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Ammonium effects on streptonigrin biosynthesis by *Streptomyces flocculus*

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SUMMARY

A defined medium containing glucose and ammonium as the sole carbon and nitrogen sources was developed to support growth and streptonigrin production. In this defined medium, increased initial levels of ammonium resulted in increased growth suggesting that nitrogen is the growth limiting nutrient. In some cases, increased initial ammonium levels resulted in decreased specific streptonigrin productivity, suggesting that nitrogen regulatory mechanisms may adversely affect streptonigrin biosynthesis. This suggestion that nitrogen regulation adversely affects antibiotic biosynthesis is further supported by results from two studies in which the ammonium supply to the cells was controlled. In the first study, streptonigrin productivity and final titer were enhanced by the addition of an ammonium trapping agent. In the second experiment, when ammonium chloride was fed slowly throughout the course of cultivation, the production phase was lengthened and the maximum antibiotic concentration was enhanced compared to the batch controls containing either the same initial or the same total ammonium chloride levels. Although our results indicate streptonigrin production may be subject to nitrogen regulatory mechanisms, the effect of nitrogen on streptonigrin production cannot be strictly correlated to the extracellular ammonium concentration. In fact, we observed that when ammonium was depleted from the medium, streptonigrin production ceased.

INTRODUCTION

Streptomyces flocculus (ATCC 13257) produces the antitumor antibiotic streptonigrin. Gould and coworkers have shown that streptonigrin is derived biosynthetically via the shikimic acid pathway. Specifically, the quinoline quinone portion of streptonigrin is derived via aminoanthranilic acid [10] and erythrose-4-phosphate [8] while tryptophan is incorporated into the phenylpicolinic acid moiety via β-methyltryptophan [9].

Previous studies indicated that Emerson medium yielded the highest streptonigrin titers of the various complex media tested (D.L. Hartley, Ph.D. thesis, University of Maryland at Baltimore, 1982), but the regulation of production in defined media has not been reported. The present study was initiated to develop a simple defined medium and to identify the key nutritional parameters regulating streptonigrin biosynthesis.

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MATERIALS AND METHODS

Medium and culture conditions. A spore suspension of *Streptomyces flocculus* (ATCC 13257) was prepared by washing spores from an agar slant with 10 ml of sterile water. The spores were vacuum filtered through Whatman # 1 filter paper and stored in glycerol (30% v/v) at −20°C.

Seed was prepared by adding 5 ml of the stored spore suspension to 120 ml of Emerson medium which contains 10 g/l glucose, 4 g/l beef extract (Difco Laboratories), 4 g/l Gelysate Peptone (Baltimore Biological Laboratories), 1 g/l yeast extract (Difco Laboratories), 2.5 g/l NaCl. After culturing the seed for 48 h, 7.5 ml of the seed was transferred into each experimental flask, which contained 150 ml of basal medium (30 g/l glucose, 75 mM potassium phosphate at pH 7.2, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, 0.4 g/l $CaSO_4 \cdot 2\,H_2O$, 0.01 g/l $FeSO_4 \cdot 7\,H_2O$, 1.9 g/l NaCl,

0.9 g/l KCl). The NH_4Cl concentration in the defined medium was varied as indicated in the text. Salts, phosphates and NH_4Cl were autoclaved together. Glucose was autoclaved separately and added aseptically. All cultures were incubated in one liter flasks at 29°C and 180 rpm on a rotary shaker.

For the ammonium ion trapping experiments, the defined medium was altered to contain an additional 8.7 g/l K_2HPO_4 and 13.7 g/l $MgSO_4 \cdot 7\,H_2O$.

For the ammonium chloride feeding study, the defined medium containing an initial ammonium chloride concentration of 0.5 g/l was periodically supplemented with additions of NH_4Cl to supply nitrogen while maintaining low, but finite concentrations of this nutrient. A concentrated NH_4Cl stock solution (232 g/l) was used for these additions, and thus a negligible volume change resulted in this culture.

Analytical procedures. For streptonigrin determination, a 10 ml sample was removed from the flasks and centrifuged at 2800 rpm at room temperature. Two ml of the supernatant was removed and the pH adjusted to 4.0 with 6 N HCl. Two ml of hexane was added to the sample to extract compounds observed to interfere with the assay. The tubes were vortexed and centrifuged and the hexane layer was removed and discarded. Two ml of butyl acetate was added to the aqueous fraction to extract the streptonigrin. The absorbance of the butyl acetate extract was measured at 375 nm using a Gilford System 2600 UV spectrophotometer against a butyl acetate blank.

The identity of the 375 nm absorbing material was confirmed using high performance liquid chromatography on a Beckman system consisting of a 421 controller, a 114 pump, a variable wavelength detector, a 5 μ Ultrasphere-octyl column (0.46 × 25 cm) and a Hewlett Packard 3392A integrator. Two ml of the organic layer from the streptonigrin extraction was evaporated, and the residue redissolved in methanol for injection. The mobile phase consisted of 60% acetonitrile and 40% phosphoric acid (8 mM) at a flowrate of 1 ml/min. Only one major peak was detected by the UV detector at 375 nm, and the retention time of this peak (5.4 min) corresponded to that of a pure streptonigrin standard.

The concentration of extracellular nitrogen was determined for cell-free supernatants using the ninhydrin method [6]. Standard curves were prepared for ammonium chloride concentrations from zero to 1 mmol.

For dry weight determination, 10 ml of sample was centrifuged, and the supernatant was removed from the cells. The cells were washed with 10 ml of distilled water and recentrifuged. The cells from this wash were then transferred to preweighed dishes and dried at 85°C for 48 h.

RESULTS

Preliminary studies (unpublished results) indicated that varying the initial glucose level from 10 to 50 g/l did not affect the specific streptonigrin productivity (i.e. the amount of streptonigrin produced per cell per day). Similar results were obtained in studies with varying initial phosphate concentrations. When the initial phosphate level was increased from 25 to 100 mM, the specific productivity remained constant. Thus, phosphate and glucose do not appear to have a specific effect on streptonigrin production.

Fig. 1 shows typical time profiles for cultures grown in a defined medium containing varying concentrations of ammonium chloride. It can be seen from this figure that increasing the initial nitrogen level by increasing the

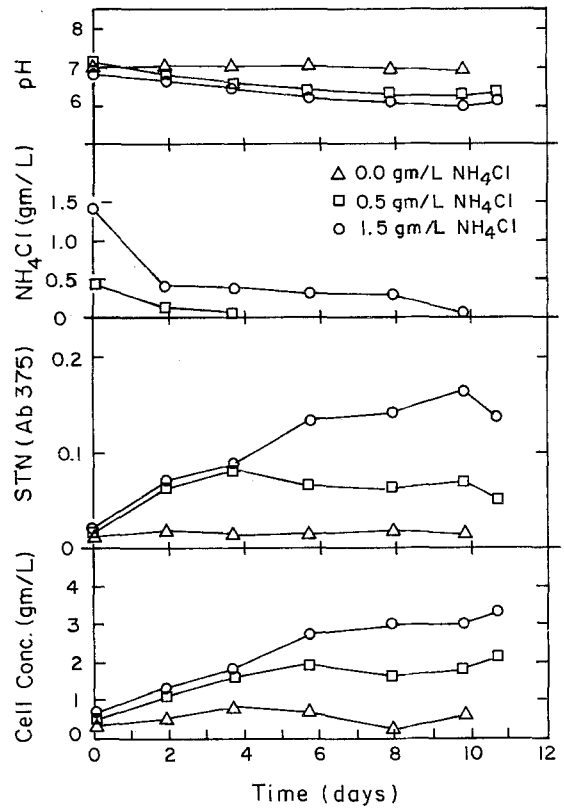

Fig. 1. Fermentation profiles for cultures grown with different initial NH_4Cl concentrations.

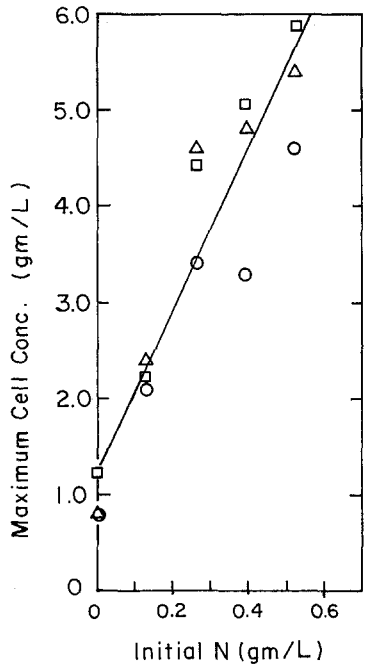

Fig. 2. Effect of initial nitrogen concentration on growth (0.1 g/l N corresponds to 0.39 g/l NH_4Cl). As indicated, data are shown for three separate experiments in which different inocula were used.

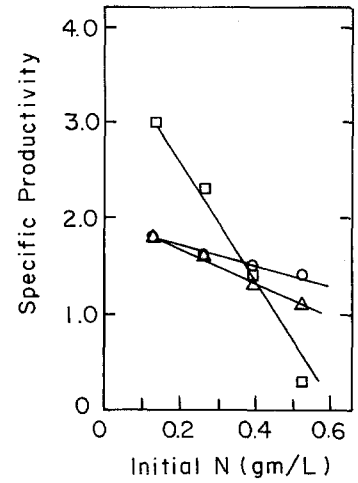

Fig. 3. Effect of initial nitrogen concentration on specific streptonigrin productivities. As indicated, data are shown for three separate experiments in which different inocula were used. Productivity units of (ΔAbs/day)/(g cells/l) × 100 are used.

NH_4Cl concentration from 0 to 1.5 g/l resulted in increased growth. This effect is summarized in Fig. 2 which shows a direct relationship between the maximum cell concentration and the initial nitrogen level for concentrations of 0–0.5 g nitrogen/l (0–2 g/l NH_4Cl). Increasing growth with increasing initial nitrogen concentrations suggests that nitrogen availability limits growth. If it is assumed that all of the nitrogen supplied to the cells is incorporated into cell mass, then the slope calculated from Fig. 2 would be the inverse of the cellular nitrogen content. This slope suggests a cellular nitrogen content of 12% which is similar although slightly higher than the 9.4% reported by Bushell and Fryday for *Streptomyces cattleya* [5].

In addition to increased growth, Fig. 1 shows that cultures grown in the presence of NH_4Cl produced more streptonigrin than cultures grown in the absence of NH_4Cl. However, when the rate of production was normalized using an average cell concentration, an inverse relationship was observed between the specific productivity and the initial nitrogen concentration (Fig. 3). Although the trends in Fig. 3 are reproducible, the magnitude of the change in specific productivity with increasing initial ammonium varied. In fact, two of the three experiments of Fig. 3 showed only a small decrease in specific productivity with increasing initial nitrogen. Thus, these results are somewhat inconclusive in terms of establishing that this fermentation is sensitive to negative nitrogen regulation by ammonium nitrogen.

Although increasing initial nitrogen levels may adversely affect the specific productivity, nitrogen appears

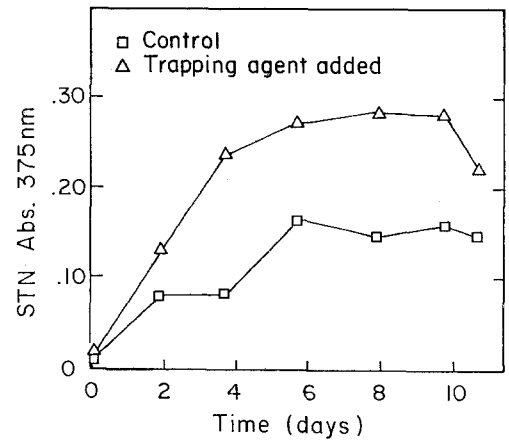

Fig. 4. Effect of ammonium trapping agent on streptonigrin production. To bind ammonium ions, 13.7 g/l $MgSO_4 \cdot 7\ H_2O$ and 8.7 g/l K_2HPO_4 were added to the experimental culture. Both the control (no trapping agent) and the experimental cultures contained an initial NH_4Cl concentration of 2 g/l.

to be required for continued streptonigrin production. This apparent requirement can be seen from Fig. 1 which shows that streptonigrin production by the cultures ceased when ammonium was exhausted from the medium.

To further examine the effects of nitrogen on streptonigrin production, alternative experiments were conducted in which the inorganic nitrogen supply was controlled. The goal of these studies was to supply sufficient nitrogen to meet the growth and production requirements of the cells while limiting their exposure to high nitrogen levels and thus minimizing the adverse effects of nitrogen regulation.

In the first study, magnesium sulfate and potassium phosphate were added to the medium to trap ammonium ions in an insoluble complex, $MgNH_4PO_4$. Fig. 4 shows that the experimental flask, containing magnesium phosphate and 2 g/l initial ammonium chloride, exhibited an increase in the maximum amount of streptonigrin produced relative to the control flask. Although the streptonigrin production period in the experimental flask was not lengthened beyond the 6-day period observed under control conditions, the rate of streptonigrin production, as calculated from the slope of the production curve, was increased by the addition of the trapping agent. Specific productivities could not be calculated because insolubles in the nitrogen trapping experiment interfered with the measurement of dry cell concentrations. However, if it is assumed that cell growth is not stimulated by the addition of the trapping agent, then the increase in streptonigrin production in these cultures would have resulted from an increase in the specific productivity (i.e. an increase in productivity per cell).

The second method used to control the supply of nitrogen was to initially supply the culture with 0.5 g/l NH_4Cl and then to periodically feed small amounts of ammonium throughout the course of the experiment. For comparison of the results from the nitrogen-fed culture, it is important to recognize that there are two possible controls: a batch culture containing the same initial nitrogen level and a batch culture containing the same total nitrogen as the ammonium-fed culture. It should be noted that the difference in pH between the nitrogen-fed culture and the cultures containing a single initial ammonium chloride addition was small (< 0.5 units) throughout the experiment. Fig. 5 shows that upon depletion of nitrogen from the batch culture containing the same initial nitrogen level (0.5 g/l NH_4Cl), growth and production were observed to cease. Since nitrogen was never depleted from the ammonium-fed culture, the growth and streptonigrin production phases were extended. Fig. 5 also shows that growth in the nitrogen-fed culture was similar to that for the batch control containing the same total nitrogen level. However, streptonigrin production in the nitrogen-fed culture was again greater than in this control. In comparison to either batch control, the increase in streptonigrin production in the nitrogen-fed culture appears to be due to an extension of the production phase and not to a large increase in specific productivity.

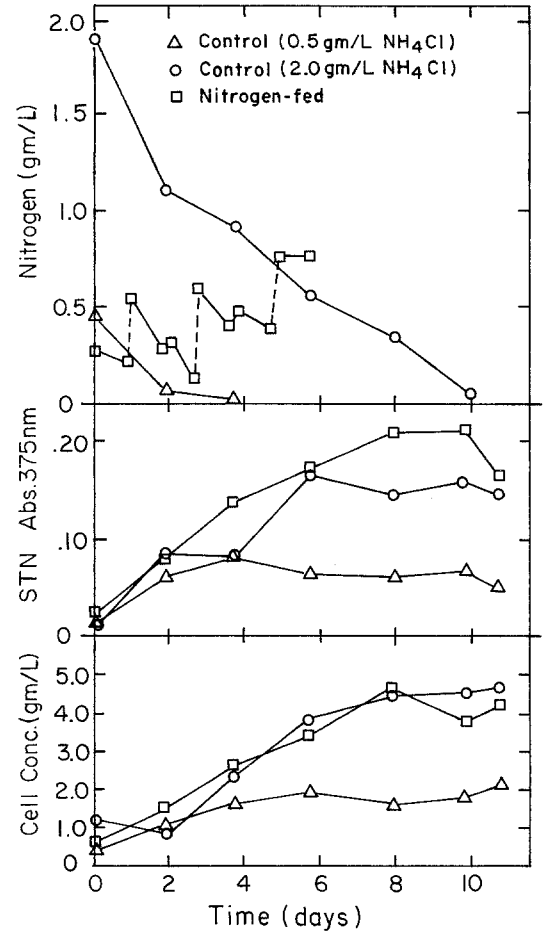

Fig. 5. Effect of ammonium feeding on growth and streptonigrin production. An initial NH_4Cl level of 0.5 g/l was used for the nitrogen-fed culture and for the control containing the same initial nitrogen level. Since a total of 1.7 g/l of NH_4Cl was added to the nitrogen-fed culture, a second control containing an initial NH_4Cl level of 2 g/l is also shown.

DISCUSSION

Streptonigrin production, like other secondary metabolites, exhibits different patterns of growth and production depending upon the nutritional content of the culture medium. When *Streptomyces flocculus* was grown in a complex medium, the doubling time of the cells was 12 h (data not shown), and streptonigrin production often followed the period of rapid growth [11]. In contrast, in the chemically defined medium, growth was slower (e.g. doubling times of two days), while streptonigrin was produced during the period of active growth. This observation, that secondary metabolite production occurs during or after growth depending on whether a defined or complex medium is used, has been reported for other antibiotic producing systems [2,13,16].

With respect to nutritional regulation, it is generally believed that secondary metabolite production commences when growth becomes limited by a specific nutrient [7]. In the defined medium which we developed, growth of *S. flocculus* is limited by nitrogen availability (Fig. 2). Therefore, it might be expected that nitrogen depletion would trigger the onset of streptonigrin production and that increased initial nitrogen levels would delay the onset of secondary metabolite biosynthesis. However, in our studies, measurement of the extracellular ammonium level showed that production occurred while ammonium was present and that increased initial levels of ammonium did not delay the commencement of streptonigrin production. Although contradictory to traditional generalizations, the observation that production in a defined medium occurs before depletion of the growth limiting nutrient is consistent with the observation that growth and production occur simultaneously.

A common observation in secondary metabolite producing systems is that increased concentrations of the growth limiting nutrient decrease antibiotic production or the rate of production per gram of cells. In the streptonigrin producing cultures of *S. flocculus*, increased initial nitrogen concentrations appeared to decrease the rate of antibiotic produced per gram of cells although the magnitude of this effect was sometimes small (Fig. 3). This rather small effect on streptonigrin biosynthesis may be due to the narrow range of initial nitrogen levels studied (0–0.5 g nitrogen/l). Other studies which have suggested nitrogen regulation of secondary metabolism have suggested the adverse effect occurs at nitrogen concentrations exceeding 0.6 g nitrogen/l [1,12]. Our efforts to examine higher initial nitrogen levels were plagued by problems associated with maintaining a reasonably constant pH while using NH_4Cl as the sole nitrogen source.

We therefore attempted to confirm the regulatory effects of ammonium ion by slowly supplying nitrogen to the culture. The use of magnesium phosphate as an ammonium trapping agent resulted in an increased rate of streptonigrin production (Fig. 4). The use of ammonium trapping agents in the production of tylosin [14], cephalosporin [3] and leucomycin [15] also resulted in increased antibiotic production. In the production of lincomycin [20], which is phosphate regulated, complexing phosphate as magnesium ammonium phosphate was also shown to increase antibiotic titers. Secondly, when ammonium chloride was slowly fed to the culture, the production period was extended, and streptonigrin titers were increased (Fig. 5). These results lend support to a negative regulatory role for ammonium ions as suggested by the initial data (Fig. 3).

However, it must be noted that the possible nitrogen regulatory effects on streptonigrin production cannot be strictly correlated with the extracellular concentration of NH_4Cl (Fig. 1). Similar observations have been noted in other systems. Shapiro and Vining observed that when ammonium was added to chloramphenicol producing cultures in a defined medium, antibiotic biosynthesis ceased for a constant 6-h period regardless of the amount of ammonium added [19]. Also, Brana et al. [4] reported that although cephalosporin production is nitrogen regulated, the effects cannot be strictly correlated to the intracellular levels of either ammonium or direct products of ammonium assimilation.

In addition to a possible regulatory role, the nitrogen source appears to be required for biosynthesis. When ammonium was depleted from the medium, streptonigrin production was observed to cease (Fig. 1), while ammonium feeding extended the production phase (Fig. 5). If growth and production are linked in this defined medium, then depletion of the growth limiting nutrient would be expected to result in the cessation of production. Also, it is possible that an available nitrogen source is required for streptonigrin biosynthesis since this antibiotic contains four nitrogen atoms. In an analogous situation, Pirt and Righelato [16] found that although penicillin production is sensitive to glucose catabolite regulation, this substrate is also required to meet the energetic and biosynthetic needs of the culture. Glucose feed rates which were insufficient in meeting the energetic and biosynthetic needs of the culture resulted in reduced penicillin production. On the other hand, Shapiro and Vining

observed that chloramphenicol, which contains two nitrogen atoms, was produced after nitrogen was depleted from a defined medium containing ammonium and/or nitrate as the sole nitrogen source [17–19].

In conclusion, the increase in streptonigrin production in response to ammonium trapping and slow feeding of ammonium chloride lends support to our initial observations of a negative correlation between streptonigrin productivity and initial ammonium levels. However, since effects of nitrogen cannot be strictly correlated to the extracellular nitrogen concentration, it would appear that ammonium may not be a direct effector regulating streptonigrin biosynthesis.

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