Characterization of a Temperature-sensitive DNA Replication Mutant of 
*Staphylococcus aureus*

By DAVID K. SUMMERS*† AND KEITH G. H. DYKE

Microbiology Unit, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, U.K.

(Received 3 November 1981; revised 18 January 1982)

A temperature-sensitive DNA replication mutant of *Staphylococcus aureus* NCTC 8325 has been isolated and characterized. After transfer to the non-permissive temperature (42 °C), DNA synthesis continued for 30 min and the mean DNA content increased by 56%. The amount of residual DNA synthesis was not reduced when the non-permissive temperature was raised, nor when chloramphenicol was added at the time of the temperature shift. During incubation at 42 °C, mutant bacteria accumulated the capacity to synthesize DNA after return to the permissive temperature (30 °C) in the presence of chloramphenicol. This capacity was lost when chloramphenicol was present at 42 °C. The properties of the mutant are consistent with a defect in the initiation of DNA replication at 42 °C.

INTRODUCTION

Most published work on DNA synthesis in *Staphylococcus aureus* has been concerned with plasmid replication (Novick, 1974; Novick et al., 1975). Measures (1973), however, isolated eight mutants of *S. aureus* PS80 which were temperature-sensitive for chromosome synthesis. These mutants were not characterized in detail but preliminary studies suggested that two of them were defective in chromosome elongation, while the remainder had defects in initiation. Thomas & Dyke (1978a) reported the isolation and mapping of four temperature-sensitive chromosome replication mutants of *S. aureus* NCTC 8325. Three of these mutations were thought to result in defects in DNA elongation while it was unclear whether the fourth (Thomas & Dyke, 1978b) was affected in initiation or elongation.

In this paper the isolation and characterization of a new temperature-sensitive DNA replication mutant, which appears to be defective in chromosome initiation, is described.

METHODS

**Bacteria.** *Staphylococcus aureus* NCTC 8325 is a naturally occurring strain and 8325 thy is a thymidine-requiring mutant isolated by H. Hackling & K. G. H. Dyke (unpublished).

**Growth and maintenance.** Bacteria were maintained on CY-agar and grown in CY-liquid medium (Novick, 1963). Inocula from stock plates were grown for 18 h at 30 °C, then diluted with fresh medium and grown to exponential phase before use. For experiments involving [*H]*thymidine incorporation, cultures were prelabelled for two generations at 30 °C before transfer to 42 °C. Where stated, chloramphenicol (Sigma) was added to CY-liquid medium at 100 µg ml⁻¹.

**Mutagenesis and screening for temperature-sensitive mutants.** Mutagenesis with ethyl methanesulphonate (EMS, Fisons) was performed according to the method of Novick (1963). Mutagenized bacteria were grown to stationary phase, diluted with fresh CY-medium and then grown to exponential phase at 30 °C. Dilutions of the culture were spread on to CY agar to give approximately 100 c.f.u. per plate. Plates were incubated at 30 °C until colonies were about 0.5 mm in diameter and then, after the positions of all visible colonies had been marked, the plates were transferred to 42 °C. After 6–8 h, the colonies which grew at 42 °C were large. Small colonies were picked and tested for temperature sensitivity by streaking on to CY-agar plates and incubating at 30 or 42 °C.

† Present address: Institute of Genetics, Church Street, Glasgow G11 5JS, U.K.
Incorporation of radioactively labelled precursors. Samples (0-5 ml) of bacterial cultures growing in the presence of radioactively labelled precursors were each added to 5 ml ice-cold 5% (w/v) trichloroacetic acid (TCA). After 30–60 min at 0°C, the samples were filtered through Whatman GF/B or GF/C glass fibre disc filters (2-1 cm diameter), washed four times with 5 ml ice-cold 5% (w/v) TCA and then four times with 5 ml 96% (v/v) ethanol. The filters were dried in vacuo at 80°C for 1 h and the radioactivity in each sample was measured in a liquid scintillation counter, after the addition of 2.5 ml 5% (w/v) butyl-PBD (Fisons) in toluene. Radioactively labelled precursors (all supplied by Amersham) were used at the following specific activities: [6-3H]thymidine, 2.5 pCi (93 kBq) ml⁻¹, 50 pg ml⁻¹; L-phenyl [2,3-3H]alanine, 2 pCi (75 kBq) ml⁻¹, 10 µg ml⁻¹; [2-14C]uracil, 0.1 µCi (3.7 kBq) ml⁻¹; [2-3H]glycine, 1 pCi (37 kBq) ml⁻¹; [2-3H]glycerol, 1 µCi (37 kBq) ml⁻¹. No unlabelled uracil, glycerol or glycine were added to CY medium.

RESULTS

Isolation of temperature-sensitive mutants

Of about 10⁵ colonies screened after EMS mutagenesis, 42 exhibited temperature-sensitive growth. These were screened for defects in DNA synthesis by measuring the ratios of [¹⁴C]uracil to [³H]thymidine incorporated by cultures of the mutants during incubation at 30 or 42°C. A mutant with a temperature-sensitive defect in DNA synthesis should show a higher ratio at 42°C than at 30°C. Two of the mutants (ts23 and ts34) had increased ratios at 42°C, but since it was discovered that ts34 continued to incorporate [³H]thymidine at 42°C for at least 2 h (data not shown), this mutant was not studied further.

Characterization of mutant ts23

When a culture of ts23 (prelabelled with [³H]thymidine for two generations at 30°C) was transferred to 42°C, incorporated radioactivity increased by 73 ± 14% (mean of 12 determinations ± s.d.) during the next 30 min, and then stopped. This could be due to a direct effect of the temperature-sensitive mutation upon DNA replication or it could be a secondary effect of a defect in some other metabolic function. To distinguish between these possibilities, the biosynthesis of protein, cell wall, membrane, RNA and DNA by ts23 and the wild-type, at 30 and 42°C, was investigated by measuring the rates of incorporation of radioactively labelled phenylalanine, glycine, glycerol, uracil and thymidine, respectively (Table 1). The results show that the rates of protein, cell wall and membrane synthesis in ts23 do not differ significantly from the wild-type. Although the rates of both DNA and RNA synthesis are reduced in the mutant at 42°C, the effect upon DNA is much greater.

Spontaneous temperature-stable revertants of ts23 were obtained at a frequency of 1.0 × 10⁻⁷. Thus the mutant phenotype probably results from a single point mutation, the primary effect of which is upon DNA synthesis.

The continuation of DNA synthesis by ts23 for 30 min after transfer to 42°C is consistent with a defect either in the initiation of chromosome replication, or in some elongation complex which is inactivated slowly at 42°C. In the latter case, increasing the non-permissive temperature above 42°C should reduce the amount of residual DNA synthesis. When thymidine incorporation by ts23 was measured for cultures transferred from 30°C to 42°C, 43.5°C or 44.25°C, there was little evidence of a decrease at higher temperatures (data not shown).

If S. aureus, like Escherichia coli, has a requirement for protein synthesis during the initiation but not elongation, of DNA replication (Lark et al., 1963), then inhibition of protein synthesis at the non-permissive temperature should decrease the amount of residual replication in a mutant with a slowly inactivated temperature-sensitive elongation function, but should not in an initiation-defective mutant. When chloramphenicol was added to a culture of ts23 simultaneously with transfer from 30 to 42°C, there was no reduction in the amount of thymidine incorporated at the non-permissive temperature (Fig. 1). Furthermore, the amount of thymidine incorporated was indistinguishable from that observed when
Table 1. *Comparison of incorporation of radiolabelled precursors by wild-type and ts23*

Cultures were prelabelled with radioactive precursors at 30 °C for two generations. Incorporation was measured at 30 °C or 42 °C over the next 2 h. Rates of incorporation (c.p.m. per ΔA_{650} = 1.0) at 42 °C were measured 1 h after transfer from 30 °C. Values given are the means from at least three independent experiments (variation between experiments <10%).

<table>
<thead>
<tr>
<th>Radiolabelled precursor</th>
<th>30 °C</th>
<th>42 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>ts23</td>
</tr>
<tr>
<td>[2-3H]Glycine</td>
<td>17000</td>
<td>17000</td>
</tr>
<tr>
<td>[2-3H]Glycerol</td>
<td>2600</td>
<td>2600</td>
</tr>
<tr>
<td>L-Phenyl[2,3-3H]alanine</td>
<td>3600</td>
<td>4000</td>
</tr>
<tr>
<td>[2-14C]Uracil</td>
<td>16000</td>
<td>16000</td>
</tr>
<tr>
<td>[6-3H]Thymidine</td>
<td>12000</td>
<td>12000</td>
</tr>
</tbody>
</table>

Fig. 1. The effect of chloramphenicol upon thymidine incorporation at 42 °C. Cultures of wild-type and ts23 bacteria, prelabelled with [3H]thymidine at 30 °C, were transferred to 42 °C (t = 0), with chloramphenicol (wild-type, ■; ts23, ○) or without chloramphenicol (wild-type, □; ts23, □). The data shown are from a single experiment but are representative of four independent experiments. Relative incorporation values were obtained by dividing the TCA-precipitated c.p.m. in each sample by the c.p.m. in a sample taken from the same culture at t = 0.

Chloramphenicol was added to a culture of wild-type bacteria simultaneously with a shift from 30 to 42 °C.

*Recovery of mutant ts23 after a period at the non-permissive temperature*

Further information about the effect upon DNA synthesis of the mutation in ts23 was obtained by measuring thymidine incorporation by the mutant at 30 °C, after a period at 42 °C. A culture of ts23 was incubated for 1 h at 42 °C before a portion of it was returned to 30 °C. Thymidine incorporation resumed after about 15 min and proceeded rapidly during the next hour, before slowing to a rate equal to that observed normally for a culture of ts23 bacteria at 30 °C. A portion of the culture which was returned to 42 °C after 15 min at
Fig. 2. A culture of ts23 was labelled with $^3$H-thymidine at 30 °C and then transferred to 42 °C. After 1 h ($t = 0$), the culture was divided into three. One part remained at 42 °C ($\square$), the second was returned to 30 °C ($O$), and the third ($\bullet$) was returned to 30 °C immediately after the addition of chloramphenicol. The data shown are typical of at least five independent experiments.

Fig. 3. A culture of ts23, labelled with $^3$H-thymidine, was transferred to 42 °C ($t = 0$). Duplicate samples were taken at 5 min intervals over the next 90 min. One was TCA precipitated immediately ($\bullet$), and the other was incubated at 30 °C with chloramphenicol for 2 h before precipitation ($O$). The data shown are typical of three independent experiments.

Fig. 4. A culture of ts23, labelled with $^3$H-thymidine, was transferred to 42 °C. Chloramphenicol was added 1 h later ($t = 0$) and samples were taken at 1 min intervals over the next 40 min. These samples were incubated for 2 h at 30 °C before TCA precipitation. Points shown are the mean of two independent experiments.

30 °C doubled its thymidine content over the next hour before incorporation stopped, whereas return to 42 °C after 30 min at 30 °C was followed by a 2.5-fold increase at the non-permissive temperature.

When, after 1 h at 42 °C, ts23 was returned to 30 °C in the presence of chloramphenicol, incorporated thymidine increased by about 150% over a period of 90 min (Fig. 2). Thus, after incubation at 42 °C, ts23 has the capacity to replicate DNA at 30 °C without further protein synthesis. The accumulation of this capacity during incubation at 42 °C was
investigated. A culture of ts23 (labelled with [\(^{3}\)H]thymidine) was transferred from 30 to 42 °C and samples were taken at 5 min intervals. Chloramphenicol was added to these and they were then incubated for 2 h at 30 °C, before incorporated radioactivity was measured (Fig. 3). Duplicate samples were immediately TCA precipitated to monitor thymidine incorporation at 42 °C. A sample to which chloramphenicol was added, but which remained at 30 °C, increased its thymidine content by 70% over the next 2 h. Incubation at 42 °C for up to 90 min progressively increased the amount of incorporation after return to 30 °C.

It is possible that, at 42 °C, ts23 synthesizes a replication complex which only becomes active after return to 30 °C. If this is so, protein synthesis at 42 °C would be required for DNA synthesis after return to 30 °C. To test this, the recovery of thymidine incorporation at 30 °C was measured in ts23 after 1 h incubation at 42 °C, followed by up to 40 min in the presence of chloramphenicol at 42 °C (Fig. 4). Not only did chloramphenicol prevent further accumulation at 42 °C of the capacity to synthesize DNA (in the absence of protein synthesis) after return to 30 °C, but the capacity which had accumulated during the first hour at 42 °C was also lost during incubation with chloramphenicol. A sample returned to 30 °C 40 min after the addition of chloramphenicol was unable to synthesize DNA.

**DISCUSSION**

Mutant ts23 did not stop DNA synthesis immediately after transfer to the non-permissive temperature, so the defect is probably not in elongation. When a culture of ts23 was labelled with [\(^{3}\)H]thymidine for two generations at 30 °C and then transferred to 42 °C, incorporated thymidine increased by 73%. Since only 75% of chromosomal DNA would have been labelled at the time of the temperature shift, the increase in incorporated thymidine is an overestimate of the increase in DNA content by a factor of 100 ÷ 75, or about 1.3. Thus the increase in DNA content at 42 °C was 73% ÷ 1.3, or 56%.

Mutants defective in initiation continue DNA synthesis at the non-permissive temperature until all rounds of replication initiated at the permissive temperature have been completed. Sueoka & Yoshikawa (1965) calculated that, after transfer of an initiation-defective mutant (with an average of one replication fork per chromosome) to the non-permissive temperature, DNA synthesis will stop after an increase of 39%. An increase of 56% (as observed for ts23) would occur if 30–40% of the chromosomes had two replication forks and the remainder had one. The replication time for the chromosome of *S. aureus* is unknown, but such a distribution of replication forks seems reasonable for bacteria which were growing in a rich medium with a mean generation time of 50 min.

An increase in the non-permissive temperature failed to reduce the amount of residual replication in ts23. This is consistent with a defect in initiation, as is the observation that residual replication was not reduced by the addition of chloramphenicol at the non-permissive temperature. Further evidence for an initiation defect was that shifting a culture of wild-type bacteria from 30 to 42 °C and simultaneously adding chloramphenicol produced the same effect as a temperature shift alone for ts23 (Fig. 1).

Mutant ts23 resumed DNA synthesis when returned to 30 °C, even when protein synthesis was inhibited by chloramphenicol. The function that is inactivated at 42 °C must, therefore, regain activity spontaneously at 30 °C. When ts23 was incubated for 1 h at 42 °C and then transferred to 30 °C, DNA synthesis resumed after approximately 15 min. If a culture was returned to 42 °C after 15 min at 30 °C, DNA synthesis was not observed at 30 °C but was observed at 42 °C. This implies that the product of the mutant gene is either involved in a pre-initiation reaction which occurs about 15 min before polymerization begins or that it renatures only slowly at 30 °C and that insufficient is present in active form to promote initiation until 15 min after return to 30 °C. The latter possibility seems unlikely, however, as DNA synthesis still took place at 42 °C when ts23 was returned to 30 °C for only 5 min (unpublished observations).
The longer that mutant ts23 was incubated at 42 °C, the more DNA replication occurred in the presence of chloramphenicol after return to 30 °C (Fig. 3). This is consistent with the continued synthesis and accumulation of essential replication proteins at the non-permissive temperature. Further evidence for this is that the accumulation of replication capacity was blocked by chloramphenicol at 42 °C (Fig. 4).

On the basis of results presented in this paper, a simple model describing the initiation of *S. aureus* chromosome replication may be constructed. It is assumed that the mutant function in ts23 is a protein (protein X) which is essential for initiation. At 42 °C, protein X is inactive, initiation is blocked and inactive initiation complex (complex C) accumulates. After return to 30 °C, protein X regains activity and interacts with complex C to form complex C(X). During the 10–15 min before DNA synthesis resumes, C(X) is modified to a form C*(X) which initiates polymerization at the origin. These processes may be summarized thus:

1. Inactive X → Active X  30 °C only
2. C + Active X → C(X)  30 °C only
3. C(X) → C*(X)  30 °C or 42 °C
4. Polymerization  30 °C or 42 °C

Replication complexes accumulated at 42 °C initiate rounds of DNA synthesis at 30 °C (at a linear rate) which will be superimposed upon exponential DNA synthesis, which resumes 10–15 min after return to the permissive temperature. Figure 5 compares the observed rate of DNA synthesis by ts23 after return to 30 °C with the theoretical result of combining exponential and linear components. The exponential rate used for the prediction was that observed in cultures of ts23 bacteria growing at 30 °C. The linear component (solid line) was assumed to be equivalent to the synthesis observed when ts23 was returned to 30 °C in the presence of chloramphenicol. There is good agreement between the prediction and the experimental results.

It was observed that incubation of ts23 at 42 °C, in the presence of chloramphenicol, reduced the capacity for DNA synthesis after return to 30 °C. This would be consistent with the model if protein X (or some component of complex C) is labile, and the accumulation of complex during incubation of ts23 at 42 °C is due to the rate of synthesis exceeding the rate of decay. Chloramphenicol would prevent complex synthesis at 42 °C, and complex already accumulated would lose activity.

REFERENCES


DNA replication mutant of S. aureus


