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Role of Uracil-DNA Glycosylase in Mutation Avoidance by *Streptococcus pneumoniae*

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Uracil-DNA glycosylase activity was found in *Streptococcus pneumoniae*, and the enzyme was partially purified. An *ung* mutant lacking the activity was obtained by positive selection of cells transformed with a plasmid containing uracil in its DNA. The effects of the *ung* mutation on mutagenic processes in *S. pneumoniae* were examined. The sequence of several *malM* mutations revertible by nitrous acid showed them to correspond to A · T→G · C transitions. This confirmed a prior deduction that nitrous acid action on transforming DNA gave only G · C→A · T mutations. Examination of *malM* mutant reversion frequencies in *ung* strains indicated that G · C→A · T mutation rates generally were 10-fold higher than in wild-type strains, presumably owing to lack of repair of deaminated cytosine residues in DNA. No effect of *ung* on mutation avoidance by the Hex mismatch repair system was observed, which means that uracil incorporation and removal from nascent DNA cannot be solely responsible for producing strand breaks that target nascent DNA for correction after replication. One *malM* mutation corresponding to an A · T→G · C transition showed a 10-fold-higher spontaneous reversion frequency than other such transitions in a wild-type background. This "hot spot" was located in a directly repeated DNA sequence; it is proposed that transient slippage to the wild-type repeat during replication accounts for the higher reversion frequency.

Uracil-DNA glycosylases hydrolyze the *N*-glycosidic bond between uracil and deoxyribose in DNA. First observed in *Escherichia coli* (33), these enzymes are found in many different procaryotic and eucaryotic cells. Genes encoding the enzyme from several sources—including *E. coli* (11, 54), *Saccharomyces cerevisiae* (42), and human placenta (41)—have been cloned and sequenced. The results show the enzyme proteins from these diverse sources to be homologous and highly conserved, with >50% of the amino acid residues identical within a protein segment the size of the *E. coli* enzyme.

One function of uracil-DNA glycosylase is the repair of DNA in which cytosine residues were spontaneously converted to uracil by deamination. Measurement in vitro of the rate of cytosine deamination in DNA at elevated temperatures (35) indicated that such deamination under physiological conditions could contribute significantly to spontaneous mutations in vivo. However, in *E. coli* these mutations only appear in *ung* mutants that have lost the glycosylase activity and therefore cannot remove uracil, which is the first step in restoring the cytosine residue (12, 13).

The glycosylase can also remove uracil residues that are incorporated into DNA from dUTP precursors during replication. Although such incorporation occurs in wild-type cells, the extent of incorporation of uracil is greatly increased in *dut* mutants of *E. coli*, and its removal is blocked in *ung dut* double mutants (53). These cells are viable even though as much as 15% of the thymine residues in their DNA are substituted by uracil (55). The function, therefore, of the incorporation of uracil and its removal soon after synthesis of DNA in wild-type cells remains obscure. One possible function is a role in DNA mismatch repair.

A heteroduplex DNA mismatch repair system that is present in *Streptococcus pneumoniae* corrects DNA mismatches that result from either genetic transformation or mistakes in replication (reviewed in reference 8). The action of this system, called Hex, in the two cases results in lower transformation frequencies and avoidance of mutations, respectively. Targeting of the strand to be corrected, which is either the donor strand in the transformation product or the nascent strand after DNA replication, appears to be based on the presence of breaks in that strand. The Hex system of *S. pneumoniae* is closely related to the Mut system of *E. coli* and *Salmonella typhimurium* (15, 43). Although the latter system can be directed by DNA methylation, this effect appears to be mediated by the production of strand breaks at unmethylated sites (24, 32). Inasmuch as a mismatch repair system closely related to Hex and Mut is also found in *Saccharomyces cerevisiae* (19, 40, 44), homologous systems may be common among both procaryotes and eucaryotes. If, as postulated for *S. pneumoniae*, they depend on breaks for nascent-strand targeting, their presence might provide another role in mutation avoidance for the ubiquitous uracil-DNA glycosylase (9, 24).

Our present concept of DNA replication predicts breaks in the lagging strand but not in the leading strand at the replication fork (18). However, observations for two bacterial species indicate that, in vivo, both nascent strands are fragmented (51, 53). A conceivable mechanism for introduction of breaks is the incorporation of uracil, followed by sequential action of uracil-DNA glycosylase and apyrimidinic (AP) endonuclease. The *exoA* gene of *S. pneumoniae* was recently shown to produce an AP-endonuclease (45). It appears to be an essential gene, perhaps because its product is routinely required to repair DNA after replication.

The object of the present work was to investigate the presence and possible roles of uracil-DNA glycosylase in mutation avoidance in *S. pneumoniae*. The enzyme was found in this species, and an *ung* mutant lacking it was isolated. A series of mutations in the *malM* gene of *S. pneumoniae*, which encodes an amylomaltase and is essen-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or relevant features	Reference[a]
S. pneumoniae		
470	*thy-7 ltr-1 vlt-3 str nov*	27
533	*sul-d sul-a str bry nov ery*	26
593	*endA1*	28
606	*malM596 hexA4 trt-1 exoA2*	28
709	*malM567 hexA4 trt-1 endA1 exoA2*	38
706	*malM597 hexA4 nov endA1 exoA2*	
1130	*malM564 ery trt-1 endA14 exoA5*	
1140	*malM567 hexA4 trt-1 endA1 exoA2 ung-1*	
1170	*malM596 hexA4 trt-1 exoA2 ung-1*	
1174	*malM567 trt-1 endA1 exoA2*	
1181	*malM567 trt-1 endA1 exoA2 ung-1*	
E. coli		
BL21(DE3)	*hsdS gal* (*int*::P_{lacUV5}-T7 gene*1 imm21 nin5*)	52
BW313	*dut ung*	20
H560rev	*thyA arg met endA*	29
Plasmids		
pJS3	Derived from pLS1-pC194 composite, Cm^r	3
pLS1	pMV158 derivative, Tet^r	50
pLS21	Hybrid of pLS5 and pET-5, Tet^r Amp^r	29
pLS70	$malM^+$ cloned in pMV158, Tet^r $malM^+$	50
pLS141	$hexA^+$ cloned in pMV158, Tet^r $hexA^+$	2

[a] From this work if no reference is indicated.

tial for maltose utilization, were obtained previously (22). These mutations were further characterized with respect to base changes, and their spontaneous reversion frequencies were determined in the presence of various combinations of *ung* and *hex* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Some strains and all plasmids used in this work are listed in Table 1. Other strains, containing only the markers indicated in Table 4, are not listed here. The strains of *S. pneumoniae* were derivatives of the nonencapsulated wild-type strain R6. Mutations *endA* and *exoA* affect the genes encoding the major endonuclease and the major exonuclease, respectively, of *S. pneumoniae* (45, 46). Although *exoA2* affects both the exonuclease and AP-endonuclease activities of the ExoA enzyme, 20 to 30% of these activities remain (28, 45). Samples of *Bacillus subtilis* BD1134 and its bacteriophage PBS2, the DNA of which contains uracil instead of thymine, were generously provided by Bruce Duncan. The *E. coli* strains were used as hosts for pLS21 and pJS3.

Growth and transformation of bacteria. Cultures of *S. pneumoniae* were grown at 37°C in a casein hydrolysate-based medium (22). Sucrose at 0.2% was the source of energy, except that maltose at 0.2% was used for selection of Mal^+ CFU. Competence for transformation was achieved by growing cultures to 10^8 CFU/ml, diluting samples in fresh medium, and incubating them at 30°C for 20 min (25). After treatment with DNA for 40 min at 30°C, the cultures were incubated at 37°C for 2 h prior to selection. Cultures were treated with chromosomal or plasmid DNA at 0.1 to 1.0 μg/ml. Transformants of *S. pneumoniae* were selected in agar medium with chloramphenicol at 2.5 μg/ml, tetracycline at 1 μg/ml, novobiocin at 10 μg/ml, or streptomycin at 100 μg/ml. Hex mismatch repair phenotypes were ascertained by treating a culture with tester DNA from strain 533 and comparing transformation to novobiocin and streptomycin resistance, which in a Hex^+ strain correspond to low- and high-efficiency markers, respectively.

E. coli was grown in L broth and transformed by the method of Hanahan (16). Transformants of *E. coli* were selected in medium containing chloramphenicol at 5 μg/ml or ampicillin at 50 μg/ml. Strain BD1134 of *B. subtilis* was grown in TY broth (48) and infected with PBS2. Bacteriophage were grown and counted by plaque assay, as described by Raimondo et al. (47), and then purified by the method of Yamamoto et al. (56).

DNA and plasmid preparation. Chromosomal DNA was prepared from *S. pneumoniae* by the procedure of Berns and Thomas (4). Thymidine-labeled DNA was prepared from strain 470 as described previously (27), except that phenol instead of chloroform was used for deproteinization. Cultures were grown with [*methyl*-^{3}H]thymidine, and the DNA had a specific activity of 74,000 cpm/μg.

Purified plasmids were prepared by the procedure of Currier and Nester (10). Crude plasmid preparations, called alkaline lysates, were obtained from *E. coli* by the method of Birnboim and Doly (5) and from *S. pneumoniae* by a modification of that method (50). Analytical gel electrophoresis of plasmids was carried out in 1% agarose gels with staining by ethidium bromide at 1 μg/ml.

[^{3}H]uracil-containing DNA was prepared from PBS2 phage grown at 37°C in 200 ml of TY broth containing 1 mCi of [^{3}H]uridine. DNA was released from phage particles by addition of 5 mM EDTA and 0.3% Sarkosyl. After treatment with phenol, the purified DNA was dialyzed against 10 mM Tris hydrochloride (pH 8.0) containing 0.1 mM EDTA. The specific radioactivity of the [^{3}H]uracil-containing DNA was 7,600 cpm/μg. Denatured DNA was prepared by heating a sample for 5 min at 100°C.

Uracil-DNA glycosylase assay. Cell extracts of *S. pneumoniae* containing native proteins were prepared either by lysis with Triton X-100 (30) or by rupture in a French pressure cell (37) as described before. Uracil-DNA glycosylase activity was determined by incubating samples of extract at 30°C in 50 μl of a mixture containing 50 mM Tris hydrochloride (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.5 μg of [^{3}H]uracil-containing DNA. Reactions were terminated by sequential addition of 25 μl of 0.4% bovine serum albumin and 25 μl of 30% trichloroacetic acid; after 20 min at 0°C, the mixture was centrifuged, and 90 μl of the supernatant fluid was taken for scintillation counting. One unit of uracil-DNA glycosylase activity corresponds to 1 nmol of uracil released per min at 30°C.

Protein concentration in extracts and fractions was determined by the method of Lowry et al. (39) or Bradford (6).

Reversion frequency of *malM* mutations. Reversion frequencies were measured as the proportion of maltose-utilizing CFU giving large colonies in mutant populations grown from small inocula to approximately 10^{10} cells. Base changes corresponding to various mutations were determined by transferring the mutation to the *mal* recombinant plasmid pLS70 by chromosomal facilitation (38) and sequencing a portion of the mutant plasmid as described previously (24).

RESULTS

Demonstration of uracil-DNA glycosylase in *S. pneumoniae*. To test for uracil-DNA glycosylase activity, samples of a

TABLE 2. Uracil-DNA glycosylase in *S. pneumoniae*

Source of DNA substrate	Form of DNA	% Radioactivity released by indicated amount of cell extract[a]:		
		0.04 μl	0.20 μl	1.00 μl
[^{3}H]uracil-labeled phage PBS2[b]	Native	1.3	7.7	54.4
	Denatured	6.1	52.3	61.5
[^{3}H]thymidine-labeled *S. pneumoniae*	Native	0.0	0.1	0.1
	Denatured	0.2	0.2	0.7

[a] Percentage of total radioactivity rendered acid soluble by an extract of *S. pneumoniae* 1130, which contained 9 mg of protein per ml, after subtraction of the value for an enzyme blank.

[b] Label from [^{3}H]uridine enters both uracil and cytosine residues to give approximately half of the total label in each (55).

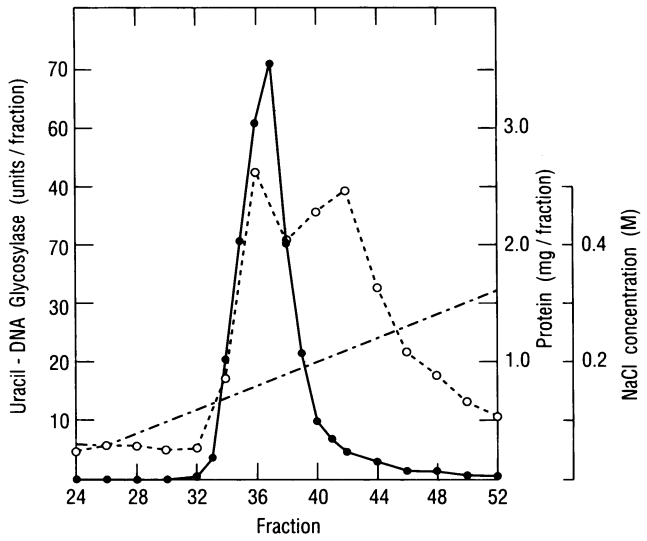

FIG. 1. DEAE-cellulose chromatography of uracil-DNA glycosylase. Cells from a 1.6 liter culture of *S. pneumoniae* 1130 grown to an OD_{650} of 0.7 were lysed with Triton X-100 in a volume of 20 ml. DNA was precipitated by addition of 2 g of polyethylene glycol 6000 and 2 M NaCl. After centrifugation, the supernatant fluid was dialyzed against DEAE column buffer (50 mM Tris hydrochloride [pH 7.6], 50 mM NaCl, 6 mM β-mercaptoethanol, 5% glycerol) and applied to a DEAE-cellulose column (2 by 40 cm). The column was eluted with a linear gradient of NaCl in the same buffer. Fractions of 6 ml were collected. Symbols ●, uracil-DNA glycosylase; ○, protein; –·–·–, NaCl concentration.

crude extract of *S. pneumoniae* 1130 were incubated with [^{3}H]uracil-containing DNA from phage PBS2 of *B. subtilis* in the presence of 1 mM EDTA. With increasing amounts of extract, as much as 60% of the tritium could be released in an acid-soluble form (Table 2). Tritium remaining in the DNA was presumably present in cytosine derived from the [^{3}H]uridine used to label the phage DNA (55). Single-stranded DNA appears to be a better substrate for the pneumococcal enzyme than double-stranded DNA, inasmuch as the rate of uracil release from heat-denatured DNA was approximately five times greater than that from native DNA.

The observed release of uracil did not result from generalized hydrolysis of the substrate. Under conditions in which uracil was released, only negligible amounts of [^{3}H]thymidine-labeled DNA were rendered acid soluble by extracts of strain 1130 (Table 2). This strain carries the *endA14* mutation, which makes it highly defective (<0.1% of wild-type activity) in the major DNase activity of *S. pneumoniae* (28). However, strains 593 (*endA1*) and 606 (*endA*$^+$), containing 10 and 100% of the wild-type level of DNase, respectively, also gave no release of thymidine label in the presence of 1 mM EDTA (data not shown). These strains exhibited the same specific activity of uracil-DNA glycosylase as strain 1130, which was approximately 2 nmol of uracil released per min at 30°C per mg of protein in crude extracts (see Table 3, below).

The uracil-DNA glycosylase activity of *S. pneumoniae* was fractionated by ion-exchange chromatography on DEAE-cellulose (Fig. 1). A crude extract of strain 1130 was applied to the column in the presence of 0.05 M NaCl, and the column was eluted with a gradient of NaCl from 0.05 to 0.4 M. A single peak of glycosylase activity was observed; it eluted at 0.15 to 0.20 M NaCl. This suggested that only a single such enzyme was present and that it should be possible to obtain a single-step mutant lacking the activity.

Isolation of an *ung* mutation. The approach to obtaining mutants of *S. pneumoniae* lacking the uracil-DNA glycosylase consisted of two steps: (i) chemical mutagenesis of a population of Ung$^+$ cells containing the enzyme and (ii) positive selection of Ung$^-$ mutants by transformation with a plasmid containing uracil in place of thymine. This approach was used by Burgers and Klein to obtain Ung$^-$ mutants of *S. cerevisiae* (7). In the transformation of *S. pneumoniae*, only single-stranded segments of donor DNA enter the cells (21). Therefore, plasmid transfer requires the interaction of complementary strands from two entry events (49), as shown in Fig. 2. In wild-type cells, removal by the glycosylase of uracil residues in the single strands of donor DNA would render the reconstituted plasmid subject to hydrolysis by an AP-endonuclease, which could prevent establishment of the plasmid (Fig. 2B). An AP-endonuclease activity that makes a single-strand break at an apurinic or apyrimidinic site was recently reported for the product of the *exoA* gene of *S. pneumoniae* (45). Two nearby lesions on opposite strands could give a double-strand break fatal to the plasmid. In an Ung$^-$ cell, uracil would remain in the plasmid DNA and the plasmid would be established normally (Fig. 2A). Positive selection for the plasmid marker should therefore select cells carrying an *ung* mutation.

To obtain uracil-containing plasmids for the transformation step, two plasmids that can replicate in both *S. pneumoniae* and *E. coli* were examined. One was pLS21, an 8.1-kb hybrid plasmid that contains replication functions from both the streptococcal plasmid pLS1 and the *E. coli* plasmid pBR322; the other was pJS3, a 4.1-kb recombinant plasmid that contains only the pLS1 replicon, which itself allows replication of *E. coli* (29). Both plasmids were prepared in *E. coli* BW313 (*dut ung*), in which up to 15% of the thymine residues in DNA are substituted by uracil (55). Plasmids pLS21 and pJS3, grown in *E. coli* Ung$^+$ Dut$^+$ strains BL21(DE3) and 560rev, respectively, and therefore containing no uracil, readily transformed *S. pneumoniae* to tetracycline resistance and chloramphenicol resistance, respectively, at frequencies of 1,000 and 20,000 transformants per ml, respectively. In both cases, the uracil-substituted plasmids gave <10 transformants per ml. Because pJS3 gave higher frequencies of transfer, it was chosen for the selective system.

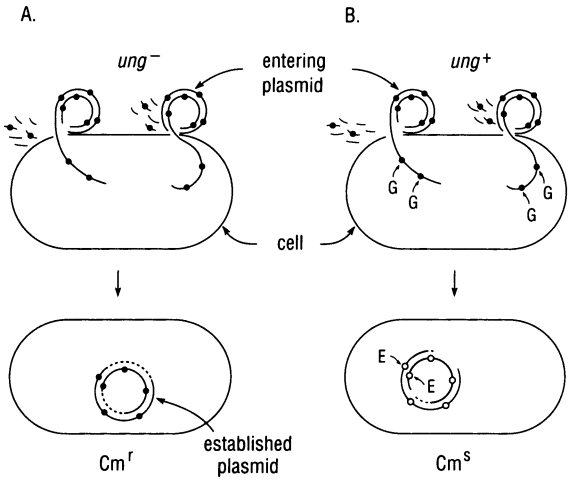

FIG. 2. Selection of *ung* cells by establishment of a uracil-containing donor plasmid. Such selection was accomplished either after mutagenesis or after transfer of an *ung* marker by transformation. Establishment of a plasmid, such as pJS3, requires reconstitution from two plasmid strands that enter the cell via the transformation pathway. (A) Plasmid DNA containing uracil is not affected in an *ung* cell, and complementary strands can reconstitute an intact plasmid, which confers resistance to chloramphenicol. (B) In an *ung*$^+$ cell, the uracil-DNA glycosylase removes uracil residues from entering DNA. On subsequent interaction of complementary donor strands, the apyrimidinic sites would be subject to endonucleolytic cleavage or would block DNA replication, preventing plasmid establishment. Symbols: ●, uracil residue in DNA; ○, apyrimidinic site; G, uracil-DNA glycosylase; E, AP-endonuclease; – – –, repair replication to reconstitute plasmid.

S. pneumoniae 606 was mutagenized by treating 5×10^8 CFU in 10 ml of growth medium with 0.5 mg of *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine for 40 min at 37°C, which reduced the number of viable CFU to 5×10^4. The cells were removed by centrifugation and grown to competence. After transformation with uracil-containing pJS3 (PJS3-U), three chloramphenicol-resistant colonies were obtained. Crude extracts of all three showed very low activities for uracil-DNA glycosylase. The mutation in one isolate was designated *ung-1*, and the enzyme activity measured for that strain is compared with that of *ung*$^+$ strains in Table 3. The mutant exhibited less than 0.5% of the wild-type activity.

Uracil-DNA glycosylase activity from wild-type cells was fractionated by agarose gel filtration on a column of Bio-Gel A-0.5m (data not shown). The activity appeared as a single peak with a possible shoulder toward the low-molecular-weight side. From the position of elution and previous calibration of the column with protein molecular weight standards, the activity corresponds to a protein of 25 to 50 kDa. Extracts of the *ung-1* mutant, when similarly fractionated, gave no detectable activity in any fraction. Although crude extracts of mutant cells reproducibly released uracil at a rate approximately 0.5% that of the wild type, mutant fractions showed <0.2% of the activity at the position of the peak. It is likely that the *ung-1* mutant contains a single mutation affecting the enzyme, because transfer of the mutation to other strains by transformation occurred at a frequency expected for a single event and gave rise to transformants as defective as the original *ung-1* mutant isolate.

TABLE 3. Uracil-DNA glycosylase activity in *ung*$^+$ and *ung* mutant strains of *S. pneumoniae*

S. pneumoniae strain	Genotype	Uracil-DNA glycosylase sp act (U/mg)
593	*endA1*	2.16
1130	*exoA5 trt-1 ery endA14 malM564*	2.04
606	*exoA2 trt-1 hexA4 malM596*	2.23
1170[a]	*exoA2 trt-1 hexA4 malM596 ung-1*	0.01

[a] Contains plasmid pJS3.

Transfer of markers and construction of strains. To test the effect of the *ung-1* mutation on mismatch repair and mutation frequency, it was necessary to introduce the mutation into various strains containing *hex* and *malM* alleles. The same positive selection method used to isolate the mutant was used to select transformants in a second-round transformation with pJS3-U after initially transforming cells of the genotype desired with chromosomal DNA from the *ung-1* mutant strain. Because this selection was for chloramphenicol resistance, the pJS3 plasmid in the original *ung-1* strain had to be removed. One way this was done was by transfer into the strain of pLS1, which is incompatible with pJS3 and which confers tetracycline resistance. After 40 generations of selection with only tetracycline, pJS3 was lost. With this new strain, 1170(pLS1), we confirmed the selective effect of pJS3-U transformation for Ung$^-$ cells. The frequency of establishment of pJS3-U in the Ung$^-$ recipient 1170(pLS1) was 1,000-fold greater than in the Ung$^+$ strain 606(pLS1). Another way that pJS3 was eliminated from *ung-1* strains was by "curing" with ethidium bromide, as previously reported for *S. pneumoniae* (31). In this case, cured clones were distinguished as small colonies in contrast to plasmid-containing large colonies when plated in agar containing the marginally selective chloramphenicol concentration of 1.0 μg/ml.

Using donor DNA from either cured or pLS1-containing *ung-1* strains, we transferred the *ung-1* marker into four different *malM* strains; in another case (*malM510*), we transferred the *mal* marker into a *mal*$^+$ derivative of an *ung-1* strain. All five *malM* mutations were point markers that from their low transformation efficiencies were thought to correspond to transition mutations (22, 24). With *mal* cultures that were not highly competent for transformation, it was easier to transform the *hex* mutant derivative than the *hex*$^+$ to Ung$^-$, which suggests that the *ung-1* mutation itself is a low-efficiency marker. In several instances, therefore, instead of transferring *ung-1* into a *hex*$^+$ strain, we put the *hex*$^+$ allele into the *ung-1 hex* strain. This was conveniently done by using pLS141, which contains the cloned *hexA*$^+$ gene (2), since over half the cells treated in this first round with the homogeneous plasmid DNA were transformed to Hex$^+$, and Hex$^+$ clones were readily obtained by testing only a dozen clones by transformation in a second round with a tester DNA containing both high-efficiency and low-efficiency markers (2). Various strains were then examined for reversion of the *malM* marker.

Spontaneous mutation frequencies in the presence of *ung* and *hex* mutations. DNA sequence analysis of *malM* mutations *malM537* and *malM567* previously showed them to result from G · C→A · T mutations (24). Mutations *malM510*, *malM532*, and *malM597* were selected for this work because their revertibility with nitrous acid indicated that they resulted from A · T→G · C transitions (22). These mutations were passed into pLS70, which contains the

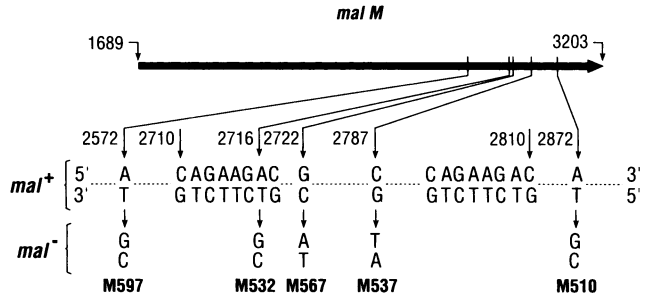

FIG. 3. Map of *malM* mutations and corresponding base changes. Heavy arrow indicates the amylomaltase structural gene of *S. pneumoniae*, which extends from nucleotide positions 1689 to 3203. Vertical marks indicate sites of mutations examined in this work. Nucleotide positions of these sites, and of a directly repeated sequence which includes one of them, are indicated. Parts of the wild-type DNA sequence are shown together with the base changes giving rise to the mutations.

cloned *malM* gene, by chromosomal facilitation of plasmid establishment, as described previously (38). Determination of the DNA sequence of these mutant alleles showed that they in fact, resulted from A · T→G · C mutations (Fig. 3). These data corroborate the earlier deduction (22) that nitrous acid treatment of transforming DNA in *S. pneumoniae* gives rise only to G · C→A · T and not to A · T→G · C mutations.

Figure 3 depicts the *malM* structural gene for the pneumococcal amylomaltase (24) and gives the location in terms of nucleotide position as well as the nature of the base change for each of the mutations examined. In the following analysis, it is assumed that large-colony revertants to Mal$^+$ indicate back-mutations to wild type at the original mutation site. Supporting this assumption is the observation that nitrous acid-induced reversions that restored the original codon gave large colonies, whereas one that altered the original amino acid residue in amylomaltase gave small colonies (22, 24).

We shall consider first the effect of the *ung-1* mutation on spontaneous reversion of *malM* mutations in a *hex*$^+$ background (Table 4). The presence of plasmid pJS3 had no significant effect on *malM* reversion frequency. Four of the *malM* mutations, that is, all except *malM532*, gave spontaneous reversion frequencies of 2.0×10^{-9} to 1.2×10^{-8} in Ung$^+$ cells. In the case of the two G · C→A · T mutations, *malM537* and *malM567*, similarly low reversion frequencies were observed in the Ung$^-$ background. However, the two A · T→G · C mutations, *malM510* and *malM597*, gave higher reversion frequencies, 5.8×10^{-8} and 2.8×10^{-8}, respectively, in Ung$^-$ cells. These values are 12-fold and 7-fold higher, respectively, than the corresponding frequencies in Ung$^+$ cells. It thus appears that the uracil-DNA glycosylase of *S. pneumoniae* is responsible for reducing the spontaneous frequency of G · C→A · T mutations by a factor of about 10. This mutation avoidance presumably results from removal of deaminated cytosine residues in DNA, as has been postulated for *E. coli* (13, 35).

One A · T→G · C mutation, *malM532*, did not give a higher reversion frequency in the Ung$^-$ background (Table 4). However, this mutation already showed an elevated reversion frequency in Ung$^+$ cells (3.7×10^{-8} and 6.0×10^{-8}). Against such a background, an additional mutation frequency of approximately 2×10^{-8} resulting from the absence of uracil-DNA glycosylase would not be noticeable. What might be the basis for the higher spontaneous reversion frequency of *malM532*? This mutation occurred at position 2716, which falls in the wild type within an 8-base direct repeat (Fig. 3). We propose that the high reversion frequency at this site results from slippage of the DNA template during replication so that the wild-type repeat is copied in place of the mutant and that the template then slips back to continue replication past the mutated site. With replication proceeding in the direction from the mutant repeat to the wild-type repeat, if the nascent strand slips forward along the template to the wild-type repeat, the mutant site would be replicated as wild-type sequence. If the nascent strand then slips back to the mutant repeat but past the mutant site, its continued synthesis would produce a wild-type allele. If the template does not slip back, a deletion bordered by the direct repeats would result. Such a deletion

TABLE 4. Reversion frequencies of *malM* mutations in Ung$^+$ and Ung$^-$ strains[a]

Hex phenotype	*malM* allele	Base change[b]	Position[c]	Plasmid in strain	Reversion frequency[d]	
					Ung$^+$	Ung$^-$
+	510	A · T→G · C	2872	None	4.5×10^{-9}	
				pJS	4.8×10^{-9}	*5.8×10^{-8}*
+	532	A · T→G · C	2716	None	3.7×10^{-8}	
				pJS3	6.0×10^{-8}	3.9×10^{-8}
+	537	C · G→T · A	2787	None	4.0×10^{-9}	
				pJS3	2.0×10^{-9}	1.1×10^{-9}
+	567	G · C→A · T	2722	None	1.2×10^{-8}	4.5×10^{-9}
				pJS3	5.0×10^{-9}	4.0×10^{-9}
+	597	A · T→G · C	2752	None	6.1×10^{-9}	
				pJS3	3.8×10^{-9}	*2.8×10^{-8}*
−	567	G · C→A · T	2722	None	9.4×10^{-7}	2.3×10^{-6}
				pJS3	5.9×10^{-7}	6.9×10^{-7}
−	597	A · T→G · C	2752	None	3.8×10^{-7}	
				pJS3	1.8×10^{-7}	2.1×10^{-7}

[a] Ung$^-$ strains carried the *ung-1* mutation. All strains were ExoA$^+$ except for those with the *malM567* mutation, which carried *exoA2*. Hex$^-$ phenotype was conferred by *hexA4* (2).
[b] From wild-type to mutant.
[c] According to DNA numbering of reference 24.
[d] Average of Mal$^+$ CFU frequencies in four independent cultures grown from small inocula to ~2.5×10^9 CFU. Values significantly higher in Ung$^-$ strains are italicized.

(in the wild-type strain) was in fact observed; it corresponds to *malM564* (24, 36).

One of the objectives of this work was to see whether uracil-DNA glycosylase action was necessary for mutation avoidance by the Hex mismatch repair system. In Table 4 it can be seen that reversion frequencies for *malM567* and *malM597* were 50-fold to 100-fold higher in Hex^- strains than in Hex^+ strains, in keeping with earlier results (24). It is apparent from Table 4 that the *ung-1* mutation has no effect on Hex reduction of mutation frequency. This is most clearly evident with the G · C→A · T forward mutation, *malM567*, which is not complicated by the *ung-1* effect on the G · C→A · T reverse mutation due to cytosine deamination. That the latter effect was not seen for *malM597* in the comparison of Ung^- to Ung^+ in a Hex^- strain is not surprising because, conferring a mutation frequency <20% of that in Hex^- cells, it would be masked by the Hex^- effect.

DISCUSSION

Uracil-DNA glycosylases from eucaryotic sources, such as *S. cerevisiae* and humans, are remarkably similar to the *E. coli* enzyme, with which they share 53 and 56% identity in amino acid sequence, respectively (41). The primary protein products for the two eucaryotic enzymes are initially larger, 40 and 34 kDa, respectively, as opposed to 26 kDa for *E. coli*. However, these initial products may be processed to give active enzyme proteins closer to 26 kDa in size (41). The apparent universality of this structure for uracil-DNA glycosylase makes it likely that the enzyme from *S. pneumoniae* will be similar. In fact, the physiological properties of the pneumococcal enzyme, such as activity in the absence of divalent cations and preference for single-stranded DNA, are similar to those of the *E. coli* enzyme (33, 34). From the behavior of the pneumococcal enzyme in gel filtration, it may be similar in size to the *E. coli* glycosylase as well. The specific activities of uracil-DNA glycosylase in crude extracts of the two bacterial species are also similar (34).

This work adduced no evidence for a role of uracil-DNA glycosylase in Hex mismatch repair. Other mechanisms for producing strand breaks in newly synthesized DNA may exist. This appears to be the case for *E. coli* as well, since breaks were found to occur in both newly synthesized strands even in *ung* mutants (53). Preliminary results indicated the presence of a hypoxanthine-DNA glycosylase in *S. pneumoniae*, which was also previously reported for *E. coli* (17). Perhaps hypoxanthine, like uracil, gets incorporated into nascent DNA. If so, this enzyme could be responsible for the putative single-strand breaks used by the Hex system for targeting nascent DNA. It may be that this or another alternative mechanism is responsible for strand targeting by the Hex system. Also mismatch repair may use breaks produced by either the alternative pathway or the uracil-DNA glycosylase pathway. Mutation avoidance by the Hex system might not be affected unless both pathways were blocked.

Any glycolytic pathway for producing strand breaks would also require AP-endonuclease function. The *exoA2* mutant (or other leaky *exoA* mutants available) does not provide a good test for this function because it contains 30% of the wild-type activity. In fact, comparison of *malM567* reversion in an *exoA2* background (this study) and in a wild-type background (reference 22, in which *malM567* is called E7) showed no difference in mutation rates. A true test for an AP-endonuclease function in mismatch repair would require a null mutation of *exoA*.

Lack of the uracil-DNA glycosylase increased G · C→A · T mutation rates in *S. pneumoniae* by a factor of approximately 10. This effect, attributed to repair of deaminated cytosine residues in enzyme-containing cells, was similar in magnitude to that observed in *E. coli*. In wild-type cells of *E. coli*, methylated cytosine residues in DNA are mutational hot spots because on deamination they give rise to thymine, which cannot be recognized by the glycosylase (12). Comparison of frequencies of G · C→A · T transitions at such methylated cytosines with those at unmethylated sites indicated an approximately sixfold increase; this difference disappeared in an *ung* mutant of *E. coli* (12). Reversion frequencies at the *trpA446* site of *E. coli* increased by 30-fold in an *ung* mutant (13). The effect found for *S. pneumoniae* falls between these values. It should be pointed out that any putative role for uracil-DNA glycosylase in Hex repair would be independent of its role in repair of deaminated cytosines. The former acts on newly synthesized DNA, whereas the latter acts on old DNA.

Inasmuch as DNA in *S. cerevisiae* is not methylated, it was predicted that the Pms mismatch repair system in this species, like the Hex system, would depend on strand breaks in nascent DNA for strand targeting (23). Interestingly, spontaneous mutation rates for forward mutations in this yeast were increased by 10- to 100-fold in *ung* cells (7). This was interpreted to represent failure to correct cytosine deamination (7), but it could represent failure of strand targeting in mismatch repair. Supporting this possibility is the fact that *ung* cells held for long periods, which would be expected to accumulate lesions in old DNA, showed mutation frequencies similar to those of growing cells. Also, in *E. coli* forward mutation rates were not significantly increased in *ung* cells (12). Testing of defined mutational transitions in *S. cerevisiae* should resolve this question. Although the level of uracil incorporation into DNA of *S. cerevisiae* is low, <0.1% of thymine residues (7), levels in this range would be sufficient for strand targeting in mismatch repair if uracil incorporation were the sole source of strand breaks for the Pms system.

In an early study of forward and reverse mutagenesis in the *malM* locus by treatment of transforming DNA with nitrous acid, it was deduced from the mutagenic pattern that nitrous acid induced predominantly G · C→A · T mutations (22). This was amply confirmed by DNA sequencing in the present work. In particular, those forward mutations that had been found to be revertible by nitrous acid—*malM510, malM532*, and *malM597*—were shown to correspond to A · T→G · C transitions. It was originally suggested that G · C→A · T transitions predominate because G is deaminated more rapidly than A or C (22). In view of the present results, an alternative explanation is also possible; that is, only G deamination is not repaired; deamination of A and C, which gives hypoxanthine and uracil, respectively, would be repaired by action of glycosylases in the cell. The determination of base changes for these three *malM* mutations, all of which were previously shown to be low-efficiency markers (22), also extends the data showing that transition mismatches, G · T and A · C, are well recognized and corrected by the Hex mismatch repair system (14, 24).

Spontaneous mutations generally occur more frequently at some sites than at others. The molecular basis for such mutational hot spots is often obscure. However, as indicated above, DNA cytosine methylation is one cause of hot spots. Another, for deletion mutations, can be short direct repeats of DNA sequence (1, 36). In this work we have uncovered another possible mechanism for base change mutational hot

spots, slippage during replication between direct repeats with but a single mismatch, which would revert that mismatch. Most cases of such slippage during replication would presumably result in deletion of the segment bordered by the repeated sequences. In a fraction of such cases, however, the site of the mutation in the upstream repeat may be replicated at the downstream repeat and then slip back to the upstream repeat, so that a mutational change at the site occurs without any deletion. The requirement for double slippage may make such events rare; in fact, the reversion frequency at the *malM532* site was only 4×10^{-8}, but this was 10-fold higher than the spontaneous frequency for other base change mutations.

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