneous rifampin-resistant colonies. Subsequently, the sequences were analyzed to ascertain the mutations responsible for the resistance to rifampin. Table 3 shows the mutations, their positions, the amino acid changes, and the distribution of the mutations.

Overexpression of tr-HexA resulted in 10-fold more rifampin-resistant mutants. Deletions and base substitutions were observed for both the spontaneous and tr-HexA expression-induced mutants, although the distributions of the mutations were different. Most strikingly, the most abundant mutation observed in the revertants obtained after tr-*hexA* induction was not observed at all in the spontaneous mutant group and *vice versa* (Table 3). The rifampin resistance experiments suggest that tr-HexA has a clear preference for G · C → A · T and A · T → G · C transitions, while it does not stimulate G · C → T · A transitions, which appeared to occur only in the spontaneous mutant collection.

Effect of environmental conditions on the mutation frequency. Next, the effect of environmental stress conditions on mutation frequencies was assessed. For this analysis the G-stretch reporter strain was exposed to several stress conditions that were chosen on the basis of their relevance for fermentation processes. Subsequently, the MelA-positive revertant frequency was determined.

Growth at temperatures ranging from 25°C to 42°C (the optimum growth temperature for *L. plantarum* is 30°C, and no growth was observed at 45°C), under low-pH conditions, with high salt concentrations (NaCl), or under starvation conditions did not significantly alter the mutation frequency. Nor did stationary-phase incubation (72 h) have an effect on the mutation frequency. However, incubation with high levels (>4 mM) of hydrogen peroxide did result in a significant increase in the mutation frequency (Fig. 3A). Incubation for 30 min in a buffer containing 6 mM H_2O_2 resulted in a 100-fold increase in the mutation frequency, which was comparable to the effect of overexpression of tr-*hexA*. However, this increase coincided with a nearly 1,000-fold decrease in viability (Fig. 3B). These results indicate that the number of revertants per survivor and therefore the actual mutation frequency are increased dramatically by peroxide treatment, whereas the total number of revertants that could be recovered decreased substantially.

Cross-protection. De Angelis et al. (11) have shown that the viability of heat-adapted *L. plantarum* cells improves when they are exposed to stress conditions (e.g., heat shock). To determine if heat-adapted cells exhibit a similar viability response when they are exposed to hydrogen peroxide and to determine the effect of hydrogen peroxide on the mutation frequency, the *L. plantarum* G-stretch reporter strain was grown at 42°C and subsequently exposed to high levels of hydrogen peroxide.

When *L. plantarum* was adapted to 42°C, there was an approximately 100-fold-smaller decrease in viability upon exposure to hydrogen peroxide at a concentration of 5 mM (Fig. 3B). The effect of adaptation to 42°C on the mutation frequency was also dramatic. Incubation of the heat-adapted cells with 5 mM H_2O_2 resulted in a mutation frequency similar to the original background mutation frequency (at 30°C with no H_2O_2). This corresponds to a 50-fold decrease in the mutation frequency compared to the mutation frequency of non-heat-

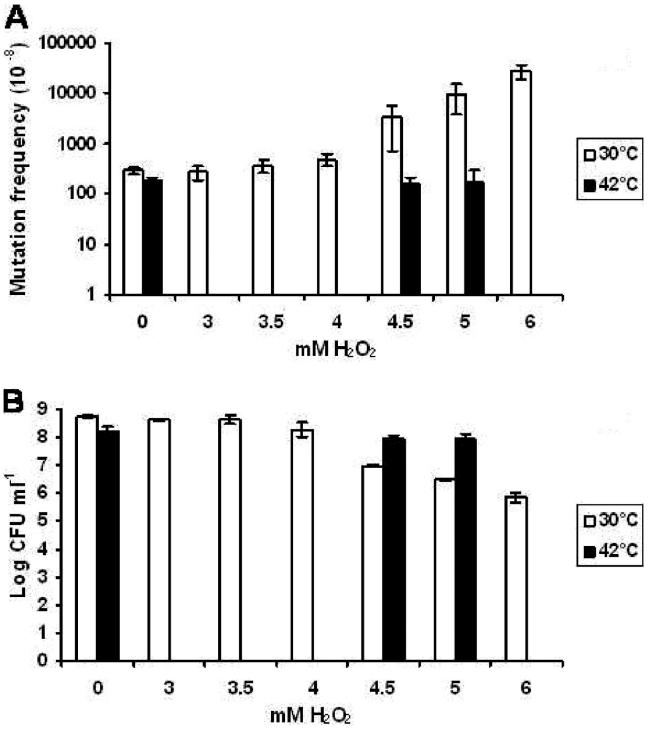

FIG. 3. Effect of hydrogen peroxide and cross-protection. *L. plantarum* NZ1807(pNZ8154) grown at either 30°C or 42°C was incubated with hydrogen peroxide (0 to 6 mM, 30 min at 30°C), after which the mutation frequency (A) and viability (B) were determined. The positive effects of adaptation to 42°C (cross-protection) on the mutation frequency (A) and viability (B) when cells were exposed to high levels of hydrogen peroxide are shown.

adapted cells exposed to 5 mM H_2O_2 (Fig. 3A). These results indicate that so-called cross-protection (11) not only is reflected by survival rates but also extends to the capacity of cells to prevent or counteract DNA damage, including increases in the mutation frequency due to environmental conditions.

DISCUSSION

A mutator reporter system for *L. plantarum* that can be used for accurate quantitative analysis of the mutation frequency was developed and used to assess the frequencies of spontaneous mutations, as well as the frequencies of mutations that are increased by genetic or environmental intervention. Unfortunately, only the G-stretch reporter strain could be used in this study. The other *L. plantarum* reporter strains, which would have enabled qualitative analysis of the mutations, could not be used to determine the mutator frequency efficiently, because the background revertant levels were below the detection limit, which was 1 revertant per 10^{11} cells. Nevertheless, these reporter strains could potentially be used in work on alternative mutator genes.

The *L. plantarum hexA* gene displays sequence homology with the *mutS* gene of *E. coli*, a truncated form of which was shown to increase the mutation frequency in this host (51). Conditional expression of the analogous truncated form of the *hexA* gene product in *L. plantarum* supported the hypothesis that this protein has a similar mutator role in this Gram-positive host. The increase in the mutation frequency due to tr-HexA was 20-fold greater than the increase resulting from incubation with the known chemical mutagen NTG. NTG induces a relatively wide spectrum of mutations by alkylating purines and pyrimidines. Most of the mutations induced by NTG are G · C → A · T transitions, but deletions and frameshifts occur to a lesser extent (24, 38). The mutation frequencies were determined with the G-stretch reporter strain, and the mutated *melA* gene in this reporter strain can be reactivated only by a single-base frameshift mutation. Therefore, the preference of NTG for G · C → A · T transitions could explain the lower mutation frequency compared to the frequency obtained with tr-HexA expression. However, the rifampin resistance experiments indicated that tr-HexA also has a preference for G · C → A · T and A · T → G · C transitions, which differs from the findings for its homolog in *E. coli*. Yang et al. determined that the truncated MutS protein of *E. coli* was a strong mutator with respect to frameshifts and a moderate mutator for base substitutions. It was also determined that the truncated MutS of *E. coli* increased the mutation frequency approximately 940-fold (Lac^+ revertant reporter system), which is almost 10 times greater than the effect of tr-HexA. However, tr-HexA increased the number of rifampin-resistant mutants 10-fold and increased the number of truncated MutS mutants of *E. coli* only 5-fold (51).

The methyl-directed mismatch repair protein MutS is involved in recognition and repair of mismatched bases and small insertion or deletions and in this way limits spontaneous mutation. In *E. coli*, repair is initiated when the dimeric MutS protein recognizes and binds to mismatched base pairs or an insertion or deletion consisting of up to four nucleotides. Binding of MutS to a mismatch is followed by ATP-dependent binding of a homodimer of the ATPase MutL. Binding of the endonuclease MutH to the MutS-MutL complex triggers ATP binding by MutL, which enhances the endonuclease activity of MutH. The strand specificity of mismatch repair resides in MutH, which recognizes a hemimethylated GATC sequence and cleaves only the unmethylated daughter strand. The GATC site can be more than1,000 bp from and on either side of the mismatch. Both MutS and MutL are also essential in the subsequent excision and resynthesis of the daughter strand (2, 28, 37, 50). The N-terminal truncated form of the mismatch repair protein MutS lacks the intact mismatch recognition domain (28, 50). Therefore, overexpression of the truncated form of MutS could exert negative dominance over MutS, perhaps by competing with wild-type MutS for association with MutL (51). In the N-terminally truncated form of the *L. plantarum hexA* gene, the homolog of the *E. coli mutS* gene, the mismatch recognition domain was also removed, suggesting that this gene functions as a mutator gene via a mechanism similar to that of MutS.

Overexpression of the tr-*hexA* gene in the reporter strains is under control of the nisin-controlled gene expression system. Hence, the expression of the tr-*hexA* mutator gene and, as a result, also the mutator frequency can be regulated by addition of nisin (Fig. 1) (33). Consequently, these strains exhibit a conditional mutator phenotype and could be used as conditional mutators in future studies, e.g., for rapid strain improvement based on *in vivo* mutagenesis combined with high-throughput function-screening assays (43).

Stress-induced mutations can participate in adaptive evolution for survival under stress conditions encountered in industrial processes, and accumulation of such mutations can contribute to instability of desired functional properties of the microbial strains used. Therefore, the effect of conditions encountered during industrial fermentation processes on the frequencies of mutation was examined using the G-stretch mutation reporter strain. While growth at different temperatures, at low pH, with high salt concentrations, or under starvation conditions did not affect the mutation frequency significantly, incubation of cells with high levels of hydrogen peroxide led to a 100-fold increase in the mutation frequency. This increase in the mutation frequency can be attributed to the direct mutagenic effect of hydrogen peroxide, which belongs to a group of chemicals known as reactive oxygen species. Hydrogen peroxide can react with metal ions like Fe^{2+}, yielding hydroxyl radicals that can damage DNA, proteins, and membranes (15). *L. plantarum* has been shown to produce hydrogen peroxide as a by-product during fermentation, and the concentration depends on the level of aeration during fermentation but can be up to 9 mM (4, 36, 40, 52). Many other lactobacilli that could be present with *L. plantarum* in an industrial process, such as *Lactobacillus bulgaricus* subsp. *delbrueckii*, are also known to produce hydrogen peroxide (30, 49, 52). Moreover, oxidative stress could also be encountered during processing due to the processing conditions used. Notably, the hydrogen peroxide effect on the mutation frequency in *L. plantarum* could be counteracted almost completely by adaptation of the cells to 42°C prior to exposure to hydrogen peroxide. Previously, heat adaptation has been shown to provide cross-protection in *L. plantarum* cells against subsequent stress challenges, and the effects include increased viability upon exposure to extreme heat shock (72°C for 90 s) and improved growth at pH 5 and in

the presence of high salt concentrations (up to 6% NaCl) (11). Our findings expand the cross-protective effects of heat adaptation to protection against the specific mutagenic challenge resulting from exposure to hydrogen peroxide.

It is well established that bacterial stress responses rely on coordinated expression of genes that alter different cellular processes, like cell division, DNA metabolism, housekeeping, membrane composition, and transport (46). Integration of these stress responses is accomplished by networks of regulators which allow the cell to react to various and complex environmental shifts (46). Since stress defenses are coordinated by such integrated regulation systems, the general stress response to the mild heat shock when organisms are grown at 42°C results in cells that are protected better against high levels of hydrogen peroxide.

These results indicate that only a few stress conditions that may be encountered by *L. plantarum* during industrial fermentation processes appear to have a strong mutation frequency-enhancing effect (only one of the stress conditions tested had an effect). Nevertheless, specific conditions (e.g., exposure to oxidative stress conditions) may still negatively influence strain stability by increasing the random mutation frequency. However, cultures may (potentially) be protected against such detrimental consequences by specific pretreatments that harness the protective capacity of the general stress response networks to prevent or counteract the consequences of environmentally induced increased mutation frequencies. Thus, such pretreatments can be employed in industrial production to enhance the robustness of a strain, both through increasing its survival capacity under harmful conditions (classical cross-protection) and by increasing its genetic stability.

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