Isolation and Transduction Analysis of Temperature-sensitive Mutants of *Staphylococcus aureus* Defective in DNA Replication

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Four temperature-sensitive mutants of *Staphylococcus aureus* with defects affecting DNA synthesis have been isolated and partially characterized. They fall into two groups: three have defects either in elongation of DNA or synthesis of its precursors; the fourth has properties inconsistent with a defect in either elongation or initiation. Transduction analysis indicated that the mutation in this fourth mutant is unlinked to the mutations in the other three, which are all clustered on one side of a gene conferring resistance to novobiocin.

INTRODUCTION

Studies of DNA replication in *Staphylococcus aureus* have been largely confined to investigation of the biochemistry and genetics of plasmid replication. Mutants with defects in both plasmid and chromosomal genes specific for plasmid replication have been isolated (Novick, 1967, 1974) and partially characterized (Novick *et al.*, 1974). The dependence of plasmid replication on genes also involved in chromosome replication and the nature of genes involved in chromosome but not plasmid replication have not, however, been studied in detail. Measures (1973) isolated a number of temperature-sensitive *dna* mutants in *S. aureus* ps80 and Raftery (1974) partially characterized one of these, whose properties indicated a defect in initiation. None of these defects could be suppressed by the presence of the plasmid pI₉₇₈₉ and, in the initiation mutant, plasmid replication was affected more rapidly than chromosome replication when the bacteria were placed at the restrictive temperature. The mutants were not, however, fully characterized nor were the mutations mapped. It was thus impossible to estimate the number of genetic loci affected by these defects.

In work designed to investigate these questions more thoroughly in *S. aureus* NCTC 8325, temperature-sensitive mutants with defects in chromosome replication have been isolated and characterized. In this paper the isolation and transduction analysis of four such mutants are described.

METHODS

Bacteria. *Staphylococcus aureus* NCTC 8325 and derived strains were used. 8325 Nov^R was isolated after ethyl methanesulphonate (EMS) mutagenesis by selection with novobiocin on CY agar by a disc diffusion method (2 µg novobiocin per disc). 8325 Spc^R was isolated similarly with 40 µg spectinomycin per disc. 8325 *ade*, 8325 *gua* and 8325 *pur* were isolated after EMS mutagenesis by replica plating colonies grown on complete PN medium (Pattee & Neveln, 1975) to PN medium lacking adenine, guanine or both. 8325 *thy* was a thymidine-requiring derivative obtained by the method of Stacey & Simson (1965). All mutants were isolated by H. Hackling & K. G. H. Dyke (unpublished).

Maintenance and growth of cultures. Strain 8325 was maintained on CY agar (Novick, 1963). For 8325 Nov^R

this was supplemented with 5 μg novobiocin ml^{-1} ; for 8325 Spc^{R} , with 200 μg spectinomycin ml^{-1} . 8325 *thy* was maintained on Difco AOAC defined medium agar with 10 μg thymidine ml^{-1} ; 8325 *ade*, 8325 *gua* and 8325 *pur* were maintained on CY agar. Bacteria were normally grown in CY medium (Novick, 1963) at 30 °C. Inocula from agar stock plates were grown for 15 to 18 h and then diluted into fresh, warmed medium and grown into exponential phase.

Bacteriophage. Phage 53 of the International Phage Typing Series was used.

Propagation of bacteriophages. CY medium (5 ml) was mixed with 10 ml of phage buffer (Novick, 1963), and then bacteria [final density about 5×10^7 colony-forming units (c.f.u.) ml^{-1}] and phage 53 were added to give an input ratio of about 0.5. Incubation with gentle shaking, at 30 °C, was continued until the medium cleared; then more bacteria were added to give 2.5×10^8 c.f.u. ml^{-1} and incubation was continued until the medium cleared again. The resultant phage preparation contained 3×10^9 to 5×10^9 plaque-forming units (p.f.u.) ml^{-1} ; contaminating bacteria were removed by passage through a sterile 24 mm Millipore filter (0.45 μm pore size).

Transduction. A 15 h culture of recipient bacteria was diluted to give about 5×10^8 c.f.u. ml^{-1} in 20 ml of CY medium. After incubation at 30 °C for 1 h, the bacteria were harvested by centrifugation and resuspended in 2 ml of warmed CY medium and 4 ml of phage buffer. The suspension was divided into two, and 0.1 ml of transducing phage preparation was added (input ratio 0.01 to 0.05) to one half. Both suspensions were then incubated at 30 °C with gentle shaking for 30 min to allow phage adsorption. Sodium citrate (final concentration 0.4 M) was then added, to prevent further phage adsorption, and the suspension was centrifuged. After washing with CY medium (plus 0.2 M-sodium citrate) and recentrifuging, the bacteria were finally resuspended in CY medium (plus 0.2 M-sodium citrate) and incubated for a further 2 to 3 h at 30 °C with aeration to allow phenotypic expression of transduced genes; they were then plated on selective plates. The bacterial suspension without transducing phage was a control for contaminants which might grow on the selective plates. In addition 0.1 ml samples of transducing phage preparations were always plated to check for contamination.

Mutagenesis and selection of mutants temperature-sensitive for growth. EMS mutagenesis was carried out according to Novick (1963). Bacteria grown into stationary phase after mutagenesis were diluted into fresh medium, grown into exponential phase at 30 °C and plated on CY agar to give approximately 100 colonies per plate. Incubation at 30 °C was continued until colonies were about 0.5 mm in diameter (18 h); then, after marking the position of all visible colonies, the plates were transferred to 42 °C. After 6 to 8 h colonies able to grow at 42 °C were large; colonies remaining small were picked and tested for temperature-sensitivity by streaking on plates at 42 and 30 °C.

Incorporation of radioactively labelled precursors. To measure the incorporation[†] of [radioactively labelled precursors into macromolecules, 1.0 ml samples of cultures of bacteria growing in the presence of these precursors were removed and 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.

RESULTS

Isolation of temperature-sensitive mutants with defects affecting DNA replication

After EMS mutagenesis, approximately 10^6 colonies were screened and 96 mutants that were temperature-sensitive for growth were isolated. A group of 80 of these mutants was screened for defects in DNA synthesis by a rapid method based on that of Karamata & Gross (1970): incorporation of [^3H]thymidine and [^{14}C]phenylalanine was measured at 42 °C to determine whether DNA synthesis was more defective than protein synthesis at this temperature. The other 16 mutants were screened by an analogous method measuring incorporation in parallel cultures of [^3H]thymidine and [^3H]uracil at 30 and 42 °C. The first group yielded 11 putative Dna mutants and the second, three. Further investigation of incorporation of phenylalanine, glycine, glycerol, uracil and thymidine by these mutants, as measures of biosynthesis of protein, protein and cell wall, membrane, RNA and DNA, respectively, was carried out at 30 and 42 °C. On the basis of (i) normal incorporation of these precursors at 30 °C with the wild type (WT) as control, (ii) primary defects only in thymidine incorporation at 42 °C, and (iii) sustained viability on agar plates at 30 °C, four mutants were selected for further study, three from the first group (ts39, ts53 and ts70) and

Table 1. Rate of incorporation of radioactively labelled precursors of macromolecules into 8325WT and temperature-sensitive mutants at 30 and 42 °C

Strain and temperature	$10^{-3} \times \text{Rate of incorporation (c.p.m. per } A_{675}^{1.0\text{ cm}} = 2.5)^*$				
	[U- ¹⁴ C]-Phenylalanine	[2- ³ H]-Glycine	[2- ³ H]-Glycerol	[5- ³ H]-Uracil	[6- ³ H]-Thymidine
WT 30 °C	6	60	35	45	90
42 °C	6	50	30	35	80
ts39 30 °C	6	40	30	45	70
42 °C	6	50	40	40	37.5† 5‡
ts53 30 °C	6.3	40	20	50	100
42 °C	6.5	40	25	40	10
ts70 30 °C	5	50	35	60	70
42 °C	5	60	40	45† 15‡	10
ts11 30 °C	5	80	60	10	90
42 °C	5	60	40	8	1

* Precursors were present at the following activities and concentrations: [U-¹⁴C]phenylalanine, 0.1 $\mu\text{Ci ml}^{-1}$, 50 $\mu\text{g ml}^{-1}$; [2-³H]glycine, 1 $\mu\text{Ci ml}^{-1}$, 100 $\mu\text{g ml}^{-1}$; [2-³H]glycerol, 2.5 $\mu\text{Ci ml}^{-1}$, no exogenous source other than in CY medium; [5-³H]uracil, 1 $\mu\text{Ci ml}^{-1}$, no exogenous source other than in CY medium; [6-³H]thymidine, 1 $\mu\text{Ci ml}^{-1}$, 10 $\mu\text{g ml}^{-1}$.

† Initial rate. ‡ Final rate.

one from the second (ts11). Table 1 shows the rates of incorporation of these precursors by 8325WT and the four derived temperature-sensitive strains, at 30 and 42 °C. All five strains showed similar rates of incorporation of phenylalanine, glycine and glycerol per unit increase in absorbance. WT, ts39 and ts53 incorporated uracil at similar rates at 30 and 42 °C while ts70 showed a decreasing rate of uracil incorporation at 42 °C, although incorporation was normal at 30 °C. This defect at 42 °C, however, appeared after the defect observed in DNA synthesis (see below) and was much smaller. ts11 showed normal uracil incorporation during rapid screening but in further tests uracil incorporation was poor, compared with WT, at both 30 and 42 °C. However, since the mean generation time at 30 °C was 90 min compared with about 48 min for WT, uracil incorporation could be correlated with growth rate assuming that RNA represents a lower proportion of the dry weight at lower growth rates. In support of this explanation is the observation that with subculturing over about 6 months the mean generation time decreased to about 51 min, and uracil incorporation became the same as in WT. These variations did not affect DNA synthesis.

The characteristics of thymidine incorporation per unit increase in absorbance by these strains are shown in Fig. 1. The incorporation by all strains was similar at 30 °C. ts39 showed considerable residual incorporation after transfer to 42 °C, but ts53, ts70 and ts11 all showed a rapid reduction in the rate of incorporation under these conditions. To test that this reduced incorporation was not due to defective uptake of thymidine at 42 °C, DNA was estimated by the method of Burton (1956). Typical results are shown in Fig. 2. These confirmed that DNA synthesis in the temperature-sensitive strains was reduced at 42 °C but normal at 30 °C. ts39 did not show a significant reduction in the rate of DNA synthesis at 42 °C until about 30 min after transfer, by which time the DNA content had almost doubled. After about 90 min the DNA content had increased by 150 to 200%. The amount of DNA synthesized in ts39 was not significantly reduced by transfer to 44 °C instead of 42 °C. In ts53, DNA synthesis showed a reduced exponential rate on transfer to 42 °C and the DNA content increased by about 90% in 90 min. ts70 and ts11 showed virtually no increase in DNA for about 45 min after transfer to 42 °C, but subsequently there was some increase in DNA content.

It was concluded that ts53, ts70 and ts11 probably have defects either in elongation enzymes or in precursor synthesis. The properties of ts39 cannot easily be explained either

Fig. 1. Incorporation of [6-³H]thymidine into 8325WT, ts39, ts53, ts70 and ts11. Cultures (15 h) of these strains were diluted into fresh, warmed CY medium containing 10 μg thymidine ml^{-1} and grown into exponential phase at 30 °C. The cultures were then diluted to about $A_{675\text{nm}}^{1.0\text{cm}} = 0.1$ in the same medium, and [6-³H]thymidine (final concn 1 $\mu\text{Ci ml}^{-1}$) was added. Incorporation and $A_{675\text{nm}}^{1.0\text{cm}}$ at 30 and 42 °C were then determined periodically for between 90 and 120 min. Incorporation at 30 °C (open symbols) and 42 °C (closed symbols): (a) $\circ$, $\bullet$, WT; $\square$, $\blacksquare$, ts39; (b) $\circ$, $\bullet$, ts53; $\square$, $\blacksquare$, ts70; $\triangle$, $\blacktriangle$, ts11. (The mean generation time of WT at 42 °C was 26 min.)

Fig. 2. DNA synthesized by 8325WT, ts39, ts53, ts70 and ts11 after transfer to 42 °C as estimated by the method of Burton (1956). Cultures (15 h) were diluted into fresh, warmed CY medium and grown into exponential phase at 30 °C. The cultures were then diluted to about $A_{675\text{nm}}^{1.0\text{cm}} = 0.25$ and transferred to 42 °C. Samples (10 ml) were withdrawn every 15 min for 90 min and their DNA contents were determined. $\circ$, WT; $\square$, ts39; $\bullet$, ts53; $\triangle$, ts70; $\blacktriangle$, ts11.

in this way or by supposing it is an initiation-defective mutant, since the residual synthesis after transfer to 42 °C is too extensive to result simply from completion of rounds of replication likely to have been already initiated at 30 °C under these conditions (Sueoka & Yoshikawa, 1965). Possible explanations include defects in synthesis of new replication complexes or temperature-sensitive termination of chromosome replication.

Transduction analysis of the mutations in ts39, ts53, ts70 and ts11

The frequencies of reversion to temperature-stability of ts11 and ts70 were low (about 1 in 10^8). ts39 and ts53 had higher reversion frequencies (1 in 10^6 for ts53; 1 in 5×10^6 for ts39); moreover, when 10^6 to 10^7 bacteria were spread on a plate, in addition to the revertants a large number of tiny, slow-growing colonies appeared. Since the frequency of

Table 2. *Linkage of nov to the ts53, ts70 and ts11 mutations*

Transductional recipient*	Fraction of novobiocin-resistant transductants that were temperature-stable	Fraction of temperature-stable transductants that were novobiocin-resistant
ts11	52/115 (45 %)	94/138 (68 %)
ts70	58/124 (47 %)	94/143 (66 %)
ts53	49/92 (53 %)	ND
ts39	0/93 (0 %)	ND

ND, Not done.

* Donor was phage 53 propagated on 8325 Nov^R.

transduction of chromosomal genes in *S. aureus* is quite low (< 1 in 10^6), only ts70 and ts11 were suitable as recipients in transduction analysis of the temperature-sensitive mutations.

Transducing phage preparations, propagated on WT, ts39, ts53, ts70 and ts11, were used to transduce temperature-stability into ts70 or ts11. Initial experiments showed that phage propagated on ts39 gave as many temperature-stable transductants as phage propagated on WT. The controls of phage propagated on ts70 into ts70 (recipient) or phage propagated on ts11 into ts11 (recipient) gave about 1 % of this number. Phage propagated on ts53 or ts11 into ts70 (recipient) or phage propagated on ts53 and ts70 into ts11 (recipient) gave 1 to 10 %, and the numbers obtained were not significantly different from the number of revertants obtained on control plates. It was tentatively concluded that the ts53, ts70 and ts11 mutations were clustered and that ts39 was at least 1 % of the chromosome away (a minimum estimate of the amount of DNA likely to be carried by the transducing phage). Alternatively the results could be explained by supposing that the different phage preparations, although having similar titres of p.f.u. ml⁻¹, differed in efficiency of transduction.

To check this a genetic marker linked by transduction to the ts53, ts70 and ts11 mutations was sought. By using as a donor a novobiocin-resistant mutant of 8325WT, we found that a gene conferring resistance to novobiocin (*nov*) was about 50 % cotransduced with these three mutations (Table 2). However, when a phage preparation propagated on 8325 Nov^R was used to transduce novobiocin resistance into ts39, none of the transductants was temperature-stable. These results confirm that the ts53, ts70 and ts11 mutations are close together and not linked to the mutation in ts39. They cannot, however, eliminate the possibility that the gene conferring novobiocin resistance separates one of the three *Dna* mutations from the other two.

We settled this point in the following way. Novobiocin resistance was transduced into ts11, ts53 and ts70 and novobiocin-resistant strains which were still temperature-sensitive were selected. Phage propagated on each and on 8325 Nov^R were used to transduce either temperature stability or novobiocin resistance into ts11 or ts70 (Table 3). Although the frequency of transduction to novobiocin resistance was much the same for all four phage preparations, the frequency of temperature-stable transductants with phage propagated on ts11 Nov^R, ts70 Nov^R or ts53 Nov^R was reduced by at least 95 % compared with the frequency with phage propagated on 8325 Nov^R, and those few colonies that were found may be revertants rather than transductants. It was concluded that the mutations in ts53, ts70 and ts11 are all closely clustered on one side of the *nov* locus.

Similar transductional analysis with phage propagated on 8325 Spc^R, 8325 *thy*, 8325 *ade*, 8325 *gua* or 8325 *pur* failed to show any linkage of these loci with ts53, ts70 and ts11 mutations.

Table 3. Average number of transductants per plate when phage 53 propagated on 8325 Nov^R or temperature-sensitive mutants of 8325 Nov^R was used to transduce novobiocin resistance or temperature stability into ts70 or ts11 in the same experiment

Transducing phage 53 propagated on donor strain:	Recipient and selected marker			
	ts70		ts11	
	Temperature stability	Novobiocin resistance	Temperature stability	Novobiocin resistance
8325 Nov ^R	115	50	86	100
ts70 Nov ^R	1	50	4	195
ts11 Nov ^R	4	60	1	150
ts53 Nov ^R	3	34	6	100
Control (no phage 53)	6	0	5	0

DISCUSSION

On the basis of both phenotypic and genetic characterization the four mutants tested fall into two groups. The mutation in ts39, which is not genetically linked to the mutations in the other mutants, has a phenotype which falls into neither of the classical *dna* mutant categories of elongation or initiation-defective (Hirota, Ryter & Jacob, 1968). The considerable residual DNA synthesis after transfer to 42 °C (150 to 200% increase) is much greater than the 30 to 40% expected for an initiation-defective mutant with a pair of replication forks on each chromosome (Sueoka & Yoshikawa, 1965). An increase of the order of 200% would be expected if there were about 30 replication forks evenly distributed on each chromosome. Possible explanations for the phenotype may be defects in the synthesis of replication elongation sites or a defect in termination of replication.

ts11, ts53 and ts70 all contain temperature-sensitive defects which are very rapidly expressed on transfer to 42 °C, and the mutations in these mutants are close together on the chromosome. It is remarkable that three out of the four mutations studied should show such close linkage. It may be chance, but another explanation might be that this is a mutational hot spot. It is interesting that in studies of *dna* loci in *Escherichia coli*, mutations at certain loci have been isolated in much greater numbers than at others (Wechsler & Gross, 1971); out of five *dna* mutants isolated and studied by Kohiyama (1968), two were *dnaA* mutants.

Alternatively there may be a cluster of *dna* genes in this region. Such an explanation would be plausible if the *nov* locus linked to these *Dna* mutations conferred resistance through alteration of a gene product involved directly in DNA replication. Novobiocin is known to be a selective inhibitor of DNA replication in *E. coli* (Smith & Davies, 1967; Standenbauer, 1975) and the site of action of novobiocin has been shown to be a DNA gyrase enzyme (Gellert *et al.*, 1976). However, resistance to novobiocin may also result from altered permeability to the drug; the above linkage might therefore be fortuitous. The explanation must await further investigation of the nature of resistance to novobiocin conferred by the *nov* gene used here.

Examination of previous transductional mapping in *S. aureus* (Ritz & Baldwin, 1962; Pattee *et al.*, 1974) allows an estimation of the separation of the ts11, ts53 and ts70 mutations from each other and from the *nov* locus. It appears that the ts11, ts53 and ts70 mutations may all be in the same gene although crossover can occur between the mutations, and that this gene may be four to five genes away from the *nov* locus. The locus containing the ts11, ts53 and ts70 mutations has been tentatively designated *dnaA*.

Previously, in *S. aureus*, transductional linkage has been demonstrated between genes in the same operon (Ritz & Baldwin, 1962; Kloos & Pattee, 1965; Tien & White, 1968; Barnes, Bondi & Fuscaldo, 1971) and between functionally related cistrons (Pattee *et al.*,

1974). The addition of *dna* loci to the genetic map of *S. aureus* is useful, especially because some mutants show reversion frequencies which should allow them to be used in transformation mapping as well. If the *nov* locus is the same as that used by Pattee & Neveln (1975) in transformational analysis then, tentatively, we can place this *dnaA* locus between *hisGbl5* and *nov* or between *nov* and *pur-102*. The finding that *dnaA* is not linked by transduction to *ade*, *gua* or *pur* does not necessarily indicate a position between *hisGbl5* and *nov* since either these purine auxotrophs may not be at the *pur-102* region (another *pur* locus exists) or the distance between *nov* and *pur-102* may be too great to show linkage of *dnaA* to both.

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