Plasmid Replication in a Temperature-sensitive Chromosome Replication Mutant of Staphylococcus aureus

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Replication of the antibiotic resistance plasmids pI258, pT10501 and pC221 has been investigated in a mutant of Staphylococcus aureus NCTC 8325, which is temperature-sensitive for the initiation of chromosome replication. Replication of pI258 stopped rapidly at the non-permissive temperature, whilst replication of pT10501 and pC221 continued (although at a lower rate than in the wild-type). It is proposed that the product of the mutant gene may be required directly for pI258 replication, but not for replication of pT10501 or pC221.

INTRODUCTION

Studies of plasmid replication in Staphylococcus aureus have concentrated on the characterization of mutants in which plasmid, but not chromosome, replication is thermosensitive. Mutations affecting plasmid replication have been found to map on both the chromosome and plasmid (Novick, 1974). Little work has been published on the relationship between plasmid and chromosome replication. Thomas & Dyke (1978) reported that replication of plasmids pT10501 and pI258 stopped at the non-permissive temperature in each of four chromosome replication mutants. These workers concluded that, unless it could be demonstrated that plasmid replication can proceed in the absence of chromosome replication, it would not be possible to decide whether the temperature-sensitive gene products were required directly for plasmid replication.

This paper describes an investigation of plasmid replication in the initiation-defective chromosome replication mutant ts23 (Summers & Dyke, 1982).

METHODS

Bacteria. Staphylococcus aureus NCTC 8325 is a naturally occurring strain in which no plasmids have been detected (Novick & Brodsky, 1972); 8325 ts23 is a thymidine non-requiring revertant of the temperature-sensitive chromosome initiation mutant, 8325 thy ts23 (Summers & Dyke, 1982).

Plasmids. Plasmid pI258 has a molecular weight of 18.6 × 10^6 (Novick et al., 1979) and confers resistance to penicillin, erythromycin, mercury and a number of inorganic ions. Plasmid pT10501 has a molecular weight of 2.9 × 10^6 (Summers & Dyke, unpublished) and specifies tetracycline resistance. Plasmid pC221 (Novick & Bouanchaud, 1971) also has a molecular weight of 2.9 × 10^6 and specifies chloramphenicol resistance.

Growth and maintenance of cultures. Bacteria were maintained on CY-agar (Novick, 1963) supplemented with cadmium acetate (50 μg ml⁻¹) for strains carrying pI258 and tetracycline (5 μg ml⁻¹) for strains carrying pT10501. Inocula from stock plates were grown overnight in CY-liquid medium, then diluted with fresh medium and grown into exponential phase before use.

Sucrose velocity gradient centrifugation. Cultures were labelled with [6-³H]thymidine and [2-¹⁴C]thymidine. Samples were lysed and analysed on neutral sucrose gradients by the method of Sheehy & Novick (1975). Gradients were fractionated by puncturing the bases of the tubes with a hypodermic needle and collecting two-drop fractions on strips of Whatman GF/B paper. The strips were soaked in 5% (w/v) TCA for 30 min at 4 °C, then washed with 5% TCA and 96% (v/v) ethanol, before drying in vacuo for 1 h at 80 °C. The strips were cut into fractions and inserted into scintillation vials. Incorporated radioactivity was determined in a liquid scintillation counter after the addition of 2.5 ml of 5% (w/v) butyl-PBD (Fisons) in toluene.

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Quantitative measurement of plasmid DNA. Sucrose gradient centrifugation was used to measure radioactively-labelled plasmid DNA as a proportion of total labelled DNA. Unless otherwise stated, results are given as the mean ± S.D. of at least three independent estimations.

Electrophoresis and fluorography. Plasmid DNA was purified using the method of Birnboim & Doly (1979). Electrophoresis was in vertical gel slabs consisting of 0.85% agarose in Tris/borate buffer (Greene et al., 1974). Gels were stained with propidium iodide (1 µg ml⁻¹) and photographed through a red filter by UV transillumination, using Ilford FP4 or Polaroid Type 664 film. Fluorography of dried gels was by the method of Chamberlain (1979), using sodium salicylate as a water-soluble fluor. Exposure was for one week at −70°C with Fuji RX Medical NIF X-ray film which was pre-fogged to increase sensitivity and obtain a linear response (Laskey & Mills, 1975). Exposed film was developed in Kodak D-19 X-ray film developer and fixed in two consecutive baths of Kodak FX-40 X-ray liquid fixer.

RESULTS

Replication of pI258 and pTI0501 at 30°C

Plasmid-bearing cultures of 8325 and 8325 ts23 were labelled with [³H]thymidine at 30°C and their DNA was analysed on neutral sucrose gradients. There was no detectable difference between the profiles from wild-type or temperature-sensitive bacteria for either pI258 or pTI0501 (results not shown). The proportions of TCA-precipitated radioactivity recovered in the plasmid peaks were 2.0 ± 0.4% for pI258 and 2.8 ± 0.6% for pTI0501.

Replication of pI258 at 42°C

Replication of pI258 at 42°C was compared in wild-type and ts23 bacteria. Cultures were incubated with [¹⁴C]thymidine at 30°C, then transferred to 42°C and incubated with [³H]thymidine for 1 h. When cell lysates were analysed on neutral sucrose gradients, pI258 banded between fractions 17 and 27 (Fig. 1). Plasmid DNA from the wild-type was labelled with both ¹⁴C and ³H. It contained 2.0 ± 0.2% of total TCA-precipitated ³H and 2.0 ± 0.2% of precipitated ¹⁴C recovered from the gradient. There was no distinct ³H-labelled peak on gradients prepared from ts23, but the presence of plasmid DNA was demonstrated by a ¹⁴C-labelled peak which contained 1.0 ± 0.2% of total ¹⁴C recovered.

Replication of pTI0501 at 42°C

A similar technique was used to investigate replication of pTI0501 at 42°C. Plasmid DNA banded between fractions 26 and 34 on neutral sucrose gradients (Fig. 2). Plasmid DNA from lysates of wild-type cells accounted for 2.8 ± 0.3% (two estimations) of total TCA-precipitated ³H recovered from the gradient and 2.9 ± 0.3% (two estimations) of total ¹⁴C. Gradients prepared from lysates of ts23 bacteria contained, in the plasmid peak, 7.3 ± 1.5% of total ³H and 4.3 ± 0.9% of total ¹⁴C.

One explanation of the increased proportion of radioactivity in the plasmid peak of ts23 is that pTI0501 replication continued at 42°C, after chromosome replication had stopped. If this were so, plasmid replication should account for an even higher proportion of total DNA synthesis between 30 and 60 min after transfer to 42°C, since very little replication of the chromosome occurs during this period in ts23 (Summers & Dyke, 1982). To test this, cultures were labelled with [¹⁴C]thymidine at 30°C, as before, and then transferred to 42°C; [³H]thymidine was added 30 min after transfer. Samples were analysed after incubation for a further 30 min at 42°C. For the wild-type, 3.2 ± 0.3% of total TCA-precipitated ³H and 2.6 ± 0.3% of ¹⁴C were found in the plasmid peak. For ts23, 13 ± 2.3% of ³H and 4.3 ± 0.7% of ¹⁴C were in the plasmid peak.

Analysis of lysates by gel electrophoresis

A proportion of plasmid DNA may co-sediment with the chromosome during sucrose or caesium chloride gradient centrifugation (Measures, 1973; Novick & Bouanchaud, 1971). The proportion of chromosome-associated plasmid is known to be altered by incubation of at least one temperature-sensitive chromosome replication mutant at the non-permissive temperature
Fig. 1. Incorporation of radioactive thymidine (c.p.m.) into pI258 at 30°C and 42°C. Cultures of 8325(pI258) and 8325(pI258) ts23 were incubated with [2-14C]thymidine [2μCi ml⁻¹ (75 kBq ml⁻¹); 1 μg ml⁻¹] for two generations at 30°C. They were then transferred to 42°C and [6-3H]thymidine [50 μCi ml⁻¹ (1.85 MBq ml⁻¹); 1 μg ml⁻¹] was added. After 1 h, samples (0.1 ml) were analysed on 10–30% neutral sucrose gradients. ○, ●, [14C]Thymidine incorporation; □, ■, [3H]thymidine incorporation. Open symbols, wild-type; filled symbols, ts23.

Fig. 2. Incorporation of radioactive thymidine (c.p.m.) into pT10501 at 30°C and 42°C. Experimental details were as described in the legend to Fig. 1, except for the strains used. ○, ●, [14C]Thymidine incorporation; □, ■, [3H]thymidine incorporation. Open symbols, strain 8325(pT10501); filled symbols, strain 8325(pT10501) ts23. Actual amounts of [3H] incorporated into the plasmid peaks were: wild-type, 4400 c.p.m.; ts23, 1400 c.p.m.

(Thomas & Dyke, 1978). To check that the increase in the proportion of [3H]-labelled DNA in the pT10501 peak, reported here for ts23 at 42°C, was not a centrifugation artefact, agarose gel electrophoresis was used to separate plasmid from chromosomal DNA.

Cultures of wild-type and ts23 bacteria, carrying pI258 or pT10501, were labelled with [3H]thymidine between 30 and 60 min after transfer to 42°C. Plasmid DNA was purified by the method of Birnboim & Doly (1979) and analysed on agarose gels (Fig. 3). Two plasmid-specific bands were seen for pI258 (P1 and P2) and three for pT10501 (T1, T2 and T3). The remaining bands were also present in extracts prepared from a plasmid-free strain and were assumed to contain chromosomal DNA. Fluorography demonstrated that radioactivity had been incorporated into pI258 by the wild-type (Fig. 3). Very little had been incorporated by ts23, even though the specific activity of [3H]thymidine added to the culture of ts23 was 10 times greater than that added to the wild-type. Plasmid pT10501, however, had been labelled in both wild-type and ts23. These results confirm, qualitatively, the results of the gradient experiments.

Replication of pC221 in ts23

This method was also used to investigate replication of a 2.9 × 10⁶ Dal chloramphenicol resistance plasmid, pC221. Four plasmid-specific bands were observed (C1, C2, C3 and C4) and [3H] was incorporated into these at 42°C by both ts23 and the wild-type (Fig. 3).
Fig. 3. Incorporation of [3H]thymidine into plasmids at 42 °C. Plasmid-containing cultures of 8325 wild-type and ts23, growing exponentially at 30 °C, were transferred to 42 °C at $A_{600}^\text{nm} = 0.15$ (approx.). After 30 min, [6-3H]thymidine was added at specific activities of 5 µCi ml$^{-1}$ (185 kBq ml$^{-1}$) (1 µg ml$^{-1}$) to wild-type cultures and 50 µCi ml$^{-1}$ (1.85 MBq ml$^{-1}$) (1 µg ml$^{-1}$) to ts23. After a further 30 min at 42 °C, samples (1.5 ml) were taken and plasmid DNA was purified by the method of Birnboim & Doly (1979). The DNA was run on a 0.85% agarose gel and fluorographed. (a) Plasmid-free control; (b) pL258; (c) pT10501; (d) pC221. Lane 1, wild-type; lane 2, ts23.
Plasmid replication in S. aureus

DISCUSSION

Taking $3.5 \times 10^9$ Dal as the molecular weight of DNA per cell at 30°C (Della Latta et al., 1978) and the molecular weights of pl258 and pT10501 as $18.6 \times 10^6$ and $2.9 \times 10^6$, respectively, the mean copy numbers per cell (calculated from the results of the sucrose gradient experiments) are four for pl258 and 32 for pT10501. These figures may be underestimates as all plasmid DNA may not be free to sediment in the plasmid peak.

Replication of pl258 takes about 5 min at 37°C (Sheehy & Novick, 1975). If this time is similar for bacteria growing at 30°C, with a mean generation time of 50 min, 10% of plasmid molecules will be replicating at any moment in an unsynchronized culture. By the reasoning of Sueoka & Yoshikawa (1965), if initiation is blocked, plasmid synthesis will stop after an increase of 4%. When ts23 was transferred to 42°C, chromosomal DNA increased by about 50% (Summers & Dyke, 1982) so, if the ts23 gene product is required for initiation of both chromosome and pl258 replication, the ratio of plasmid to chromosome synthesis should be lower in ts23 at 42°C than in the wild-type. The results described in this paper are consistent with this. An alternative explanation is that the product of the mutant gene is not required directly, but that initiation of pl258 replication does not occur in the absence of chromosome initiation. This possibility cannot be excluded as pl258 replication has not been demonstrated in the absence of chromosome replication.

When a culture of ts23, containing pT10501, was incubated with $[^3H]$thymidine at 42°C, the proportion of radioactivity in the plasmid peak was significantly greater than when ts23 was incubated at 30°C or when the wild-type was incubated at 42°C. It is known that incubation of at least one chromosome replication mutant of S. aureus at the non-permissive temperature, increases the amount of plasmid DNA which sediments in the plasmid peak on sucrose gradients (as opposed to sedimenting in association with the chromosome or cell membrane). This results in an apparent increase in the ratio of plasmid to chromosomal DNA synthesis (Thomas & Dyke, 1978). If this were the only cause of the increased proportion of $^3$H recovered from the pT10501 peak after incubation of ts23 at 42°C, the fraction of total $^{14}$C in the plasmid peak (incorporated at both 30°C and 42°C) would equal the fraction of total $^3$H (incorporated at 42°C only). This is not so (Figs 1 and 2). A simple change in sedimentation properties cannot, therefore, account for the result.

A more plausible hypothesis is that the ts23 gene product is not required for pT10501 replication, which therefore continues after chromosome replication has stopped. A quantitative estimate of pT10501 synthesis in ts23 at 42°C may be made from the data of Fig. 2. The mean generation time of wild-type bacteria at 42°C is 25 min, so the total DNA content must increase fivefold in 1 h. The ratio of plasmid to total DNA synthesis by the wild-type was the same at 30°C and 42°C, so plasmid DNA must also have increased fivefold in 1 h. This is equivalent to each plasmid molecule present at the time of the temperature shift, giving rise to four additional molecules at 42°C. This synthesis is represented by the incorporation of 4400 c.p.m. Under the same conditions, ts23 incorporated 1400 c.p.m. into the plasmid peak, so this represents the synthesis of 1-3 additional molecules (or a 2-3-fold increase in plasmid DNA) at 42°C. Thus pT10501 replication must have been initiated in ts23 at 42°C, although the amount of plasmid DNA synthesis was less than in the wild-type.

Gel electrophoresis confirmed the results obtained in the sucrose gradient experiments. It was also shown that pC221 behaved similarly to pT10501 in ts23 at 42°C. Both plasmids are small, multicopy replicons which carry a single antibiotic resistance determinant and might be expected to employ the same components of the chromosome replication machinery for their own replication.

This is the first report of S. aureus plasmid replication in the absence of chromosome replication. It demonstrates that at least some plasmids do not require the full range of chromosome replication functions. In the light of these results, it appears that the failure of pT10501 to replicate, at the non-permissive temperature, in the chromosome replication mutants described by Thomas & Dyke (1978) was due to a requirement for the products of the mutant genes rather than to stringent coupling between chromosome and plasmid replication.
REFERENCES


