Demonstration of the occurrence of spontaneous mutations in nondividing cell populations
INTRODUCTION

Spontaneous mutations are thought to arise as errors in replication of the genetic material when cells increase their numbers. These errors that are either blind to or escape the correction mechanisms then become fixed as mutations in the genome. Can spontaneous mutations also arise in nondividing cells where there is no net DNA replication? An early study by Ryan in 1955 suggested that His\(^+\) revertants could arise in populations of nondividing His\(^-\) cells of *Escherichia coli* starved for the amino acid (204). Recent interest in this question was revived by Cairns et al. in 1988 with the controversial claims, not only that mutations can occur in starving cell populations that are subjected to non-lethal selections but also that such mutations are "directed" to the loci under selection (40).

The major difficulty in studying the existence of stationary-phase mutations is that the same mutations could also have occurred when the cells were dividing to give rise to the population in the stationary phase, so that it is only by the time of appearance of the mutants that the two classes can be separated. In the results presented in this chapter, an approach that uses a spatial rather than temporal distinction between these two classes of mutants was used. For this, a novel conditional-lethal strategy was devised to selectively eliminate revertants for a particular mutation that arose during the exponential growth of a population, so that any revertants that arose in the population in stationary phase could then be studied.

In this strategy, the parental population of cells is able to grow under both restrictive and permissive conditions whereas mutants at a particular locus arising in that population are killed under the former condition and survive under the latter (Fig. 3.1A). Thus, if the parental population is grown to stationary phase under the restrictive condition, all mutants of the class under study that arose during the phase of exponential growth will be killed. If the population is then subjected to selection under permissive conditions, the only mutants that would survive and be scored are those which arose after shift to the latter condition, i.e., in the stationary phase.

The above strategy was used in two different ways to examine the occurrence of stationary-phase mutations (Fig. 3.1B). Both involve Lac\(^-\) parental populations of cells grown as colonies on agar plates, and the mutations under study are reversions to Lac\(^+\), with independent revertants being scored as papillae on the surface of the colonies. In the first, the target reversion is the precise excision of a transposition-defective insertion element (encoding Kan\(^r\)) situated in the coding region of the *lacZ* gene (*lacZ::Tnl10dKan*); conditional
Fig. 3.1

A.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Parent</th>
<th>Mutant/Revertant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permissive</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Restrictive</td>
<td>+++</td>
<td>KILLED</td>
</tr>
</tbody>
</table>

**Fig 3.1A Conditional-lethal strategy.** At the permissive condition both parent and the revertants of that particular strain can grow whereas at the restrictive condition only the parent can grow, but not the mutant. +++ means growth.

B.

<table>
<thead>
<tr>
<th>Parent</th>
<th>Example I</th>
<th>Example II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>lacZ::Kan, Kan' (Ts)</td>
<td>lacZ (any), galE (Ts)</td>
</tr>
<tr>
<td>Mutant</td>
<td>lac+, kan' (Ts)</td>
<td>lac+, galE (Ts)</td>
</tr>
<tr>
<td>Growth medium</td>
<td>primary carbon source + lactose + Kan</td>
<td>primary carbon source + lactose</td>
</tr>
<tr>
<td>Permissive condition</td>
<td>30°</td>
<td>30°</td>
</tr>
<tr>
<td>Restrictive condition</td>
<td>42°</td>
<td>42°</td>
</tr>
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**Fig. 3.1B Two examples of conditional-lethal strategy.** In both systems the reversions of a lac− parent are studied. The strategy is described in detail in text.
lethality of Lac\(^+\) revertants (on Kan-containing medium) was achieved with the presence of a Kan\(^r\) (Ts) allele at another site so that the Lac\(^+\) revertants are Kan\(^r\) at 42\(^\circ\) and Kan\(^r\) at 30\(^\circ\) (while the Lac\(^-\) population is Kan\(^r\) at both temperatures).

In the second example, a temperature-sensitive \textit{galE} mutation was used to study the stationary-phase reversion of a variety of Lac\(^-\) mutations to Lac\(^+\); it is known that Lac\(^+\) \textit{galE}, but not Lac\(^-\) \textit{galE}, strains are killed upon exposure to lactose [because of the generation of galactose from lactose in the Lac\(^+\) \textit{galE} cells, to which the \textit{galE} mutants are sensitive, (10)]. Using both the strategies described above, it has been demonstrated in this study that several kinds of Lac\(^-\) mutations can revert to Lac\(^+\) in nondividing populations of cells within a colony.

The results presented in this chapter are organized into two parts. Part A describes the first application in the conditional-lethal strategy, i.e., the use of Kan\(^r\) (Ts) system in demonstrating that spontaneous precise excision events occur in nondividing cells. In Part B, the isolation of a temperature-sensitive \textit{galE}(Ts) mutation and its use in the study of stationary-phase mutagenesis is described.

**RESULTS**

3.1 **Part A: Use of Kan\(^r\) (Ts) system to demonstrate the occurrence of spontaneous precise excision events in nondividing cells**

3.1.1 **Construction of strain GJ1823**

Strain GJ1823 was constructed from the wild-type prototrophic \textit{E.coli} strain MG1655 (19) in two successive steps of P1 phage transduction (also described in Section 5.1), the first one involving the transfer of \textit{zbi-900::Tn10dKan(Ts)} i.e., Kan\(^r\) (Ts) mutation situated in the 17 min region from strain GJ1170 with Kan\(^r\) selection at 30\(^\circ\); and the second involving the transfer of a \textit{lacZ4525::Tn10dKan} insertion situated in the 8 min region from GJ513 with Kan\(^r\) selection at 42\(^\circ\). Both the Kan\(^r\) (Ts) as well as the \textit{lacZ4525::Tn10dKan} (hereinafter referred to as \textit{lacZ::Kan}) mutations had been obtained earlier by other workers in this laboratory.

Strain GJ1823 forms Lac\(^-\) colonies and is capable of giving rise to Lac\(^+\) revertants following spontaneous precise excisions of the Tn10dKan element inserted in the \textit{lacZ} gene. However, on Kan-supplemented plates this strain can give rise to Lac\(^+\) reversions only at 30\(^\circ\) but not at 42\(^\circ\), because of the remaining Kan\(^r\) (Ts) allele on the chromosome.
3.1.2 Strategy to study stationary-phase mutagenesis

Strain GJ1823 was used to study stationary-phase mutagenesis utilizing the conditional-lethal selection strategy (as described in the introduction). In this strategy, the Lac− population of GJ1823 cells would be grown to stationary-phase at 42° on Kan-supplemented medium, so that the spontaneous Lac+ revertants that arise in the log-phase of growth would be killed; the plates would then be temperature-downshifted to 30°, wherein the Lac+ revertants, if any, that would arise subsequently would be selectively recovered [because of the presence of the KanT (Ts) allele] and they would represent stationary-phase excision mutations. These Lac+ mutants can be scored as papillae on the surface of the colonies grown on medium containing lactose as a second carbon source (which remains unutilized by the Lac− population).

3.1.3 Conditional-lethal phenotype of KanT (Ts) allele in strain GJ1823

On Lac+ papillation medium supplemented with Kan, the frequency of Lac+ papillae in colonies of GJ1823 at 42° was less than 1% that at 30° and also less than 1% that at 42° itself in the absence of Kan. These values provide an estimate of mutations resulting from (i) reversions of KanT (Ts) to KanT, by either mutation or gene conversion, and (ii) duplications of lacZ::KanT (with one copy subsequently reverting to Lac+ by precise excision while the other confers KanT at 42°).

3.1.4 Lac+ papillation assay of GJ1823 in nondividing cells

Lac+ papillation assays in nondividing cells of GJ1823 were done as described in Section 2.7.15. The GJ1823 cultures were grown at 42° in LB broth containing Kan and they were plated after appropriate dilution so as to get approximately 20 to 25 single colonies per plate (petri dish of 85 mm diameter) on LB medium supplemented with Kan, Xgal and 0.1% lactose. The plates were incubated either continuously at 30° (control) or at 42° for 3 days, followed by 5-7 days at 30°. The plates were scored for Lac+ papillation patterns, and quantitative analysis of the distribution of papillae was also done. The Lac+ papillation patterns in colonies of strain GJ1823 [KanT (Ts) lacZ::KanT] is shown in Fig. 3.3A. As explained in the following section, the papillae that arose in the central zone of the colony could be considered as the reversions that had occurred predominantly in the nondividing cells. However, differentiating the peripheral zone of dividing cells versus central zone of nondividing cells in single colonies was arbitrary, and for the purpose of clear demarcation
between these two zones, tex mutations, which increase the Lac\textsuperscript{+} reversions predominantly in dividing cells, were used as described below.

3.1.5 Use of tex mutations to demarcate zones of dividing and nondividing cells in colonies

The kinetics of growth of bacterial colonies on a solid surface have been described earlier (188, 243). Unlike those grown in liquid medium, cells in a colony do not exhibit a homogeneous stationary phase; instead, the central zone is comprised predominantly of nondividing cells following local exhaustion of nutrients in the medium beneath the colony, while the periphery represents a zone of actively dividing cells. To quantitate the relative proportions of dividing and nondividing cells in different zones of any colony incubated at 30° (after initial growth at 42°), Lac\textsuperscript{+} papillation itself was used as a marker. This was achieved by using tex mutator mutations (described in Chapter 5), which increase the frequency of lacZ::Kan\textsuperscript{r} precise excisions during exponential growth. None of the previously described tex mutations (such as mutH, -L, -S, uvrD or dam) had a sufficiently strong Tex phenotype for the Tn10dKan element and hence the new tex mutations (such as ssb-200 and uup-351) described in Chapter 5 were used for this purpose. As explained in Chapter 5, these newly obtained tex mutants exhibited a 5- to 12-fold increase in lacZ::Tn10dKan excision frequency (Table 5.1). The isolation and characterization of new tex alleles obtained in this study are described in detail in Chapter 5.

One of them (ssb-200) is in the gene encoding single-stranded DNA binding protein and creates a glycine\textsuperscript{+}to aspartate amino acid substitution at position 4 in the SSB protein, and another is an insertion mutation in the uup gene (uup-351::Tn10dTetl).

3.1.6 Demonstration of lacZ::Kan\textsuperscript{r} precise excisions in nondividing cells

The strain GJ1823 and its ssb (GJ1854.1, described in Chapter 5) derivative along with GJ1886 (carrying uup-351 mutation, described in Chapter 5) were plated on LB medium supplemented with Kan, Xgal and lactose (as described in Section 3.1.4). The Lac\textsuperscript{+} papillation patterns in single colonies were examined either after continuous incubation at 30° or after temperature downshift from 42° to 30° (Fig. 3.3A). As expected, papillae in colonies of the tex strains continuously grown at 30° occurred in much larger numbers than in the tex\textsuperscript{+} isogenic strain, and they were uniformly distributed over the colonies (top row, Fig. 3.3A). This pattern was altered to that of an annulus or ring in the mutator colonies that were grown initially at 42° and then shifted to 30° (bottom row, Fig. 3.3A). This pattern change itself
provides a validation of the conditional-lethal phenotype in the Lac\(^+\) revertants (with loss of papillae in the central zones of colonies on the temperature-shifted plates testifying to the efficacy of the killing of the corresponding Lac\(^+\) revertants that had arisen during the growth of these cells at 42\(^\circ\)).

To quantitate the distribution of papillae in different zones of a colony, the ratio of the number of papillae in the peripheral zone to that in the central zone of the temperature-shifted colonies for each strain was calculated. The ratio of the colony diameter at the time of temperature downshift to that at the end of incubation at 30\(^\circ\) was determined and that ratio was approximately 0.65. On photographs of these plates (continuously incubated at 30\(^\circ\) and temperature-shifted), each colony was demarcated into a peripheral zone and a central zone by concentric circles drawn to the same ratio, and the number of papillae in each zone was counted. Values for several individual colonies were then used to calculate the mean ratio of peripheral to central zone papillation for a strain, and the values are given in Table 3.1.

For the tex strains, the ratios of peripheral- to central zone papillation in the colonies subjected to temperature shift was around 5. If one assumes that the vast majority of papillae in the tex strains represent mutations that have occurred in growing cells, the distribution ratios for these strains suggest that at least 80% of all cell divisions in colonies following the temperature shift have occurred in the peripheral zone. This conclusion is in accordance with the results from earlier studies on colony growth kinetics.

If one further assumes that all lacZ::Kan\(^r\) precise excisions even in the tex\(^+\) parent occur only in dividing cells, one expects that the ratio of distribution of papillae would be similar for both tex\(^+\) and tex isogenic strains, although the absolute numbers of papillae would be much higher in the tex strains. However, this was not the case, as seen in Fig. 3.3A. Colonies of the tex\(^+\) strain that were incubated first at 42\(^\circ\) and then at 30\(^\circ\) did not exhibit an annular distribution pattern of papillae, and the results in Table 3.1 indicate that the ratio of peripheral to central zone papillation for this strain was only about one sixth that for the ssb strain.

The annular pattern of papillation observed in tex strains could be explained as an artifact, if these strains were to exhibit differential viability compared to its isogenic wild-type strain at 42\(^\circ\). However, control experiments (involving measurements of viability in colonies grown on medium without lactose supplementation) indicated that there was no
change in the viability of the stationary-phase populations between the tex+ and tex mutant strains.

The results above therefore indicate that only about 15% of the central zone papillation in the tex+ colonies may be accounted for on the basis of the excision frequency for dividing cells and from the number of dividing cells in this zone. The balance of 85% can hence be interpreted as the excision events that have occurred in nondividing cells of the central zone; or at the very least, they can be interpreted to be the reversions that are blind to the mutator effects of two different tex mutations, ssb and uup.

3.2 Part B: Isolation of galE(Ts) mutation and its application to study stationary-phase mutagenesis

3.2.1 Rationale to use the galE(Ts) strategy

The above described strategy involving Kanf (Ts) allele was useful for studying a certain class of mutational events, i.e., transposon precise excisions. In order to generalize the conditional-lethal strategy for studying the reversions of any "loss of function" mutations in the lacZ gene, another system was developed. For this purpose, a temperature-sensitive galE mutation was obtained and used in the present study. The rationale for employing a galE(Ts) mutation to examine lacZ reversions in nondividing cells is as follows: galE mutants are extremely sensitive to galactose (as they accumulate a toxic intermediate, UDP-galactose generated during the galactose metabolism), to the extent that Lac+ galE mutants are killed on exposure to lactose (because of galactose released upon lactose hydrolysis that is mediated by LacZ); on the other hand, Lac− galE mutants are lactose-resistant (10). Thus a strain that is Lac− and galE(Ts) will grow normally at restrictive temperature but all the Lac+ revertants that arise during growth would be killed; any revertants that arise after shifting the plates to the permissive temperature would be able to grow and they represent stationary-phase events.

3.2.2 Isolation of galE(Ts) mutation

Strain GJ2202 carries a lacZ(Am) mutation on F' 128, as well as a missense mutation (galE28) in galE which is linked 50% to the Kanf (Ts) mutation at min 17. Spontaneous Gal+ revertants of GJ2202 were selected at 30° on minimal-A plates supplemented with galactose as the sole carbon source. Approximately 150 Gal+ mutants so obtained were then screened for a Gal− Gal+ phenotype at 42° (as described in Section 2.7.11C), and in this manner seven temperature-sensitive mutants were obtained. One of the putative temperature-sensitive galE+
pseudorevertants so identified was designated GJ2203, and its \textit{galE(Ts)} allele was designated \textit{galE516(Ts)}.

3.2.3 Conditional-lethal phenotype of \textit{galE516(Ts)} mutant

To demonstrate that the \textit{galE516} mutation was effective in mediating galactose-induced lysis at 42°, the following experiment was done. GJ2203 [also carrying a multicopy plasmid bearing \textit{galE516(Ts)} allele, which is described in Section 3.2.4 below] was grown in minimal A-Casamino acids to mid-log phase at 30° and after addition of 0.1% D-galactose the culture was immediately split equally into two flasks; one flask was incubated at 30° and the other was shifted to 42°. At regular intervals, the viable counts (cfu/ml) of bacteria in each flask were determined. The data in Fig. 3.2 clearly demonstrate that the \textit{galE(Ts)} mutation was effective in inducing galactose mediated lysis of >99.9% of the cells at 42°, but had no effect at 30°.

On solid Lac\textsuperscript+ papillation media, it was expected that the strain GJ2203 can papillate only at 30° but not at 42°, because of the presence of the \textit{galE(Ts)} allele. For reasons that are not completely understood (which may perhaps be related to catabolite repression) the Lac\textsuperscript+ papillation studies employing \textit{galE(Ts)} mediated lethality could not be performed on LB-based media because galactose-sensitivity of GJ2203 could not be demonstrated in such media. Hence, the papillation studies on the \textit{galE(Ts)} strains were done on minimal synthetic medium supplemented with Casamino acids as the sole carbon source (a comparatively poorer medium than LB), and lactose which serves both as the alternative carbon source for the growth of the Lac\textsuperscript+ revertants and also for generating galactose by hydrolysis and thus causing the conditional-lethal phenotype.

However, when strain GJ2203 was used in papillation studies, it was found that even at 30°, it exhibited few and very small-sized papillae, presumably because the \textit{galE(Ts)} allele was not sufficiently Gal\textsuperscript+ at the permissive temperature to allow good growth of the papillae. The following manipulation was therefore undertaken to improve the papillation efficiency at the permissive temperature.

3.2.4 Obtaining a multicopy \textit{galE516(Ts)} plasmid by homologous recombination

In an attempt to overcome the above mentioned problem, the \textit{galE516(Ts)} mutation was transferred on to a multicopy plasmid as follows. In the first step, plasmid pHYD601 (which is a pBR322 derivative encoding Amp\textsuperscript+ and carrying \textit{\Delta galET\textsuperscript+ K\textsuperscript+} region) that had been constructed earlier in this laboratory, was introduced into the strain GJ2203. In the second
Fig. 3.2 Conditional-lethal phenotype of \textit{galE} (Ts) mutant, strain GJ2203 carrying pHYD603. Viable counts (cfu/ml) were determined as a function of time after addition of 0.1% D-galactose to the culture growing at 30° and split into two halves: one-half continuing to be incubated at 30° (o) and the other shifted to 42° (●). The time of addition of galactose is marked by an arrow.
step, a phage P1 lysate prepared on a GJ2203/pHYD601 transformant was used to transduce GJ2202 (which has galE28 on the chromosome, and is therefore Gal−) simultaneously to Amp′ and Gal+ at 30°, by selecting for transductants on galactose-minimal A plates containing ampicillin. In this way, plasmid derivatives on to which the galE516(Ts) mutation had been transferred by homologous recombination from the chromosome of GJ2203 were obtained. One such plasmid was designated as pHYD603. Strain GJ2203/pHYD603 exhibited satisfactory Lac+ papillation at 30° and continued to be Gal− Gal+ at 42°.

3.2.5 Isolation of spontaneous galP and mgl mutations

Unexpectedly, colonies of the galE(Ts) strain GJ2203 exhibited poor growth at 42° on lactose-supplemented medium, even though the strain is Lac− and is hence not expected to generate galactose from lactose. It was found that these Lac− cells were being killed at 42° by the galactose released following lysis of Lac+ mutants arising in the parental Lac− population (i.e., a bystander effect).

To circumvent this problem, two mutations that abolish the transport of galactose were introduced into strain GJ2203. These two mutations are in galP and mgl genes that encode the two known galactose transporters, galactose permease and methyl galactoside permease respectively. The galE(Ts) strains were rendered mutant successively in galP and mgl by selection, respectively, for resistance to 1 mM 2-deoxy galactose (181) at 30° and resistance to lysis by 0.1% D-galactose at 42° on minimal medium supplemented with Casamino acids. The first selection yielded exclusively galP mutants, while in the second selection the galP mgl mutants were screened from others carrying mutations in the galETK operon (which would also be Gal+) because only the former (test for lactose sensitivity was done after making the strain Lac+ by conjugation with a HfrC donor KL226) would be sensitive to lactose at 42° (because of the internally generated galactose by hydrolysis of lactose). At 42°, these galP mgl strains were now resistant to exogenous galactose but remained sensitive to endogenously released galactose from lactose hydrolysis (in the Lac+ revertant derivatives).

Although Lac+ papillation efficiency in the galE(Ts) derivatives constructed above was still only 50% of that with the Kan′ (Ts) approach, it was now possible to extend the studies on mutations in nondividing cells to the analysis of reversions of various Lac− mutations other than the lacZ::Kan′ insertions.
3.2.6 Construction of \textit{galE(Ts)} strain derivatives

Derivatives of \textit{galE(Ts)} strains were constructed carrying one of the following mutations: a chromosomal \textit{lacZ::Tn10dKan} insertion, the \textit{lacI33::lacZ} frameshift mutation either on an F' or on the chromosome; or an amber mutation at codon 17 of \textit{lacZ}, once again either on an F' or on the chromosome. Strains bearing the \textit{lacZ(Am)} allele can revert to Lac\textsuperscript{+} by base changes either at codon 17 itself or at amber suppressor loci that together represent all the eight possible transitions and transversions.

The derivatives carrying the F'-borne mutations were constructed from strains CSH143 [a strain containing an F' 128 with \textit{lacZ(Am)} allele] and FC82 [a \textit{trp} auxotroph having an F' 128 with \textit{lacI33::lacZ} allele]; whereas the chromosomal \textit{lac} mutation-bearing derivatives were constructed from strain CSH142 [a \textit{Δlac-pro} strain] as follows. All three strains were used as recipients in a P1 transduction wherein the \textit{galE516(Ts)} allele was transferred with the aid of Kan\textsuperscript{r} (Ts) marker at min 17. Derivatives of all the above strains having \textit{galE(Ts)} alleles were then made \textit{galP} and \textit{mgl} as described in Section 3.2.5. The \textit{galE(Ts)} \textit{galP mgl} strains having the \textit{lacZ(Am)} and \textit{lacI33} alleles located on F' were designated as GJ2207 and 2214 respectively. The \textit{galE(Ts)} \textit{galP mgl} derivative of the \textit{Δlac-pro} strain CSH142 which was used for the construction of chromosomal \textit{lac} mutant derivatives was designated as GJ2218.

Strain GJ2218 was first made Lac\textsuperscript{+} Pro\textsuperscript{+} by conjugation with a HfrC donor strain KL226, so as to construct GJ2219. Subsequently, the frameshift, amber and Kan\textsuperscript{r} insertion mutations in \textit{lac} were each introduced into GJ2219 by P1 transduction (the first two in linkage, respectively, with the \textit{zaj-3099::Tn10Kan} insertion from CAG18594 and \textit{lacI3098::Tn10Kan} insertion from CAG18420) with selection for Kan\textsuperscript{r} at 42\degree. The strains bearing \textit{lacZ::Kan\textsuperscript{r}} insertion, amber mutation and frameshift allele on the chromosome were designated as GJ2220, 2231 and 2235 respectively. All these strains were finally transformed with the multicopy \textit{galE(Ts)}-bearing plasmid pHYD603 to ampicillin resistance, and then used in papillation studies.

3.2.7 Lac\textsuperscript{+} papillation studies in \textit{galE(Ts)} derivatives

Derivatives of \textit{galE(Ts)} \textit{galP mgl} strains carrying the multicopy plasmid pHYD603 were grown overnight in LB broth containing ampicillin. A 3- to -5 \textmu l volume of suspension containing \textasciitilde10\textsuperscript{4} cells was then spotted on to the surface of minimal A plates supplemented with ampicillin, Casamino acids (plus tryptophan in the case of FC82 derivatives) and 0.1%
lactose, at a density not exceeding 20 spots per plate (of 85 mm diameter). The Lac+ papillae that had grown on each spot were visualized by the gentle addition of 20 μl of Xgal solution (0.5 mg/ml), followed by 20 μl of 1 M sodium carbonate once sufficient blue coloration had developed.

Colonies of the above strains were examined for their Lac+ papillation patterns either after continuous incubation at 30° or following temperature-shift from 42° to 30° (Figs. 3.3B to 3.5). As described earlier in Section 3.1.6, the ratios of the peripheral to the central zone papillae were calculated and these values are given in Tables 3.1 and 3.2. Apart from the wild-type strains, the following mutant derivatives were also constructed and their papillation patterns were compared.

(1) Mutations in mutator loci [the ssb-200 mutation for chromosomal lacZ::Kan', mutS for both F' and chromosomal lacI33::lacZ allele and both mutS and mutY for F' and chromosomal lacZ(Am) allele]; (2) the recA56 mutation (which abolishes all activities of RecA protein); and (3) for strains bearing the lac point mutations on the F', a traD mutation which abolishes F-mediated conjugation. The ssb-200, recA56 and traD mutations were transduced by linked phage P1 transductions with the use of zjc-904::Tn10dTet (from GJ1889), srl-300::Tn10 (from GJ216) and zzf-902::Tn10dCm (from GJ2209), respectively. Strains were made mutS and mutY by Tet' transduction from CSH115 and CSH117 respectively, since the mutator mutations in these two strains have been caused by Tn10 insertions in the concerned genes.

Based on the papillation patterns of the above strains, the following conclusions could be drawn.

i. An apparent annular papillation pattern was observed following temperature-shift for the ssb lacZ::Kan' strain (Fig. 3.3B) as well as for the mutY derivatives of lacZ(Am) [on both F' (Fig. 3.4) and chromosome (Fig. 3.5)]. The calculated ratios of peripheral to central zone papillae were also high for these colonies (Tables 3.1 and 3.2). These results confirm the efficacy of the galE(Ts) mutation in eliminating Lac' revertants at the restrictive temperature.

ii. The observations made earlier for wild-type chromosomal lacZ::Kan' excision [in the Kan' (Ts) strain] were essentially reproduced also in the galE(Ts) derivative (Fig. 3.3B and Table 3.1), thereby reinforcing the conclusion that such excisions are able to occur in nondividing cells.
iii. The F' derivative (without mutator mutation) carrying \textit{lacI33::lacZ} yielded very large numbers of papillae on continuous incubation at 30° and marked central zone papillation on the temperature-shifted plates (Fig. 3.4). The Lac\textsuperscript{+} papillation pattern for the episomal \textit{lacZ(Am)} mutation also resembled that for the frameshift allele, but the absolute numbers of papillae were smaller (Fig. 3.4). In both cases, the calculated ratios of peripheral to central zone papillae in the temperature-shifted colonies were low and similar to the values reported for chromosomal \textit{lacZ::Kan\textsuperscript{r}} excision, indicative therefore of reversions occurring in nondividing cells (Table 3.2).

iv. In their chromosomal location, the frameshift and the amber \textit{lac} mutations reverted to Lac\textsuperscript{+} at a low frequency, but examination of sufficient numbers of temperature-shifted colonies clearly established the occurrence of central-zone papillation (Fig. 3.5), and an associated low zonal distribution ratio of papillae (Table 3.2), for both of them.

v. Mutations in \textit{recA} or \textit{traD} were associated with a considerable decrease in reversion frequencies for either point mutation on the F' (Fig. 3.4).

vi. Unlike \textit{mutY} and \textit{ssb}, which gave annular papillation on the temperature-shifted plates of \textit{lacZ(Am)} and \textit{lacZ::Kan\textsuperscript{r}} derivatives, respectively, the \textit{mutS} mutation appeared to give an increased central-zone papillation for both the \textit{lacZ(Am)} and \textit{lacI33::lacZ} targets after temperature-shift (Fig. 3.4 and 3.5). This was reflected also in the low ratio of peripheral to central zone papillae in the temperature-shifted \textit{mutS} colonies (Table 3.2). The data therefore suggested that the reversion frequency for each of these mutations in nondividing cells was elevated in the absence of methyl-directed mismatch repair.

The \textit{galE(Ts)} strain carrying both the \textit{lacI33::lacZ} mutation on the F' and the mutator \textit{mutS} allele grew very poorly on lactose-supplemented plates at 42° and the reason for this might be that under these conditions the frequency of reversion to Lac\textsuperscript{+} (and consequent release of galactose by lysis) during growth at 42° is so high that bystander killing occurs despite the presence of \textit{galP} and \textit{mgl} mutations. Thus, the effect of mutator mutation may be additive to the F' position effect on increase in the reversion frequency of the frameshift \textit{lac} allele.
Fig. 3.3A Lac⁺ papillation patterns in the Kan⁺ (Ts) lacZ::Kan⁺ strains GJ1823 (w.t), GJ1854.1 (ssb-200), and GJ1886 (uup)

Fig. 3.3B Lac⁺ papillation patterns in the galE (Ts) lacZ::Kan⁺ strains GJ2220 (w.t), and GJ2222 (ssb-200)
Fig. 3.4 Lac+ papillation patterns in galE(Ts) strains carrying lac point mutations on F'. Strains GJ2207 and GJ2214 and their recA, traD, mutS and mutY derivatives were spotted on Lac+ papillation medium and incubated as described in the text. All strain derivatives carried the multicopy galE(Ts) plasmid pHYD603.
Fig. 3.5 Lac\(^+\) papillation patterns in galE(Ts) strains carrying chromosomal lac point mutations. Strains GJ2231 [lacZ (Am)] and GJ2235 [lacI33::lacZ] and their mutS and mutY derivatives were examined by Lac\(^+\) papillation spot assay. All strains carried pHYD603 plasmid.
Table 3.1  Ratio of papillae in peripheral zone to central zone for temperature-shifted colonies of lacZ::Kan<sup>r</sup> strains

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Conditional-lethal strategy</th>
<th>Kan&lt;sup&gt;r&lt;/sup&gt; (Ts)</th>
<th>galE(Ts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil (wild-type)</td>
<td></td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>ssb-200</td>
<td></td>
<td>9.0</td>
<td>7.5</td>
</tr>
<tr>
<td>uup-351</td>
<td></td>
<td>4.8</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined

Table 3.2  Ratio of papillae in peripheral zone to central zone for temperature-shifted colonies of strains carrying lacZ(Am) or lacI33::lacZ mutations<sup>a</sup>

<table>
<thead>
<tr>
<th>Mutation</th>
<th>lacZ(Am) on</th>
<th>lacI33::lacZ on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F'</td>
<td>Chromosome</td>
</tr>
<tr>
<td>Nil (wild-type)</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>mutS::Tn10dTet</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>mutY::Tn10dTet</td>
<td>4.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains are those described in the legends to Figure 3.4 (lac point mutations on F') and Figure 3.5 (same mutations on the chromosome). Ratios were calculated as described in the text.

<sup>b</sup> ND, not determined.
DISCUSSION

3.3 Chromosomal and F' mutations occur in nondividing cells

A conditional-lethal strategy for elimination of log-phase reversions in a population, in combination with a means to quantitate the relative distribution of dividing cells in different zones of a colony at the permissive temperature, was used to demonstrate that reversion to Lac⁺ of a chromosomal lacZ::Kan' insertion and of two other lac mutations, both on the chromosome and on an F' can occur in nondividing cells. The argument for their occurrence is based on spatial patterns of Lac⁺ papillation in colonies and is primarily statistical.

These observations support the idea that the mutations above have arisen in nondividing cells and not merely in stationary-phase cells, for the reason that the central-zone reversions are blind to the effects of several mutator mutations that increase the reversion frequency at the target loci during exponential growth. The distinction may be important, because stationary-phase broth cultures have been shown to exhibit considerable cell turnover, with "killer" mutants growing at the expense of the rest of the population (249).

Deletions of the transposon prophage Mu (reviewed in 218) and transposition of IS30 are known to be stimulated in stationary-phase cells (180). The Tn10dKan element used in this work is also derived from a transposon, but there are important mechanistic differences between transposition and precise excision. Tn10dKan is incapable of autonomous transposition, and furthermore precise excision is entirely dependent only on host-encoded functions; on the other hand, the former events are transposition related (69, 90). As shown for Tn10dKan in this study, precise excisions of Tn3 and IS150 have also been reported to be stimulated in the stationary phase (20, 173).

3.4 A common mechanism for mutations in nondividing cells

Of the various reversions examined, it was found that the frequency of stationary-phase reversion to Lac⁺ was most pronounced for the chromosomal lacZ::Kan' and for the F' frameshift lac mutations. It is known that these two events are particularly sensitive to replication slippage and methyl-directed mismatch repair, and hence it is likely that these two mechanisms could contribute (additively) to the occurrence of spontaneous mutations in nondividing cells.

The results obtained in this study suggest that a mutation in mutS increases central-zone papillation on the temperature-shifted plates for both lac133::lacZ and lacZ(Am)
derivatives. This finding is consistent with earlier work, and supports the hypothesis that methyl-directed mismatch repair is an important error-correction mechanism in nondividing cells (20, 85, 125, 219). The result obtained with the mutY mutator strain suggests that it is a mutator only in dividing cells as an annular pattern is seen on the temperature-shifted plates.

The conditional lethal strategy developed here can also be used to screen for mutants with altered central-zone papillation (which represents the stationary-phase mutation frequency); such strains may facilitate a genetic dissection of mechanisms that underlie spontaneous mutagenesis in nondividing cells.