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Nutritional control of actinorhodin production by *Streptomyces coelicolor* A3(2): suppressive effects of nitrogen and phosphate

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Summary. Actinorhodin production in *Streptomyces coelicolor* A3(2) was relatively insensitive to the carbon source concentration but was elicited by nitrogen or phosphate depletion, or by a decline in the growth rate. In starch-glutamate media with nitrogen limitation, increasing the nitrogen supply delayed the onset of antibiotic synthesis and, at concentrations above 30 m*M*, decreased its rate. In a similar medium with phosphate limitation, increasing the initial phosphate concentration delayed actinorhodin formation and, above 2.5 m*M*, reduced the rate of synthesis. Experiments in which actinorhodin synthesis was elicited by phosphate depletion at various nitrogen concentrations demonstrated strong suppression by residual glutamate. Cultures in which actinorhodin biosynthesis was initiated by nitrogen depletion were not similarly suppressed by increasing amounts of residual phosphate. The results suggest that actinorhodin production in *S. coelicolor* A3(2) responds to interacting physiological controls, notable among which is nitrogen catabolite regulation.

Introduction

There is a large body of information describing the effects of nutrition and environment on secondary metabolism. In general it demonstrates that the formation of secondary metabolites is a response to reduced growth opportunities and is normally suppressed when an organism is growing at its full potential (Demain et al. 1983; Vining 1986). Restriction of growth allows the genetic information for secondary processes to be expressed and the level of expression is subject to a variety of subsidiary influences (Bhatnagar et al. 1988). Nutrition plays an important role in the onset and intensity of secondary metabolism, not only because limiting the supply of an essential nutrient is an effective means of restricting growth but also because the choice of limiting nutrient can have specific metabolic and regulatory effects. Although the overall consequences of nutritional stress have now been described for numerous secondary processes, they have not been linked, except in a speculative way, to molecular control mechanisms (Vining and Doull 1988).

Information on the molecular genetics of antibiotic biosynthesis is accumulating rapidly and the regulatory elements that control gene expression in these secondary metabolic pathways are becoming known. One of the products on which studies have focused is actinorhodin. This isochromanequinone antibiotic is produced by *Streptomyces coelicolor* A3(2) and related species via a polyketide intermediate. Progress in determining the sequence of reactions by which it is formed has complemented advances in the molecular biology of its production (Malpartida and Hopwood 1986). However, relatively little is known at the physiological level about control of actinorhodin biosynthesis.

Where the production of polyketide metabolites has been investigated, nutrient limitation has been reported to influence yields. Polyene and tetracycline synthesis is elicited by phosphate depletion and is depressed by certain nitrogen and carbon sources (Martin 1983; Behal 1987). Anthracyclines are produced in response to nitrogen limitation (Dekleva et al. 1985) while phosphate, ammonia and readily assimilated carbon sources must be depleted to optimize production of several macrolide antibitotics (Ōmura and Tanaka

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1986). With the goal of relating the wealth of phenomenological observations on secondary metabolism to the emerging understanding of genetic systems in streptomycetes, we have examined nutritional effects on actinorhodin formation.

Materials and methods

Culture and growth conditions. Streptomyces coelicolor A3(2) was provided by D. A. Hopwood of the John Innes Institute, Norwich, UK, and was maintained on sporulation agar (Ochi 1986). Spore inocula were prepared as described by Doull and Vining (1989a). To prepare vegetative inocula, spores and mycelium from a plate culture on MYM agar (Stuttard 1982) were added to 50 ml of J medium (Doull and Vining 1989b) in a 250-ml erlenmeyer flask. The culture was incubated for 48 h at 30° C on a rotary shaker (220 rpm); the washed mycelium, resuspended in the same volume of water was used (1% v/v) as the inoculum. Actinorhodin production cultures were incubated under the conditions used to prepare the vegetative inoculum except that about one hundred 0.3-mm diameter glass beads were added to each flask (Doull and Vining 1989a).

Media. The basal medium for actinorhodin production contained (per litre): morpholinopropanesulphonic acid (MOPS), 21 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 9 mg; $CaCl_2$, 1 mg; NaCl, 1 mg; and the trace mineral salt solution (4.5 ml) described by Chatterjee and Vining (1981). In addition, phosphate as a 7:3 (w/w) mixture of K_2HPO_4 and KH_2PO_4, a carbon source and a nitrogen source were added as required in each experiment. The pH was adjusted to 6.5 with NaOH after addition of supplements.

Analyses. Values are averaged from 4–8 replicated cultures. Culture samples (2.5 ml) were diluted and adjusted to pH 12 (± 1) with NaOH. The suspension was then clarified by centrifugation and the absorbance of the solution was measured at 640 nm (OD_{640}) in a spectrophotometer (Spectronic 21, Bausch and Lomb, Rochester, NY, USA; 10-mm internal diameter cuvettes). The actinorhodin content of the culture was calculated by using the specific extinction ($E_{1\%,1\,cm} = 355$) at 640 nm determined for an alkaline solution of actinorhodin (Doull and Vining 1989a). Growth was measured as the dry weight of washed cell material collected from a sample by filtration. The diameter of mycelial wefts was measured as described previously (Doull and Vining 1989a).

Phosphate was determined by the method of Herbert et al. (1981). Glutamate was measured as described by Shapiro and Vining (1983) except that samples were not pretreated to remove ammonium. To determine the amount of residual starch in cultures, a sample (25 µl) of the supernatant solution was mixed with 5 ml phosphate buffer, pH 6.8, then treated with an aqueous solution (0.5 ml) of iodine (0.38 g/l) and KI (1.2 g/l). The absorbance at 730 nm was related to that given by known concentrations of starch.

Results

Culture conditions

In starch-glutamate media under optimized growth conditions, cultures of *S. coelicolor* A3(2) form small, loosely aggregated and well dispersed mycelial wefts; biomass accumulates rapidly and high biomass yields are attained (Doull and Vining 1989a). When a spore inoculum is used to seed defined media of this general composition, actinorhodin can be produced in a fully biphasic pattern (Doull and Vining 1989a, b). To ensure that the inoculum size and type were not limiting factors in the present study, the size of mycelial wefts, the growth rate and yield, and the amount and pattern of actinorhodin production were compared in cultures initiated from various amounts of spore and vegetative inocula in a basal medium with 15 m*M* phosphate, 60 m*M* glutamate and 5% starch.

No appreciable differences were found in cultures receiving fresh spore suspensions or spores stored frozen in glycerol, and above a threshold value the size of spore inocula was not important. With sizes below the threshold, the diameters of mycelial wefts were similar but growth rates and biomass accumulation became progressively lower. With a vegetative inoculum the weft diameter was approximately three times that in cultures initiated from spores. Although the maxima for biomass accumulation and actinorhodin production were comparable to those in cultures given spore inocula above the threshold size, the growth rate was much lower. In the following physiological experiments, therefore, all production cultures were inoculated with spores at a concentration above the threshold level.

Carbon sources

In a basal medium with 15 m*M* phosphate and 60 m*M* glutamate, varying the starch concentration from 1%–5% had little effect on the time at which actinorhodin production began (Fig. 1). Starch was exhausted between 48 and 72 h in cultures receiving 3% or less but remained in excess, even after 96 h where the initial concentration was 4% or 5%. In all cultures, unused phosphate and glutamate were present at the onset of antibiotic synthesis. The results indicated that at a concentration of 5% the primary carbon source was in excess and did not suppress actinorhodin production; they also suggested that actinorhodin biosynthesis is not initiated by carbon source depletion.

Nitrogen sources

A basal medium with 5% starch, 15 m*M* phosphate and glutamate was used to examine the ef-

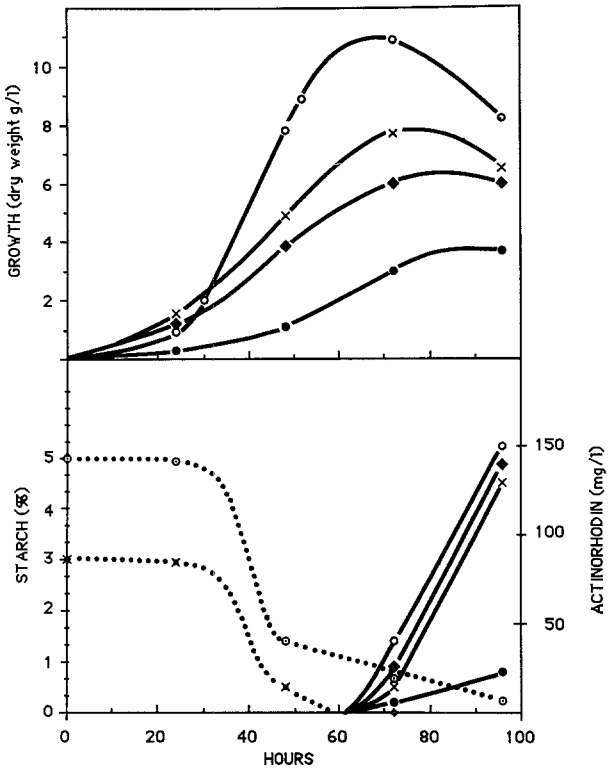

Fig. 1. Growth, starch consumption and actinorhodin production in a basal medium with 15 m*M* phosphate, 60 m*M* glutamate and various starch concentrations: —●—, 10 g/l; —×—, 20 g/l; —◆—, 30 g/l; —○—, 50 g/l. Residual starch is plotted with *dotted lines*. Some data have been omitted for clarity

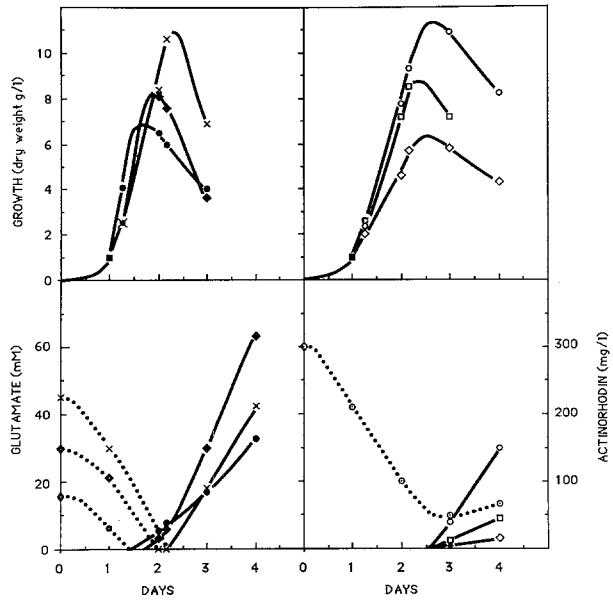

Fig. 2. Growth, glutamate consumption and actinorhodin production in a basal medium with 5% starch, 15 m*M* phosphate and various concentrations of glutamate: —●—, 15 m*M*; —◆—, 30 m*M*; —×—, 45 m*M*; —○—, 60 m*M*; —□—, 75 m*M*; —◇—, 90 m*M*. Residual glutamate is plotted with *dotted lines*. Some data have been omitted for clarity

fect of varying the nitrogen source concentration. In cultures with 15–60 m*M* glutamate, maximum biomass increased with increasing nitrogen availability (Fig. 2). At 75 and 90 m*M* glutamate, biomass accumulation was progressively diminished; the pH of the latter cultures increased to 7.7 at 2 days, despite the presence of MOPS buffer in the media. In cultures with 15–45 m*M* glutamate, initiation of actinorhodin synthesis coincided with depletion of the nitrogen source. Cultures receiving 60–90 m*M* glutamate retained excess nitrogen in the broth throughout the period under investigation; in each medium, actinorhodin production began at the growth maximum; its rate showed a roughly inverse relationship to the residual glutamate content of the broth at that time. At all glutamate concentrations, excess phosphate and starch were present throughout the fermentation.

Varying nitrogen concentration with low phosphate

To determine the effect on actinorhodin biosynthesis of varying the nitrogen concentration in a medium with a low phosphate content, cultures were grown in a basal medium with 5% starch, 2.5 m*M* phosphate and glutamate at concentrations between 30 and 75 m*M*. In all cultures the supply of phosphate was exhausted by 48 h with unused glutamate remaining (Table 1). Actinorhodin production began shortly afterwards in cultures given 30 m*M* glutamate and with longer delays in those given higher nitrogen concentrations. The initial rate of biosynthesis, measured during the first 6 h, decreased substantially in cultures with increasing amounts of residual glutamate.

Table 1. Effect of varying the glutamate concentration in cultures of *Streptomyces coelicolor* A3(2) supplied with limiting phosphate[a]

Glutamate concentration		Actinorhodin production	
Initial (m*M*)	At idiophase onset (m*M*)	Time of onset (h)	Initial specific rate (μg/mg/h)
30	2.0	52	1.5
45	9.0	54	1.2
60	14.0	64	0.7
75	26.0	64	0.05

[a] The basal medium contained 5% starch and 2.5 m*M* phosphate

Varying phosphate concentration in media where nitrogen is in excess

The effect of varying the phosphate concentration from 0.6 to 15 m*M* was first examined in a medium with carbon and nitrogen sources (5% starch, 60 m*M* glutamate) optimized for growth of *S. coelicolor* A3(2) (Fig. 2). As anticipated (Doull and Vining 1989a), biomass yields increased progressively to a maximum at 10–15 m*M* phosphate (Fig. 3). At phosphate concentrations up to 5 m*M*, actinorhodin production began close to the time when phosphate was exhausted from the medium. In cultures which received 10 or 15 m*M*, unused phosphate was present during the 4 days of observation and actinorhodin production began at the peak in biomass. The initial rate of production was highest with 2.5 m*M* phosphate. In all of these cultures, analyses showed starch and glutamate to be present in excess.

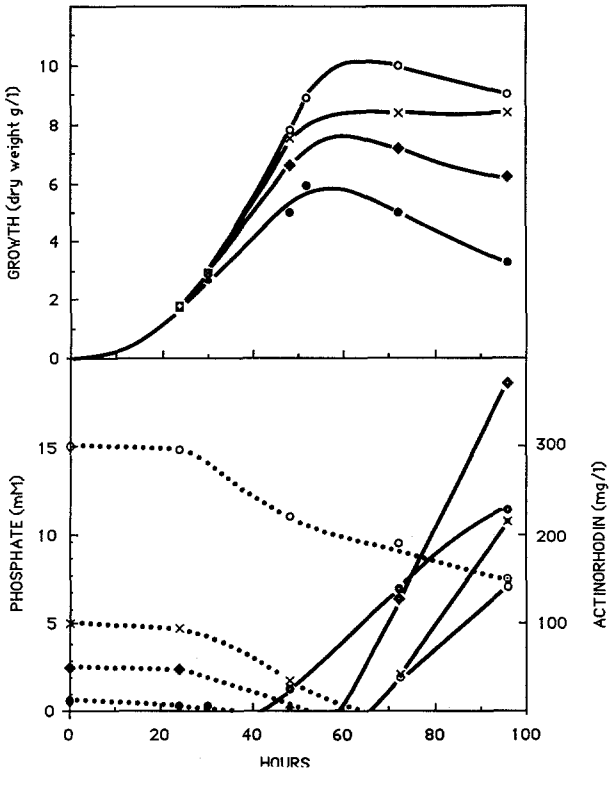

Fig. 3. Growth, phosphate consumption and actinorhodin production in a basal medium with 5% starch, 60 m*M* glutamate and various concentrations of phosphate: —●—, 0.6 m*M*; —◆—, 2.5 m*M*; — × —, 5 m*M*; —○—, 15 m*M*. Residual phosphate is plotted with *dotted lines*. Some data have been omitted for clarity

Table 2. Effect of varying the phosphate concentration in cultures of *S. coelicolor* A3(2) supplied with limiting glutamate[a]

Phosphate concentration		Actinorhodin production	
Initial (m*M*)	At idiophase onset (m*M*)	Onset time (h)	Initial specific rate (μg/mg/h)
3	0.0	48	1.5
6	1.2	51	1.7
9	3.7	51	2.0
12	5.6	52	2.2
15	6.5	52	1.3

[a] The basal medium contained 5% starch and 30 m*M* glutamate

Varying phosphate in media where nitrogen is limiting

Cultures were grown in a basal medium containing 5% starch, 30 m*M* glutamate and phosphate concentrations ranging between 3 and 15 m*M*. Under these conditions, actinorhodin synthesis was elicited at almost the same time (48–52 h) by depletion of nitrogen in media with different amounts of residual phosphate (Table 2). The rate of production showed small increments as the initial phosphate concentration was raised to 12 m*M*, but decreased at 15 m*M*.

Discussion

Actinorhodin production in cultures of *S. coelicolor* A3(2) is not strongly influenced by the kind of carbon source provided (Doull and Vining 1989b). It is supported well in media containing starch, a substance that promotes rapid biomass formation, and is not delayed or suppressed by excess starch. The results suggest that actinorhodin biosynthesis is not subject to carbon catabolite repression. In contrast, phosphate and glutamate negatively control formation of the antibiotic. During rapid growth in the presence of an excess of these two nutrients, actinorhodin is not produced. Depletion of either nutrient elicits production but is not an essential condition for this event. Actinorhodin production is initiated by termination of rapid growth in a culture, even when both of these suppressive nutrients are present.

High concentrations of glutamate in the culture medium at the onset of production decrease the rate of actinorhodin formation, suggesting

that nitrogen limitation is necessary for either full expression of the biosynthesis genes or full enzyme activity. The overall results indicate that control of actinorhodin biosynthesis is a multifunctional process in which limiting nutrients such as nitrogen and phosphate play different roles and in which the growth rate itself may play a part.

In cultures initiated from a vegetative inoculum, most defined media give only partially biphasic patterns of growth and actinorhodin production; for complete suppression of actinorhodin synthesis during the trophophase, a complex medium capable of supporting very rapid biomass accumulation is required (Doull and Vining 1989b). The early formation of actinorhodin leading to phase overlap in cultures initiated from a vegetative inoculum was attributed to a shift-down effect in seed mycelium which had been grown in a rich, complex medium. The results of the present investigation are consistent with this interpretation in that actinorhodin biosynthesis is shown to be elicited by a decrease in growth rate, even when critical nutrients (phosphate and nitrogen) are in excess. A blurred distinction between growth and secondary metabolism is probably due not only to the regulatory consequences of shifting down to a less rich medium but also to the tendency of *S. coelicolor* A3(2) to grow as compact mycelial pellets in which the interior is at least partly nutrient starved (Doull and Vining 1989a).

One way in which nutrients are known to suppress secondary metabolic pathways is by inhibiting key enzyme reactions (Lubbe et al. 1985). A second way is by repressing synthesis of the secondary pathway enzymes. Repression has been demonstrated in the candicidin producer, *S. griseus*, from which a phosphate-regulated promoter sequence for the *pab* genes involved in antibiotic synthesis has been cloned (Martin et al. 1988). Preliminary experiments (data not included) in which a labeled *act*I gene coding for polyketide synthase (Malpartida et al. 1987) has been hybridized with the total RNA fraction from *S. coelicolor* A3(2) indicate that synthesis of a specific mRNA for actinorhodin is associated with rapid formation of the antibiotic. A situation in which excess glutamate not only repressed synthesis but also lowered the activity of enzymes for actinorhodin formation might resemble that described by Brana et al. (1986) for cephalosphorin biosynthesis in resting cells of *S. clavuligerus* where inhibition by glutamate was postulated to be due to its effect on transport or metabolic activity.

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