The Effects of Temperature on Growth and Production of the Antibiotic Granaticin by a Thermotolerant Streptomycete

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The synthesis of granaticin, a polyketide-derived antibiotic synthesized as a secondary metabolite by Streptomyces thermoviolaceus strain NCIB 10076, was studied at different growth temperatures. Quantitative measurements of the antibiotic made during batch fermentations showed that the yield was greatest at 45 °C, whereas the rate of synthesis was most rapid at 37 °C. The timing of the appearance of granaticin in culture could not be assigned to any particular phase of growth or to de-repression due to depletion of any particular nutrient. However, at all temperatures, appearance of the antibiotic coincided with a rise in ammoniacal nitrogen presumably due to deamination of glutamate, the carbon source for growth. We have previously shown that production of the antibiotic is pH sensitive and that some carbon sources result in higher titres than others. This paper examines the effect of temperature on the physiology of growth and on antibiotic production in more detail under conditions that also allow an exact measurement of granaticin yield.

INTRODUCTION

Taxonomic studies have revealed that many Streptomyces are able to grow at 45 °C (Williams et al., 1983). However until recently there were relatively few reports concerning the ability of species from this group to grow at temperatures in excess of 50 °C. Goodfellow et al. (1987) surveyed 50 thermophilic streptomycetes from a range of habitats which they were able to assign to a number of clusters in a numerical phenetic study that identified some features of their physiology and metabolic versatility. Such work is valuable in view of the commercial advantages of thermophilic species. These include faster reaction times and reduced cooling costs for large fermentations which make the thermophilic streptomycetes potentially useful vehicles for antibiotic production at high temperatures. Streptomyces thermoviolaceus is a particularly useful model system for such studies because it grows over a temperature range of around 25 to 58 °C (Edwards & Ball, 1987) and produces granaticin, a pigmented and pH sensitive antibiotic (Maehr et al., 1979) which is synthesized via the polyketide pathway (Arnone et al., 1979; Snipes et al., 1979). Snipes et al. (1979), using 13C-labelled acetate and glucose, showed that granaticin is produced by mixed biogenesis from eight acetate units and one dideoxyglucose moiety. This agrees with other radiolabelling work that showed polyketide antibiotics to be acetate derived (Floss et al., 1985). Using a 18O2 atmosphere, Floss et al. (1985) also demonstrated that no molecular oxygen was incorporated into the molecule, indicating a direct cyclization of the polycetlyl chain rather than a classical hydroxylation using molecular oxygen, followed by a dehydration to the lactone.

METHODS

Organism and culture conditions. Streptomyces thermoviolaceus subsp. thermoviolaceus NCIB 10076 was maintained on complex solid medium that contained (g l−1): glucose, 4; malt extract, 10; yeast extract, 4; and after adjusting to pH 7.2, agar, 20. After 48–72 h incubation at 50 °C the aerial mycelium bearing grey spores had
formed and the spores were resuspended in 30% (v/v) glycerol and stored at −70 °C. Defined salts medium contained (g l−1): glutamate (sodium salt), 9.35; MOPS [3-(N morpholino)propanesulfonic acid], 5.23; (NH₄)₂SO₄, 2; MgSO₄. 7H₂O, 1; K₂HPO₄, 1; CaCl₂. 2H₂O, 0.05 and 1 ml l−1 of a trace element solution that contained 1 g l−1 of each of the following: MnCl₂. 4H₂O, ZnSO₄. 7H₂O, FeSO₄. 7H₂O. The resultant solution was adjusted to pH 7.0 prior to autoclaving for 15 min at 121 °C and 15 p.s.i. (103.5 kPa). Inocula were raised from spores by adding 1 ml of spore suspension to 100 ml of salts medium in 500 ml flasks. These were incubated with shaking for 10, 12, 14, 20 or 24 h for the temperature series 55, 50, 45, 37, 30 °C respectively after which time they were used to inoculate the fermenter (at the appropriate temperature).

Fermentations. These were carried out in an LH 500 series fermenter using a 2 litre vessel with a working volume of 1800 ml and equipped with baffles and pH control to 7.0 using 1 M-H₂SO₄ as the titrant. Dissolved oxygen was contained (g 1-l) were used to inoculate the fermenter (at the appropriate temperature). Oxygen consumption detected by an Ingold oxygen electrode probe linked to a chart recorder. Samples were removed at regular intervals during the growth period and the following analytical determinations carried out.

Measurements in culture samples. (a) Biomass determination. Culture samples (25 ml) were harvested and pelleted by centrifugation at 1800 g for 10 min and the pellet washed with water, resuspended in 5 ml of distilled water and placed in a pre-dried, pre-weighed foil cap and dried to constant mass at 105 °C in an oven.

(b) Granaticin. Cell free supernatant (25 ml) was lowered to pH 4.0 using dilute HCl and then extracted twice in 0.5 vol. (12.5 ml) of ethyl acetate, until no colour was visible in the aqueous phase. This was evaporated to dryness under vacuum, resuspended in a similar volume of ethyl acetate and dried again. The resulting residue was resuspended in 0.5 vol. (12.5 ml) of 0.1 M-HCl made up in ethanol. The absorbance was then read, against a suitable blank, at 223 nm using a Perkin-Elmer Lambda-5 spectrophotometer and the concentration of the antibiotic calculated using the mM absorption coefficient calculated from the purified sample.

(c) Glutamate. This was measured in cell free supernatants prepared from different stages of growth using the L-glutamic acid colorimetric method supplied in kit form by Boehringer Mannheim (Ruf & Siepe, 1983).

(d) Ammoniacal nitrogen determination. Cell free supernatants prepared from different stages of growth were analysed for ammoniacal nitrogen using the indolphenol colorimetric determination (Wainwright & Pugh, 1973).

Purification of granaticin. The pH of 60 litres of whole broth that contained granaticin was adjusted to pH 4.0 using HCl. The antibiotic was extracted twice using 30 litres of ethyl acetate until the aqueous layer was colourless. The extract was evaporated to dryness under vacuum and the residue taken up in 2 litres of alkaline water and adjusted to pH 9 with NaOH. This was back extracted with 400 ml of ethyl acetate to remove lipid residue left in the aqueous layer. Granaticin is relatively insoluble in ethyl acetate at pH 9 and remains primarily in the aqueous layer. The pH was then lowered to 4 and the granaticin extracted into 2 litres of ethyl acetate. This was evaporated to dryness, redissolved in a similar volume and again evaporated to remove the last traces of water. The remaining residue was then dissolved in 10 ml of fresh ethyl acetate and hexane added until a precipitate was formed. This precipitate was filtered in a Buchner funnel under vacuum and washed twice with hexane to remove any remaining impurities. The resulting powder was then desiccated and stored for future use.

RESULTS

Method for estimation of granaticin

Before the effects of temperature on granaticin production could be assessed a reliable means of estimating the amount of antibiotic in culture samples was required. A spectrum of purified granaticin dissolved in acidified ethanol (0.1 M-HCl in ethanol) is shown in Fig. 1. Peaks are apparent at 223, 285, 410, 525 and 562 nm with the largest being at 223 nm. From this spectrum, using an M₅ value of 444-l (St Pyrek et al., 1969), an absorption coefficient (mM) of 22.8 was calculated. This was used to determine the granaticin yields in all further work.

Activities of cultures at low and high growth temperatures

Fermentations were set up at different temperatures with glutamate as carbon source. Previous work had indicated that glutamate resulted in high titres of granaticin (James & Edwards, 1988). This growth substrate could act as both the sole carbon and nitrogen source and supported growth and antibiotic production (results not shown). However, it was noted that inclusion of (NH₄)₂SO₄ in salts media containing glutamate did not result in lower yields of granaticin compared with glutamate alone. This indicates that, under these growth conditions,
antibiotic production is not repressed by ammoniacal nitrogen. This is unlike *S. fradiae*, which is stimulated to produce tylosin by glutamate, but inhibited by the presence of ammonia in the medium (Vu-Trong & Gray, 1987). Two batch fermentations representative of a low (30 °C) and high (50 °C) growth temperature are shown in Figs 2(a) and (b) respectively. Previous work has shown that with glutamate as carbon source, growth and secondary metabolism were accompanied by a rise in pH (James & Edwards, 1988). This is due partly to the removal of glutamic acid and partly to the secretion of ammoniacal nitrogen into the medium. At 30 °C biomass reached a maximum value of 3.7 g l⁻¹ after 55 h, with a doubling time of around 4.2 h up to 26 h, but thereafter growth slowed down. The cessation of the more rapid growth phase was accompanied by the appearance of granaticin in culture supernatants; its synthesis continued up to 50 h and then the level decreased with time. Respiratory rate, an indirect measure of growth, increased exponentially up to around 53 h but then declined at the same time as the apparent degradation of granaticin was occurring, but perhaps more significantly at the time when the carbon source had nearly been exhausted. Glutamate disappearance was also measured and it is noteworthy that at the time when the level of granaticin was maximal some 30% of the initial amount of glutamate remained unused. Ammoniacal nitrogen rose at approximately the same time as granaticin synthesis. The levels up to around 44 h remained constant at 15 mmol l⁻¹, which was the concentration of ammonia initially supplied as (NH₄)₂SO₄ in the growth medium. This result indicates that under these cultural conditions *S. thermoviolaceus* meets its nitrogen requirement predominantly via the catabolism of glutamate (Fig. 2a).

At and above 50 °C the overall trends in the different parameters tested (Fig. 2b) were the same as those described for the fermentation at 30 °C. The major differences were that the reaction times were much faster and granaticin production was greatest when the glutamate had been completely depleted. Growth at 50 °C was rapid and uninterrupted up to the peak biomass of 3.7 g l⁻¹ at 9 h. The oxygen uptake rate increased with an identical gradient (Fig. 2b) until the peak biomass was reached, then dropped rapidly. The peak biomass coincided with substrate
Fig. 2. Typical batch fermentations for *S. thermoviolaceus* grown at (a) 30 °C or (b) 50 °C in a glutamate/salts medium. Samples were removed during growth in order to determine the amounts of biomass (●), glutamate (□), granaticin (■), and ammoniacal nitrogen (▲). The in situ respiratory activity (○) was also measured before removal of each sample.

Exhaustion. Granaticin appeared after 6 h growth and its production continued well after all the carbon source had been consumed. At 30 °C granaticin synthesis occurred when there was still an appreciable concentration of glutamate remaining (Fig. 2a). The ammoniacal nitrogen also followed antibiotic synthesis more closely at this temperature. Finally, it is noteworthy that once the carbon source had been completely utilized there was a rapid fall in biomass indicative of cell lysis.

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Figs 3(a) and (b) summarize the results for some of the parameters tested in Fig. 2(a) and (b) for fermentations cultured at temperatures ranging from 30–55 °C. The generation time of *S. thermoviolaceus* grown at 30 °C was approximately 4 h and fell with increasing incubation temperature to around 1 h at 50 °C. At 55 °C, which is near to the maximum temperature (58 °C) for growth, it rose to 3 h, indicating that cellular metabolism was becoming thermosensitive. Yield of biomass decreased up to 45 °C, a temperature at which the yield of granaticin was at its highest. Conversely at 50 °C the yield of biomass increased whilst that of granaticin fell from around 0.37 mM to 0.14 mM (Fig. 3a). Fig. 3(b) confirms that the optimum temperature for yield of antibiotic is 45 °C whether it is expressed in terms of granaticin per unit biomass formed or per unit substrate consumed. The production rate however is more rapid at 37 °C.
Antibiotic synthesis in S. thermoviolaceus

Fig. 3 Summary of the effects of growth temperature on the parameters shown in Fig. 2 for cultures grown in the glutamate salts medium. (a) Biomass yield (●), granaticin yield (●), and doubling times for growth (○). (b) Rate of granaticin appearance (▲) and the yield of granaticin in terms of carbon source (▲) or biomass (□). The results in (a) and (b) are typical of at least two separate experiments.

DISCUSSION

This work has demonstrated that S. thermoviolaceus makes a useful model for studying secondary metabolism and in particular the regulation of a polyketide-derived product. It grows rapidly on a simple salts medium with an appropriate carbon source and produces a coloured and easily quantified antibiotic. Granaticin is preferentially synthesized at temperatures as high as 55 °C but optimally at 45 °C. This shows that S. thermoviolaceus possesses a termotolerant polyketide pathway. Although the rate of production of the secondary metabolite is fastest at 37 °C the yield is not as high as that seen at 45 °C. We attribute this to a preference for producing biomass at 37 °C and examination of Fig. 3(a) shows that there is an inverse relationship between cell yield and titre of antibiotic over the temperature range 30 to 50 °C. What is particularly interesting about the quantities of granaticin produced at the different temperatures is that a preference for biomass rather than granaticin is shown at the two extremes of the temperature range (30 and 50–55 °C), while at the intermediate temperatures there is a preference for the antibiotic. At 37 °C there is only 75% of the quantity of biomass produced at either 30 or 50 °C whilst there is twice as much antibiotic. At 45 °C the antibiotic is produced earlier in the fermentation (results not shown) and its synthesis is preferred. At 50 °C, more than at the other temperatures, the commitment is to rapid biomass formation until substrate exhaustion (Fig. 2b), with a rapid doubling time of 72 min. Earlier work reveals that S. thermoviolaceus has a thermosensitive respiratory chain and that there is significant loss of NADH oxidase activity above 40 °C (Edwards & Ball, 1987). This would suggest that other factors may also become limiting towards the maximum growth temperature of 58 °C and would explain slower growth rates with reduced growth yields and low antibiotic titres at 55 °C.

At 30 °C, and to a lesser extent at 50 °C, granaticin is detectable in the culture supernatant before all the glutamate has been utilized. In fact antibiotic synthesis commences up to 1 h earlier because the cell suspension is always seen to turn blue before granaticin is detectable in the culture supernatant. Therefore, secondary metabolism commences during the period of
active growth and not when the cells have stopped growing. This is interesting in the light of earlier work suggesting that secondary products are synthesized principally during 'stationary phase' cultures (Demain, 1972; Demain et al., 1979). These reviews also mention that in ideal situations 'trophophase' and 'idiophase' are separate but can overlap in practice. The growth associated antibiotic production reported here is much more in line with findings of workers using continuous culture methods to study the production of antibiotics in the streptomycetes (Siktya et al., 1961; Trilli et al., 1987; Rhodes, 1984) where growth associated antibiotic production could be demonstrated. In some cases as growth rate increased so did antibiotic titre, which suggests that the classification of phases into idio- and trophophase may be misleading.

Measurements of ammoniacal nitrogen show that this parameter mirrors that of antibiotic production, especially at higher temperatures. The fact that increased ammoniacal nitrogen release corresponds to the release of antibiotic and the reduced growth rate would tend to reflect the flux of glutamate-derived carbon and energy through secondary pathways. The synthesis of granaticin when glutamate is used as the sole carbon source is therefore complex and must involve gluconeogenesis in order to provide dideoxyglucose. In addition it may well involve a number of anaplerotic reactions to provide the appropriate glycolytic and Krebs cycle intermediates, in particular either pyruvate or acetyl-CoA for the synthesis.

As noted earlier the doubling time for S. thermoviolaceus at 50 °C is 72 min which, in addition to the ability to rapidly deplete available substrates, would give the organism a selective advantage in a competitive environment. S. thermoviolaceus is isolated readily from horse manure (Desai & Dhala, 1967) and granaticin has been shown to inhibit RNA synthesis by interference with leucyl-tRNA synthetase in Gram-positive bacteria, notably Bacillus spp. (Ogilvie et al., 1975; Weiser et al., 1977). It could be postulated therefore that if granaticin were synthesized in the environment it would confer a selective advantage for the survival and proliferation of S. thermoviolaceus over facultatively thermophilic Bacillus spp. growing in the same environment.

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REFERENCES


