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Genetics

5-Methylcytosine is not a mutation hot spot in nondividing *Escherichia coli*

(very short patch mismatch repair/deamination rates/starvation/DNA synthesis)

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ABSTRACT **Spontaneous deamination of 5-methylcytosine (5meC) causes hot spots of C·G → T·A mutations in *Escherichia coli* and in human cells. In *E. coli*, the resulting T·G mispairs can be corrected to C·G by very short patch (VSP) repair, which requires the product of gene *vsr*. Mutation hot spots in genes of replicating *vsr*$^+$ bacteria are attributable to low Vsr activity. To determine the rate of deamination of 5meC and the efficiency of VSP repair in nondividing bacteria, we used kanamycin-sensitive (Kan^S) lysogens containing a λ*kan*$^-$ prophage. Deamination of a 5meC in the *kan*$^-$ gene resulted in mutation to kanamycin resistance (Kan^R). Lysogens containing a single λ*kan*$^-$ prophage per bacterial genome were grown in synthetic medium with limiting amino acids and stored at 15°C or 37°C. In the absence of VSP repair, Kan^R mutants accumulated at the rate of approximately 1.3×10^{-7} per bacterium per day at 37°C. This is similar to the 5meC → T mutation rate reported for DNA in solution. In *vsr*$^+$ bacteria, the Kan^R accumulation rate was 3×10^{-9} per bacterium per day, which is not significantly higher than the rate observed when the target cytosine was unmethylated. The increase in Kan^R mutants was barely detectable in *vsr*$^+$ cultures stored at 15°C for 4 months. It is likely that mutation hot spots at 5meC in rapidly dividing cells are attributable to insufficient time for T·G correction in the interval between deamination of 5meC and subsequent DNA replication. DNA synthesis occurred in bacteria starved for amino acids and this synthesis was not highly mutagenic.**

Although DNA is a relatively stable biological molecule, the cytosine moieties are subject to spontaneous deamination to uracil. Uracil is removed from DNA by uracil glycosylase, which appears to be present in all living cells. The DNA of many bacterial and eukaryotic species contains 5-methylcytosine (5meC) in addition to cytosine. Deamination of 5meC produces thymine, which is not recognized by uracil glycosylase and consequently can result in C → T mutations. In *Escherichia coli* and related members of the *Enterobacteriaceae,* the product of gene *dcm* methylates the second cytosine in the sequence 5′-CC(A or T)GG-3′ (1). 5meC is the site of mutation hot spots in gene *lacI* of *E. coli* and gene *cI* in λ prophage (2, 3). In mammals, most cytosine methylation occurs in CpG doublets, and C → T mutation at these sites accounts for almost 50% of p53 mutations in colon cancer cells (4). In addition, about one-third of single base pair mutations causing genetic disease are attributable to deamination of 5meC (5). Since deamination of 5meC is expected to be time-dependent, it is a potential source of mutations that may contribute to the senescence of cells and individuals (6).

The accumulation of C → T mutations at 5meC can be used to study the rate of spontaneous deamination at physiological temperatures. The rate of mutation at 5meC has been determined for plasmids (7) and also at 5-hydroxymethylcytosine (5OHmeC) in bacteriophage particles (8). We know of no studies of the rate of deamination of 5meC in stationary phase bacteria, which may be compared with nondividing cells of higher organisms. In starved *E. coli*, condensation of the DNA into a nucleoid is correlated with the production of large amounts of histone-like proteins, and bacteria become more resistant to stresses (for a review, see ref. 9). Spermine and spermidine, for example, protect DNA from single-strand breaks produced by singlet molecular oxygen (10). We wished to determine whether the 5meC in the DNA of starved bacteria was as susceptible to deamination as DNA in solution or packaged in phage capsids.

Adjacent to *dcm* on the bacterial chromosome is gene *vsr*, whose product is required for the correction of the T·G mispair present after deamination of 5meC (11). Vsr nicks 5′ to the mispaired T, which is recognized when it is in contexts related to the methylatable sequence. *In vitro*, only Vsr is required for nicking, but in bacterial cultures, specific T·G repair is reduced severalfold by mutations in genes *mutL* or *mutS*. DNA polymerase I and ligase accomplish the removal and resynthesis of a few nucleotides 3′ to the nick; the entire process is called VSP (very short patch) repair (reviewed in ref. 12). The existence of mutation hot spots at 5meC in cultures of replicating bacteria (2, 3) shows that a considerable fraction of thymines arising by deamination are not corrected by VSP repair. However, in its absence, the frequency of mutants at 5meC increases about 10-fold, so VSP repair obviously has an important antimutagenic function (3). In crosses between phage λ mutants, T·G mispairs can occur in DNA heteroduplex molecules. VSP repair of T·G mismatches present in the appropriate contexts can be increased by the presence of a multicopy *vsr*$^+$ plasmid, and by agents that decrease the rate of phage replication (13). In nature, enteric bacteria proliferate slowly in the intestines of their hosts or languish in ponds or waterways, where nutrients may be scarce. The average doubling time for enteric bacteria in nature is estimated to be about 40 hr (14). One might anticipate that the probability of VSP repair of T·G mismatches resulting from deamination of 5meC would be higher in nonreplicating than in rapidly dividing bacteria. On the other hand, proteins that accumulate during the stationary phase may decrease the accessibility of T·G mispairs, reducing the efficiency of VSP repair. A 5-fold accumulation in *E. coli* of the DNA-binding protein H-NS during stationary phase is correlated with a significant reduction in transcription of some genes, attributable to promoter blocking (15). Consequently, Vsr activity, which is apparently

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Abbreviations: 5meC, 5-methylcytosine; VSP, very short patch; Kan^S, kanamycin sensitive; Kan^R, kanamycin resistant; 5OHmeC, 5-hydroxymethylcytosine.

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suboptimal in replicating bacteria, might be reduced further in stationary phase cells.

We have found that in nondividing *E. coli* lacking VSP repair, the rate of mutation at 5meC (which reflects the deamination rate) was very similar to the deamination rate of 5meC in DNA in solution. The *in vivo* mutation rate also approximated the rate of mutation due to deamination of 5OHmeC in T4 bacteriophage particles. A 50-fold reduction in the rate of mutation at 5meC in Vsr^+ bacteria indicates that VSP repair continued during storage at 37°C. The phenotypic expression of kan^+ mutations in nondividing bacteria is evidence that both DNA and protein synthesis occurred during starvation.

MATERIALS AND METHODS

Bacteria, Plasmids, and Media. Bacterial strains and plasmids are listed in Table 1. M9 salts and LB (Luria broth) medium were as described (18). Supplemented M9 medium (M9S) contained 1% glucose and 0.6 mg/ml Bacto casamino acids. Ampicillin (50 mg/ml) was added to media for bacteria containing amp^R plasmids. LA (Luria agar) plates contained LB with 1% Bacto agar. Kan plates (LA plus 50 μg/ml kanamycin sulfate) were stored at 4°C for a period not exceeding 10 days.

Culture and Storage Conditions. Preliminary experiments indicated that lysogenic bacteria stored in M9 medium, with growth limited by the availability of required amino acids, survived longer than bacteria stored in LB (data not shown). Individual flasks containing 50–200 ml of M9S medium were inoculated with fewer than 10^7 kanamycin-sensitive (Kan^S) bacteria to avoid introducing kanamycin-resistant (Kan^R) mutants. Incubation was at room temperature until the bacterial concentration reached about 10^7/ml. Cultures were then shaken at 37°C until the turbidity remained constant for 60 min (bacterial concentration approximately 10^9/ml). Four individual 50-ml cultures or four 50-ml portions of larger cultures were stored in glass bottles with loose caps in incubators at 37°C or 15°C. Cultures were shaken only before each sampling to resuspend settled cells; no additional ampicillin was added to plasmid-carrying cultures during the storage period.

Detection of Kan^R Mutants. For zero-time assays, samples (1–10 ml) from Kan^S cultures were concentrated by centrifugation to approximately 0.2 ml, spread on Kan plates, and incubated at 37°C. Samples from stored cultures were also assayed after growth in broth, as described below. In the case of cultures with a low frequency of Kan^R mutants (i.e., cultures stored at 15°C), the large number of bacteria spread per plate resulted in a thin lawn of bacterial growth in addition to discrete Kan^R colonies.

Table 1. Bacterial strains, plasmids, and phage

	Relevant genotype	Ref.
Strain		
GM30	dcm^+, vsr^+, *supE44*, *thr-1*, *leuB6*, *hisG4*	13
GM31	As GM30, but *dcm-6*	1
GM2142	As GM31, but $supE^+$	13
RP4182	Δ(*dcm*, *vsr*) his^-, $trpA^-$	16
Plasmid		
pDCM21	dcm^+, amp^R	11
pKanS-D94	kan^S, amp^R	17
Phage		
λKanS	kan^S, $cIind^-$	This paper
λN⁻KanR	*Nam53*, *cIts857*, kan^R	This paper

RESULTS

A Selective System for Measuring Rates of Spontaneous Mutation at 5meC. The *kan* gene from Tn*5* codes for a kanamycin–neomycin phosphotransferase. A small number of Kan^R bacteria can be detected among a large number of Kan^S bacteria by plating on LA medium containing kanamycin. A site for cytosine methylation by Dcm was created in kan^+; as shown in Fig. 1, a codon for leucine (no. 94) was replaced with a proline codon, resulting in loss of enzyme activity (17). An *Xho*I fragment containing the mutant *kan* gene from plasmid KanS-D94 (17) was cloned into the unique *Xho*I site of phage λ DNA. A $cIind^-$ derivative of this construct, designated λKanS, was used to prepare lysogens containing a single prophage per bacterial chromosome (19). Deamination of 5meC in the Pro codon in the prophage's kan^- gene produces T paired with G. If VSP repair replaces the mismatched T with C before the strand replicates, mutation to kan^+ is avoided. Replication of the T-containing strand changes the anticodon for Pro to the anticodon for Leu, and leads to expression of the Kan^R phenotype (Fig. 1).

Survival of Kan^S and Kan^R Lysogens During Storage. To validate use of frequencies of Kan^R mutants in stored Kan^S cultures for the calculation of mutation rates, we determined whether Kan^R mutants had a selective advantage or disadvantage under the storage conditions. Spontaneous Kan^R mutants were obtained by plating an aliquot of one of the lysogens used in this study, GM31(λKanS)pDCM21, on Kan plates. Ten Kan^R colonies were used as the inoculum for a Kan^R culture. The Kan^S and derived Kan^R lysogens were then grown under the conditions used for storage experiments. Before storage, Kan^R bacteria were added to an aliquot of the Kan^S culture to produce a ratio of about 1 Kan^R per 10^6 Kan^S bacteria. The mixture, and another aliquot of the Kan^S culture, were both stored at 37°C for 9 days, during which time the number of viable bacteria declined 63%. The frequency of Kan^R in the mixture was corrected for new mutations by subtracting the frequency of Kan^R mutants in the Kan^S culture. In the stored mixed culture, the (corrected) frequency of Kan^R bacteria was 7.9×10^{-7} on day 1 and 6.1×10^{-7} on day 9, indicating no selection for or against the mutant bacteria. A similar experiment was performed with a mixture of Kan^R and Kan^S

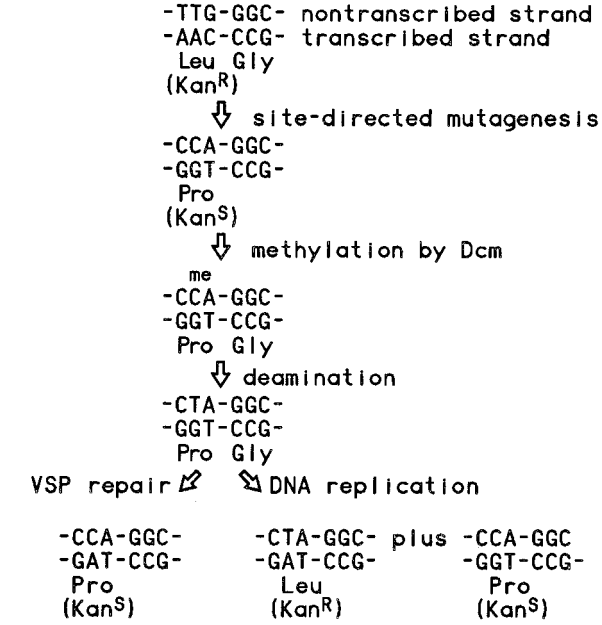

FIG. 1. Genetic assay for deamination and VSP repair at 5meC in the *kan* gene.

bacteria stored at 15°C. After storage for 21 days (64% survival), there was no significant change in the ratio of Kan^R/Kan^S lysogens in the mixture (data not shown).

Expression of New Kan^R Mutations in Stored Cultures. It is important to keep in mind that the target cytosine in these studies was on the nontranscribed DNA strand. Deamination of 5meC in the *kan*$^-$ gene, producing thymine, results in a kan$^+$ anticodon only after DNA replication replaces the G in the transcribed strand with A. We anticipated that expression of the Kan^R phenotype in starved bacteria would require growth in a supplemented medium to allow DNA replication, transcription, and subsequent synthesis of the active kanamycin phosphotransferase. Bacterial samples from stored cultures were diluted 2-fold in LB and incubated at 37°C with shaking. The concentrations of Kan^R and Kan^S bacteria were determined immediately before dilution ("zero-point" mutants), and at intervals during incubation in LB. The highest frequency of Kan^R mutants usually was obtained after incubation for 60–90 min. Longer incubation times gave lower mutant frequencies due to bacterial division and segregation of chromosomes containing *kan*$^+$ and *kan*$^-$ alleles (Fig. 1). Fig. 2 presents typical data showing the delayed expression of kan$^+$ mutations arising in cultures stored at 37°C. At each sampling time, approximately one-half of the *total* number of mutants (i.e., mutants detected after incubation in LB for the optimal period) were expressed before transfer to a rich medium. The data points presented in Figs. 3–7 represent the maximal Kan^R frequencies obtained *after* incubation of samples in LB.

To determine whether (limited) growth of Kan^S bacteria on Kan plates was responsible for the zero point Kan^R mutants, lysogens that had been stored at 37°C for 13 days were spread on Kan plates and then washed off the plates immediately after plating (zero-time) or after incubation at 37°C. The number of colony-forming bacteria relative to zero-time was reduced 75% after 90 min and 99% after 120 min, indicating that Kan^S bacteria did not undergo cell division. We then considered the possibility that although they did not divide, starved Kan^S bacteria might have survived on Kan plates long enough to replicate their DNA and express any accumulated *kan*$^+$ mutations. To detect possible phenotypic expression of kanamycin resistance in bacteria on Kan plates, we employed strain GM2142, a nonlysogenic derivative of the Vsr$^-$ strain GM31. Bacteria that had been stored at 37°C for 15 days were mixed with λN$^-$KanR and plated immediately on Kan plates, or

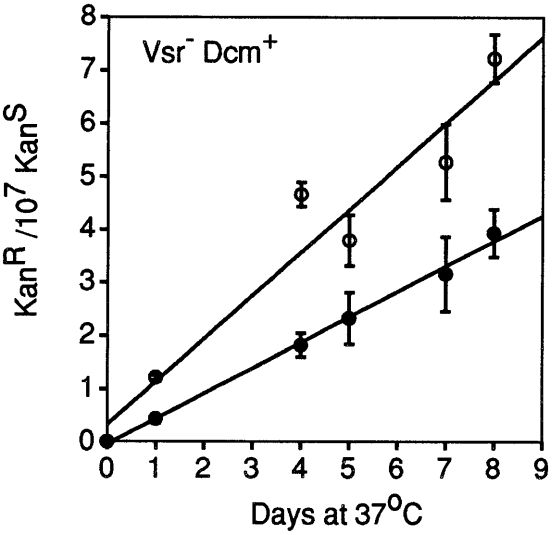

FIG. 2. Increase in the frequency of Kan^R mutants during storage of GM31(λKanS)pDCM21 at 37°C. Solid symbols, zero-point mutants; open symbols, Kan^R mutants after incubation of stored bacteria in LB. Curve fitting was by the method of least squares.

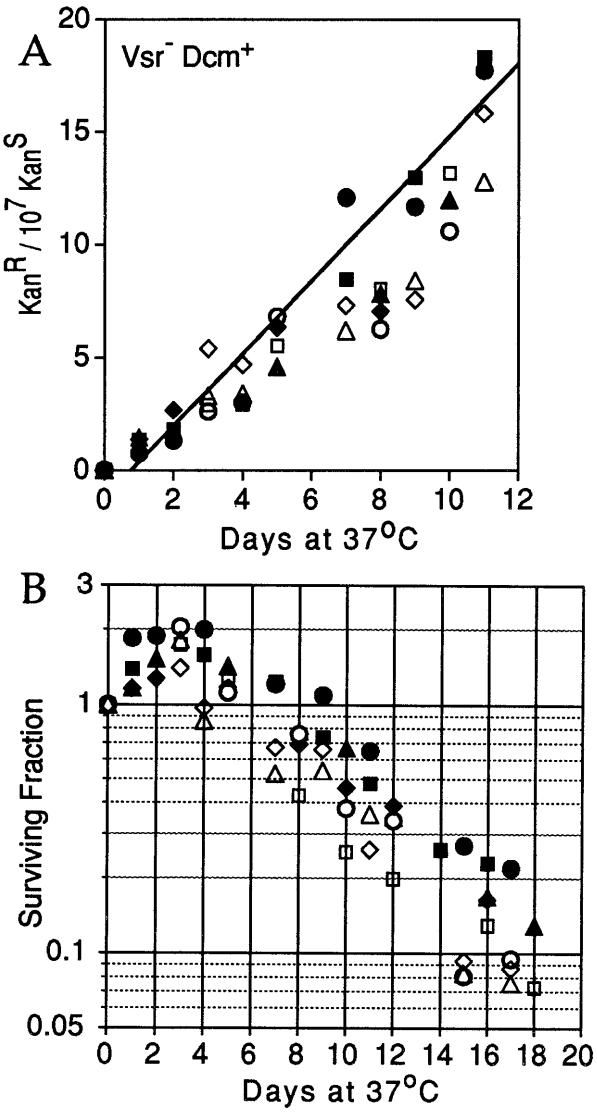

FIG. 3. (*A*) Increase in the frequency of Kan^R mutants in GM31(λKanS)pDCM21 at 37°C. Solid symbols, cultures stored in the M9S growth medium; open symbols, portions of the same cultures resuspended in M9 before storage. (*B*) Survival of the cultures shown in *A*. For cultures shown as solid symbols, the average number of Kan^S per ml decreased from 1.3 to 1×10^9 between day 1 and day 7. In the same interval, the average number of Kan^R per ml increased from 179 to 962.

plated after growth in LB. λ*N*$^-$ mutants form plasmids in GM2142 but do not kill the host. Kan^R colonies appeared on Kan plates only when the infected bacteria were first allowed to grow in LB for at least 60 min (data not shown). The time required for 90% of λ particles to adsorb and transfer their DNA through the bacterial membrane is approximately 5 min at 37°C (20). Since such rapid transfer can occur even through isolated bacterial membranes (21), it is very likely that only a few minutes were required for λ DNA to enter the bacteria. Because newly injected *kan*$^+$ genes were not expressed in bacteria spread on Kan plates, we concluded that the Kan^R mutants detected before growth in supplemented medium resulted from mutation expression during storage, and not after plating on the selective medium.

Mutation at 5meC in Kan^S Bacteria Stored at 37°C. The increase in frequency of Kan^R mutants during storage was studied using two different lysogenic strains in which the target cytosine was methylated but VSP repair was absent. Strain GM31 (Fig. 3) carries the *dcm-6* mutation that destroys

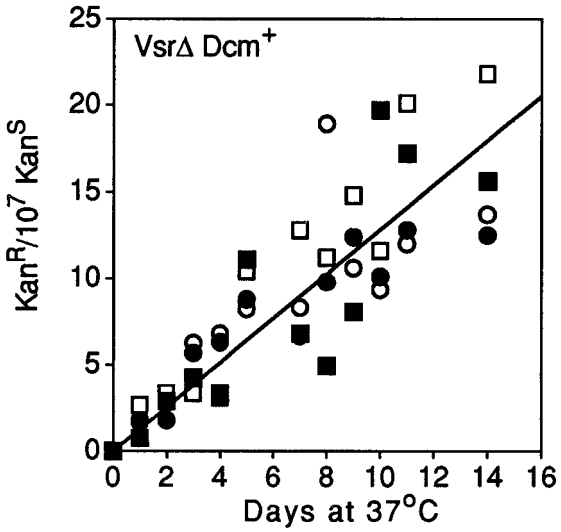

FIG. 4. Increase in the frequency of Kan^R mutants in RP4182(λKanS)pDCM21 cultures stored at 37°C. Open and solid symbols refer to two aliquots of the same culture that were stored separately.

methylase activity and reduces VSP repair by 90% (22). A second Kan^S lysogen (Fig. 4) has a deletion that removes *dcm, vsr*, and adjoining genetic material. In both lysogenic strains, cytosine methylase was provided by *dcm*$^+$ on plasmid pDCM21. Data points indicate Kan^R frequencies corrected for Kan^R mutants present at the start of storage. An unexpected finding was large variation between individual cultures, and even between portions of the same culture stored separately. Survival of GM31 lysogens is shown in Fig. 3*B*; similar survival curves were obtained for all cultures stored at 37°C (data not shown). The bacterial concentration doubled during the first 1–2 days of storage, and began to decline after 4–5 days.

During 11 days of storage at 37°C, the frequency of Kan^R mutants increased at approximately the same rate in the two Vsr$^-$ lysogens (Figs. 3*A* and 4). The similarity of mutation rates in the two strains indicates that there was no significant VSP repair in the *dcm-6* bacteria. Reliable data for longer storage times were difficult to obtain because of plasmid loss; all plotted data are from culture samples in which 90% or more of the bacteria retained the *dcm*$^+$ plasmid, and cell survival was 10% or more.

C → T Mutation in the Absence of Cytosine Methylation. Cultures of *dcm-6* lysogens were stored at 37°C and assayed for Kan^R frequency at weekly intervals. Over a period of 28 days, the frequency of Kan^R mutants increased at the rate of 1.8×10^{-9} per lysogen per day (Fig. 5*A*). Kan^R bacteria accumulated at a similar rate in lysogenic cultures containing a control plasmid (data not shown). Mutation to kanamycin resistance in bacteria in which the target cytosine is not methylated is attributable to replication errors, to deamination of cytosine to produce uracil (which is usually removed by the product of the *ung* gene), or to other DNA damage.

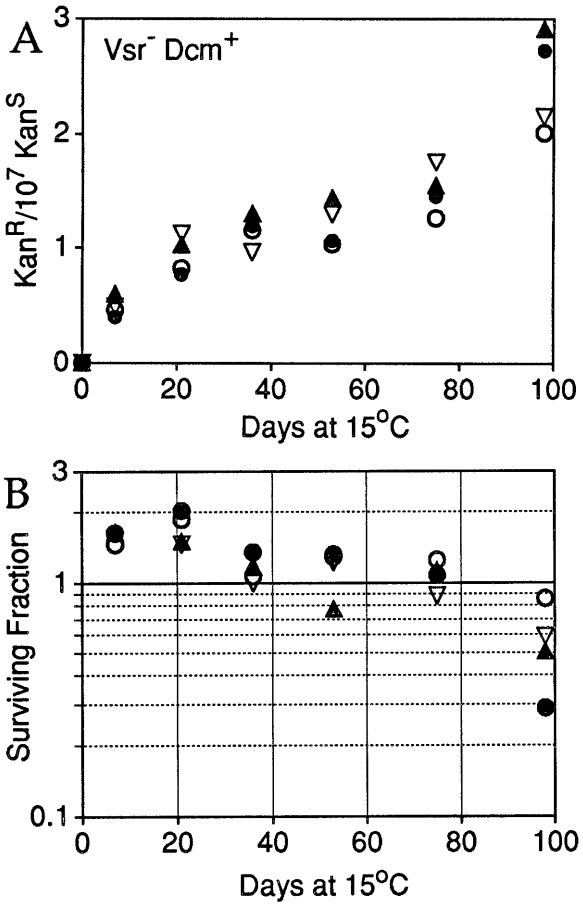

FIG. 6. (*A*) Increase in the frequency of Kan^R mutants in GM31(λKanS)pDCM21 cultures stored at 15°C. (*B*) Survival of the cultures shown in *A*. Between days 7 and 53, the average number of Kan^S per ml decreased from 1.3 to 1×10^9/ml, whereas the average number of Kan^R per ml increased from 43 to 63.

Mutation to Kan^R in *dcm*$^+$*vsr*$^+$ Lysogens. Kan^R mutants accumulated at approximately the same rate in cultures containing wild-type *dcm* and *vsr* (Fig. 5*B*) as in *dcm*$^-$*vsr*$^-$ cultures

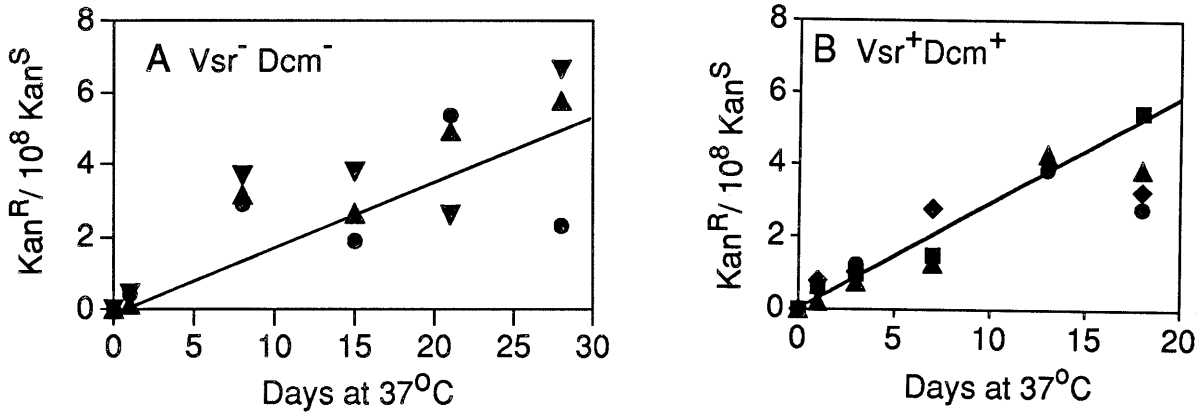

FIG. 5. (*A*) Increase in the frequency of Kan^R mutants in GM31(λKanS) cultures stored at 37°C. (*B*) Increase in the frequency of Kan^R mutants in GM30(λKanS) cultures stored at 37°C.

Table 2. Summary of mutation rates

Temperature	Bacterial genome	Plasmid	*kan*$^+$/*kan*$^-$/day*
37°C	*dcm-6*	p*dcm*$^+$	1.1×10^{-7}
37°C	Δ*dcm*, *vsr*	p*dcm*$^+$	9.3×10^{-7}
37°C	*dcm*$^+$, *vsr*$^+$		2.0×10^{-9}
37°C	*dcm-6*		1.3×10^{-9}
15°C	*dcm-6*	p*dcm*$^+$	1.8×10^{-9}
15°C	*dcm*$^+$, *vsr*$^+$		1.0×10^{-10}
15°C	*dcm-6*		ND

ND, Not detectable.
*Corrected for an average of 1.4 chromosomes per Kan^S bacterium.

(Fig. 5*A*). To confirm the persistence of cytosine methylation in the stored bacteria, DNA was extracted from a culture after storage for 20 days and tested for sensitivity to cleavage by *Eco*RII, which cuts only unmethylated CC(A or T)GG sequences. No digestion by *Eco*RII was detected, indicating that extensive demethylation had not occurred.

Mutation at 5meC at 15°C. In *dcm-6* lysogens containing pDCM21 that were stored at 15°C, we observed a doubling of bacterial concentration during the first 21 days. (Fig. 6*B*). During this period, the rate of appearance of Kan^R mutants was 5×10^{-9} per bacterium per day (Fig. 6*A*). In the period from 21 to 75 days, this rate decreased to 1×10^{-9} per bacterium per day. Between days 98 and 123, when bacteria started to die, Kan^R mutants appeared at the faster rate observed during the initial 21 days of storage. Thus, the rate of appearance of Kan^R mutants at 15°C was less than 1/10th the rate observed at 37°C.

Fig. 7 shows that *dcm*$^+$*vsr*$^+$ cultures accumulated Kan^R mutants at the rate of about 1.3×10^{-10} per bacterium per day, which is at the limit of detectability in our system. Thus, VSP repair must be functioning at 15°C, a temperature which approximates some normal environments of *E. coli*, such as sewers or ponds. We were unable to detect any increase in Kan^R mutants in *dcm-6* lysogens stored at 15°C for 140 days (data not shown).

DISCUSSION

The Rate of Deamination of 5meC Is Similar in Starved Bacteria and dsDNA Stored *in Vitro*. We have studied the rate of appearance of Kan^R mutants in populations of nondividing bacteria in which deamination of a 5meC results in mutation from kanamycin sensitivity to resistance. To allow phenotypic expression of all mutations, starved bacteria were cultured in a rich medium for a short time before plating on the selective medium. The frequency of Kan^R mutants can be equated with the 5meC deamination frequency if the starved bacteria each contained only one copy of the *kan* gene. When *E. coli* were grown to stationary phase in M9 medium, the majority of bacteria contained only one chromosome equivalent, and 35–40% contained two chromosomes (23). At 37°C, Kan^R mutants accumulated at a constant rate of $1.3–1.5 \times 10^{-7}$ per bacterium per day. Correction of this mutation rate to reflect the presence of two chromosomes in one-third of the bacteria gives a deamination rate of about 1×10^{-7} per 5meC per day. This is quite similar to the rate of mutation (6×10^{-8} per 5meC per day) observed by Shen *et al*. (7) at a 5meC in plasmid DNA stored at 37°C. In an earlier study, Baltz *et al*. (8) studied reversion of a T4 *rII* mutation, which required a C·G to T·A transition (8). In T4, all cytosines are replaced by 5OHmeC. Deamination of 5OHmeC produces 5-hydroxyuracil, which mispairs with guanine, resulting in a transition mutation. In the phage stock stored at 37°C, *rII*$^+$ mutations appeared at a rate of 3×10^{-7} per phage per day, which is about 3-fold higher than the rate we observed for mutation at 5meC.

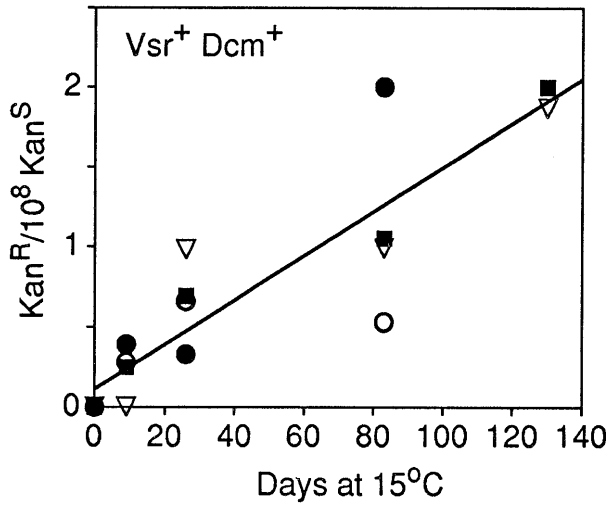

FIG. 7. Increase in the frequency of Kan^R mutants in GM30(λKanS) stored at 15°C.

It is not possible to attribute the relatively small differences in deamination rates at 37°C in DNA, phage particles, and bacteria to a specific factor. The fact that the mutation rate at 5meC was as high in starved bacteria as in plasmid DNA suggests that the bacterial DNA was not protected from deamination by histone-like proteins. The context of the methylated cytosine was different in each study, and context influences the deamination rate (8, 24). DNA configuration, e.g., tight packing inside a phage capsid, may also alter the probability of deamination. The low rate of mutation at 5meC in Vsr$^-$ bacteria stored at 15°C was unexpected, and suggests that a repair system other than VSP may be available, or that the poststorage growth period was not sufficiently long to allow expression of all *kan*+ mutations.

VSP Repair Is Efficient in Starved Bacteria. The presence of *vsr*$^+$ caused a 50-fold reduction in the rate of spontaneous mutation at the site of a 5meC (Table 2). This reduction is even greater than the 20-fold reduction in mutation at 5meC observed in replicating *E. coli* when excess Vsr$^+$ was present (3). The rate of mutation to Kan^R in nondividing *dcm*$^+$*vsr*$^+$ (wild-type) bacteria at 37°C was only 50% higher than the mutation rate in *dcm-6* lysogens, where *kan*$^+$ mutations are caused by events other than deamination of 5meC. Because of the scatter in the data points, this difference may not be significant. At 15°C, VSP repair reduced mutation at 5meC to a barely detectable level. Thus, 5meC is not a hot spot for spontaneous mutation in nondividing Vsr$^+$ bacteria.

VSP repair in wild-type *E. coli* is largely dependent on MutS and MutL, since loss of either function reduces the frequency of VSP repair significantly (25, 26). MutL and MutS are essential for the correction of DNA replication errors (reviewed in ref. 27). It has been suggested that DNA replication accompanied by a shortage of MutS and/or MutL could account for the high rate of mutation observed in some starved bacteria (28–30). MutS protein appears to be very unstable in bacteria grown to maximum density in an enriched mineral salts plus glucose medium (31). However, in our experiments, growth was limited by the availability of required amino acids. The low rate of mutation at 5meC, which we observed in wild-type bacteria, suggests that levels of MutS and MutL adequate for VSP repair were available, or that these proteins were not required for VSP repair under starvation conditions. Furthermore, the low mutation rate observed in stored *dcm*$^-$ cultures (see below) would not be expected in the absence of replication error correction.

DNA and Protein Synthesis in Nondividing *E. coli*. Mutation in nondividing *E. coli* was studied intensively by F. J. Ryan more than 40 years ago (32). Although he failed to obtain

direct evidence for DNA synthesis in starved bacteria, Ryan deduced that some DNA turnover must occur (33). Recent studies of mutation in starved bacteria suggest strongly that DNA synthesis in nondividing cells can account for these mutations (34, 35). Expression of *kan*$^+$ mutations in the experiments reported here required both DNA synthesis (to transfer the new information to the transcribed strand) and protein synthesis (to manufacture the active enzyme that confers kanamycin resistance). Data in Fig. 2 show that the *total* number of Kan^R mutants that were present at day t were detectable as zero-point mutants at approximately day $t + 3$. Thus, the starved bacteria must have replicated the mutation site once every 3 days. Since no increase in genome equivalents per cell occurred in bacteria stored under similar conditions (23), we are led to conclude that there was DNA turnover in the nondividing bacteria. DNA synthesis in our starved bacteria obviously was not very error-prone, since in Dcm$^-$ bacteria, *kan*$^+$ mutations occurred at a rate of less then 2×10^{-9} per bacterium per day at 37°C and less than 2×10^{-10} per bacterium per day at 15°C. The high mutation rates responsible for "adaptive mutation" (36) must be attributed to factors other than DNA replication during starvation *per se.*

Slow T·G Repair Accounts for Frequent Mutation at 5meC in Rapidly Dividing Bacteria and Eukaryotic Cells. In higher organisms as well as bacteria, a directed system of mismatch repair detects mispairs caused by replication errors, and removes several hundred nucleotides, including the mispaired base, from the newly synthesized strand. This strand is identified by the presence of nicks or the absence of methylation at specific sequences (27). In addition, human cells contain a glycosylase that recognizes the T·G mispair and preferentially removes the T (37). If, as in *E. coli*, the specific enzyme for T·G recognition is in short supply, replication may occur before removal of the T produced by deamination of 5meC. In tissues where cells divide rarely if at all, there is a long period between gene replications and one would expect little mutation at 5meC. Inefficiency of T·G correction can account for mutation hot spots at 5meC that occur in gene p53 in tumors of organs whose cells are dividing rapidly: colon, stomach, endometrium. Mutation at 5meC is less frequent in tumors of organs whose cells divide less frequently (4). These differences in mutation frequency are not attributable to differences in cytosine methylation (38). A very efficient system of biased correction of T·G to C·G would be mutagenic, because it would compete with mismatch repair of replication errors (12). Thus, mutation hot spots at 5meC may be an unavoidable consequence of cytosine methylation in cells that frequently divide.

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