Characterization of *Lactococcus lactis* UV-Sensitive Mutants Obtained by ISS1 Transposition

PATRICK DUWAT,* ARMELLE COCHU, S. DUSKO EHRLICH, AND ALEXANDRA GRUSS

Génétique Microbienne, Institut National de la Recherche Agronomique, 78352 Jouy en Josas Cedex, France

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Studies of cellular responses to DNA-damaging agents, mostly in *Escherichia coli*, have revealed numerous genes and pathways involved in DNA repair. However, other species, particularly those which exist under different environmental conditions than does *E. coli*, may have rather different responses. Here, we identify and characterize genes involved in DNA repair in a gram-positive plant and dairy bacterium, *Lactococcus lactis*. Lactococcal strain MG1363 was mutagenized with transposition vector pGh* 

The results indicate that UV resistance of the lactococcal strain can be attributed in part to DNA repair but also suggest that other factors, such as cell envelope composition, may be important in mediating resistance to mutagenic stress.

Exposure of bacteria to DNA-damaging agents such as UV light and oxygen or drugs such as mitomycin (MC) may have deleterious effects on the bacterial cell, particularly on chromosomal structure and replication. In response, bacteria have evolved a diverse array of enzymatic pathways which are specialized in the removal of damaged DNA. Since the current model for prokaryotic DNA repair is based on DNA metabolism of enteric bacteria (principally *Escherichia coli*), further studies of nonenteric microbes will be useful in determining the extent to which repair mechanisms are conserved.

Although numerous DNA repair processes identified in *E. coli* are common to other bacteria, differences have also been noted. Photoreactivation systems are lacking in many bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Deinococcus radiodurans*, and *Neisseria gonorrhoeae* (11, 27). In some bacteria, including enteric bacteria closely related to *E. coli*, an inducible mutagenesis DNA repair response may be lacking (22, 58, 59). The species *D. radiodurans* presents a remarkably high UV resistance, which is attributed to the presence of two UvrABC-like excision repair systems (48). In contrast, no system similar to excision repair has been identified in *Pasturella haemolytica* (42). Methyl-directed mismatch repair (49) and very-short-patch repair systems may also be different among organisms, since neither adenine nor cytosine methylation is universal (25). These few examples illustrate the considerable variations that exist among DNA repair systems of different bacterial species.

Little is known about DNA repair in *Lactococcus lactis*, a gram-positive plant and dairy microorganism. It was reported that unlike *E. coli*, *L. lactis* has no inducible error-free repair system for alkylation damage (2). Only three functionally conserved genes directly involved in DNA repair have been characterized in *L. lactis*: (i) the fpg gene codes for an N-glycosylase, which releases the 8-oxoguanine from oxidatively damaged DNA (19); (ii) the recAB genes code for the major exonuclease of the cell, which is the functional equivalent of the *E. coli* RecBCD or *B. subtilis* AddAB enzymes (20); and (iii) the recA gene encodes a highly conserved protein, which is central to DNA repair pathways (17). RecA is a recombinase (for a review, see reference 54) and, as shown for *E. coli* and many other organisms, a regulator of a set of genes which are principally implicated in DNA repair (for a review, see reference 65). The recA gene of *L. lactis* encodes a protein that is 56% identical to the *E. coli* RecA protein (16). As expected, the lactococcal recA gene is necessary for DNA recombination, repair after mutagenic stress (17), and induction of lysogenic phage (7). It has also been implicated in the function of an abortive phage infection mechanism (21, 29). We recently found that in *L. lactis*, recA is also needed for a full response to oxidative and thermal stress conditions (18). Implication of the recA gene in heat shock response has not been reported for other microorganisms and may be an example of species particularity of regulation.

To further characterize the role of recA and to identify other genes involved in DNA repair in *L. lactis*, we isolated and characterized DNA damage-sensitive insertion mutants; the affected genes are called urs (for UV sensitive). Seven strains mutated in identified genes (*polA, hexB, deoB, dldD, gerC, and gidA*) or in a non-identified gene (urs-75) were characterized and are described here.

**MATERIALS AND METHODS**

**Bacteria, cell culture, and plasmids.** *E. coli* TG1 (57) [supE hsdS thi Δ(lac proAB) F’ (traD6 proAB ΔlacF lacZAM15)] and TG1 (TG1 containing a chromosomal copy of the pWV01 repA gene [kindly provided by K. Lennhouts]) and *L. lactis* MG1363 (a derivative of strain ML3 cured of plasmids and prophages [23]) VEL1122 (a recA derivative of MG1363 containing a tetracycline resistance [Tc] marker [18]), and CLS65-5 (erythromycin resistance [Em] marker chromosomally integrated in MG1363 [41]) were used in this study. *E. coli* cultures were grown on Luria-Bertani medium (47) at 30 or 37°C as needed, and erythromycin (150 μg/ml) was added as required. *L. lactis* cultures were grown at 30 or 37°C, as specified, in M17glu (M17 medium [62] to which 1% [wt/vol] glucose was added after the medium was autoclaved). Erythromycin (3 μg/ml), streptomycin (150 μg/ml), rifampin (50 μg/ml), chloramphenicol (5 μg/ml), and tetracycline (5 μg/ml) were added as needed for *L. lactis*. Adenine, cytosine, guanine, and thymine were added to the growth media to give a final concentration of 300 μg/ml.
guanine, and thymine (15 μg/ml per base) were added, when indicated, for growth of the wild-type (wt) and deoB strains in liquid or solid M17gulu medium. The plasmids used were (i) theta replicating plasmid pIL253 (Em’60) and pIL205 (Cmr’40); (ii) thermosensitive (ts) plasmids pG’h’host5 (Em’44), and pG’h’host2 (Em’45), referred to as pG’h’Rif’RifN’ (Em’); pG’h’host5 containing a 1.7-kb EcoRI fragment of the IL1403 histidine operon and pHist-MG (Em’; pG’h’host5 containing the corresponding PCR-amplified 1.7-kb fragment of the M1363 histidine operon cloned into the EcoRI site). DNA manipulations. Cloning, plasmid and chromosomal DNA extraction, restriction analyses, DNA transformation and Southern hybridizations were performed by published methods (16, 31, 39, 53, 57).

Synthetic oligonucleotides and PCR. Single-stranded DNA oligonucleotide primers complementary to DNA sequences in the histidine operon of IL1403 were synthesized in our laboratory on a Beckman synthesizer. The forward primer was 5’TATGGAATTTTGAATTCATGAC-3’ (corresponding to published DNA sequence positions 3972 through 4000), and the reverse primer was 5’TGATGAGGATTATTTTAGAATTCTTAGAC-3’ (corresponding to published DNA sequence positions 5721 through 5697). These primers were used to PCR amplify (56) a fragment of the histidine operon of the M1363 chromosome. Reactions (15 s at 94°C, 15 s at 55°C, and 30 s at 72°C for 30 cycles) were performed on a Perkin-Elmer Cetus 2400 apparatus with the Boehringer Tag DNA polymerase as recommended by the manufacturer.

Insertional mutagenesis and isolation of MC- and UV-sensitive mutants. Mutagenesis with pG’h’S was performed as previously described (45). The clones selected on M17gulu plates containing 100 μg/ml of Hph were streaked on M17gulu plates containing 500 μg/ml of Rif. After 2 days of growth, the clones scored as MC sensitive were grown in liquid medium and then streaked on solid medium to test for UV sensitivity.

Characterization of MC- and UV-sensitive mutants. Recovery of chromosomal targets of transposon insertion in the MC- and UV-sensitive clones was performed as described previously (45). Transposon insertion results in integration of the entire plasmid, flanked by duplicate copies of IS41 (41). The IS41 flanking sequences were recovered on replicative plasmids by digesting total chromosomal DNA with unique restriction enzymes (HindIII and EcoRI) and transforming ligated DNA in the TG1 or TG1rep. The 38 possible ISS1-flanking sequences, 33 were cloned in this way on the excised pG’h’S vector. Fivejunctions could not be cloned, possibly due to their toxic effects or to DNA instability in E. coli. For each clone, a DNA sequence of 300 to 500 bp of chromosomal proximal DNA (adjacent to ISS1) was used to perform DNA and protein homology comparisons in databases by using the BLAST programs. Three analyses allowed us to identify 11 of the target genes, although for 3 of them, homology to known genes was revealed with only one of the two ISS1-flanking sequences (Table 1). The other sequences showed no significant homology to sequences present in databases. Mutants with mutations in the putative genes polA, hexB, deoB, dldD, gerC, gidA, and uvs-75, were further analyzed. These mutants were selected by excision of the transposon vector (45) (see Materials and Methods); the excised strains were used for all further experiments.

UV sensitivity tests. UV survival curves were generated to estimate the relative sensitivity of mutant strains (Fig. 1). The mutants showed varied responses to UV irradiation. None of them was as sensitive as the VEL1122 recA strain. Three of the mutants, with mutations in polA, hexB, and deoB, have increased sensitivity at all tested UV doses compared to the wt strain (Fig. 1A); the other four, with mutations in dldD, gerC, gidA, and uvs-75 (Fig. 1B), were UV sensitive only at high irradiation doses (50 J/m²).

As the UV test was performed on rich media, it was possible that toxic compounds were generated during irradiation. To verify that the death of the gerC, dldD, and uvs-75 mutants was really due to UV, irradiation was performed in nonreactive medium (Ringer’s solution). The results (not shown) were essentially identical to those observed when the mutants were irradiated on the plates. These results indicate that the mutants sensitive to both low- and high-dose UV irradiation may play a direct role in DNA metabolism while those sensitive to only high-dose irradiation may reflect the permeability of the cell to UV light.

Homologous recombination test. The UV sensitivity of the strains may be due to an incapacity of the mutants to promote recombination. Homologous recombination was therefore examined by measuring the frequencies of plasmid integration into the chromosome; the plasmid used, pHist-MG, is st and
contains a 1.7-kb chromosomal DNA fragment of the MG1363 histidine operon. We observed that efficiency of pHist-MG integration by homologous recombination was reduced 10- to 300-fold in the L. lactis gidA, polA and uvs-75 mutants compared to the wt strain (Table 2). It is notable that the gidA and uvs-75 mutant strains are only partially UV sensitive yet are significantly reduced in their capacity to promote homologous recombination.

**Detailed mutant analyses.** Sequence similarities between the interrupted genes and previously described genes (Table 1) allowed us to assign putative functions and/or gene names to the mutated genes under study. Six of these mutants plus one with unknown function were further analyzed to determine their roles in DNA repair in L. lactis.

(i) **polA**. The PolI bacterial DNA polymerases are multifunctional enzymes which generally have polymerase and exonuclease activities. A potential transcription initiation site (TTTTT-GA-17N-TAAAAA) is found 60 bp upstream of the ATG start of the lactococcal polA gene. This potential promoter is surrounded by three different sets of direct repeats, which may suggest that the promoter undergoes transcriptional regulation. Sequence analysis shows that a potential open reading frame (ORF) is present 220 bp upstream of and diverging from polA. In E. coli, the PolI protein is composed of two different functional domains (10, 35). The C-terminal domain, known as the Klenow fragment, has polymerase activity, as well as a 3’-to-5’ exonuclease activity. This latter activity is not present in the S. pneumoniae PolI protein (14), and similarities between L. lactis PolI and its pneumococcal homolog (43) make it likely that the 3’-to-5’ exonuclease activity is also absent from the lactococcal protein. The 500-amino-acid N-terminal domain endows the enzyme with 5’-to-3’ exonuclease activity. As this activity has been shown in E. coli to be necessary for growth in rich medium (34), and as ISS1 is inserted in the lactococcal polA gene at nucleotide 1755 of the coding sequence (equivalent to protein sequence position 558), we think it likely that the truncated gene produces a protein fragment which retains this 5’-to-3’ exonuclease activity.

(ii) **hexB**. The general mismatch repair system, called Hex in S. pneumoniae (12) and Mut in E. coli (49), acts on recombination intermediates as a DNA replication editor, correcting potentially mutagenic mismatches. Hex and Mut eliminate DNA heteroduplexes containing mismatches during homologous recombination in conjugation or transformation. In S. pneumoniae or E. coli, inactivation of the Hex or Mut system also confers a mutator phenotype. Isolation of the hexB mutant of L. lactis indicates that the polA mutant is at least affected in its polymerase activity. Given the properties of the L. lactis polA mutant described above, we believe that PolI, as in other microorganisms, is a multifunctional enzyme involved in DNA repair processes such as excision repair, in chromosomal replication for the processing of Okazaki fragments, and in homologous recombination.

It was shown that the PolI enzyme in B. subtilis is necessary for initiation of replication of the replicating plasmid pAMβ1 (9). To further study the lactococcal polA mutant phenotype, we tested whether it affects pAMβ1 replication in L. lactis. The transformation efficiency of the pAMβ1 derivative pII253 was compared with that of the rolling-circle plasmid pG+ host5 in a wt strain and in the polA mutant. We observed comparable transformation efficiencies for the two types of plasmids in the wt strain. However, no transformants were obtained with 1 μg of pII253 in the polA strain despite efficient transformation of the pG+ host5 (5 × 10^4 transformants per μg). These results confirm that polA is necessary for pAMβ1 replication in L. lactis and indicate that the polA mutant is at least affected in its polymerase activity. Given the properties of the L. lactis polA mutant described above, we believe that PolI, as in other microorganisms, is a multifunctional enzyme involved in DNA repair processes such as excision repair, in chromosomal replication for the processing of Okazaki fragments, and in homologous recombination.

(iii) **mutL**. Inactivation of the Mut system also confers a mutator phenotype. Isolation of the mutL mutant of L. lactis shows increased homologous recombination and spontaneous mutation rates, as is observed for the analog mutants in E. coli and...
S. pneumoniae. A 1.7-kb fragment of the histidine operon of L. lactis subsp. lactis IL1403 is 95% identical to the corresponding DNA fragment of MG1363 (data not shown), used above in the plasmid homologous recombination test. Plasmid pHist-IL, containing this DNA fragment, was used to evaluate the effect of mismatches on plasmid integration frequencies in the wt and hexB L. lactis subsp. cremoris strains. Integration of the pHist-MG plasmid occurred at the same frequencies as in the wt strain, indicating that homologous recombination was not affected in the hexB strain (Table 3). However, the frequency of integration in the hexB strain is about 10 times greater than in the wt strain, indicating that the Hex system in L. lactis also aborts recombination events between DNA molecules with mismatches. The partial effect of the hexB mutation on homologous recombination may be allele dependent, because ISS1 insertion occurred within the last third of the protein, and may thus result in a truncated peptide with partial activity, as the remaining N-terminal region of HexB presents the highest homology to the MutL protein of E. coli. The intermediate phenotype may also be due to the HexA protein, which binds mismatches (33) and may interfere with the strand transfer processes.

The role of HexB in mismatch repair was further confirmed by comparing the spontaneous mutation rates of wt and hexB strains, determined by the appearance of Rif r mutants. The hexB mutant exhibits a significant increase in spontaneous mutation frequency (about 130-fold) compared to the wt strain (Table 3).

All these data confirm that a system equivalent to the Hex system of S. pneumoniae exists in L. lactis. The considerable homeologous recombination that occurs in the wt strain may indicate that, as in S. pneumoniae, the Hex system in L. lactis can be titrated and hence inactivated if sufficient homeologous DNA is present in the cell.

(iii) deoB. The deo genes are involved in purine and pyrimidine salvage pathways (50). These genes are necessary for the assimilation of exogenous free bases or nucleosides from the environment and for the reutilization of bases or nucleosides provided by nucleotide turnover. Analysis of the lactococcal sequence allowed us to identify a consens us ribose binding site (GGAGAGA) and two potential consensus promoters upstream of the translation start of the deoB gene. The sequence encoding the C-terminal end of an unidentified protein (67 amino acids), followed by a transcription terminator, is found upstream of deoB, and two putative genes, orfC and deoD, follow deoB (Fig. 2). As no terminator and no promoter could be found in the deoB-orfC and orfC-deoD intergenic regions, we think that deoB, orfC, and deoD are organized in an operon in L. lactis. No gene homologous to orfC was previously described in the deoB-deoD operon of B. subtilis or E. coli. We believe it likely that transposon insertion in deoB leads to inactivation of the entire operon.

deo mutants acquire purine or pyrimidine only by de novo synthesis or by utilization of exogenous bases (26). To test if the UV sensitivity of the deoB strain is due to a depletion of the purine and/or pyrimidine pools, we performed UV sensitivity tests with deoB cells grown in M17glu medium supplemented with all four bases or not supplemented. Addition of the four bases to the medium restored complete UV resistance to the deoB strain (Fig. 3). Based on these observations, we propose that the UV sensitivity of the deoB strain is due to a lack of nucleotides and possibly to a diminished capacity to reutilize the bases provided by DNA and RNA degradation following UV stress. It was previously observed that a mutation in a uracil salvage pathway of B. subtilis (upp) confers a UV-sensitive phenotype (30, 50). We predict that nucleotide addition would also restore UV resistance to that strain and that DNA repair functions are normal in both B. subtilis upp and L. lactis deoB mutants.

(iv) dlTD. The DlTD enzyme is implicated in cell wall synthesis and is necessary for the esterification of lipoteichoic and cell wall teichoic acids (52). The deduced amino acids sequence from DNA upstream of the putative lactococcal dlTD gene is highly homologous to the DlTD enzyme of B. subtilis, encoded by ipe-4R. Therefore it is likely that, as in Lactobacillus casei and B. subtilis, the dlT genes of L. lactis are organized in an operon.

Inactivation of dlT genes in Lactobacillus casei affects cell
growth and morphology (28). We observed that the L. lactis dltD mutant grows slowly and forms longer chains than the wt strain (data not shown), suggesting that dltD inactivation may affect the organization and structure of the cell wall. The following results support this suggestion: (i) a conjugation test with the self-mobilizable plasmid pIL205 revealed that transfer frequency of pIL205 was 30 times lower in the mutant strain (data not shown), suggesting that the mutant strain could be made electrocompetent without the usual addition to the medium of glucose, an inhibitor of lactococcal cell wall biosynthesis (data not shown). Thus, phenotypic differences of the dltD mutant, which include UV sensitivity, may indicate that the cell wall is altered in this strain and may have a diminished ability to protect the cell from environmental aggressions.

(v) gerC. GerC enzymes are involved in the biosynthesis of isoprene, a component of the cell membrane (66). Sequence analysis show that the ISS1 insertion occurred in an ORF which is homologous to the B. subtilis gerC A ORF (25% identity and 61% similarity). A second ORF, which is homologous to the B. subtilis GerCC protein (33% identity and 55% similarity), was identified 200 bp downstream of the ISS1 insertion site. The gerC locus of B. subtilis is organized as an operon containing three gerC genes (A, B, and C). We suspect that in L. lactis, the gerC operon comprises only two genes, gerCA, and gerCC, because a gerCB analog was previously isolated in L. lactis (24) on a DNA fragment having no homology overlap with that characterized here. As with dltD, we observed that transfer of the self-mobilizable plasmid pIL205 with a gerC recipient was less efficient than with a wt recipient (transfer frequency in the gerC recipient, 1.3 \times 10^{-8}). Similarly, we explain the UV sensitivity of the gerC mutant by an alteration in envelope or membrane organization. To our knowledge, this is the only report of a UV-sensitive phenotype of a gerC mutant; this phenotype may be consistent with the capacity of isoprene and isoprene derivatives to absorb UV radiation. It was previously reported that UV radiation affects membrane permeability in E. coli (61). Our data suggest that the converse may also be true. It will also be of interest to determine whether this UV-sensitive phenotype is specific to L. lactis.

(vi) gidA. gidA genes are very well conserved and are generally localized near the chromosomal replication origin (51). However, no clear function has been attributed to their products. In E. coli, gidA gene inactivation reduces the growth rate and causes filamentation of the cell in medium containing glucose (64). A role for gidA in recombination or in UV resistance has not been previously reported. Further characterization, such as complementation experiments, will be necessary to determine whether the observed phenotype is due to the gidA mutation itself or to a polar effect of the ISS1 insertion.

(vii) uvs-75. No significant homology could be found between uvs-75 and known genes or ORFs. However, sequence analysis revealed that the encoded protein contains highly hydrophobic domains (data not shown) and thus is likely to be a membrane protein. In addition, ISS1-flanking DNA sequences indicate that uvs-75 is in an operon (data not shown). Inactivation of uvs-75 resulted in intermediate UV sensitivity and decreased plasmid recombination compared to those in the wt strain. Analysis of downstream sequences revealed an ORF transcribed in the direction opposite to that of uvs-75. The ORF is homologous to the sequences encoding eucaryotic proteins implicated in DNA repair, NucR (63) and Htf9C (8). We consider it possible that the phenotypes of the uvs-75 mutant are due to a polar effect of the ISS1 insertion on the expression of the downstream gene.

TABLE 2. Homologous recombination frequencies in L. lactis UV-sensitive mutants

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Recombination frequencya in:</th>
<th>Mutagenesis frequencya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>recA</td>
</tr>
<tr>
<td>pG&lt;sup&gt;+&lt;/sup&gt;host5</td>
<td>7.5 \times 10^{-5}</td>
<td>2.8 \times 10^{-6}</td>
</tr>
<tr>
<td>pHist-MG</td>
<td>5.6 \times 10^{-2}</td>
<td>3.6 \times 10^{-6}</td>
</tr>
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\(^a\) Recombination frequencies were measured as in Table 2 and correspond to the means of at least three independent experiments. Standard deviations are less than 20% of the given values.

\(^b\) Spontaneous mutation frequencies were measured as the number of Rif<sup>r</sup> colonies at the nonpermissive temperature for plasmid replication (37.5°C) divided by the total number of colonies on nonselective medium and correspond to the means of at least three independent experiments. Standard deviations are less than 20% of the given values.

DISCUSSION

In the present study, we isolated MC- and UV-sensitive insertional mutants of L. lactis. All the mutants are independent, and ISS1 flanking-sequence data allowed us to identify 11 of the 19 target genes. Of the remaining mutated genes, four lack homology with known genes and four were not studied here. Of the seven stabilized mutant strains presented and characterized here, all are sensitive to high doses of UV (50 J/m<sup>2</sup>) but only three (the polA, hexB, and deoB mutants) are also sensitive to low UV doses. Homologous recombination (as tested by plasmid recombination) is affected only in polA, gidA and uvs-75 mutants.

Three classes of mutations are discerned. The first class (hexB, polA, and deoB) corresponds to expected mutations affecting DNA metabolism. The second class (gerC and dltD...
mutants) presumably affects cell envelope organization; mutations in this class of genes were not previously described as conferring UV sensitivity. As both of these genes are part of an operon, the UV-sensitive phenotype may also involve downstream genes because of a possible polar effect of the ISS1 insertion. Our results concerning this class are consistent with previous observations that envelope integrity and DNA repair capacities are somehow related. For example, (i) after SOS induction in E. coli, the majority of the RecA protein is found associated with the membrane (36); (ii) chemical modification of membrane lipids may induce the SOS response (4); (iii) in E. coli recA or lexA mutants, membrane permeability is modified (38); and (iv) during the E. coli SOS response, septum formation is inhibited, showing that cell division arrest is associated with repair (65). It remains to be tested whether gerC and dltD mutants of E. coli or B. subtilis are UV sensitive. A third class, composed of the genes not studied further, including arcD (a component of an arginine/ornithine antiporter), bgaA (encoding a 6-phospho-beta-glucosidase), hgp (unknown function), metB (coding for a protein involved in methionine metabolism), proA (coding for a protein involved in proline metabolism) and gidA, corresponds to genes with no known link to UV resistance. It has been observed that in E. coli, mutations affecting sugar metabolism or transcription may modify recombination frequencies (5, 55) and that phosphate starvation induces some SOS genes (15). Our data indicate that alterations in general metabolism or the energy level of the cell may change its DNA repair capacities.

Only a few genes directly involved in DNA repair were identified in this mutagenesis. We do not have an explanation for this result. However, stress pathways seem to be more interactive in L. lactis than in E. coli, so that stresses present during the screening, such as oxygen or temperature, may affect the selection. For example, the UV-sensitive recA mutant survives poorly in the presence of either oxygen or elevated temperature (18). This may explain why we did not isolate certain mutants in the present study. It will be interesting to perform the same type of mutagenesis under anaerobic conditions or at 30°C to determine whether more genes directly involved in DNA repair are obtained.

The results presented here reveal elements in the DNA damage response which have not been previously described. It remains to be determined whether the roles of the identified genes in DNA repair are particular to L. lactis. Furthermore, characterization of gene promoters (such as that of polA) and of the nonidentified mutants will be important in unraveling the regulation of DNA damage response in L. lactis.

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45. Maguin, E., H. Prévost, S. D. Ehrlich, and A. Gruss. 1996. Efficient inser-