JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1561-1563

Vol. 127, No. 3
Printed in U.S.A.

Isolation of Temperature-Sensitive Mutants of R Plasmid by In Vitro Mutagenesis with Hydroxylamine

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Received for publication 1 April 1976

Temperature-sensitive mutants were isolated from a tetracycline resistance plasmid, pSC101, after mutagenesis with hydroxylamine. They were divided into three classes according to their growth characteristics. They include mutants defective in the replication of plasmid deoxyribonucleic acid and strains having a mutation in the drug resistance gene.

Temperature-sensitive (Ts) mutants provide a useful probe for studies on the replication of deoxyribonucleic acid (DNA). There is some difficulty, however, in isolating such mutants in plasmids since they usually exist in multicopies within a cell. Therefore, plasmid DNA was mutagenized in vitro and returned to host cells, and then the temperature-sensitive characteristics were examined. With this method, we succeeded in isolating 17 temperature-sensitive mutant plasmids, designated pHS1 through pHS17, from a tetracycline resistance plasmid, pSC101 (1).

Bacteria carrying pSC101 were grown, and closed circular plasmid DNA was isolated by lysozyme-Sarkosyl treatment and dye-buoyant density centrifugation. The DNA was treated with hydroxylamine by a modification of the Tessman method for phage (5). At appropriate times, samples were withdrawn and applied to $CaCl_2$-treated *Escherichia coli* KH39 *supD*(Ts) (3). This strain was selected as the host since it carries temperature-sensitive suppressor I (4), thus allowing the isolation of amber mutants in addition to true temperature-sensitive mutants. After incubation at 30°C for 3 h in broth, cells were plated on tetracycline-containing nutrient agar plates (5 μg/ml) and incubated at

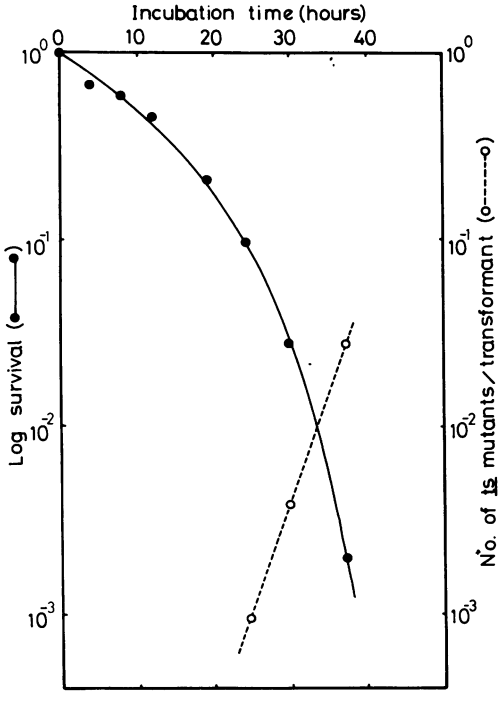

FIG. 1. *Survival of pSC101 plasmid DNA and appearance of temperature-sensitive mutants after treatment with hydroxylamine. Closed circular pSC101 DNA (20 μg/ml) was incubated at 37°C with 0.4 M hydroxylamine in 0.05 M sodium phosphate buffer (pH 6.0)-1 mM ethylenediaminetetraacetate (total volume, 1 ml). At the times indicated, the solution was diluted fivefold and dialyzed against 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0)-1 mM ethylenediaminetetraacetate-0.02 M NaCl in the cold. A 0.1-ml volume of DNA solution (ca. 0.4 μg) was added to 0.2 ml of $CaCl_2$-treated cells (2 × 10^9 cells) in 0.1 M $CaCl_2$, and the mixture was kept at 0°C for 60 min. The $CaCl_2$-treated cells were prepared from E. coli KH39 by the procedure of Lederberg and Cohen (3). After a 3-min heat pulse at 30°C, the mixture was added to 2.7 ml of broth and aerated for 3 h at 30°C. Portions (1 ml) were plated on nutrient agar containing about 5 μg of tetracycline per ml, and the plates were incubated at 30°C for 2 days. Colonies larger than about 0.5 mm in diameter were counted as transformants. The temperature-sensitive mutant isolation technique was as described in the text. Symbols: ●, survival of plasmid DNA to yield transformants; ○, number of temperature-sensitive mutants per transformant.*

30°C. Colonies formed were counted as transformants.

For the selection of temperature-sensitive mutants, transformants were picked with a toothpick onto two nutrient agar plates without the drug; one plate was incubated at 30°C and the other was incubated at 42°C. Cells grown at 30 or 42°C were picked from the edge of colonies to two tetracycline-containing plates, which were then incubated at 30 and 42°C, respectively. Clones that produce colonies at 30°C but not at 42°C in the presence of the drug were selected as temperature-sensitive mutants. The frequency of appearance of temperature-sensitive mutants increased with time of mutagenesis, whereas the total number of transformants decreased progressively (Fig. 1).

Plasmid DNAs of 10 mutants isolated from the 24.5- and 30-h mutagenizations were transferred to strain Om84 (*supD*) by transformation, and the transformants were subjected to growth tests under four different conditions. Colonies produced at 30 and 42°C in the absence of tetracycline were streaked onto two tetracycline-containing plates, one of which was incubated at 30°C and the other at 42°C. All of the clones examined produced colonies at 30°C (Fig. 2a) but not at 42°C (Fig. 2d) in the presence of tetracycline. Since this host carries no temperature-sensitive suppressor gene, the mutants seem to be temperature sensitive owing to a plasmid missense mutation but not to *supD* (Ts).

From the growth patterns (Fig. 2), the temperature-sensitive mutants can be divided into

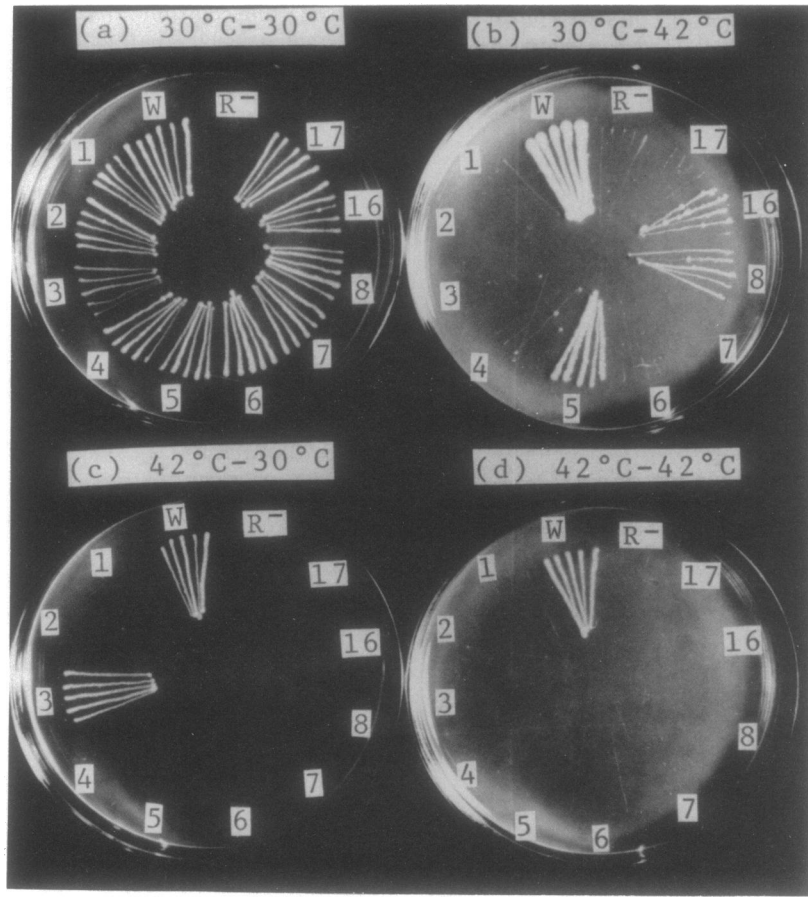

FIG. 2. *Effect of temperature on the growth of E. coli Om84 (supD)-harboring plasmid mutants. Cells were grown in nutrient agar at 30°C (a and b) or 42°C (c and d). After 2 days of incubation, cells at the edge of colonies were streaked, by using toothpicks, on tetracycline-containing plates and incubated at 30°C (a and c) or 42°C (b and d). (a) Preincubation without tetracycline at 30°C and incubation with it at 30°C. (b) Without tetracycline at 30°C and with it at 42°C. (c) Without tetracycline at 42°C and with it at 30°C. (d) Without tetracycline at 42°C and with it at 42°C. R⁻, Cells carrying no plasmid; W, cells carrying pSC101, wild type; 1 through 17: cells carrying temperature-sensitive mutant plasmids pHS1 through pHS17.*

three classes. (i) Cells harboring plasmid mutant pHS1, pHS2, pHS4, pHS6, pHS7, or pHS17 fail to produce colonies on tetracycline-containing plates at both 30 and 42°C once they have been grown at 42°C in the absence of the drug. These mutants seem to be unable to replicate or segregate their DNA at high temperature and are thought to be similar to plasmid pSC201 isolated by Kretshmer et al. (2). (ii) Cells harboring pHS3 produce colonies on tetracycline-containing plates at 30°C but not at 42°C irrespective of preincubation temperature. This may indicate a temperature-sensitive mutation in the tetracycline resistance gene(s). (iii) Cells harboring pHS5, pHS8, or pHS16 cannot grow on a tetracycline-containing plate if they are preincubated at 42°C without the drug, but they do produce colonies even at 42°C when preincubated at 30°C. The nature of this type of mutation is not clear. It might be a leaky, replication-defective mutation, but there is a possibility that it is an entirely different type, such as one that interferes with host cell growth at high temperature.

In vitro mutagenesis of plasmid DNA has been applied successfully for the isolation of temperature-sensitive λ*dv* mutants (T. Hashimoto and K. Matsubara, unpublished data) and may be applicable to other plasmids.

We thank H. Hashimoto and Y. Ike for their advice concerning the preparation of tetracycline-containing medium, K. Matsubara and K. Hasunuma for helpful suggestions, and S. N. Cohen, T. Nagata, and H. Iyehara for providing bacterial strains.

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