Isolation and characterization of temperature-sensitive mutants of *Streptomyces coelicolor* A3(2) blocked in macromolecular synthesis

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A collection of temperature-sensitive mutants of *Streptomyces coelicolor* A3(2) was isolated. The majority of the mutants showed an osmotically remedial phenotype. Mutants defective in macromolecular synthesis were identified and characterized further. Four mutants were found in which DNA replication was defective, but which continued to synthesize RNA and protein at the restrictive temperature (39 °C). The kinetics of cessation of DNA synthesis allowed a tentative identification of slow (initiation) and fast (elongation) stop *dna* mutants. The inhibition of DNA replication in the four mutants was found to be reversible on returning to the permissive temperature (30 °C), but only after a delay of about 2 h. Three other mutants were identified which showed not only cessation of DNA replication at the restrictive temperature, but also defects in other macromolecular synthesis events.

Introduction

One approach to the study of essential cell functions is to use conditional-lethal mutants. The isolation of mutants which are defective in DNA synthesis in some restrictive environment, usually high temperature, was a prerequisite for the analysis of the complex events of DNA replication and the cell division cycle of *E. coli* (reviewed by McMacken *et al.*, 1987). More recently, a similar strategy has been applied to investigate the novel problems posed by *Caulobacter crescentus*, a bacterium which undergoes cell differentiation; temperature-sensitive DNA replication and cell division mutants have been used to investigate the central role of the cell cycle in controlling morphogenesis (Newton & Ohta, 1990).

Our interest lies in the streptomycetes, a group of bacteria which have a complex life cycle exhibiting both morphological and metabolic differentiation. A substrate mycelium of branching hyphae, which results from vegetative growth, gives rise to aerial mycelium which subsequently forms spores. Aerial hypha formation is accompanied by physiological changes, in particular the production of a number of secondary metabolites.

Morphological and physiological differentiation appear to be temporally coordinated, and may be initiated in response to nutrient limitation (Braña & Demain, 1988; de los Reyes-Gavilan *et al.*, 1991). Evidence for

such interlinked regulation has come additionally from the study of pleiotropic mutants (*bld* and *afs* mutants) which are defective in sporulation and antibiotic production (reviewed by Chater, 1989).

It is an over-simplification to state that there is a clear demarcation between primary growth and the secondary metabolic phase in *Streptomyces*. However, since one criterion for primary growth of bacteria is an increase in cellular DNA content, it has been proposed that the cessation of DNA replication marks the end of this phase (Martin & Demain, 1980), at least in liquid culture. Defining the mechanisms that control chromosome replication may be important in understanding the onset of morphological and biochemical differentiation in *Streptomyces*.

Another challenging aspect of studying the control of DNA replication in *Streptomyces* is related to the mycelial nature of this organism. The branching hyphae are the result of growth without cell separation. Septa are not abundant, and each compartment of the vegetative hyphae contains multiple copies of the chromosome (Prosser *et al.*, 1988); only the spores contain a single copy. This can be compared with events in *E. coli*, in which the cell cycle involves coordinated DNA replication, cell division and separation (Von Meyenburg & Hansen, 1987).

In the case of *Streptomyces*, our knowledge of macromolecular synthesis in general, and DNA replication in particular, is limited (Granozzi *et al.*, 1990).

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Moreover, the available data are mainly concerned with events occurring during spore germination (Hardisson *et al.*, 1980). Our long term objective is to gain an understanding of the control of DNA replication in *Streptomyces coelicolor* A3(2), genetically the best characterized streptomycete (Hopwood *et al.*, 1985). In this report we describe the isolation and characterization of a number of temperature-sensitive mutants of *S. coelicolor* which are defective in macromolecular synthesis.

Methods

Bacterial strains. *Streptomyces coelicolor* A3(2) strain MT1110 was used for the isolation of the ts mutants. This strain (isolated in this laboratory) is an SCP1⁻, SCP2⁻ derivative of the wild-type prototrophic strain 1147 (Hopwood, 1959).

Growth media and culture conditions. Mannitol soya agar medium (MS) was used to prepare spore stocks as described by Hobbs *et al.* (1989). Auxotrophic mutants were detected using minimal medium (Hopwood *et al.*, 1985). Oxoid nutrient agar was used when screening colonies for the ts phenotype. Difco nutrient agar, which contains no added NaCl, was chosen for testing the osmotic remediality of the ts mutants. The measurement of the uptake of radiolabelled compounds was carried out using liquid cultures in YPGS and YPG, which are modified YEME media (Hopwood *et al.*, 1985). YPGS (3 g l⁻¹ Difco yeast extract, 5 g l⁻¹ Difco Bacto-peptone, 10 g l⁻¹ glucose and 25% sucrose, w/v) was used for the non-osmotic remedial mutants and YPG (YPGS lacking sucrose) for the osmotic remedial mutants.

The standard growth temperature was 30 °C. A permissive temperature of 30 °C and a restrictive temperature of 39 °C were used when screening for the ts mutants.

Mutagenesis and isolation of ts and auxotrophic mutants. Spores of strain MT1110 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at a concentration of 2 mg ml⁻¹ at pH 8 (Hopwood *et al.*, 1985). Single colonies arising from mutated spores were grown to sporulation at 30 °C on MS then 'tooth-picked' onto the appropriate test media. Duplicate Petri dishes were incubated at 30 °C and 39 °C; colonies which failed to grow at 39 °C but grew at 30 °C were rechecked. The putative ts mutants were subjected to two further rounds of single colony isolation, sporulation and temperature-sensitivity testing before being designated as ts mutants. Auxotrophic mutants were screened at 30 °C.

Measurement of macromolecular synthesis. DNA, RNA and protein syntheses were monitored by measuring, respectively, the incorporation into acid-insoluble material of [methyl-³H]thymidine [specific activity 40 Ci mmol⁻¹ (1.48 TBq mmol⁻¹); 1.0 µCi ml⁻¹ in the growth medium], [2-¹⁴C]uracil [specific activity 55 mCi mmol⁻¹ (2.04 GBq mmol⁻¹); 100 nCi ml⁻¹ in the growth medium] and [³⁵S]methionine [specific activity 1000 Ci mol⁻¹ (37 TBq mmol⁻¹); 1.0 µCi ml⁻¹ in the growth medium].

Spores were inoculated into 250 ml baffled flasks containing 50 ml liquid medium (YPGS or YPG) supplemented with the required radioactive precursors and incubated at 30 °C on an orbital shaker. Growth was monitored as OD₆₀₀ (Cecil spectrophotometer); the temperature shift was carried out when cultures reached OD₆₀₀ = 0.02–0.05. At this stage, flasks were transferred to a 39 °C water bath for 15 min, with shaking (the culture reached a temperature of 39 °C after approximately 5 min), and were then placed in a 39 °C orbital shaker.

To measure the radioactive incorporation, four 0.5 ml samples were taken at each time point, the samples being dealt with as two pairs. One

pair was added to 0.5 ml aliquots of cold 10% (w/v) trichloroacetic acid (TCA) and cooled on ice for 2 h then washed (see below). The other samples were added to 0.5 ml aliquots of 2 M-NaOH and incubated overnight at 37 °C. After incubation, 0.5 ml cooled 50% (w/v) TCA was added, and the samples cooled on ice for 2 h before washing. All the samples were washed with cold 5% (w/v) TCA on glass-fibre filters (Whatman GF/C). After drying the filters, the radioactivity in the acid-insoluble fraction was counted in a RackBeta II liquid scintillation counter (LKB) using Optiphase 'Hisafe' 3 scintillation fluid (LKB). The data on the incorporation of [³H]thymidine into DNA were derived from NaOH-treated samples, whereas the data for [³⁵S]methionine and protein were derived from non-NaOH-treated samples. Data on [¹⁴C]uracil incorporation into RNA were obtained by subtracting the c.p.m. in NaOH-treated samples from the c.p.m. in non-NaOH-treated samples. Each experiment was carried out at least three times to confirm the reproducibility of the results shown. All the radioactive compounds were obtained from Amersham International.

Results and Discussion

Isolation of temperature-sensitive (ts) mutants

All of the mutants described here were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of spores. 50000 spore clones, which grew at the permissive temperature of 30 °C, were screened for ts growth at 39 °C and/or auxotrophy at 30 °C. 102 mutants which were ts on nutrient agar were identified. This frequency (0.2%) was comparable to the observed frequency of non-ts auxotrophs in this experiment (0.3%).

Growth medium suppression of ts phenotype

The temperature-sensitive phenotype of these mutants was tested on several different media. No differences were observed between minimal and complex media; however, on the addition of sucrose (25%, w/v) to the media, only 12 of the 102 ts mutants failed to grow at 39 °C. Replacement of the sucrose with iso-osmolar KCl (4.1%, w/v) gave similar results. The ability of sucrose or KCl to suppress the ts phenotype suggests that the majority of the ts mutants are osmotically remedial. Osmotic suppression occurs with ts mutants of many bacteria and fungi (reviewed by Csonka, 1989), including ts *dna* mutants of *E. coli* (Chu *et al.*, 1977; Ricard & Hirota, 1969). This remediality probably reflects the fact that the structures of such mutant proteins are unusually sensitive to the solute composition of the cytoplasm, which is in part determined by the external osmolarity.

During the course of the media testing, it was noted that 48 h growth at 30 °C in the presence of KCl or sucrose resulted in enhanced production of the red pigment, undecylprodigiosin, when compared with the results obtained on non-supplemented solid media. This phenomenon was apparent with the parent strain as well as the mutants. It is likely that the overproduction of

undecylprodigiosin is due to a 'proline effect'. Measures (1975) found that osmotic stress resulted in large increases in the intracellular levels of proline in a variety of bacteria; more recently, this has been shown with *Streptomyces griseus* as well (Killham & Firestone, 1984). A study of the synthesis, uptake and breakdown of proline by *S. coelicolor* has demonstrated that the intracellular concentration of this amino acid can directly influence the level of synthesis of undecylprodigiosin; excess proline can be shunted into the production of this secondary metabolite (D. A. Hodgson, personal communication). Thus a high salt concentration in the growth medium may result in the intracellular accumulation of proline and enhanced production of undecylprodigiosin.

Identification of macromolecular synthesis mutants

In order to identify any mutants defective in macromolecular synthesis, liquid culture experiments were carried out at the permissive and restrictive temperatures to follow the incorporation of [^3H]thymidine, [^{14}C]uracil and [^{35}S]methionine into DNA, RNA and protein, respectively.

DNA synthesis mutants

Four mutants were found in which DNA replication is defective, but which continue to synthesize RNA and protein at 39 °C. None showed a cold-sensitive growth phenotype at 20 °C. In ts-38, a non-osmotically remedial ts mutant, the incorporation of [^3H]thymidine into DNA continued for 2 h after the shift from 30 °C to 39 °C but then ceased, whereas the incorporation of [^{14}C]uracil and [^{35}S]methionine proceeded for at least 5 h after switching to the restrictive temperature (Fig. 1a). Cessation of DNA synthesis was not observed in cultures maintained at 30 °C (data not shown). The effect of the culture temperature on DNA synthesis of the wild-type strain in YPGS media is shown in Fig. 1(b). The doubling time of the wild-type strain in this medium at 39 °C is about 90 min. The growth kinetics of ts-38 in YPGS medium were also monitored by optical density readings; the OD₆₀₀ continued to increase for a minimum of 5 h after transfer to the restrictive temperature. This pattern of cessation of DNA replication has been described as 'slow stop' for ts mutants of other bacterial species (Hirota *et al.*, 1970; Wechsler & Gross, 1971) and implies that ts-38 may be defective in the initiation of DNA replication. Samples of YPGS cultures of the wild-type parent and ts-38 were examined by phase contrast microscopy. Two hours after the switch to 39 °C, the mycelium of both strains exhibited an increased frequency of branching as compared to that seen in 30 °C cultures.

Fig. 1. Macromolecular synthesis of ts-38 and the parent strain MT1110. Spores were inoculated into YPGS medium containing the specified radiolabelled compounds at 30 °C. Cultures were shifted to 39 °C as indicated. Incorporation of the radiolabelled compounds into TCA-precipitable material was determined. (a) ts-38: ●, [^3H]thymidine; □, [^{14}C]uracil; and △, [^{35}S]methionine. (b) MT1110: ●, [^3H]thymidine.

The three other ts DNA replication mutants isolated in this study (ts-66, ts-102 and ts-114) all showed an osmotic remedial ts phenotype, necessitating the use of media without sucrose (YPG) for the labelling studies. Initial experiments determined whether the temperature shift to 39 °C had any observable effect on DNA replication of the wild-type strain in YPG medium. After the switch to 39 °C, DNA synthesis was found to be impaired for up to 3 h, then the incorporation of [^3H]thymidine started to increase again as in the 30 °C control culture (Fig. 2). Similar results were obtained in experiments monitoring protein synthesis. This behaviour may reflect a heat shock response by the organism. In a recent study, E. Barratt (personal communication) has demonstrated that a high sucrose concentration in the medium protects *S. lividans* against the effects of thermal shock. It seems possible that this effect, like the suppression of the ts mutations, is due to a decreased level of protein denaturation at high osmotic pressures.

Labelling studies on ts-66 showed that [^3H]thymidine incorporation stopped within 20 min of raising the temperature to 39 °C, and there was also some evidence of a net decrease in DNA content (Fig. 3a). RNA and protein synthesis continued for approximately 3 h then

Fig. 2. DNA synthesis of the parent strain MT1110. Spores were inoculated into YPG medium containing $[^3\text{H}]$ thymidine at 30°C . The culture was shifted to 39°C as indicated. Incorporation of $[^3\text{H}]$ thymidine into TCA-precipitable material was determined.

Fig. 3. Macromolecular synthesis of (a) ts-66 and (b) ts-102. Spores were inoculated into YPG medium containing $\bullet$, $[^3\text{H}]$ thymidine; $\square$, $[^{14}\text{C}]$ uracil; or $\triangle$, $[^{35}\text{S}]$ methionine at 30°C ; cultures were shifted to 39°C as indicated. Incorporation of the radiolabelled compounds into TCA-precipitable material was determined.

the incorporation of $[^{14}\text{C}]$ uracil and $[^{35}\text{S}]$ methionine levelled off. $[^3\text{H}]$ Thymidine incorporation continued for about 30 min after switching ts-114 to 39°C . Protein and RNA were synthesized for a few hours, but at a reduced linear rate.

The kinetics of cessation of DNA synthesis observed for ts-66 and ts-114 contrasts with those for ts-38 and we propose, by analogy with other systems (Wechsler &

Gross, 1971), that the former two mutants are defective in the elongation step of DNA replication. The pattern of macromolecular synthesis shown by these mutants is markedly different from that of the ts mutant of *Streptomyces hygroscopicus* described by Süss *et al.* (1988), which showed an immediate cessation of both DNA and RNA synthesis on transfer to the restrictive temperature.

In the fourth mutant, ts-102, DNA synthesis continued for about an hour after transfer to the restrictive temperature, then stopped. The incorporation of $[^{35}\text{S}]$ methionine was maintained for 2.5 h, and that of $[^{14}\text{C}]$ uracil for about 3.5 h before reaching a plateau (Fig. 3b). This mutant exhibits a phenotype which is intermediate between those of ts-38 and ts-66, and the nature of the lesion in DNA replication is unclear. *E. coli* ts mutants collected by Kohiyama *et al.* (1966) also contained examples with such behaviour. The mutation in ts-102 appears to have an effect independent of temperature, since the doubling time of this strain in YPG at 30°C was about 100 min, whereas under the same growth conditions the wild-type, ts-66 and ts-114 had doubling times of about 80 min.

The absence of sucrose, which is used to produce more dispersed growth, from the liquid medium YPG made detailed microscopic examination of the cultures problematic. Moreover, after transfer to the restrictive temperature, the mycelium of these three ts mutants formed dense clumps, whereas in the 30°C control cultures, the mycelium formed loose clumps with numerous freely extending hyphae. The wild-type strain underwent a similar but transient morphological response to the increased temperature, which corresponded to the period of impaired macromolecular synthesis described above. After about 3 h at 39°C , the appearance of the mycelium 'reverted' to that of the 30°C culture.

In *Streptomyces*, hyphal extension occurs by tip growth (Prosser *et al.*, 1988), whereas DNA synthesis appears to take place throughout the vegetative mycelium (Kummer & Kretschmer, 1986; Prosser *et al.*, 1988). Aggregations of nucleoids increase in frequency and size with increasing distance from the hyphal tip and they occur close to branching points, supplying lateral branches with nucleoids (Kretschmer, 1987; Prosser *et al.*, 1988). Correct segregation of nucleoids into branches would appear to be essential if exponential growth of the organism is to be maintained. Understanding mycelial growth and the development of a regulated branching mycelium requires knowledge of the mechanisms controlling cell wall synthesis, septation, branching and normal DNA replication in *Streptomyces*; the ts DNA synthesis mutants described here may be useful in this context.

Fig. 4. Reinitiation of DNA synthesis of ts-66 on return to the permissive temperature. Spores were inoculated into YPG medium containing [³H]thymidine at 30 °C; the culture was shifted to 39 °C for approx. 3 h, then returned to 30 °C as indicated. The incorporation of [³H]thymidine into TCA-precipitable material was determined.

Double temperature shift experiments

Double temperature shift experiments were undertaken to determine whether or not the block in DNA synthesis observed in the four ts mutants was reversible. Cultures were incubated at 30 °C for 18 h, transferred to 39 °C for 3 h and then returned to 30 °C. The inhibition of DNA replication in all four mutants was found to be reversible on returning to the permissive temperature, but only after a delay of about 2 h. Fig. 4 shows the effect for mutant ts-66. The majority of DNA initiation mutants of *E. coli* show a delayed resumption of DNA synthesis in the same type of experiments (Hanna & Carl, 1975); the results with ts-38 are thus comparable. However, ts-66 and ts-114 did not show the rapid reinitiation of DNA synthesis which is typical of *E. coli* DNA elongation mutants (Chu *et al.*, 1977); instead DNA replication recommenced about 2 h after the return to the permissive temperature.

Data from double temperature shift regimes are potentially important when considering reciprocal shift experiments which can provide insight into the timing and hierarchy of successive cell cycle events. This concept was developed by Hereford & Hartwell (1974) in their analysis of the yeast cell cycle, and has been applied subsequently to *Caulobacter crescentus*. Osley & Newton (1980) examined how the steps required for DNA replication and cell division, which appear to regulate differentiation in *Caulobacter*, are temporally controlled and organized. In the case of *S. coelicolor*, our aim would be to use reciprocal shift experiments to probe the possible inter-relationships between the control of DNA replication, the cell cycle and secondary metabolite formation.

Fig. 5. Macromolecular synthesis of (a) ts-98 and (b) ts-45. Spores were inoculated into YPG medium containing ●, [³H]thymidine; □, [¹⁴C]uracil or Δ, [³⁵S]methionine at 30 °C; the culture was shifted to 39 °C as indicated. Incorporation of the radiolabelled compounds into TCA-precipitable material was determined.

Macromolecular synthesis mutants with more complex phenotypes

Three other mutants were identified which showed not only cessation of DNA replication at the restrictive temperature, but also defects in other macromolecular synthesis events.

In the case of ts-98, incorporation of [³H]thymidine and [¹⁴C]uracil stopped after 30 min at 39 °C, whereas [³⁵S]methionine incorporation continued for 3 h, although at a reduced rate (Fig. 5a). One possible interpretation is that ts-98 is the equivalent of a *dnaK* mutant since these labelling kinetics are similar to those observed with a ts *dnaK* mutant of *E. coli* (Itikawa & Ryu, 1979).

Ts-60 showed rapid cessation of protein and RNA synthesis (within 15 min of transfer to 39 °C), DNA synthesis continued for about 1 h (data not shown). The most likely classification of ts-60 is as an RNA synthesis mutant, perhaps with the mutation affecting RNA polymerase (Ishihama *et al.*, 1980). However when ts-60 was tested in a double temperature shift experiment, no reversibility of the inhibition of macromolecular synthesis was observed, even 6 h after a return to 30 °C. This pattern does not correspond to that obtained with *E. coli* RNA polymerase mutants, which show a rapid recovery

Table 1. *Properties of ts macromolecular synthesis mutants of S. coelicolor*

Mutant	Osmotically remedial	Patterns of macromolecular synthesis at 39 °C			Possible lesion by analogy with <i>E. coli</i>
		DNA	RNA	Protein	
38	No	Stops after 2 h	Continues for 5 h	Continues for 5 h	DNA initiation
66	Yes	Stops within 20 min	Continues for 3 h	Continues for 3 h	DNA elongation
102	Yes	Stops after 1 h	Continues for 3.5 h	Continues for 2.5 h	DNA replication
114	Yes	Stops after 30 min	Continues for 2.5 h	Continues for 2.5 h	DNA elongation
45	Yes	Stops after 1 h	Stops after 1 h	Stops after 1 h	<i>adk</i> -like
60	Yes	Continues for 1 h	Stops within 15 min	Stops within 15 min	RNA synthesis
98	Yes	Stops after 30 min	Stops after 30 min	Continues for 3 h	<i>dnaK</i> -like

of RNA synthesis on transfer to the permissive temperature (Yoshinaga & Sugiura, 1977). The lack of reversibility of the ts-60 RNA synthesis lesion after transfer from restrictive to permissive temperatures was reflected in a loss of viability. The ability to form colonies on nutrient agar at 30 °C declined ten fold after 3 h exposure to 39 °C; such a loss of viability was not observed with the ts DNA synthesis mutants.

In temperature shift experiments on ts-45, synthesis of DNA, RNA and protein stopped after an hour at 39 °C (Fig. 5b). Incorporation of [¹⁴C]uracil into RNA resumed rapidly on return to 30 °C, whereas DNA and protein synthesis were not reinitiated within 3 h of transfer to the permissive temperature. The apparently simultaneous cessation of DNA, RNA and protein synthesis may point to a mutation in an energy-generating system rather than directly in one of the macromolecular synthesis pathways. Another possibility is that ts-45 is similar to the *dnaW* mutant of *E. coli* (Blinkowa & Gawecka, 1979), although the latter exhibits much more rapid inhibition of macromolecular synthesis at 42 °C. The *dnaW* mutation has been shown subsequently to be an allele of the *adk* (adenylate kinase) gene (Henson *et al.*, 1982). Interestingly, double temperature shift experiments with the *dnaW* mutant produced reinitiation results similar to those obtained with ts-45; i.e. RNA synthesis started rapidly on transfer to 30 °C, but the inhibition of DNA and protein synthesis was reversible only after a long delay (Blinkowa & Gawecka, 1979).

Clearly, further analysis of these three mutants will be required before their specific lesions can be identified and this may have to await the accumulation of more data concerning the primary metabolism of *Streptomyces*.

Conclusions

We believe this to be the first report of the isolation and preliminary characterization of a set of ts macromolecular synthesis mutants of *S. coelicolor*. The properties of

these mutants are summarized in Table 1. Hopwood (1966) reported the isolation of ts mutants of *S. coelicolor* for gene mapping purposes; auxotrophic and 'indispensable' ts mutants were identified but the latter were not characterized further. More recently, a single ts *dna* mutant of *S. hygroscopicus* has been described (Süss *et al.*, 1988), but the rapid cessation of both DNA and RNA synthesis at the restrictive temperature is evidently different from the patterns observed with the four mutants which we have classified as *dna* mutants. These *S. coelicolor* ts mutants have considerable potential as tools for the study of many areas of streptomycete molecular biology. By analogy with other micro-organisms (McMacken *et al.*, 1987; Newton & Ohta, 1990), they should prove useful in elucidating the regulation of macromolecular synthesis. DNA replication and its possible involvement in the control of secondary metabolite production are primary targets for such studies. The tentative identification of both slow (initiation) and fast (elongation) stop mutants should facilitate the cloning of some of the essential genes required for DNA replication in *S. coelicolor*. Future work will also include an investigation of plasmid replication in the *dna* mutants, and probing of possible integrative suppression events arising from interactions between the *Streptomyces* chromosome and the large linear plasmid SCP1.

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