Properties of a Temperature-sensitive Mutant of *Staphylococcus aureus* Defective in DNA Replication and Cell Division and Replication of Plasmids in the Mutant

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The properties of a temperature-sensitive mutant (ts39) of *Staphylococcus aureus* NCTC 8235 are described. After transfer to the restrictive temperature (42 °C), absorbance increased 10- to 20-fold but DNA content did not increase beyond 150 to 200 % and cell division continued at a greatly reduced rate. On transfer back to the permissive temperature, both cell division and DNA synthesis resumed if the transfer occurred after less than 120 min at 42 °C. Resumption of DNA replication was blocked by chloramphenicol (100 µg ml⁻¹). The results are discussed with reference to possible defects in DNA replication.

Replication of the plasmids pI₂₅₈ and pT₁₀₅₀₁ and the chromosome were affected to a similar extent in ts39. Growth at 42 °C resulted in the appearance of an increased amount of pI₂₅₈ DNA in a form that sedimented slowly in a sucrose gradient.

INTRODUCTION

Four mutants of *Staphylococcus aureus* NCTC 8325 that are temperature-sensitive for DNA replication have been described (Thomas & Dyke, 1978). In mutant ts39, which is phenotypically and also genotypically distinct from the other three as shown by transduction analysis, the DNA content increased by 150 to 200 % after transfer to the restrictive temperature (42 °C). The kinetics of the residual DNA synthesis in ts39 were inconsistent with a partial defect in elongation. Also, the amount of synthesis was consistent with an initiation defect only if more than three cycles of chromosome replication were occurring at one time (Sueoka & Yoshikawa, 1965), or if the initiation defect was only partial, allowing further rounds of replication to be initiated after transfer to 42 °C. Although possible, it is unlikely that three cycles of chromosome replication were occurring at one time, since the generation time of ts39 at 30 °C is 50 min. [In *Escherichia coli*, the mean generation time must be shorter than 40 min under normal conditions before even two rounds of replication overlap (Cooper & Helmstetter, 1968).]

Other possible explanations are that the temperature-sensitive defect affected either the synthesis of elongation enzymes or the termination of replication. Since termination of replication is required for cell division in *E. coli* (Marunouchi & Messer, 1973), a possible way to distinguish between these explanations would be to investigate cell division at the restrictive temperature.

In this paper we describe experiments to investigate both DNA synthesis and cell division in mutant ts39. The results are discussed in relation to possible explanations for the temperature-sensitive phenotype.

We have also investigated how replication of plasmids conferring resistance to tetracycline (pT₁₀₅₀₁) or the ability to synthesize penicillinase (pI₂₅₈) is affected by this defect, since little is known about the dependence of staphylococcal plasmid replication on functions involved in

chromosome replication. These plasmids have molecular weights of about 3×10^6 and 18.6×10^6 , respectively, and are therefore typical of the plasmids in *S. aureus* (Lacey, 1975).

METHODS

Bacteria. *Staphylococcus aureus* NCTC 8325, a naturally occurring strain in which no plasmids have been detected, (Novick & Brodsky, 1972) was used. Strain 8325WT was the non-temperature-sensitive parent strain carrying no plasmids; ts39 was a temperature-sensitive mutant (Thomas & Dyke, 1978). Strains 8325WT(pI₂₅₈) and ts39(pI₂₅₈) were constructed by transduction (Thomas & Dyke, 1978) of plasmid pI₂₅₈ (Novick, 1974) from strain 258(pI₂₅₈) (Mitsuhashi *et al.*, 1965). Strains 8325WT(pT₁₀₅₀₁) and ts39(pT₁₀₅₀₁) were obtained by transduction of plasmid pT₁₀₅₀₁ from strain ps80(pT₁₀₅₀₁). The original strain carrying pT₁₀₅₀₁ was supplied by Dr E. H. Asheshov. The presence of this plasmid confers resistance to tetracycline ($5 \mu\text{g ml}^{-1}$).

Growth and maintenance. Bacteria were maintained on CY agar (Novick, 1963), supplemented with cadmium acetate ($53 \mu\text{g ml}^{-1}$) for strains carrying pI₂₅₈, and with tetracycline ($5 \mu\text{g ml}^{-1}$) for strains carrying pT₁₀₅₀₁. Inocula from stock plates were grown in CY medium (Novick, 1963) at 30°C for 15 to 18 h and then diluted into fresh medium and grown into exponential phase before radioactive labelling.

Estimation of bacterial numbers and viable counts. To disrupt clumps of bacteria for both bacterial number and viable count estimation, suspensions were sonicated for 60 s using an MSE probe sonicator with a 0.9 cm diam. stainless-steel probe, which at maximum tuning operated at 0.8 A. This period of sonication gave the maximum increase in colony forming units. For bacterial number determinations, samples were mixed with an equal volume of 0.15 M-NaCl/0.015 M-NaN₃ (to halt growth) and counted in a Thoma circular counting chamber (Gallenkamp). Since viewing was by oil immersion and a phase contrast microscope, the normal rigid glass coverslip was replaced by a thin glass one (Chance Propper, no. 1½). To average out distortions, two different coverslips were used in succession for each sample. The number of colony forming units (c.f.u.) per ml was determined by plating 0.1 ml samples of dilutions of sonicated cultures on CY agar.

Incorporation of [³H]thymidine into DNA. Samples (0.5 ml) from cultures of bacteria growing in the presence of [³H]thymidine (The Radiochemical Centre, Amersham) were mixed with an equal volume of ice-cold 10% (w/v) trichloroacetic acid (TCA). After 30 min at 0°C , the samples were filtered on to 2.1 cm GF/C discs (Whatman), washed with 4×5 ml ice-cold 5% (w/v) TCA and 4×5 ml ethanol, and dried at 80°C under reduced pressure. The radioactivity in each sample was determined in a liquid scintillation counter after addition of 2.5 ml of 5% (w/v) 2-(4'-*tert*-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (butyl-PBD) in toluene.

Estimation of plasmid DNA. [³H]Thymidine and [¹⁴C]thymidine were from The Radiochemical Centre, Amersham. Cultures growing at 30°C were diluted into CY medium to give $A_{675\text{ nm}}^{1\text{ cm}} = 0.2$, then [³H]thymidine or [¹⁴C]thymidine (final concn $1 \mu\text{Ci ml}^{-1}$, $2 \mu\text{g ml}^{-1}$) was added and incubation was continued for two generations at either 30 or 42°C . The bacteria were harvested, lysed, and plasmid DNA was separated on linear sucrose gradients by the method of Sheehy & Novick (1975).

RESULTS

DNA synthesis in ts39

After transfer to the restrictive temperature (42°C or higher), the DNA content in ts39 increases by 150 to 200% (Thomas & Dyke, 1978). This could be the result of completion of replication already initiated before transfer. To test this, the amount of DNA synthesis that occurred after the addition of chloramphenicol to exponentially growing bacteria was determined, since this antibiotic prevents the initiation of new rounds of replication (Maaløe & Hanawalt, 1961). Incorporation of [³H]thymidine into the wild type at 30°C in the presence of chloramphenicol was less than that into ts39 after transfer to the restrictive temperature (Fig. 1). The residual DNA synthesis at 30°C after addition of chloramphenicol was similar in the wild type and ts39 (data not shown). Therefore, if DNA synthesis after addition of chloramphenicol estimates the completion of all rounds of replication already in progress, then ts39 cannot be an initiation-defective mutant.

If the phenotype of ts39 results from partially or slowly inactivated enzymes at 42°C , then raising the temperature should either reduce the residual DNA synthesis or decrease the time needed to reach a plateau. Increasing the restrictive temperature to 45°C resulted in a

Fig. 1

Fig. 2

Fig. 1. Effect of restrictive temperature and chloramphenicol on DNA synthesis. Strains 8325WT and ts39 growing exponentially at 30 °C in CY medium (plus 10 μg thymidine ml^{-1}) were diluted in similar medium to the same density ($A_{675\text{ nm}}^{1\text{ cm}}$ = about 0.1) and [^3H]thymidine was added to a final concn of 1 $\mu\text{Ci ml}^{-1}$. Three 10 ml cultures of 8325WT were treated in the following way: (i) transferred to 42 °C (○); (ii) retained at 30 °C (□); (iii) retained at 30 °C but chloramphenicol was added (final concn 100 $\mu\text{g ml}^{-1}$) (■). One 10 ml culture of ts39 was transferred to 42 °C (●). Samples (0.5 ml) were withdrawn periodically and the incorporated radioactivity was determined.

Fig. 2. Effect of chloramphenicol on recovery of DNA synthesis in ts39 when transferred to 30 °C after a period at 42 °C. Absorbance (○, ●) and DNA content (□, ■), as estimated by the method of Burton (1956), were determined after transfer to 42 °C, and upon return after 75 min to 30 °C without (○, □) or with (●, ■) chloramphenicol (100 $\mu\text{g ml}^{-1}$).

Table 1. Increase in absorbance and number of viable bacteria in cultures of the wild type and ts39 after transfer to 42 from 30 °C

Strain	After transfer from 30 to 42 °C		After 90 min at 42 °C	
	$A_{675\text{ nm}}^{1\text{ cm}}$	Viable count (c.f.u. ml^{-1})	$A_{675\text{ nm}}^{1\text{ cm}}$	Viable count (c.f.u. ml^{-1})
8325WT	0.161	1.1×10^8	1.26	7.6×10^8
ts39	0.168	1.0×10^8	1.32	2.2×10^8

plateau, but incorporation of [^3H]thymidine was similar to that at 42 °C during the first 45 min after transfer (data not shown). It was concluded that the bulk of residual DNA synthesis at the restrictive temperature was unlikely to be due to either a partially or a slowly inactivated enzyme.

When a culture of ts39 was transferred back to 30 °C after 75 min at 42 °C, DNA synthesis resumed at a doubling time of about 35 to 40 min (Fig. 2). This is somewhat faster than the rate of synthesis usually found during exponential growth at 30 °C (doubling time 45 to 50 min) and is consistent with the occurrence of either multiple initiations or of some synchronization of the replication process. The rate of increase of absorbance of the culture showed a lag on transfer back to 30 °C (Fig. 2). Addition of chloramphenicol to the culture at the time of transfer from 42 to 30 °C prevented the resumption of extensive DNA synthesis (Fig. 2), indicating that protein synthesis is required.

Cell division in ts39

For about 70 to 90 min after transfer to 42 °C, the increase in absorbance of cultures of ts39 and the wild type was similar; in the same period, the number of viable bacteria increased about seven-fold for the wild type, but only about twofold for ts39 (Table 1).

Fig. 3

Fig. 3. Absorbance and bacterial number of cultures of 8325WT and *ts39* at 30 and 42 °C. Strains 8325WT (●, ■) and *ts39* (○, □) growing exponentially at 30 °C in CY medium were diluted to $A_{675\text{ nm}}^{1\text{ cm}}$ = about 0.05 and absorbance (○, ●) and bacterial number (□, ■) were determined periodically. After 60 min, the cultures were transferred to 42 °C.

Fig. 4

Fig. 4. Effect of chloramphenicol on recovery of cell division in *ts39* when transferred to 30 °C after a period at 42 °C. Absorbance (○, ●) and bacterial number (□, ■) were determined after transfer to 42 °C, and upon return after 75 min to 30 °C without (○, □) or with (●, ■) chloramphenicol ($100\text{ }\mu\text{g ml}^{-1}$).

Determinations were carried out on four separate occasions. The variation in results was as high as twofold between the number of viable bacteria per unit of absorbance for different cultures but within a single experiment the variation was less ($\pm 20\%$).

Similar results were obtained in experiments in which total numbers of bacteria were compared with absorbance (Fig. 3). With the wild type, the absorbance increased faster than the number of bacteria for about 90 min after transfer to 42 °C. When this culture was diluted, the absorbance and bacterial number increased in parallel so that the ratio of absorbance to bacterial number remained constant over many generations (data not shown). In contrast, the rate of division of *ts39* decreased soon after transfer to 42 °C although the absorbance continued to increase quite rapidly for about 90 min before becoming constant or declining slightly. After 90 min at 42 °C, dilution of *ts39* into fresh medium at 42 °C did not result in any increase of absorbance or in numbers of bacteria.

When cultures of *ts39* were returned to 30 °C after incubation for various periods at 42 °C, the ratio of numbers of bacteria to absorbance returned to the value obtained for growth at 30 °C. This return occurred even in the presence of chloramphenicol, although further growth was blocked (Fig. 4). This contrasts with the effect of chloramphenicol on DNA synthesis on return to 30 °C after 75 min at 42 °C when most DNA synthesis was inhibited.

Exposure of a growing culture of *ts39* to 42 °C for 120 min followed by a return to 30 °C resulted in neither an increase of absorbance nor an increase in bacterial numbers over 90 min. Thus, there seems to be some irreversible change in *ts39* during prolonged incubation at 42 °C.

Replication of plasmids in ts39

To estimate the effect of the temperature-sensitive defect on plasmid replication at both the permissive and non-permissive temperatures, bacteria were labelled with [$6\text{-}^3\text{H}$]thy-

Table 2. Percentage of total incorporated [^3H]thymidine recovered in plasmid peaks

Plasmid-bearing strains were analysed after labelling at 30 or 42 °C for 90 min.

Labelling temp.	Percentage of total incorporated c.p.m. in plasmid peak			
	pI_{258} *		pT_{10501} †	
	8325WT	ts39	8325WT	ts39
30 °C	1.5 ± 0.4	1.7 ± 0.5	2.2 ± 0.2	2.8 ± 0.35
42 °C	1.3 ± 0.5	4.4 ± 1.5	1.4 ± 0.3	1.5 ± 0.4

* Average of 10 experiments. † Average of 3 experiments.

midine ($1\ \mu\text{Ci ml}^{-1}$, $2\ \mu\text{g ml}^{-1}$) while growing at 30 °C or after transfer to 42 °C. Samples were then lysed and analysed on neutral sucrose velocity gradients by the method of Sheehy & Novick (1975). This procedure separates plasmid DNA from chromosomal DNA. The percentage of incorporated radioactivity found in the plasmid peak was determined (Table 2) and then the relative rate of plasmid and chromosome replication was estimated. After 90 min labelling at 30 °C (about two generations), the plasmid peak for pI_{258} from the wild type contained 1.5% of the incorporated thymidine and slightly more when ts39 was the host. The corresponding values for pT_{10501} were 2.2 and 2.8% (Table 2).

When the labelling experiment with pI_{258} was done at 42 °C, the proportion of radioactivity in the plasmid remained the same in the wild type but was increased about threefold in the temperature-sensitive mutant. With pT_{10501} there was a similar reduction (to 1.4 and 1.5%, respectively) in both the wild type and ts39. The experiments with pI_{258} were done ten times and those with pT_{10501} three times. There was a significant variation in the results for pI_{258} , but in experiments in which the results for the wild type were high, the results for ts39 were also high, so the variation was less than indicated by the limits shown in Table 2. It was considered possible that the defect affects replication of the chromosome and pT_{10501} similarly, but has less effect on replication of pI_{258} .

To test this hypothesis, the wild type and ts39, each carrying pI_{258} , were labelled with [$2\text{-}^{14}\text{C}$]thymidine ($1\ \mu\text{Ci ml}^{-1}$, $2\ \mu\text{g ml}^{-1}$) over two generations at 30 °C, and then either transferred to 42 °C and labelled as above with [^3H]thymidine or harvested and mixed with bacteria labelled separately with [^3H]thymidine at 42 °C as above. By comparing the $^3\text{H} : ^{14}\text{C}$ ratio in the plasmid peak and total radioactivity, differences between plasmid and chromosome replication could be detected. Under both procedures, the plasmid and total $^3\text{H} : ^{14}\text{C}$ ratios for the wild type were similar (plasmid $^3\text{H} : ^{14}\text{C}$ /total $^3\text{H} : ^{14}\text{C}$ was 1.18 for prelabelled bacteria transferred to 42 °C and 1.14 for separately labelled bacteria). When prelabelled ts39(pI_{258}) was transferred to, and labelled at 42 °C, the plasmid $^3\text{H} : ^{14}\text{C}$ /total $^3\text{H} : ^{14}\text{C}$ was 1.08, but when the prelabelled bacteria were mixed with ts39(pI_{258}) labelled separately at 42 °C for 90 min, this ratio was 1.93. The quantities of bacteria used in each case were strictly comparable. These results at 42 °C indicate that growth of ts39(pI_{258}) did not produce a differential effect on the rates of replication of plasmid and chromosome but rather allowed a greater percentage of plasmid DNA to be recovered.

DISCUSSION

Since increasing the restrictive temperature from 42 to 45 °C does not greatly reduce the residual DNA synthesis in ts39, this residual synthesis does not appear to result from a protein slowly inactivated by heat. The temperature-sensitive protein is therefore not likely to be directly involved in elongation. A number of observations suggest that the defect in ts39 does not directly affect the initiation of replication. As already discussed, it is hard to explain the extensive residual DNA replication (150 to 200%) in terms of an initiation defect. A

standard method for determining the amount of DNA synthesis expected from completion of rounds of replication already initiated is to add chloramphenicol ($100\text{ }\mu\text{g ml}^{-1}$) or starve for required amino acids, to inhibit initiation, and measure residual DNA synthesis (Maaløe & Hanawalt, 1961; Lark, 1966). Using this method, we have established that ts39 is not completely defective in initiation (Fig. 1). However, chloramphenicol also inhibits termination of replication (Marunouchi & Messer, 1973) and may also cause replication to terminate part way through a round of replication (Lark, 1973; Evans & Eberle, 1975). Therefore, the observation that there is more residual DNA synthesis in ts39 at $42\text{ }^{\circ}\text{C}$ than when chloramphenicol is added to the wild type at $30\text{ }^{\circ}\text{C}$ must be interpreted with reservations.

The inhibition of cell division soon after transfer to $42\text{ }^{\circ}\text{C}$ is inconsistent with a defect in initiation of DNA replication since theoretical arguments and experimental results (Beyersmann, Schlicht & Schuster, 1971) indicate that cell division equivalent to completed rounds of replication should continue at the restrictive temperature. However, in a temperature-sensitive mutant of *E. coli*, CRT83, which phenotypically and genotypically is a *dnaA* initiation-defective mutant (Kohiyama, 1968; Hirota, Ryter & Jacob, 1968), it has been found that cell division is halted immediately on transfer to the restrictive temperature (Katchatourians & Clark, 1970). It may be possible, therefore, for initiation-defective mutants to affect division in the same way as proposed for termination-defective mutants.

Therefore, while it is difficult to reconcile the properties of ts39 with an initiation defect, this possibility cannot be ruled out. For instance, since cell division in ts39 is not completely blocked, it may be that the defect in DNA replication is incomplete. Thus the characteristics of ts39 could be compared to those of a 'non-classical' initiation-defective mutant of *E. coli*, FA21, when incubated at $39\text{ }^{\circ}\text{C}$ (Fangman & Novick, 1968). This analogy is only partially successful since at higher temperatures DNA synthesis in FA21 is much reduced, in contrast to that in ts39, and in FA21 division is less affected than DNA synthesis at the restrictive temperature.

Alternative explanations may be either a membrane defect resulting in reduced synthesis of elongation complexes and slower division, or a partial defect in termination or segregation of daughter chromosomes. The first alternative is based on the knowledge that the replication complex in *S. aureus* is associated with the membrane (Measures, 1973). A defect in a membrane protein whose synthesis is required for division might result in a rapid effect on the division process and also might interfere with the replication of DNA progressively, for example by interacting with the putative elongation complex. The second alternative – a partial defect in termination or segregation – is an interesting possibility but one which it is difficult to substantiate. A termination defect may explain why, on transfer of the mutant to $30\text{ }^{\circ}\text{C}$ after a period at $42\text{ }^{\circ}\text{C}$, chloramphenicol inhibits DNA synthesis but not cell division. The termination defect could be removed on return to $30\text{ }^{\circ}\text{C}$ by a process not requiring protein synthesis, for example by a reversible change of conformation; thus termination and segregation could occur, and this might allow cell division without the need for DNA synthesis. Both these alternative explanations are highly speculative.

It is not possible to draw any firm conclusions as to the nature of the defect in ts39 but it does not conform to known properties of other bacterial *dna* mutants. Whatever the explanation for the defect, it does provide scope for further investigation of the relationship between cell division and the synthesis of replicons.

The observation that replication of the chromosome and the plasmids pI₂₅₈ and pT₁₀₅₀₁ are affected to a similar extent by the temperature-sensitive defect in ts39 indicates either that this mutation has occurred in a gene supplying a product required by the chromosome and the plasmid, or that replication of these two entities is coupled in some other way. It is not possible to distinguish between these possibilities with the data available. Similar problems of interpretation have been encountered in studies on *E. coli* (Arai & Clowes, 1974).

It is interesting that growth at the restrictive temperature results, on lysis, in release of a larger than normal amount of pI₂₅₈ DNA in a slowly sedimenting form. That this does not

occur with pT₁₀₅₀₁ may indicate differences between the intracellular associations of the two types of plasmid, for example membrane attachment, as has been previously found (Measures, 1973). Possible explanations for the alteration are changes in chromosome or membrane structure during growth at the restrictive temperature. Preliminary experiments (unpublished) support the second suggestion. The defect may also have some effect at the permissive temperature which would explain the higher yield of plasmid DNA in the mutant (Table 2). The high percentage of the total [³H]thymidine found in the pI₂₅₈ peak after growth at 42 °C confirms the findings that penicillinase plasmids in *S. aureus* are multicopy plasmids (Chopra, Bennett & Lacey, 1973; Ruby & Novick, 1975; Rush, Novick & Delap, 1975). It also confirms that this method for isolation of plasmid DNA is very dependent on association of plasmid DNA with chromosomal and/or membrane material, the nature of which is at present unknown.

The fact that pT₁₀₅₀₁ is not naturally temperature-sensitive indicates that it is not similar to the plasmid pT₁₆₉ (Novick *et al.*, 1975).

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